Gene expression of antioxidant enzymes and heat shock proteins in tropical seagrass *Thalassia hemprichii* under heat Stress

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ABSTRACT: During tidal exposure, intertidal seagrass is susceptible to extreme radiation doses, desiccation, thermal stress and excess light leading to membrane and protein degradation. Plants have developed various mechanisms in heat stress adaptation including induction of heat shock proteins (HSPs). Under elevated temperature, production of reactive oxygen species (ROS) also increase and plants must activate their antioxidant defense and protect themselves from heat stress. Here we explore from a molecular perspective the ability of *Thalassia hemprichii* to activate heat stress response mechanism. Expression of several antioxidant genes (Mn-Sod, Apx, and Cat) and some heat shock protein genes (Hsp70, Hsp81, and sHsp-cp) were observed. The qPCR approach was adapted to understanding the molecular mechanisms of physiological performance from gene expression studies. These finding reported that Mn-Sod, Apx, Cat, Hsp70 and Hsp81 genes were significantly overexpressed as a result of heat stress. However, sHsp-cp was down-regulated.

KEY WORDS: Antioxidant, Gene expression, Heat shock protein, Heat stress, ROS, Seagrass, *Thalassia hemprichii*.

INTRODUCTION

Global warming that occurred in the last few decades has not only affected the life of terrestrial organisms but also has an impact on coastal organisms (Rowan, 2004; Stillman, 2003). This condition not only causes a rise in the air temperature, but also the temperature of the seawater globally (Hanumappa and Nguyen, 2010). Low tide during the day in the shallow waters of the coastal ecosystem triggers heat stress (Anthony *et al.*, 2007). These heat stresses could be the condition of the exposure of the organism due to the tides, the solar radiation and the high temperature of seawater in the pool (Berkelmans, 2002; Rasheed and Unsworth, 2011).

Seagrass is one of the key factors in ecosystems that act as spawning, feeding, nursing, and sheltering various types of coastal organisms (Orth *et al.*, 2006). The presence of disorder in the form of biotic dams abiotic on the seagrass could have a direct impact on the various communities that inhabit the seagrass itself. Unfortunately, the seagrass ecosystem has been reported lost in some areas throughout the hemisphere (Waycott *et al.*, 2009). Collier and Waycott (2014) noted that high temperatures during low tide can lower the rate of growth and even induce seagrass mortality. Several seagrass species were also reported to have increased the percentage of mortality by the end of summer (Reusch *et al.*, 2005; Marba and Duarte, 2010). Orth *et al.* (2006) highlighted the critical need for a targeted global conservation effort to protect, monitor, and successfully manage seagrasses.

Molecular indicators provide the earliest possible evidence of stress and imminent mortality (Hoffmann and Daborn, 2007), far exceeding that of morphological and physiological indicators (Hasegawa *et al.*, 2000). To date, however, molecular indicators have not yet been used with seagrasses. For example, of the 49 seagrass indicators that have been used across 42 European monitoring programs, none was molecular (Marba *et al.*, 2013). This is not to say that molecular indicators have been 'off the radar' in the seagrass world, rather, molecular indicators have long been the 'holy grail' of seagrass molecular biologists (Macreadie *et al.*, 2014). Progress toward developing molecular indicators of heat stress in seagrasses has been remarkably rapid over the past 12 years. Arguably the first major development was by Ransbotyn and Reusch (2006), who performed real-time quantitative polymerase chain reaction (RT-qPCR) to identify housekeeping (control) genes and assess gene expression levels in the Northern Hemisphere seagrass species *Zostera marina* under heat stress and control conditions. Other researchers used antioxidants and heat shock proteins as molecular markers to determine the occurrence of stress on the seagrass. The superoxide dismutase (Sod) gene was reported to have significantly increased expression in *Zostera marina* treated with heat wave (26°C). Even the Sod gene experienced a significant increase in expression before the treatment reached the heating temperature (19°C; Winters *et al.*, 2011). Shinozaki *et al.* (2015) observed that SOD is the first enzyme in the ROS detoxification process (converting an O_2^- to H_2O_2) and by the enzyme catalase (CAT) and ascorbate peroxidase (APX) to convert H_2O_2 into H_2O molecules. The expression of the Hsp70,

Table 1. List of reference and genes of interest analyzed in *Thalassia hemprichii*

Gene name		Primers sequence 5'->3' (F/R)	Size	Tm (°C)	GC (%)
housekeeping **(reference)**					
Elf4A	F	ATTCAGCAGGCACAGTCTGG	20	60.60	55.00
	R	AGTACCAAAGCCTGGCACTC	20	60.50	55.00
Tbp	F	TACAGGCCCGTAATGCAGAG	20	60.50	55.00
	R	GCAGCAAGCTTCGAGTGTTC	20	60.50	55.00
Gapdh	F	AGGTTCTTCCTGCTTTGAATG	21	57.40	42.86
	R	CTTCCTTGATTGCTGCCTTG	20	58.40	50.00
18s	F	CGAGACCTCAGCCTGCTAAC	20	62.50	60.00
	R	AAGATTACCCAAGCCTGTCG	20	58.40	50.00
antioxidant (target)					
MnSod	F	ATGGGTGTGGCTTGCTTA	18	53.90	50.00
	R	ATGCATGCTCCCATACATCT	20	56.40	45.00
Apx	F	GCAGATTTAATTGCTGTTGCCG	22	60.30	45.45
	R	ACGGAAGCAACTCTTTAGGC	20	58.40	50.00
Cat	F	AGCGAGCTGCTCATTCTCTG	20	60.50	55.00
	R	AGACATGGCCGGAAGACATC	20	60.50	55.00
heat shock protein (target)					
Hsp70	F	AGGACCAGCGATCGGTATTG	20	60.50	55.00
	R	CCTCTCGGAGTCCGTGAAAG	20	62.50	60.00
Hsp81	F	TATCGCTGCTGGAGCTGATG	20	60.50	55.00
	R	GATCCACCAGCCTGTGACTC	20	62.50	60.00
sHsp-cp	F	CCTGCCTCATCGTCCTCATC	20	62.50	60.00
	R	CGCTCCTCCATCGACATCTC	20	62.50	60.00

Hsp90, sHsp gene experienced an increase in the expression of the high temperature stress in *Posidonia oceanica* (Marlin-Guirao *et al.*, 2016), Hsp70 and Hsp80 in *Zostera marina* (Bergmann *et al.* 2010) and Hsp70, Hsp80, and sHsp in *Zostera noltii* (Massa *et al.*, 2011). However, a study of the molecular response of seagrass under heat stress is currently only limited to seagrass that has habitat in temperate climates.

Thalassia hemprichii (Ehrenb.) Aschers. is seagrass which inhabited in tropic climate. It is most widely-distributed in the South East Asia water, especially in Indonesia water (Aswandy & Azkab 2000). It means that *T. hemprichii* may be used as an ideal model that could describe the ecological conditions of tropical coastal waters. Thus, it is necessary to conduct a study on the molecular response of *T. hempricii* as a tropical seagrass model under heat stress. The purposes of this study were to evaluate the expression of antioxidants and heat shock proteins genes of *T. hemprichii* under heat stress treatment. This study is concerning to genes expression of Mn-Sod, Apx, Cat, Hsp70, Hsp81, and sHsp-cp as molecular indicators which response to heat stress. We argued that molecular indicators for early detection of chronic stress in seagrasses will significantly improve the effectiveness of seagrass management and conservation efforts.

MATERIALS AND METHODS

Culture conditions and stress treatment

The experiment was conducted in hydroponic culture system under aquarium conditions at Laboratory of Plant Physiology, Department Biology Faculty of Sciences and Technology Airlangga University. Samples *Thalassia hemprichii* (10–5 cm from base to the tip of

leaf) were obtained from the north coast of Lamongan (6°52'40.8"S 112°12'50.5"E). The experimental design adopted from Purnama *et al.* (2015). A total of 10 samples *Thalassia hemprichii* were divided into 2 treatment groups: heat (treatment with thermal stress) and normal (control at ambient temperature). Before heat treatment, seagrass samples were acclimatized for one week in laboratory conditions (light intensity of 250-µmol m^{-2} s^{-1} at a temperature of 26 ± 2°C). The temperature in the heat stress treatment aquaria was gradually increased from 28°C to 41.5°C (increased 1.5°C per day over 8 days). The heat source was from the water heater connected to the digital Thermostat STC 100 which will maintain the temperature. Seagrass in aquaria without thermal stress kept with each set of an experiment as a control. The sampling of second youngest leaves was randomly picked from all group. Each group of approximately 5 leaf samples from 5 individual plant from both groups were collected, cleaned from epiphytes, and blotted using tissue paper. Samples were put in a tube and immediately frozen in liquid nitrogen for further analysis.

RNA extraction and cDNA synthesis

Approximately 100 mg leaf sample was used for RNA extraction. The RNA extraction was performed using RNAprep PureKit for Plant (Tiangen) according to manufacturer's protocol. The RNA quality was verified using 3 µL of RNA extraction on denaturing 1% agarose gels and quantification was performed with a spectrophotometer at 260 and 280 nm. Subsequently, RNA (5 ng) was reverse-transcribed in complementary DNA (cDNA) with the iScript using Eppendorf® mastercycler personal. TM cDNA Synthesis Kit (Bio-Rad).
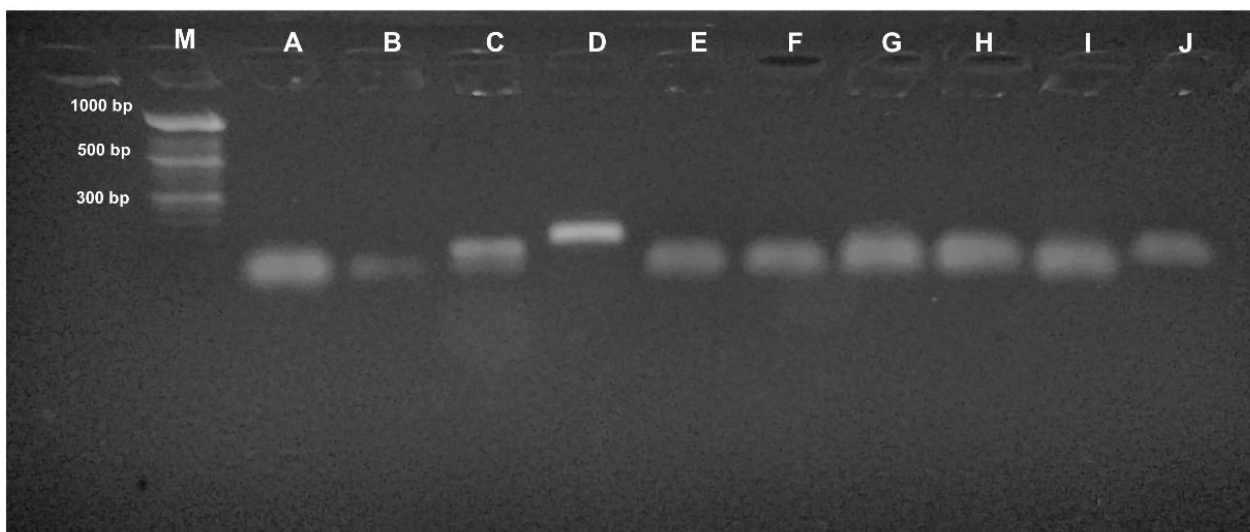
Fig. 1. Agarose gel electrophoresis analysis shows ten genes are well amplified. M: marker; **A**: Elf4A; **B**: Tbp; **C**: Gapdh; **D**:18s; **E**: Mn-Sod; **F**: Apx; **G**: Cat; **H**: Hsp70; **I**: Hsp81; **J**: sHsp-cp.

Primer design

Primers (Table 1) were designed using Primer3 (Rozen & Skaletsky, 2000). Selection conditions included: product size (100–200 bp), TM (57–60°C) and GC content (40–60%); these conditions facilitated cross-comparison of assays and assured similar PCR efficiencies (Ransbotyn and Reusch, 2006). At least one of the primers was placed across an exon-exon boundary to avoid genomic amplification Sequence alignments of the homologous sequences from both *Zostera marina* and *Posidonia oceanica* together were performed by CLUSTALW (EMBL-EBI) embedded in BioEdit. The only BLAST hits with p < 0.001 were considered.

Quantitative real-time PCR (qRT-PCR)

The mRNA expression level of *Thalassia hemprichii* on different treatment was performed on a Bio-Rad CFX96™ Real-Time PCR System (Bio-Rad) using the SsoFast™ Evagreen® Supermix (Bio-Rad). Reactions were run in duplicates using 5 biological replicates. Reactions (20 μl) included 10 μl SsoFast™ Evagreen® Supermix, 350 nM forward and reverse primers, 50 ng μl^{-1} cDNA template, and DEPC-treated water until the volume reaches 20 μl. Thermocycling conditions included: 3 min hot start at 95°C, 45 main cycles of 30 sec at 95°C, and 1 min at 58°C. To verify that the reaction yielded only a single product, a dissociation protocol (58°–95°C, increase 0.5°C each 5 sec) was followed using the above thermocycling profile (data not shown) and visualized by 1% agarose gels.

Initial work included screening potential of the best reference gene, performed by excel-base program NormFinder (Andersen *et al.*, 2004). Relative messenger RNA (mRNA) transcription levels were based on the ΔΔCt method (Livak & Schmittgen, 2001) using the

Bio-Rad CFX Manager. Primer efficiencies were assumed to E = 2 (%E = 100%).

RESULTS

The electrophoresis results showed that the ten primers used specifically amplified a specific region and produced a single product. Primer pairs were designed to amplify products with product sizes ranging from 100–200 bp so that the band position is still below the 500 bp marker (Fig. 1) According to NormFinder, Elf4A gene was the best gene that could be used as normalizator for target gene subjected to heat stress, with stability value 0.005 (see Fig. 2). As for the combination of two genes as normalizator were Elf4A and Tbp genes with a stability value of 0.014. In this study, the combination of Elf4A and Tbp gene expression regulation was used as a reference gene to normalize the target genes

As shown in Fig. 3, generally all genes are overexpressed due to heat stress except sHsp-cp. The relative expression of Mn-Sod was 3.02-fold, Apx was 4.93-fold and Cat was 1.72-fold. The relative expression Hsp70 was up-regulated by 1.71-fold and Hsp81 by 2.78-fold. While the sHsp-cp gene was down-regulated by -2.21-fold. Expression of Cat, Apx, and Hsp81 had a significant increase in regulation even at the level α: 0,01. While the expression of Mn-Sod and Hsp70 were recorded a significant increase in regulation at α: 0.05. Nevertheless, the declined sHsp-cp gene expression did not show a significant difference (P> 0.05).

DISCUSSION

The transcription of heat shock proteins (HSPs) under the control of heat stress transcription factors
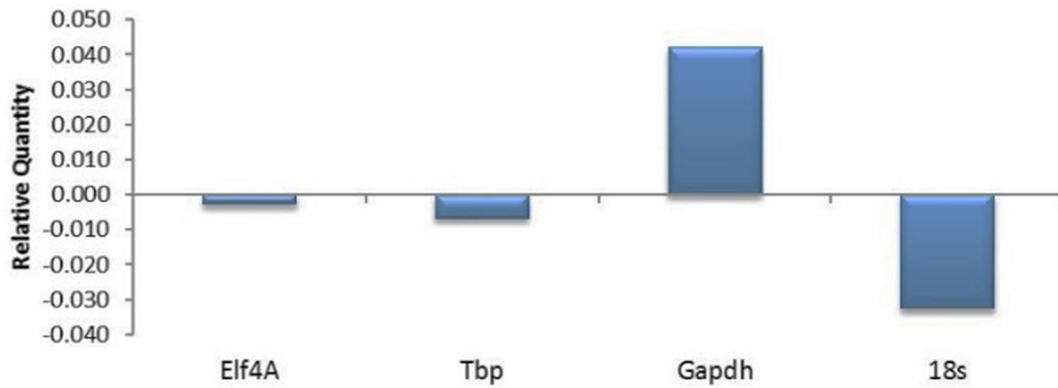
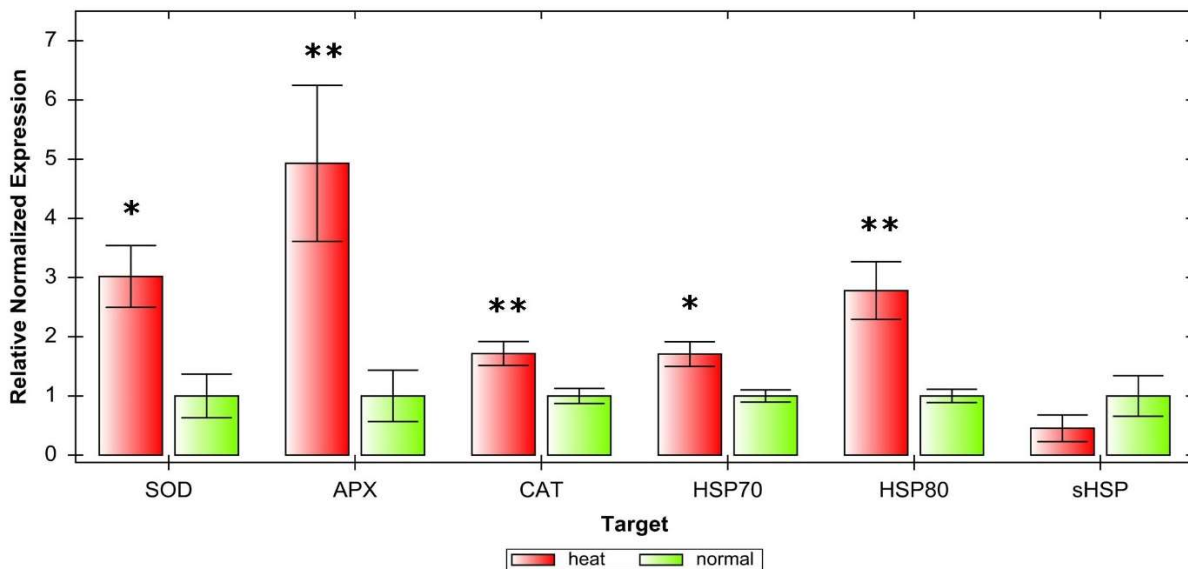**Fig. 2.** The relative quantity value of all four housekeeping genes from the analysis results using NormFinder.

Fig. 3 Relative expression of antioxidant and heat shock protein genes of ***Thalassia hemprichii*** in both thermal treatments. Each value is shown as Mean ± Standard Error. *: significant at α: 0.05; **: significant at α: 0.01.

(HSFs) plays a fundamental role in the heat stress response (HSR) and the acquired thermotolerance in plants. The HSP70 proteins are central components of the cellular network of molecular chaperones and folding catalysts. Overexpression of Hsp70 due to heat stress also reported by Sung *et al*. (2001) on *Arabidopsis thaliana*. In this study, expression of Hsp70 of *T. hemprichii* had increased by 2–20 folds after high-temperature treatment. The HSP70 family members have an important role in preventing aggregation and facilitating the process of refolding proteins during heat stress (Wang *et al*., 2004). The HSP70 assists protein-folding processes by a transient association of their substrate binding domain with short hydrophobic peptide segments within their substrate proteins. The substrate binding and release cycle are driven by the switching of HSP70 between the low-affinity adenosine triphosphate bound state and the high-affinity adenosine diphosphate bound state (Mayer and Bukau, 2005). Lee

and Schöffl (1996) suggested that plants with increased Hsp70 gene expression are positively correlated with its ability to tolerate increased temperature so that Hsp70 becomes a potential biomarker candidate for the presence of abiotic stress (Ireland *et al*., 2004).

A significant increase in expression also occurred in Hsp81 genes that is a member of the HSP90 protein family (Shanker and Venkateswarlu, 2011). The HSP90 protein, as well as HSP70, is an ATP-dependent molecule chaperone (Pearl and Prodromou, 2006). The main function of HSP90 is to regulate the key protein folding in signal-transduction, cell cycle control, protein degradation and protein traffic (Wang *et al*., 2004). HSP90 interacts only with the relatively well-folded proteins involved in transcription settings and signal transduction pathways. Furthermore, the HSP90 function requires the formation of complexes composed of several chaperones, including HSP70 and HSP40 proteins. This suggested close cooperation between

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chaperon molecules (Zhao and Houry, 2005). Queitsch *et al.* (2002) reported that HSP90 also played a role in morphological changes and adaptation of *Arabidopsis thalia*. Marlin-Guirao *et al.* (2016) reported that Hsp70 and sHsp were overexpressed in seagrass *Posidonia oceanica* because of stress. While Hsp90 was also found overexpressed by both *Cymodocea nodosa* and *Posedonia oceanica*. Similar findings also noted by Bergmann *et al.* (2010) that the Hsp80 gene was overexpressed in *Zostera marina* due to high-temperature exposure.

In contrast to the expression of Hsp70 and Hsp81, the expression of sHsp-cp had downregulation although not significantly. The chloroplast-localized small heat shock proteins (sHsp-cp; 21–30 kDa) plays a crucial role in thermolabile protein protection of PS II reaction centers (Wang and Luthe 2003). However, sHSP was unable to facilitate the protein folding process by itself but merged with other chaperone complexes (Wang *et al.*, 2004). Scarpeci *et al.* (2008) reported that Hsp70 and sHsp17.6B-CI together were expressed when increased temperature occurred. The decrease in sHsp-cp gene expression in this study was thought to be due to the already damaged photosynthetic apparatus so that the function of sHSP-cp to protect the photosynthetic apparatus was not applicable.

During stress, ROS is produced larger in organelles where electron transport occurs, such as in mitochondria, chloroplasts or peroxisome (Impa *et al.*, 2012). ROS plays a pivotal role in the response of various stresses (Torres and Dang, 2005). Differences in cellular ROS levels are considered as signals to induce expression of various gene networks in plants. The *Arabidopsis thaliana* plant has a network of at least 152 genes, in the form of ROS-scavenging and ROS-producing protein. Gene networks are interconnected allegedly and have different roles in controlling plant acclimatization and tolerance in stress condition (Mittler *et al.*, 2004). To suppress the accumulation of ROS in cells, plants developed an enzymatic antioxidant defense system (ROS-scavenging) including superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) (Du *et al.*, 2013).

Expression of manganese superoxide dismutase (Mn-Sod) gene was increased significantly due to high-temperature treatment in this study. The SOD enzyme is considered the first defense against the accumulation of ROS by converting superoxide anions (O_{2-}) to hydrogen peroxide (H_2O_2) and oxygen (Bowler *et al.*, 1992). The SOD-Mn enzyme was reported to play a central role in the response and tolerance of *Zostera marina* because of high temperatures (Winters *et al.*, 2011; Liu *et al.*, 2016). Tutar *et al.* (2017) also reported that SOD-Mn was an antioxidant enzyme that significantly increased in expression during high-temperature stresses on

Cymodocea nodosa and *Posidonia oceanica*. The enzyme SOD-Fe only increased on the seagrass species *Posidonia oceanica*. Furthermore, the expression of Sod gene had increased at a temperature of 10–15°C. However, when the temperature raised to 30°C, the expression of the Sod gene weakens significantly (Liu *et al.*, 2016). The Mn-SOD is essentials antioxidant enzyme that detoxifies the free radical superoxide, the major by-production mitochondrial respiration (Candas and Li, 2014). Hence, higher expression of Mn-Sod gene could indicate that high accumulation of ROS in mitochondria.

Expression of Apx gene on *Thalassia hemprichii* was significantly increased. Expression of Apx gene tended to be the highest over other antioxidant expressions (Mn-Sod and Cat). APX enzyme converts peroxide (H_2O_2) to H_2O by oxidizing ascorbate to monodehydroascorbate (MDA). Furthermore, with the presence of NAD(P)H, monodehydroascorbate (MDA) reductase converts MDA into ascorbate (Apel and Hirt 2004). Peroxides are produced by either chloroplast, mitochondrial or peroxisomal organelles during high-temperature stresses. Peroxide compounds are also produced by the conversion of superoxide anion (O_{2-}) by SOD enzyme (Bowler *et al.*, 1992). The high expression of Apx gene also caused by HsfA4a transcription factor acted as the presence of H_2O_2 molecules (Miller and Mittler 2006). The promoter region of the Apx gene has a short segment called heat shock element (HSE). The HSE has a nGAAnnTCCn sequence. The sequence could be induced by either H_2O_2 and Hsf in order for the Apx gene to be transcribed (Rizhsky *et al.*, 2004).

The CAT enzyme has a similar role to APX for eliminating the ROS as H_2O_2 into H_2O and O_2 in the cell. CAT converts molecules into H_2O and O_2 molecules (Hajiboland, 2012). Peroxides are produced in peroxisome during photorespiration process as the reaction to convert Glycolate into Glyoxylate (Heldt, 2005). It seemed that the expression of CAT illustrates the abundance H_2O_2 in the peroxisome. Expression of Cat gene in *Thalassia hemprichii* also showed an increase in regulation due to high-temperature treatment. Liu *et al.* (2016) reported the opposite condition, the expression of Cat genes was decreased in *Cymodocea nodosa*. While in another seagrass species, *Posidonia oceanica*, there was no change of expression of Cat genes due to high-temperature treatment.

CONCLUSION

These findings highlight the molecular responses of *Thalassia hemprichii* against heat stress. In facts, response and survival of heat stress are complex phenomena in seagrass. Despite recent advances in our understanding of the molecular mechanisms involved in

heat stress sensing in plants, many questions remain unanswered. Further, these genes could be assessed to other different stresses such as desiccation, exceed light moreover combination stress. However, mRNA abundance and protein levels are not always correlated, especially for low copy numbers of mRNAs (potentially very important for regulation). To better understand the process of stress tolerance and to develop strategies to improve stress resistance, the genomic approaches need to be complemented by analyses of the plant protein by performing Western Blot.

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