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Integrated miRNA-mRNA analysis uncovers immediate-early response to salinity stress in gill-derived cell line of *Gymnocypris przewalskii*

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Abstract

Salinity adaptation is an important issue in aquaculture. Understanding the immediate-early response to salinity stress helps in comprehending this process. In vitro experiments using cell lines can explain cell-independent reactions without the involvement of hormones in vivo. In this study, salinity stress experiments were conducted using cell line derived from the gills of *Gymnocypris przewalskii* (GPG cell line) to isolate immediate-early response-related genes and miRNAs using transcriptomics, followed by bioinformatics analysis. The results showed that intracellular free Ca^{2+} appeared to be a key factor in cell sensing and initiating downstream cell signaling in response to external salinity. Additionally, cell apoptosis was the most common feature of salinity stress, with multiple signaling pathways involved in salinity-induced cell apoptosis. Furthermore, MiRNAs played a crucial role in the rapid response to salinity stress by selectively inhibiting the expression of specific genes. Additionally, for the first time in the *G. przewalskii* genome, Tf2 and TY3 families of transposons were found to have responsive roles to the external salinity stress. This study contributes to a better understanding of osmotic sensing in *G. przewalskii* and provides theoretical assistance for improving salinity adaptation in aquaculture fish species.

Keywords *Gymnocypris przewalskii*, Salinity, Cell line, Apoptosis, miRNA, Transcriptome

Introduction

Osmoregulatory mechanisms underlying the adaptation of migratory fish to different salinity levels have been an important area to study. Previous studies have shown that osmoregulation in fish is a complex physiological process that occurs at the biological and cellular tissue level, and it is mainly mediated by the kidney, gill, and intestine [1–3]. The ability of euryhaline or migratory fish to adapt to different osmotic levels is mainly due to the osmoregulatory mechanism that helps the fish maintain a constant body osmolarity [4]. In vivo studies have demonstrated that the stress response in fish is a two-step process.

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Upon sensing external stress signals, fish initiate a primary response that transmits information to the central nervous system and triggers the release of hormones from the endocrine organs, such as the pituitary [5–7]. In the secondary response, these released hormones elicit physiological and biochemical reactions. For instance, osmotic stress significantly elevates blood cortisol levels in fish, which in turn activates osmosis-related transcription factor 1 (OSTF1) that promotes the expression of genes involved in osmoregulation.

G. przewalskii, a member of the subfamily Cyprininae and the genus*Gymnocypris*, is a fish species that inhabits the saline lakes of the Qinghai-Tibet Plateau, specifically Qinghai Lake (salinity 9–11‰). This species exhibits migratory spawning behavior and can tolerate significant salinity fluctuations during this period, making it an excellent biological model for studying changes in salinity. In vivo studies of salinity stress in *G. przewalskii* have identified numerous genes involved in homeostasis, hormone synthesis, defense response, complement activation, and reproductive development [8]. However, since studies at the organismal level often integrate both independent cellular responses and intercellular signaling processes, the independent processes by which cells sense osmotic stimuli and trigger cellular responses are still unclear.

Gills are the primary osmoregulatory tissue in fish, accounting for approximately 50% of the contact interface between the fish and its surrounding environment. In response to the need for osmosis between the blood and the external aqueous environment, gills have evolved into a highly vascularized epithelium with a large surface area. In freshwater-domesticated fish, the epithelial cells of the gills actively transport sodium and chloride ions from freshwater into the blood to compensate for the passive loss of ions in the urine or on the body surface. In contrast, seawater-domesticated fish can compensate for water loss by swallowing seawater and simultaneously secreting excess Na^+ and Cl^- through the gills. Additionally, it has been found that the epithelial cells of the gills can enhance the passage of small molecules through a remodeling process in response to osmosis [9]. Numerous in vivo studies have focused on osmoregulation in the gills of euryhaline fish [10–14]. These studies have primarily examined osmoregulatory mechanisms in vivo but have not been able to elucidate the active adaptation process of gill tissue to osmotic stress.

To clarify the initial process of active adaptation of gill cells to salinity, in this study, we identified rapidly adaptive genes and regulatory miRNAs during osmosis using gill-derived cell lines from *G. przewalskii* (GPG cell line) (Wei, et al. 2022) and transcriptome sequencing. These results will contribute to our understanding of the osmosensing and signal transduction processes in fish. Additionally, given the increasing salinity of Qinghai Lake, understanding the adaptation mechanisms of this species is also significant for conservation of *G. przewalskii.*

Materials and methods

Cell morphology and apoptosis detection

The fish needed for the experiments were provided by the Rescue and Rehabilitation Center of Naked Carps in Lake Qinghai (Xining, China). Gills from 3 juvenile fishes (3-month-old, 5±1.5 cm in length and 2.0–4.5 g in weight) were dissected after euthanasia with a lethal dose (80.0 mg/L) of MS-222 (Sigma, Shanghai, China) and used for primary cell culture according to the method of Wei et al. (2022). In detail, under sterile conditions, gills were minced and cultured in DMEM (high sugar) medium (Gibco, New York, USA) supplemented with 15% fetal bovine serum (FBS) (Sanger, Shanghai, China) and penicillin-streptomycin solution (300 units/ml) (Gibco, New York, USA). After cell migration and reaching 50% confluence in the flask, the cells were digested with 0.5% trypsin (Gibco, New York, USA) and subcultured into new flasks at a ratio of 1:2 with 4 ml of growth medium, including 85% DMEM (high sugar) medium, 15% FBS, and penicillin-streptomycin solution (100 units/ml). GPG cells (10^{5h} passage) were plated at a concentration of 1×10^4 cells per well in 6-well plates (Corning, New York, USA). When the cells reached 80–90% confluence, 1.5 ml of complete medium supplemented with 0.4% and 0.8% sodium chloride was added to simulate osmotic stress. After 24 h of salinity simulation, the morphology of the cells was observed using a microscope and apoptotic cells were stained with the TUNEL Apoptosis Detection Kit (Red fluorescence) (Beyotime, Shanghai, China). Images were taken using the Nikon inverted microscope (Eclipse Ts2). During cell culture and processing, the incubator parameters were set to 25 °C and 5% CO_2.

Osmotic stress simulation

GPG cells (10^{5h} passage) were plated at a concentration of 1×10^4 cells per well in 6-well plates. When the cells reached 80–90% confluence, 1.5 ml of complete medium supplemented with 0.8% sodium chloride was added to simulate osmotic stress in 6-well plates. Cells were collected at 0, 3, 6, and 9 h (GPG-0 h, GPG-3 h, GPG-6 h, and GPG-9 h) after osmotic stress using cell scraper (Corning, New York, USA), and were immediately frozen in liquid nitrogen with untreated cells (Control) until RNA-seq. To ensure the accuracy of sequencing, each treatment included three independent cell samples.

Cell transcriptome sequencing and analysis

Library construction and sequencing

Complete medium supplemented with 0.8% sodium chloride was used to simulate osmotic stress, as it can significantly change the morphology of GPG cells and induce apoptosis. Total RNA was extracted from the cells (Invitrogen, Carlsbad, CA, USA) and used for library construction. For microRNA library construction, RNA molecules in the size range of 18–30nt were enriched from total RNA using polyacrylamide gel electrophoresis (PAGE). The NEBNext Multiplex Small RNA Library Prep Set (NEB #7300, New England Biolabs, Ipswich, MA, USA) was used for 3' and 5' adapter ligation and reverse transcription, and 140–160 bp size products were enriched to generate a cDNA library.

For mRNA sequencing, mRNA was enriched using Oligo(dT) beads (Dynabeads™ Oligo(dT)25, Invitrogen, MA, USA). The enriched mRNA was fragmented into short fragments (Invitrogen, MA, USA) and reversely transcribed into cDNA using the NEBNext Ultra RNA Library Prep Kit for Illumina (NEB #7530, New England Biolabs, Ipswich, MA, USA). A bases were added to the purified double-stranded cDNA fragments, and the cDNA fragments were ligated to Illumina sequencing adapters and PCR amplified to generate a cDNA library. The cDNA library from microRNA and mRNA was sequenced using an Illumina Novaseq6000 by Gene Denovo Biotechnology Co. (Guangzhou, China).

Differential expressed genes and miRNA determination

In the transcriptome analysis, sequencing reads were mapped to the reference genome using HISAT2 [15]. After filtration with SOAPunke software to remove reads with adaptors, > 0.1% unknown bases (N), and low-quality reads (percentage of base that quality is lesser than 20 is greater than 50% in a read) [16], the mapped reads of each sample were assembled by using StringTie v1.3.1 [17, 18] in a reference-based approach. Meanwhile, those assembled transcripts found in the sequencing results but not included in the reference genome were defined as novel genes. For each transcription region, an FPKM (fragment per kilobase of transcript per million mapped reads) value was calculated to quantify its expression abundance and variations, using RSEM software [19]. In the following analysis, principal component analysis (PCA) was performed with R package models (http://www.r-project.org/) in this experience to test the repeatability of samples. RNA differential expression analysis was performed by DESeq2 software between two different groups [20].

For miRNA, raw reads were further filtered by the SOAPunke software (v1.5.2, Beijing Genomics Institute, Shenzhen, China) to remove low-quality reads, reads without 3'adapters or containing 5'adapters, reads containing 3' and 5' adapters but no small RNA fragment between them, reads containing polyA in small RNA fragment, and reads shorter than 18nt (not including adapters). Then, the filtered reads were aligned with small RNAs in GeneBank database (Release 209.0), Rfam database (Release 11.0), and reference genome to remove rRNA, scRNA, snoRNA, snRNA, and tRNA. These obtained clean tags were then searched against miRBase database (Release 22) and reference genome to identify existing miRNAs and candidates [21]. MiRNA differential expression analysis was performed by edgeR software after calculation and normalization according to TPM value (TPM = Actual miRNA counts/Total counts of clean tags*10^6). Two software programs, Miranda (Version 3.3a) and TargetScan (Version 7.0), were used to predict target genes.

In these process, the differential gene expression levels were assessed using t-tests. The genes with a false discovery rate (FDR) < 0.05 and a fold change ≥ 2 were considered DEGs. MiRNAs with a fold change ≥ 2 and a p-value < 0.05 in a comparison were defined as significant DEmiRNAs.

Bioinformatics analysis

To examine the expression pattern of DEGs, the expression data of each sample were clustered by Short Time-series Expression Miner software (STEM) [22]. In the process, Maximum Unit Change in Model Profiles between Time Points was set as 1, Maximum output profiles number was set as 20 (similar profiles will be merged), and Minimum ratio of fold change of DEGs was no less than 2.0. The clustered profiles with p-value < 0.05 were considered as significant profiles. Then, Gene Ontology (GO) [23] was used to complete gene functional classification, and KEGG database [24] was used to identify enriched metabolic pathways and signal transduction pathways. The calculated p-values were subjected to FDR correction, and GO terms and Pathways with FDR ≤ 0.05 were defined as significantly enriched.

Validation of the role of voltage-gated calcium ion channels in GPG cell salinity sensing

Three types of culture media were prepared: Control (the complete medium, C), Salinity (the complete medium supplemented with 0.8% sodium chloride, S), and Salinity-Nifedipine (the complete medium supplemented with 0.8% sodium chloride and 100 μm Nifedipine, SN). When GPG cells were seeded in 6-well plates and reached 80–90% confluency, they were treated with these three culture media. After 6 h, the cells were collected using a cell scraper and immediately frozen in liquid nitrogen for RNA-seq. Due to the need to dissolve Nifedipine in DMSO, an equal volume of DMSO was added to each treatment group to minimize errors.

Assuming that salinity induces changes in membrane potential, thereby activating voltage-gated calcium ion channels and triggering the release of Ca^{2+} as a second messenger to induce the expression of numerous genes, the addition of the voltage-gated calcium ion channel inhibitor Nifedipine should inhibit the release of intracellular Ca^{2+} and reverse the expression regulation of genes induced by Ca^{2+} as a second messenger. Therefore, the DEGs with opposing expression trends between the C-vs-S group and the S-vs-SN group were selected as the target genes for sequencing and bioinformatics analysis.

miRNA-mRNA pairs identification and network construction

STRING was used to analyze the protein interaction network of differential genes [25]. For the species included in the database, Cytoscape was used to construct the interaction network diagram [26]. For the species not included in the database, the interaction network was constructed using reference species included in the STRING database after alignment.Differentially expressed genes were compared with the predicted DEmiRNA targeting genes, and Pearson correlation coefficient between miRNA and target gene was calculated. MiRNA-target gene pairs with negative expression correlation, correlation coefficient<-0.7, and P value < 0.05 were screened out and used to construct a network diagram with Cytoscape.

Reverse transcription and quantitative real-time PCR

To verify the accuracy of the transcriptome sequencing, Real-time qPCR was performed using the TB Green® Premix DimerEraser™ (Perfect Real Time) (Takara, Dalian, China) in CFX Connect Real-Time PCR Detection System (Bio-rad, California, USA).

Twelve genes, including ARHGEF11 (rho guanine nucleotide exchange factor 11 isoform X11), Etv5 (ETS translocation variant 5-like), FOSL2 (fos-related antigen 2-like isoform X1), hes4-b (transcription factor HES-4-B-like protein), jdp2 (jun dimerization 2-like protein), JUN (transcription factor AP-1-like), mafb (transcription factor MafB-like), POL (uncharacterized protein), sgk1 (Serine/threonine-protein kinase Sgk1), SUPT3H (transcription initiation protein SPT3 homolog isoform X2), Tf2-12 (Transposon Tf2-6 polyprotein), and TY3B-1 (uncharacterized protein), were randomly selected for qRT-PCR verification in GPG cells under salinity stress. Meanwhile, fourteen miRNAs, including miR-1246-z, novel-m0073-3p, novel-m0076-3p, miR-194-x, novel-m0356-3p, miR-210-x, miR-21-y, miR-199-z, miR-147-y, novel-m0116-5p, miR-3966-z, miR-10,955-z, miR-7550-z, and novel-m0275-5p, were also selected for qRT-PCR verification in those cells.

In study of validation of the role of voltage-gated calcium ion channels in GPG cell salinity sensing, we randomly selected SPOP (speckle-type POZ protein), ADAMTS8 (A disintegrin and metalloproteinase with thrombospondin motifs 8-like), SBDS (ribosome maturation protein SBDS), nfil3 (nuclear factor interleukin-3-regulated protein-like), Foxl1 (forkhead box protein L1-like), Rbm28 (RNA-binding protein 28-like), CTSS (cathepsin S-like), MCL1 (induced myeloid leukemia cell differentiation protein Mcl-1 homolog), hes1-a (transcription factor HES-1-like protein), hes1-a1, REXO1 (RNA exonuclease 1 homolog isoform X1) and REXO1a for qRT-PCR verification.

Primers were tabulated in Table S1, and the quality of the PCR primers was confirmed by melting curve analysis. Data were expressed as means ± SE (Standard Error of Mean) of three independent experiments.

Result

Apoptosis detection

After 24 h of salinity simulation, significant morphological differences were observed in GPG cells. Cells added with 0.8% NaCl in the culture medium exhibited more cell membrane shrinkage and detachment. When stained using the TUNEL method, cells treated with 0.8% NaCl showed obvious fluorescence staining, indicating the occurrence of apoptosis in GPG cells induced by the addition of 0.8% NaCl in the culture medium (Fig. 1).

Expression profiling analysis of mRNA-seq data

A total of 49,058 genes were identified in GPG cells after merging the filtered data from the four groups, including 3,544 novel genes in addition to the reference genes (Table S2). Meanwhile, PCA analysis showed that different replicates in each group presented a clustered distribution (Fig. 2A). Acute salinity treatment induced significant changes in gene expression in GPG cells within 0–9 h. Compared to the control group, the number of differentially expressed genes (DEGs) continued to increase within 9 h, from 3,048 DEGs at 3 h after stress to 8,539 at 9 h. Among these three groups, there were 2,513 co-expressed genes. The response of cells to salinity gradually decreased over time, and the DEGs between groups were 3,048, 799, and 215, respectively (for control vs. GPG-3 h, GPG-3 h vs. GPG-6 h, and GPG-6 h vs. GPG-9 h groups) (Fig. 2B).

Enrichment analysis of the 2513 co-expressed DEGs identified biological processes, molecular functions, and cell signaling pathways that were continuously altered up to 9 h after stress. GO enrichment analysis showed that these DEGs were significantly enriched in DNA integration and transmembrane transport cellular processes. Molecular functions, such as motor activity, transcription factor activity, and transporter activity, were also significantly enriched (Fig. 3A). KEGG analysis showed that salinity significantly affected the NF-kappa B signaling

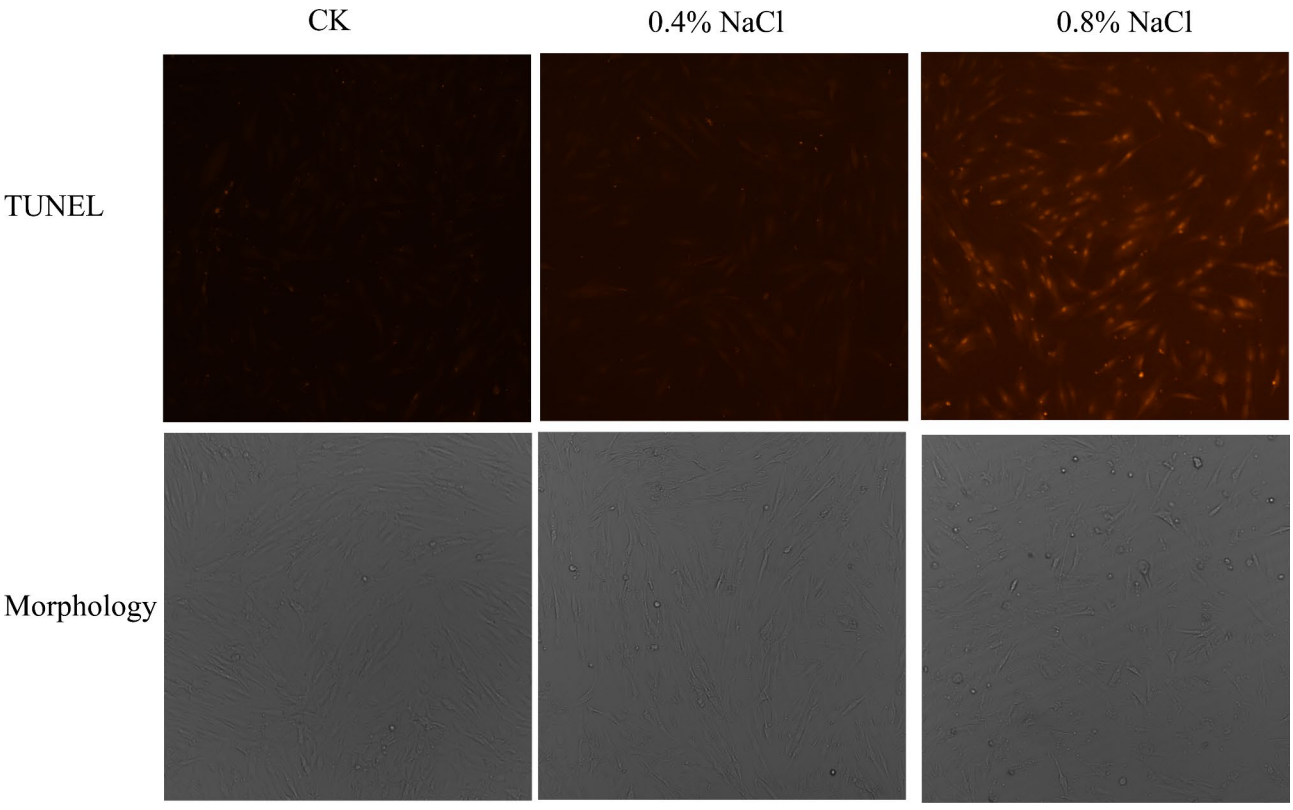

Fig. 1 Apoptosis detection under salinity stress. Red fluorescence indicates the signal of cell apoptosis

pathway, TNF signaling pathway, IL-17 signaling pathway, cGMP-PKG signaling pathway, and MAPK signaling pathway in GPG cells. At the same time, the aldosterone-regulated sodium reabsorption, apoptosis, protein digestion and absorption, cardiac muscle contraction, mineral absorption, ABC transporters, complement and coagulation cascades, endocrine and other factor-regulated calcium reabsorption, and insulin secretion-related pathways were also regulated (Fig. 3B).

Gene clustering and functional enrichment of DEGs

The DEGs isolated in this experiment were clustered into 20 profiles using the Short Time-series Expression Miner (STEM) software. The most represented clusters were profile 0, 17, 9, and 2 (p-value < 0.05). In profile 19 and 0, the expressions of genes displayed a stepwise increase (1251 DEGs) or stepwise decrease (profile 0, 3306 DEGs) after salinity stress. In profile 17, the expression of 3105 gene transcripts increased in the first 3 h but plateaued until 9 h. And in profile 2, 351 genes showed a downward trend of gene expression pattern in the first 3 h and plateaued until 9 h (Fig. 4). Those genes were selected for further bioinformatics analysis.

In profile 17, the 3105 salinity-sensitive up-regulated genes were significantly enriched in items related to DNA integration, G-protein coupled receptor signaling pathway, Transmembrane transport, and Inositol phosphate metabolic process. In KEGG enrichment analysis, the enriched items included the Calcium signaling pathway, CGMP-PKG signaling pathway, Neuroactive ligand-receptor interaction, NF-kappa B signaling pathway, CAMP signaling pathway, Endocrine and other factor-regulated calcium reabsorption, MAPK signaling pathway, TNF signaling pathway, and ABC transporters (Figure S1). As for the DEGs in profile 19, their functions were associated with Transcription factor activity, Transporter activity, and gene expression regulation processes (Figure S2).

The 351 DEGs in profile 2 were significantly down-regulated only within 3 h after salinity stress and remained at low levels during the later time periods. These genes are considered typical salinity-sensitive down-regulated genes. GO enrichment analysis revealed their significance in the biological processes of cell-cell adhesion, primary metabolic process, and DNA integration. The molecular biological function of poly(A) RNA binding was also significantly down-regulated (Figure S3). However, these 351 DEGs did not show enrichment in any KEGG pathways.

In profile 0, the 3306 DEGs were mainly located in the Intracellular and Nucleus compartments. They were enriched in biological terms related to Protein modification, Rho protein signal transduction, Transcription, Small GTPase mediated signal transduction, and

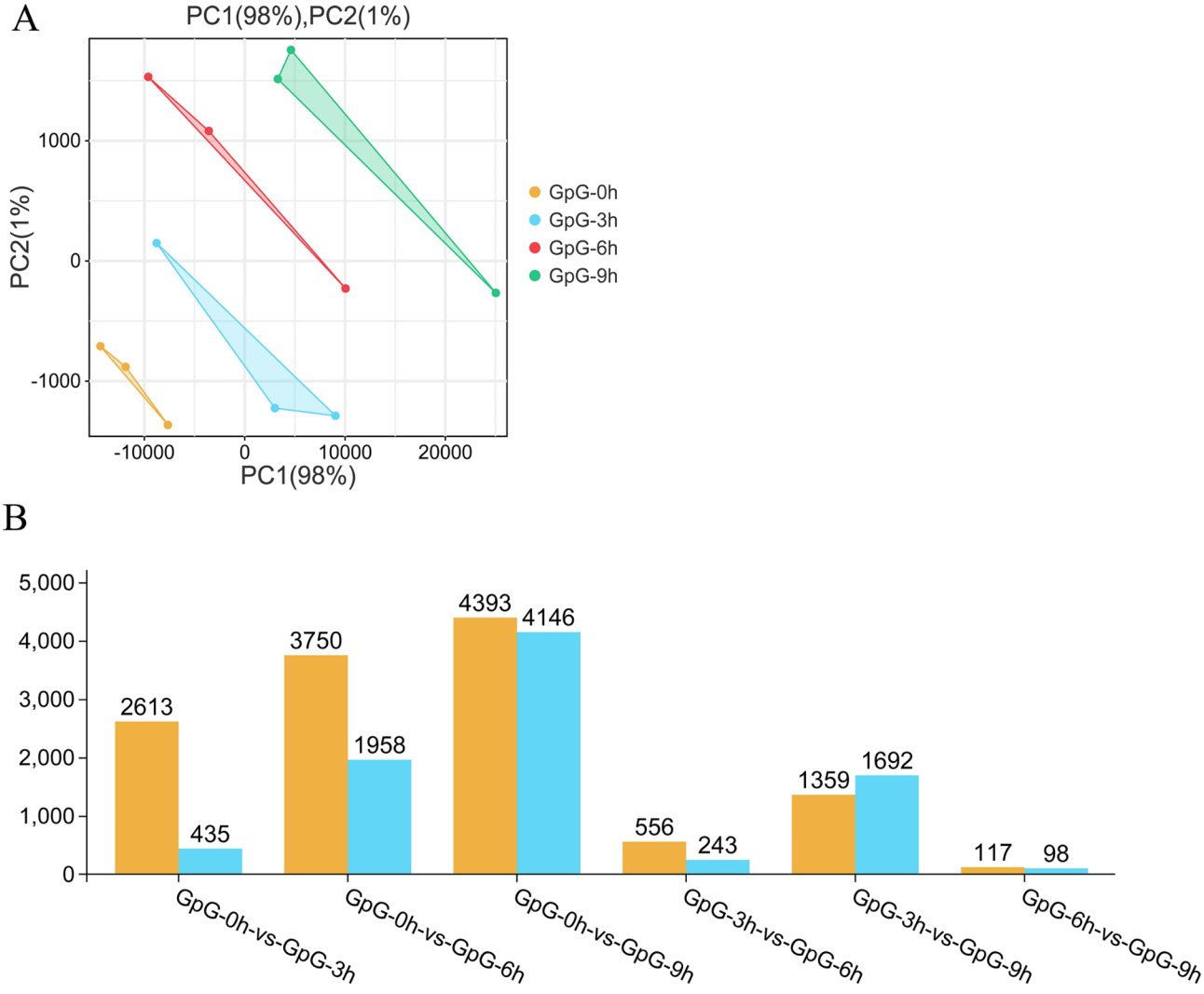

Fig. 2 PCA analysis of DEGs in different groups (A) and DEGs statistics (B). Orange represents upregulated genes in pairwise comparisons, while blue represents downregulated genes

Regulation of apoptotic process. In the protein modification process, molecular functions such as Protein kinase activity, Methyltransferase activity, Transferase activity, Protein phosphorylation, and Ubiquitinyl hydrolase activity were significantly enriched (Figure S4A). Additionally, the enriched KEGG pathways included Ubiquitin-mediated proteolysis, Hippo signaling pathway, Wnt signaling pathway, MAPK signaling pathway, MTOR signaling pathway, P53 signaling pathway, RNA degradation, TGF-beta signaling pathway, and Autophagy (Figure S4B).

The role and effects of Ca2+ in salinity perception

In the S group, there were 3566 upregulated DEGs and 3757 downregulated DEGs after 6 h of stress. In the SN group, there were 3538 upregulated DEGs and 3993 downregulated DEGs after 6 h of stress. There were a total of 282 DEGs shared between the S and SN groups, including 72 upregulated genes and 210 downregulated genes (Fig. 5A). Using the target gene isolation method, a total of 128 target DEGs were identified, accounting for 45% of the total differentially expressed genes in the S-vs-SN group. Among them, under the condition of 0.8% NaCl stress, 9 significantly downregulated DEGs could be reversed by 100μM voltage-gated calcium ion channel inhibitor Nifedipine. Additionally, 119 upregulated DEGs due to salinity were reversible by 100μM Nifedipine (Fig. 5B). In the subsequent enrichment analysis, these 128 target genes were enriched in multiple terms related to the salinity response in GPG cells. This further confirms the importance of voltage-gated calcium ion channels as crucial proteins in the early cellular sensing of external salinity. (Figure 6A and B).

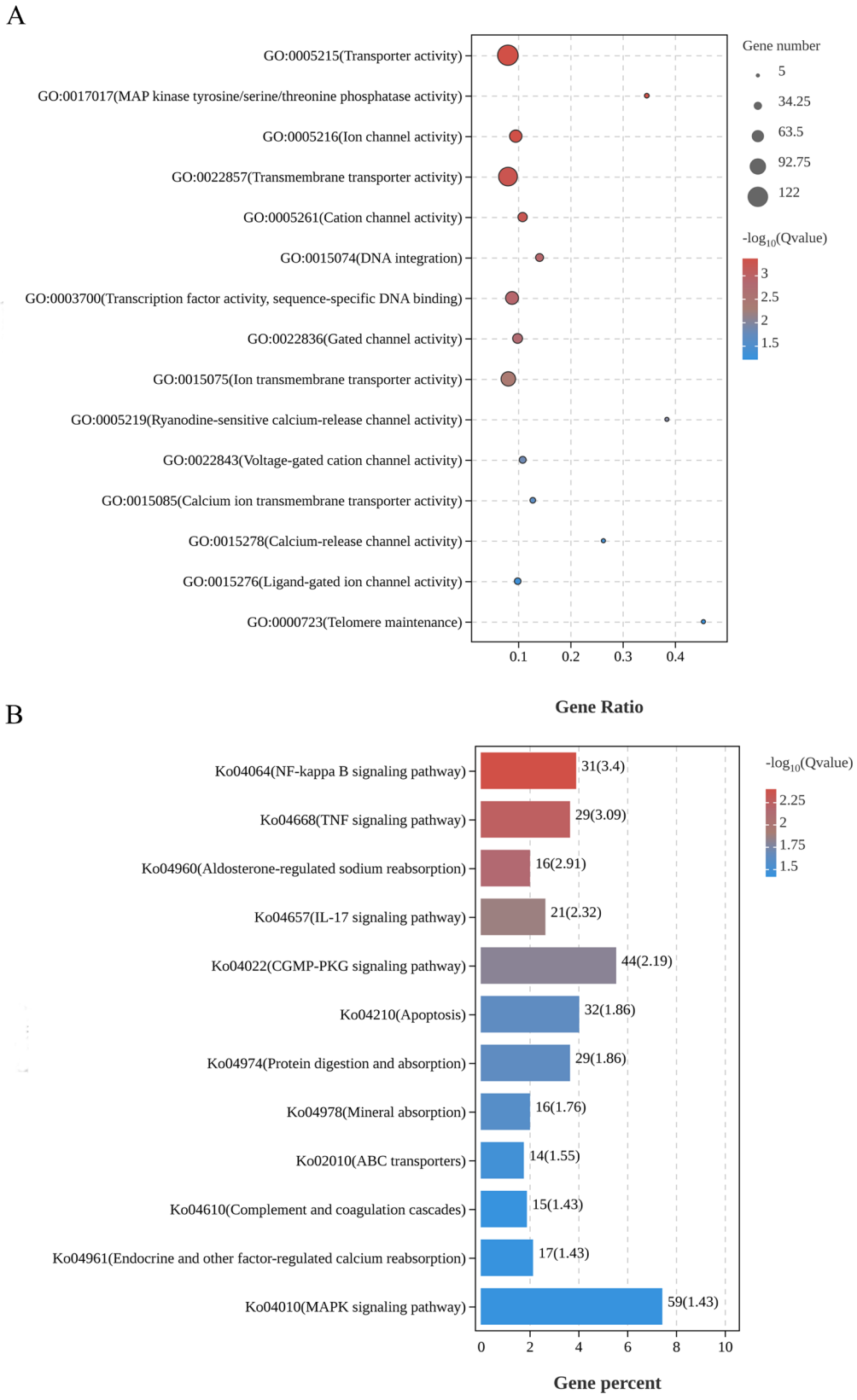

Fig. 3 GO (A) and KEGG pathway (A) enrichment analysis of the co-expressed DEGs in groups

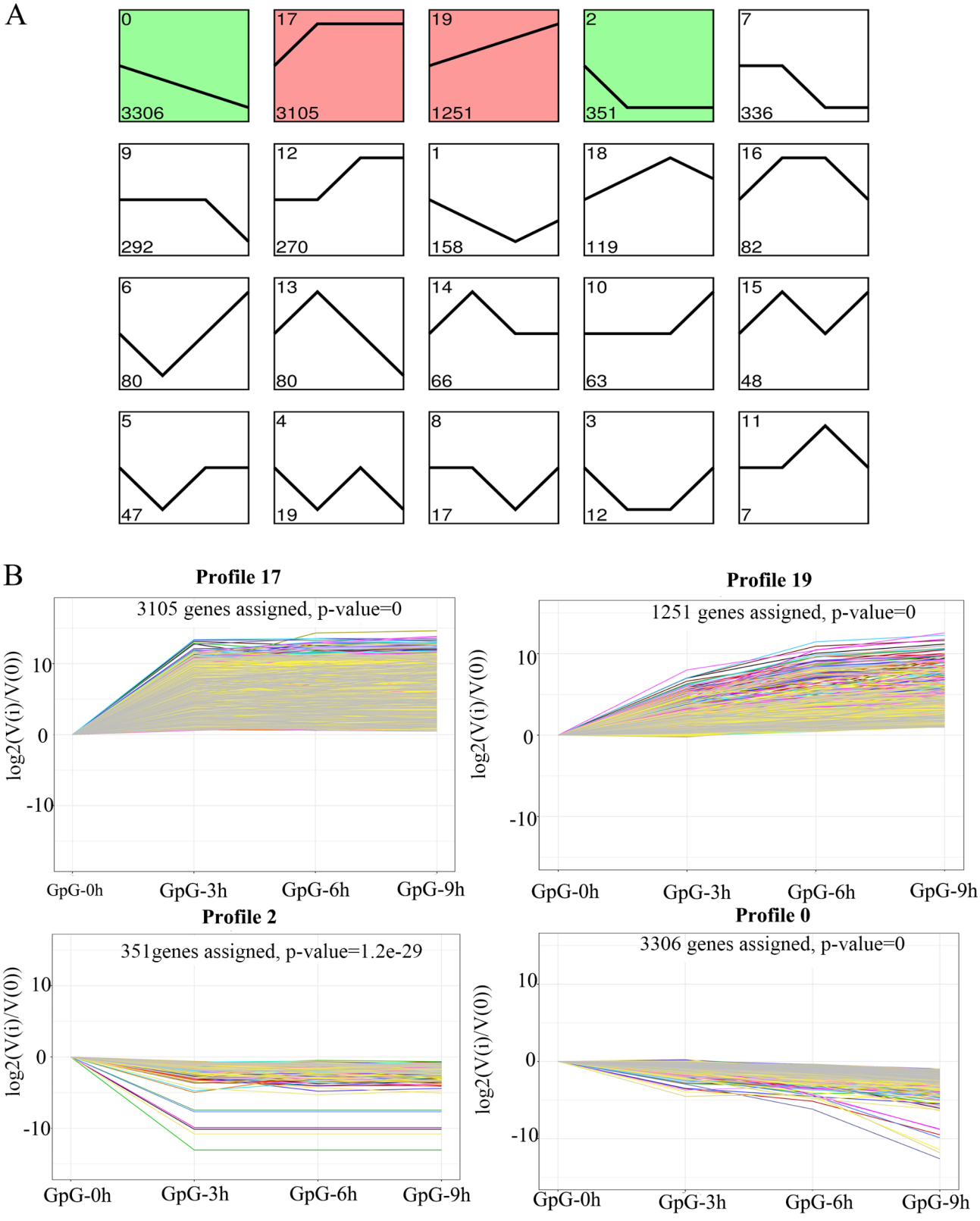

Fig. 4 Trend analysis of differentially expressed genes. A: The distribution of DEGs across 20 clustering patterns, where the number in the top left corner represents the clustering profile, and the number in the bottom left corner represents the number of DEGs within that clustering type. B: Four significant clustering profiles

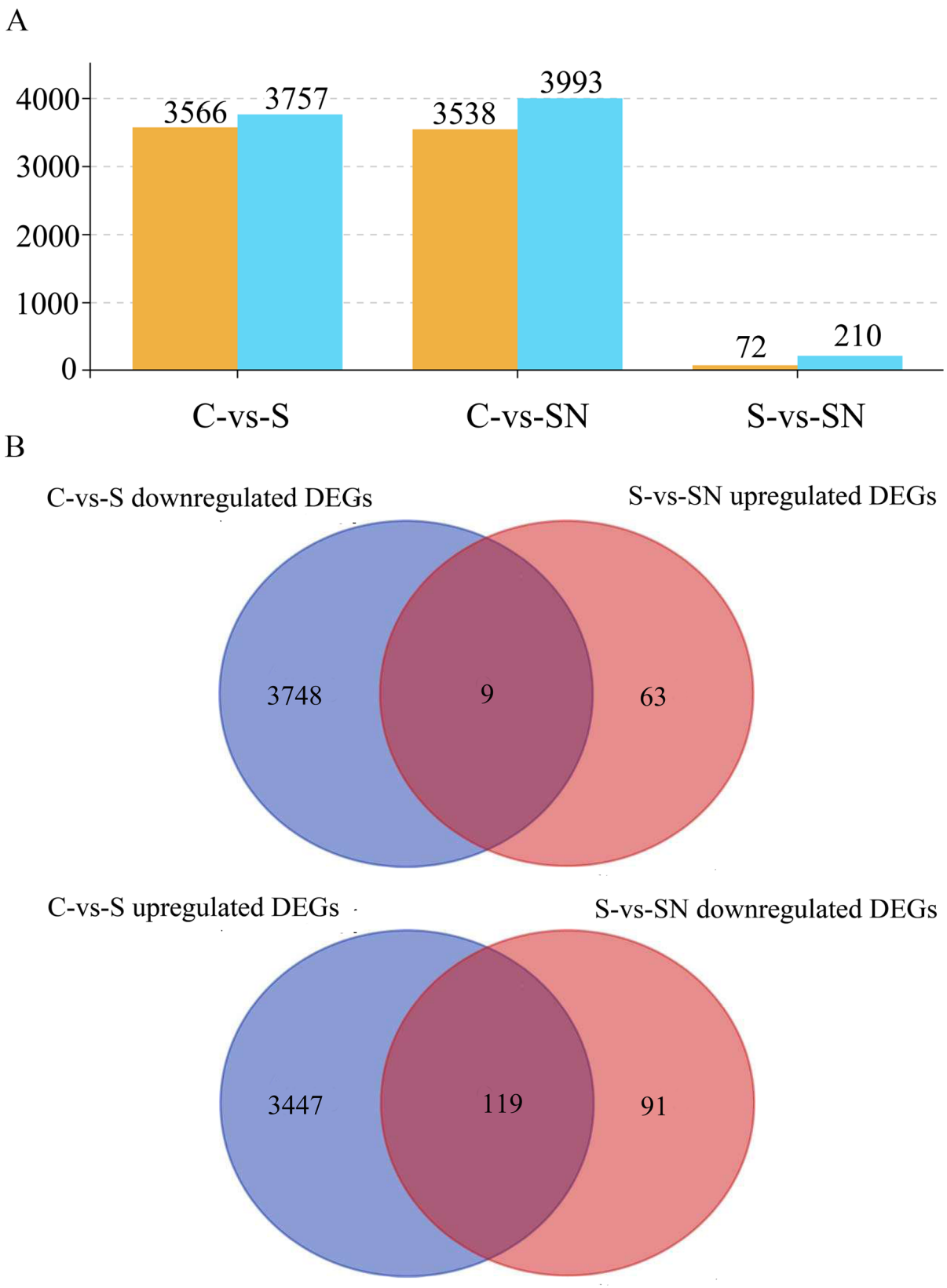

Fig. 5 Statistics of DEGs between groups and target genes identification. A: Statistics of differentially expressed genes between groups. Orange represents upregulated genes in pairwise comparisons, while blue represents downregulated genes. B: Identification of genes reversed by Nifedipine

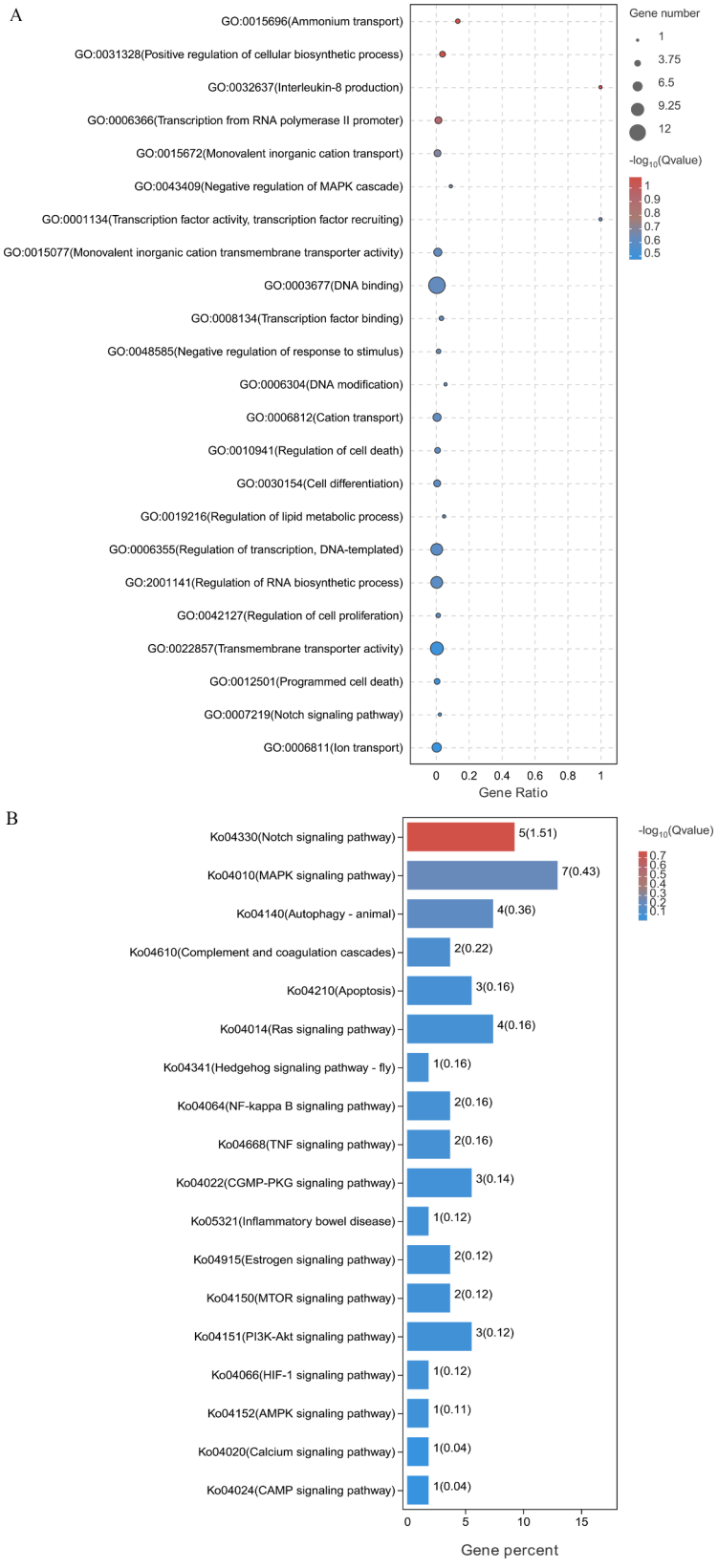

Fig. 6 GO (A) and KEGG pathway (A) enrichment analysis of the target DEGs responsed to Nifedipine addition

miRNA-mRNA pairs identification

In this study, a similar number of miRNAs were identified in different replicates, with a total of 2010 miRNAs identified (Figure S5A). PCA analysis further confirmed the good reproducibility of the samples (Figure S5B). Moreover, the number of DEmiRNAs in each comparison group was positively correlated with the number of mRNAs, with the Control-VS-GPG-9 h group having the highest number of DEmiRNAs, totaling 482 (Figure S5C). Based on the correlation analysis between miRNAs and target genes, as well as the analysis of their targeting relationship, Pearson correlation coefficient was calculated. A total of 31,801 miRNA-mRNA pairs were identified with a correlation coefficient less than −0.7 and $p<0.05$, including 509 DEmiRNAs and 2119 DEGs (Figure S5D).

Involvement of miRNA in functional alterations induced by salinity stress

To further elucidate the impact of miRNAs on cellular biological functions, differentially expressed genes (DEGs) enriched in apoptosis (GO: 0042981), transport (GO: 0055085), and DNA integration processes (GO: 0015074) within the GPG-3 h group were selected to construct regulatory networks of hub genes and miRNAs.

In the apoptosis process, the top 5 hub genes were identified using CytoHubba as luo008571 (FADD), luo007079 (apaf1), luo005446 (apaf1), luo018624 (CFLAR), and luo018619 (CFLAR). Through the analysis of miRNA-mRNA target gene associations, it was found that luo018619 (CFLAR) and luo008571 (FADD) could be regulated by miRNAs. There are 29 miRNAs involved in regulating luo018619 (CFLAR) and 9 miRNAs involved in regulating luo008571 (FADD) (Fig. 7, Table S3).

The expression of cell membrane ion transport proteins is not only a stress response to salinity stress, but also regulates other adaptive processes in cells. The top 5 hub DEGs enriched in transmembrane transport-related process, were luo018946 (HCN4) and luo037719 (CACNA1G), which were significantly downregulated, and luo030728 (CACNA1I), luo031159 (Kcna3) and luo019077 (slc15a2), which were upregulated (Fig. 8). In addition, luo019077 (slc15a2) and luo018946 (HCN4) were important miRNA-regulated target genes, which indicated that miRNA is important regulatory factor in ion transport processes (Table S4).

DEGs enriched in the DNA integration process include luo000844 (Tf2-11), luo041489 (POL), luo041079 (TY3B-G), luo040327 (POL), luo004286 (Tf2-12), luo009006 (ARHGEF11), and luo010751 (POL). As shown in Fig. 9, luo000844 (Tf2-11) and luo004286 (Tf2-12) were found to interact with each other among the proteins related to DNA integration. Furthermore, the expression activity of

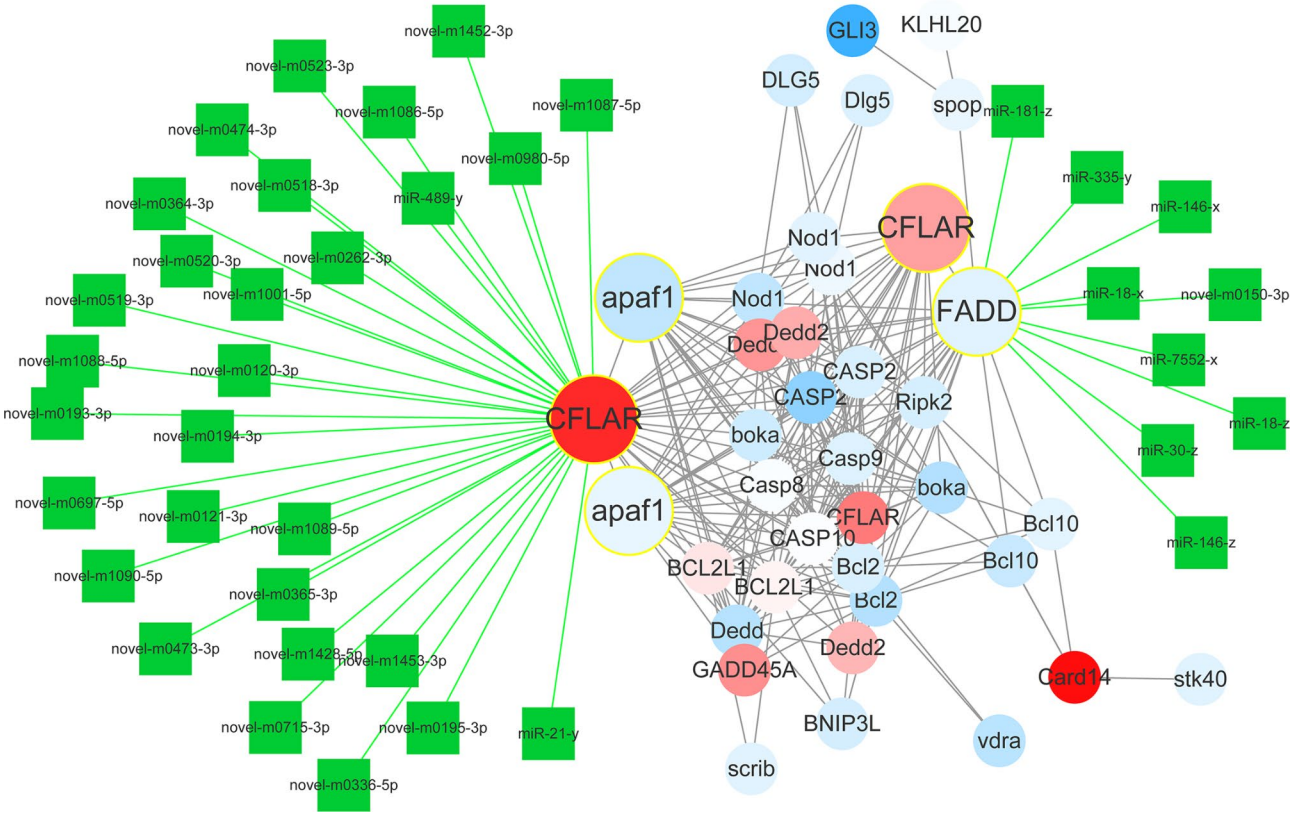

Fig. 7 Network diagram of key mRNA and miRNA during apoptosis in cells

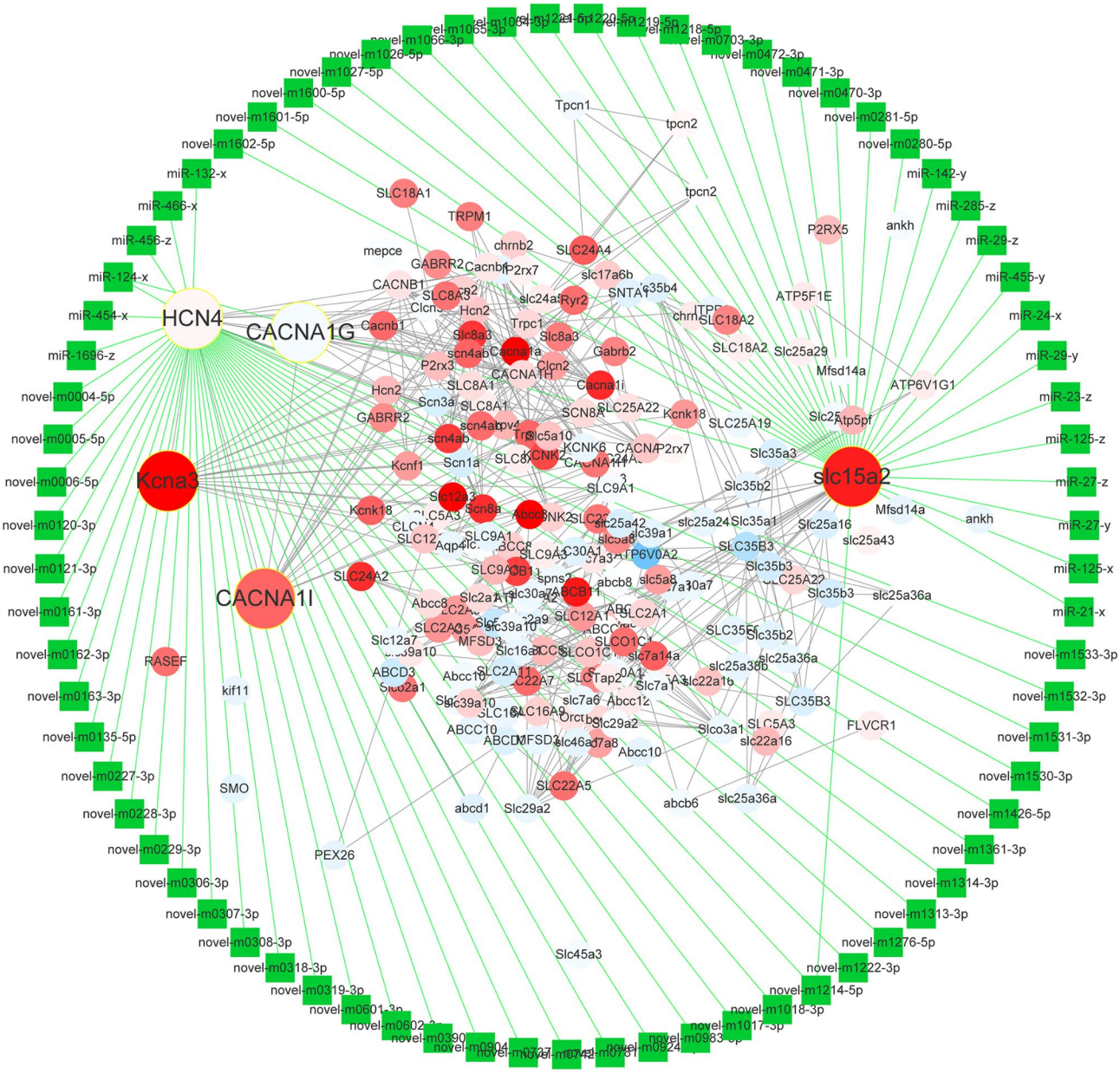

Fig. 8 Network diagram of key mRNA and miRNA during transmembrane transport in cells

the Tf2 family transposons was found to be regulated by the miRNAs listed in Table S5.

Discussion

In order to investigate how gill cells perceive salinity and elicit physiological responses, we designed the relevant components of this study. Given that the in vitro responses of gill cells differ from those of the whole organism, it was essential to determine an effective concentration for salt treatment. Through the observation of the apoptosis process in gill cells under varying salinity conditions, we identified 0.8% NaCl as the optimal treatment concentration. Subsequently, we utilized transcriptome sequencing to identify rapid response genes within 3 to 9 h following treatment. We hypothesize that these rapid response genes are closely associated with the gill cells' ability to detect changes in external salinity and activate corresponding signaling pathways. During this investigation, we identified rapid response genes and related miRNAs that are linked to apoptosis, ion transport, and DNA integration. Notably, through the analysis of signaling pathways, we discovered that the intracellular release of Ca^{2+} may serve as a critical factor in transducing extracellular stimuli into intracellular signals. To further validate the role of Ca^{2+} in this process, we treated GPG cells under saline conditions with the

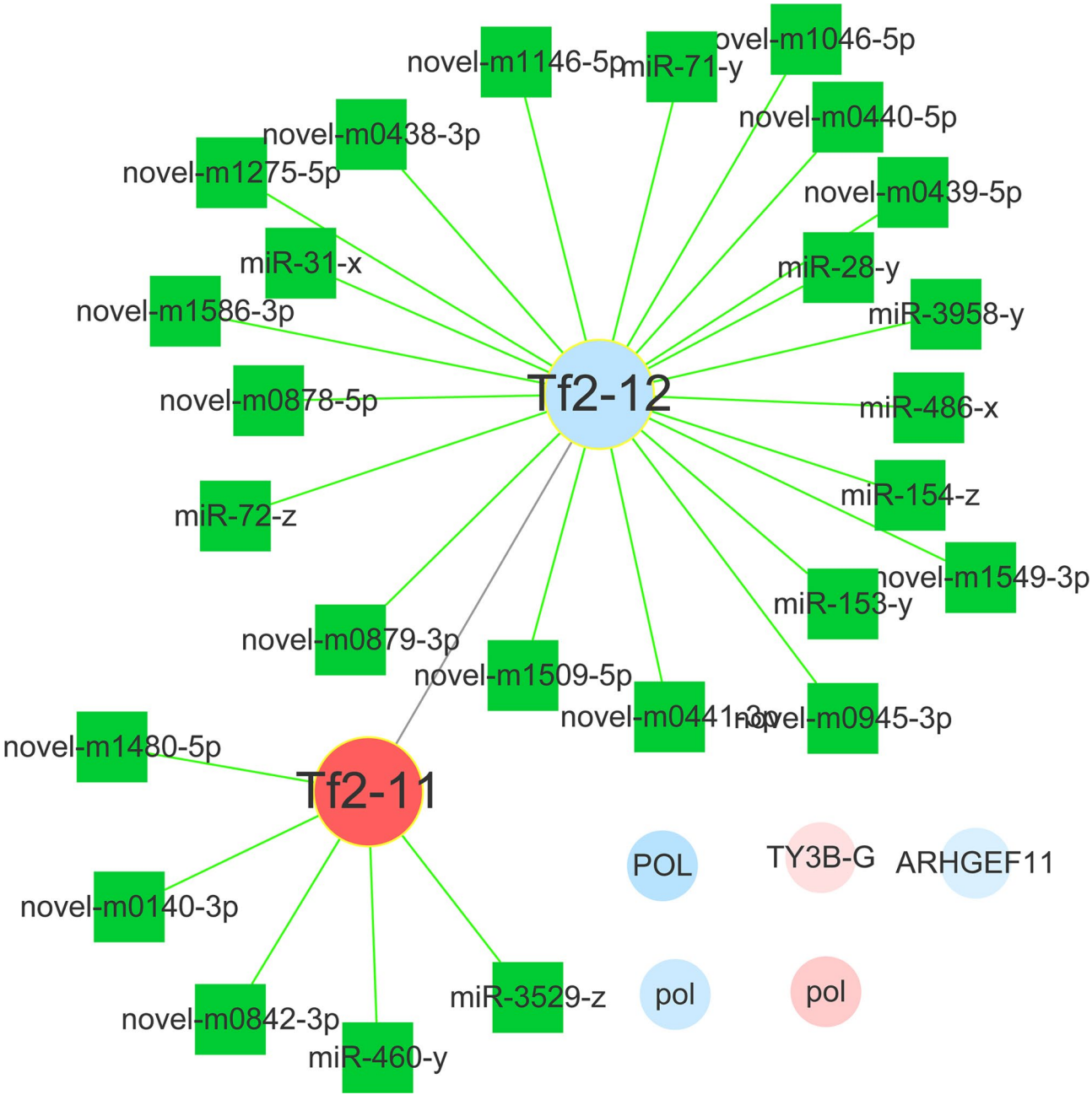

Fig. 9 Network diagram of key mRNA and miRNA relating with DNA integration

voltage-gated calcium ion channel inhibitor Nifedipine. The results indicated that the expression trends of 128 salinity-responsive genes could be reversed by Nifedipine, providing further confirmation of the functional significance of Ca^{2+}.

Salinity response and voltage-gated calcium ion channel

The GPG cell line was derived from the gill tissue of *G. przewalski*, an indigenous euryhaline species from Qinghai Lake on Qinghai-Tibet Plateau. In this experiment, the cell line showed rapid response to salinity and apoptosis similar to that in vivo. This cell line can be used as a cell model in studies on salinity adaptation of plateau fishes. In 9 h after acute salinity stress, the cells exhibited a typical signal amplification process. Within 3 h of acute salinity stress, the expression of transmembrane transporters significantly increased, and the expression of DNA integration, Apoptosis, Telomere maintenance and poly (A) RNA binding related genes significantly changed. These biological processes are obviously less related to the increased expression of transcription factors than proteins modification. Protein modification

related DEGs were significantly enriched in the stepwise decrease cluster, indicating the regulation of protein expression through protein modification mainly occured in the initial stage and gradually decreased in salinity acclimation. It was speculated there must be a signaling molecule that could activate protein modification in such a short period. Combined with the rapid response DEGs (showing increased or decreased trend in first 3 h) enrichment pathways, we suspected that Ca^{2+} was most likely to act in regulating osmosensor to initiate cell downstream responses.

Ca^{2+} is a highly versatile intracellular signaling molecule, and changes in intracellular Ca^{2+} concentration can regulate many cellular functions. Extracellular signaling molecules, such as cortisol and angiotensin II, have been shown to be involved in osmoregulation by increasing intracellular calcium concentration [27, 28]. In vitro, hypotonic stress can also increase the content of intracellular Ca^{2+} in cultured gill cells, and intracellular calcium can be used as the main node to participate in osmotic regulation through transcription factors with a variety of calcium-binding proteins [29]. In GPG cells, the salinity induced immediate-early response genes were significantly enriched in Calcium signaling pathway and Endocrine and other factor-regulated calcium reabsorption pathways indicated that Ca^{2+}, as a osmotic sensing molecule, participated in the early osmotic regulation of gill cells of *G. przewalski* in Qinghai Lake.

To further elucidate the role of Ca^{2+} channels in this process and downstream response proteins, Ca^{2+} channel inhibitor in combination with salinity stress were employed to determine downstream proteins. Assuming that salinity induces changes in membrane potential, thereby activating voltage-gated calcium ion channels and triggering the release of Ca^{2+} as a second messenger to induce the expression of numerous genes, the addition of the voltage-gated calcium ion channel inhibitor Nifedipine should inhibit the release of intracellular $Ca^{2+}$and reverse the expression regulation of genes induced by Ca^{2+} as a second messenger. After using the voltage-gated calcium channel inhibitor Nifedipine, it was found that a concentration of 100μM Nifedipine could inhibit the cellular response processes induced by salinity stress and reverse the gene expression trends caused by salinity stress. Although the addition of Nifedipine only altered the expression of 128 genes, 45% of these genes were target genes. This indicates that voltage-gated calcium ion channels are important channel proteins for cells to sense changes in external salinity. It is likely that changes in external salinity first alter the membrane potential of the cell membrane, thereby activating voltage-gated calcium ion channels, changing the concentration of intracellular free calcium ions, and causing independent cellular responses to salinity. However, the number of genes in the salinity stress response reversed by a concentration of 100μM Nifedipine is not as high as expected. This could be attributed to the diversity of Ca^{2+} channel types and the selection of Ca^{2+} inhibitor types and concentrations.

Cell apoptosis

Cell apoptosis is an important process for fish to adapt to changes in salinity, and in vivo experiments on various fish species have shown that salinity induces changes in gill filaments through the process of cell apoptosis [30]. In our previous study, we simulated salinity stress and found that 7 days of 0.9% salinity stress caused changes in the expression of various apoptosis-related proteins in the gill tissues of Qinghai Lake naked carp [8]. Additionally, we observed changes in the shape of gill filaments and the number of chloride cells in Qinghai Lake naked carp gill slices after long-term salinity stress. In this study, we investigated the acute response of the Qinghai Lake naked carp GPG cell line to salinity, and the results showed that an increase in salinity also induced the apoptosis process in GPG cells, indicating the existence of a hormone-independent cell apoptosis process in GPG cells.

Furthermore, we analyzed the DEGs significantly enriched in the apoptosis process and found that multiple pathways were involved in the salinity-induced cell apoptosis process, including the Calcium signaling pathway, NF-kappa B signaling pathway, MAPK signaling pathway, TNF signaling pathway, and P53 signaling pathway (Figure S6). These pathways were significantly enriched in profile 17, which consisted of salinity-sensitive up-regulated genes that responded within 3 h after salinity stress. This suggests that GPG cells promote the expression of relevant genes in these pathways through protein modification regulation, thereby inducing cell apoptosis. In Fig. 7, it was found that the core genes that can induce apoptosis included luo008571 (FADD), luo007079 (apaf1), luo005446 (apaf1), luo018624 (CFLAR) and luo018619 (CFLAR). FADD (Fas-associated death domain protein) is a protein involved in cell signaling and apoptosis (programmed cell death). It acts as an adaptor molecule that links death receptors (such as the Fas receptor) to downstream signaling mechanisms. Its most important function is to initiate the process of cell apoptosis by transmitting death receptor signals to the apoptotic pathway. When specific ligands activate death receptors, FADD binds to the death domain of the receptors, allowing inactive caspase-8 or caspase-10, i.e., initiator cysteine proteases, to be recruited and activated. This leads to the formation of the death-inducing signaling complex and subsequent activation of the caspase cascade, ultimately resulting in cell death [31]. Apaf-1 (Apoptotic Protease Activating Factor 1) is a key effector of cell apoptosis, primarily involved in the activation

and regulation of the apoptotic pathway. When cells are subjected to stress or damage, Apaf-1 binds to ATP and cytochrome C to form the Apaf-1 complex, which activates caspase-9, a cysteine protease with apoptotic activity, ultimately executing cell death. In addition, Apaf-1 interacts with members of the Bcl-2 family to regulate mitochondrial function and maintain cell survival and homeostasis [32].

Osmotic adaptation

In osmotic adaptation, the balance of cellular osmotic pressure is primarily achieved through the expression of various transmembrane transport proteins for ions. The top 5 hub genes identified through the PPI network include luo018946 (HCN4), luo037719 (CACNA1G), luo030728 (CACNA1I), luo031159 (Kcna3), and luo019077 (slc15a2) (Fig. 8). CACNA1 (Calcium voltage-gated channel subunit alpha1) protein is an alpha-1 subunit of voltage-gated calcium ion channels, which play a crucial role in regulating calcium ion permeability on the cell membrane. In cells, CACNA1 protein plays an important role in regulating intracellular calcium ion concentration [33]. HCN4 (Hyperpolarization-activated cyclic nucleotide-gated potassium channel 4) protein is an ion channel protein that exhibits hyperpolarization activation, meaning the channel opens at negative potentials, resulting in nonspecific Na^+ and K^+ ion transmembrane transport [34]. Kcna3, also known as Potassium Voltage-Gated Channel Subfamily A Member 3, encodes a protein of voltage-gated potassium channels, which form ion channels in the cell membrane and regulate the stability of the cell membrane potential [35]. Slc15a2, also known as Peptide Transporter 2, encodes a peptide transporter protein that functions in transporting small peptide substances into cells across the cell membrane [36].

Among these, four core genes respond to changes in cell membrane potential, highlighting the significant role of membrane potential in the transmembrane transport of substances following salinity stress. Multiple studies have indicated that changes in extracellular salinity can alter the membrane potential of cells [37, 38]. Considering the characteristics of Ca^{2+} release within cells, it is hypothesized that changes in extracellular salinity likely first alter the membrane potential difference across the cell membrane, leading to the release of Ca^{2+}, which subsequently triggers downstream cellular responses.

Gene integration and telomere maintenance are important responses of cellular adaptation to salinity

Osmotic stress could affect telomere maintenance. Telomeres are located at the end of linear chromosomes and consist of short tandem DNA repeats and a large number of related proteins. The presence of telomeres distinguishes the natural ends of chromosomes from random DNA breaks, thus stabilizing the chromosome structure. The telomere maintenance system is a biomarker of human health, and it has been proven that glucocorticoids, reactive oxygen species (ROS), mitochondria, and inflammation play key roles in telomere maintenance [39, 40]. Telomere length is important for maintaining stem cell populations and maintaining tissue homeostasis required for cell division [41]. Osmotic stress altered the expression of 5'-to-3' DNA helicase (PIF1) gene and up-regulated the expression of protection of telomeres protein 1 (POT1) in GPG cells. POT1 could help telomerase being recruited to telomeres, and exhibit a significant effect on telomere lengthening [42]. Up-regulation of this protein can help to inhibit DNA damage caused by osmotic stress, but the regulatory mode behind it remains to be further studied.

DNA integration in GPG cells was related with saline stress. The karyotypes and genomes of fish have higher diversity and evolutionary specificity, and their transposon content has also increased compared with mammals [43]. There are a large number of transposable elements in the salmon genome, some of which can be activated by external stimuli [44]. In this study, we found that the expression of several Transposon in GPG cells was affected by external salinity, including Transposon Ty3-I Gag-Pol polyprotein (TY3B-G), transposon TF2-type (Tf2). After the orogeny, *G. przewalskii* gradually evolved from a freshwater fish to a species that can adapt to the saline and alkaline water of Qinghai Lake. A variety of evolutionary signatures have been identified in the genome of *G. przewalskii* [45], some of which may be associated with salinity-mediated transposition.

miRNA plays a crucial role in the rapid response to salinity stress

miRNAs are a class of small non-coding RNA molecules that are widely regarded as important regulators of gene expression. In fish, miRNAs play a key role in molecular regulatory mechanisms and can modulate the transcription and translation processes of target genes, thereby regulating important physiological processes such as growth and development, immune response, and reproductive control in fish [46–48]. MiRNAs also play an important regulatory role in osmotic stress in fish. For example, these miRNAs can regulate the expression of water channel proteins, such as AQP, affecting the balance of intracellular and extracellular water and helping fish adapt to osmotic stress environments [49]. MiRNAs can also regulate the expression of Na^+/K^+-ATPase, influencing the balance of sodium and potassium ions inside and outside fish cells, thereby regulating ion balance under osmotic stress [50]. In this study, we evaluated the roles of miRNAs in the rapid response to salinity stress and found that miRNAs extensively participate in the

expression of genes involved in the early osmoregulated response of GPG cells, serving as an important mechanism for mediating the regulation of gene of immediate-early response to salinity stress.

Conclusion

To further understand the signaling transduction process of *G. przewalskii* under salinity stress, we performed transcriptome sequencing on GPG cell line (the gill cell line from *G. przewalskii*) to identify rapidly adaptive genes and regulatory miRNAs during osmotic stress. RT-PCR indicated the reliability of the RNA-Seq expression analysis (Figure S7). The results showed that: ① Within 3 h after salinity stress, there were significant changes in gene expression, with significant changes in proteins involved in protein modification, indicating that the changes in expression levels of rapidly responsive genes during early salinity stress may be the result of protein modification in cell signaling pathways. ② After salinity stress, the Ca^{2+} signaling pathway was significantly affected, and voltage-gated calcium ion channel appeared to be a key factor in cell sensing and initiating downstream cell signaling in response to external salinity. ③ Cell apoptosis was the most typical feature of salinity stress, and KEGG enrichment results showed that multiple signaling pathways were involved in salinity-induced cell apoptosis, including the Calcium signaling pathway, NF-kappa B signaling pathway, MAPK signaling pathway, TNF signaling pathway, and P53 signaling pathway. ④ For the first time in *G. przewalskii* genome, Tf2 and TY3 families of transposons were found to be responsive roles to the external environment. ⑤ miRNAs play a crucial role in the rapid response to salinity stress by selectively inhibiting the expression of specific genes.

Supplementary Information

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Supplementary Material 1

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Not applicable.

Author contributions
FW implemented the whole experiments and prepared the original draft. XZ, FJ, QY, YC, MZ and MC participated in cell culture, validation work, and data processing. JL provided available experimental methods and was a major contributor in writing the manuscript.

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Data availability
The reported sequences data from cells under osmotic stress was archived in the Sequence Read Archive (SRA) with accession number PRJNA1066629. Data in validation of the role of voltage-gated calcium ion channels in GPG cell could be achieved with PRJNA1066630.

Declarations

Conflict of interest
The study authors certify that they have NO affiliations with, or in volvement in, any organization or entity with any financial or non- financial interest in the subject matter or materials discussed in this manuscript.

Ethics approval and consent to participate
The present study was performed in strict accordance with the Standard Operation Procedures of the Guide for the Use of Experimental Animals of Qinghai University. The research protocol was reviewed and approved by the Ethical Committee of Qinghai University. Female triploid rainbow trout were obtained from local fisheries (Qinghai, China).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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