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Response of the PI3K-AKT signalling pathway to low salinity and the effect of its inhibition mediated by wortmannin on ion channels in turbot *Scophthalmus maximus*

Wenxiao Cui[1,2] | **Aijun Ma**[1,3] | **Xinan Wang**[1,3]

[1]Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Shandong Key Laboratory of Marine Fisheries Biotechnology and Genetic Breeding, Qingdao Key Laboratory for Marine Fish Breeding and Biotechnology, Qingdao, China

[2]National Demonstration Center for Experimental Fisheries Science Education, Shanghai Ocean University, Shanghai, China

[3]Laboratory for Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science and Technology, Qingdao, China

Correspondence
Aijun Ma, Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Shandong Key Laboratory of Marine Fisheries Biotechnology and Genetic Breeding, Qingdao Key Laboratory for Marine Fish Breeding and Biotechnology, No.106 Nanjing Road, Qingdao 266071, Shandong, China.
Email: maaj@ysfri.ac.cn

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Abstract

The PI3K-AKT signalling pathway is a crucial part of the signal transduction network and is involved in many important physiological processes and the stress response to environmental alteration. In this study, gill and kidney transcriptome data demonstrated that the PI3K-AKT signalling pathway plays an important role in the stress response of the turbot *Scophthalmus maximus* when exposed to low salinity. Low-salinity stress led to down-regulation of gene expression, protein content and phosphorylation of AKT1 at Thr308 of the PI3K-AKT signalling pathway. However, the abundance and phosphorylation of AKT1 protein at Ser473 and Thr308 were detected only in the gill and not in the kidney. Inhibition of the PI3K-AKT signalling pathway mediated by wortmannin showed that wortmannin remarkably inhibited *PI3K* gene expression and protein synthesis as well as phosphorylation of AKT1 at Thr308 with or without low-salinity stress. In the context of pathway inhibition, suppressed expression levels of ion channel genes demonstrated that the PI3K-AKT signalling pathway acts on osmoregulation by positively regulating ion transport.

KEYWORDS

ion channels, low salinity, PI3K-AKT signalling pathway, turbot *Scophthalmus maximus*, wortmannin

1 | INTRODUCTION

Salinity is a major environmental factor that affects the growth, reproduction and survival of organisms (Birrer, Reusch, & Roth, 2012). Aquatic organisms, especially fish, respond to fluctuations in osmotic pressure with compensatory molecular adaptations that enable them to remodel homeostasis of osmotically disturbed aspects of cell structure and function (Kültz & Burg, 1998). The adaptive and acclimatory responses of fish to salinity stress depend on powerful networks that consist of osmosensing and osmotic stress signalling transduction (Fiol & Kültz, 2007). The crucial function of these networks is to integrate signals from multiple osmosensors and generate an amplified output stimulus to control the appropriate effector mechanisms (Fiol & Kültz, 2007; Seale, Richman, Hirano, Cooke, & Grau, 2003). The osmosensory signal transduction network in fishes is complex and consists of calcium, MAPK (mitogen-activated protein kinase), 14–3–3 and other macromolecular-activated signalling pathways (Kültz, 2012).

The PI3K-AKT signalling pathway is a well-known player in a variety of biological processes that are critical in mediating many aspects of cellular function, including cell growth, metabolism and survival (Franke, 2008). The core components of the pathway, namely phosphatidylinositide 3-kinases (PI3Ks) and AKT (protein kinase B), have been shown to regulate cell proliferation, survival and metabolism and have therefore been the focus of many studies in this field (King, Yeomanson, & Bryant, 2015; Wu et al., 2016). PI3Ks are a unique family of intracellular lipid kinases that phosphorylate the 3′-hydroxyl group of the inositol ring of phosphatidylinositides on the plasma membrane. PI3K signalling plays a key role in different cellular processes such as metabolism, inflammation, cell survival, motility and cancer progression (Vanhaesebroeck, Guillermet-Guibert, Graupera, & Bilanges, 2010). AKT (now also called AKT 1) is a serine/threonine-specific protein kinase that plays a key role in multiple cellular processes, including glucose metabolism, apoptosis, cell proliferation, transcription and cell migration. AKT1 also is involved in cellular survival pathways by inhibiting apoptotic processes. Additionally, it can induce protein synthesis pathways and is therefore a key signalling protein in cellular pathways (Chen et al., 2001).

Although these functions are well researched, recent studies have highlighted the significance of the PI3K-AKT signalling pathway in development and cellular differentiation in pluripotent stem cells (Yu & Cui, 2016). The importance of the signalling pathway response to stress also is receiving attached more attention. Transcriptome profiling and molecular pathway analysis of genes in association with salinity adaptation in Nile tilapia (*Oreochromis niloticus*) suggested that the chief sub-networks contained in the stable-then-change category were part of the PI3K signalling pathway (Xu et al., 2015). Research has shown that the PI3K-AKT pathway responds to and is activated by changes in membrane pressure mediated by osmotic stress (Munnik & Meijer, 2001). Hypertonic saline, on the other hand, attenuates pulmonary apoptosis through a PI3K-AKT-independent mechanism in rats (Jaskille, Koustova, Kirkpatrick, Rhee, & Alam, 2004). Brain transcriptome profiling analysis of Nile tilapia (*O. niloticus*) under long-term hypersaline stress showed that the PI3K-AKT signalling pathway is the upstream pathway for modulation of osmoregulation (Yan et al., 2018).

When exposed to salinity stress, turbots are able to maintain homeostasis by performing various physiological functions, which implies that they use of a wide variety of biological processes (Fouchs et al., 2009; Ollivier et al., 2006). A number of studied focus on the osmoregulatory mechanism of turbot. For example, a genome scan of natural populations of turbot living in different environmental conditions was performed to identify genes with a salinity adaptive genetic pattern (Vilas et al., 2015). The turbot's osmoregulatory capability was characterized by ionic and water branchial fluxes, gut and kidney exchanges, morpho-structural modification (particularly of the gill), enzyme activities of the main target organs and hormonal control (which was also investigated following transfer to hypo-saline environments) (Gaumet, Boeuf, Severe, Roux, & Mayer-Gostan, 1995; Imsland, Gunnarsson, Foss, & Stefansson, 2003; Tytler & Ireland, 1995). Additionally, the energy metabolism and growth factors in turbot exposed to the different salinity treatments have been studied (Dietz, Stiller, Griese, Schulz, & Susenbeth, 2013; Imsland, Björnsson, Gunnarsson, Foss, & Stefansson, 2007). However, few studies have focused on signal transduction, especially the PI3K-AKT signalling pathway, and their role in osmoregulation in turbot remains unclear.

The turbot *Scophthalmus maximus* is a stenohaline teleost species that maintains a constant internal milieu of 384 mOsm kg^{-1} compared with the external seawater, which is 1,109 mOsm kg^{-1} (Carruthers, 1944; Robinson, 1996). Therefore, it is very important to study osmotic signal transduction of turbot under hypotonic conditions. The goal of this study was to evaluate the role of the PI3K-AKT signalling pathway in the osmoregulatory organs (gill and kidney) of turbot in response to osmotic stress. The results will provide greater understanding of the signal transduction process that occurs under hypo-salinity stress in turbot and other homologous species.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

All experimental treatments for artificially cultivated fish were performed according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, China. The study protocol followed the recommendations of the Experimental Animal Ethics Committee, Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, China.

2.2 | Transcriptome data

The gene expression profiles of transcriptome data (SRP153594, PRJNA596587) were generated previously using an Illumina Hiseq

TABLE 1 Gene primers used for qRT-PCR

Gene name	Forward primer	Reverse primer
PI3K	TGTGAACTGTCCATTGCCGA	CTGGTCCAGGCGCTCAAATA
AKT1	AGCGACAGACATGCGAAGAA	TCACGACAGGAATCCACTGC
AQP1	GTCGCAGCAGCTCTCGTCTAC	CCGCTGACCAGGACCTTCATG
AQP11	GCAGGTGGCAGTATGACAGGAG	AGACCAGACAGTACTCCAGGAAGG
NHE	GCCAACATCTCCCACAAGTCC	GACGAAGGTCCAGTTCCAGGC
NKA	GTGGGCCGATTTGATCATTTGC	CCTGGGCTGCGTCGAATGATAAG
14–3–3	TTGCCATGCTGGACACCGTTAAG	CGCCATCACCTTCGCCATCAC
NAC	GCAACAAAGGACCCGTAAAGT	GACCAAACACCCAGGACAGAC
β-actin	CATGTACGTTGCCATCCAAG	ACCAGAGGCATACAGGGACA

2000 platform (Cui et al., 2019). The transcriptome data set included three hypo-salinity (5‰)- and three seawater (30‰)-treated kidney and gill samples of turbot (total length 10.14 ± 0.29 cm) exposed to treatments for 24 hr respectively. The experimental facilities and conditions were photoperiod 14-hr light: 10-hr dark; temperature, 14.0 ± 1.0°C, pH 8.1. Reference genome and gene model annotation files were downloaded directly from the genome website (http://denovo.cnag.cat/genomes/turbot/) (Figueras et al., 2016). An index of the reference genome was built using Hisat2 v2.0.4, and paired-end clean reads were aligned to the reference genome using Hisat2 v2.0.4. Differential expression analysis of the two conditions/groups (three biological replicates per condition) was performed using the DESeq2 package. Genes with an adjusted *p*-value <.05 after analysis were assigned as differentially expressed genes (DEGs). We used KOBAS software to test the statistical enrichment of DEGs in Kyoto Encyclopedia of Genes and Genomes pathways.

2.3 | Fish

Healthy juvenile turbots (body weight 33.11 ± 1.21 g) were obtained from Yantai Tianyuan Aquaculture Co., Ltd. Prior to the experiment, fish were fed with a commercial diet two times daily (8:00 and 18:00) at 1% of their body weight; during the experiment, they were not fed. All experiments were conducted in accordance with the principles and procedures approved by the Institutional Animal Care and Use Committee of the Yellow Sea Fisheries Research Institute (Qingdao, China).

2.4 | Response of the PI3K-AKT signalling pathway to low salinity

To detect the response of the PI3K-AKT signalling pathway in turbot to low salt stress, the salinity experiment was performed with three different salinities (5, 10 and 30 ppt, 30 ppt was the control group). In this experiment, healthy individuals were stocked in same tanks (60 cm × 50 cm × 45 cm) with flow-through seawater at a stocking density of 20 fish per tank for 2 weeks to adapt them to the recirculating experimental facilities and conditions (photoperiod 14-hr light: 10-hr dark; temperature, 14.0 ± 1.0°C, pH 8.1). The nine tanks then were divided into three groups (triplicate tanks for each salinity), and the water in the tanks in each salinity group was replaced with seawater of the appropriate different salinity. Experimental salinities were achieved by mixing bore-well water with dechlorinated tap water. At 1, 5, 12 and 24 hr, three fish from each tank (nine individuals per group at each time point) were anaesthetized with 100 μl/L MS222 (Maya Reagent, China) and then sacrificed. Gill and kidney tissues were sampled from each fish, and the fresh tissues were frozen in liquid nitrogen and stored at −80°C for subsequent experiments (qPCR, enzyme-linked immunosorbent assay, SDS-PAGE and Western blotting).

2.5 | Inhibition of the PI3K-AKT signalling pathway

We conducted an inhibition experiment, in which the PI3K-AKT signalling pathway was inhibited by wortmannin to test the effect of the PI3K-AKT signalling pathway on osmoregulation in turbot. Wortmannin is a potent, selective and irreversible PI3K inhibitor that binds to the p110 catalytic subunit of PI3K. Wortmannin was purchased from Solarbio. It was dissolved at 0.4 mg/ml in DMSO and diluted with 0.9% NaCl following the manufacturer's protocol. In the first set of experiments, turbots (n = 6) were given a single bolus intramuscular injection of 0.7 mg/kg wortmannin (Ng, Tsao, Nicklee, & Hedley, 2001) and killed at 1 hr and 4 hr (three individuals at each time point). This part of experiment was triplicate, and turbot injected with normal saline acted as the control group. In the second set of experiments, 0.7 mg/kg wortmannin was administered to six fish via intramuscular injection, and the fish were exposed to salinity stress (5‰). Three fish then were sacrificed at 1 hr and 4 hr to determine whether inhibition of the pathway was independent of salinity treatment. A pre-test was conducted to determine whether there was a toxic effect. This part of experiment was triplicate, and turbot injected with normal saline acted as the control group. All fish were anaesthetized with 100 μl/L MS222 (Maya Reagent, China) before sampling. The gill and kidney of each animal were harvested for quantitative real-time (qRT)-PCR, enzyme-linked immunosorbent assay (ELISA), SDS-PAGE and Western blotting. The experimental facilities and conditions are described in section 2.3.

2.6 | qRT-PCR

Total RNA was extracted from tissues using an RNA prep pure Tissue Kit (Tiangen). RNA purity was checked using the NanoPhotometer® spectrophotometer together with agarose gel electrophoresis. First-strand cDNA was synthesized using TranScript Frist-Stand cDNA Synthesis SuperMix (Beijing TransGen Biotech Co. Ltd.). The PI3K sequence in genome (GenBank: CP026244.1) was used to design primer. All primers (Table 1) for qRT-PCR were designed using Primer5.0 software. The specificity and efficiency of all primers for qRT-PCR were validated by sequencing and then blast to NCBI and by running serial dilutions and creating a calibration curve respectively. The housekeeping gene β-actin was used to adjust the gene expression data in order to keep the correct rate of pre-analysis gene expression data (Cui et al., 2019). qRT-PCR was performed following the protocol provided by the *TransStart* Top Green qPCR SuperMix Kit (Beijing TransGen Biotech Co. Ltd.) on a 185–5096 CFX96 Real-Time PCR Detection System (Beijing yue da Biotechnology Co. Ltd.). The $2^{-\Delta\Delta Ct}$ method was used to analyse the expression of genes (Schmittgen & Zakrajsek, 2000).

2.7 | SDS-PAGE and western blotting

Total protein extracted from gill and kidney tissues with RIPA (Solarbio) were diluted to an equal concentration with phosphate-buffered saline (PBS). A homogenate aliquot then mixed with Laemmli buffer (1:5 ratio) containing 100 mmol/L DTT was

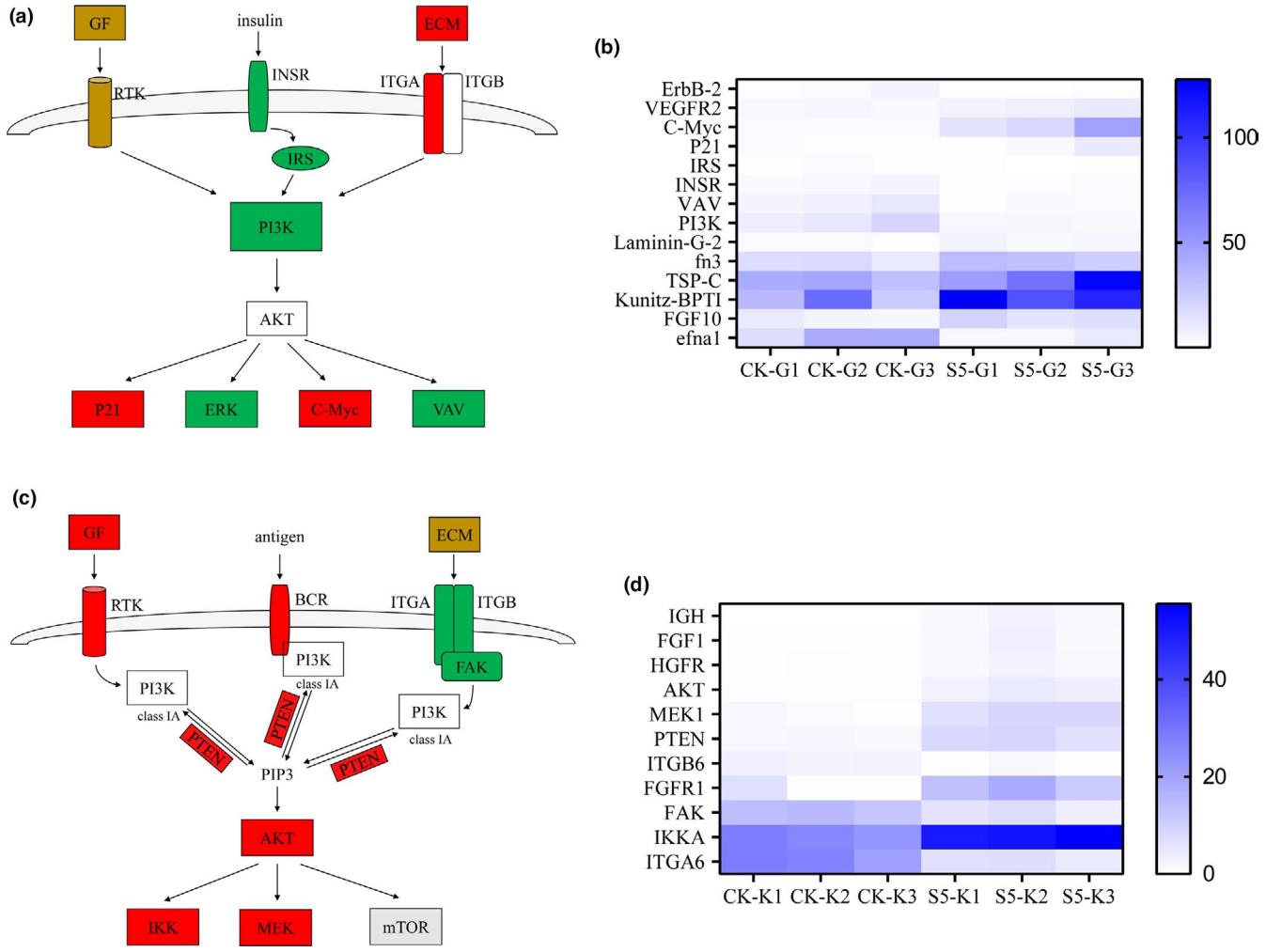

FIGURE 1 The schematic diagram of the PI3K-AKT signalling pathway on transcriptome. (a) The schematic diagram and (b) the heat map of DEGs of the PI3K-AKT signalling pathway on gill transcriptome. (c) The schematic diagram and (d) the heat map of DEGs of the PI3K-AKT signalling pathway on kidney transcriptome. *ErbB-2*, erythroblastosis oncogene B, *VEGFR2*: Vascular endothelial growth factor receptor 2, *IRS*, Insulin receptor substrate, *INSR*: Insulin receptor, *VAV*, Rho-associated protein kinase 1(ROCK1), *fn3*, Fructosamine-3, *TSP_C*, thrombospondin C, *FGF10*, Fibroblast growth factor 10, *efna1*, Ephrin-A1, *GF*, growth factor, *RTK*, growth factor receptor (receptor of tyrosine kinase), *BCR*, immunoglobulin heavy chain (B cell receptor), *ECM*, von Willebrand factor (extracellular matrix), *ITGA*, integrin alpha, *ITGB*, integrin beta, *FAK*, focal adhesion kinase, *PTEN*, phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase, *IKK*, inhibitor of nuclear factor kappa-B kinase, *MEK*, mitogen-activated protein kinase, Red colour: up-regulated, Green colour: down-regulated, Yellow colour: up- or down-regulated, Blue: Reads number in transcriptome

denatured by heating to 100°C for 7 min before loading on a 12% SDS-PAGE gel. Proteins were separated and electrotransferred to a PVDF membrane for 1 hr at 15 mA/gel. Immunoblotting using a rabbit polyclonal anti-AKT1 (AKT1, phosphor-Ser473, phosphor-Thr308) antibody (at 1:1,000 dilution) (Sangon) was performed and detected with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (Sangon) (Wang, Hu, Niu, & Chen, 2016). The antibody bands were visualized by chemiluminescence detection using Western Lightning Plus ECL (PerkinElmer) on a Tanon-5200 system.

2.8 | Protein content changes of PI3K

Detection of protein concentration of PI3K was performed following the protocol supplied by the manufacturer of the FISH PI3K ELISA KIT (JianglaiBio). The gill and kidney tissues were washed three times and then homogenized with PBS buffer on ice. After centrifugation, supernatants, controls and standards were incubated with PI3K detector (anti-zebrafish) labelled by HRP for 60 min on a detection plate before the solution was discarded and a secondary detector was added. The last step included a colour development phase that was stopped after approximately 15 min. Absorbance was read using an ELISA plate reader at a wavelength of 450 nm (Thermo Scientific).

2.9 | Statistical analysis

All statistical analyses were performed using SPSS19.0 for Windows. Results are expressed as mean values with their standard error mean (*SEM*). Normality and homoscedasticity assumptions were tested prior to the analysis. One-way analysis of variance (ANOVA) was employed to distinguish statistical differences between different treatment and control groups. Tukey HSD tests were then used to find significant groupings within the data set. In all analyses, the significance level was set at $p = .05$.

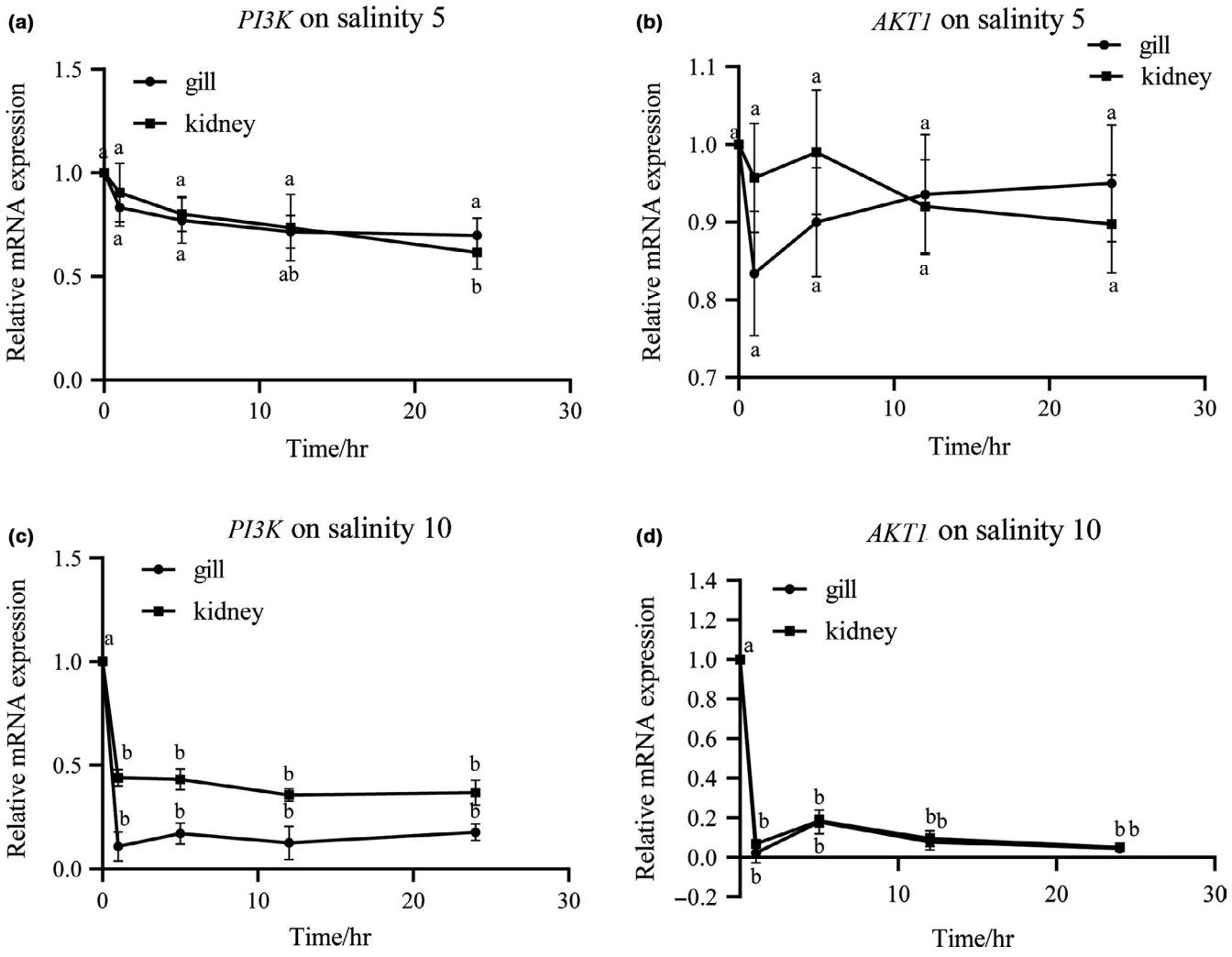

FIGURE 2 Gene expression after low-salinity stress. (a) Relative mRNA expression of *PI3K* on salinity 5, (b) relative mRNA expression of *AKT1* on salinity 5, (c) relative mRNA expression of *PI3K* on salinity 10 and (d) relative mRNA expression of *AKT1* on salinity 10. '0' represents control group. Different letters represent significant differences ($p < .05$) between the columns

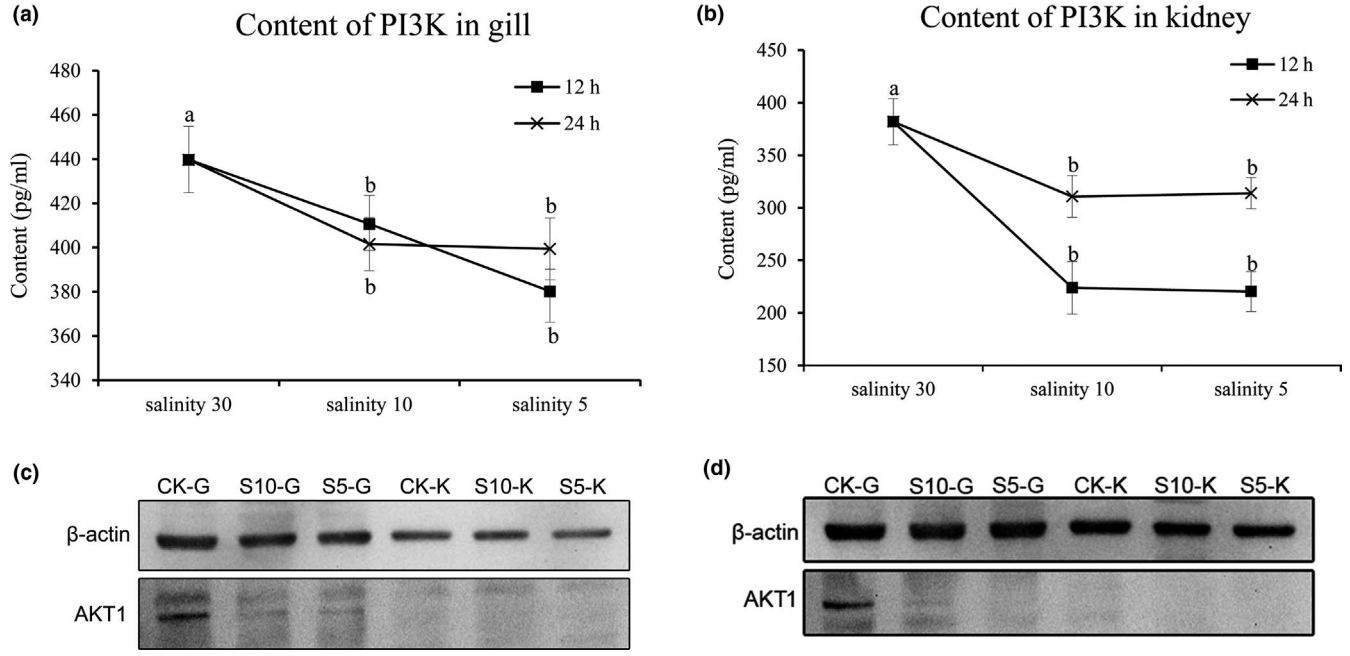

FIGURE 3 Protein content after low-salinity stress. (a) Content of PI3K in gill, (b) content of PI3K in kidney, (c) protein levels of AKT1 on 24 hr and (d) protein levels of AKT1 on 12 hr. CK, control group, G, gill, K, kidney, S10, salinity 10 ppt, S5, salinity 5 ppt, 'h' represents hour

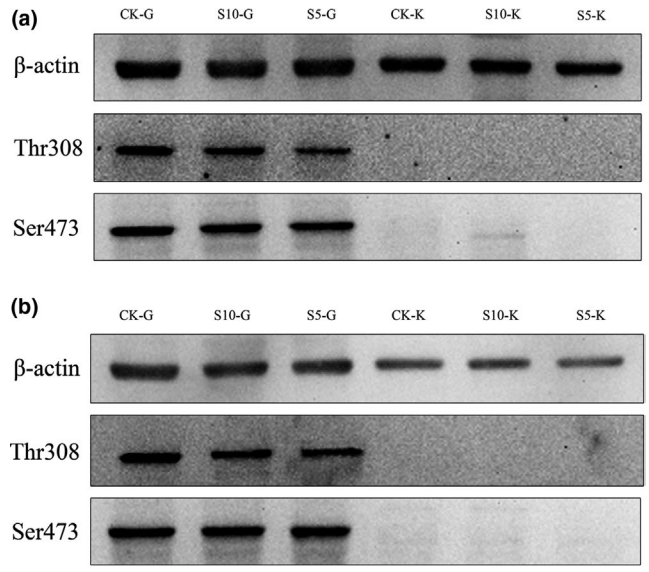

FIGURE 4 Phosphorylation levels of AKT1 under low salinity for 12 hr (a) and 24 hr (b). Different letters represent significant differences ($p < .05$) between the columns. CK, control group, G, gill, K, kidney, S10, salinity 10 ppt, S5, salinity 5 ppt. Thr308: phosphorylation of AKT1 at Thr308, Ser473: phosphorylation of AKT1 at Ser473

3 | RESULTS

3.1 | Transcriptome analysis revealed the role of the PI3K-AKT signalling pathway

Transcriptome data from the gill demonstrated that the core gene *PI3K* was down-regulated under hypo-salinity stress, the expression of genes coding signalling factors activating the PI3K-AKT signalling pathway was particularly affected, and the downstream genes were differentially expressed compared with the control group (Figure 1a,b). High throughput sequencing revealed that in the kidney, the expression level of the core gene *AKT1* in the PI3K-AKT signalling pathway was significantly up-regulated, transcripts coding important activators or inhibitors were changed, and the important gene *PTEN* in the pathway was also up-regulated. The amounts of transcripts of downstream genes *IKK* and *MEK* of the signalling pathway also changed (Figure 1c,d). These results show that the PI3K-AKT signalling pathway played an important role in the hypo-osmotic regulation of turbot at the transcriptome level.

3.2 | Response of the PI3K -AKT signalling pathway to hypo-salinity stress

Expression of core genes was analysed to investigate the response patterns of the PI3K-AKT signalling pathway to hypo-salinity stress. First, gene expression profiling was conducted to study the response to low salinity at the transcriptional mRNA level. The mRNA expression of gene *PI3K* in the gill and kidney was slightly down-regulated in a linear manner over time (Figure 2a). The time-course curves of transcript levels indicate that the abundance of *AKT1* fluctuated to maintain its relatively stability and original level in the osmotic regulatory organs of specimens exposed to salinity 5 despite slight down-regulation (Figure 2b). However, the expression levels of *PI3K* and *AKT1* first significantly decreased ($p < .05$) and then remained relatively stable in the gill and kidney at salinity 10 (Figure 2c,d).

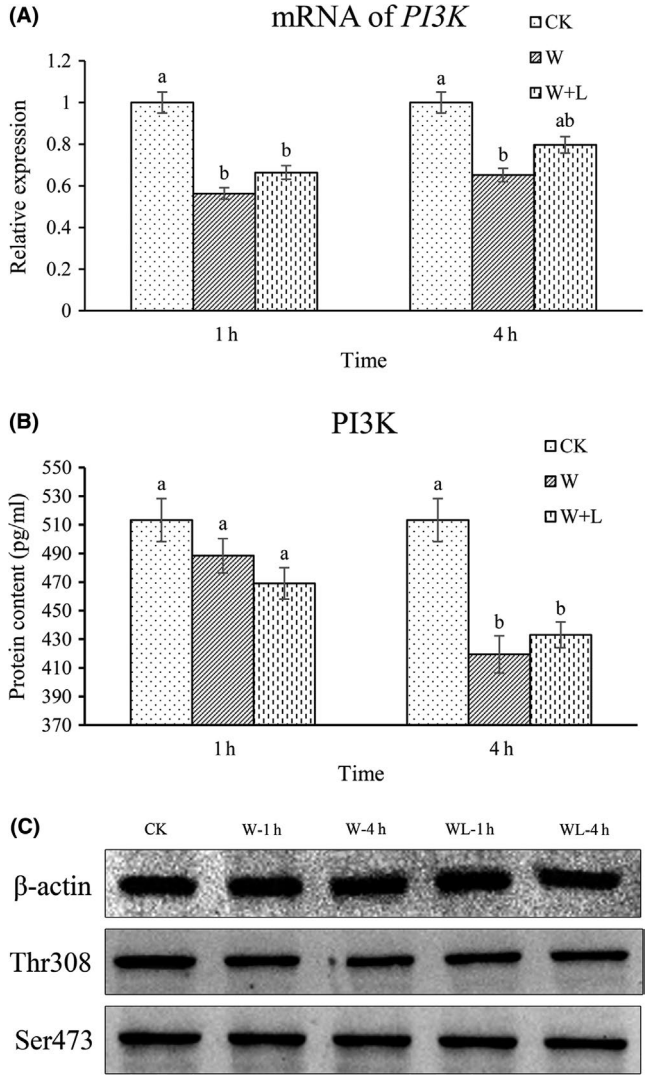

FIGURE 5 Changes of PI3K-AKT signalling pathway after wortmannin administration. (a) The relative expression of *PI3K*, (b) protein content of PI3K and (c) phosphorylation levels of AKT1. Thr308: phosphorylation of AKT1 at Thr308, Ser473: phosphorylation of AKT1 at Ser473. CK, control group, G, gill, K, kidney, S10, salinity 10 ppt, S5, salinity 5 ppt, 'h' represents hour. W, wortmannin, L, low salinity, different letters represent significant differences ($p < .05$) between the columns

The ELISA was conducted to assess how PI3K protein expression patterns respond to low-salinity stress. With the decrease in salinity, protein contents of PI3K in the gill gradually decreased, whereas in the kidney, they first decreased and then stabilized (Figure 3a,b). However, the results of SDS-PAGE and Western blotting showed that the protein levels of AKT1 in the gill were down-regulated at 12 hr and 24 hr under low-salinity stress, but no AKT1 protein was detected in the kidney (Figure 3c,d).

We also examined phosphorylation levels of AKT1, which is a well-established downstream target of PI3K, to identify the activity of the signalling pathway. In the gill, phosphorylation levels of AKT1 at Thr308 were remarkably inhibited under low salinity at 12 hr and 24 hr, with greater inhibition at salinity 5 than at salinity 10. Phosphorylation at Ser473 was slightly decreased at 12 hr and remained stable at 24 hr in the gill. Phosphorylation of AKT1 at Ser473 and Thr308 was not detected in the kidney in all treatments (Figure 4).

3.3 | Effects of PI3K-AKT signalling pathway inhibition mediated by wortmannin on ion channels

To investigate the effects of the PI3K-AKT signalling pathway on the ion channels involved in osmoregulation, the inhibition experiment using wortmannin was performed. As AKT1 is not activated in the kidney, only the gills were used for this experiment. Expression of PI3K was significantly ($p < .05$) inhibited after wortmannin administration at 1 hr and 4 hr in the regular salinity treatment. Moreover, the expression of PI3K was remarkably decreased at 1 hr and 4 hr after wortmannin injection and salinity stress treatment (Figure 5a). Additionally, protein content changes of PI3K were also detected after treatment. The ELISA assay showed that PI3K protein contents were decreased in the turbot gill in all treatments, and at 4 hr, the protein content was significantly ($p < .05$) reduced (Figure 5b). To further detect signalling pathway inhibition, phosphorylation of AKT1 in gill was evaluated. The levels of phosphorylated pAKT1-Thr308 were remarkably inhibited after wortmannin treatment with or without low salinity. However, phosphorylation of AKT1 at Ser473 remained relatively constant after wortmannin administration with and without low salinity (Figure 5c). Taken together, these results showed that the PI3K-AKT signalling pathway was inhibited after wortmannin administration with or without low salinity.

Expression of ion channel genes was evaluated to study the effects of the inhibition of the PI3K-AKT signalling pathway on osmoregulation. qRT-PCR demonstrated that genes *AQP1*, *AQP11*, *NKA*, *NHE1*, *14–3–3* and *NAC* were significantly ($p < .05$) down-regulated at 1 hr and 4 hr with or without low salinity after inhibition of the PI3K-AKT signalling pathway (Figure 6).

4 | DISCUSSION

It has been well recognized that the changes of many environmental factors in water, such as salinity, have important impacts on the homeostasis of marine teleost fish (Edwards & Marshall, 2012; Whittamore, 2012). Principles and patterns of osmoregulation have been identified in many euryhaline fish (Edwards & Marshall, 2012; Kültz, Chakravarty, & Adilakshmi, 2001), but the signal transduction network composed of signalling pathways is poorly understood. The PI3K-AKT signalling pathway, which is a crucial part of signal transduction network, has been found to be involved in many important physiological processes and stress responses to environmental fluctuations in parameters such as salinity (Cui et al., 2019; Yan et al., 2018; Yu & Cui, 2016). The results of the present study demonstrated for the first time that the PI3K-AKT signalling pathway is involved in osmoregulation in turbot.

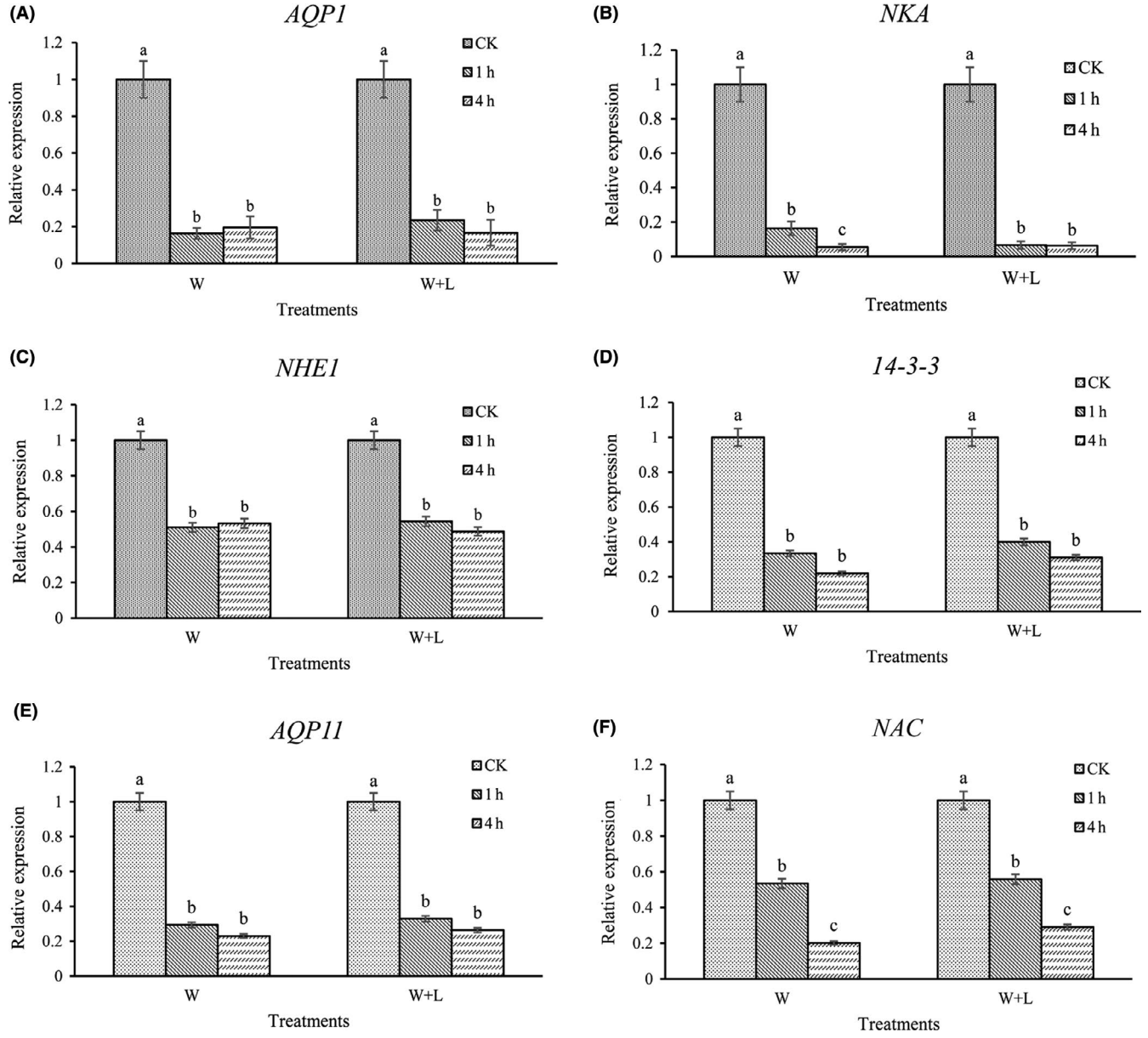

FIGURE 6 Expression of ion channels genes after wortmannin administration. *AQP1*, Aquaporin 1, *NKA*, Na^+-K^+-ATPase, *NHE1*, Na^+-H^+ exchanger 1, *AQP11*, Aquaporin 11, *NAC*, Na^+/Cl^- cotransporter, different letters represent significant differences ($p < .05$) between the columns

Although the specific mechanism has not been studied in fish, many transcriptome data sets have shown that the PI3K-AKT signalling pathway responds to and is activated by salinity changes (Munnik & Meijer, 2001; Yan et al., 2018). Consistent with previous research (Munnik & Meijer, 2001; Yan et al., 2018), the transcriptome data in this study showed that the expression of core genes in the gill and kidney changed after turbot was exposed to hypo-salinity stress and that the PI3K-AKT signalling pathway played an important role in the stress response to low salinity. Differences in activation factors and downstream effectors of the PI3K-AKT signalling pathway between transcription in the gill and kidney demonstrated that the PI3K-AKT signalling pathway is involved in regulating a wide array of physiological processes and in the difference in osmoregulation between the gill and kidney (Brazil, Yang, & Hemmings, 2004; Edwards & Marshall, 2012; Kültz, 2015). This result also verified the functional diversity of the PI3K-AKT signalling pathway in different cells (Franke, 2008).

To deal with stress, the PI3K-AKT signalling pathway minimizes the effects of altering environmental factors by regulating gene expression (Jaskille et al., 2004). As a result, hypertonic saline attenuates pulmonary apoptosis through a PI3K-AKT-independent mechanism by decreasing the phosphorylation of AKT (Jaskille et al., 2004). Additionally, brain transcriptome data demonstrated that the PI3K-AKT signalling pathway modulates osmoregulation of Nile tilapia *O. niloticus* as it plays an integral role in mediating signal transduction (Yan et al., 2018). In the present study, gene expression, protein content and levels of phosphorylated pAKT1-Thr308

of the PI3K-AKT signalling pathway were down-regulated in the gill under hypo-salinity stress. This may be because low-salinity stress can lead to inhibition of the PI3K-AKT signalling pathway, which may maintain the normal function of tissues by up-regulating apoptosis and clearing cells with impaired function to some extent (Wang et al., 2016). Although the gill and kidney of turbot both are important organs involved in osmotic regulation, the content and phosphorylation of AKT1 protein at Ser473 and Thr308 were detected only in the gill. A possible explanation for this difference is that varying and isoform-specific types of AKT may be present in different tissues, and different AKT isoforms are associated with distinct biological processes (Franke, 2008). Generally, maximum activation of AKT requires both Thr308 phosphorylation and phosphorylation at a second site (Ser473) by PDK2, a kinase whose identity remains unclear (Liang & Slingerland, 2003). However, the phosphorylation levels of AKT1 at Thr308 were remarkably inhibited under low salinity, whereas phosphorylation at Ser473 remained relatively stable in the gill of turbot. These results agree with those of a previous study, which reported that this AKT1 modification (inhibition of phosphorylation at Thr308) was sufficient to activate the mammalian target of rapamycin complex 1 (mTORC1) by direct phosphorylation and that activation of mTORC1 resulted in increased protein synthesis and cell survival by direct phosphorylation of its effectors (Martini, Santis, Braccini, Gulluni, & Hirsch, 2014). In summary, in turbot, the PI3K-AKT signalling pathway responds to low-salinity stress by reducing gene expression, protein production and phosphorylation of AKT1 at Thr308.

The effects of the PI3K-AKT signalling pathway on the ion channels involved in osmoregulation in turbot were studied by the inhibiting PI3K-AKT using wortmannin. Consistent with Ng et al. (2001), the present study showed that wortmannin remarkably inhibited PI3K gene expression, protein synthesis and phosphorylation of AKT1 at Thr308. The relatively stable phosphorylation of AKT1 at Ser473 after wortmannin administration and low-salinity treatment also demonstrated that the PI3K-AKT signalling pathway mediates osmotic regulation through phosphorylation of AKT1 at Thr308. After wortmannin administration, the mRNAs of genes coding proteins AQP1 for water transport (Chandy, Zampighi, Kreman, & Hall, 1997; Kültz et al., 2001), NKA for Na^+ and K^+ transport (Hu, Chu, Yang, & Lee, 2017), NHE1 for Na^+ and H^+ exchange (Baumgartner, Patel, & Barber, 2004), 14–3–3 for salinity adaptation (Miao, Long-Tao, Bin-Peng, Hang-Jiao, & Xiao-Ling, 2012), AQP11 for the transport of water and small neutral solutes across cell membranes (Ishibashi, Koike, Kondo, Hara, & Tanaka, 2009) and NAC for Na^+/Cl^- cotransport were down-regulated (Inokuchi, Hiroi, Watanabe, Lee, & Kaneko, 2008). These results demonstrated that the PI3K-AKT signalling pathway acts on osmoregulation by positively regulating ion transport, as was also reported by Gavello, Carbone, and Carabelli (2016), who showed that PI3K is an effective factor for ion channel regulation. Leshem, Seri, and Levine (2007) also found that salinity stress responses, such as increased plasma membrane endocytosis, are coordinated by phospholipid-regulated signalling pathways. This also represents transcriptional regulation of ion channels genes modulated by the PI3K-AKT signalling pathway (Ballou, Lin, & Cohen, 2015). Chung et al. (2010) suggested that the activation of the phosphoinositide PI3K/AKT pathway by the over-expression of active kinases can recruit 14–3–3 proteins.

5 | CONCLUSION

In summary, transcriptome data from the gill and kidney of turbot demonstrated that the PI3K-AKT signalling pathway plays an important role in the stress response induced by exposure to low salinity. Low-salinity stress leads to down-regulation of gene expression, protein content and phosphorylation of AKT1 at Thr308 of the PI3K-AKT signalling pathway in the gill of turbot. Inhibition of the PI3K-AKT signalling pathway mediated by wortmannin showed that wortmannin remarkably inhibited *PI3K* gene expression, protein synthesis and phosphorylation of AKT1 at Thr308 with or without low-salinity stress. In the context of pathway inhibition, suppressed expression levels of ion channel genes demonstrated that the PI3K-AKT signalling pathway acts on osmoregulation by positively regulating ion transport.

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CONFLICT OF INTEREST

All the authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS

Wenxiao Cui participated in the experimental design, investigations, data analyses and interpretation, and drafted the manuscript. Aijun Ma and Xinan Wang participated in the experimental design and coordinated the study.

ETHICAL APPROVAL

The research did not need any ethical approval to be conducted.

DATA AVAILABILITY STATEMENT

The authors confirm that all data generated or analysed during this study, and the data supporting the findings of this study are available within the article.

ORCID

Aijun Ma https://orcid.org/0000-0002-1964-467X

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