



# Calcium-Calmodulin-Involved Heat Shock Response of *Neoporphyra haitanensis*

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Increasing global temperatures have seriously affected the sustainable development of *Neoporphyra haitanensis* cultivation. Although several pathways are reported to be involved in the response of *N. haitanensis* to heat stress, it is unknown which ones are activated by signal transduction. Previously, we detected a large influx of calcium ions ( $\text{Ca}^{2+}$ ) in *N. haitanensis* under heat stress. In this study, we further investigated the specificity of  $\text{Ca}^{2+}$  signaling and how is it transduced. Transmission electron microscopy and Fv/Fm analyses showed that the  $\text{Ca}^{2+}$  signal derived from extracellular  $\text{Ca}^{2+}$  formed at the early stage of the response to heat stress, and the signal was recognized and decoded by *N. haitanensis* calmodulin (*NhCaM*). In yeast two-hybrid assays, DnaJ, a voltage-dependent anion channel, and a bromodomain-containing protein interacted with PhCaM1 *in vivo*. The transcript levels of the genes encoding these proteins increased significantly in response to heat stress, but decreased upon inhibition of NhCaM1, indicating that these interacting factors were positively related to NhCaM1. Additionally, a comparative transcriptome analysis indicated that  $\text{Ca}^{2+}$  signal transduction is involved in phosphatidylinositol, photosystem processes, and energy metabolism in *N. haitanensis* under heat stress. Our results suggest that  $\text{Ca}^{2+}$ -CaM plays important roles in signal transduction in response to heat stress in *N. haitanensis*.

**Keywords:** calcium signal, calmodulin, heat stress, *Neoporphyra haitanensis*, yeast two-hybrid

## INTRODUCTION

Global climate change-driven increases in seawater temperature seriously affect the sustainable development of *Neoporphyra/Neoporphyra*, which has the highest commercial value per unit mass (approximately \$523 per wet metric ton) among all cultivated seaweed species (FAO, 2017). *Neoporphyra haitanensis*, a typical warm temperate zone species originally found in Fujian Province, is widely cultivated along the coast of South China. The output of *N. haitanensis* reached 150,000 tons (dry weight) in 2020 in China (China Fishery Bureau, 2021). Due to importantly economic and ecologic value, the market for this commodity is likely to increase dramatically in the future. However, *N. haitanensis* farms are often affected by sustained high temperatures that are induced by the ‘temperature rebound’ in autumn (Xu et al., 2013; Wang et al.,

2018; Zheng et al., 2020), especially in 2021 (unpublished). Persistent high temperatures make thalli susceptible to disease, premature aging, and eventual decay, leading to a significant drop in yield (Yan et al., 2009). Therefore, studying the molecular mechanisms of the response to heat stress and cultivating new heat-tolerance varieties are necessary.

Previous studies have indicated that the thalli of *N. haitanensis* resist high temperatures by inhibiting photosynthesis, protein synthesis, and energy metabolism to reduce unnecessary energy consumption, which in turn facilitates the rapid establishment of acclimatory homeostasis (Xu et al., 2013; Shi et al., 2017; Wang et al., 2018; Chang et al., 2021). Accordingly, the response to heat stress in *N. haitanensis* involves multiple stress resistance pathways. However, little is known about how these resistance pathways are activated. The calcium ion (Ca<sup>2+</sup>) is an astonishingly versatile signaling molecule that is poised at the core of a sophisticated network of signaling pathways (Dodd et al., 2010). Transient, sustained, or oscillatory rises in the cytosolic Ca<sup>2+</sup> concentration serve as a second messenger (McAinsh and Pittman, 2009; Thor, 2019). Our previous study found that heat stress significantly increased Ca<sup>2+</sup> influx, while the expression level of heat shock proteins (HSPs) genes (*HSP22* and *HSP70*) showed downregulated after the Ca<sup>2+</sup> influx was inhibited by verapamil, indicating that the Ca<sup>2+</sup> signal generated by the plasma membrane Ca<sup>2+</sup> channel is crucial for *N. haitanensis* responses to heat stress (Zheng et al., 2020). However, the temporal specificity of calcium signaling is unclear in *N. haitanensis* under heat stress.

Furthermore, the information encoded within these transient changes in Ca<sup>2+</sup> contents is decoded and transmitted by a toolkit of Ca<sup>2+</sup>-binding proteins that regulate transcription *via* Ca<sup>2+</sup>-responsive promoter elements and regulate proteins *via* phosphorylation (Dodd et al., 2010). Calmodulins (CaMs) is a universal Ca<sup>2+</sup> sensor among eukaryotes (Rhoads and Friedberg, 1997). As a downstream target protein of Ca<sup>2+</sup>, CaM acts as an intermediary in Ca<sup>2+</sup>-mediated reactions, and regulates physiological and biochemical processes in cells by binding to intracellular enzymes or proteins. In this way, CaM plays an important role in cell proliferation, differentiation, and apoptosis, and participates in signal transduction of abiotic stress stimuli (Pawar et al., 2008). Our previous study suggested that CaM could act as a Ca<sup>2+</sup> signal decoding protein in *N. haitanensis* in response to heat stress (Zheng et al., 2020). However, CaM has neither transcription factor nor enzyme activity, nor does it serve as a structural protein. Instead, it is the downstream CaM-binding proteins that regulate physiological functions. Therefore, identifying the proteins that interact with CaM is an important way to reveal the physiological function of CaM, and is the key to deciphering the Ca<sup>2+</sup>-CaM signal transduction system in *N. haitanensis* under heat stress.

Therefore, the aim of this study was to 1) investigating the temporal specificity of the Ca<sup>2+</sup> signaling response to heat stress by comparing the quantum yield (Fv/Fm) of thalli in seawater with that of thalli in Ca<sup>2+</sup>-free seawater, and by observing the distribution of Ca<sup>2+</sup> by scanning electron microscopy; 2) identifying proteins that interact with NhCaM by yeast two-

hybrid analyses; 3) determining which pathways were activated downstream of Ca<sup>2+</sup> signaling under heat stress through transcriptome analysis. The present results provide new insights into the signal transduction mechanism of *N. haitanensis* under heat stress.

## MATERIALS AND METHODS

### Growth Conditions and Treatments

The heat-resistant strain Z-61 of *N. haitanensis* (Chen et al., 2008) was selected and purified by the Laboratory of Germplasm Improvements and Applications of *Neoporphyra* at Jimei University, Fujian, China. For the control, Z-61 thalli were cultured in natural seawater supplemented with Provasoli's enriched seawater medium at 21°C under a 10-h light/14-h dark photoperiod [50-60 μmol (m<sup>-2</sup> s<sup>-1</sup>)]. The growth medium was refreshed every 2 days.

The seawater temperature exceeds 30°C during 'temperature rebound' in autumn, the season of the cultivation of *N. haitanensis*. Thus, in order to screening heat treatment condition, the Fv/Fm of the thallus was detected at six temperatures (30°C, 32°C, 34°C, 36°C, 38°C, 40°C). The results showed that Fv/Fm of the thalli had no change at 30°C for 48 h, while it decreased significantly at 1 h of 34°C - 40°C. However, Fv/Fm of thalli at 32°C showed no significant change within 12 h, but decreased rapidly after 12 h (**Figure S1**). Therefore, we selected 32°C as heat stress condition to investigate signal transduction that usually occurred at the early stage of stress. Healthy thalli with a length of (15 ± 1) cm were selected for heat treatment (32°C), and each group had three biological replicates. The composition of artificial seawater, in g/L, was as follows: NaCl 26.73, MgCl 2.26, MgSO<sub>4</sub> 3.25, CaCl<sub>2</sub> 1.15, NaHCO<sub>3</sub> 0.2, KCl 0.72, NaBr 0.058, H<sub>3</sub>BO<sub>3</sub> 0.058, Na<sub>2</sub>SiO<sub>3</sub> 0.0035, KAl (SO<sub>4</sub>)<sub>2</sub>·12H<sub>2</sub>O 0.023, LiNO<sub>3</sub> 0.0013, NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> 0.0040. For Ca<sup>2+</sup>-free seawater, CaCl<sub>2</sub> was omitted.

### Chlorophyll Fluorescence Analysis

A Diving Pulse Amplitude Modulation fluorometer (Diving-PAM, Walz, Effeltrich, Germany) was used to measure the ratio of variable chlorophyll fluorescence to maximal chlorophyll fluorescence (Fv/Fm). Thalli were acclimated in darkness for 10 min, and then chlorophyll fluorescence was measured using a slightly modified version of a previously published method (Yu and Guy, 2004).

### Transmission Electron Microscope Analyses

The method of transmission electron microscope analyses was a slight modification on the basis of publication by Ouyang et al. (2011). A 1 cm<sup>2</sup> piece blade was cut from the middle of the thallus and fixed for 6–10 h in 2.5% glutaraldehyde + 2% paraformaldehyde fixative dissolved in 0.2% potassium pyroantimonate buffer (pH 7.6). The sample was washed three times with 2% potassium pyroantimonate phosphate buffer for 20 min each time. Then, the sample was fixed in 1% osmium and

2% potassium pyroantimonate phosphate buffer at room temperature in the dark for 7 h. The sample was washed three times with 2% potassium pyroantimonate phosphate buffer solution for 15 min each time, and then washed three times with 0.1 M phosphate buffer solution for 15 min each time. Dehydration, infiltration, embedding, and slicing were carried out using established methods.

### Isolation of Full-Length *NhCaM1* cDNA, and Sequence Analysis

Through searches of sequences at the NCBI database, *NhCaM1* was screened out from the transcriptome of *N. haitanensis*. The full-length *NhCaM1* sequence was cloned by 5'-rapid amplification of cDNA ends (5'-RACE) using the SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA), according to the manufacturer's instructions. Multiple amino acid alignments were performed using Clustal X, and phylogenetic trees were constructed by the maximum likelihood method using MEGA6.0 software (Tamura et al., 2013).

### Quantitative Real-Time-PCR

Purified RNA (1 μg) was used as the template for synthesizing cDNA with the PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). The cDNA solutions were diluted to 5 ng μL<sup>-1</sup> with nuclease-free water for subsequent quantitative real-time (qRT)-PCR assays using the Step One Plus real-time PCR system (Applied Biosystems, Foster City, CA, USA) using the SYBR Premix Ex Taq kit (Takara). The gene encoding ubiquitin conjugating enzyme (*UBC*) was used as an internal control (Li et al., 2014). The PCR procedure was that recommended by the manufacturer. The primers used are listed in **Supplementary Table S1**.

### Yeast Two-Hybrid Assay

The yeast two-hybrid assay was performed using the Clontech Yeast Two-Hybrid System (pGBKT7 and pGADT7). The full-length *NhCaM1* sequence was cloned into pGBKT7. The negative controls were the pGBKT7-LAM control vector and the pGADT7-T control vector. The pGBKT7-53 control vector and pGADT7-T control vector were used as positive controls for self-activation and toxicity tests of decoy genes. The positive blue clones on SD/-Trp/-Leu/X-α-Gal/AbA plates were transferred to SD/-Trp/-Leu/-His/-Ade/X-α-Gal/AbA medium for rigorous screening. Blue positive clones were selected for one-to-one verification. The transcript levels of the interacting genes were detected under heat stress (32°C) and after treatment with the CaM inhibitor trifluoperazine (TFP) at 8 M.

### Transcriptome Analysis

*N. haitanensis* thalli were incubated with 100 μM verapamil, an inhibitor of Ca<sup>2+</sup> channels, for 2 h before heat treatment (32°C). After 15 min of heat treatment, total RNA was extracted using the E.Z.N.A. plant RNA Kit (OMEGA Bio-Tek, Norcross, GA, USA). Seaweeds cultured in normal seawater served as the CK. Thalli were also cultured in Ca<sup>2+</sup>-free seawater under heat stress for 15 min and 3 h. Three biological samples were collected for each experimental group. *De novo* transcriptome assembly and annotation were

completed by GENE DENOVO Biotechnology Co. Ltd. (Guangzhou, China). The transcript level of genes was calculated based on reads per kb per million reads (RPKM) values. Differentially expressed genes were identified by comparing RNA-seq data between groups and between samples using DESeq2 and edgeR software (Robinson et al., 2010; Love et al., 2014), respectively. Genes showing two-fold changes were scored as differentially expressed on the basis of a false-discovery rate of 0.05. BlastX was used to compare unigene sequences with those in protein databases NR, SwissProt, KEGG, and COG/KOG (e value < 0.00001), and functional annotation information for the most similar proteins was assigned to unigenes.

### Statistical Analyses

Data were subjected to one-way analysis of variance. Differences were considered significant at  $P \leq 0.05$ . Unless otherwise indicated, all reported *P*-values refer to the results of one-way ANOVA followed by least significant difference (LSD) tests.

## RESULTS

### Fv/Fm of *N. haitanensis* Thalli Supplemented With Ca<sup>2+</sup>

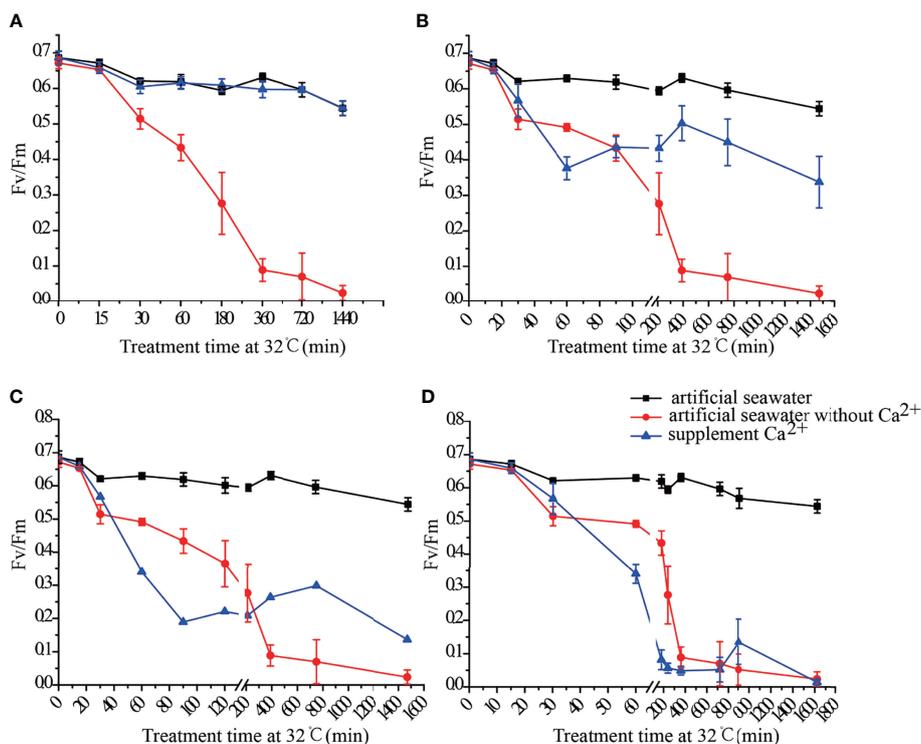
When Ca<sup>2+</sup> was added into artificial seawater without Ca<sup>2+</sup> at 15 min of the heat treatment, the thalli Fv/Fm values were restored to the normal level (**Figure 1A**). When Ca<sup>2+</sup> was added into artificial seawater without Ca<sup>2+</sup> at 30 min and 1 h of the heat treatment, the Fv/Fm values of the thalli recovered somewhat, but did not return to the normal levels (**Figures 1B, C**). The addition of Ca<sup>2+</sup> into artificial seawater without Ca<sup>2+</sup> at 3 h of the heat treatment did not improve the heat resistance of thalli, and the Fv/Fm value decreased to 0.1 (**Figure 1D**).

### Physiological Phenotypes of *N. haitanensis* Thalli in Ca<sup>2+</sup>-Free Seawater

Under heat stress, the growth of thalli stagnated, and those in the Ca<sup>2+</sup>-free seawater began to bleach and decay. This was indicative of severe damage to pigments and photosystems. Thalli in the Ca<sup>2+</sup>-free seawater became transparent by 36 h of heat stress. Lesions did not appear until 24 h of heat treatment in artificial seawater (**Figures S2A–E**). After disinfection, thalli were cultured in natural seawater, artificial seawater, or Ca<sup>2+</sup>-free seawater at 22°C. Within 12 h, there was no difference in Fv/Fm between thalli cultured in natural seawater and those cultured in artificial seawater, while the Fv/Fm values were decreased slightly in thalli cultured in Ca<sup>2+</sup>-free seawater (**Figure S2F**). Under heat stress (32°C), the thalli cultured in both natural seawater and artificial seawater showed slightly decreased Fv/Fm values, while those cultured in Ca<sup>2+</sup>-free seawater showed a sharp decrease in Fv/Fm, dropping to less than 0.1 after 12 h. The thalli were on the point of death at 12 h of heat treatment in Ca<sup>2+</sup>-free seawater (**Figure S2F**).

### Distribution of Ca<sup>2+</sup> in *N. haitanensis* Thalli as Determined by Electron Microscopy

At 30 min of heat stress, a large amount of Ca<sup>2+</sup> was present in the cell wall, cell membrane, and cell interior (**Figures 2A, D**).



**FIGURE 1** | Fv/Fm of *N. haitanensis* thalli after adding Ca<sup>2+</sup> at 15 min (A), 30 min (B), 1 h (C) and 3 h (D) of heat treatment.

At 6 h of heat stress, no Ca<sup>2+</sup> was present in the cell wall or cell membrane (Figures 2E, F). In the thalli cultured in Ca<sup>2+</sup>-free seawater, fewer Ca<sup>2+</sup> aggregates were present in the cell wall and cell membrane, but Ca<sup>2+</sup> was distributed inside the cell (Figures 2G, H).

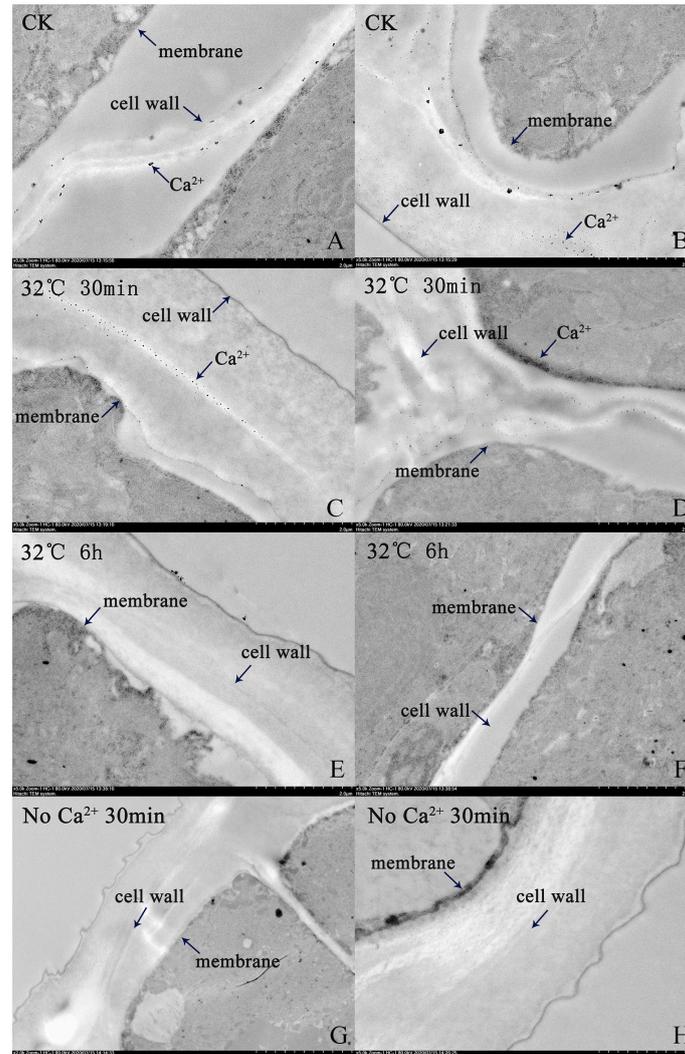
### Sequence Analysis of *NhCaM1*

The transcript levels of genes encoding CaM1 and CaM2 were detected in thalli cultured in seawater and Ca<sup>2+</sup>-free seawater under heat stress (32°C) for 15 min, 30 min, 45 min, 1 h, 3 h, 6 h, 12 h, 18 h, and 24 h. Under heat stress, the transcript levels of genes encoding CaM1 and CaM2 were up-regulated, and CaM1 was up-regulated earlier than CaM2. In the thalli cultured in Ca<sup>2+</sup>-free seawater, the expression of these genes was inhibited to varying degrees (Figure 4). To explore the regulation of *NhCaM1* during the response of *N. haitanensis* to heat stress, we isolated a 450-bp sequence of *NhCaM1* (Figure S3). A BLAST analysis revealed that the protein encoded by this gene contained four 'EF-hand' domains, which are characteristic of CaM proteins in other species and are involved in binding Ca<sup>2+</sup>. On the basis of these findings, we speculated that this gene encodes CaM in *N. haitanensis*, so it was designated as *NhCaM1*. This sequence has been submitted to the GenBank database under the accession number KU179277. Analyses using ExPASy ProtParam indicated that *NhCaM1* encodes a protein consisting of 149 amino acids with the formula C<sub>723</sub>H<sub>1138</sub>N<sub>184</sub>O<sub>253</sub>S<sub>11</sub>, a theoretical molecular weight of 16.8 kDa, and a theoretical isoelectric point 4.09. The protein was predicted to have 14

positively charged amino acid residues (Arg and Lys), 38 negatively charged amino acid residues (Asp and Glu), an instability coefficient of 36.43, and a GRAVY score of 0.543. The results showed that *NhCaM1* is stable and hydrophilic. Swiss-Model was used to predict the third-order spatial structure of *NhCaM1*. No signal peptides or transmembrane domain sequences were found by PrediSi, but three serine and three threonine residues were identified as potential phosphorylation sites by TMHMM.NetPhos2.0. Additionally, the expression of *NhCaM1* and *NhCaM2* showed down-regulation in Ca<sup>2+</sup>-free seawater under heat stress (Figure 3).

### Screening for *PhCaM1*-Interacting Proteins

The full-length ORF of *PhCaM1* was cloned into the pGBKT7 bait vector to screen the *P. haitanensis* Y2H cDNA library under heat stress conditions. Before screening, the autoactivation and toxicity of the clone were tested in yeast. The yeast cells harboring the *PhCaM1* clone grew slightly and remained blue on SD/-Leu/-Trp/-his/x- $\alpha$ -gal medium, but not on SD/-Leu/-Trp/-His/-Ade/X- $\alpha$ -gal/AbA medium, confirming that pGBKT7-CaM1 had no autoactivation in these Y2H Gold yeast strains. 30 positive clones were obtained by screening the library three times, and 25 clones were successfully sequenced after removing failures of PCR amplification and sequencing. 7 sequences of these 25 sequences can be annotated. Thus, these annotated 7 sequences were selected for one-to-one validation. After BLAST comparisons at the NCBI, three had annotations:



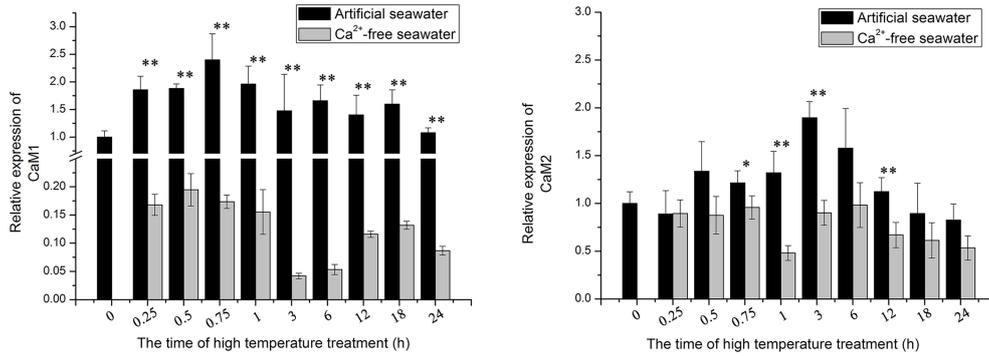
**FIGURE 2** | Transmission electron micrographs showing distribution of Ca<sup>2+</sup> in *N. haitanensis* thalli under heat stress. (A, B) Control group. (C, D) Heat treatment for 30 min. (E, F) Heat treatment for 6 h. (G, H) Heat treatment for 30 min in Ca<sup>2+</sup>-free seawater.

DnaJ (also referred to as HSP40), the voltage-dependent anion-selective channel (VDAC), and a bromodomain-containing protein (BCP) (Figure 4A). Additionally, we detected up-regulation of DnaJ, VDAC, and BCP under heat stress, especially at the early stage of the response. Treatment with TFP resulted in down-regulation of all three genes, as did culturing of thalli in Ca<sup>2+</sup>-free seawater (Figure 4B).

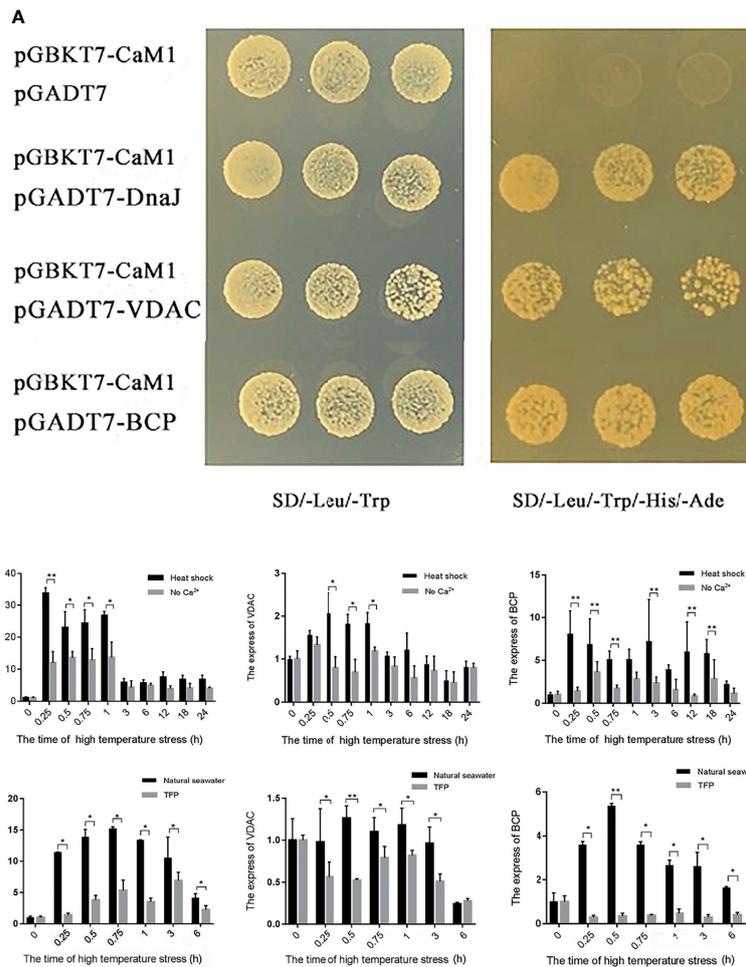
### Differentially Expressed Genes (DEGs) Analysis

At 15 min of heat treatment, 1,747 genes were up-regulated and 2,273 genes were down-regulated, while at 15 min after verapamil treatment, 3,904 genes were up-regulated and 5,021 genes were down-regulated (Figure S4). Further analysis showed that three CaM genes were down-regulated by inhibition of Ca<sup>2+</sup> channels by verapamil, while one gene was up-regulated under

heat stress (Figure 5). The transcript levels of 24 of 34 DEGs encoding HSPs, including five HSP40 (DnaJ) genes, six HSP20 genes, four HSP90 genes, nine HSP70 genes, were also up-regulated in thalli at 15 min of heat treatment, while the expressions of these genes were inhibited by verapamil. Whereas, the increases in the transcript levels of three HSP20 genes, two HSP 70, two HSP40, and one HSP90 gene were higher after inhibition of Ca<sup>2+</sup> influx than in the heat treatment. Transcripts of key genes related to phosphatidylinositol signaling, mainly including three phosphoinositide 4-kinase (PI4K) genes, two phospholipase C (PLC) genes, two phosphatidylinositol-4-phosphate 5-kinase (PIP5K) genes, one phosphoinositide 3-kinase (PI3K) gene, were not detected or down-regulated at 15 min of heat treatment, but they were significantly up-regulated in thalli treated with verapamil. Compared to 15 min heat treatment, the transcript levels of 26



**FIGURE 3** | Transcript levels of *NhCaM* in *N. haitanensis* at different times under heat stress. \* and \*\* represent significant difference ( $P < 0.05$ ) and extremely significant difference ( $P < 0.01$ ) between the same time point in different treatment groups, respectively.



**FIGURE 4** | Screening for *NhCaM1* interaction partners by yeast two-hybrid analysis, and gene transcript levels in thalli of *N. haitanensis* under different treatments. **(A)** Screening for proteins that interact with *NhCaM1* in *N. haitanensis* under heat stress by yeast two-hybrid analysis; **(B)** Transcript levels of genes encoding proteins that interact with *NhCaM1* in *N. haitanensis* thalli under different treatments. TFP: trifluoperazine, a CaM inhibitor. \* and \*\* represent significant difference ( $P < 0.05$ ) and extremely significant difference ( $P < 0.01$ ) between the same time point in different treatment groups, respectively.



several minutes to dozens of minutes, while that caused by hypoxia stress lasts for several hours. For *N. haitanensis*, supplementation with Ca<sup>2+</sup> at the early stage (15 min) of heat stress was able to restore the normal growth of thalli, but the heat tolerance of thalli could not recover when supplementation with Ca<sup>2+</sup> after 30 min of heat stress. Therefore, Ca<sup>2+</sup> signaling plays a role in the early response to heat stress in *N. haitanensis*.

## Identification of CaM-Interacting Proteins Reveals Potentially Pathways Regulated by Ca<sup>2+</sup> Signaling

Sensing of transient (Ca<sup>2+</sup>)<sub>cyt</sub> increase required for downstream stress-responses is mediated by Ca<sup>2+</sup>-binding sensor proteins. Our previous study revealed that CaM, a small acidic protein represents a universal Ca<sup>2+</sup> sensor across eukaryotes (Reddy et al., 2002), are able to decipher Ca<sup>2+</sup> signaling and then activated resistant genes in *N. haitanensis* under heat stress (Zheng et al., 2020). However, CaM has neither transcription factor nor enzyme activity, nor does it serve as a structural protein. It regulates physiological functions *via* downstream CaM-binding proteins. Therefore, the present study identified the proteins that interact with CaM to investigate how CaM regulates thermotolerance of *N. haitanensis*. The result of 'one to one' verification by yeast two-hybrid assay showed that the CaM of *N. haitanensis* interacts with DnaJ, VDAC, and BDC proteins in response to heat stress (Figure 4).

One adverse impact of high temperatures on cells is the production and accumulation of denatured or misfolded proteins. HSPs can facilitate protein folding and stabilize partially unfolded proteins. The most conserved HSPs are molecular chaperones that prevent the formation of non-specific protein aggregates and help proteins to acquire and retain their native structures (Richter et al., 2010). HSPs has been demonstrated that contribute to the tolerance of *N. haitanensis* to various abiotic stresses, including heat stress (Wang et al., 2018), hypersaline stress (Wang et al., 2019; Wen et al., 2020), and hyposaline stress (Wang et al., 2020). As a molecular chaperone widely found in plant cells, DnaJ is a member of the HSP40 family and functions as a molecular chaperone to maintain intracellular protein homeostasis (Hennessy et al., 2005). The transcript level of gene encoding HSP40 were found to be significantly up-regulated in response to heat stress in *N. haitanensis*, but either CaM inhibitors or the absence of Ca<sup>2+</sup> inhibited the expression of genes encoding HSPs, and this reduced the heat resistance of *N. haitanensis* (Figures 4, 5). This suggested that *NhCaM1* is functional upstream of the induction of HSPs, and can bind HSP40 to regulate the response of thalli to heat stress, which is consistent with previous study. Such as, the expression of *OsCaM1-1* and *OsHSP17* reached their maximal expression levels rapidly within 30 min heat shock treatment in rice (*Oryza sativa* L). Meanwhile, Over-expression of *OsCaM1* induced the expression of *AtHSP* at a non-inducing temperature and enhanced intrinsic heat tolerance in Arabidopsis (Wu et al., 2012). In transgenic Arabidopsis plants, *UidA* reporter gene expression directed by *AtHSP18.2* promoter was affected by Ca<sup>2+</sup> and CaM inhibitors (Liu et al., 2005). Ca<sup>2+</sup>-CaM

regulates gene expression *via* mediating the phosphorylation status of heat shock factors, which in turn modulates the expression of HSPs genes for heat shock signal transduction (Liu et al., 2007; Liu et al., 2008; Wu et al., 2012). Therefore, Ca<sup>2+</sup>-CaM signal transduction system is crucial for activation of HSPs to maintain protein homeostasis during the response of *N. haitanensis* to heat stress.

Several Ca<sup>2+</sup> transport systems across the endothelium control the concentration of Ca<sup>2+</sup> in the cytoplasm or mitochondria (Ginzel et al., 2001). VDAC are highly conserved proteins that exist in all eukaryotes and are the main protein components of the mitochondrial outer membrane (Colombini, 2012). As core members of mitochondrial metabolism and ion signaling, VDACS are involved in the exchange of compounds between the cytoplasm and the membrane, including ions such as Ca<sup>2+</sup>, metabolites such as succinate or ATP, and larger molecules such as tRNA or DNA that regulate the exchange of substances in response to cellular signals and stress (Hombler et al., 2012). Ca<sup>2+</sup> can pass through the mitochondrial outer membrane *via* VDACS, thus confirming their role in regulating Ca<sup>2+</sup> homeostasis (Ginzel et al., 2001). A previous study found that a VDAC of *Arabidopsis thaliana* interacts with the Ca<sup>2+</sup> sensor calcineurin-B-like proteins 1 under cold stress (Li et al., 2013). In the present study, we found that the transcript level of VDAC, interacting with *NhCaM1* in *N. haitanensis*, was significantly inhibited by inhibition of CaM under heat stress. It is possible that CaM regulates the transport of substances into and out of mitochondria through VDACS in *N. haitanensis* under heat stress.

As members of bromodomain and extra-terminal motif protein family, BCPs mediates a wide range of biological processes (Fujisawa and Filippakopoulos, 2017; Ren et al., 2017). Such as, many modifications (phosphorylation, acetylation, ubiquitination) and transcriptional co-activation are involved in the regulation of transcription by polyprotein complexes containing BCPs (Denis, 2001a). BCPs affect histone and chromatin organization *via* interactions between the bromodomain and acetylated lysine residues. Lysine acetylation is a histone modification that forms a stable epigenetic mark on chromatin for BCPs to dock and in turn modulates gene transcription (Denis, 2001b; Hassan et al., 2007). Under heat stress, the interaction between *NhCaM1* with BCP might ensure the diversity, speed, and flexibility of transcriptional responses so that the thalli of *N. haitanensis* can actively respond to heat stress. However, the type of BCP and how it interacts with acetylated histidine residues in *N. haitanensis* are yet to be verified.

## Downstream Response Pathways Regulated by Ca<sup>2+</sup> Signaling Under Heat Stress in *N. haitanensis* Phosphatidylinositol Signals in Response to Ca<sup>2+</sup> Signaling

The phosphatidylinositol (PI) signaling system is significant for cells to response to different biotic and abiotic stresses, and interaction with Ca<sup>2+</sup> signaling network (Berridge et al., 1998;

Lin et al., 2004). The present result showed that under heat stress, the expression of PLC, PI4K, and PI5K exhibited up-regulation after inhibition of Ca<sup>2+</sup> channels (**Figure 5**). PI can be phosphorylated at 4' position of the inositol ring by PI4Ks, which resulting in production of two second messenger precursors (phosphatidylinositol 4-phosphate (PI4P)) and phosphatidylinositol-4,5-bisphosphate (PI<sub>(4,5)</sub>P<sub>2</sub>) (Xu et al., 1992). Nilius et al. (2006) revealed that PI<sub>(4,5)</sub>P<sub>2</sub> strikingly improved activity of TRPM4, that depolarizes the plasma membrane and regulates Ca<sup>2+</sup> influx, by increasing the channel's Ca<sup>2+</sup> sensitivity. The loss of *OsPLC4* compromised the increase of inositol triphosphate and free cytoplasmic Ca<sup>2+</sup> in *Oryza sativa cv*, and inhibited the induction of genes involved in Ca<sup>2+</sup> sensor and salt stress (Deng et al., 2019). Similarly, *OsPLC1* preferred to hydrolyze PI4P and elicited salt stress-induced Ca<sup>2+</sup> signals (Li et al., 2017). Additionally, PI signaling can also regulate HSPs activity under abiotic stresses. It has been demonstrated that accumulation of the small heat shock proteins (HSP18.2 and HSP25.3) was downregulated in a PLC mutant line of Arabidopsis (*atplc9*) and was upregulated in the overexpressed lines after heat shock (Zheng et al., 2012). This might interpret why the transcripts of few HSPs genes were up-regulated after the inhibition of Ca<sup>2+</sup> signaling. Thus, it has been speculated that in the absence of extracellular Ca<sup>2+</sup> influx signals, *N. haitanensis* activated PI signaling earlier to regulate the response to heat stress through increasing the concentration of intracellular Ca<sup>2+</sup> or other ways.

### Changes in Photosynthesis in Response to Ca<sup>2+</sup> Signaling

*N. haitanensis* captures light via phycoerythrin (PE), phycocyanin, and allophycocyanin. The up-regulated expression of a PE precursor of *N. haitanensis* was detected after 15 min of heat stress, while they were down-regulated after Ca<sup>2+</sup> influx was inhibited. It suggested that increased synthesis of PE was regulated by the extracellular Ca<sup>2+</sup> signal, which helped the thalli capture light and positively respond to heat stress. Additionally, Ca<sup>2+</sup> can help protect the photosynthetic system from photoinhibition under heat stress by accelerating the repair of subunits in the photosystem II reaction center (Yang et al., 2013; Yang et al., 2015). *In vivo*, the degree of damage under stress depends on the balance between damage and repair processes, especially in the light system (Mohanty et al., 2007). After only 15 min of heat treatment, some proteins in the photosystem were up-regulated and the dimer structure was stabilized to allow photosynthesis to continue normally in *N. haitanensis*. In contrast, in the absence of the extracellular Ca<sup>2+</sup> signal, photosystem of thalli could not be repaired in a timely manner.

### Energy Metabolism in Response to Ca<sup>2+</sup> Signaling

The stability of energy supply under stress is closely related to stress resistance. *N. haitanensis* can resist stress by increasing energy metabolism, mainly the glycolytic pathway and pentose phosphate pathway (Wang et al., 2018; Wen et al., 2020). Here, we also found similar results, however, the main genes, such as, Fructose 6-phosphate kinase (PFK), Fructose-bisphosphate

(FBP) aldolase, phospholipase c (PLC) of glycolytic pathway showed downregulated under heat stress after inhibition of Ca<sup>2+</sup> channels by verapamil (**Figure 5**). Among these genes, higher activity of FBP aldolase stimulates the glycolytic pathway by catalyzing a reversible cleavage reaction of fructose-1, 6-bisphosphate into two trioses: glyceraldehydes-3-phosphate and dihydroxyacetone phosphate (Matsumoto and Ogawa, 2008). He et al. (2012) found that exogenous Ca<sup>2+</sup> significantly elevated the quantity of the FBP aldolase, which alleviated the effects of hypoxic stress on cucumber roots. Meanwhile, the glycolytic pathway (initiated with sucrose synthase forming UDP-glucose and fructose and completed with alcohol dehydrogenase) were up-regulated *via* Ca<sup>2+</sup> elevation under hypoxic stress (Igamberdiev and Hill, 2018). Therefore, the Ca<sup>2+</sup> signal is involved in regulating the glycolytic pathway to provide energy and the related carbon skeletons for key metabolic processes in the response of *N. haitanensis* to heat stress.

## CONCLUSIONS

Our results show that Ca<sup>2+</sup>-CaM is involved in the early response of *N. haitanensis* to heat stress. It is crosstalk with PI signaling and is crucial for triggering HSPs to maintain protein homeostasis, regulating the transport of substances into and out of mitochondria through VDACs, and activating glycolysis to provide energy and the related carbon skeletons during the response of *N. haitanensis* to heat stress. These results increase our understanding of the molecular mechanisms associated with Ca<sup>2+</sup> signal transduction in *N. haitanensis* under heat stress.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI [accession: PRJNA725630].

## AUTHOR CONTRIBUTIONS

CTX, HYZ, and WLW designed the experiments. HYZ and WLW, carried out the experiments. HYZ, WLW, CTX, CSC, KX, YX, and DHJ analyzed the data, interpreted the results. HYZ, WLW, and CTX drafted the manuscript. All the authors reviewed and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2022.875308/full#supplementary-material>

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