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Proteomic Response to Environmental Stresses in the Stolon of a Highly Invasive Fouling Ascidian

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Ascidians, particularly those highly invasive ones, are typical fouling organisms to cause significantly negative ecological and economic influence in coastal ecosystems. Stolon, which is the unique structure of some solitary ascidians to complete the essential process of adhesion, possesses extremely high tolerance to environmental stresses during biofouling and invasions. However, the mechanisms underlying environmental tolerance remain largely unknown. Here, we used the quantitative proteomics technology, isobaric tags for relative and absolute quantitation (iTRAQ), to investigate the molecular response to environmental challenges (temperature and salinity) in the stolon of a highly invasive fouling ascidian, *Ciona robusta*. When compared with the control, a total of 75, 86, 123, and 83 differential abundance proteins were identified under low salinity, high salinity, low temperature, and high temperature stress, respectively. Bioinformatic analyses uncovered the key pathways under both temperature and salinity stresses, including "cytoskeleton," "signal transduction," and "posttranslational modification," which were involved in stolon structure stability, protein synthesis, and stress response activation. Under the low salinity stress, the "extracellular matrix" pathway was identified to play a crucial role by regulating cell signal transduction and protein synthesis. To deal with the high salinity stress, stolon could store more energy by activating "carbohydrate/lipid transport" and "catabolism" pathways. The energy generated by "lipid metabolism" pathway might be beneficial to resist the low temperature stress. The upregulation of "cell cycle" pathway could inhibit cell growth, thus helping stolon conserve more energy against the high temperature stress. Our results here provide valuable references of candidate pathways and associated genes for studying mechanisms of harsh environmental adaptation and developing antifouling strategies in marine and coastal ecosystems.

Keywords: ascidian, biofouling, invasive species, proteomics, salinity, temperature, environmental stress

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INTRODUCTION

Biofouling, the undesired adherence of fouling organisms on various submerged surfaces, is one of the most concerned environmental issues in global aquatic ecosystems (Bellard et al., 2013; Ricciardi et al., 2017; Briski et al., 2018). More than 4000 species have been recorded as biofoulers in both marine and freshwater ecosystems (Nakano and Strayer, 2014). Among these diverse

organisms, mussels, barnacles, ascidians, sea stars, and tube worms are the most representative taxa (Flammang et al., 2015; Li X. et al., 2021). These taxa usually use their specialized organs/structures, such as mussel byssus (Andrade et al., 2015), sea star and sea urchin tube feet (Santos et al., 2013), ascidian stolon (Li et al., 2019), and barnacle base plate (von Byern and Grunwald, 2010), to firmly adhere to the surfaces of underwater substrates to achieve their successful biofouling. In order to solve the biofouling problem, it is a prerequisite to understanding the adhesive processes of these organs/structures and the associated mechanisms, as well as environment-organism interactions that can affect the adhesive abilities of fouling taxa (Li et al., 2019). In the last decade, numerous studies have confirmed that the underwater adhesion is largely biomacromolecule-mediated, and remarkable adhesive proteins, such as mussel foot proteins (Mfps), barnacle cement proteins, sea star footprint proteins, and sea urchin tube feet cement secreted by the adhesive organs/structures of these fouling organisms, have been identified as the crucial elements for marine biofouling (von Byern and Grunwald, 2010; Hennebert et al., 2012; Santos et al., 2013; Zhang et al., 2017b). Despite the fact that environmental changes also largely determine the successful biofouling (Chen et al., 2021), limited information is available on their influential mechanisms, particularly on the complex interaction mechanisms between environmental factors and adhesion-related proteins.

Studies have illustrated that environmental changes can affect biofouling by altering the expression and functions of adhesion-related proteins. For example, the fouling ability of the barnacle *Balanus amphitrite* decreased with temperature increased from 15 to 25°C, and such decreased ability of biofouling was owing to the expression changes of cement proteins (Johnston, 2010). The expression of some Mfps reduced significantly in the foot of *Mytilus coruscus* exposed to higher temperature, thus affecting the byssus production and further weakening biofouling (Li Y. F. et al., 2020). Other environmental stressors, such as pH (ocean acidification), nanoparticles, and microplastics, can also weaken the fouling strength by influencing adhesion-related protein expression (Hu et al., 2015; Scott et al., 2019; Khan et al., 2020; Shi et al., 2020). In addition, studies found that several metabolic pathways associated with adhesion were also involved in the response of the fouling organs/structures of marine organisms to environmental changes. "Osmoregulation" and "cell cycle" were identified as crucial common pathways in the feet of *Mytilus galloprovincialis* and *Mytilus trossulus* under salinity stresses (Lockwood and Somero, 2011). The pathways of "metabolic pathway," "focal adhesion," and "cytoskeleton" participated in the response to cadmium challenges in the foot of *Perna viridis* (Zhang et al., 2017c). Available evidence from these studies suggests that anti-stress strategies and associated adhesion-related protein responses should be taxa- or even organ/structure-specific in marine biofoulers, and more efforts are needed to comprehensively investigate common and specific mechanisms underlying environmental challenges.

Ascidians such as *Ciona*, *Styela*, *Botrylus*, and *Didemnum* are fouling taxa in coastal ecosystems (Dijkstra and Simkanin, 2016). Even worse, many species of these taxa are highly invasive, largely spreading the negative effects of biofouling and further threatening local biological communities and global industries including underwater facilities, shipping, and aquaculture (Cahill et al., 2012; Zhan et al., 2015; Yan et al., 2017; Kim et al., 2019). Among ascidians, *C. robusta* is a notorious invader for its extremely high biofouling capacity mainly derived from the rootlike fouling structure, the stolon (Li et al., 2019). Stolon can enlarge the binding area between the ascidian body and substrate surface by releasing adhesive proteins to enhance the interfacial adhesion (Pennati and Rothbächer, 2014; Ueki et al., 2018). The stolon can still maintain its structural stability and underwater adhesive ability under various challenges including temperature and salinity stresses, which has been used repeatedly when studying the response mechanisms of ascidians to environmental stresses (Renborg et al., 2014; Hawes et al., 2018; Huang et al., 2019; Li et al., 2019). Former studies have demonstrated that *C. robusta* body displayed high tolerance to temperature and salinity stresses, which were related to multiple layers of mechanisms such as rapid microevolution and adaptation, phenotypic plasticity, and metabolism trade-off (Renborg et al., 2014; Dijkstra and Simkanin, 2016; Hawes et al., 2018; Huang and Zhan, 2020; Chen et al., 2021). As a special adhesive structure, it remains unknown how *C. robusta* stolon, particularly the adhesion-related proteins in stolon, actively respond to environmental stressors. Recently, several adhesion-related proteins have been identified from *C. robusta* stolon using mass spectrometry technologies (Ueki et al., 2018; Li et al., 2019), bringing us an opportunity to reveal the molecular response mechanisms of *C. robusta* stolon to environmental challenges.

Isobaric tags for relative and absolute quantitation (iTRAQ) has been widely applied in analyzing the effects of environmental changes on fouling organisms at the proteomics level, mainly because such technique has outstanding accuracy, high-throughput, strong sensitivity, and dynamic detection ability (Han et al., 2013; Ji et al., 2014; Zhang et al., 2015; Tang et al., 2020). With this technique, this study aimed to reveal the proteomic response of *C. robusta* stolon to temperature and salinity challenges. We detected the changes in protein expression in the *C. robusta* stolon exposed to different temperature and salinity stresses. Subsequently, the functional annotation and network analyses on these stress-related proteins were conducted to reveal the common and differential response mechanisms of this adhesive structure to environmental changes.

MATERIALS AND METHODS

Animal Collection and Experimental Design

Ciona robusta adults (average length of 6 cm) adhered on scallop cages were collected from the Longwangtang Aquaculture Farm, Dalian, Liaoning Province, China (38°49′′N, 121°24′′E) in September 2019. Collected ascidians were acclimatized in the filtered (the size of the filter membrane is 0.45 μm) and aerated seawater at 22 ± 1°C, 30 ± 1 psu, and pH 8.1 ± 0.1 (natural conditions at the collection site) for 1 week. They were fed with the dried algae powder mixture of *Spirulina* sp. and *Chlorella* sp. daily. After acclimation, we conducted a preliminary experiment

to select suitable exposure time and level for temperature and salinity stresses. The temperature gradient were designed as 5, 10, 30, and 40°C, while the salinity gradient were designed as 12, 20, 40, and 50 psu according to the results obtained from previous investigations (Carver et al., 2006; Bouchemousse et al., 2016; Chen et al., 2018). Ascidian individuals were exposed to the different gradients of environmental stresses for 1 week. The analysis results showed that exposing *C. robusta* to the levels of 12 psu, 40 psu, 10°C, 30°C for 120 h could represent the low salinity, high salinity, low temperature and high temperature stresses on this species. After preliminary experiment, the healthy ascidians with obvious stimuli responses were randomly assigned to five groups: control (C, 22°C/30 psu), high salinity (HS, 22°C/40 psu), low salinity (LS, 22°C/12 psu), high temperature (HT, 30°C/30 psu), and low temperature (LT, 10°C/30 psu). Each exposure group contained three separate 20 L tanks as replicates and 40 individuals in each replicate tank (**Figure 1**). After exposure for 120 h, the stolon that was still attached to the substrate surface was dissected from 18 individuals in each tank and pooled together as a mixed biological replicate. A total of 270 stolon tissues (18 individuals × 3 replicates × 5 groups) were collected, and the residual seawater on the sampled stolon surface was removed with the sterilized absorbent paper. The stolon samples were then rapidly frozen with liquid nitrogen and preserved at −80°C until proteomics analysis.

Protein Preparation and Isobaric Tags for Relative and Absolute Quantitation Labeling

To extract the total proteins, the collected samples were ground into powder with liquid nitrogen. A total of 3 mL radio-immunoprecipitation assay buffer was added to the powder and homogenized with a glass homogenizer. After centrifugation at 16,000 *g* for 30 min, the supernatant was deposited by adding fourfold volume of cold acetone containing 10 mM DTT at 20°C for about 3 h. Another centrifugation at 20,000 *g* for 30 min was performed at 4°C and the supernatant was then discarded. The collected precipitate was resuspended with 800 μL of cold lysate containing a solution of 8 M urea, 30 mM 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES), 1 mM PMSF, 2 mM EDTA, and 10 mM DTT, and then incubated at 56°C for 1 h (Lopez et al., 2017). The iodoacetamide was rapidly added into the resuspended sample to obtain the solution with the final concentration of 55 mM by incubating it at room temperature for 1 h in the dark. Following a second round of centrifugation at 20,000 *g* for 30 min at 4°C, the protein-containing supernatant was collected and stored at −80°C for subsequent analyses.

The purity of extracted stolon proteins was analyzed using the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method (Li et al., 2019), while the protein concentration was determined using the Bradford method (Bradford, 1976). Subsequently, the filter-aided sample preparation method was used for protein digestion. In brief, a total of 100 μg of stolon proteins was added into the 10 K tube for ultrafiltration. A total of 200 μL of 50% triethylammonium bicarbonate (TEAB) buffer was added to the obtained solution and then centrifuged at 14,000 *g* for 40 min at 4°C, and this step was repeated once. The protein mixture was then treated with 1 μg/μL of Sequencing Grade Modified trypsin (Promega, Madison, WI, United States) at 37°C for 24 h. The target peptides were lyophilized with a lyophilizer and collected into a new centrifuge tube. Finally, the peptide samples were redissolved in 50% TEAB buffer. Sample labeling was performed using an iTRAQ 8-plex reagent kit (AB Sciex, Framingham, MA, United States) following the manufacturer's instructions. The isotopes 116, 117, 118, 119, and 121 were selected to label the stolon proteins in the control, LS, HS, LT, and HT groups, respectively.

Mass Spectrometry Analysis

A set of iTRAQ-labeled samples were combined, desalted, and vacuum-dried. The labeled peptide mixture was dissolved with buffer A (10 mM ammonium formate, pH = 10) and separated with a reversed phase C18 column (75 μm × 10 cm, 5 μm, 300 Å, Agela Technologies) mounted on an ultimate 3000 nano LC system (Dionex, Sunnyvale, CA, United States). Finally, a total of 16 fractions were harvested and freeze-dried. Each fraction was re-dissolved with 5 μL 0.1% formic acid and passed through a Nano LC-MS/MS system at a flow rate of 300 nL/min. The separated compounds were then eluted into an Electrospray Ionization Orbitrap of the Q-Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA, United States), setting in positive ion mode and data-dependent manner with full MS scan at 350–2,000 m/z, full scan resolution at 70,000, and MS/MS scan resolution at 17,500. The minimum signal threshold for MS/MS scan was set at 1E + 5 and the isolation width was set at 2 Da.

Differential Abundance Proteins Identification

The raw data of MS analysis was converted into files with mgf format using the Proteome Discoverer 1.4 software (Thermo Fisher Scientific Inc., Bremen, Germany). The obtained clean data was searched using MASCOT software (Matrix Science, London, United Kingdom; version 2.3.0) to identify proteins. The protein database for *C. robusta* in UniProt[1] was used as the reference database for protein identification as described by a previously published protocol (Li et al., 2019). The summed intensities of the matched spectrum were used for quantizing the relative expression ratios of proteins, and one protein was quantified using at least two spectra. Subsequently, these ratios were transformed to $\log_2$ intensities (Kuplik et al., 2019). The protein abundance differences between the exposed groups (LS, HS, LT, and HT) and the control group were evaluated by using *t*-test method combined with the Benjamini–Hochberg correction (Ji et al., 2014). The DAPs, including the up-regulated and the down-regulated proteins, were identified using p-value < 0.05 and fold change ≥ 1.2 or ≤ 0.83, respectively. The raw data obtained from LC-MS/MS have been uploaded to the public repository iProX (ID: IPX0002919000).

[1] https://www.uniprot.org/taxonomy/7713

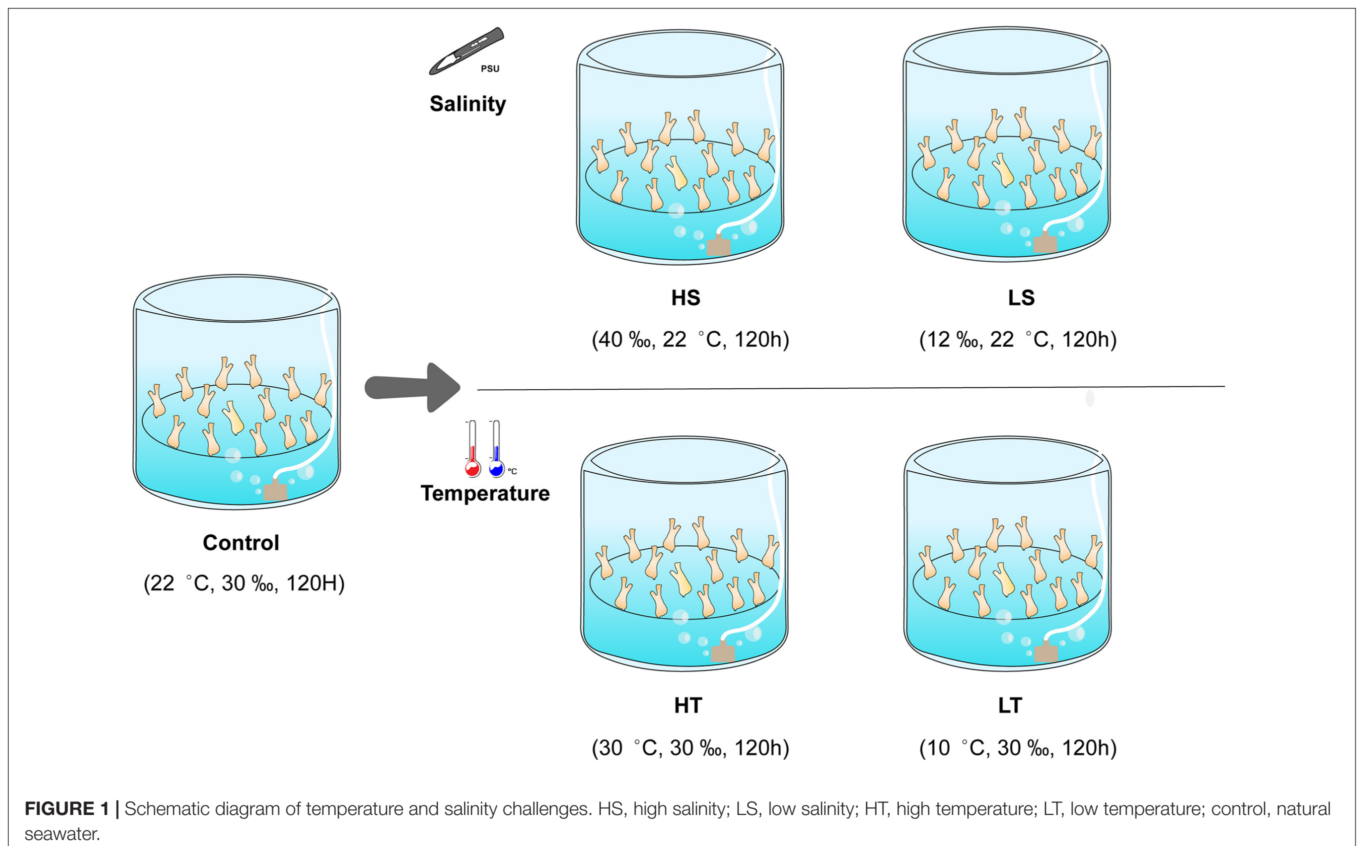

FIGURE 1 | Schematic diagram of temperature and salinity challenges. HS, high salinity; LS, low salinity; HT, high temperature; LT, low temperature; control, natural seawater.

Bioinformation Analysis

The functional annotation of the obtained DAPs was performed using OmicsBean online program[2], which includes the enrichment functions of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG, Gene and Consortium, 2000; Kanehisa et al., 2008). After removing the invalid values and antilibrary data, a total of 75, 86, 123, and 83 DAPs in the four groups were identified. The hypergeometric test was used to find the significantly enriched GO terms and KEGG pathways with the criterion of p-value < 0.01. Clusters of Orthologous Groups (COG) enrichment was also employed to functionally classify the DAPs based on the orthology concept (Tatusov et al., 2000). The Venn diagram online tool[3] was used to analyze the overlapped DAPs among different exposure groups. According to the Venn results, the coexisting DAPs of salinity and temperature stresses were evaluated by protein–protein interactions (PPI). The PPI network analysis was conducted to further clarify the inter-relationships and expression patterns of the DAPs related to salinity/temperature challenges by using the Search Tool for the Retrieval of Interacting Genes[4] (version 11.0) and Cytoscape software[5] (version 3.7.2) with default parameters.

[2]http://www.omicsbean.cn
[3]http://bioinformatics.psb.ugent.be/webtools/Venn/
[4]http://string.embl.de
[5]http://www.cytoscape.org

RESULTS

Stolon Protein Analysis

A total of 33,068 MS/MS counts were generated from the stolon across all five groups (control included). By searching against the UniProt database, 3,930 unique peptides and 1,083 proteins were identified (**Supplementary Table 1**). A total of 597 (approximately 55.12%) of the identified proteins had at least two unique peptides. Our results illustrated that most of the proteins (approximately 75%) were composed of 100–700 amino acids (**Supplementary Figure 1**), while 87.63% of the identified proteins contained less than seven peptides (**Supplementary Figure 2**). The molecular weight of most of the identified proteins was less than100 kDa, accounting for 80% of the total proteins. Moreover, 15 proteins with low molecular weight (<10 kDa) and 135 proteins with high molecular weight (>100 kDa) were identified (**Supplementary Figure 3**). The distribution of protein coverage illustrated that the coverage with less than 5, 5–15, 15–30, and 30–100%, accounting for 37.65, 36.93, 18.12, and 7.30% of the total proteins in stolon, respectively (**Supplementary Figure 4**).

Differential Abundance Proteins Identification

For salinity stresses, a total of 75 DAPs including 49 up-regulated and 26 down-regulated proteins were identified from the stolon exposed to LS, while a total of 86 DAPs including 54 up-regulated

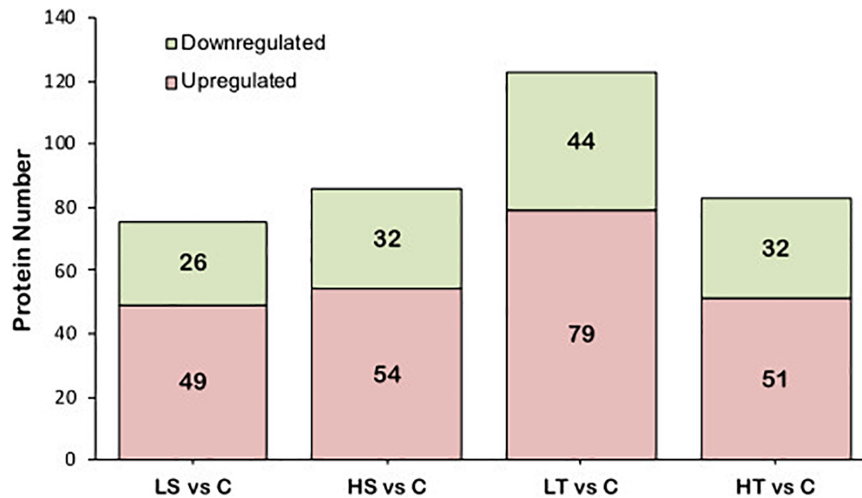

FIGURE 2 | Analysis of the differential abundance proteins in the stolon of *Ciona robusta* exposed to temperature and salinity stresses. HS, high salinity; LS, low salinity; HT, high temperature; LT, low temperature; C, natural seawater control.

and 32 down-regulated proteins were identified from the stolon exposed to HS. For temperature stresses, a total of 123 DAPs containing 79 up-regulated and 44 down-regulated proteins were identified from the stolon exposed to LT, while a total of 83 DAPs containing 51 up-regulated and 32 down-regulated proteins were identified from the stolon exposed to HT (**Figure 2**). Venn analysis showed that a total of 27 DAPs were overlapped in both HS and LS groups, while 48 DAPs were overlapped in both HT and LT groups (**Figure 3**). The detailed information on these DAPs is shown in **Supplementary Table 2**.

Enrichment Analysis for the Differential Abundance Proteins

Gene Ontology enrichment analysis on the DAPs showed that these proteins were enriched into three categories (**Figure 3** and **Supplementary Tables 3–6**): biological process (BP), cell component (CC), and molecular function (MF). The "Cytoskeleton" term was significantly enriched in both HS and LS groups. The terms "extracellular region," "cellular ion homeostasis," "cellular cation homeostasis," and "extracellular matrix structural constituent" were only enriched in the LS group (**Figure 4A**). The terms "cytoskeletal part," "cytoskeleton organization," and "proteolysis" were only enriched in the HS group (**Figure 4B**). Meanwhile, the terms "cytoskeleton," "polymeric cytoskeletal fiber," and "carbohydrate binding" were enriched in both HT and LT groups. The terms "extracellular space," "calcium ion binding," and "enzyme inhibitor activity" were enriched in the LT group (**Figure 4C**). The significantly enriched terms in the HT group were "microtubule," "cell cycle," and "nucleoside-triphosphatase activity" (**Figure 4D**).

By performing KEGG pathway enrichment analyses, the "extracellular matrix (ECM)" pathway was specifically enriched in the stolon exposed to LS (**Figure 5A**), whereas the "transport and catabolism" and "signal transduction" were the most significant pathways enriched in the stolon exposed to HS (**Figure 5B**). Furthermore, two pathways, including "signal transduction" and "lipid metabolism," were significantly enriched in the stolon exposed to LT (**Figure 5C**), whereas the pathways were "signal transduction" and "cell growth and death" in the HT group (**Figure 5D**).

The DAPs identified from the stolon exposed to LS, HS, LT, and HT were enriched into 16, 18, 19, and 17 COG categories, respectively (**Figure 6**). Among these, the most significant categories in the LS group were "signal transduction mechanisms," "cytoskeleton," "extracellular structures," and "posttranslational modification, protein turnover, chaperones" (**Figure 6A**), whereas the most significant ones in the HS group were "cytoskeleton," "signal transduction mechanisms," and "posttranslational modification, protein turnover, chaperones" (**Figure 6B**). Meanwhile, the most significant categories in the LT group were "posttranslational modification, protein turnover, chaperones," "signal transduction mechanisms," and "cytoskeleton" (**Figure 6C**), whereas they were "cytoskeleton," "posttranslational modification, protein turnover, chaperones," "cell cycle control, cell division, chromosome partitioning,"

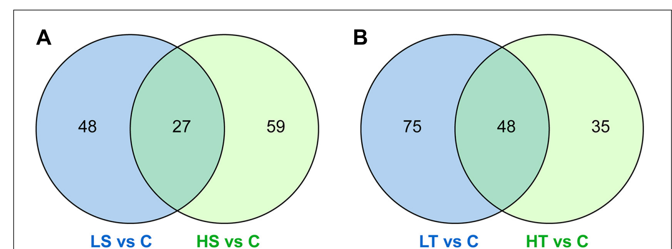

FIGURE 3 | Venn diagrams showing the common differential abundance proteins identified from the stolon of *Ciona robusta* exposed to salinity and temperature stresses. **(A)** Salinity stresses and **(B)** temperature stresses. HS, high salinity; LS, low salinity; HT, high temperature; LT, low temperature; C, natural seawater control.

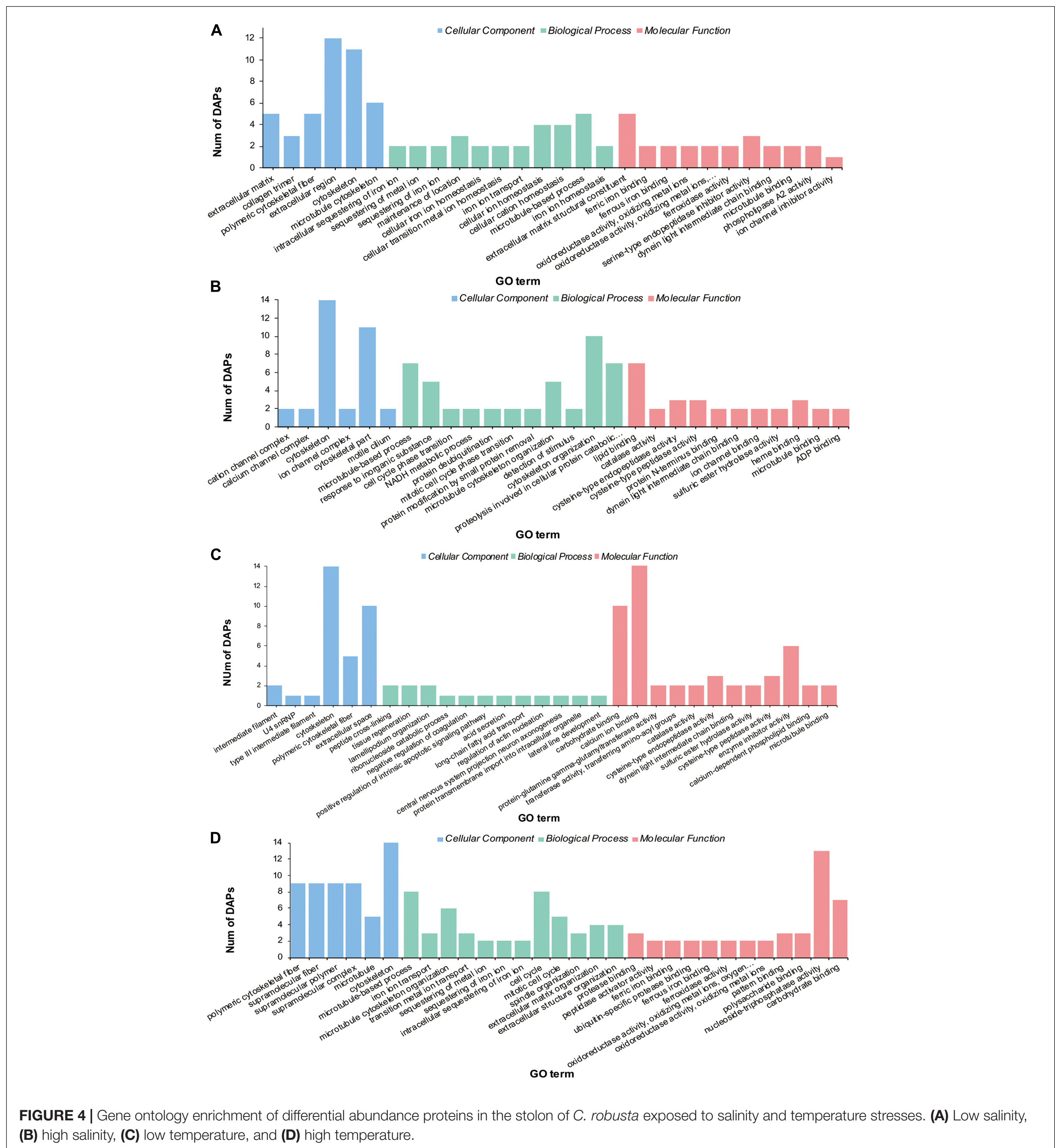

FIGURE 4 | Gene ontology enrichment of differential abundance proteins in the stolon of *C. robusta* exposed to salinity and temperature stresses. **(A)** Low salinity, **(B)** high salinity, **(C)** low temperature, and **(D)** high temperature.

"inorganic ion transport and metabolism," and "signal transduction mechanisms" in the HT group (**Figure 6D**).

Protein Interaction Analysis

A total of 27 and 48 DAPs were identified by using the PPI method from the stolon exposed to salinity and temperature stresses, respectively (**Figure 7**). After hiding the disconnected nodes, only 22 and 29 proteins were correlated to each other in the salinity and temperature networks, respectively. Among these DAPs, one KEGG pathway "ribosome" was significantly enriched under high and low salinity stresses, while two KEGG pathways, "ribosome" and "phagosome," were significantly enriched under high and low temperature stresses, respectively.

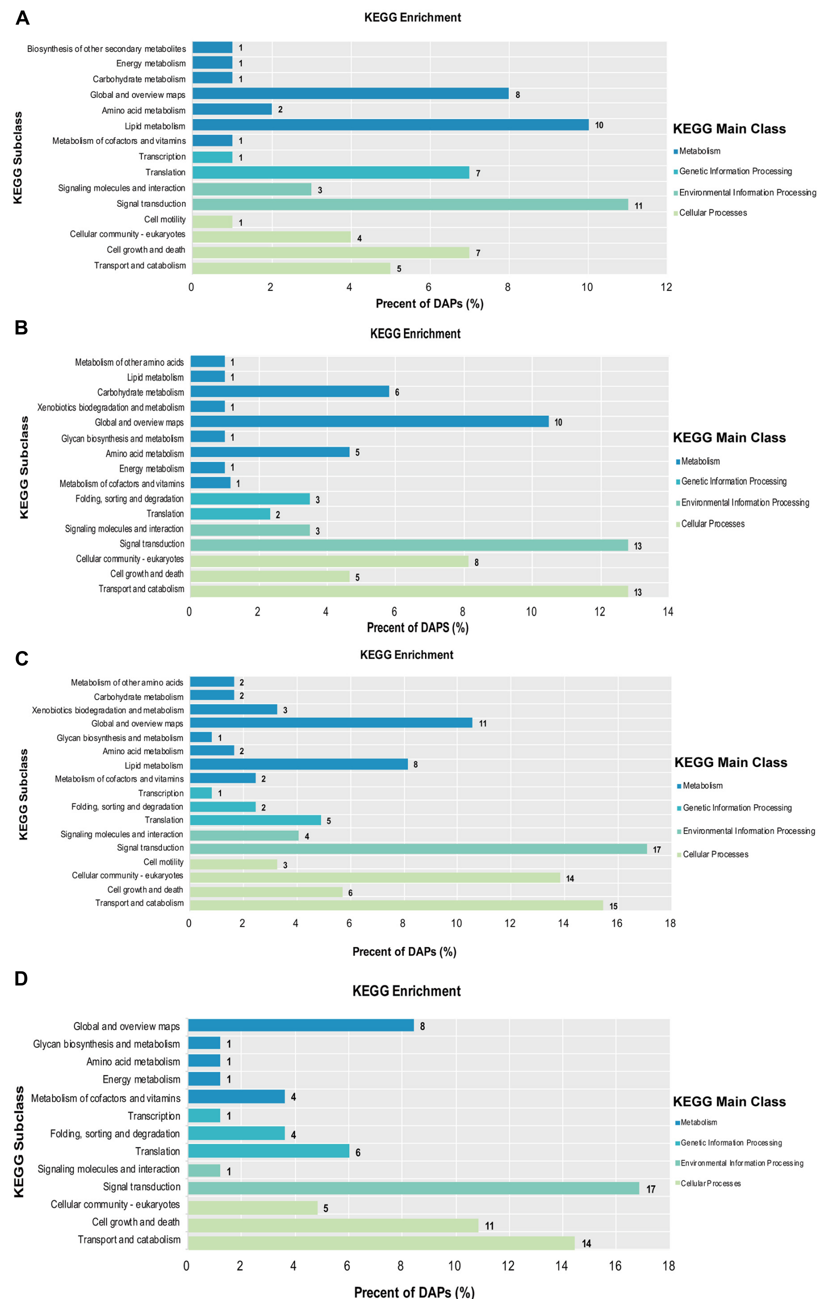

FIGURE 5 | Kyoto encyclopedia of genes and genomes enrichment analysis of the differential abundance proteins in the stolon of *C. robusta* exposed to salinity and temperature stresses. **(A)** Low salinity, **(B)** high salinity, **(C)** low temperature, and **(D)** high temperature. The number represents the number of proteins enriched to the pathways.

DISCUSSION

Proteomic Response to Salinity Stress

In aquatic species, salinity is a common environmental stressor that can remarkably affect metabolism and osmotic regulation of various organisms (Vargas-Chacoff et al., 2015). Marine animals can maintain their most critical physiological functions through multiple molecular mechanisms when exposed to salinity stresses, despite that there are alterations in osmotic pressure and cytoplasmic composition in their cells (Rautsaw et al., 2020). In our study, the identified DAPs in *C. robusta* exposed to both low and high salinity stresses were significantly enriched in the pathways "posttranslational modifications (PTMs)," "cytoskeleton," and "signal transduction," suggesting the involvements of these pathways in the responses of stolon to salinity stress.

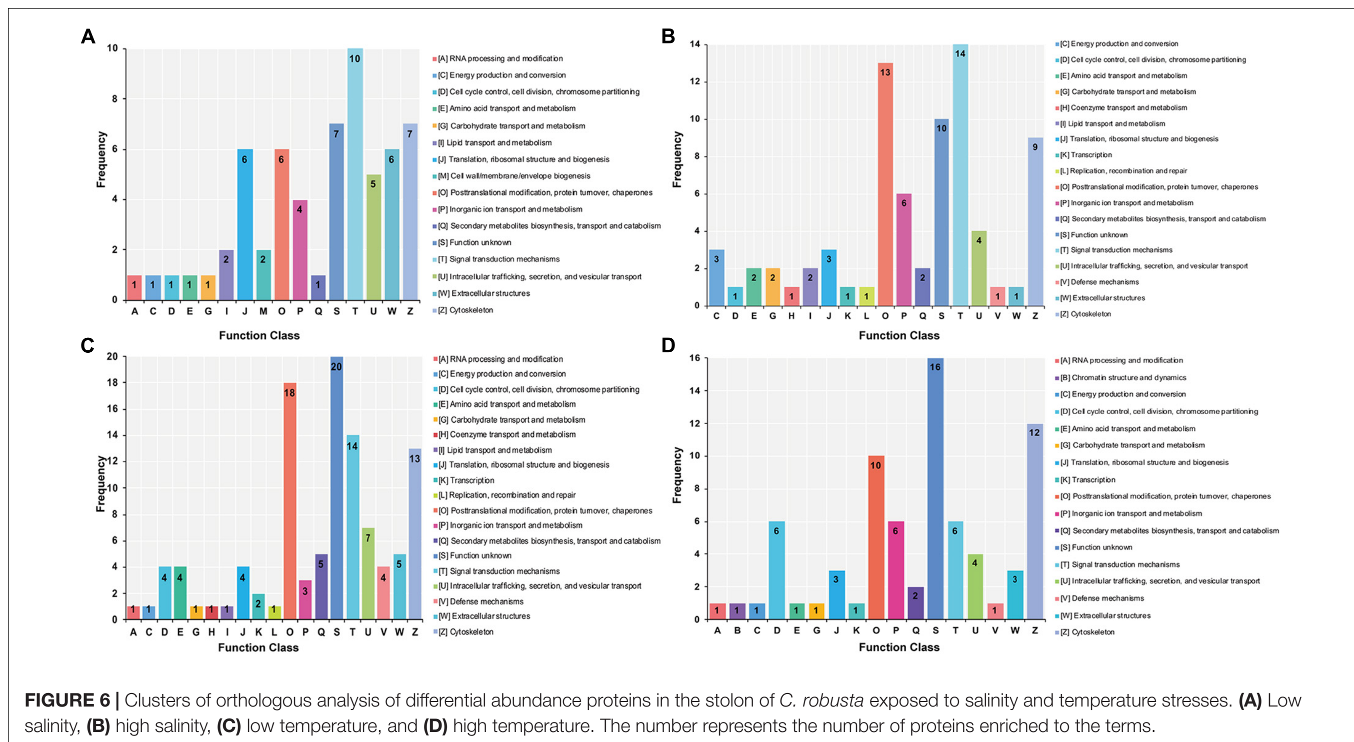

FIGURE 6 | Clusters of orthologous analysis of differential abundance proteins in the stolon of *C. robusta* exposed to salinity and temperature stresses. **(A)** Low salinity, **(B)** high salinity, **(C)** low temperature, and **(D)** high temperature. The number represents the number of proteins enriched to the terms.

During their synthesis, package, and release, proteins are successively modified. The PTMs, such as phosphorylation, glycosylation, and hydroxylation, are crucial for controlling protein conformation and increasing proteome complexity in organisms (Cloutier and Coulombe, 2013). PTM is indeed a common characteristic of numerous adhesive proteins of marine organisms (Flammang et al., 2015). In these PTMs, protein glycosylation has emerged as an important biochemical process in marine bioadhesion. An example is the marine mussel adhesive protein Pvfp-1, which has extensive threonine O-glycosylation (Zhao et al., 2009). Protein hydroxylation was found in all the plaque proteins (Mfp-1 to Mfp-6) of mussels and cement proteins (Pc-1 and Pc-2) of tubeworms (Waite et al., 2005; Lee et al., 2011). In addition, phosphoproteins have also been detected from the mussel foot proteins such as Mcfp-5 and Mcfp-6 and tubeworm cement proteins such as Pc-3A and Pc-B (Zhao et al., 2005; Zhao and Waite, 2006). Our results here indicated that the response of the *C. robusta* stolon to salinity stresses might be regulated by PTMs (**Figure 6**). Such a finding provides new evidence of PTMs to implicate in stolon attachment processes of *C. robusta*, although the PTMs have not been detected in the adhesive proteins of this species so far.

We found that several DAPs associated with salinity stresses were significantly enriched in the "cytoskeleton" term (**Figure 4**). Cytoskeletal proteins provide structural organization to cells in eukaryotic organisms (Bogatcheva and Machado, 2020). They are involved in transmitting important regulatory signals during cell activation, keeping the cellular structure stable, and maintaining signal communication between different cells (Bogatcheva and Machado, 2020). Furthermore, the cytoskeletal proteins may have major roles in adjusting biological rhythms by sensing environmental parameters (Artigaud et al., 2014). Therefore, *C. robusta* may adjust its cytoskeleton management strategy when exposed to salinity stresses, thereby consolidating the cell growth efficiency and further maintaining the structural stability of the stolon. This may be a fact that the stolon can secret some adhesive proteins that permanently glue the tunicate to the substrate under harsh salinity stresses.

Signal transduction pathway is a vital biochemical process in which cells convert extracellular signals from the surrounding environments into intracellular specific reactions (Kling, 1998). The signal transduction is initiated as one of the classic strategies to deal with environmental salinity changes in most aquatic animals (Buckley et al., 2006; Zhang et al., 2015; Dou et al., 2018; Li Y. et al., 2021). In our study, the identified DAPs were significantly enriched in "signal transduction" pathway, indicating the importance of signal transduction in coping with salinity stresses (**Figure 5**). Indeed, previous studies have confirmed that signal transduction could help aquatic organisms perceive their environmental changes (van der Geer, 2013). Moreover, a study showed that signal transduction-related pathways, such as G protein-coupled receptor, Ras GTPase, and P13K/Akt/mTOR pathways, could regulate the response of the oyster *Crassostrea gigas* to salinity stimulation (Zhang et al., 2015). Li Y. et al. (2021) reported that the MAPK signaling pathway was activated in the gills of the razor clam *Sinonovacula constricta* after exposure to salinity stresses (Li Y. et al., 2021). All these findings suggest that signal transduction-related pathways were activated under salinity challenges. Our results here provide one more layer of evidence to support this conclusion, further confirming that the *C. robusta* stolon could respond to salinity stresses by activating various signal transduction pathways.

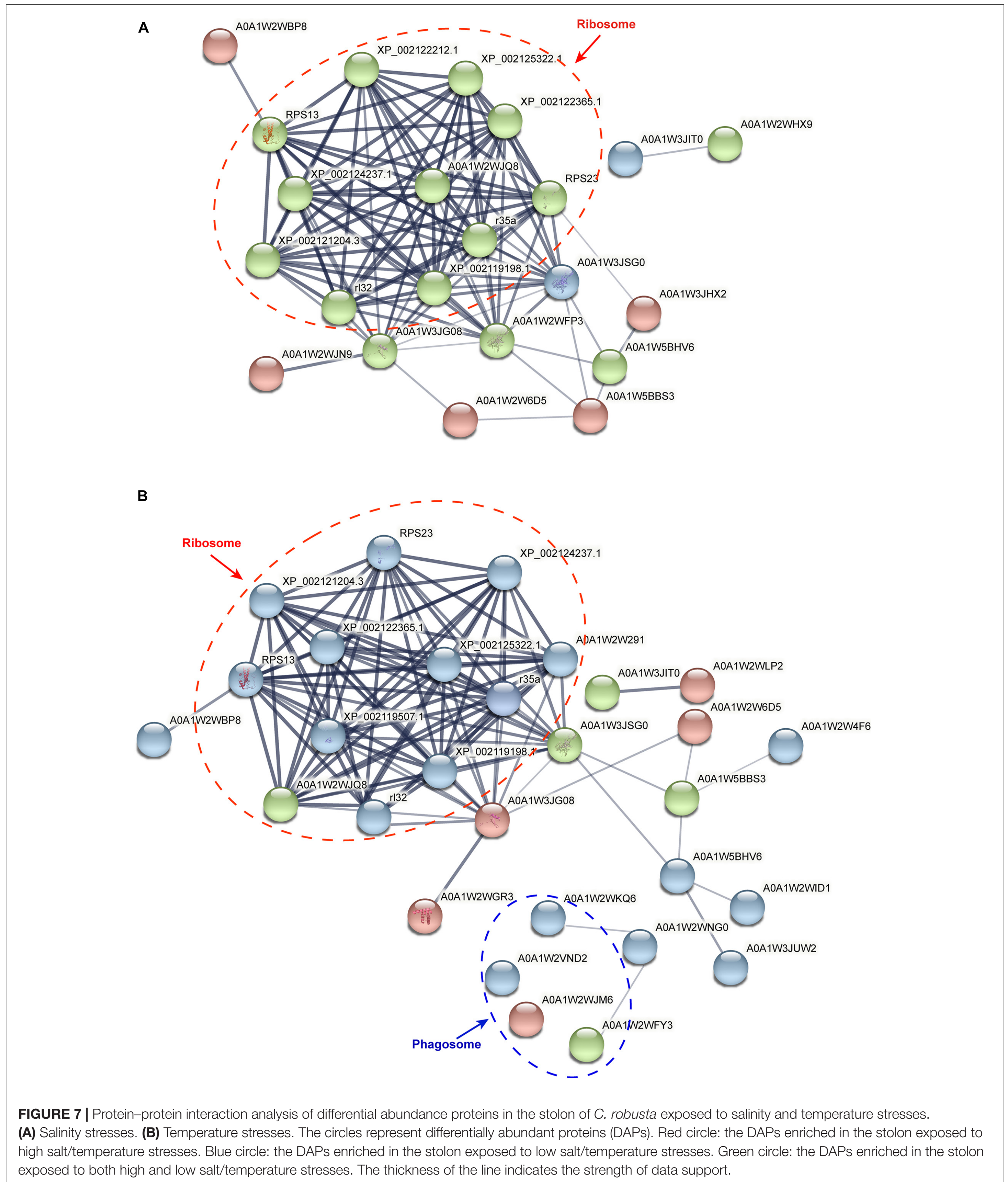

FIGURE 7 | Protein–protein interaction analysis of differential abundance proteins in the stolon of *C. robusta* exposed to salinity and temperature stresses. **(A)** Salinity stresses. **(B)** Temperature stresses. The circles represent differentially abundant proteins (DAPs). Red circle: the DAPs enriched in the stolon exposed to high salt/temperature stresses. Blue circle: the DAPs enriched in the stolon exposed to low salt/temperature stresses. Green circle: the DAPs enriched in the stolon exposed to both high and low salt/temperature stresses. The thickness of the line indicates the strength of data support.

The "extracellular matrix (ECM)" term was significantly enriched in the stolon exposed to low salinity. ECM is a complex and dynamic structure that provides the scaffold wherein cells are located. Apart from its function as the principal scaffold of cells, ECM provides the signals regulating cell behaviors and triggers multiple biological activities that are essential for tissue

morphological homeostasis (Theocharis et al., 2016). A previous study revealed that *Botrylloides nigrum* and *Botryllus planus* ascidians exhibited dramatic morphological response to low salinity stresses, including the expansions of cloacal cavities and distensions of pharyngeal baskets and neural glands (Dijkstra and Simkanin, 2016). Similarly, the changes in the ECM of *C. robusta* stolon might enhance its defense ability to low salinity stresses by regulating morphological homeostasis of stolon. In addition, the ECM in stolon might not only provide physical scaffolds but also regulate many cellular processes by inducing growth, migration, differentiation, and morphogenesis to respond to salinity changes, and this has been reported in the rough skin sculpin *Trachidermus fasciatus* and the mud crab *Scylla paramamosain* (Theocharis et al., 2016; Ma et al., 2018; Zhang et al., 2020).

We also observed the significant enrichment of the DAPs associated with the "transport and catabolism" in the stolon exposed to high salinity stress. An earlier study on the sea cucumber *Holothuria leucospilota* showed that the protein-dominated catabolism was significantly enhanced under low salinity stresses, whereas the carbohydrate- or lipid-dominated catabolism was significantly enhanced under high salinity stresses (Yu et al., 2013). The proteomic response of *C. robusta* stolon to higher salinity might be consistent with that of sea cucumber, and the response mechanisms of stolon to hypersaline stresses likely rely on the activation of carbohydrate/lipid transport and catabolism that can produce energy.

Proteomic Response to Temperature Stress

Temperature is known to significantly affect marine organisms' physiology (Pineda et al., 2012; Dong et al., 2019; Irvine et al., 2019). Indeed, temperature changes can affect the adhesive organs/structures of marine organisms. A typical case is byssus adhesion reduction in mussels caused by heat treatment, which has been considered an antifouling strategy for mussels (Perepelizin and Boltovskoy, 2011). Our results here illustrate that the stolon of *C. robusta* was also influenced by temperature changes at the molecular level. We found the significant enrichment of the term "cytoskeletal proteins" in *C. robusta* stolon exposed to temperature challenges (**Figure 4**). Similarly, earlier analyses showed that the changes in the abundance of cytoskeletal proteins in the ascidians *Ciona intestinalis* and *Ciona savignyi* were closely associated with thermal stresses (Serafini et al., 2011). Studies on marine teleosts and invertebrates also documented the recombination of the temperature-induced cytoskeletal structure (Vornanen et al., 2005; Buckley et al., 2006; Lockwood et al., 2010; Tomanek and Zuzow, 2010; Jayasundara et al., 2015). Such available evidence suggests that the cytoskeletal proteins in the *C. robusta* stolon might be involved in temperature stress response through two mechanisms: the alterations in cytoskeletal protein abundance and recombination of the cytoskeletal structure.

Molecular chaperones are involved in the folding or unfolding of proteins and the assembly or disassembly of larger macromolecular complexes (Cloutier and Coulombe, 2013). They are found in all cell types in animals, and their activities are tightly regulated to maintain normal cell functions. Previous studies showed that the higher steady-state levels of molecular chaperones might underlie the capacity of *C. intestinalis* to out-compete *C. savignyi* in warm habitats (Serafini et al., 2011). Meanwhile, many molecular chaperones, including heat shock proteins 24, heat shock proteins 90, and calcium-binding protein, were likely to act as chaperones for maintaining the cytoskeletal structure under temperature stresses, preventing the aggregation of the denatured proteins (Lockwood et al., 2010; Lopez et al., 2017). The significant enrichment of chaperoning molecules in our study demonstrates that homeostasis regulation is a crucial mechanism for the *C. robusta* stolon to respond to temperature stresses. These molecular chaperones likely help the *C. robusta* stolon achieve stable cytoskeletal structure.

Besides cytoskeleton and molecular chaperones, the term of "calcium-binding proteins" was also significantly enriched in the *C. robusta* stolon under both low and high temperature stresses. Ca^{2+} is a highly universal intracellular signal regulating several molecular pathways and cellular processes, including cell proliferation, excitability, exocytosis, and transcription (Berridge et al., 2003; Clapham, 2007). Intracellular calcium-binding proteins can be divided into two categories. One category consists of proteins with Ca^{2+}-binding function, which can transport Ca^{2+} across cell membranes and thus irreversibly regulate the concentration of Ca^{2+} in the surrounding environments. Another category can decode Ca^{2+} signals into functionally specific signals (Carafoli et al., 2001). An investigation showed that the signaling triggered *via* regulating the intracellular Ca^{2+} concentration may be involved in the thermal response of the oyster *C. gigas* (Zhang et al., 2015). We cannot determine the specific functions of calcium-binding proteins in *C. robusta* stolon, but the enrichment of these proteins in our study suggests that calcium signaling in the stolon should be one of the important molecular mechanisms in response to temperature stresses.

The "phagosome" pathway was enriched in the stolon exposed to both low and high temperature stresses in our study (**Figure 7**). Phagocytosis is an important congenital defense mechanism for macrophages against bacterial infections (Pradhan et al., 2018). In eukaryotes, phagosomes can engulf potential pathogenic microorganisms and apoptotic cells through binding to the lysosome (Kinchen and Ravichandran, 2008). The enrichment of DAPs in the "phagosome" pathway under temperature stresses implies that temperature might be a remarkable environmental factor leading to the apoptosis of stolon cells in *C. robusta*. The phagosome is a highly dynamic organelle, even in healthy individuals, and it can phagocytose billions of dead cells per day. However, under stress conditions, they were more inclined to eliminate apoptotic cells (Dean et al., 2019). Therefore, the phagosome may assist in maintaining the homeostasis of stolon cells and the structural stability *via* phagocytizing apoptotic cells in the *C. robusta* stolon.

For most organisms, the dynamic equilibrium of lipid metabolism is the fundamental physiological status in maintaining vital activities (Gu et al., 2017). This means that some lipids are constantly oxidized to meet metabolic needs,

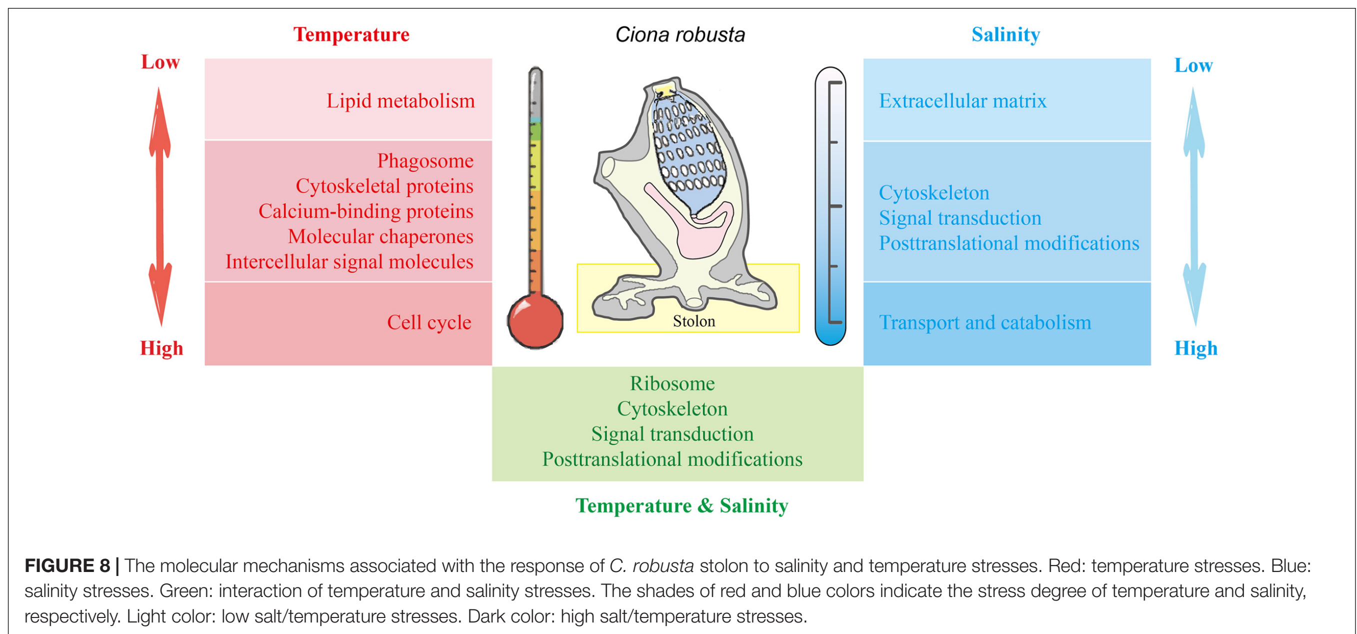

FIGURE 8 | The molecular mechanisms associated with the response of *C. robusta* stolon to salinity and temperature stresses. Red: temperature stresses. Blue: salinity stresses. Green: interaction of temperature and salinity stresses. The shades of red and blue colors indicate the stress degree of temperature and salinity, respectively. Light color: low salt/temperature stresses. Dark color: high salt/temperature stresses.

whereas others are synthesized and stored (Zhang et al., 2017a; Gyamfi et al., 2018). Chilling stress is a common challenge to many aquatic organisms, and responding to chilling stress requires lipid metabolic reprogramming. Eventually, animals usually generate more energy by degrading some lipids to resist cold stresses (Brodte et al., 2008). The enrichment of the "lipid metabolism" pathway has been found in many marine organisms such as *Eleginops maclovinus*, *M. galloprovincialis*, and *Pelteobagrus vachelli* in response to low temperature stresses (Brodte et al., 2008). Our study illustrated that "lipid metabolism" was the only pathway enriched in the *C. robusta* stolon exposed to low temperature. Mechanistically, lipid metabolism may provide more energy for the *C. robusta* stolon by suppressing the lipogenesis capacity and enhancing the lipolysis capacity during chilling stresses. Unlike the mechanism by which the stolon responded to low temperature, the "cell cycle" term exhibited a significant enrichment in the stolon in response to high temperature stress. Heat is a common stress during ascidian invasions and failure to adapt to this stressor can result in programmed cell demise and decreased viability (Carafoli et al., 2001). Previous studies found that marine organisms could respond to heat stress *via* the arrested cell cycle progression. For example, the marine teleost *Gillichthys mirabilis* could conserve energy through inhibiting body cell growth and proliferation to cope with thermal challenges (Buckley et al., 2006). *C. robusta* may follow this mechanism and its stolon can inhibit cell growth and eliminate the damaged cells, assisting stolon in preserving energy to defend against high temperature stresses.

Common Response to Both Types of Environmental Stresses

Despite the stress-specific pathways observed in this study, the response to both temperature and salinity stresses was not completely independent. Venn diagram analysis revealed that "cytoskeleton," "signal transduction," and "posttranslational modification" were the overlapping pathways in both salinity and temperature stresses. The common response was also detected by several studies, showing that ascidians mitigated adverse environmental challenges through similar mechanisms (Serafini et al., 2011; Huang et al., 2019; Li H. et al., 2020; Wei et al., 2020; Chen et al., 2021).

Based on PPI analysis, the DAPs enriched in "ribosome" pathway were changed significantly under both salinity and temperature stresses, indicating that these stresses may affect protein synthesis and metabolism in *C. robusta* stolon (**Figure 7**). Marine organisms usually rely on the regulation of proteins related to stress response, such as cytoskeletal proteins and adhesive proteins, to maintain cellular homeostasis and limit the adverse effects of harmful environmental stimuli (Vind et al., 2020). Cytoskeletal proteins, which are used to sustain the stable state of cellular/organ structure and communication, are synthesized by ribosomes (Bashline et al., 2014; Bogatcheva and Machado, 2020). Additionally, the proteins related to adhesive functions in marine fouling organisms are also synthesized by ribosomes (Lafontaine and Tollervey, 2001; Jimenez et al., 2019). Previous studies have indicated the presence of adhesive proteins synthesized by ribosomes in the stolon of *C. robusta* (Li et al., 2019). The enrichment of "ribosome" pathway in *C. robusta* may be helpful for the stolon to synthesize some proteins associated with the defense against salinity and temperature stresses.

CONCLUSION

By using iTRAQ technique, our study revealed the dynamic proteomic response to salinity and temperature stresses in the stolon of the highly fouling ascidian *C. robusta*. Functional enrichment analysis recovered common and

challenge-specific response pathways (**Figure 8**). In addition to the common pathways such as "cytoskeleton," "signal transduction," and "posttranslational modification," "extracellular matrix," "carbohydrate/lipid transport and catabolism," "lipid metabolism," and "cell cycle" play crucial roles in dealing with the stresses of low salinity, high salinity, low temperature, and high temperature, respectively. The findings here illustrate that *C. robusta* stolon could respond to environmental challenges by developing complex and diverse molecular mechanisms. The pathways and associated proteins obtained in this study provide candidate references for further studies of molecular mechanisms of marine biofouling, local environmental adaptation during invasions, and formulating antifouling strategies in marine and coastal ecosystems.

DATA AVAILABILITY STATEMENT

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the iProX partner repository (Ma et al., 2019) with the dataset identifier PXD026891.

AUTHOR CONTRIBUTIONS

AZ, SL, and XL conceived the study. XL and SL designed the experiments, conducted the experiments, analyzed the data, and wrote the manuscript. JC and RF analyzed the data. All authors contributed to the revisions of this 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.2021.761628/full#supplementary-material

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