Comparative transcriptomic analysis of *in situ* and onboard fixed deep-sea limpets reveals sample preparation-related differences

Highlights

- Deep-sea sampling without RNA *in situ* fixation influenced the gene expression greatly.
- Concomitant sampling stress perturbed various life activities of deep-sea limpets.
- Providing a high-quality *in situ* transcriptome of limpet *Bathyacmaea lactea*.

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Comparative transcriptomic analysis of \textit{in situ} and onboard fixed deep-sea limpets reveals sample preparation-related differences

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SUMMARY

Precise gene expression reflects the molecular response of deep-sea organisms to their harsh living environments. However, changes in environmental factors during lifting samples from the deep sea to a research vessel can also affect gene expression. By using the transcriptomic approach, we compared the gene expression profiles of the onboard fixed with the \textit{in situ} fixed samples of the deep-sea limpet \textit{Bathyacmaea lactea}. Our results revealed that the concomitant stress during conventional deep-sea sampling without RNA \textit{in situ} fixation greatly influenced the gene expression. Various biological activities, such as cell and tissue structure, lysosomal activity, fluid balance, and unsaturated fatty acid metabolism, were perturbed, suggesting that the sampling stress has exerted systemic impacts on the life of the limpets. These findings clearly illustrate that deep-sea samples without RNA \textit{in situ} fixation can easily lead to biased results in gene expression analysis, which requires to be appropriately addressed in future studies.

INTRODUCTION

Approximately 88.3\% of the ocean is deeper than 1000 m, commonly known as the deep sea (Weatherall \textit{et al.}, 2015), which is characterized by high hydrostatic pressure, low temperature (except for the hydrothermal vents), deficient food supply, and total darkness, and has therefore been considered hostile to life (Rex and Etter, 2010). However, both microorganisms and macroorganisms have been discovered in the deep sea, which is actually the largest habitat on Earth (Ramirez-Llodra \textit{et al.}, 2010). The deep sea supports various ecosystems with high biodiversity and biomass, such as hydrothermal vents, cold seeps, and whale falls (Kiel, 2016). Deep-sea organisms must have evolved various adaptive strategies to survive and thrive under such harsh environmental conditions. High-quality samples and free access to their genetic information are necessary to obtain a comprehensive and in-depth knowledge of the molecular mechanisms underlying deep-sea adaptation. Nevertheless, deep-sea sampling has always been quite technically challenging because of the great depth and far distance from land.

Owing to the rapid development of deep-sea sampling tools and techniques during the past decades (Clark \textit{et al.}, 2016), obtaining samples from deep-sea ecosystems has become easier. Furthermore, many studies have been conducted to obtain the genomic information of deep-sea organisms and explore the deep-sea adaptation mechanisms through genome, transcriptome, and proteome sequencing and analyses (Lan \textit{et al.}, 2018; Sun \textit{et al.}, 2017; Wang \textit{et al.}, 2019a; Yang \textit{et al.}, 2020). However, most of these studies were based on deep-sea samples obtained by conventional deep-sea sampling method, that is, the samples were fixed on the board of a research vessel after retrieval from the seafloor. For organisms that have adapted well to the deep sea, the harsh local environment has been optimum for them. Sampling these organisms from the seafloor to the surface might cause inevitable stress because of the changes in the ambient environment. Hence, conventionally obtained faunal samples can hardly reflect their natural physiological and biochemical status as they were inhabiting the deep sea. It is worth mentioning that some researchers realized this issue and tried to obtain in situ fixed deep-sea samples and advocated for the utilization of \textit{in situ} fixed deep-sea samples in their studies (Chen \textit{et al.}, 2021; Gao \textit{et al.}, 2019; Mat \textit{et al.}, 2020; Motoki \textit{et al.}, 2020; Sanders \textit{et al.}, 2013; Sun \textit{et al.}, 2020; Wang \textit{et al.}, 2019b; Watsuji \textit{et al.}, 2014; Wei \textit{et al.}, 2020). Nevertheless, studies that explicitly compare the biological difference between in situ fixed and conventional sampled deep-sea fauna are still lacking.
The cold seep is a typical chemosynthetic ecosystem in the deep sea, where reduced sulfur compounds and methane emanate from the seafloor to the water (Feng et al., 2018; Levin, 2005). In addition to the general deep-sea environmental stressors mentioned earlier, the organisms inhabiting cold seeps also need to cope with extra stressors, such as low oxygen and potentially harmful substances in the seepage fluid (McMullin et al., 2000). The deep-sea patellogastropod limpet Bathyacmaea lactea is one of the dominant macrobenthos in the Haima cold seep in the South China Sea (Liu et al., 2020), and no evidence of harboring endosymbionts in B. lactea has been reported until now. Given its small size and open-shell structure, B. lactea is relatively easier to be in situ fixed during deep-sea sampling than the others because it does not need to be cracked. In this study, two methods were used for sampling this limpet species (Figure 1): the conventional onboard fixation method in which samples are fixed on board by RNA stabilizing solution after retrieval and the in situ fixation method in which the samples are fixed on the seafloor.

Figure 1. Pictures showing the two sampling methods applied in this study
(A) Schematic of the two sampling methods. 
(B) An on-site photograph of the tuck net filled with the deep-sea limpet Bathyacmaea lactea attached to the mussel shells (indicated with red arrows). Inset: dorsal view of a B. lactea specimen. Scale bar, 0.5 cm.
(C) An on-site photograph of the in situ fixation. Bathyacmaea lactea attached on mussel shells (indicated with red arrows) were fallen off and in situ fixed in the sampling chamber (indicated with a white dotted box) fully filled with in-house RNA stabilizing solution.

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before suffering sampling stress. Further comparative transcriptomic analysis was performed on the limpet samples fixed with different methods to investigate the influences of deep-sea sampling stress on their gene expression at the transcriptional level.

RESULTS AND DISCUSSION

Transcriptome sequencing, assembly, and annotation

A total of 27,674 nonredundant transcripts with predicted open-reading frames were obtained by using 648,458,172 reads from seven individuals of *B. lactea* for transcriptome assembly, with an N50 value of 3610 bp (Table S1). The Benchmarking Universal Single-Copy Orthologs (BUSCO) assessment results show 98% completeness (single copy: 84.1% and duplicated: 13.9%) for the predicted transcripts. The functional annotation of the transcripts indicated that 23,327 transcripts had hits to the NCBI nonredundant (NR) database; 17,160 to the EggNOG database; 16,462 to Gene Ontology (GO) items; and 8424 to the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Figure S1). To the best of our knowledge, this is the first report on the *in situ* transcriptome of *B. lactea*.

Differential gene expression analysis between the *in situ* and onboard fixed samples

The expression level of each transcript was quantified in seven individuals. The principal component analysis (PCA) result shows a clear separation between the *in situ* fixed and onboard fixed groups along PC1, explaining the 48.2% variance (Figure 2A). Although variability also exists among replicates of the same group, we consider it acceptable, as these samples grew in a volatile wild environment that might result in individual differences. Moreover, replicates among the onboard fixed group showed more variability than that of *in situ* fixed group (Figure 2A), possibly because of having suffered the sampling stress.

Differential gene expression analysis was performed between the two groups to investigate the impacts of sampling stress during retrieval from the seafloor on the gene expression of cold seep-adapted limpets. A total of 3,436 (12.4%) differentially expressed genes (DEGs) were identified, with 1,858 upregulated and 1,578 downregulated in the onboard fixed limpets compared with *in situ* fixed ones (Figure 2B). This result indicated that sampling stress indeed led to dramatic changes in the gene expression of the deep-sea limpets during the ~80 min sampling process. During the conventional deep-sea sampling process, the samples were exposed to the ambient environment during retrieval from the seafloor to the surface. As a result, the samples suffered from decreased hydrostatic pressure, increased water temperature, increased dissolved oxygen, and decreased salinity (Figure S2). Other environmental parameters, such as hydrogen sulfide, metal ion, and pH, might also have changed. In a recent study conducted under laboratory conditions, few (~ 0.1%) DEGs were identified in the shallow-water sea cucumber *Apostichopus japonicus* after the hydrostatic pressure changed in the first hour (Chen et al., 2020), and in another study conducted in a cold seep, approximately 40 min of decompression from the 1,119 m depth resulted in 337 DEGs in the gill.
of the deep-sea mussel *Gigantidas platifrons* (formerly “*Bathymodiolus* platifrons”) (Chen et al., 2021). Therefore, we speculate that the large number of DEGs identified in this study might be induced by combined factors rather than a single factor during a sampling process of only ~80 min.

**Effect of the sampling stress caused differential gene expression on the deep-sea limpets**

GO and KEGG enrichment analyses were performed on the identified DEGs to discover the biological activities and metabolic pathways influenced by the DEGs induced by sampling stress. GO enrichment analysis of the upregulated DEGs in the onboard fixed group identified 24 significantly enriched GO terms, including structural molecule activity (GO:0005198), transmembrane transporter activity (GO:0022857), ribosome (GO:0005840), translation (GO:0006412), cellular biosynthetic process (GO:0044249), and plasma membrane (GO:0005886) (Table 1). GO enrichment analysis of the downregulated DEGs in the onboard fixed group resulted in 27 GO terms, including catalytic activity (GO:0003824), hydrolase activity (GO:0016787), peptidase activity (GO:0008233), small molecule metabolic process (GO:0044281), lipid metabolic process (GO:0006629), and lysosome (GO:0005764) (Table 2). KEGG enrichment analysis of all the DEGs identified 14 significantly enriched pathways, which were involved in transport and catabolism (lysosome [ko04142]); glycan biosynthesis and metabolism (glycosaminoglycan degradation [ko00531], other glycan degradation [ko00511]); carbohydrate metabolism (pentose and glucuronate interconversions [ko00040], amino sugar and nucleotide sugar metabolism [ko00520]); amino acid metabolism (histidine metabolism [ko00340], glycine, serine, and threonine metabolism [ko00260]); digestive system (protein digestion and absorption [ko04974]); cell growth and death (ferroptosis [ko04216]); lipid metabolism (biosynthesis of unsaturated fatty acids [ko01040]); endocrine system (PPAR signaling pathway [ko03320], renin-angiotensin system [ko04614]); and metabolism of cofactors and vitamins (one carbon pool by folate [ko00670], retinol metabolism [ko00830]) (Figure 3). Many affected biological functions

### Table 1. GO enrichment analysis of the upregulated transcripts in the onboard fixed group

| GO ID       | GO description                              | Category               | FDR        |
|-------------|---------------------------------------------|------------------------|------------|
| GO:0005198  | Structural molecule activity                | Molecular Function     | 7.86E-7    |
| GO:0003735  | Structural constituent of ribosome          | Molecular Function     | 7.35E-4    |
| GO:0022857  | Transmembrane transporter activity          | Molecular Function     | 7.69E-4    |
| GO:0005215  | Transporter activity                        | Molecular Function     | 7.69E-4    |
| GO:0005840  | Ribosome                                    | Cellular Component     | 7.69E-4    |
| GO:0032501  | Multicellular organismal process            | Biological Process     | 4.96E-3    |
| GO:0006518  | Peptide metabolic process                   | Biological Process     | 6.07E-3    |
| GO:0006412  | Translation                                 | Biological Process     | 6.07E-3    |
| GO:1901566  | Organonitrogen compound biosynthetic process| Biological Process     | 6.07E-3    |
| GO:0034645  | Cellular macromolecule biosynthetic process | Biological Process     | 6.07E-3    |
| GO:1901576  | Organic substance biosynthetic process      | Biological Process     | 6.07E-3    |
| GO:0044249  | Cellular biosynthetic process               | Biological Process     | 6.07E-3    |
| GO:0043043  | Peptide biosynthetic process                | Biological Process     | 6.07E-3    |
| GO:0043604  | Amide biosynthetic process                  | Biological Process     | 6.07E-3    |
| GO:0043603  | Cellular amide metabolic process            | Biological Process     | 6.07E-3    |
| GO:0009059  | Macromolecule biosynthetic process          | Biological Process     | 6.07E-3    |
| GO:0044271  | Cellular nitrogen compound biosynthetic process | Biological Process | 6.07E-3    |
| GO:0043228  | Nonmembrane-bounded organelle               | Cellular Component     | 9.89E-3    |
| GO:0043232  | Intracellular nonmembrane-bounded organelle  | Cellular Component     | 9.89E-3    |
| GO:0005576  | Extracellular region                        | Cellular Component     | 9.89E-3    |
| GO:0050877  | Nervous system process                      | Biological Process     | 1.73E-2    |
| GO:0003008  | System process                              | Biological Process     | 1.98E-2    |
| GO:0005886  | Plasma membrane                             | Cellular Component     | 4.58E-2    |
| GO:0016020  | Membrane                                   | Cellular Component     | 4.58E-2    |
and metabolic pathways were identified, suggesting that the sampling stress during retrieval from the seafloor exerted a systemic influence on the vital activity of the sampled deep-sea limpets.

**Cell and tissue structure**

GO enrichment analysis showed that cell structure maintenance, transmembrane transport, and biosynthetic process-related activities were upregulated (Table 1), whereas catabolism- and metabolic-process-related activities were downregulated potentially because of the sampling stress (Table 2). Collagen is the main structural protein in the extracellular matrix of diverse connective tissues, playing important roles in tissue morphogenesis and the maintenance of tissue structural integrity (Gelse et al., 2003). Previous studies uncovered that some collagens are positively selected in the deep-sea fish Aldrovandia affinis (Lan et al., 2018) and the deep-sea alvinocaridid shrimp Shinkaicaris leurokolos (Zhu et al., 2020), suggesting their potential roles in deep-sea adaptation. Herein, 86 collagen-encoding transcripts were identified, with 19 upregulated and 4 downregulated in the onboard fixed group (Table S2). The alteration of the expression levels of such a large number of collagen-encoding transcripts suggests that sampling stress, especially the pressure and temperature variations, will likely have an impact on the cell and tissue structure.

**Lysosomal activity and fluid balance**

KEGG enrichment analysis indicated that lysosome (ko04142) was the most affected pathway (Figure 3), implying that lysosomal activity was greatly altered in the cell of the limpets that suffered from sampling stress during retrieval from the seafloor.
stress. Lysosome (ko04142) was also significantly enriched in the enrichment analysis of the downregulated DEGs (Figure S3A), suggesting that the lysosomal activity might be downregulated in onboard fixed limpets. In addition, the renin-angiotensin system (ko04614) pathway, which is mainly engaged in osmoregulation, was also significantly enriched (Figure 3), suggesting that the sampling stress might perturb the fluid balance of the deep-sea limpets (Salzet et al., 2001), which would be caused by decompression-associated osmotic pressure change or by dehydration when the remotely operated underwater vehicle (ROV) was retrieved on board (Nobata et al., 2013; Yancey et al., 2014).

Unsaturated fatty acid metabolism

High hydrostatic pressure and low temperature are the key limiting factors in the colonization of deep-sea organisms (Brown and Thatje, 2014). Increased hydrostatic pressure and decreased temperature can reduce the fluidity of biological membranes, which are mainly composed of lipid bilayers and various proteins, leading to their dysfunction (Balny et al., 2002; Hazel, 1995; Kato et al., 2002; Marques et al., 2003). Many deep-sea organisms are known to rely on a large proportion of unsaturated fatty acids to cope with high hydrostatic pressure and low-temperature-caused rigidity of the membranes (Parzanini et al., 2018; Van Campenhout et al., 2016; Wang et al., 2019a). Deep-sea vent shrimps exposed to atmospheric pressure exhibit a lower level of unsaturated fatty acids than those kept under natural high pressure (Shillito et al., 2020). When the shallow-water amphipod Eogammarus possjeticus was exposed to high pressure, the expression of fatty acid desaturase and the elongation of the very long-chain fatty acids protein (ELOVL) involved in the production of unsaturated fatty acids increased (Chen et al., 2019b). As the biosynthesis of unsaturated fatty acids (ko01040) pathway regulating the production of unsaturated fatty acids was significantly enriched from all the DEGs (Figure 3), and the fatty acid metabolism (ko01212) pathway was significantly enriched from the downregulated DEGs as well (Figure S3A), the expression of fatty acid desaturase and fatty acid elongation protein was investigated. Four fatty acid desaturase-encoding transcripts and three fatty acid elongation protein-encoding transcripts were downregulated in the onboard fixed group (Figure 4A, Table S3), indicating that the biosynthesis of unsaturated fatty acids in the deep-sea limpets decreased during retrieval, which might have been caused by the decreased hydrostatic pressure along with the increased ambient temperature during the sampling process.

Figure 3. A bubble diagram showing the enriched pathways obtained in the KEGG enrichment analysis of all the differentially expressed genes between the in situ and onboard fixed groups.
Chemical defense reactions

Many marine animals, such as squids release secretions in defense against predator attacks (Wood et al., 2010), and sea anemones maintain their venom quality and quantity when suffering from environmental stressors (Hoepner et al., 2019). Among all the DEGs identified between the onboard and in situ fixed groups, 14 toxin-encoding transcripts and eight cysteine-rich venom protein-encoding transcripts were significantly upregulated during the retrieval process of deep-sea limpets from the seafloor (Figure 4B, Table S4). We speculated that sampling stress might also trigger their chemical defense reactions, resulting in the increased expression of toxin- and cysteine-rich venom protein-encoding transcripts. These reactions might be mediated by pathways involved in neural signal transduction, such as neuroactive ligand-receptor interaction (ko04080) and calcium signaling pathway (ko04020), which were significantly enriched from the upregulated DEGs (Figure S3B).

Insights into deep-sea adaptation through the in situ fixed limpet transcriptome

The highly expressed transcripts of a transcriptome are likely to play decisive roles. The top 10% most abundant transcripts (top 2,767 transcripts ranked by transcripts per million [TPM] values) of the in situ fixed B. lactea transcriptome were applied to the KEGG enrichment analysis to investigate the pathways involved in their environmental adaptation to the seep habitat. Results showed that 19 pathways were significantly enriched, several of which have been reported to play potential roles in deep-sea adaptation, such as lysosome (ko04142), metabolism of xenobiotics by cytochrome P450 (ko00980), glutathione metabolism (ko00480), and fatty acid elongation (ko00062) (Figure 5A).

Lysosome (ko04142) is the most significantly enriched pathway in the KEGG enrichment analysis of the top 10% most abundant transcripts in the in situ fixed B. lactea transcriptome (Figure 5A). A total of 64 transcripts mapped to lysosome (ko04142), including lysosomal acid hydrolases, lysosomal membrane proteins, other lysosomal enzymes and activators, mannose-6-phosphate receptor, clathrins, adaptor protein complex 3 (AP-3), and V-ATPase, were highly expressed (Figure 5B and Table S5), suggesting high lysosomal activity in the limpet living in the natural cold seep environment. High lysosomal activity
has been reported in the symbiotic organs of several cold-seep symbiotic species, including mussels (Yu et al., 2019; Zheng et al., 2017), clams (Ip et al., 2021; Lan et al., 2019), and tubeworms (Sun et al., 2021), and was deduced to digest the symbionts in the specialized bacteriocytes for nutrition and to control the symbiont populations as well. Nevertheless, the limpets of *Bathyacmaea* are considered to mainly graze the bacterial film on the substrates they attached to (i.e., mainly mussel shells) for nutrition (Chen et al., 2019a; Liu et al., 2020), implying that the high lysosomal activity detected in *B. lactea* might play different roles. However, 45 of the 63 highly expressed transcripts were significantly downregulated in the onboard fixed group (Figure 5B), indicating that the role of high lysosomal activity might be interrupted by sampling stress, which is consistent with the result that lysosome (ko04142) was the most significantly enriched pathway in the KEGG enrichment analysis of all the DEGs (Figure 2A) and downregulated DEGs (Figure S3A).

Lysosomes are ubiquitous cellular organelles known as the waste disposal system involved in the degradation and recycling of the cellular waste derived from both extracellular and intracellular regions, and growing evidence shows that they are also involved in many other cellular processes, including secretion, metabolic signaling, plasma membrane repair, and response to environmental cues, playing vital roles in maintaining cellular and organismal homeostasis (Ballabio and Bonifacino, 2020; Settembre et al., 2013).

In cold seeps, stressful environmental conditions might increase the production of intracellular waste (e.g., damaged organelles, oxidized lipids, and misfolded proteins) and the encounter with the pathogens of local organisms. Therefore, the high lysosomal activity would be conducive to increasing the efficiency of cellular clearance and reusage of the breakdown products for imperative nutritional needs. We assume that the high lysosomal activity might be a normalcy in the cold-seep-adapted nonsymbiotic invertebrates, which is a universal strategy for maintaining cellular and organismal homeostasis under the harsh environmental conditions of seep areas. However, various stressors can influence the function of lysosomes. For example, chemical contaminant treatments significantly decrease the lysosomal stability in molluscan hepatopancreas (Shaw et al., 2019), and the acute thermal stress on the deep-sea sponge holobiont causes significant lysosomal destabilization (Strand et al., 2017). Deep-sea sampling stress may have perturbed the lysosomal stability of the deep-sea limpets and thus decreased the lysosomal activity.

**Figure 5. KEGG enrichment analysis of the top 10% most abundant transcripts in the in situ fixed limpet transcriptome**

(A) Bubble diagram showing the enriched pathways of the top 10% most abundant transcripts.

(B) Heatmap showing the expression pattern of the top 10% highly expressed transcripts mapped to the lysosome (ko04142) pathway. Differentially expressed transcripts are in black and those not are in gray.
In the Haima cold seep, *B. lactea* usually attaches to the shells of the bathymodioline mussel *Gigantidas haimaensis* harboring methane-oxidizing endosymbionts, which makes use of methane emitted from the seafloor as the energy resource (Xu et al., 2019). Under such a condition, these limpets are also exposed to methane along with other toxic substances from their ambient environment and thus need to evolve suitable strategies for detoxification. Considering that the metabolism of xenobiotics by cytochrome P450 (ko00980) and glutathione metabolism (ko00480) pathways were significantly enriched from the top 10% most abundant transcripts, we investigated the expression of the mixed-function oxygenase (MFO) system components cytochrome P450 (CYP), conjugating enzyme glutathione S-transferase (GST), antioxidant enzymes superoxide dismutase (SOD), and glutathione peroxidase (GPX) (Lee, 1981; Ramos and Garcia, 2007; Xiao et al., 2020). Seven transcripts of CYP, twelve transcripts of GST, three transcripts of SOD, and one transcript of GPX ranked in the top 10% (Figure 6 and Table S6), indicating high MFO system and antioxidant enzyme activities in the limpet *B. lactea*, which might be responsible for the xenobiotic detoxification.

Conclusions

In this study, we successfully sampled the in situ fixed deep-sea limpet *B. lactea* and obtained its first in situ transcriptome. Comparative transcriptomic analysis of the in situ and onboard fixed samples revealed that the concomitant stress during conventional deep-sea sampling without in situ fixation affected their gene expression. Furthermore, sampling stress exerted systemic influences on the life of the sampled deep-sea limpets by perturbing the cell and tissue structure, lysosomal activity, fluid balance, and unsaturated fatty acid metabolism. These findings reveal that conventionally sampled samples from deep sea without RNA in situ fixation might lead to biased results in transcriptomic analyses and suggest that in situ fixed deep-sea samples are highly demanded for mRNA quantitative-analysis-based studies.

Limitations of the study

Due to the small size (0.5–0.8 cm) of *B. lactea* we collected, the whole body of each individual, rather than the dissected tissues, was used for transcriptome sequencing and downstream data analyses. This sampling strategy may bias the expression levels of some genes that actually exhibit opposite expression...
patterns in different tissues. Nevertheless, we consider such impacts to be limited and would not influence the main findings of this work.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.104092.

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**AUTHOR CONTRIBUTIONS**

P-YQ and JS conceived this project. GY and TX collected the samples. GY and YL performed the bioinformatics analyses and drafted the manuscript. TW helped to prepare the figures. All authors contributed to the manuscript writing and approved it for submission and publication.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Chemicals, peptides, and recombinant proteins | | |
| Ammonium sulfate | Xilong Scientific | Cat#12600901 |
| EDTA | Solarbio Life Sciences | Cat#E8040-100g |
| Sodium citrate | Xilong Scientific | Cat#10201601 |
| TRizol | Thermo Fisher Scientific | Cat#15596018 |
| RNAlater | Thermo Fisher Scientific | Cat#AM7021 |
| Deposited data | | |
| Raw RNA-seq data of six limpet Bathyacmaea lactea | This study | NCBI under BioProject PRJNA765439 |
| Software and algorithms | | |
| Trimmomatic version 0.39 | Bolger et al. (2014) | N/A |
| Trinity version 2.8.5 | Grabherr et al. (2011) | N/A |
| Salmon version 1.2.1 | Patro et al. (2017) | N/A |
| TransDecoder version 5.5.0 | https://github.com/TransDecoder/TransDecoder/wiki | N/A |
| CD-HIT version 4.8.1 | Fu et al. (2012) | N/A |
| BUSCO version 3.0.2 | Waterhouse et al. (2018) | N/A |
| OmicsBox version 1.4.11 | Biobam | N/A |
| RNA-seq 2G | Zhang et al., 2017 | N/A |

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Pei-Yuan Qian (boqianpy@ust.hk).

Materials availability
All unique/stable reagents generated in this study are available from the lead contact without restriction.

Data and code availability
- All raw sequencing data and the transcriptome assembly of B. lactea were deposited to NCBI under BioProject PRJNA765439.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS
This study was based on wild deep-sea limpet B. lactea, and no experimental models were used.

METHOD DETAILS

Deep-sea sampling and fixation
Limpets of B. lactea were collected from a single colony in the Haima cold seep (~1400 m depth) in South China Sea by the ROV Haima 2 onboard the R/V Haiyangdizhi6 during the HYDZ6-202005 cruise in August 2020. CTD (Sea-Bird, Bellevue, WA, USA) data showed that the seawater temperature increased from 3.0°C to 30.7°C, the dissolved oxygen increased from 1.8 mg/L to 5.2 mg/L, and the salinity changed from 34.6 to 33.4 at the sampling site (Figure S2). Two methods were used for sample fixation. 1) Onboard fixation: G. haimaensis mussels attached
with limpets were wrapped up with a tuck net held by the ROV manipulator arm and placed into the sample basket of the ROV (Figure 1B). The tuck net was transferred into the laboratory on board after the ROV was retrieved, and the limpets were immediately fixed by RNAlater (Thermo Fisher Scientific, Waltham, MA, USA) and frozen with liquid nitrogen after 4°C overnight. The sampling process from the seafloor to the laboratory took approximately 80 min. In situ fixation: G. haimaensis mussels attached with limpet B. lactea were cracked slightly by the ROV manipulator arm before they were placed into the sampling chamber fully filled on board with ~12 L in-house RNA stabilizing solution to preserve their RNA in situ (Figure 1C). The chamber was sealed by closing the lid and returned to the sample basket of the ROV. After the ROV was retrieved on board, the sampling chamber was transferred to the lab on board. The in situ fixed limpets were transferred to the RNAlater (Thermo Fisher Scientific, USA) immediately and frozen with liquid nitrogen after 4°C overnight. All the limpets were stored at −80°C until usage. The in-house RNA stabilizing solution was prepared as previously described (Mat et al., 2020) with 700 g of ammonium sulfate, 40 mL of 0.5 M EDTA, 25 mL of 1 M sodium citrate, and 935 mL of distilled water; the pH was adjusted to 5.2, and the solution was stored in a 4°C cold room in the ship before the ROV dive.

RNA extraction and sequencing

Four individuals of B. lactea with in situ fixation and three with onboard fixation were used in this study. The whole body tissue of each individual was used for RNA extraction using TRIzol Reagent (Thermo Fisher Scientific) in accordance with the manufacturer’s protocol. The quality and quantity of the extracted RNA were measured by the Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA). The mRNA of each individual was enriched by Oligo-dT probes and used for cDNA synthesis and eukaryotic library construction. All cDNA libraries were sequenced on an Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA) to yield paired-end reads with a length of 150 bp in Novogene (Beijing, China).

Transcriptome assembly and annotation

Adaptors and low-quality bases of the raw reads were trimmed by Trimmomatic version 0.39 (Bolger et al., 2014) with the following setting: “ILLUMINACLIP: TruSeq3-PE-2.fa:2:30:10:2:keepBothReads LEADING:3 TRAILING:3 MINLEN:36”. The obtained clean reads of all the individuals were used for the de novo assembly of the transcriptome by Trinity version 2.8.5 (Grabherr et al., 2011). Salmon version 1.2.1 (Patro et al., 2017) was used to quantify the expression of each assembled transcript. The transcripts with a transcript per million value below 0.1 were removed. TransDecoder version 5.5.0 (https://github.com/TransDecoder/TransDecoder/wiki) was used to predict the open reading frame of the transcripts. Potential isoforms of the protein sequences were removed using CD-HIT version 4.8.1 (Fu et al., 2012) with c set to 0.95. The completeness of the assembled transcriptome was assessed using BUSCO version 3.0.2 to search against the metazoa_odb10 database (Waterhouse et al., 2018). The predicted protein sequences were used for functional annotation by searching their predicted protein sequences against the NCBi Non-Redundant (NR) databases using BLASTp version 2.10.0+ with an E-value cut-off of 1 x 10^-5, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database via KEGG Automatic Annotation Server (KAAS), and the Gene Ontology (GO) via OmicsBox version 1.4.11 (BioBam, Valencia, Spain).

QUANTIFICATION AND STATISTICAL ANALYSIS

Gene expression analysis

Differential expression analysis was performed between four in situ and three onboard fixed limpets using DESeq2 (Love et al., 2014) implemented in RNA-seq 2G (Zhang et al., 2017) on the basis of the mapped reads count. Genes with a significant false discovery rate (FDR) value less than 0.05 and a fold change larger than two were identified as DEGs. The script “abundance_estimates_to_matrix.pl” implemented in Trinity (Grabherr et al., 2011) was used to generate a matrix of TMM-normalized expression values. This matrix was used for PCA by Past version 4.03 (Hammer et al., 2001). The significance of the comparison in PCA was further examined using PERMANOVA on a Bray-Curtis dissimilarity matrix (implemented in Past version 4.03) calculated for TMM-normalized expression values.

Enrichment analyses

The GO terms of the DE-Gs were enriched using the Fisher Exact Test implemented in OmicsBox 1.4.11 (BioBam). The KEGG pathways were enriched with the cumulative hypergeometric distribution method implemented in OmicShare online tool version 6.3.0 (http://www.omicshare.com/tools).