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Alternative splicing (AS) mechanism plays important roles in response to different salinity environments in spotted sea bass

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ABSTRACT

Salinity represents a critical environmental factor for fishes, and it can directly influence their survival. Transcriptomic analysis at the gene expression level has been extensively conducted to identify functional genes or pathways involved in salinity adaptation in numerous euryhaline fishes. However, the post-transcriptional regulation mechanism in response to salinity changes remains largely unknown. Alternative splicing (AS), the main mechanism accounting for the complexity of the transcriptome and proteome in eukaryotes, plays essential roles in determining organismal responses to environmental changes. In this study, RNA-Seq datasets were used to examine the AS profiles in spotted sea bass (*Lateolabrax maculatus*), a typical euryhaline fish species. The results showed that 8618 AS events were identified in spotted sea bass. Furthermore, a total of 501 and 162 differential alternative splicing (DAS) events were characterized in the gill and liver under low- and high-salinity environments, respectively. Based on GO enrichment results, DAS genes in both the gill and liver were commonly enriched in 8 GO terms, and their biological functions were implicated in many stages of gene expression regulation, including transcriptional regulation and post-transcriptional regulation. Sanger sequencing and qPCR validations provided additional evidence to ensure the accuracy and reliability of our bioinformatic results. This is the first comprehensive view of AS in response to salinity changes in fish species, providing insights into the post-regulatory molecular mechanisms of euryhaline fishes in salinity adaptation.

1. Introduction

Environmental salinity could directly impact the survival rate, growth performance and physiological functions of fishes [1]. The vast majority of fish species are stenohaline living in either freshwater or seawater, and depend on stable water salinity to maintain constant osmolality in their body fluids [2]. On the contrary, there are several euryhaline fishes with evolved physiological mechanisms that allow them to tolerate and even thrive in water with highly fluctuating salinity [2,3].

With the development of high-throughput sequencing technologies, transcriptomic analyses have been extensively performed to identify functional genes and pathways involved in salinity adaptation and osmoregulation in euryhaline fishes, such as threespine stickleback (*Gasterosteus aculeatus*) [4], black-chinned tilapia (*Sarotherodon melanotheron heudelotii*) [5], milkfish (*Chanos chanos*) [6], Japanese eels (*Anguilla japonica*) [7], Nile tilapia (*Oreochromis niloticus*) [8], striped catfish (*Pangasianodon hypophthalmus*) [9] and spotted sea bass (*Lateolabrax maculatus*) [10]. Based on their studies, numerous differentially expressed genes have been identified that are mainly involved in ion transportation, energy metabolism, signal transduction, and structural organization [6,9]. Nevertheless, few studies have focused on post-transcriptional regulation after salinity adaptation in aquatic organisms [11,12].

Alternative splicing (AS), the process of pre-mRNA splicing in different arrangements to produce structurally and functionally distinct mRNA and protein variants, is considered one of the most extensively post-transcriptional mechanisms accounting for the complexity of the transcriptome and proteome in eukaryotic organisms [13]. For example, >90% of multiexonic genes in humans (*Homo sapiens*) are alternatively spliced [14], and the *Neurexin-1-α* gene of mouse (*Mus musculus*) can produce 247 unique alternatively spliced variants [15]. Studies have shown that AS events are species- and tissue-specific cues, and are modulated in response to external stimuli [15–17]. The pre-mRNA splicing reaction is generally carried out by spliceosomes (multicomponent ribonucleoprotein complexes) consisting of five small nuclear ribonucleoproteins (snRNPs), namely, U1, U2, U4, U5 and U6, and a multitude of non-snRNP splicing factors, such as *serine- and arginine-rich proteins* [18,19]. The generation of mature mRNA needs to recognize exon-intron splice sites accurately and remove introns precisely from pre-mRNAs, which are generally regulated by various *cis*- and *trans*-

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regulatory elements [18,20,21]. The splice variants from the same pre-mRNA may translate into different proteins, exhibit distinct subcellular localization or possess various biological functions [22]. For instance, two *Bcl-x* isoforms in fruit fly (*Drosophila melanogaster*) could inhibit or activate cell apoptosis [23,24]. Additionally, over one-third of AS events could result in the insertion of an in-frame premature termination codon within the transcript, which would trigger mRNA degradation by the process of nonsense-mediated decay (NMD) [25,26]. AS-coupled NMD, as a frequent mechanism, contributes to the regulation of the number of functional transcripts and gene expression in eukaryotic organisms [13,25].

Although the specific biological function of most alternative spliced mRNA remains unknown, a few studies have demonstrated that AS in aquatic species could be a tightly regulated process in response to environmental stresses. For example, 103 genes from the heart of Nile tilapia undergo differential usage of exons and splice junction events after acute hypoxia stress, including *elongation factor 2 kinase* and *NADH dehydrogenase Fe-S protein 1*, which has been demonstrated to be related to the control of gene expression in response to environmental stresses [27]. AS events were reported to be greatly induced (over 20% increase) by bacterial infection in channel catfish (*Ictalurus punctatus*) [28]. In addition, *heat shock transcription factor* gene has been found to be differentially alternatively spliced after heat stress in channel catfish [21]. However, few studies have focused on salinity-related AS events in fishes.

Spotted sea bass is considered one of the most important and promising mariculture species in East Asia [29]. Moreover, it is a typical euryhaline teleost, capable of tolerating a wide range of salinity ranging from 0 to 45 ppt [12,30]. Thus, spotted sea bass represents an excellent model to investigate the osmoregulation process, including the AS mechanism, during salinity adaptation. In this study, RNA-Seq datasets were employed to identify and characterize AS profiles in spotted sea bass under normal physiological conditions and determine the differentially alternative splicing (DAS) events in response to different salinity environments. This study will provide insights into the molecular basis for further study of the AS mechanism in salinity adaptation in fishes.

2. Materials and methods

2.1. Ethics statement

Ethics approval for this study was obtained from the Institutional Review Board at Ocean University of China (Permit Number: 20141201) and all participants provided written informed consent. This study was not involved in endangered or protected species and experiments were performed in accordance with relevant guidelines.

2.2. Identification of AS events in spotted sea bass

To identify AS profiles of spotted sea bass, Illumina RNA-Seq data were downloaded from the NCBI Sequence Read Archive (SRA) database. In detail, the transcriptomic data was generated from samples (PRJNA407434) pooled by 12 tissues of spotted sea bass adult (body length: 31.56 cm; body weight: 590.99 g). The total RNA from 12 tissues, including brain, heart, gill, blood, liver, spleen, skin, muscle, head kidney, kidney, intestine and gonad, were extracted separately. Then, equal amounts of RNA of each tissue (500 ng per tissue) were pooled as one sample for the following library construction and sequencing. The reference genome information of spotted sea bass was also downloaded from the NCBI database (PRJNA407434) for the following bioinformatics analysis.

First, the quality of Illumina paired-end raw data was evaluated by FastQC v0.11.8 [31]. The data was then subjected to adaptor trimming and quality filtering (quality score <20 and read length shorter than 35 were removed) using Trimmomatic v0.39 [32]. The trimmed data were aligned to the reference genome of spotted sea bass using TopHat2 v2.1.1 with default parameters [33]. The aligned results were filtered, and only unique aligned reads were retained for subsequent analysis. Reference-based transcript assembly was performed using Cufflinks v2.2.1 [34]. Transcripts from each sample were merged into a single dataset using Cuffmerge v2.2.1. ASTALAVISTA v4.0 was employed to determine the types of AS events [35], which were classified into six groups based on specific code, including exon shipping (ES), intron retention (IR), alternative 3′ splice sites (A3SS), alternative 5′ splice sites-A5SS (A5SS), mutually exclusive exon (ME) and others (OT).

2.3. Identification of AS events in different salinity environments

AS analysis in gill and liver tissues of spotted sea bass under different salinity environments was conducted using the RNA-Seq data. For the liver data (PRJNA347604), spotted sea bass fingerlings (body weight: 10.66 ± 0.05 g) were randomly transferred to low salinity (LS, 5 ppt) and high-salinity environments (HS, 30 ppt) in triplicate tanks. After 60 days of breeding, 6 individuals per tank were anesthetized with MS-222 and sampled immediately for liver tissues. The RNA of liver tissues was extracted, and equal amounts of RNA from 6 individuals per tank were pooled as one sample. A total of 6 samples (3 replicated samples × 2 treatment groups) were sequenced on the Illumina HiSeq 2500 platform, and 125 bp paired-end reads were generated. For the gill data (PRJNA515986), spotted sea bass adults (body weight: 158.23 ± 18.77 g) were randomly transferred to freshwater (FW, 0 ppt) and seawater environments (SW, 30 ppt) in triplicate tanks. After 30 days of breeding, 3 individuals per tank were anesthetized and rapidly sampled for gill tissues. Then, total RNA of gill tissues was extracted, and equal amounts of RNA of 3 individuals from the same tank were pooled as one sample, and a total of 6 samples were sequenced on the Illumina HiSeq 4000 platform generating 150 bp paired-end reads.

The bioinformatic analysis pipeline to examine AS events in different salinity environments was the same as the method mentioned above (Section 2.2) before transcript assembly. It has been demonstrated that sequence depth was significantly positively correlated with AS genes and event numbers [36]. Thus, the same number of unique aligned reads from each group were used for transcript assembly using Cufflinks v2.2.1 [34]. Then, ASTALAVISTA v4.0 was utilized to determine AS events of each sample. Only AS events detected in at least two replicated samples in the same group were considered stable AS events for subsequent analyses.

2.4. Identification of differential alternative splicing (DAS) events and differentially expressed genes (DEGs)

rMATS v4.0.2 was utilized to detect and define five classical types, including ES, IR, A3SS, A5SS and ME, of DAS events by computing the inclusion level from two-group RNA-Seq data with replicates [37]. In detail, rMATS used a hierarchical framework to simultaneously model the variability among replicates and the estimation uncertainty of inclusion and exclusion isoform proportions in individual replicates [37]. Mature mRNAs including or excluding additional sequences were regarded as inclusion or exclusion isoforms, respectively [38]. The software uses a likelihood-ratio test to calculate the *P*-value and false discovery rate (FDR) of the inclusion levels between two sets of RNA-Seq datasets. FDR < 0.05 was set as the threshold for DAS events, and DAS genes were also determined.

The bioinformatic analysis pipeline to identify differentially expressed genes (DEGs) in gill and liver under different salinity environments was the same as the method mentioned above (Section 2.2) before transcript assembly. Based on the aligned reads, the DEGs were determined using Cuffdiff v2.2.1 with the threshold of |log2(Fold change)| > 1 and FDR < 0.05.

2.5. Functional enrichment analysis and protein-protein interaction (PPI) network construction

All DAS genes were scanned against the Swiss-Prot protein and Nr database using BLAST v2.2.31 with e-value 1e-5 [39]. The Gene Ontology (GO) terms of DAS genes were annotated using Blast2GO [40]. GO term and KEGG pathway enrichment analysis were conducted using clusterProfiler R package [41]. The enriched GO terms in the gill and liver were ranked by *P*-value. PPI networks were constructed using STRING v11.0 (http://string-db.org/) with default parameters. Additionally, Maximal Clique Centrality (MCC) method of cytoHubba module in Cytoscape software was performed to determine the hub genes in the networks [42].

2.6. Validation experiments by Sanger sequencing and qPCR

Total RNA was extracted from gill tissues of FW- and SW-groups individuals using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. The RNA concentration and quality were assessed by a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and its integrity was evaluated by 1% agarose gel electrophoresis. RNA was reverse transcribed to cDNA using the PrimeScriptTM RT reagent kit (Takara, Otsu, Japan). Transcript-specific primers (Supplementary Table 1) were designed based on a unique region of four selected inclusion isoforms of DAS genes, including probable ATP-dependent RNA helicase DDX5 (*ddx5*), heterogeneous nuclear ribonucleoprotein L (*hnpl*), arginine/serine-rich protein 1 (*rsrp1*) and mitogen-activated protein kinase (*mapk14a*), using Primer 5 software. RT-PCR amplification was performed as follows: initial denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, 55–60 °C for 30 s and 72 °C for 10 min. PCR products with expected sizes were sequenced by the Sanger method to confirm the sequence accuracy of the inclusion isoforms (BGI company, Guangdong, China). In addition, qPCR was performed to detect the expression levels of the abovementioned four inclusion isoforms under different salinity environments to validate our analysis results. The 18S rRNA was set as the internal reference gene. 10× diluted cDNA was used as a template. qPCR was performed on the Applied Biosystems 7300 (Applied Biosystems, CA, USA) using the SYBR Premix Ex TaqTM Kit (Takara, Shiga, Japan). qPCR consisted of 2 μl of cDNA, 10 μl SYBR premix Ex Taq, 0.4 μl forward primers, 0.4 μl reverse primers, 0.4 μl ROX reference dye, and 6.8 μl ddH_2O in a final volume of 20 μl. qPCR was repeated in triplicate (technical replicates) and was run in accordance with this procedure: 95 °C for 30 s and 40 cycles of 95 °C for 5 s, 55 °C for 30 s, and 72 °C for 30 s. The expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The expression levels are shown as the mean ± standard deviation.

3. Results

3.1. Overview of AS events in spotted sea bass

A summary of the RNA-Seq datasets used in this study is shown in Supplementary Table 2. Based on the genomic information and RNA-Seq samples pooled with 12 tissues, a total of 8618 AS events were identified in spotted sea bass adults. According to different splicing patterns, these AS events can be roughly divided into six types: ES, IR, A3SS, A5SS, ME and OT (Fig. 1A). Strikingly, the most enriched type of AS event was ES, accounting for 24.39%, followed by IR (23.13%), OT (19.10%), A5SS (17.65%), A3SS (14.16%) and ME (1.57%) (Fig. 1B; Supplementary Table 3).

It was noted that 8618 AS events were derived from 4302 genes. An UpSet plot was constructed to intuitively visualize the intersecting sets of each gene and AS types (Fig. 2). The results showed that more than half of the AS genes (2738, 63.65%) only generated single AS events, including 769 IR events, 651 ES events, 498 A3SS events, 417 A5SS events, 362 OT events and 41 ME events that were produced from a single gene. In contrast, the remaining genes produced at least two AS events, some of which even harbored six types of AS events, such as *TANK-binding kinase 1-binding protein 1* involved in innate immune response, *nuclear protein MDM1 essential* for mitotic growth, and *cGMP-specific 3′,5′-cyclic phosphodiesterase* playing roles in signal transduction.

To investigate the distribution of AS events and genes in the reference genome of spotted sea bass, a Circos plot was constructed (Fig. 3), and the AS event density (AS event number/gene number) was calculated in numerous 0.2 Mb bins on each chromosome (Chr). The average AS event density was approximately 2 per bin (0.2 Mb). It was obvious that there were several AS hot bins (bins with AS events density > 5, Fig. 3). For instance, nearly 10 AS events density were detected in the bin ranging from 23.8 to 24.0 Mb in Chr 8, which suggested that only 3 genes could produce 30 AS events. GO term enrichment analysis was performed to provide insight into the probable function of genes in hot bins. The results indicated that these genes were associated with cellular component assembly (GO:0022607) and cellular component biogenesis (GO:0044085) for biological process, plasma membrane part (GO:0044459) for cellular component, and protein binding (GO:0005515) for molecular function (Supplementary Fig. 1).

3.2. AS event profiles in liver and gill under different salinity environments

The detailed AS event profiles of liver and gill tissues in different salinity environments are shown in Fig. 4 and listed in Supplementary Table 4. In the gill (Fig. 4A), a total of 2169 and 2635 AS events were identified in the FW and SW groups, respectively. In the liver (Fig. 4B), 917 AS events were detected in the LS group compared with 1184 in the HS group. In brief, despite tissue differences, a significantly larger

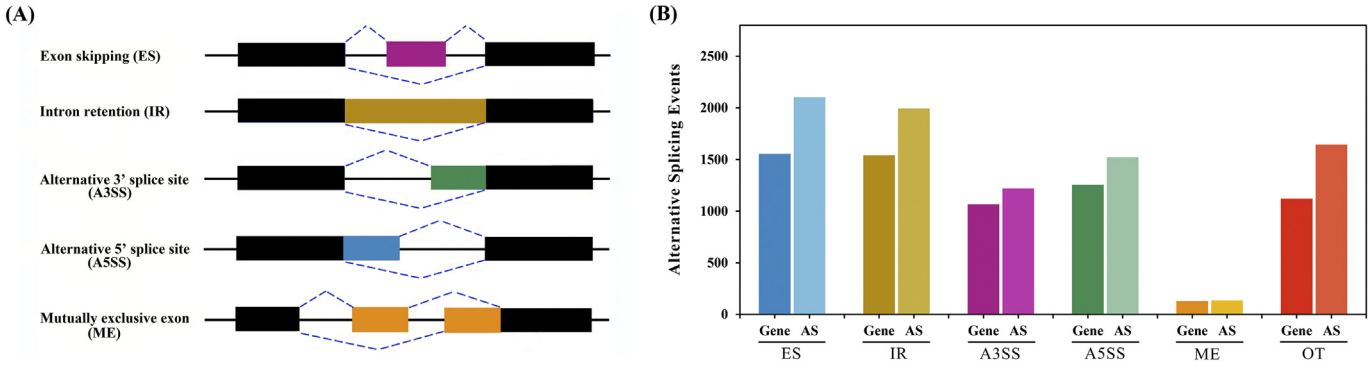

Fig. 1. Statistics of AS events. (A) Illustration of five classical AS events. (B) Number of AS events and involved genes detected in the 12 pooled tissues of spotted sea bass.

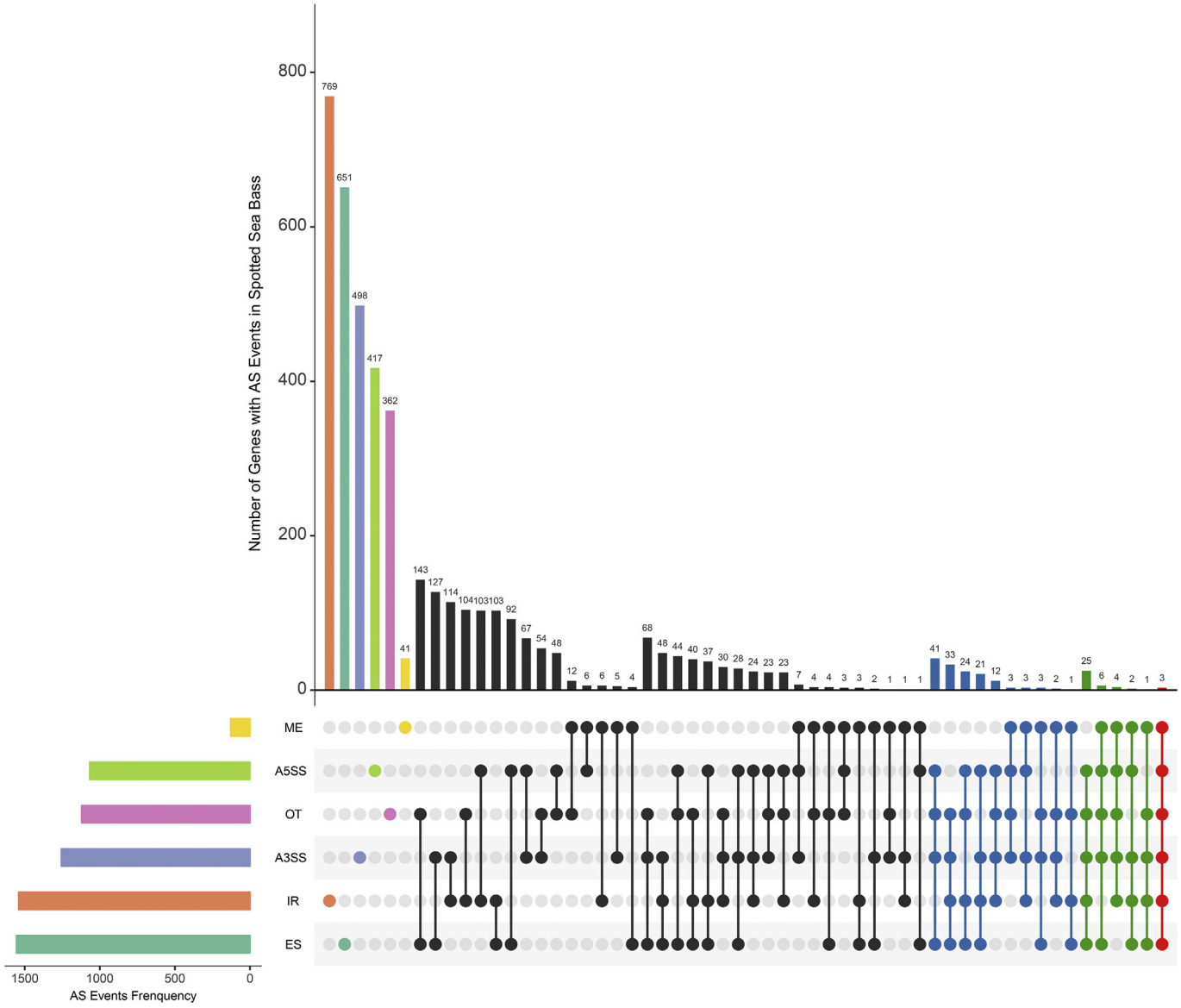

Fig. 2. UpSet plot of interactions between six types of AS events and involved genes. The upper and left bar plots represent the number of genes with AS events. Genes with a single AS event are marked with respective colors. Genes with more than three types of AS events are shown with blue, green and red dotted lines.

number of AS events were observed under high-salinity environment in comparison with low-salinity environment.

3.3. DAS events and DEGs in the gill and liver under different salinity environments

DAS events were determined by comparing the RNA-Seq datasets of triplicate samples of gill and liver tissues in different environments using rMATS. A total of 501 and 162 DAS events were identified in the gill and liver, which were generated from 432 and 149 genes, respectively (Table 1). Their detailed information is summarized in Supplementary Table 5. Several genes have been demonstrated to be potentially involved in osmoregulation in fishes (Supplementary Table 6), including *sodium/hydrogen exchange transporter 2* (ion transport), *sodium/hydrogen exchange transporter 3* (ion transport), *sodium- and chloride-dependent taurine transporter* (amino acid transport), *mitochondrial glutamate carrier 1* (amino acid transport), *mitogen-activated protein kinase 14a* (signal transduction), *mitogen-activated protein kinase kinase kinase 8* (signal transduction) and *acyl-coenzyme A thioesterase 8* (energy metabolism). Additionally, it was notable that 19 DAS genes were detected in both gill and liver tissues (Supplementary Table 7).

To determine the relationships between DEG and DAS genes, a total of 495 and 524 DEGs were identified in gill and liver tissues under different salinity environments, respectively. As shown in Supplementary Fig. 2, 29 genes were both differentially expressed and alternative spliced in gill under different salinity environment, while only 1 gene was commonly detected in liver. The detail information of these overlap genes were summarized in Supplementary Table 7.

3.4. Functional enrichment analysis of DAS genes

To illustrate the potential functional characteristics of the identified DAS gene sets in the gill and liver, GO enrichment tests were performed, and the top 30 enriched GO terms, ranked by *P*-value, are shown in Fig. 5. Several immune-related GO terms were enriched in the gill, including leukocyte differentiation (GO:0002521), lymphocyte differentiation (GO:0030098), hematopoietic or lymphoid organ development (GO:0048534) and tumor necrosis factor production (GO:0032640). Catabolic process-related GO terms were discovered in the liver,

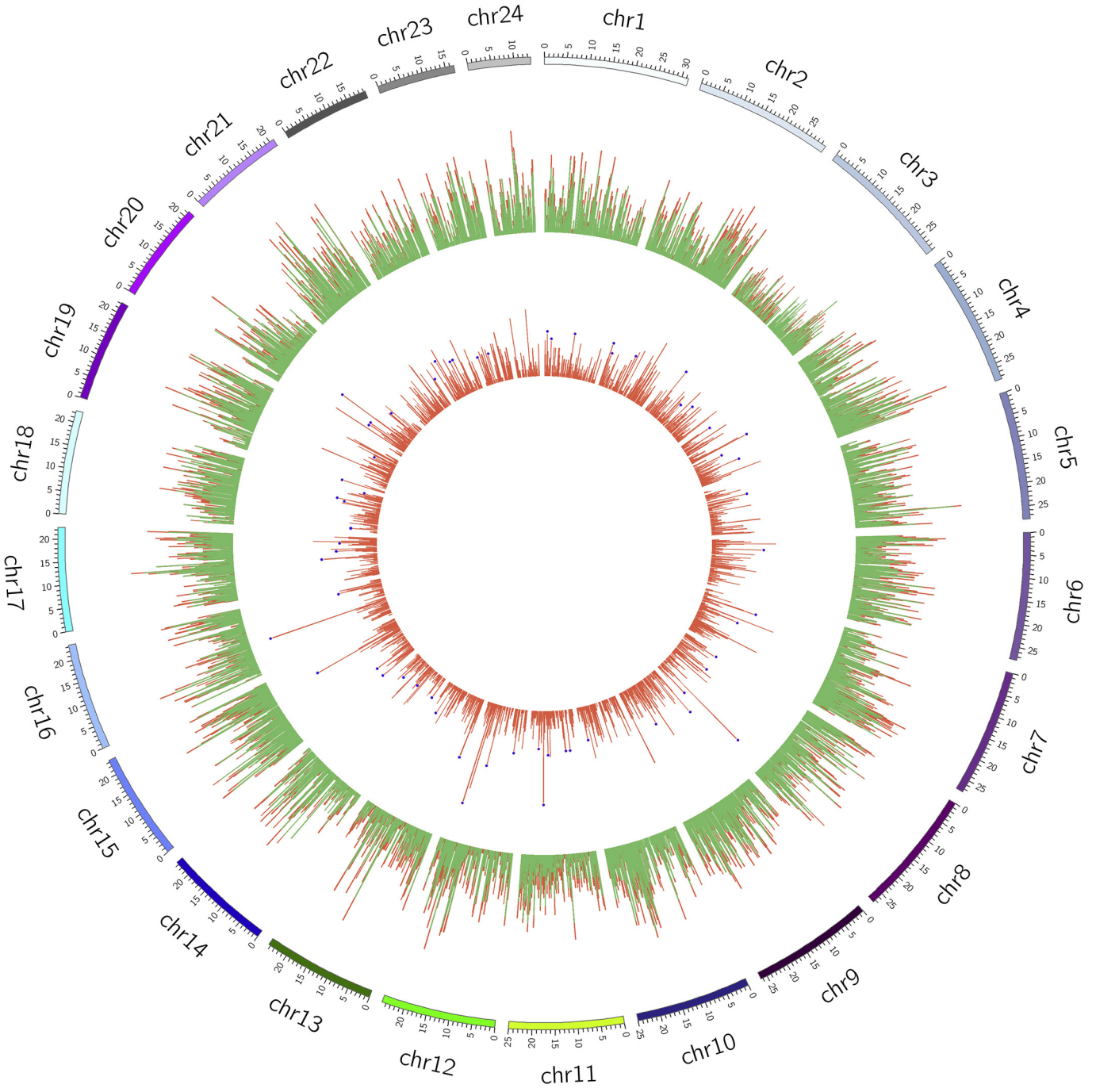

Fig. 3. Distribution of alternative splicing events and genes in the reference genome of spotted sea bass. In the inner, the orange bars represented the AS event density (AS event number/AS gene number) of each bin (0.2 Mb) in chromosomes. Hot bins (AS events density > 5) are marked with blue dots. In the outer, the orange and green bars were used to show the percentage of genes with AS events in each bin (0.2 Mb). The orange and green bars represented the genes with AS events or not, respectively.

including protein metabolic process (GO:0019538), cellular nitrogen compound metabolic process (GO:0034641), nuclear-transcribed mRNA catabolic process (GO:0000956), primary metabolic process (GO:0044238), aromatic compound catabolic process (GO:0019439), nucleobase-containing compound catabolic process (GO:0034655), cellular nitrogen compound catabolic process (GO:0044270) and heterocycle catabolic process (GO:0046700). The results suggested a tissue-specific biological function of DAS genes. Additionally, it was notable that DAS genes in gill and liver were commonly enriched in 8 GO terms for biological processes, including RNA processing (GO:0006396), mRNA metabolic process (GO:0016071), mRNA processing (GO:0006397), RNA splicing via spliceosome (GO:0000398), RNA splicing via transesterification reactions with bulged adenosine as nucleophile (GO:0000377), RNA splicing via transesterification reactions (GO:0000375), regulation of mRNA metabolic process (GO:1903311) and regulation of mRNA processing (GO:0050684).

A Chord plot was constructed to obtain a further understanding of the functions of the 8 most commonly enriched GO terms and their DAS genes (Fig. 6). Strikingly, a total of 53 DAS genes were identified to be associated with these common terms. Of the 53 DAS genes, 6 genes, including *hnpl*, *nudt21*, *ddx5*, *cherp*, *ptbp2* and *ctu2*, were shared in both gill and liver, while 10 genes were only detected in liver, and 37 were gill-specific (Fig. 6). The detail information of the 53 DAS genes were listed in Supplementary Table 8.

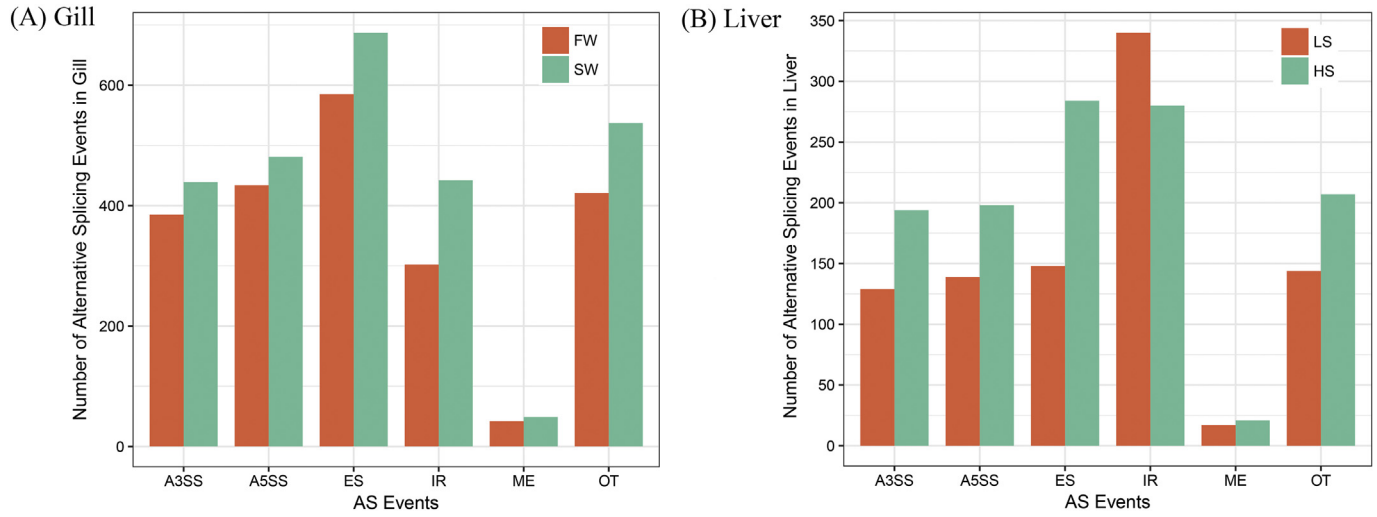

Fig. 4. Statistics of AS events in the gill (A) and liver (B) of spotted sea bass under different salinity environments.

Additionally, as shown in Supplementary Fig. 3, DAS genes were enriched in RIG-I-like receptor signaling pathway in gill and splicesome in liver with $P < 0.05$. PPI networks were constructed to investigate the interaction relationships among DAS genes in gill and liver tissues, respectively (Supplementary Fig. 4; Supplementary Table 8). Based on the MCC method, the hub DAS genes, highly connected with other genes in the networks, were identified in gill (*ddx5*, *cherp*, *nudt21*, *hnpl*, *hnpc*, *hnnph1*, *gtf2f2*, *ybx1*, *u2af1*, *u2surp*, *tra2a*, *srm2*, *ppil6*) and liver (*ddx5*, *cherp*, *nudt21*, *hnpl*, *upf1*, and *ncbp1*), which were paralleled with the results of GO enrichment.

3.5. Function categories and schematic model for DAS genes of commonly enriched GO terms

GO enrichment analysis provided the initial overview of functional categories for DAS genes in commonly enriched GO terms. However, due to the incomplete annotation of reference proteins in the databases, the specific biology functions of these DAS genes were further investigated by reviewing the literature. It is noted that these DAS genes were involved in various links of gene expression regulation, which were further classified into transcriptional regulation and post-transcriptional regulation (Fig. 7). For example, for gene transcription, *gt2f2* and *gt2h3* were reported to be involved in transcription initiation [43,44], while *cherp*, *ddx5*, *mapk14a*, *sltm* and *ncor1* were demonstrated to play a part in regulating the activities of RNA polymerase [45–49]. Several DAS genes in our study work for RNA processing and maturation, such as *u2af* and *usp3* for spliceosome assembly [43,44], *hnpl*, *ptbp2* and *srek1* for pre-mRNA splicing [50–52] and *tnrc6a* and *nudt21* for mRNA polyadenylation [53,54]. Additionally, *cetn2* and *chtop* are involved in mRNA export from the nucleus to the cytoplasm [55,56]. Other DAS genes, such as *celf1*, *ncbp1*, *upf1* and *ago2*, were implicated in the gene translational regulation [52,57–60], especially *ncbp1* and *upf1*, which were previously reported to act on NMD (Fig. 7). In general, our results suggest that these DAS genes are involved in many processes of gene expression regulation, and their specific biological functions are discussed in detail in the Discussion section.

Table 1
DAS events in the liver and gill of spotted sea bass under different salinity environments. The numbers separated by a colon represent the DAS number with higher inclusion levels in the FW vs. SW and LS vs. HS groups.

AS events	FW vs SW (gill)	LS vs HS (liver)
Exon skipping	228 (110:118)	33 (23:10)
Intron retention	87 (36:51)	96 (88:8)
Alternative 5′ splice site	69 (33:36)	15 (10:5)
Alternative 3′ splice site	88 (47:41)	16 (9:7)
Mutually exclusive	29 (13:16)	2 (1:1)
Total	501	162

3.6. Experimental validation

Four DAS genes, including *ddx5*, *hnpl*, *mapk14a* and *rsrp1*, were selected to validate the accuracy and reliability of the bioinformatics analysis results. Specific primers of the inclusion isoforms were designed, and their RT-PCR products were sequenced (Fig. 8A and Supplementary Table 9). The sequencing results were in accordance with our bioinformatics analysis results. Additionally, qPCR analysis was performed to detect the expression levels of *ddx5*, *mapk14a*, *hnpl* and *rsrp1* in the gills of the SW and FW groups (Fig. 8B). The qPCR results showed similar patterns with expression levels predicted by RNA-Seq, suggesting the reliability of our study.

4. Discussion

In our study, a total of 8618 AS events were identified in spotted sea bass. Of 4302 AS genes, 2730 (63.65%) AS genes only generated single AS events, and the remaining genes contained at least 2 AS events, suggesting that genes producing multiple AS events were widespread in spotted sea bass. In addition, numerous hot bins of AS events were identified in the reference genome of spotted sea bass. Based on the GO enrichment results of genes in hot bins, it indicated that AS mechanism may play important roles in modulating the functional characteristic of genes involved in cellular component assembly, organization regulation and protein binding in spotted sea bass.

Salinity is regarded as one of the most important environmental factors for fish distribution and survival because fishes are exposed directly to water environments with varying salinities. To adapt to different salinity environments, euryhaline fishes have evolved sophisticated osmoregulation strategies. In recent years, a series of functional osmoregulatory genes have been identified in euryhaline fish species, and their involvement in response to salinity changes has been investigated [10,30,61–63]. However, as an important mechanism for modulating gene expression and function, there have been few studies on salinity-responded AS mechanisms in fishes. AS, a critical post-transcriptional mechanism, not only plays an important role in increasing the diversity and complexity of the transcriptome and proteome in eukaryotic organisms but also participates in stress responses [13,26]. In our study, a genome-scale analysis of AS events was performed in the gill and liver of spotted sea bass under low- and high-salinity

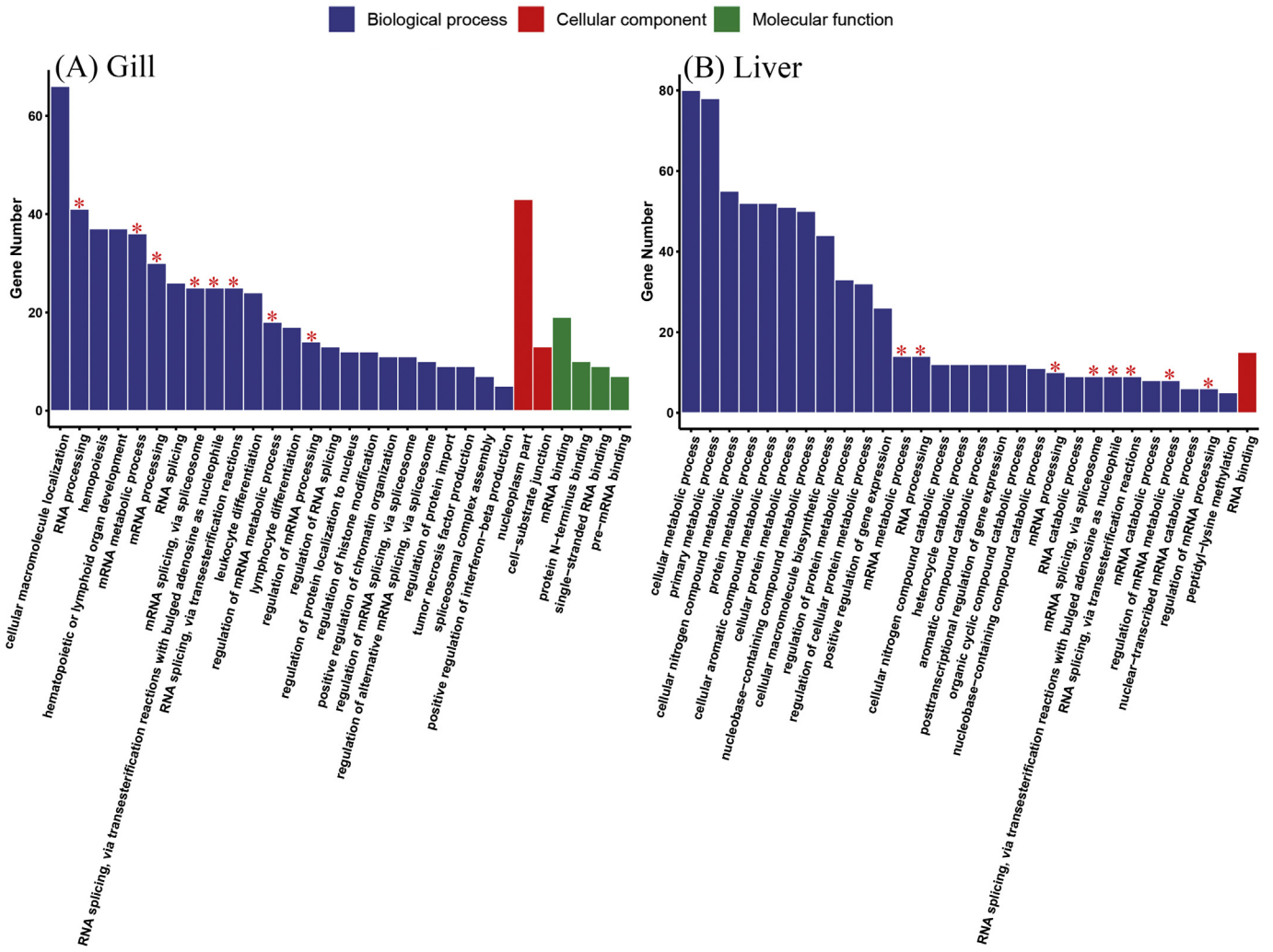

Fig. 5. Top 30 of enriched GO terms of DAS genes in the gill (A) and liver (B) of spotted sea bass under different salinity environments. Common GO terms in gill and liver are marked with red stars.

environments using RNA-Seq data, which provide insight into the global dynamics of AS in response to different salinity conditions.

Notably, significantly larger number of AS events was found in both the gill and liver under the high-salinity environment, indicating that hypertonic conditions may induce the generation of AS events in spotted sea bass. It has been proved that the best living condition for spotted sea bass is the brackish water environment, as the calculated isotonic point is 11.4, which is closer to the low-salinity condition than the high-salinity condition [64]. Moreover, our previous study showed that high-salinity conditions would cause greater changes in the internal osmotic pressure and cause challenges for spotted sea bass [30]. The phenomenon of induced environmental stress-responding AS events has been reported previously in fish species. For example, heat stress could stimulate an increase in 492 AS events (29.2%) in the liver of channel catfish [21]. A total of 103 differentially spliced genes were significantly induced by acute hypoxia stress in the heart of Nile tilapia [27]. These studies and our results in the current study demonstrated that generation of more AS events could be a general regulatory mechanism in response to environmental stressors in fishes.

rMATS was employed to identify a catalogue of DAS genes in gill and liver tissues under different salinity environments by the likelihood test. For function enrichment analysis, it is worth noting that 8 enriched GO terms were shared in both the gill and liver, which consisted of 53 DAS genes. Of these DAS genes, several common DAS genes (*ddx5*, *hnpl*, *cherp* and *nudt21*) detected in both gill and liver were also recognized as the hub genes in PPI networks, which suggested their crucial roles in salinity adaptation in spotted sea bass. The 53 DAS genes are involved in many stages of gene expression regulation, from gene activation in the nucleus to mRNA translation and decay (Fig. 7). Based on their respective function, DAS genes were further classified into transcriptional regulation and post-transcriptional regulation. Their potential functions are discussed below.

4.1. DAS genes evolved in transcription regulation

In eukaryotes, the major part of the genome, including all coding genes, is transcribed by RNA polymerase II [65]. As a member of the DEAD box family, *ddx5* has been reported to play important roles in interacting with RNA polymerase II holoenzyme and regulating transcriptional activation [46]. *cherp* is an integral endoplasmic reticulum (ER) membrane protein that not only mediates cell calcium homeostasis but also induces an increase in the largest subunit of RNA polymerase II, which is a key part of RNA transcription [45]. In contrast, the *ncor1* gene acts as a general inhibitor of transcription through impeding the access of basal transcription factors [48,49]. *Dedd2* is a component of the transcription factor TFIIIC, an important regulator of the transcription of tRNA, 5S rRNA by RNA polymerase III [66]. Both *gtf2f2* and *gtf2h3* are subunits of transcription initiation factor IIF (TFIIF), which shows ATP-dependent DNA-helicase activity and has an essential role in transcription initiation [43,44].

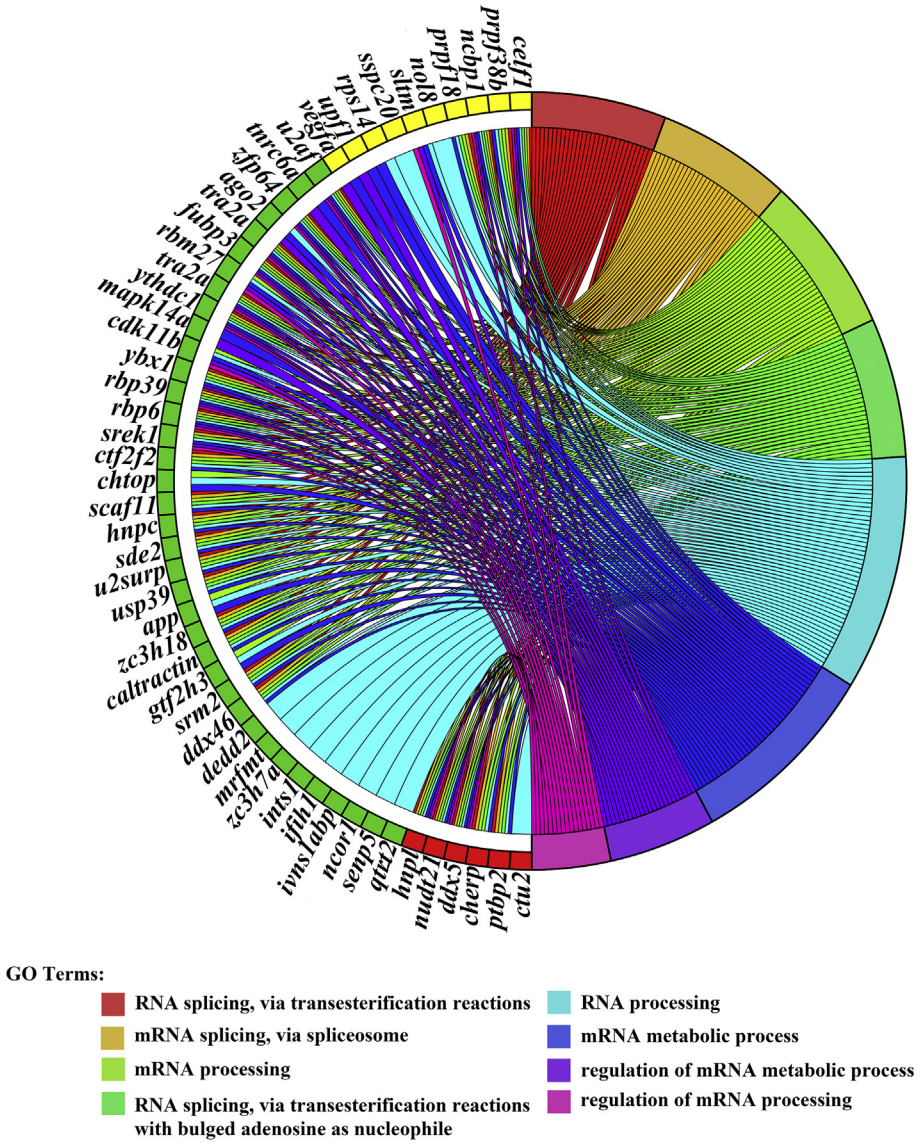

Fig. 6. DAS-enriched GO terms identified in both gill and liver in different salinity environments and their related DAS genes. DAS genes were linked with their involvement in function categories via various color ribbons. Gill- and liver-specific DAS genes are marked with yellow and green, respectively. Common DAS genes that existed both in gill and liver are represented with red. The full names of these DAS genes are listed in Supplementary Table 8.

4.2. DAS genes evolved in post-transcriptional regulation

Several DAS genes are involved in post-transcriptional regulation, including pre-mRNA processing and downstream events such as translation and mRNA decay. For the regulation of spliceosome assembly and splicing process, *ddx46*, *srek1*, *prpf38*, *srrm2*, *ints1 tra2a*, *hnpc* and *u2af* genes have been shown to play important roles in spliceosome assembly or directly regulate the activities of some elements in spliceosomes [52,67–71]. As shared DAS genes in the gill and liver, *hnpl* and *ptbp2* were demonstrated to play negative roles in pre-mRNA splicing. For example, *ptbp2* is a kind of RNA-binding protein that binds to intronic polypyrimidine tracts and mediates the negative regulation of exon splicing [51]. Serine phosphorylation of *hnpl* could inhibit binding of the large subunit of *u2af* [50]. Additionally, *tnrc6a* and *nudt21* could influence mRNA polyadenylation, which shortens nuclear-transcribed mRNA poly(A) tailing [54] and activates polyadenylation processing [53], respectively.

The transport of RNA molecules from the nucleus to the cytoplasm is fundamental for gene expression, which was generally mediated by an important export complex called TREX-2, several components of which, including *cetn2* and *chtop* [55,56], were also differentially spliced under different salinity environments in our study. In addition, some DAS genes in our results were involved in mRNA translation and NMD processes. Through AS events, mRNA transcripts could encode proteins with subtle or opposing functional differences that have profound biological consequences [72]. In contrast, other mRNA transcripts with inserts of in-frame PTCs may encode truncated proteins or trigger transcript degradation by the NMD process [26]. This AS-coupled NMD could be a frequent mechanism for the regulation of gene expression [25]. In our results, *ncbp1* has been reported to regulate both mRNA translation and NMD processes [73]. The *ythdc1* gene can specifically recognize N6-methyladenosine (m6A)-containing mRNAs and promote mRNA translation efficiency [57], while the *ago2* gene was reported to bind with mRNA and guide translational repression [60]. A previous study demonstrated that the *celf1* gene, interacting with the 5′ region of mRNA, could promote mRNA translation and play important roles in the regulation of

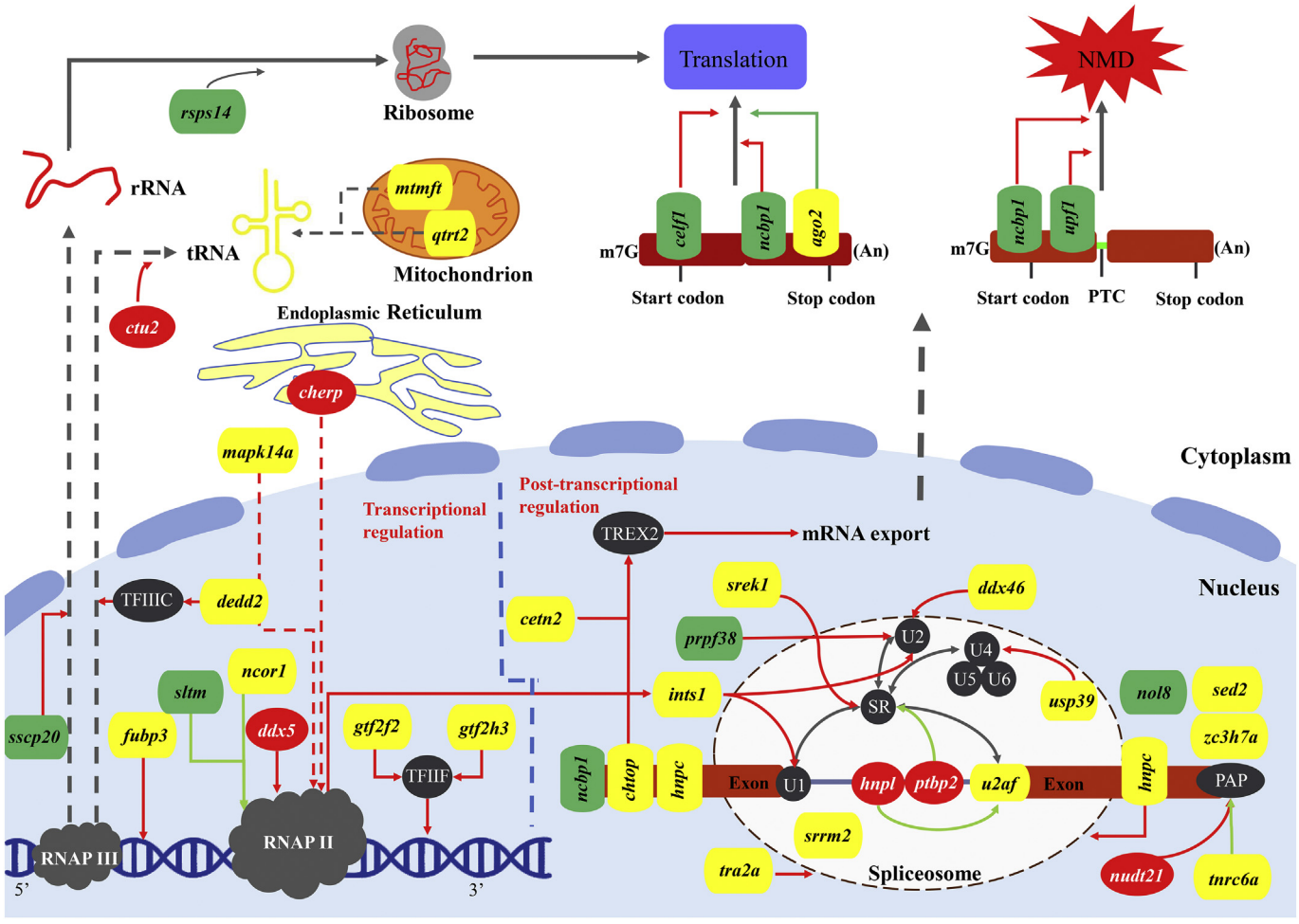

Fig. 7. Schematic diagram of DAS genes identified in different salinity environments and their respective biological functions in the regulation of gene expression. Gill- and liver-specific DAS genes are colored yellow and green, respectively. DAS genes that are commonly found in both liver and gill are marked in red. Positive regulation function is shown by red arrows, while the negative regulation function is shown in green. The dashed gray line represents transmembrane transport. The full names of these genes are listed in Supplementary Table 8.

liver development, proliferation and inflammation by the AS mechanism [58]. In our study, *celf1* was also identified as a DAS gene in the liver under different salinity environments, suggesting that it may play a role through AS in the liver in the salinity response in spotted sea bass.

As described above, several DAS genes are regulators of gene expression, which means that the AS mechanism could play important roles in gene expression regulation in salinity adaptation. However, further studies are required to investigate the specific biological functions of detected DAS events in our study.

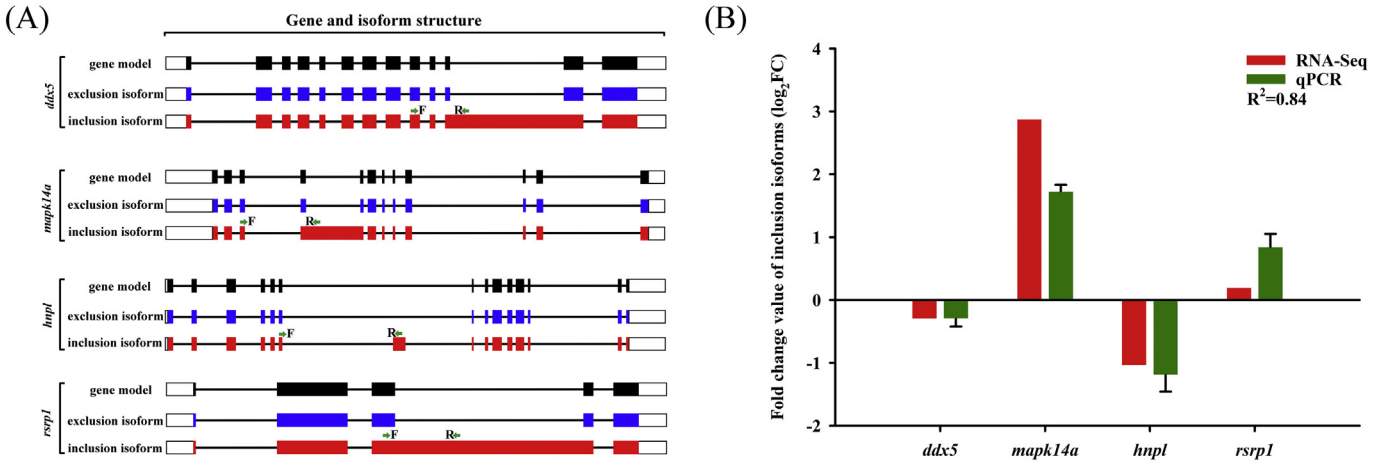

Fig. 8. Experimental validation for the DAS events. (A) Schematic diagram of predicted gene models in spotted sea bass reference genome and exclusion isoforms and inclusion isoforms of four DAS genes. (B) qPCR validations of four inclusion isoforms of DAS genes. The fold change (Log_2FC) value represents the relative expression levels of inclusion isoforms in SW in comparison with that in the FW group.

5. Conclusion

In our study, a total of 8618 AS events were determined in spotted sea bass, and DAS events were characterized in the gill and liver under low- and high-salinity environments. Our results showed that a high-salinity environment induced the generation of AS events in both the gill and liver. Moreover, DAS genes in the gill and liver were commonly enriched in 8 GO terms, and their biological functions were implicated in various stages of gene expression regulation, including transcriptional and post-transcriptional regulation from gene activation in the nucleus to mRNA translation and decay. These findings suggested that AS could be an important regulatory mechanism for spotted sea bass in adapting to different salinity environments.

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Credit author statement

Yuan Tian: Conceptualization, Methodology, Software, Writing - Original Draft; Haishen Wen: Funding acquisition; Xin Qi: Resources; Xiaoyan Zhang: Resources; Yalong Sun: Methodology, Validation; Jifang Li: Formal analysis; Feng He: Methodology; Meizhao Zhang: Methodology; Kaiqiang Zhang: Methodology; Wenzhao Yang: Visualization; Zurui Huang: Visualization; Yuhang Ren: Software; Yun Li: Conceptualization, Funding acquisition, Writing - Review & Editing.

Declaration of competing interest

The authors declared that the research was not any potential conflict of interest.

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