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Analysis and Characterization of the GABA Transaminase and Succinate Semialdehyde Dehydrogenase Genes in the Microalga *Isochrysis zhanjiangensis* in Response to Abiotic Stresses

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Abstract Gamma-aminobutyric acid (GABA), widely existing in different organisms, is rapidly accumulated in plants in response to environmental stresses. The main biosynthesis and degradation pathways of GABA constitute the GABA shunt, which is tied to the tricarboxylic acid (TCA) cycle. GABA transaminase (GABA-T) and succinate semialdehyde dehydrogenase (SSADH) are two essential enzymes for the GABA degradation pathway. While there are abundant studies on GABA shunt in higher plants at the physiological and genetic levels, research on its role in microalgae remains limited. This study aimed at exploring the function of *GABA-T* and *SSADH* genes in *Isochrysis zhanjiangensis*, an important diet microalga, under different stresses. We cloned two *GABA-T* genes, *IzGABA-T1* and *IzGABA-T2*, and one *SSADH* gene *IzSSADH* from *Isochrysis zhanjiangensis* and conducted heterologous expression experiments. The results showed that the overexpression of *IzGABA-T1* or *IzGABA-T2* enhanced the survival rates of yeast transformants under heat or NaCl stress, while the overexpression of *IzSSADH* improved yeast tolerance to NaCl stress but had no obvious effect on heat stress. Additionally, the results of quantitative real-time polymerase chain reaction (qPCR) showed that *IzGABA-T1* transcription increased in the HT (salinity 25, 35℃) and LS (salinity 15, 25℃) groups. At 24 h, the *IzGABA-T2* transcriptions increased in the HT, LS, and HS (salinity 35, 25℃) groups, but their transcription levels decreased in all groups at 48 h. *IzSSADH* transcription increased in the LS group. These results suggest that *IzGABA-T1*, *IzGABA-T2*, and *IzSSADH* are associated with temperature and salinity stresses and possess a certain preference for different stresses.

Key words abiotic stress; GABA; heterologous expression; *Isochrysis zhanjiangensis*; transcription

1 Introduction

Plants often encounter biotic and abiotic stresses that threaten their growth and development and even result in a significant reduction in crop yield (Singh and Jha, 2017). Extreme temperature, salinity, and ultraviolet radiation are the main abiotic stresses (Yuan *et al*., 2019). For example, it was reported that marine heatwaves resulted in high mortality and lowered growth of macroalgae (Gao *et al*., 2021). When *Acutodesmus dimorphus* was exposed to a high temperature of 50℃, the levels of chlorophyll *a* and carotenoids declined and the formation of reactive oxygen species (ROS) sharply rose (Chokshi *et al*., 2020). In addition, salinity stress can lead to osmotic and ionic stresses in cells, disrupting cell homeostasis (Krishna *et al*., 2019). Responding to these stresses, a series of stress resistance mechanisms have been derived in plants at morphological, physiological, molecular, and cellular levels (Imran *et al*., 2021). For instance, more osmoregulatory substances (*e.g*., proline and betaine) are produced in plants to maintain cellular osmotic balance under extreme stresses (Wai *et al*., 2020). Up to now, diverse stress resistance mechanisms have been explored in plants. The gamma-aminobutyric acid (GABA) shunt is one of them (Seifikalhor *et al*., 2019).

GABA is a non-protein amino acid that is widely distributed in animals, plants, and microorganisms (Podlešáková *et al*., 2019). Normally, the content of endogenous GABA in plants stably maintains a low level, which increases sharply under adverse conditions (Podlešáková *et al*., 2019). However, the adjustment of GABA content mainly relies on the GABA shunt, which is closely correlated with the

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tricarboxylic acid (TCA) cycle (Ansari *et al*., 2021). GABA shunt is a short metabolic pathway that works for regulating cytosolic pH and endogenous hormones, balancing carbon/nitrogen metabolism, and scavenging ROS (Ramos-Ruiz *et al*., 2019; Ansari *et al*., 2021). Therefore, it is recognized that GABA has a close relationship with multiple stress resistance systems (Balfagón *et al*., 2022). This was supported by several studies demonstrating that exogenous GABA could alleviate freezing damage in persimmon fruit (Niazi *et al*., 2021) and salt stress in lettuce (Kalhor *et al*., 2018). A previous report showed that the contents of GABA in tobacco and soybean leaves both rapidly increased after insect treatment (Bown *et al*., 2002). Similarly, a study by Chi *et al*. (2021) showed that $CaCl_2$ treatment of fresh-cut pears led to the accumulation of GABA content and enhanced the activities and transcriptions of the related enzymes of GABA shunt. Meanwhile, it was confirmed by Hijaz *et al*. (2018) that the endogenous GABA content and the expression levels of GABA shunt-related enzymes in citrus were remarkably increased with the addition of exogenous GABA.

GABA can be biosynthesized in two pathways, *i.e*., the glutamic acid decarboxylase pathway and the polyamine degradation pathway (Liao *et al*., 2017). The glutamic acid decarboxylase pathway, usually referred to as the major pathway for GABA biosynthesis, is the α-decarboxylation reaction of L-glutamate (Glu) catalyzed by glutamic acid decarboxylase (GAD) (Tang *et al*., 2020). The polyamine degradation pathway starts from the degradation of polyamine or diamine, which are catalyzed by polyamine oxidase (PAO) or diamine oxidase (DAO), and generate Δ1-pyrroline. Then GABA is biosynthesized by pyrroline dehydrogenase (PDH) with Δ1-pyrroline as the substrate (Chi *et al*., 2021). For GABA degradation, GABA is converted to succinic semialdehyde (SSA) by GABA transaminase (GABA-T). Then SSA is further converted to succinic acid with succinate semialdehyde dehydrogenase (SSADH). In the last step, succinic acid enters the TCA cycle. Generally, the glutamic acid decarboxylase pathway and the GABA degradation pathway are collectively defined as the GABA shunt, involving GAD, GABA-T, and SSADH (Tang *et al*., 2020).

Owing to the simplicity of the GABA shunt, it is regarded as one of the most promising metabolic pathways for genetic manipulation to enhance stress resistance (Fedorin *et al*., 2022). Many attempts have been made to elucidate the GABA shunt in higher plants. For instance, the deletion of the C-terminal autoinhibitory domain of GAD could dramatically increase the GAD activity and the endogenous GABA content in tomato fruits (Nonaka *et al*., 2017). Similarly, a study by Mirabella *et al*. (2008) demonstrated that the mutation of *GABA-T* caused an increase in GABA content. In addition, it was reported that the level of H_2O_2 was accumulated in *Arabidopsis thaliana* with the *SSADH* mutation (Kirch *et al*., 2004). As for microalgae, research on the GABA shunt lags significantly behind that on higher plants, and only a few studies have explored the effects of exogenous GABA on the growth, lipid content, and photosystem activity of microalgae (Ding *et al*., 2019). However, detailed studies on enzymes of the GABA shunt in microalgae have rarely been reported.

Isochrysis zhanjiangensis belongs to the Phylum Chrysophyta, and is characterized by rapid growth, easy digestibility, and high nutritional value (Zhu *et al*., 2019). Therefore, it is popular in many fields, such as the aquaculture industry, pharmaceutical industry, and functional food production (Wang *et al*., 2021; Pei *et al*., 2022). Nevertheless, the culture of *I. zhanjiangensis* in open raceway ponds has been threatened by various abiotic and biotic stresses (Richardson *et al*., 2014), particularly in southern China, where hot and rainy weather is prevalent (Li *et al*., 2022). The inappropriate environmental conditions affect the biomass yield and the biochemical composition of microalgae, while also lower the quality of microalgal products, thus impeding the large-scale production of microalgae (Otogo *et al*., 2021). Hence, it is essential to elucidate the mechanism of stress resistance in *I. zhanjiangensis*.

Based on the genomic data of *I. zhanjiangensis* previously obtained by our group, two GABA transaminase genes, *IzGABA-T1* and *IzGABA-T2*, and one succinate semialdehyde dehydrogenase gene named *IzSSADH* in the GABA shunt were retrieved and cloned. With heterologous expression in *Saccharomyces cerevisiae*, it was verified whether these genes were correlated with stress resistance. Furthermore, their transcription levels in *I. zhanjiangensis* treated with different temperatures and salinities were analyzed. This study will provide some references for a better understanding of the function of GABA shunt in microalgae.

2 Materials and Methods

2.1 Algae Strain and Cultivation Conditions

I. zhanjiangensis, provided by the Marine Biology Laboratory of Ningbo University, was cultured in NMB3# liquid medium (pH 7.2, salinity 25) at 25℃ with fluorescent light of 100 μmol photons $m^{-2} s^{-1}$ and a 12 h:12 h light:dark cycle.

2.2 Gene Cloning and Bioinformatics Analysis

I. zhanjiangensis cells reaching the middle-logarithmic growth phase were harvested by centrifuging at 6000 *g* for 10 min at room temperature. Subsequently, total RNA was extracted using the E.Z.N.A.® Total RNA Kit (Omega, Madison, WI, USA), and the quality and purity of the RNA were checked by the NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, USA). The first-strand cDNA was synthesized and served as the template for amplifying the open reading frames (ORFs) of *IzGABA-T1*, *IzGABA-T2*, and *IzSSADH*. Based on the sequence information acquired from genomic data, specific primers were designed with restriction sites adjacent to the start and stop codons, respectively (Table 1). A Kozak consensus sequence (GCC ACC) was added to the forward primer to enhance translation efficiency.

The total volume of the polymerase chain reaction (PCR) mixture was 50 μL, containing 2 μL of forward and reverse primers (10 μmol L^{-1}) each, 2 μL of cDNA, 5 μL of 10× pyrobest Buffer II, 4 μL of dNTP mixture (2.5 mmol L^{-1}), 0.4 μL of Pyrobest DNA polymerase (TaKaRa, Beijing, Chi-

na), and 34.6 μL of sterilized water. The PCR procedure consisted of one cycle of pre-denaturation at 94℃ for 5 min and 30 cycles of amplification including denaturation at 94 ℃ for 30 s, annealing at 56℃ for 30 s, and extension at 72℃ for 2 min in each cycle. The purified PCR products were individually connected to the pMD18-T vector (TaKaRa, Beijing, China) and sequenced, yielding plasmids of pMD-18T-IzGABA-T1, pMD18T-IzGABA-T2, and pMD18T-IzSSADH. Introns and exons were identified by aligning the ORF sequence to the full DNA sequence of these genes. The isoelectric point and molecular weight were determined using the online ExPASY platform (http://www.expasy.org/). The subcellular localization of proteins was predicted by iPSORT (https://ipsort.hgc.jp/) and software (http://cello.life.nctu.edu.tw/). The protein transmembrane region was predicted by the TMHMM tool (http://www.cbs.dtu.dk/services/TMHMM-2.0/). Alignment of the homologous protein sequences was performed using Clustal X 1.81. Phylogenetic analysis was conducted by the MEGA 4.0 software with the neighbor-joining method.

Table 1 Names and sequences of primers used in this study

Procedure	Primer name	Primer sequence (5'–3')	Product size†
Cloning of gene cDNA	IzGABA-T1-F-Hind III	CCCAAGCTTGCCACCATGCTTTCGTGCGCGCTG	1462 bp
	IzGABA-T1-R-Xbal	CTAGTCTAGACTAGACGCGCTCTTTCCAGTG	
	IzGABA-T2-F-BamH I	CGCGGATCCGCCACCATGTTGGAGTCTATTGTC	726 bp
	IzGABA-T2-R-EcoR I	CCGGAATTCTCAATGGCCGAGCATTTCG	
	IzSSADH-F-Hind III	CCCAAGCTTGCCACCATGATGCCTCGCACTCTGG	1606 bp
	IzSSADH-R-Xbal	CTAGTCTAGATCAGGCTTCATCAATGGCC	
Quantitative analysis of the transcriptional level	GAPDH2-F	CGTTGACTACGATACCGC	184 bp
	GAPDH2-R	CTTCTTAGCACCGCCCTG	
	IzGABA-T1-F	TGCGTCCACTCTCATCCA	148 bp
	IzGABA-T1-R	GAAATTGCGCTTCTCGGG	
	IzGABA-T2-F	TCACGCACACCCAGTAG	181 bp
	IzGABA-T2-R	ATCGAGGCAGCCAAATA	
	IzSSADH-F	AGAACGCACTACCCGCC	187 bp
	IzSSADH-R	CCAAACCATTCGCAAAA	

Note: † Here included the lengths of ORF and primers.

2.3 Function Verifying in the Yeast

The plasmids pMD18T-IzGABA-T1, pMD18T-IzGABA-T2, pMD18T-IzSSADH, and pYES2 (Invitrogen, Carlsbad, CA, USA) were synchronously double-digested with the corresponding restriction enzymes. The targeted fragments were ligated with T4 ligase and transformed into competent *Escherichia coli* cells. The positive colonies were randomly selected from the ampicillin plates for PCR verification and sequencing, generating the recombinant plasmids pYES2-IzGABA-T1, pYES2-IzGABA-T2, and pYES2-IzSSADH. And then, the empty plasmid pYES2 and recombinant plasmids were separately transformed into competent *S. cerevisiae* INVSc1 cells with the *S.c.* EasyComp Transformation Kit (Invitrogen, Carlsbad, CA, USA). After selection on uracil-deficient SC-U plates containing 100 mg L^{-1} ampicillin, yeast transformants were picked for sequencing analysis. Yeast transformants harboring pYES2, pYES2-IzGABA-T1, pYES2-IzGABA-T2, or pYES2-IzSSADH were individually cultivated in SC-U liquid medium containing 2% (w/v) glucose without uracil. After incubating at 30℃ for 12 h, the OD_{600} values of each group were determined. They were centrifuged to remove the supernatant and added the corresponding volume of fresh SC-U induction medium containing galactose (2%, w/v) until the yeast cultures reached an OD_{600} of 0.4, and NP-40 was added to each group to a final concentration of 1%. Then, they were incubated at 30℃ for 36 h to induce gene expression (Zhang *et al*., 2021). Adjusting the OD_{600} value to 0.4, the induced yeast transformants were individually treated with heat and NaCl stresses with modified protocols based on the description of Wang *et al*. (2008). For the heat treatment, the yeast transformants of 100 μL were incubated at 53℃ for 2 h, while the yeast transformants without heat stress served as controls. Then, yeast transformants without and with heat stress were individually diluted to 10^{-2}. Finally, 5 μL of each of the dilutions was spotted onto the SC-U quartile plates and incubated at 30℃. For the NaCl stress, 1 mL of yeast transformants from each group were collected by centrifuging at 2500 *g* for 2 min at room temperature. In the next step, cells that were resuspended in 1 mL of NaCl solution (5 mol L^{-1}) and those without NaCl treatment were kept at 4℃ for 24 h. Cells without and with NaCl stress were respectively diluted to 10^{-2} and spotted onto the SC-U quartile plates with equivalent amounts. Finally, they were incubated at 30℃, and the survival rates were compared at different time periods.

2.4 Analysis of Transcription Levels in *I. zhanjiangensis*

I. zhanjiangensis reaching the mid-exponential growth phase were divided into five groups, namely, the Control (salinity 25, 25℃), LT (salinity 25, 15℃), HT (salinity 25, 35℃), LS (salinity 15, 25℃), and HS (salinity 35, 25℃) groups, respectively. Other culture conditions were consistent with the pre-culture. The growth rate of *I. zhanjiangensis* was monitored daily by measuring the cell density with hemocytometers. Algal cells in different groups were harvested at 0, 24, and 48 h, respectively. The samples were instantly frozen in liquid nitrogen and stored at −80℃ for RNA extraction.

Primers for quantitative real-time polymerase chain re-

action (qPCR) were designed with Premier 5.0 (Table 1). The size of PCR products ranged from 148 to 187 bp. *GAPDH2* was selected as the internal reference gene. The amplification efficiencies of all genes were checked in advance. The cDNA synthesis and qPCR were carried out with the Perfectstart® Uni RT&qPCR kit (TransGen, Beijing, China). The reverse transcription system (20 μL) consisted of total RNA of 1 μg, gDNA remover of 1 μL, and 5×TransScript®Uni-in-One SuperMix of 4 μL. The procedure for qPCR was as follows: initial denaturation at 94°C for 30 s, 40 cycles of amplification including denaturation at 94°C for 5 s, and annealing at 60°C for 30 s. At the end of each run, the purity of the amplification product was assessed using the melting curve generated by each sample. Three biological replicates and three technical replicates were performed for each sample. The relative transcriptional levels were analyzed with the $2^{-\Delta\Delta Ct}$ method.

2.5 Statistical Analysis

All data were statistically analyzed using SPSS 20.0. ANOVA was used to analyze the significance with a Duncan's post hoc test. And $P<0.05$ was considered statistically significant. All data were displayed as mean±SD (standard deviation) ($n=3$).

3 Results

3.1 Gene Identification and Sequence Analysis

Two *GABA-Ts* and one *SSADH* were retrieved from the genomic data of *I. zhanjiangensis* and individually named *IzGABA-T1*, *IzGABA-T2*, and *IzSSADH*. The full nucleotide sequences of *IzGABA-T1* (1581 bp), *IzGABA-T2* (702 bp), and *IzSSADH* (2876 bp) were deposited in GenBank with accession numbers OQ570643, OQ570644, and OQ570645, respectively. The ORFs of these genes were 1437, 702, and 1581 bp in length, encoding 478, 233, and 526 amino acids, respectively. There were individually one, zero, and seven introns identified in the nucleotide sequences of *IzGABA-T1*, *IzGABA-T2*, and *IzSSADH*, all conforming to the GT/AG rule (Fig.1). The predicted isoelectric points of IzGABA-T1, IzGABA-T2, and IzSSADH were 6.29, 5.91, and 5.76. The predicted molecular weights of these proteins were 51.6, 25.4, and 55.4 kDa, respectively. Subcellular localization prediction showed that IzGABA-T1, IzGABA-T2, and IzSSADH were localized in the mitochondrion, cytoplasm, and mitochondrion, respectively. In addition, there were no transmembrane domains predicted in IzGABA-T1, IzGABA-T2, and IzSSADH.

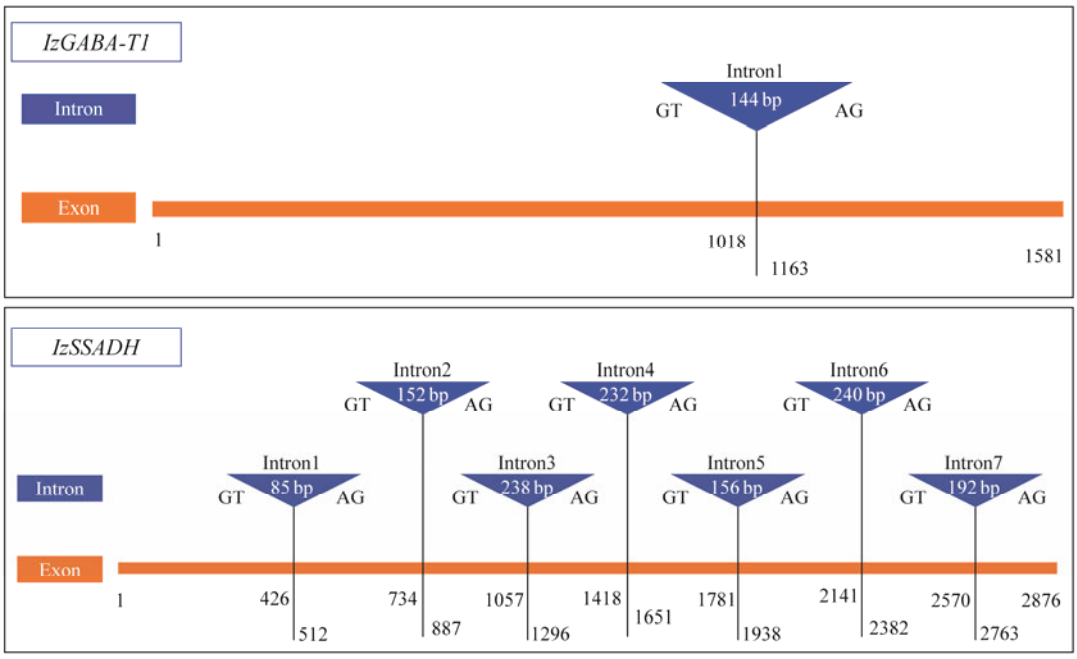

Fig.1 Distributions and lengths of introns in *IzGABA-T1* and *IzSSADH*. Blue/orange rectangles and triangles represent introns/exons.

Pairwise alignment revealed that IzGABA-T1 shared high identities (53.4%–75%) with other GABA-T proteins from different species. The lowest and highest similarities were respectively obtained with *Nannochloropsis gaditana* (53.4%) and *Symbiodinium natans* (75%). The similarity of IzGABA-T2 with others was between 48.3% and 54.5%. Among them, the highest one (54.5%) was obtained with *Solanum lycopersicum*, *Malus domestica*, *Camelina sativa*, and *Phalaenopsis equestris*, and the lowest one was with *Dendrobium catenatum* (48.3%). However, it was noteworthy that the similarity between IzGABA-T1 and IzGABA-T2 was only 49.2%. In addition, a phylogenetic tree was constructed with the 16 GABA-T proteins, as shown in Fig.2. IzGABA-T1 and *S. natans* were clustered into one branch correspondingly, while IzGABA-T2 and *N. gaditana* were clustered together. Pairwise alignment of IzSSADH with other SSADH proteins from various species also produced high identities (67.1%–75%). Additionally, phylogenetic analysis of different SSADH proteins was conducted in Fig.2.

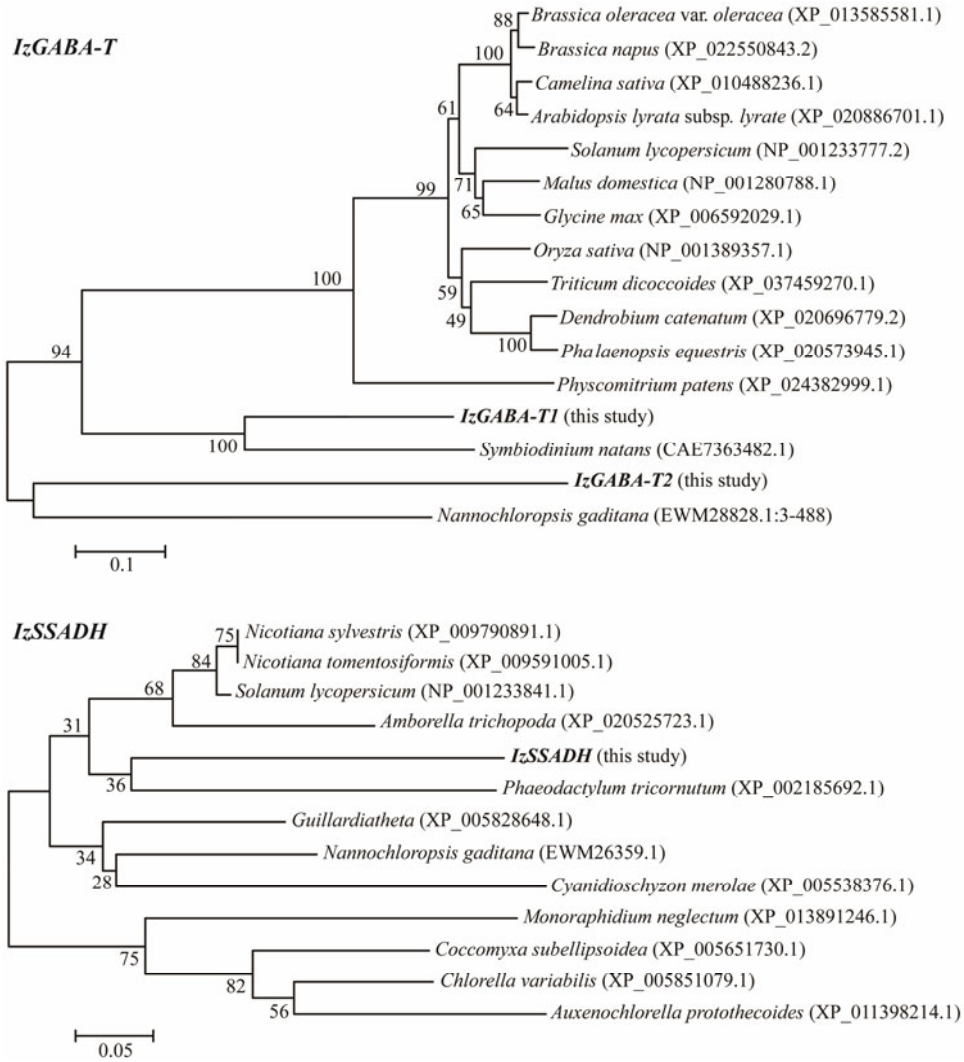

Fig.2 Neighbor-joining trees of 16 GABA-T proteins and 13 SSADH proteins from different species. Bootstrap analyses of 1000 randomized sequence replicates were conducted. The numbers at the branches represent the bootstrap values. The bracket after the species name represents the GenBank ID. The GABA-T and SSADH of *I. zhanjiangensis* are marked in bold.

3.2 Gene Function Examinations in Yeast

3.2.1 Heat stress tolerance assay

In order to clarify the functions of IzGABA-T1, IzGABA-T2, and IzSSADH, heterologous expressions of these proteins were performed in *S. cerevisiae* INVSc1, respectively. The induced yeast transformants harboring recombinant plasmids or pYES2 were respectively treated with heat and NaCl stresses. Afterwards, cells of equivalent amounts were spotted on plates, and the functions of these genes were assessed by comparing the colony numbers of the recombinant plasmid-transformed and the pYES2-transformed yeast.

At 72 h, it was observed that colonies of yeast cells without heat treatment were obviously more than those with heat treatment, which is applicable for yeast cells with both the empty vector pYES2 and recombinant plasmids. The tendencies of 96 h and 120 h were consistent with 72 h. It was suggested that the heat treatment of 53℃ for 2 h did cause serious damage to the growth of yeast. At 72 h, colonies of the heat-stressed yeast transformed with pYES2-IzGABA-T1 or pYES2-IzGABA-T2 appeared, but no colonies of heat-stressed yeast transformed with pYES2 were observed (Fig.3). The same trend was observed after 96 h and 120 h, indicating that heterologous expressions of *IzGABA-T1* and *IzGABA-T2* enhanced the heat tolerance of yeast. Moreover, the number of colonies with pYES2-IzGABA-T1 significantly exceeded that with pYES2-IzGABA-T2 under heat stress. For *IzSSADH*, no colonies appeared with yeast cells transformed with pYES2 or pYES2-IzSSADH. It could be speculated that heterologous expression of *IzSSADH* failed to improve the heat tolerance of *S. cerevisiae*.

3.2.2 NaCl stress tolerance assay

From Fig.4, it was clearly shown that colonies of yeast cells without NaCl treatment were drastically more than those with NaCl treatment at 72, 96, and 120 h, regardless of whether they harbored an empty pYES2 vector or recombinant plasmids. It was suggested that the NaCl treatment

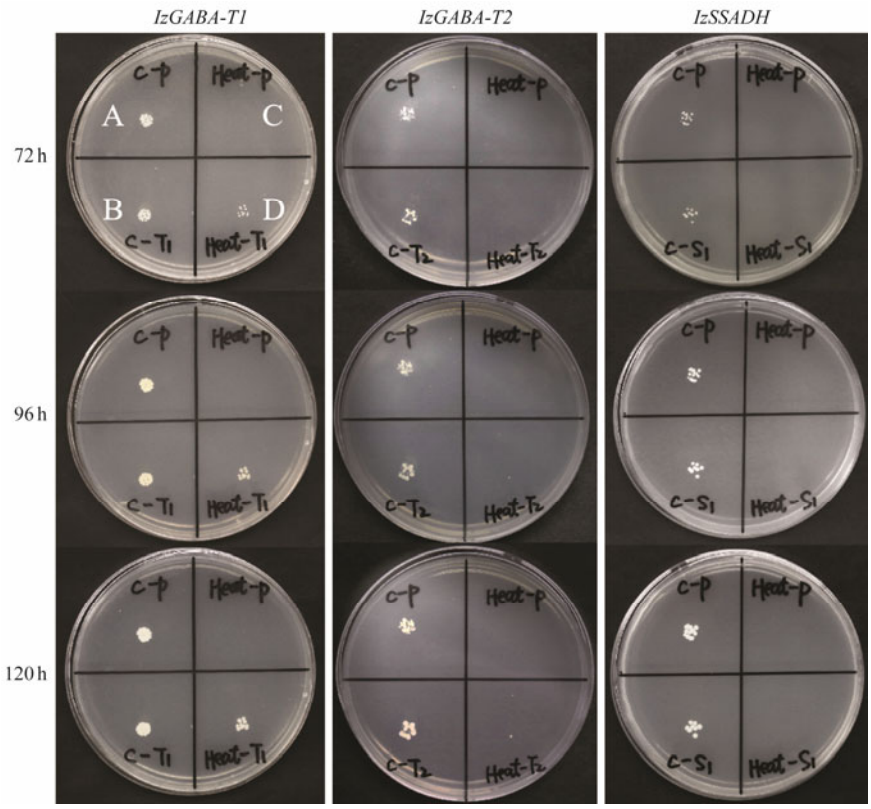

Fig.3 Growth status of yeast cells with heat treatment. Letter A (C-P) and letter C (Heat-P) represent pYES2-transformed yeast cells without or with heat stress. Letter B (C-T1/T2/S1) and letter D (Heat-T1/T2/S1) represent yeast cells transformed with recombinant plasmids without or with heat stress. P, pYES2; T1, pYES2-IzGABA-T1; T2, pYES2-IzGABA-T2; S1, pYES2-IzSSADH. Briefly, the letters A, B, C, and D are only marked on the plate in the upper left corner.

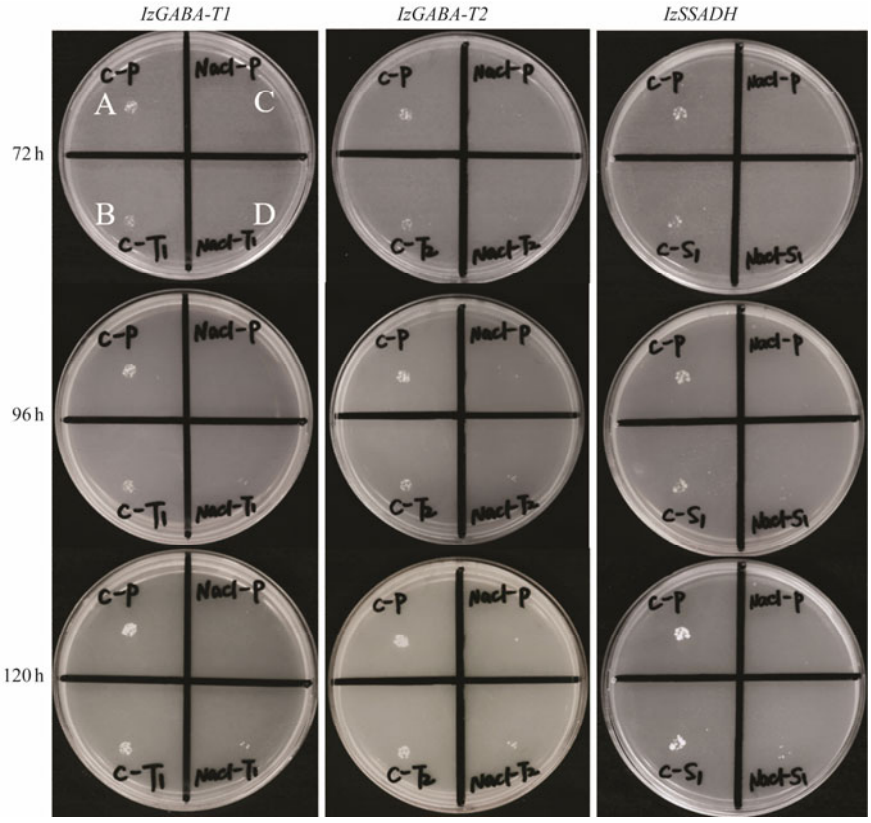

Fig.4 Growth status of yeast cells with 5 mol L^{-1} NaCl treatment. Letter A (C-P) and letter C (NaCl-P) represent pYES2-transformed yeast cells without or with NaCl stress. Letter B (C-T1/T2/S1) and letter D (NaCl-T1/T2/S1) represent yeast cells transformed with recombinant plasmids without or with NaCl stress. P, pYES2; T1, pYES2-IzGABA-T1; T2, pYES2-IzGABA-T2; S1, pYES2-IzSSADH. Briefly, the letters A, B, C, and D are only marked on the plate in the upper left corner.

(5 mol L^{-1}) did inhibit the growth of yeast. Furthermore, colonies of the NaCl-stressed yeast transformed with pYES2-IzGABA-T1, pYES2-IzGABA-T2, or pYES2-IzSSADH were obviously more numerous than those with pYES2 (Fig.4). These results indicated that the expression of *IzGABA-T1*, *IzGABA-T2*, or *IzSSADH* could improve the NaCl tolerance of yeast cells.

3.3 Algae Growth and Gene Transcription Profiling

3.3.1 Growth of *I. zhanjiangensis* under different treatments

The growth of *I. zhanjiangensis* was monitored under different treatments. At 24 h, algal growth in the LT and HT groups was inhibited significantly compared to the Control group. Instead, no significant differences were detected among the LS, HS, and Control groups. At 48 h, the growth of yeasts in LT, HT, and LS groups was dramatically lower than that of the Control group. However, the density of the HS group was slightly changed without significant differences ($P>0.05$). These findings indicated that low temperature, high temperature, and low salinity had obvious effects on the growth of *I. zhanjiangensis*, especially the low and high temperatures. However, the effect of high salinity on the growth of *I. zhanjiangensis* was not obvious (Fig.5).

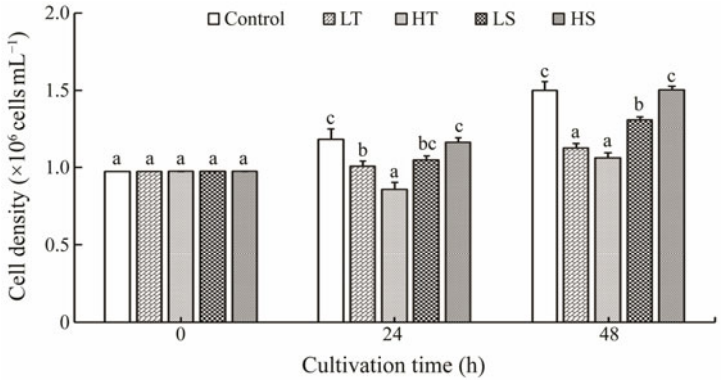

Fig.5 Growth of *I. zhanjiangensis* under different treatments. One-way ANOVA with Duncan's post hoc test was used to show the difference between various treatments. Different letters (a, b, and c) indicate significant differences ($P<0.05$). The experiment was conducted in three biological replicates. All data are displayed as mean ± SD (standard deviation) ($n=3$). Control, salinity 25, 25℃; LT, salinity 25, 15℃; HT, salinity 25, 35℃; LS, salinity 15, 25℃; HS, salinity 35, 25℃.

3.3.2 Transcription profiling analysis

To analyze the relative transcription levels of *IzGABA-T1*, *IzGABA-T2*, and *IzSSADH* under different treatments, algae cells were harvested at 24 and 48 h, and their transcription levels were analyzed using qPCR (Fig.6). At 24 h, the transcription levels of *IzGABA-T1* were significantly upregulated in the HT, LS, and HS groups by 417.9%, 42.2%, and 46.3%, respectively, compared with the control group. The transcription levels of *IzGABA-T1* in the HT group were 4.93, 3.64, and 3.54-fold of those in the LT, LS, and HS groups, respectively, while no significant difference was observed between the LT and control groups. At 48 h, *IzGABA-T1* transcription levels in the HT and LS groups were dramatically upregulated by 55.4% and 63.6% ($P<0.05$), compared with the control group. On the contrary, *IzGABA-T1* transcription was remarkably reduced in the LT group, and there was no significant difference between the HS and control groups.

The transcription levels of *IzGABA-T2* were different from those of *IzGABA-T1*. At 24 h, the transcription levels of *IzGABA-T2* in the HT, LS, and HS groups were drastically increased by 146.4%, 32.8%, and 31.4% ($P<0.05$) compared to the control group, respectively. The *IzGABA-T2* transcription in the LT group was dramatically downregulated by 28% ($P<0.05$). At 48 h, the mRNA levels of *IzGABA-T2* were significantly downregulated ($P<0.05$) in all stressed groups compared with the control group, and the lowest level was observed in the HS group.

At 24 h, the transcription levels of *IzSSADH* were significantly upregulated by 105.9% and 58.8% in the HT and LS groups, respectively ($P<0.05$), compared to the control group. *IzSSADH* transcription was dramatically decreased by 42.3% in the LT group ($P<0.05$), while high salinity seemed to have no effect on the *IzSSADH* transcription level. At 48 h, the transcription level of *IzSSADH* was remarkably upregulated by 148.4% in the LS group ($P<0.05$). However, the *IzSSADH* mRNA levels were downregulated in the LT, HT, and HS groups, which were 52.6%, 9.1%, and 30.4% of the control group, respectively.

4 Discussion

GABA, a stress-related protein, participates in fundamental metabolic pathways and responds to extreme environments in a variety of species, including mammals, plants, and microbes (Seifikalhor *et al*., 2019). It was reported that *S. cerevisiae* consistently maintained a modest amount of endogenous GABA, which increased significantly under various stresses (Cao *et al*., 2013b; Zhang *et al*., 2022). While substantial research about the GABA shunt at physiological and genetic levels has been conducted in higher plants (Liu *et al*., 2021), related research is scarce in microalgae, specifically regarding the enzymic genes of the GABA shunt. Here, two *GABA-Ts* and one *SSADH* were cloned from *I. zhanjiangensis*. Possessing mitochondrial tar-

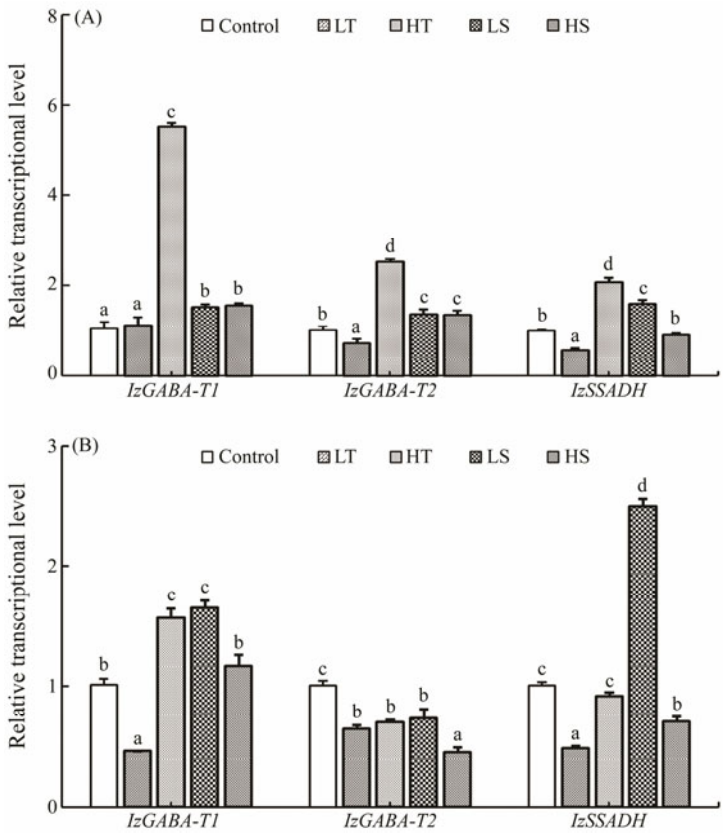

Fig.6 Relative transcription levels of IzGABA-T1, IzGABA-T2, and IzSSADH under different treatments for 24 h (A) and 48 h (B). Different letters (a, b, c, and d) indicate significant differences ($P < 0.05$). The experiment was conducted in three biological replicates. All data are displayed as mean ± SD (standard devi- ation) ($n = 3$). Control, salinity 25, 25°C; LT, salinity 25, 15°C; HT, salinity 25, 35°C; LS, salinity 15, 25°C; HS, salinity 35, 25°C.

geting peptides, IzGABA-T1 and IzSSADH are both located in the mitochondrion, while IzGABA-T2 is located in the cytoplasm. The subcellular localization analysis was consistent with previous reports (Shelp *et al*., 2012; Jalil *et al*., 2017).

Heterologous expression is considered as one effective method for the verification of gene function with different hosts, *e.g*., *E. coli* and *S. cerevisiae*. This method has previously been employed to test genes associated with stress resistance in higher plants, which proved to be successful. Yu *et al*. (2014) found that the overexpression of one betaine aldehyde dehydrogenase gene from *Ammopiptanthus nanus* enhanced the tolerance of *E. coli* to high salt and heat. In addition, the overexpression of one vascular highway 1-interacting kinase gene from date palm improved the survival rates of yeast under salinity, LiCl, and oxidative stresses (Al-Harrasi *et al*., 2020). Similarly, heterologous expression of one dehydration-responsive element-binding protein2 gene from *Eremosparton songoricum* conferred *S. cerevisiae* with higher tolerances to osmotic, salt, cold, heat, and oxidative stresses (Li *et al*., 2014). In these attempts, transformants of *E. coli* or *S. cerevisiae* were cultured on solid plates, and the performances of colonies were observed to estimate gene function. This method was also applied in the present study of stress resistance genes from *I. zhanjiangensis*.

In our study, the functions of IzGABA-T1, IzGABA-T2, and IzSSADH were validated with *S. cerevisiae* as the host. It was indicated that expressions of the *I. zhanjiangensis IzGABA-T1* and *IzGABA-T2* genes enhanced the survival rates of *S. cerevisiae* under heat and NaCl stresses. Our results were consistant with the previous studies. The deletion mutations of genes related to the GABA shunt in *S. cerevisiae* led to its growth defects under heat stress (Cao *et al*., 2013b). Likewise, a study by Jalil *et al*. (2017) demonstrated that the *GABA-T* mutant in *A. thaliana* caused reductions in chlorophyll content, GABA content, and GAD activity, showing that *GABA-T* was associated with stress tolerance. Furthermore, the function of IzSSADH was estimated by *S. cerevisiae*. It was shown that *IzSSADH* improved the ability of *S. cerevisiae* to resist the NaCl stress, but it did not work for the heat stress. However, it was reported that SSADH played a role in the resistance to heat stresses in *Arabidopsis* (Bouché *et al*., 2003). This might depend on species specificity (Breschi *et al*., 2016).

Temperature and salinity, two essential environmental factors for algal growth, have gained extensive concerns. Several studies have reported that heat and salinity stresses impact photosynthesis, antioxidant systems, and fatty acid biosynthesis in microalgae (Kan *et al*., 2012; Atikij *et al*., 2019; Shetty *et al*., 2019). However, their influences on genes of GABA shunt in microalgae have not been elucidated. They were addressed in this study. Our results showed that the transcription levels of *IzGABA-T1*, *IzGABA-T2*, and *IzSSADH* genes involved in GABA degradation changed significantly under stress conditions. Moreover, *IzGABA-*

T1 was the most active one, compared to *IzGABA-T2* and *IzSSADH*. It was inferred that the degradation of GABA increased in *I. zhanjiangensis* responding to stresses. Hence, more succinic acid was produced and supplied for the TCA cycle, which was crucial for the biosynthesis of ATP and other metabolites (Steinhauser *et al*., 2012).

The transcriptional levels of *IzGABA-T1*, *IzGABA-T2*, and *IzSSADH* genes were observed to exhibit the most drastic changes in high-temperature conditions compared to other treatments. Similar studies by Cao *et al*. (2013a) and Bouché *et al*. (2003) showed that the GABA-T deficient mutant of yeast and the *SSADH*-knockout mutant of *Arabidopsis* were sensitive to heat stress. Previous reports found that *GABA-T* transcription was upregulated under salt stress in higher plants, such as rice seedlings (Kim *et al*., 2007) and *A. thaliana* (Renault *et al*., 2010). Another study also demonstrated that *SSADH* transcription levels significantly increased with instant NaCl treatment in *Zea mays* (Fedorin *et al*., 2022). Conversely, the mutation of *GABA-T* resulted in decreased salt tolerance in *A. thaliana* (Renault *et al*., 2010). However, the opposite result was obtained in our study. Specifically, the high salinity treatment had no significant effect on the transcription levels of *IzGABA-T1*, *IzGABA-T2*, and *IzSSADH* in *I. zhanjiangensis*, while the transcription of these genes in the LS group was more active. It was speculated that the response of *I. zhanjiangensis* to high salinity stress might not rely on the GABA shunt but on other stress-resistance genes or systems.

In addition, it was observed that the transcription levels of *IzGABA-T1*, *IzGABA-T2*, and *IzSSADH* changed significantly depending on culture period and treatment method. Similar results have also been reported in many species. For example, the aldehyde dehydrogenase gene transcriptt ion showed distinction in *Syntrichia caninervis* treated with NaCl at different timepoints (Wang *et al*., 2020). The expressions of glyoxalase genes were different in *Phoenix dactylifera* treated with NaCl, methylglyoxal (MG), and H_2O_2 (Jana *et al*., 2021). In this study, the growth of most stressed groups was inhibited, compared with the control group, despite the increased transcription of GABA-related genes in response to these stresses. This might be because the changes at the cellular level lagged behind those at the molecular level (Yin *et al*., 2019). The upregulation of genes could not completely offset the negative impact of adversity.

5 Conclusions

Two *GABA-Ts* (named *IzGABA-T1* and I*zGABA-T2*) and one *SSADH* (named *IzSSADH*) were cloned in this study. Their functions were identified by different expression levels in *S. cerevisiae*. It was confirmed that *IzGABA-T1* and *IzGABA-T2* improved the tolerance of yeast to heat and NaCl stresses. And *IzSSADH* endowed yeast with a higher tolerance to resist NaCl stress. Further transcription level analysis in *I. zhanjiangensis* showed that the *IzGABA-T1* transcription increased in response to high temperature and low salinity. In addition, the transcription of *IzSSADH* was dramatically upregulated under low salinity treatment. This study will provide references for a better understanding of the role of GABA shunt in the stress resistance of microalgae.

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