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Salt tolerance candidate genes identified by QTL mapping, RNA-seq, and functional analysis in tilapia

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ABSTRACT

Salt tolerance in fish is crucial for aquaculture as it enhances survival and productivity in varying salinity conditions, thus expanding the range of viable farming environments and improving economic sustainability. Through QTL mapping and GWAS in a hybrid F_2 family of Mozambique and Nile tilapia, two large-effect QTL on chromosomes 11 and 18 were identified respectively. These two QTL explained a total of 39.9 % of the phenotypic variance. The identification of a QTL on LG11 suggests the presence of a previously unrecognized genetic factor contributing to salt tolerance. Comparative transcriptomic analysis of gill and kidney tissues between susceptible and tolerant tilapias highlights the importance of osmotic balance in regulation of salt tolerance in tilapia. Integration of QTL and RNA-seq data identified two candidate genes: acyl-coenzyme A thioesterase 5 (*acot5*) and sodium- and chloride-dependent taurine transporter (*slc6a6*) likely playing critical roles in such process. Functional analysis showed that over-expressing *acot5* or *slc6a6* in grouper kidney cells increased viability under salt stress by 4.46 % and 17.53 %, respectively. Subcellular localization revealed nuclear presence of ACOT5 and stress-induced nuclear translocation of SLC6A6. These findings highlight *acot5* and *slc6a6* as candidates for genetic manipulation and selection to enhance salt tolerance in tilapia, guiding genetic improvement efforts and promoting sustainable practices.

1. Introduction

Tilapia, a group of cichlid fish native to Africa, have become a staple in global aquaculture due to their rapid growth, adaptability to diverse environments, and high nutritional value (Webster and Lim, 2006; Yue et al., 2016). However, salinity tolerance remains a significant challenge, limiting tilapia production in brackish and marine environments. Enhancing salt tolerance in tilapia is crucial for expanding their aquaculture potential and addressing the increasing demand for seafood in regions with saline water resources (Suresh and Lin, 1992; Yue et al., 2024).

The ability of tilapia to tolerate saline environments is a complex trait influenced by multiple genetic and environmental factors (Suresh and Lin, 1992; Yue et al., 2024). Previous studies have highlighted the role of specific physiological and molecular mechanisms in salt tolerance, including ion regulation, osmoregulation, and stress response pathways (Gu et al., 2018; Huang et al., 2024; Jiang et al., 2019; Prunet and Bornancin, 1989; Rengmark et al., 2007). Despite these advancements, the identification of candidate genes and their regulatory networks remains incomplete, necessitating comprehensive genomic and functional analyses to pinpoint the genetic basis of salt tolerance in tilapia.

Quantitative trait loci (QTL) and genome-wide association study (GWAS) are powerful tools for identifying genomic regions associated with complex traits (Yanez et al., 2023; Yue, 2014), including salt tolerance. By linking phenotypic variations to specific genetic loci, they provide insights into the genetic architecture of these traits (Huang et al., 2024). However, QTL mapping and GWAS often lack the resolution needed to pinpoint individual candidate genes, making it essential to integrate additional genomic approaches. RNA sequencing (RNA-seq) offers a high-resolution method to examine gene expression profiles under varying salinity conditions (Chandhini and Rejish Kumar, 2019), enabling the identification of differentially expressed genes (DEGs) that may play crucial roles in salt tolerance (Qin et al., 2022). By combining

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QTL mapping/GWAS with RNA-seq, it is possible to narrow down candidate genes within QTL regions (Wang et al., 2021) and gain a deeper understanding of their functional roles in salt tolerance mechanisms. Functional analyses are crucial to validate the roles of these candidate genes (Li et al., 2015; Liu et al., 2022; Wang et al., 2022), which is a necessary step in translating genomic discoveries into practical applications for tilapia breeding programs. Several studies have used QTL mapping and RNA-seq to identify significant QTL associated with salt tolerance, such as LG 4 and LG 18 in Nile tilapia (Gu et al., 2018) and LG 18 in red tilapia (*Oreochromis spp*) (Jiang et al., 2019). However, these studies were limited to a single species or population and lacked functional analyses for candidate genes.

In this study, we employed an integrative approach combining QTL mapping/GWAS, RNA-seq, and functional analysis to identify and validate candidate genes associated with salt tolerance in tilapia. We mapped two significant QTL for salt tolerance on LG11 and 18, explaining 39.9 % phenotypic variation. In addition, we identified two candidate genes (i.e., *acot5* and *slc6a6*) for salt tolerance. This study not only enhances our knowledge of the molecular mechanisms underlying salt tolerance but also contributes to the development of genetically improved tilapia strains with enhanced resilience to saline environments, thereby supporting sustainable aquaculture practices in regions affected by salinity.

2. Material and method

2.1. Mapping population

All fish handling procedures adhered to the guidelines for the care and use of animals in scientific research, as established by the Institutional Animal Care and Use Committee (IACUC) of Temasek Life Sciences Laboratory, Singapore. The study was conducted under IACUC approval number TLL(F)-22–004.

The F_2 hybrid tilapia family was derived from our previous study, constructed using a female Mozambique and a male Nile tilapia as F_0 parents. Information regarding to the culture, measurement of traits except for salt tolerance, genotyping, filtering of genotypes and construction of high-density linkage map were described in detail in our previous study (Wang et al., 2024).

2.2. Phenotyping salt-tolerance trait

Initially, the F_2 fish (61.1 ± 12.5 g) were maintained in freshwater with temperature of 28–32 °C and dissolved oxygen levels above 5 mg/L. At five months post-hatch, a total of 284 individuals were transferred into a 1.2-ton water tank with freshwater maintained for two weeks prior to challenge with sea water. On the day of the challenge, the freshwater in the tank was added sea salt to increase the salinity gradually at a rate of 5 ppt per hour, until a final salinity of 35 ppt was reached. This whole experiment lasted for eight hours and 20 min, from 7:45 am to 4:05 pm. The fish were monitored throughout the experiment, and those exhibiting signs of salt intolerance—such as losing balance and lying at the bottom of the tank (even if not fully deceased)—were classified as 'dead' and were recorded. Phenotypic data collection began when the first fish exhibited these signs, marking that moment as time 0. From this point, the time for each subsequent fish that either died or exhibited similar signs was recorded. Traits were recorded as quantitative data, specifically time to death, measured from time 0 until each subsequent fish exhibited signs of salt intolerance. After the challenge experiments were terminated, all salt-tolerant survivors were collected and recorded. All these fish were tagged with electronic tags, enabling the extraction of both genotype and phenotype information obtained from our previous study (Wang et al., 2024) for later selecting fish for studying gene expression.

2.3. QTL mapping and association analyses

QTL mapping was conducted using the MapQTL6 software (Kyazma, Wageningen, Netherlands), with the multiple QTL model (MQM) algorithm, based on a linkage map constructed with 38,102 SNPs (Wang et al., 2024).The permutation tests with 1000 replications were performed and the QTL with LOD scores exceeding the 0.05 significance level threshold at the genome and chromosome levels were considered significant, as established in our prior study (Wang et al., 2019).

A GWAS was conducted using the generalized linear model (GLM) with GAPIT v3, considering genetic structure, kinship matrix, and sex as covariates (Wang and Zhang, 2021). Using this model, the heritability of salt tolerance was estimated by the proportion of phenotypic variance explained by additive genetic variance. Statistical significance of SNPs was assessed using Bonferroni corrections, as detailed in previous study (Wang et al., 2024).

2.4. Seawater challenges in F_2 family for gill and kidney RNA-seq

Following QTL mapping and GWAS analyses, salt tolerant and susceptible genotypes were determined using the most differentiated SNP within each QTL interval (Table 1). Based on their effects on salt tolerance, the alleles in LG 11 (position: 35,540,553: T/A; T: tolerant, A: susceptible) and LG 18 (position: 12,007,794: T/C; T: tolerant, C: susceptible) were used to select salt-tolerant and salt-susceptible fish (Table S1, Mozambique tilapia genome, unpublished data). Three fish with the TT genotype in both LG 11 and LG 18 were designated as the salt-tolerant group, while three fish with the AA genotype in LG 11 and the CC genotype in LG 18 were categorized as the salt-susceptible group. To ensure uniformity in salt challenge experiments, all six fish were female and derived from the hybrid F_2 family, which was constructed using the same F_0 parents as the mapping family. These fish were then transferred to a challenge tank containing seawater with a salinity of 20 ppt for 2 h. During the exposure, their behaviors were monitored, and no abnormal activities were observed post-challenge. After the salt challenge, all fish from both groups were euthanized using AQUI-S® (AQUI-S, Lower Hutt, New Zealand) following the supplier's protocol. Gill and kidney tissues were collected from both the salt-tolerant and salt-susceptible groups and preserved in TRIzol® reagent (Life Technologies, Carlsbad, USA). Total RNA was extracted from the tissues using TRIzol® reagent, according to the manufacturer's guidelines.

2.5. Gill and kidney RNA-seq library construction, sequencing and bioinformatics analysis

Total RNA (2 μg) from each gill and kidney sample was sent to NovogeneAIT Genomics (Singapore) for RNA-seq library preparation and sequencing. The mRNA libraries were prepared using the Illumina TruSeq RNA Library Prep Kit v2 and sequenced on the NovaSeq 6000 system, generating 2 × 150 bp paired-end reads. The raw reads are available in the DDBJ database under accession number PRJDB18373.

The raw sequencing reads were cleaned by eliminating low-quality reads (Q < 20) and trimming primer/adaptor sequences using the NGS QC Toolkit (Patel and Jain, 2012). The filtered reads were subsequently aligned and mapped to the Nile tilapia reference genome (O_niloticus_UMD_NMBU) using the STAR tool (Dobin et al., 2013) with default

Table 1
Summary statistics of the significant QTL for salt tolerance traits identified by QTL mapping in hybrid tilapia. Genomic positions of the significant QTL are provided with reference to the genome of Nile tilapia (version UMD_NMBU).

LG.	QTL interval (cM)	Position (bp, O_niloticus_UMD_NMBU)		PVE (%)
11	0.888–6.470	30,603,629	37,581,709	16.2
18	39.757–56.247	24,212,720	38,049,427	23.4

PVE: Phenotypic variation explained.

parameters (Table S2). The HTSeq-count software (Anders et al., 2015) was used to quantify uniquely mapped reads, enabling differential gene expression analysis in accordance with the Nile tilapia genome annotation file. Differentially expressed genes (DEGs) between the salt-tolerance group and the salt-susceptible group were identified using DESeq2 (Love et al., 2014). Genes with a fold-change (FC) cut-off value of 4 and an adjusted *P* value of less than 0.05 were considered as DEGs. The DEGs were then mapped to QTL regions on chromosomes 11 and 18 to identify candidate genes for salt tolerance. Corresponding protein sequences of the DEGs were extracted from the annotation files of the reference genome and mapped to the zebrafish protein database (Ensembl release 99) using BLASTP with an e-value threshold of 1e-10.

Gene Ontology (GO) accessions were obtained for subsequent analysis. Network analysis was performed using Metascape (Zhou et al., 2019), incorporating GO annotations and zebrafish gene function networks to elucidate interactions among various functional groups of differentially expressed genes (DEGs).

The qPCR was used to validate the expression levels of DEGs identified through RNA-seq. Eight primer pairs (Table S3) were designed to target four up-regulated and four down-regulated genes in gill and kidney tissues. The $\log_2$ fold-change values obtained from RNA-seq were compared with those from qPCR for each gene. The consistency between RNA-seq and qPCR results was assessed using *R*-squared values and *P*-values.

2.6. Cloning and transforming acot5 and slc6a6 into vectors for gene overexpression

Two pairs of primers (Table S3) were synthesized to amplify the open reading frames (ORFs), including the stop codons, of *acot5* and *slc6a6*. The PCR products for *acot5* and *slc6a6* were inserted into the *EcoRI* and *XbaI* sites of the pcDNA-3.1 vector, creating the OE-ACOT5 and OE-SLC6A6 constructs. The recombinant vectors were verified by restriction enzyme digestion and DNA sequencing. Grouper kidney cells at 80 % confluence, grown on a 6-well plate, were divided into three groups for different treatments: one transfected with one microgram of free-pcDNA (OE-control), one with one microgram of OE-ACOT5, and one with one microgram of OE-SLC6A6. Transfections were performed using Lipofectamine 3000 Reagent (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. After 24 h, G418 (500 μg/mL) was added for drug selection, which was carried out for 5 days. Following G418 selection, overexpression efficiency was evaluated by qRT-PCR.

To challenge the G418-selected grouper cells with salt, approximately 5.5×10^4 cells were seeded into each well of a 24-well plate (Thermo Fisher Scientific, USA). For the salt-challenged samples, a 100 ppt (100 g/L) seawater solution was prepared using sea salt and filtered through a syringe filter (Sigma-Aldrich, USA). To achieve a final concentration of 20 ppt sterile seawater, 100 μL of the filtered seawater solution was added to 400 μL of cell culture buffer. This mixture was then applied to the cell cultures. For the control group, 100 μL of sterile water was added to 400 μL of cell culture buffer. There were three biological replicates for each type of transfected cell (OE-Control, OE-ACOT5, and OE-SLC6A6). After 24 h, cell viability was assessed using the methylthiazol tetrazolium (MTT) assay. For this, 50 μL of MTT labeling reagent (final concentration 0.5 mg/mL) was added to each well containing 500 μL of cell medium. The plate was incubated at 28 °C in the culture incubator for 4 h. Subsequently, the media were carefully removed without disturbing the cells, and 500 μL of DMSO was added to solubilize the formazan crystals by pipetting up and down. The plate was incubated at 28 °C for 15 min to ensure complete solubilization of the purple formazan crystals. The resulting solution was then transferred to a microplate, and absorbance was measured spectrophotometrically using Infinite 200 PRO microplate reader (Tecan, France). The absorbance of the formazan product was recorded at 570 nm, with a reference wavelength greater than 650 nm.

2.7. Subcellular localization of ACOT5 and SLC6A6 after salt challenge

Primer pairs for *acot5* and *slc6a6*, designed with *XhoI* and *EcoRI* restriction sites, were used to amplify the ORFs, including the stop codons (Table S3). The PCR products were separated on a gel and purified using the QIAquick Gel Purification Kit (Qiagen, Germany), creating the recombinant vectors ACOT5-EGFP and SLC6A6-EGFP. The sequences of these constructs were verified by DNA sequencing. Transfection was carried out on cells at 90 % confluence grown on glass-bottom petri dishes (MatTek Corporation, USA) using Lipofectamine 3000 Reagent (Thermo Fisher Scientific, USA), according to the manufacturer's instructions. After 48 h, the grouper kidney cells were stained with DAPI, and GFP and DAPI signals were detected using the Zeiss Axio Observer 7 Microscope (Carl Zeiss, Germany). To examine subcellular localization after salt challenge, the transfected ACOT5 and SLC6A6 cells were treated with 20 ppt seawater. Similarly, these cells underwent DAPI staining, and both DAPI and GFP signals were detected after 24 h.

2.8. Statistical analysis

A two-tailed *t*-test was performed using Microsoft Excel (Microsoft, USA). Statistical significance was indicated by asterisks (*: $P < 0.05$, **: $P < 0.01$). All results were based on triplicate measurements.

3. Results

3.1. Significant QTL for salt tolerance

As salinity increased, the fish began exhibiting stress behaviors, such as resting at the bottom of the tank, approximately 6 h after the challenge began. Mortality occurred within a range of 0 to 167 min, with an average time to death of 90.2 ± 35.5 min, resulting in a final mortality rate of 89 % (Supplementary Fig. S1). There is no evidence of sex-specific effect on salt tolerance in the mapping family (Supplementary Fig. S2). Analysis of genetic variation using the generalized linear model revealed that the heritability (h^2) of salt tolerance was 0.809. Two large-effect QTL were identified on LG11, explaining 16.2 % of the phenotypic variation (PVE), and on LG18, explaining 23.4 % of the variation, respectively (Table 1 and Fig. 1). GWAS detected the same two large-effect QTL on LG11 and LG18, with explained variances of 16.4 % and 23.5 %, respectively (Table 2 and Fig. 1). Additionally, three chromosome-wide significant QTL for salt tolerance were identified on LG4, LG7, and LG10, with PVE of 7.7 %, 8.5 %, and 7.2 %, respectively. Based on the physical locations of the two large-effect QTLs on LG11 and LG18, respectively, sequences of 7,400,898 bp and 5,031,248 bp in length were retrieved from reference genome of Nile tilapia (version O_niloticus_UMD_NMBU) (Conte et al., 2017) to search for candidate genes for salt tolerance. These regions contained 260 and 161 genes (with different variants), non-coding RNAs and uncharacterized RNAs on LG11 and LG18, respectively. Based on the effects of SNPs on salt tolerance, alleles on LG 11 and LG 18 were used to select salt-tolerant and salt-susceptible fish for the 20 ppt seawater challenge. Kidney and gill tissues were then collected for RNA-seq analysis. (detailed information can be found in section 2.4).

3.2. Differentially expressed genes (DEGs) of gill and kidney between salt tolerance and susceptible tilapia

The 12 mRNA libraries were sequenced, generating an average of 67.8 million (M) paired-end reads per library. After filtering out low-quality reads, the average number of clean reads per library was 67.1 M. These clean reads were mapped to the Nile tilapia genome (ver-

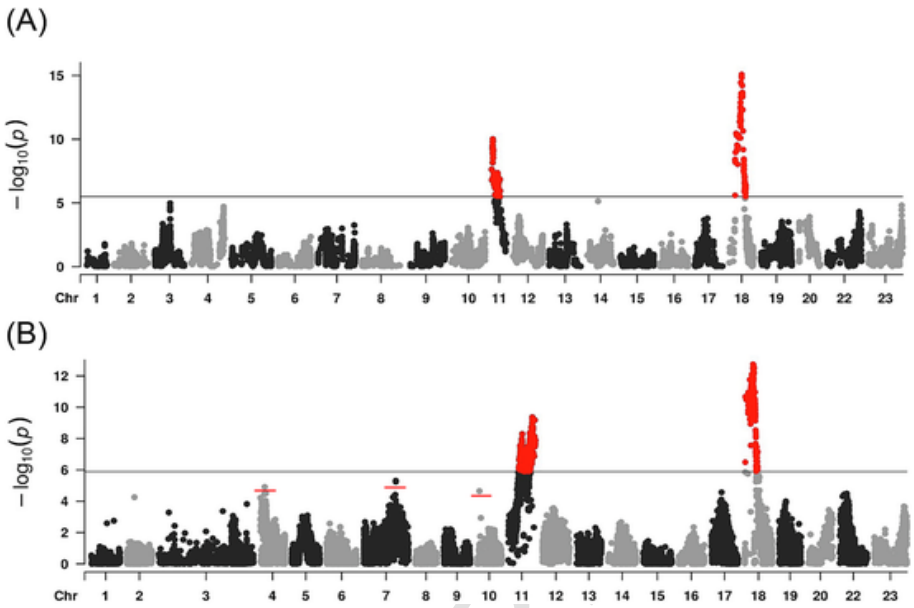

Fig. 1. (A) Genome scan of QTL for salt tolerance traits in an F_2 family of hybrid tilapia (*O. mossambicus* × *O. niloticus*). QTL with genome-wide significance is highlighted with red dots. (B) Whole genome-wide association studies on salt tolerance traits in an F_2 family of hybrid tilapia (*O. mossambicus* × *O. niloticus*). Loci with genome-wide significance are highlighted with red dots, while those with chromosome-wide significance are indicated with red horizontal lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Summary statistics of the significant QTL for salt traits identified by GWAS in hybrid tilapia. Genomic positions of the QTL are provided with reference to the genome of Nile tilapia (version UMD_NMBU).

Chrom.	Position (bp, O_niloticus_UMD_NMBU)		PVE (%)
4	6,195,480	8,346,427	7.7
7	17,972,747	18,070,691	8.5
10	2,963,327	3,063,826	7.2
11	31,481,671	38,882,569	16.4
18	24,742,265	29,773,513	23.5

PVE: Phenotypic variance explained by QTL.

sion UMD_NMBU), with approximately 92.2 % of the reads uniquely mapped (~30.9 million reads), which were used for differential expression analysis. RNA-seq analysis of the gill from salt-tolerant and salt-susceptible groups identified 154 differentially expressed genes (DEGs), with 97 up-regulated and 57 down-regulated (Fig. 2A). The transcriptome analysis of the kidney detected 4898 DEGs, including 3304 up-regulated and 1594 down-regulated genes (Fig. 2B). Validation of transcriptomic data showed high consistency with qPCR results (Pearson's $R = 0.8996$, $P < 0.0003$) (Fig. 2C). Gene ontology (GO) enrichment analysis of the up-regulated genes in the gill revealed no significant enriched terms. However, GO enrichment analysis of the down-regulated DEGs in the gill highlighted terms related to immune responses (GO:0006955). Additionally, GO analysis of all gill DEGs revealed significant terms associated with neutrophil degranulation (DRE6798695) and cilium movement (GO:0003341).

In the kidney, GO enrichment of the up-regulated DEGs highlighted terms associated with oxoacid metabolic process (GO:0043436), cilium organism (GO:0044782), alpha-amino acid metabolic process (GO:1901605), cellular modified amino acid metabolic processes (GO:0006575), and cytoskeleton in muscle cells (DRE04820) (Fig. 2D). The down-regulated DEGs in the kidney were enriched for GO terms related to the immune system processes (GO:0002376), cell cycle (GO:007049), DNA replication (GO:0006260), and nucleotide catabolic process (GO:0009166) (Fig. 2E).

3.3. Candidate genes acot5 and slc6a6 for salt tolerance

To identify candidate genes for salt tolerance in tilapia, DEGs were mapped to the two large-effect QTL regions associated with salt tolerance. In the QTL interval of ~7 Mb at LG11, 5 up-regulated and 3 down-regulated DEGs were found in the gills, and 23 up-regulated and 17 down-regulated DEGs were identified in the kidney (Fig. 3A and B, Tables S3 and S4), for example *rab25*, *perilipin-5*, and *synaptic vesicle glycoprotein 2 A*. In the QTL interval of ~5 Mb at LG18, there were 3 up-regulated and 4 down-regulated DEGs in the gills, and 34 up-regulated and 12 down-regulated DEGs identified in the kidney (Fig. 3C and D, Tables S3 and S4). GO enrichment analysis of the up-regulated DEGs located in QTL region in the kidney revealed significant terms related to acyl-CoA metabolic processes (GO:0006637), cellular lipid metabolic processes (GO:0044255), and organophosphate metabolic processes (GO:0019637). The down-regulated DEGs in the kidney were enriched for GO terms associated with the adenylate cyclase-modulating G protein-coupled receptor signaling pathway (GO:0007188) and regulation of response to external stimuli (GO:0032101) (Fig. 3E and F). Among the up-regulated DEGs in the kidney on LG 18, Acyl-coenzyme A thioesterase 5 (*acot5*) was selected as a candidate gene, showing a log (fold-change) of 15.29 in the kidney and 4.02 in the gill (4 exons and 3 introns, 7251 bp DNA and 1437 bp cds). This gene was selected not only because it is the top DEG but also due to its significant differential expression in both kidney and gill tissues, suggesting a consistent and potentially critical role in salt tolerance across different organs. Another gene, the sodium- and chloride-dependent taurine transporter (*slc6a6*) (5 exons and 4 introns, 7100 bp DNA and 540 bp cds), exhibited a log (fold-change) of 15.22 in the kidney. This gene was selected because it was the second highest DEG on LG 18 QTL interval. There were still other common DEGs in gill and kidney, including putative ferric-chelate reductase 1, thyroglobulin, sialic acid-binding Ig-like lectin, transcription factor HES-7, putative C-type lectin domain, acyl-coenzyme A thioesterase 1, myeloid-associated differentiation marker homolog (Table S4 and S5).

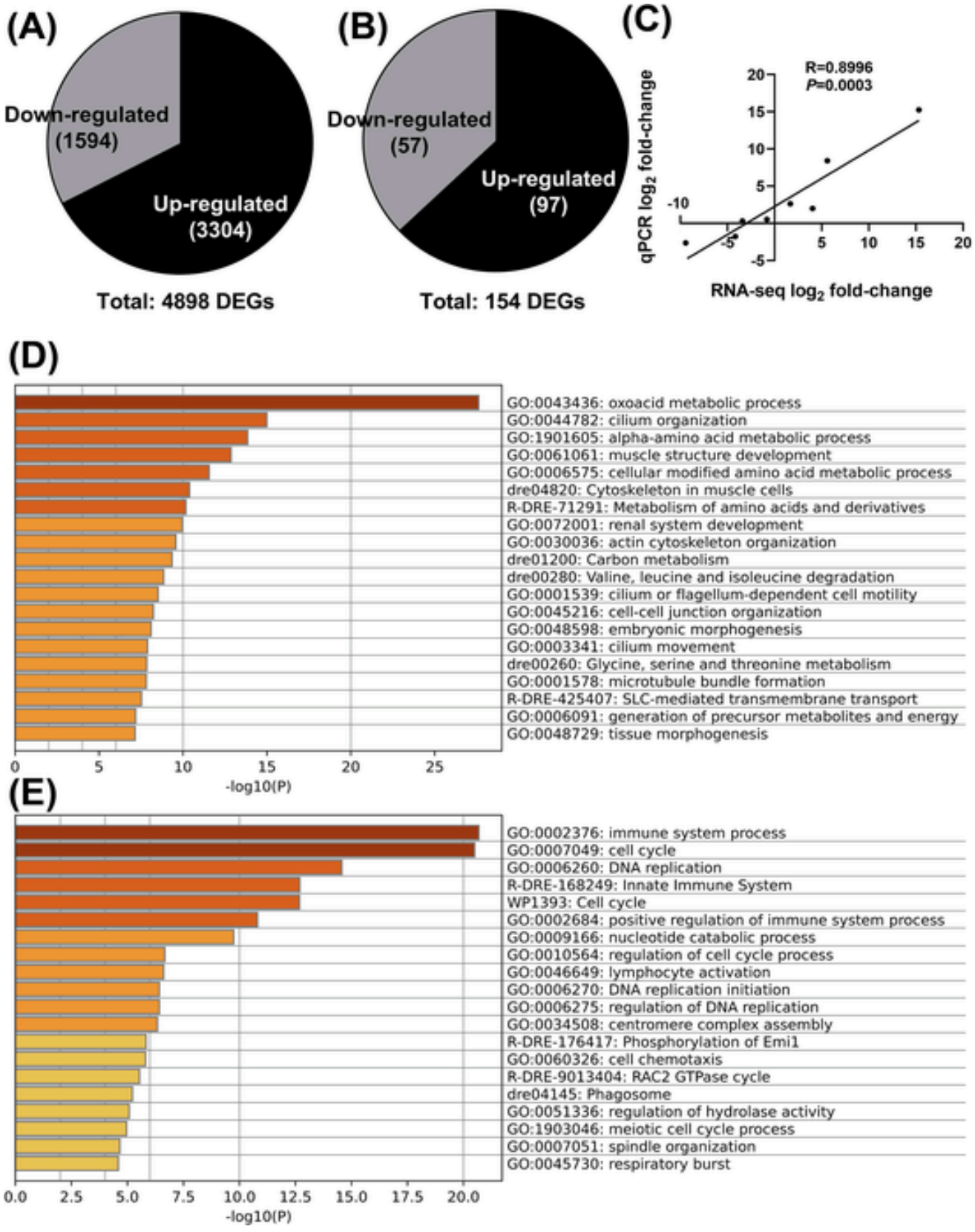

Fig. 2. The transcriptome analysis of the gill and kidney between salt-tolerance and salt-susceptible groups. (A) Venn graph of DEGs between tolerance and susceptible groups in gill. (B) Venn graph of DEGs between tolerance and susceptible groups in kidney. (C) Correlation between gene expression data generated by RNA-

Fig. 2.—*continued*
seq and qRT-PCR. (D) GO enrichment of up-regulated genes between salt-tolerance and salt-susceptible groups in the kidney. (E) GO enrichment of down-regulated genes between salt-tolerance and salt-susceptible groups in kidney.

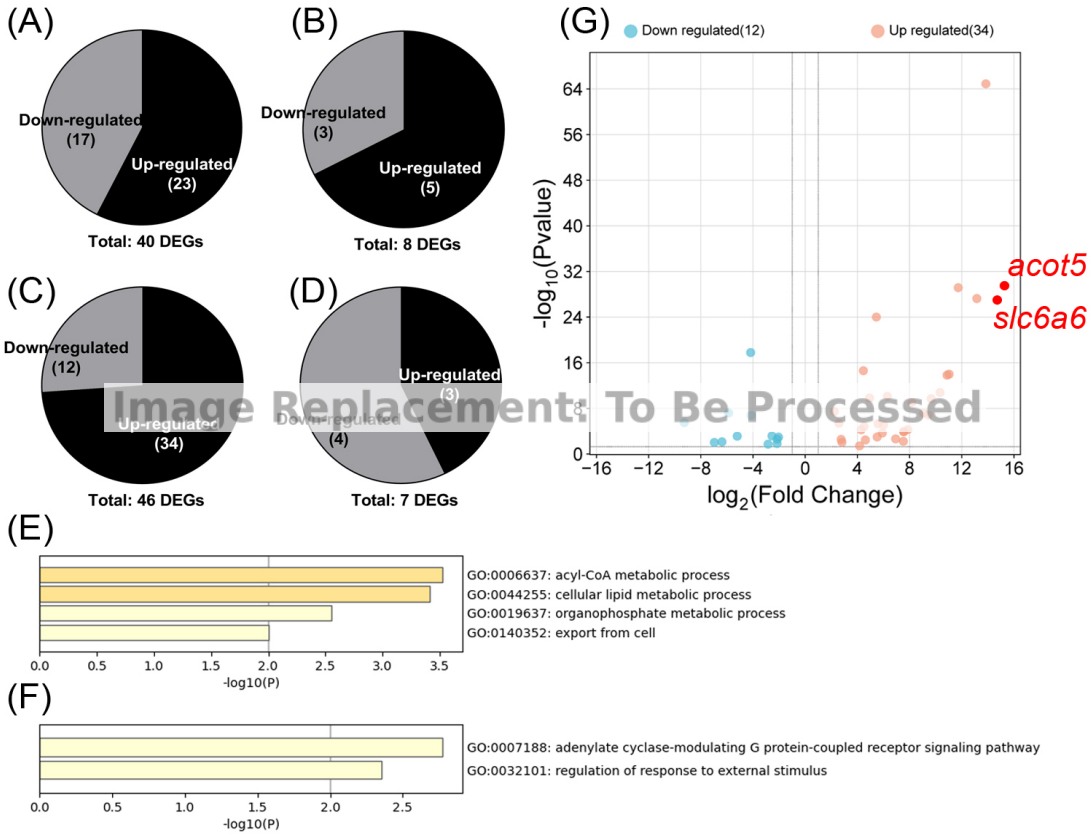

Fig. 3. Identification of candidate genes for salt tolerance in the gill and kidney. (A) Venn diagram of DEGs located in LG11 QTL in the gill. (B) Venn diagram of DEGs located in LG11 QTL in the kidney. (C) Venn diagram of DEGs located in LG18 QTL in the gill. (D) Venn diagram of DEGs located in LG11 QTL in the kidney. (E) GO enrichment of up-regulated genes located in LG18 QTL in the kidney. (F) GO enrichment of down-regulated genes located in LG18 QTL in the kidney. (G) Volcano plot of DEGs located in LG18 QTL intervals in the kidney. Red points indicated the candidate genes *acot5* and *slc6a6*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Cell viability increased in grouper cells overexpressing acot5 or slc6a6

To evaluate the effects of over-expressing *acot5* or *slc6a6* on salt tolerance, group kidney cells were transfected with the recombinant vectors OE-ACOT5 and OE-SLC6A6, respectively. Following transfection and G418 selection, cells were harvested for analysis. Relative expression levels of *acot5* and *slc6a6* were quantified using qRT-PCR, revealing a significant increase compared to cells transfected with the empty OE-control vector (Fig. 4A and B). Subsequently, the transfected cells were exposed to 20 ppt seawater for 24 h to assess cell viability. The results showed that the viability of OE-ACOT5 and OE-SLC6A6 cells was significantly higher than that of the control group, with increases of 4.46 % and 17.53 %, respectively, compared to the empty vector group (Fig. 4C).

3.5. SLC6A6 translocated from cytoplasm to the nucleus post-salt challenge

To determine the subcellular localization of ACOT5 and SLC6A6, fusion proteins ACOT5-EGFP and SLC6A6-EGFP were constructed and transfected into grouper kidney cells. Using wide-field inverted microscopy, GFP and DAPI signals were detected in both the cytoplasm and nucleus of cells transfected with empty EGFP-C1, which served as the control (Fig. 5A). The ACOT5-EGFP fusion protein was localized in the nucleus, whereas the SLC6A6-EGFP fusion protein was localized in the cytoplasm (Fig. 5A). To investigate changes in fusion protein localization following a salt challenge, cells were exposed to 20 ppt seawater. After 24 h, ACOT5-EGFP remained in the nucleus, while SLC6A6-EGFP translocated from the cytoplasm to the nucleus (Fig. 5B).

4. Discussion

4.1. Advancements and implications of mapping salt tolerance QTLs in hybrid tilapia

In this study, mapping of QTL for salt tolerance in the F_2 hybrid population of *O. niloticus* × *O. mossambicus* represents a significant advancement in understanding the genetic basis of salinity adaptation in tilapia. The F_2 generation allows for the segregation of traits inherited from both parent species, facilitating the identification of specific genetic factors influencing salt tolerance and other desirable traits (Yue et al., 2024). Moreover, the genetic diversity and trait segregation in F_2 hybrid populations enhance the accuracy of QTL mapping (Cnaani et al., 2003), crucial for pinpointing genome regions associated with salt tolerance. Previous studies have primarily focused on mapping salt tolerance within single species or population, such as Nile tilapia (Gu et al., 2018) and Malaysia red tilapia strain (*Oreochromis spp*) (Jiang et al.,

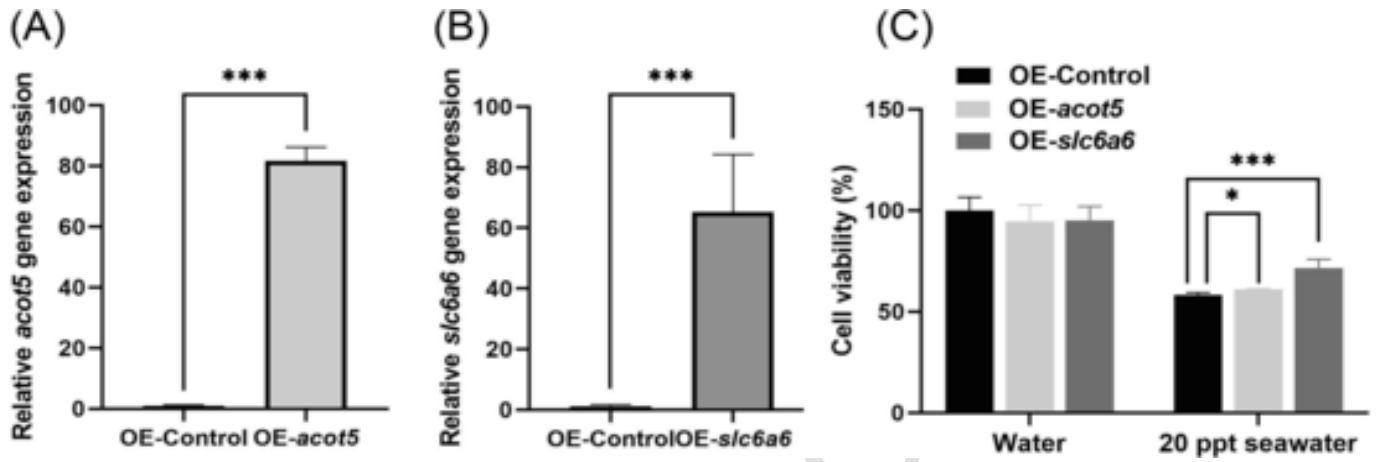

Fig. 4. Cell viability increased in the over-expressed *acot5* or *slc6a6* grouper kidney cells. (A) Relative expression level of *acot5* was significantly up-regulated in the cell transfected with OE-*acot5*. (B) Relative expression level of *acot5* was significantly up-regulated in the cell transfected with OE-*slc6a6*. (C) The viability of OE-*acot5* and OE-*slc6a6* cells was significantly higher than that of the control group. *: $P < 0.05$, ***: $P < 0.005$.

2019). In contrast, the increased variability of the hybrid F_2 generation helps identify both large- and minor-effect QTL, offering a comprehensive understanding of the genetic basis of salt tolerance (Wang et al., 2024).

In this study, the loci associated with salt tolerance were identified at LG 11 and LG 18. The identification of LG 18 was consistent with other studies of salt tolerance in Nile tilapia (Gu et al., 2018; Huang et al., 2024) and red tilapia (Jiang et al., 2019). The slight variation in QTL locations for salinity tolerance on chromosome 18 across different studies can be attributed to genetic background differences among populations or families (Huang et al., 2024). However, the consistent identification of QTL on LG18 suggests underlying conserved genetic mechanisms for salinity adaptation in various tilapia species or strains, implying a shared evolutionary ancestry. Furthermore, the conserved nature of the LG18 QTL across different tilapia species presented a valuable target for cross-species breeding programs. Breeders can utilize this information to develop strains with enhanced salt tolerance by targeting this QTL, potentially improving the robustness and productivity of tilapia in saline conditions.

Unlike the well-characterized QTL for salt-tolerance on LG 18, which has been previously reported (Gu et al., 2018; Huang et al., 2024), the QTL on LG 11 has not been highlighted in earlier studies, marking it as a unique finding in our research. This novel QTL on LG 11 suggests the presence of previously unidentified genetic factors contributing to salt tolerance. These findings provided a deeper understanding of the genetic basis of salt tolerance and indicates the potential for further research to explore specific genes and variants within this QTL. The identification of LG 11 and LG18 as critical regions for salt tolerance facilitates the application of marker-assisted selection (MAS) in breeding programs. By focusing on these linkage groups, breeders can more efficiently select for salt-tolerant individuals, thereby accelerating the development of resilient strains.

4.2. Metabolic changes and cellular changes in the gill and kidney under salinity stress

The RNA-seq analysis of gill tissue from salt-tolerant and salt-susceptible groups highlighted important insights into the biological mechanisms underlying salt tolerance in tilapia. DEGs associated with neutrophil degranulation and cilium movement were identified, indicating significant adaptations to the salt environment. The enrichment of genes involved in neutrophil degranulation, a key process in innate immunity, suggests that salt-tolerant tilapia may modulate immune functions to manage stress in high-salinity environments. The down-regulation of immune response genes may further reflect an adaptation to balance stress responses (Birrer et al., 2012). As changes in salinity can influence the immune system, enhanced immunity becomes crucial for survival in variable and potentially pathogenic saline waters (Peiseler and Kubes, 2019). Additionally, the enrichment of genes related to cilium movement underscores the importance of ciliary function in osmoregulation, essential for maintaining ion balance and expelling excess salts in high-salinity conditions (Marshall, 2011). These adaptations are vital for the survival and physiological stability of tilapia in fluctuating saline environments (Pablo et al., 2017).

In the kidney, the up-regulation of genes related to oxoacid metabolic processes suggests a metabolic shift to meet the increased energy demands of osmoregulation. Enhanced oxoacid metabolism indicates increased ATP production to support ion pumps and other mechanisms maintaining osmotic balance (McDonald, 2019; Prescott and Lloyd, 2000). Similarly, the up-regulation of genes involved in cilium organization and cytoskeletal structure highlights adaptations in fluid movement and cellular integrity under high salinity (Marra et al., 2016). The cytoskeleton plays a vital role in maintaining cell shape and integrity, especially under high stress conditions. The up-regulation of genes related to cytoskeletal organization indicates structural adaptations to withstand the osmotic pressure exerted by high salinity (Zhang, 2009). The down-regulation of immune-related genes suggests a trade-off, reallocating resources from immune functions to prioritize osmoregulation, ensuring survival in high-salinity environments. Additionally, decreased expression of genes associated with the cell cycle and DNA replication may indicate a shift towards cellular maintenance over growth (Kültz, 2005). This is a common response to environmental stress, where conserving energy and resources becomes critical. Further research should focus on validating these findings and to understand the specific roles of the identified genes in conferring salt tolerance.

4.3. Candidate genes for salt tolerance

Integrating genetic and transcriptomic data is crucial to reliably identify candidate genes associated with complex traits such as salt tolerance. This comprehensive approach combines the advantages of both GWAS and QTL mapping to pinpoint genomic regions linked to traits, while RNA-seq reveals changes in gene expression under stress conditions (Wu et al., 2023; Yang et al., 2021). The distribution of DEGs across two large-effect QTL regions suggested their potential roles in conferring salt tolerance by modulating various physiological

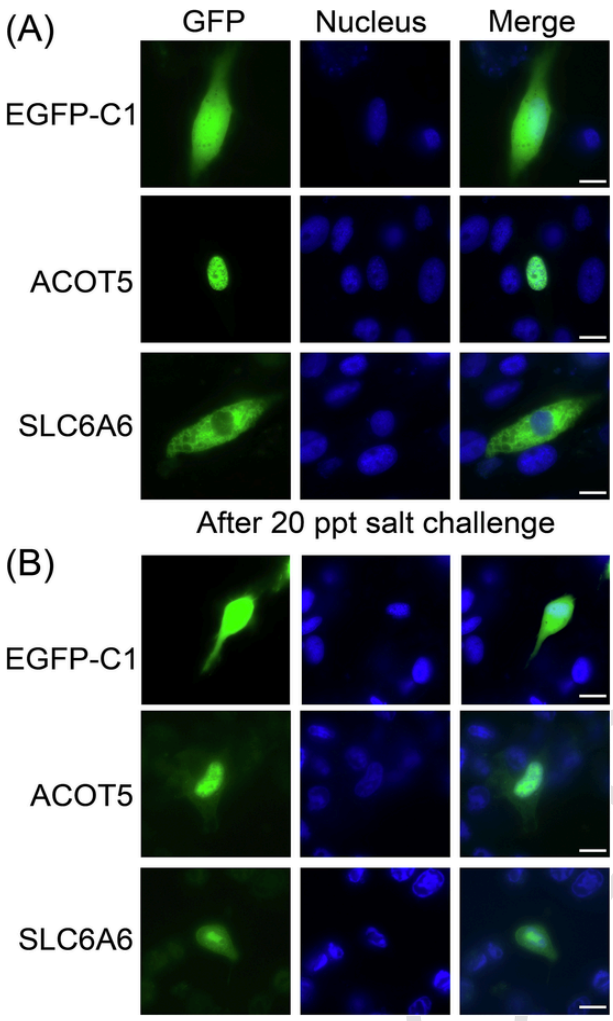

Fig. 5. (A) Subcellular localization of ACOT5 and SLC6A6 in the grouper kidney cell line. (B) Three groups of grouper kidney cells were challenged with 20 ppt seawater for 24 h. All cells were fixed with paraformaldehyde and nuclei were stained with DAPI. Bar = 10 μm.

processes. Pathways related to acyl-CoA metabolic processes, cellular lipid metabolic processes, and organophosphate metabolic processes have also been identified in the gill of salt-challenged tilapia (Qin et al., 2022), indicating their importance for energy metabolism and maintaining cellular integrity under osmotic stress. The *acot5*, an important gene in the acyl-CoA metabolic processes, emerged as a key candidate gene for salt tolerance due to its differential expression in both kidney and gill tissues. This gene has also been highlighted in other salt tolerance studies (Huang et al., 2024). The *acot5* plays a crucial role in lipid metabolism (Ellis et al., 2015), and membrane lipids are vital for osmotic adjustment, aiding in the regulation of water balance. Its function may involve enhancing water uptake and reducing water loss by accumulating osmoprotectants within the cytoplasm. Another significant candidate gene is the sodium- and chloride-dependent taurine transporter (*slc6a6*), an osmoprotectant that helps to regulate cellular osmotic balance under stress conditions (Yasunaga and Matsumura, 2014).

In addition to *acot5* and *slc6a6*, several other DEGs within the two large-effect QTL regions should be considered as potential candidates for salt tolerance. On the QTL of LG 18, genes such as the sodium/potassium transport ATPase subunit α-2 play crucial roles in maintaining ion gradients across the plasma membrane, essential for cellular ion homeostasis under salt stress (Adav et al., 2014). Zinc finger proteins, including GLIS1 and BED domain-containing protein 1-like, may regulate gene expression related to stress responses, enhancing resilience to salinity (Sun et al., 2010). In the novel QTL on LG 11, genes like *rab25*, *perilipin-5*, and *synaptic vesicle glycoprotein 2a* are implicated in membrane trafficking, lipid metabolism, and cellular communication, all vital during osmotic stress (Kessler et al., 2012; Stout et al., 2019; Wang et al., 2011). The identification of these DEGs suggests a complex network of genes contributing to salt tolerance. Future studies should investigate these genes' specific roles in enhancing osmotic stress resistance.

By acknowledging a wider range of candidate genes, we highlighted that our study not only identified *acot5* and *slc6a6* as key regulators but also opened a new path for further investigation of other potential genes to salt tolerance. This view could lead to more advantageous strategies for the genetic improvement of aquaculture species, ensuring better adaptation to saline environments.

4.4. Functional roles of acot5 and slc6a6 in enhancing salt tolerance

The results demonstrated that *acot5* and *slc6a6* play crucial roles in enhancing cellular salt tolerance. The upregulation and increased activity of *acot5* likely enhance lipid metabolism, thereby boosting energy production, which is a vital adaptive mechanism for managing salinity stress. (Gao et al., 2020). By ensuring an adequate energy supply, cells can more effectively handle the high energy demands associated with ion transport and other protective processes. Taurine is a key organic osmolyte in most cells (Schaffer et al., 2000). The results suggested the role of *slc6a6* in taurine transport, highlighting the significance of osmolytes in shielding cells from osmotic stress (Mezzomo et al., 2019). Notably, the salt-induced translocation of *slc6a6* to the nucleus may suggest an additional regulatory function, potentially involving the modulation of genes that aid in osmotic balance and stress response. The nuclear localization of *acot5* and the salt-induced nuclear translocation of *slc6a6* suggested their potential roles in regulating other genes expression in response to salt stress. This phenomenon, where proteins translocate to the nucleus to facilitate important functions during stress response, has been observed in other studies (Yang et al., 2022b). These findings imply that *acot5* and *slc6a6* might interact with nuclear components to influence the expression of stress-response genes, thereby enhancing the resilience of cell to high salinity.

Previous studies may have suggested genes from ACOT families and SLC families as candidates for salt tolerance (Gu et al., 2018; Huang et al., 2024; Jiang et al., 2019). However, these studies did not validate the role of these genes in increasing salt tolerance or study their potential mechanisms. Our study facilitates the understanding of these genes by providing functional validation through overexpression experiments and subcellular localization analysis. This comprehensive approach not only confirmed the involvement of ACOT5 and SLC6A6 in salt tolerance but also initially revealed their potential roles and mechanisms at the cellular level. Future genetic modification strategies could aim to upregulate these genes in tilapia, potentially using advanced techniques to increase their expression levels. This approach could help develop strains with enhanced salt tolerance, thereby promoting more sustainable aquaculture practices (Yang et al., 2022a).

5. Conclusion

This study advances the understanding of salinity adaptation in tilapia by identifying and mapping QTLs associated with salt tolerance, particularly on LG18, and revealing a novel QTL on LG11. Key candidate genes, *acot5* and *slc6a6,* were identified for their roles in osmotic regulation and cellular homeostasis. These findings provide crucial insights for selective breeding and genetic modifications, aiming to develop tilapia strains with enhanced resilience in saline environments.

The study also offers practical implications for improving aquaculture sustainability by broadening habitat suitability and enhancing overall productivity. Further studies on the functions of other candidate genes located in significant QTL on LGs 11 and 18 will enhance our understanding the molecular mechanisms underlying salt-tolerance of tilapia.

CRediT authorship contribution statement

Zituo Yang: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Le Wang:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Fei Sun:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Data curation. **Joey Wong:** Writing – review & editing, Writing – original draft, Validation, Methodology. **May Lee:** Writing – review & editing, Writing – original draft, Validation, Investigation, Data curation. **Shadame Yeo:** Writing – review & editing, Writing – original draft, Validation, Investigation, Funding acquisition. **Yanfei Wen:** Writing – review & editing, Investigation, Data curation. **Gen Hua Yue:** Writing – review & editing, Writing – original draft, Validation, Supervision, Software, Resources, Project administration, Conceptualization.

Declaration of competing interest

The authors declare no competing interests.

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Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2024.741762.

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