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Research Article

Identification and Verification of the Blue Pigment in the Edible Jellyfish (*Rhopilema esculentum*)

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Rhopilema esculentum is widely cultured in Chinese coastal regions and has thousand years of consumption history in China. Despite *R. esculentum* owns multiple colors (mainly ochre and blue) in aquaculture ponds, little is known about the pigments and color formation. This study identified and verified the blue pigment of *R. esculentum*. Results showed that the blue pigment of *R. esculentum* has 262 amino acids with 54.96% identity to the blue pigment protein precursor of *Rhizostoma pulmo*. The blue pigment of *R. esculentum* has Kringle and Frizzled domains, which is in accordance with rhizostomins characteristics. Compared to ochre *R. esculentum*, the expression level of blue pigment increased 3.49-fold in blue *R. esculentum*. In addition, overexpression of the blue pigment in *Rhodosporidium toruloides* made transformant cells blue under blue light excitation. Our findings provide knowledge for the blue color formation of *R. esculentum* and will benefit the color selection of *R. esculentum* with excellent traits.

1. Introduction

Rhopilema esculentum (Cnidaria, Scyphozoa, Rhizostomidae) is distributed in the seas of Korea, Japan, and China [1] and is widely cultured in coastal areas of China. As a medicine food homology jellyfish, *R. esculentum* shows potential in food, medical, and healthcare industries since it is rich in nutrient and bioactive compounds [2–6]. Due to overfishing and deterioration of the habitat, *R. esculentum* population has been decreasing [1] and fails to meet the consumers' needs in spite of stock enhancement and resource release [7]. Screening of *R. esculentum* species with excellent traits is a vital strategy to address this problem.

For aquatic animals, color is an important quality criteria and is related to perceived quality, consumer acceptability, and price paid [8, 9]. Investigating color formation of aquatic animals is beneficial for color breeding. *R. esculentum* owns multiple color phenotypes (mainly blue and ochre) in aquaculture ponds; however, the color formation of these *R. esculentum* is unknown, which hinders the process of color breeding. In jellyfish, pigments have been found associated with their color formation, including blue, brown, and magenta [10–13]. Among these pigments, blue pigments have been found in many Scyphozoan species, such as *Rhizostoma pulmo* [14], *Cassiopea xamachana* [10], *Stomolophus* sp. 2 [12], and *Stomolophus meleagris* [13]. Meanwhile, the chemical characteristics of these blue pigments have been well studied in reported Rhizostomeae jellyfish [10–13, 15]. As the scarce pigment in nature, blue pigments have great potential in aquaculture, food, health industries, and cosmetics [16, 17]. Therefore, investigating blue pigments of *R. esculentum* is not only beneficial for revealing the blue color formation but also for further exploitation and utilization of *R. esculentum* resources.

With the advent of high-throughput sequencing technologies, omics methods have been well applied in investigating

the color formation of farmed plants and animals [8, 14, 18–20]. Omics methods have been applied in investigating the life cycle and toxins of *R. esculentum* except for color formation and pigments [7, 21–23]. The potential genes and metabolics regulating the strobilation of *R. esculentum* were found by transcriptomic and metabolomic methods [21, 24]. The putative toxins of *R. esculentum* were predicted by genomic and proteinomic methods [7, 22]. In this study, the color phenotypes of ochre and blue *R. esculentum* were observed and the blue pigment of *R. esculentum* was identified and verified with the help of proteomic method. In addition, the molecular characteristics of blue pigment were analyzed. These results provide an understanding of blue color formation of *R. esculentum* and will be beneficial for the color selection of *R. esculentum* with excellent traits.

2. Materials and Methods

2.1. R. esculentum. Both adult blue and ochre *R. esculentum* were collected from the same aquaculture pond in Yingkou city, China. The outer epidermises of *R. esculentum* umbrella were extracted as experiment materials for comparative proteomic analysis. For each proteomic group, three biological replicates were conducted.

2.2. Comparative Proteomic Analysis between Blue and Ochre R. esculentum. One hundred milligram experiment materials were ground in liquid nitrogen and lysed using PASP lysis buffer (100 mM NH_4HCO_3, 8 M urea, pH 8), followed by 5 min ultrasonication on ice. Then, the lysate was centrifuged at 12,000 *g* for 15 min (4°C) and the supernatant was collected and reduced with 10 mM DTT for 1 hr at 56°C, followed by alkylation with sufficient IAM for 1 hr in the dark at room temperature. After that, precooled acetone with a fourfold volume was added into the solution and vortexed, followed by more than 2 hr incubation at −20°C. The mixed solution was centrifuged at 12,000 *g* for 15 min (4°C) and the precipitation was collected. The precipitation was washed with 1 mL cold acetone and dissolved with dissolution buffer (8 M urea, 100 mM TEAB, pH 8.5) as the protein sample.

The concentration and quality of protein sample was measured by Bradford protein quantitative kit and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), respectively. Twenty microgram protein sample was loaded onto a 12% SDS-PAGE gel and runned by electrophoresis for 110 min, including 20 min at 80 V and 90 min at 120 V. Then, the gel was stained by Coomassie brilliant blue R-250 and decolored until the bands were visualized clearly.

Protein sample was diluted to 100 μL with DB dissolution buffer (8 M urea, 100 mM TEAB, pH 8.5) and digested at 37°C for 4 hr by adding sufficient trypsin and 100 mM TEAB buffer. After that, the protein sample was digested again by 1.5 μL trypsin and 2 μL 1 M $CaCl_2$ overnight. Then, the digested sample was mixed with formic acid (adjusted to pH < 3) and centrifuged at 12,000 *g* for 5 min. The supernatant was collected, loaded to the C18 desalting column slowly, washed with washing buffer (0.1% formic acid, 3% acetonitrile) three times, and eluted with elution buffer (0.1% formic acid, 70% acetonitrile). The eluted sample was collected and lyophilized. For the labeling reaction, 100 μL 0.1 M TEAB buffer and 41 μL acetonitrile-dissolved TMT labeling reagent were added to the lyophilized sample in order. The reaction solution was mixed and shaken for 2 hr. After that, the labeling reaction was stopped by addition of 8% ammonia and all labeled sample was mixed with equal volume, desalted, and lyophilized.

The lyophilized sample was dissolved in 2% acetonitrile (adjusted pH to 10.0 using ammonium hydroxide) and centrifuged at 12,000 *g* for 10 min. The supernatant was collected and fractionated using a C18 column (Waters BEH C18, 4.6 × 250 mm, 5 μm) on a Rigol L3000 HPLC system, setting the column oven at 45°C. All fractions were dried under vacuum and resolved in 0.1% (v/v) formic acid.

EASY-nLC^{TM} 1200 UHPLC system (Thermo Fisher Scientific) coupled with Q Exactive^{TM} HF-X mass spectrometer (Thermo Fisher Scientific) was used to analyze the fractions. One microgram fractured sample was injected into a C18 Nano-Trap column (4.5 cm × 75 μm, 3 μm), and the peptides were separated in an analytical column (15 cm × 150 μm, 1.9 μm). The separated peptides were analyzed by the Q Exactive^{TM} HF-X mass spectrometer (Thermo Fisher Scientific) and the top 40 precursors with the highest abundant in the full scan were selected, fragmented, and analyzed in MS/MS.

The spectra generated by MS/MS were searched against the constructed *R. esculentum* protein database using Proteome Discoverer version 2.4 (PD 2.4), setting mass tolerance for precursor ion 10 ppm and product ion 0.02 Da. Peptide-spectrum matches (PSMs) with credibility of more than 99% were retained, and the identified protein contains at least one unique peptide. The PSMs and proteins were controlled with the false discovery rate (FDR) of less than 1.0%. *t*-test was used for quantitative analysis of protein expressions and proteins with fold change ≥1.2 or ≤0.83 ($p < 0.05$) were defined as differentially abundant proteins (DAPs). DAPs were used for cluster heat map analysis and enrichment analysis of Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways [25].

2.3. Bioinformatics Analysis of the Upregulated Proteins in Blue R. esculentum. The amino acid sequence of protein was used for bioinformatics analysis. The conserved domain of protein was analyzed using CD-Search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi); the homologous proteins were searched using NCBI-blastp (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome); the signal peptide of protein was analyzed using SignalP-5.0 (https://services.healthtech.dtu.dk/service.php?SignalP-5.0); the transmembrane region of the protein was predicted using TMHMM-2.0 (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0); sequence alignment between homologous proteins was performed using MAFFT-v7.505 (https://mafft.cbrc.jp/alignment/server/).

2.4. Characterization of the Blue Pigment in Blue R. esculentum. Among the top 10 upregulated proteins in blue *R. esculentum*, mRNA.RE15725 was considered as the

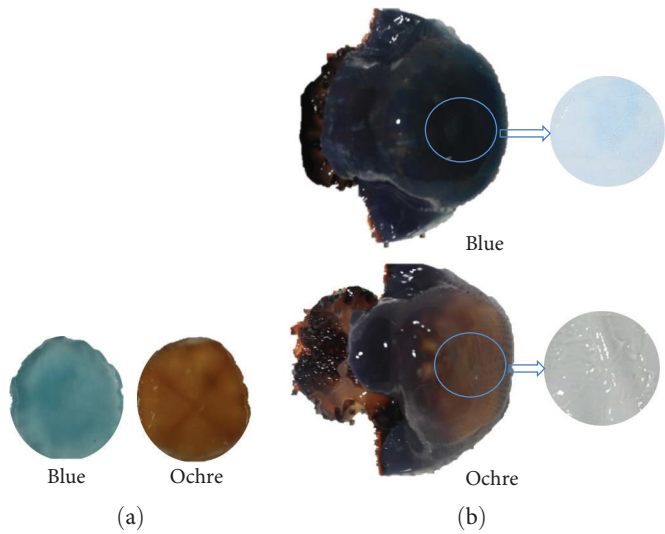

FIGURE 1: Color observation of blue and ochre *R. esculentum*. (a) Inner gastrodermis of blue and ochre *R. esculentum*. (b) Outer epidermis of blue and ochre *R. esculentum*.

potential blue pigment. After codon optimization, the potential blue pigment encoding gene was transformed to *Rhodosporidium toruloides* using Agrobacterium-mediated method [26]. Based on the vector pZPK, we construced overexpression vector pZPK-pGK-Nrs-tNOS-pXYL-Blup-tHSP (*Supplementary 1*). The colors of *R. toruloides* transformants were observed using Lecia DM 6B under blue light excitation (405 nm) and emission wavelength (480 nm).

3. Results

3.1. Body Color Observation of R. esculentum. In aquaculture ponds of Yingkou city, most *R. esculentum* are blue and ochre (*Supplementary 2*). For blue and ochre *R. esculentum*, we observed that their colors were consistent with the colors of inner gastrodermis (Figure 1). The color of inner gastrodermis is blue in blue *R. esculentum* while ochre in ochre *R. esculentum* (Figure 1). Further observation of the outer epidermis found that it is blue for blue *R. esculentum* while transparent for ochre *R. esculentum* (Figure 1).

3.2. Comparative Proteomic Analysis. A total of 3,741 proteins were identified in the outer epidermis of *R. esculentum* with an annotation ratio of 95.30%. Compared to ochre *R. esculentum*, 38 differentially expressed proteins (DEPs) were identified in blue *R. esculentum*, including 22 upregulated and 16 downregulated proteins (Figure 2(a)). Hierarchical cluster analysis of the DEPs revealed that the difference was low within groups but high between groups, indicating that the proteomic results were reliable (Figure 2(b)). GO enrichment analysis indicated that the DEPs were enriched in response to stress, metalloendopeptidase activity, pyridoxal phosphate binding, and so on (Figure 2(c)). KEGG enrichment analysis indicated that the DEPs were enriched in RNA transport, phenylalanine, tyrosine, and tryptophan biosynthesis, phenylalanine metabolism pathways, and so on (Figure 2(d)).

Further analysis showed that the 22 upregulated proteins in blue *R. esculentum* concentrated on GO terms like response to stress, translational elongation, and regulation of apoptotic process (Figure 3(a)) and KEGG pathways like Notch signaling pathway, FoxO signaling pathway, and Wnt signaling pathway (Figure 3(b)).

Among the top 10 upregulated proteins in blue *R. esculentum*, the molecular weights ranged from 11.9 to 90 kDa with fold change ranging from 1.44 to 3.96 (Table 1). mRNA.RE15725 (*Supplementary 3*) may be the potential blue pigment due to its similarity to the blue pigment protein precursor of *R. pulmo* (GenBank accession: AAS83462.1) with 54.96% identity and consistent with rhizostomins characteristics [27]. According to GO and KEGG annotations, function of mRNA.RE15725 was protein binding, while the involved metabolic pathways were unknown.

3.3. Characterization of the Blue Pigment. Sequence alignment analysis of mRNA.RE15725 and blue pigment protein precursor of *R. pulmo* revealed that they have 93 fully conserved residues, accounting for 35.50% amino acid sequence of mRNA.RE15725 (Figure 4(a)). For mRNA.RE15725, Frizzled and Kringle were adjacent to each other (Figure 4(b)). After transforming mRNA.RE15725 encoding gene into *R. toruloides*, the transformant cells turned blue under blue light excitation (Figure 5).

4. Discussion

Colors are produced by pigments and nanostructures in plants and animals [28, 29]. Cnidarian animals generated various pigments to be colorful, such as carotenoids from *Velella velella* [30], melanoid from *Pelagia noctiluca* [31], and rhizostomins from Scyphozoa [27]. Despite *R. esculentum* having multiple colors in aquaculture ponds, their pigments remain unknown. In this study, we observed that color of *R. esculentum* was consistent with the color of inner gastrodermis and the blue color of *R. esculentum* attributed to the blue pigment distributed in umbrella part. Blue pigments are relative scarcity in nature, and stable blue pigments are needed for industrial

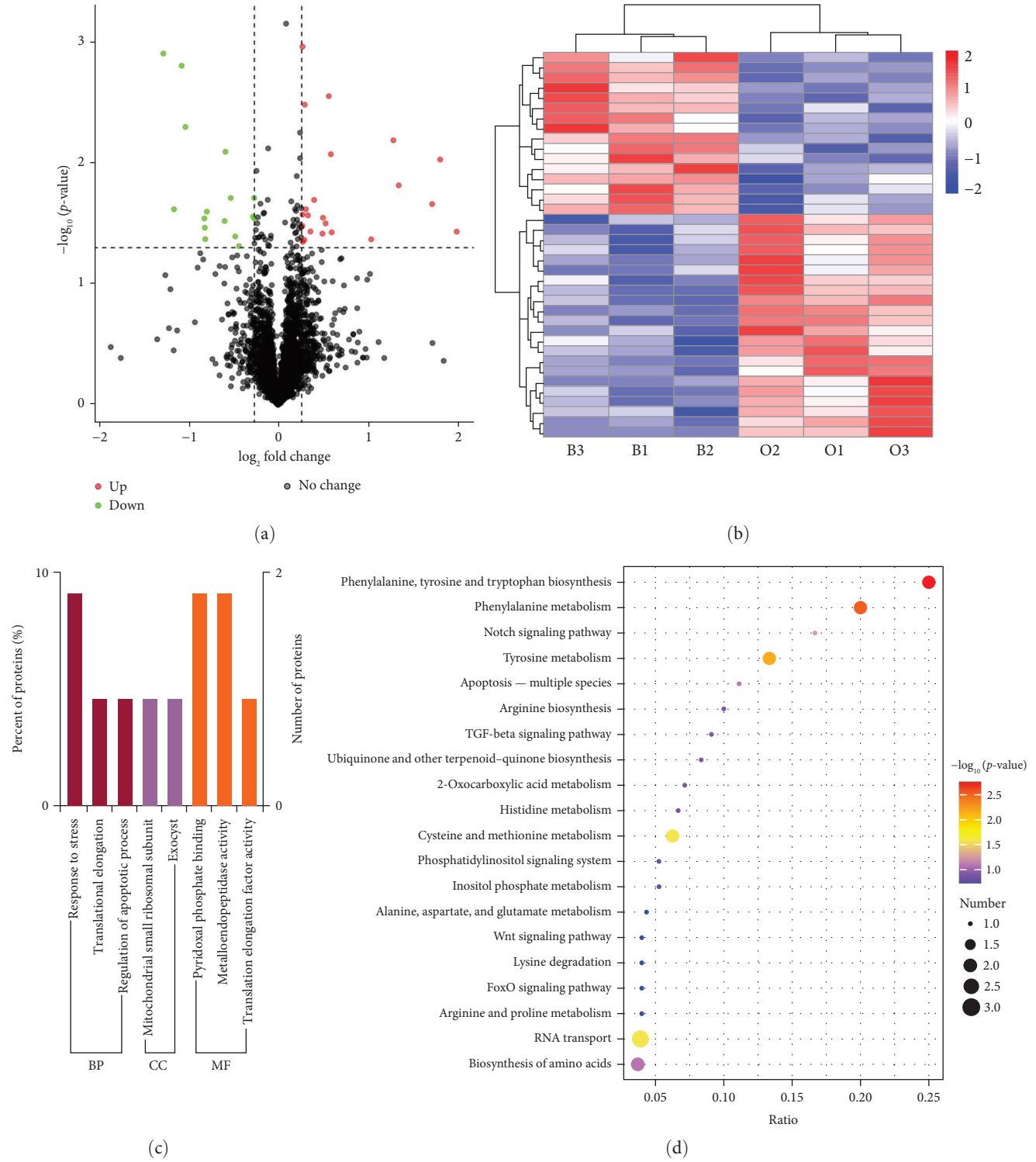

Figure 2: Comparative proteomic analysis of blue and ochre *R. esculentum*. (a) Volcano plot of the DEPs. (b) Hierarchically clustered heat map of the DEPs. (c) Enriched GO terms of the DEPs. (d) Enriched KEGG pathways of the DEPs. B1, B2, and B3 represent blue *R. esculentum* 1, 2, and 3, respectively; O1, O2, and O3 represent ochre *R. esculentum* 1, 2, and 3, respectively; BP represents biological process; CC represents cellular component; MF represents molecular function.

purposes [32, 33]. Several blue pigments have been found in Scyphozoa species, such as *Stomolophus* sp. 2, *C. xamachana*, and *R. pulmo* [10, 12, 15]. These blue pigments are proteins and play roles in biophysical and biochemical processes [27]. Despite we found the blue pigment of *R. esculentum*, little is known about the molecular background.

For identified the blue pigment in *R. esculentum*, we used comparative proteomic analysis between blue and ochre

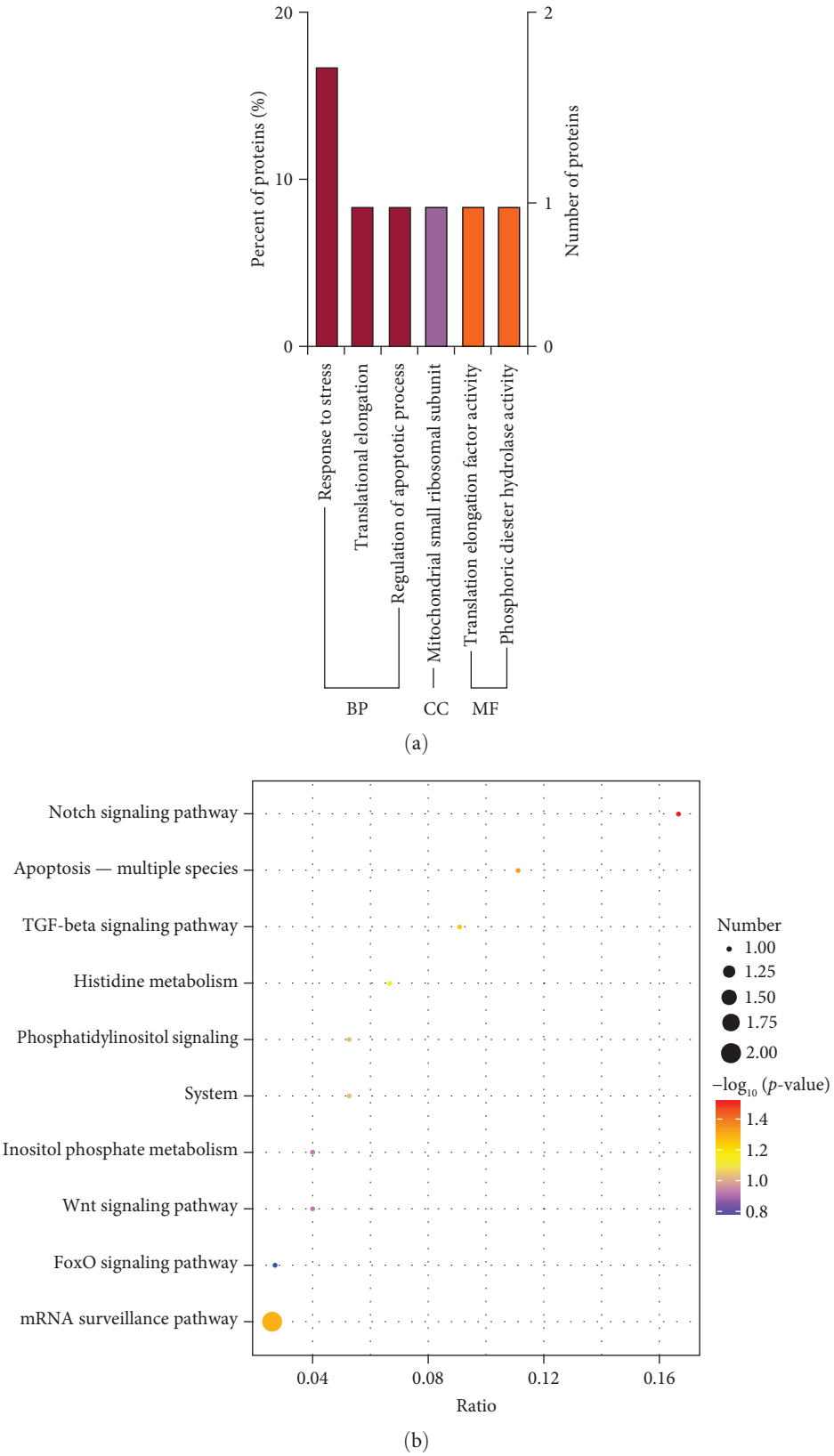

Figure 3: GO and KEGG enrichment analysis of the upregulated proteins in blue *R. esculentum*. (a) GO enrichment analysis. (b) KEGG enrichment analysis. BP represents biological process; CC represents cellular component; MF represents molecular function.

Table 1: Summaries of the top 10 upregulated proteins in blue *R. esculentum* *.

Name	ACN	MW (kDa)	FC	CD	HP (per identification)	SL
mRNA.RE16640	199	22.5	3.96	Commd superfamily	COMM domain-containing protein 4 (*Exaiptasia diaphana*) (57.29%)	–
mRNA.RE15725	262	29.3	3.49	Kringle, Frizzled	Blue pigment protein precursor (*Rhizostoma pulmo*) (54.96%)	–
mRNA.RE06462	324	36.3	3.28	–	Uncharacterized protein LOC107328915 isoform X2 (*Acropora digitifera*) (50.48%)	–
mRNA.RE12990	640	69.8	2.53	ZnMc_MMP, HX, PG_binding_1	Macrophage metalloelastase-like (*Xenia* spp. Carnegie-2017)(43.56%)	Plasma membrane protein
mRNA.RE13674	698	78.7	2.43	zf-TAZ superfamily	CREB-binding protein-like isoform X2 (*Portunus trituberculatus*) (29.63%)	Nucleus protein
mRNA.RE06167	151	16.8	2.05	USP_Like	Universal stress protein in QAH/OAS sulfhydrylase 3′ region-like (*Hydra vulgaris*) (50.68%)	–
mRNA.RE03241	284	32.4	1.51	–	–	–
mRNA.RE10340	483	54	1.50	CASc superfamily, death effector domain	CASP8 (*Sepia pharaonis*) (26.71%)	Cytoplasm protein
mRNA.RE06258	415	47.6	1.48	28S ribosomal protein S31	Mitochondrial (*Nanorana parkeri*) (36.84%) MRP-S31 superfamily	Mitochondrion protein
mRNA.RE11399	1,393	156.9	1.44	PH-GRAM1_TCB1D9_TCB1D9B, TBC, EFh superfamily	TBC1 domain family member 9B (*Hydra vulgaris*) (62.75%)	Centrosome protein

*ACN represents amino acid number; MW represents molecular weight; FC represents fold change; CD represents conserved domain; HP represents homologous protein; SL represents subcellular localization; "–" represents no results.

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mRNA.RE15725    M-KFLTAFFFLVAVGSAFIDATPVRSRLLEMLKEPVPLELEFCK-SLGYSLTSRVNFMKQ
AAS83462.1      MAKAIVAMLLLFFTNVEMTISSAVPAKMVQLLREKVPLNIEFCRLAMGYSETAKINFMQQ
                * * :.*:::*. ..  :  ::.* :::::::* ***::***: ::***  *:::*** : *

mRNA.RE15725    TNQSAVAQDYLYKALKIMHATGCSDFTKPYTCATYAPAFSKHYGALPPCRSLTRTEEQC
AAS83462.1      TNQSLVQQDRLFKALLTLSKFGCSELTEAYTCATYAPPVIAPYGALPPCRSLCKNVKGNC
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mRNA.RE15725    GNFNAAMAKLLSKGECKMTKKGGDYRGTVSKTSSGKSCQAWAAQTPHRHTKTAESHPNDD
AAS83462.1      DALTTAMAKHLANGECKMTTEGGDYRGKVSQTFDGVKCQAWDTQEPHRHSVTAKTHPNDG
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mRNA.RE15725    LTGNYCRNPDGEPNGPWCYTTTSKRWEYCDVPTCKVQFYCDYYPEASATQGCVDYKYNES
AAS83462.1      LESNYCRNPDGESKGPWCYTTSGRRWDYCAVPKCKVQFYCNYFPEPSENQGCVDYTYNRK
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mRNA.RE15725    SGNMEQVVKGNPVAPLDDWKIPKY
AAS83462.1      EAKLELLVRGNPRARIDPWAIPEY
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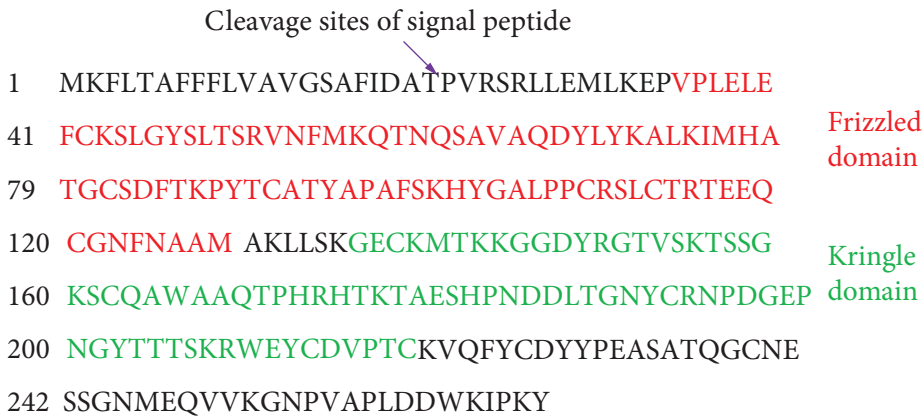
(a)



(b)

Figure 4: Functional analysis of mRNA.RE15725. (a) Amino acid sequence alignment between mRNA.RE15725 and AAS83462.1 (blue pigment protein precursor of *Rhizostoma pulmo*). (b) Functional analysis of mRNA.RE15725. "*" indicates positions, which have a single, fully conserved residue; ":" indicates that one of the "strong" groups is fully conserved; "." indicates that one of the "weaker" groups is fully conserved; the purple arrow indicated the predicted cleavage site of signal peptide; amino acid sequence highlighted in red font is Frizzled domain; amino acid sequence highlighted in green font is Kringle domain.

R. esculentum. Compared to ochre *R. esculentum*, the upregulated proteins in blue *R. esculentum* concentrated on GO terms such as response to stress, translational elongation, and regulation of apoptotic process, and KEGG pathways such as FoxO signaling and Wnt signaling; manifesting color of *R. esculentum* may be associated with stress response and signaling transduction. As the top second upregulated protein in blue *R. esculentum*, mRNA.RE15725 contains Frizzled domain, which is in accord with characteristics of rhizostomins [27] and associated with Wnt signal transduction pathway [34]. In addition, mRNA.RE15725 is homologous to the blue pigment protein precursor of *R. pulmo* with 54.96% identity and made *R. toruloides* cells blue by overexpression. Therefore, we hypothesized that mRNA.RE15725 was the blue pigment of *R. esculentum*.

Functions of blue pigments have been investigated in Scyphozoa species [12, 15, 35]. For example, blue pigment from *Stomolophus* sp. 2 contained Zn^{2+} and Cu^{2+} and has broad temperature adaptability (10–50°C) [12]; blue pigment from *R. pulmo* was involved in the evolution track of morphogenesis [15]; blue pigment from *C. xamachana* may play functional roles in mitigating excessive solar radiation and metals reservoir [35]. According to the findings in *C. xamachana* and *Stomolophus* sp. 2 [12, 35], blue pigment from *R. esculentum* may be involved in metal storage since metalloendopeptidase activity is enriched in blue *R. esculentum*. In addition, functions of blue pigments have been studied in other aquatic animals and plants. In *Sander vitreus*, concentration of blue pigments (sandercyanin) was higher in summer than other seasons, which may play roles in countershading and photoprotection [36]. Except for greening oysters, blue pigments produced by *Haslea karadagensis* showed multiple biological activities, including inhibiting the growth of marine bacteria and fungi and antiviral activity [37]. Considering the previous studies and the above results, blue pigment of *R. esculentum* may play roles in stress response, signal transduction, and photoprotection.

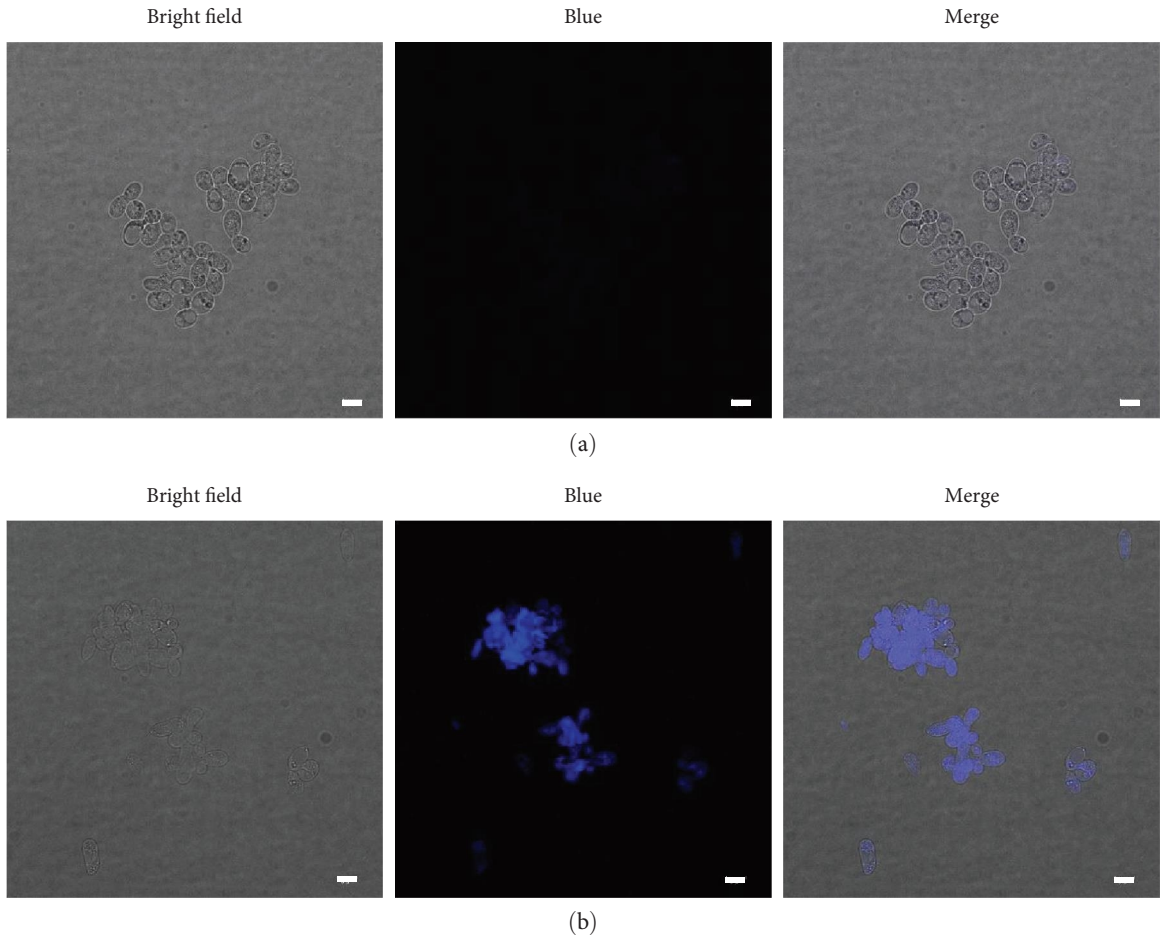


FIGURE 5: Microscopy observation of *R. toruloides* cells under 405 nm blue light excitation. (a) Wild type strain. (b) *R. toruloides* transformant with mRNA.RE15725 overexpression. Scale bar, 5 μm.

5. Conclusion

This study found that colors of blue and ochre *R. esculentum* are consistent with colors of inner gastrodermis and identified the blue pigment of *R. esculentum*, which may be associated with stress response and signal transduction in *R. esculentum*. These results explored our understanding of pigments and color formation in *R. esculentum* and provided valuable information for development and utilization of *R. esculentum* resources.

Data Availability

The raw data of proteomics are deposited in the iProX with the accession number of IPX0005689000 (http://www.iprox.cn/page/project.html?id=IPX0005689000).

Ethical Approval

All experiments in this study were conducted according to guidelines of Animal Experiments Ethics Committee of Liaoning Ocean and Fisheries Science Research Institute.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

YFL and ZZ developed the initial idea and designed the study. BC, YLL, QG, XB, and JZ performed the experiments. BC and YLL performed the data analysis. BC wrote the initial manuscript. All authors contributed to the article and approved the submitted version.

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Supplementary Materials

Supplementary 1. Map of overexpression vector pZPK-pGK-Nrs-tNOS-pXYL-Blup-tHSP.

Supplementary 2. Photo of *Rhopilema esculentum* harvest in Yingkou city, China.

Supplementary 3. The complete amino acid sequence of mRNA.RE15725.

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