# The full-length transcriptome of *Spartina alterniflora* reveals the complexity of high salt tolerance in monocotyledonous halophyte

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## ABSTRACT

*Spartina alterniflora* (*Spartina*) is the only halophyte in the salt marsh. However, the molecular basis of its high salt tolerance remains elusive. In this study, we used PacBio full-length single molecule long-read sequencing and RNA-seq to elucidate the transcriptome dynamics of high salt tolerance in *Spartina* by salt-gradient experiments (0, 350, 500 and 800 mM NaCl). We systematically analyzed the gene expression diversity and deciphered possible roles of ion transporters, protein kinases and photosynthesis in salt tolerance. Moreover, the co-expression network analysis revealed several hub genes in salt stress regulatory networks, including protein kinases such as *SaOST1*, *SaCIPK10* and three *SaLRRs*. Furthermore, high salt stress affected the gene expression of photosynthesis through down-regulation at the transcription level and alternative splicing at the post-transcriptional level. In addition, overexpression of two *Spartina* salt-tolerant genes *SaHSP70-I* and *SaAF2* in Arabidopsis significantly promoted the salt tolerance of transgenic lines. Finally, we built the SAPacBio website for visualizing the full-length transcriptome sequences, transcription factors, ncRNAs, salt-tolerant genes, and alternative splicing events in

*Spartina*. Overall, this study sheds light on the high salt tolerance mechanisms of monocotyledonous-halophyte and demonstrates the potential of *Spartina* genes for engineering salt-tolerant plants.

Running title: *Spartina* transcriptome under salt stress

*Keywords*: *Spartina alterniflora*; high salt tolerance; ion transporter; protein kinase; regulatory hub genes; alternative splicing; single molecule real-time sequencing

## INTRODUCTION

Over 20% of the world's irrigated land suffers from soil salinization, and salt stress challenges the survival of plants through affecting their development and reproduction (Deinlein et al., 2014; Roy, Negrao, & Tester, 2014). However, only ~2% of terrestrial plants are salt-tolerant halophytes with strong ability to survive in concentrations of NaCl over 200 mM (Mangu et al., 2015). *Spartina alterniflora* (*Spartina*) is the only halophytic plant that can survive in highly saline environment of salt marshes (Subudhi & Baisakh, 2011). Intraspecific variation studies reveal that the strong saline environmental adaptation of *Spartina alterniflora* may be largely due to the greater ion selectivity to the uptake of potassium and excluding sodium (Bradley & Morris, 1991; Smart & Barko, 1980), which results in a lower $Na^+/K^+$ ratio in *Spartina alterniflora* compared to the brackish water plant- *Spartina patens* (Hester, Mendelssohn, & McKee, 2001). Moreover, *Spartina alternifora* also develops specialized salt glands to secret ions (Nestler, 1977; Subudhi & Baisakh, 2011). Furthermore, cell membranes actively exclude salt out of cells to avoid the toxic effects of them inside cells (Subudhi & Baisakh, 2011). Therefore, *Spartina*

*alterniflora* exhibits a delicate ion exclusion system from physiology aspects to survive hash saline environment and it is a good model plant to study high salt tolerance. However, the molecular basis of salt tolerance in *Spartina alterniflora* remains unexplored, and the post-transcriptional regulation of high salt tolerance has not been characterized in plants.

Ion transporters play important roles in response to salt stress (J. K. Zhu, 2002, 2016). SOS1 (Salt Overlay Sensitive 1) is the most well-known $Na^+/H^+$ antiporter that is used to extrude $Na^+$ from root cells into the soil and load $Na^+$ into the xylem for long-distance transportation from roots to leaves via transpiration systems (Yang & Guo, 2018; Y. Zhang et al., 2018; J. K. Zhu, 2002, 2016). Briefly, excessive salt stress triggers cytoplasmic $Ca^{2+}$ signaling, which is then sensed by SOS3 (J. K. Zhu, 2016). SOS3 then interacts and activates the CBL-interacting protein kinase SOS2 (J. K. Zhu, 2016). The activated SOS2 then phosphorylates and activates the $Na^+/H^+$ antiporter SOS1 (J. K. Zhu, 2016). HKT1 has an antagonistic function with SOS1 on long-distance $Na^+$ transport and prevents $Na^+$ transport into leaves (J. K. Zhu, 2016). Lower $Na^+/K^+$ ratio is very important in plant salt tolerance (G. Chen et al., 2015; Y.

Zhang et al., 2018; X. Zhu et al., 2018) and ion transporters from Shaker family are responsible for potassium uptake and transport (Barragan et al., 2012; Lacombe et al., 2000). AKT1 and AKT2 are two important potassium transporters from the Shaker family that function for both potassium influx and efflux (Y. Zhang et al., 2018). KAT2 and KAT1 interacted with each other to form inward $K^+$ channels (Nieves-Cordones et al., 2014). SKOR plays a role in delivering $K^+$ from the root stelar cells toward shoots (Gaymard et al., 1998). The $K^+/H^+$ antiporter NHX1 and NHX2 mediate the $K^+$ uptake from cytosol to vacuolar and regulate reservoir dynamics of $K^+$ (Barragan et al., 2012). However, none of these ion transporters has been characterized in *Spartina* and their regulatory roles in high salt tolerance are largely unexplored.

Protein phosphorylation is one important post-translational regulation mechanism and is regulated by protein kinases (Y. Ding et al., 2015). SOS2 represents one of the $Ca^{2+}$ dependent protein kinases, including 10 SnRK2 and 25 SnRK3 in Arabidopsis (J. K. Zhu, 2016). In the SnRK2 family, SnRK2.10 is rapidly activated in Arabidopsis roots under salt stress (McLoughlin et al., 2012) and OST1 regulates cold stress by

phosphorylating C-repeat binding factors such as ICE1 under cold stress (Y. Ding et al., 2018; Fujii, Verslues, & Zhu, 2011). In the SnRK3 family, the loss of Arabidopsis SnRK3.16/CIPK1 and SnRK3.14/CIPK6 causes osmotic or salt stress sensitive phenotype (D'Angelo et al., 2006; Tripathi, Parasuraman, Laxmi, & Chattopadhyay, 2009). In addition, the leucine-rich repeat receptor protein kinase (LRRs) was also reported in response to environment stresses. For example, MIK2 is correlated with mild salt tolerance by the natural variation study (Van der Does et al., 2017). The overexpression of PnLRR-RLK27 from Antarctic moss confers salt tolerance (J. Wang et al., 2017). On the contrary, Arabidopsis LRR-like kinase AtRPK1 and OsRPK1 are negatively correlated with salt tolerance (Shi et al., 2014). However, it remains elusive whether protein kinases involve in high salt stress associated regulatory network.

Transcriptome sequencing has been widely adopted to explore the gene expression dynamics in response to salt stress from different species (Bushman, Amundsen, Warnke, Robins, & Johnson, 2016; Liu et al., 2019; Mutwakil et al., 2017; Qiu et al., 2017). However, the transcriptome studies in high salt tolerance from halophyte are

relative limited and most of them mainly focus on dicotyledonous halophytes (Lee et al., 2013; Subudhi & Baisakh, 2011; Y. Zhang et al., 2008). Moreover, although the genome-wide transcriptome reprogramming under salt stress has been reported (Liu et al., 2019), the reprogramming under different salinity stress conditions remains unexplored. In addition, alternative splicing (AS) is one important post-transcriptional regulation that plays critical roles in eukaryotic pre-mRNA processing and contributes to diversifying the transcriptome and proteome (Deng & Cao, 2017). AS is affected by cold and heat stress in plants, and more importantly, ~10% of AS events are associated with salt stress in Arabidopsis (F. Ding et al., 2014; Srivastava, Lu, Zinta, Lang, & Zhu, 2018). Mutations of splicing factors such as SKIP, Sm-like protein 5, and SKB1 lead to the increase of AS events, especially intron retention (IR), under salt stress (Cui, Zhang, Ding, Ali, & Xiong, 2014; Feng et al., 2015; Z. Zhang et al., 2011), indicating the important function of AS in response to salt stress. However, how AS responds to salt stress in halophytes remains unclear. The main difficulty in analyzing transcriptome in *Spartina* is largely attributed to its complex genome with aneu-hexaploid (2n = 6x = 62) and the lack of high quality reference genome

(Renny-Byfield et al., 2010). Fortunately, the recently developed single-molecule long-read sequencing technology (SMRT) by the Pacific Biosciences (PacBio) platform can sequence full-length mRNA isoforms without *de novo* assembly, which has been widely adopted to profile full-length transcripts in *Salvia miltiorrhiza*, *Sorghum bicolor*, *Phyllostachys edulis*, *Drynaria roosii*, maize, wheat and strawberry (Abdel-Ghany et al., 2016; An, Cao, Li, Humbeck, & Wang, 2018; Dong et al., 2015; Y. Li, Dai, Hu, Liu, & Kang, 2017; Sun et al., 2018; B. Wang et al., 2016; T. Wang et al., 2017; Xu et al., 2015). These pioneering studies provide elegant solutions for the study of biological mechanisms in poorly-annotated species.

In this study, we used PacBio SMRT sequencing and RNA-seq to get insight into the high salt tolerance mechanism of *Spartina alterniflora.* Full-length reference transcript sequences of *Spartina* were obtained. We uncovered the crucial role of ion transporters associated with the high salt tolerance and identified hub genes in the high salt tolerant regulatory network. The involvement of alternative splicing in high salt tolerance was also documented. Furthermore, candidate transcripts for salt tolerance of *Spartina* were functionally analyzed in Arabidopsis. We also built the

146 SAPacBio website (http://plantpolya.org/SAPacBio/) for visualization and query of
147 full-length transcriptome sequences in *Spartina*. Overall, our results provide new
148 insights into the high salt tolerance regulatory mechanism of halophytes and
149 demonstrate the great potential of *Spartina* to provide genetic resources for
150 engineering salt-tolerant plants.
151

152 **MATERIALS AND METHODS**

153 **Plant Materials**

154 *Spartina alterniflora* seeds were collected in Ganyu County (E119º16'; N34º46'),
155 Jiangsu Province, China. *Spartina* seedlings were grown on half-strength Hoagland
156 liquid medium for 6 weeks in a growth chamber under a 16-hour light (80 µmol
157 $m^{-2}s^{-1}$)/8-hour dark regime at 26ºC (day) and 16ºC (night). Plants with similar vigor
158 were selected and salinized for 24 hours, in triplicate, at different NaCl concentrations
159 (0, 350, 500, and 800 mM). These concentrations were selected to mimic
160 non-salt-stressed conditions, low, medium, and high-salinity stress, since the lethal
161 salt concentration for *Spartina* germination is ~1000 mM NaCl (Subudhi & Baisakh,

2011), and the average salinity level of the sea is ~599 mM (3.5%) NaCl. Total RNAs were isolated (Tiangen, Cat.DP441) and high-quality samples (RNA integrity number > 8) were used to prepare libraries for experimental validation.

**Library construction and sequencing**

Pacbio SMRT and RNA-seq (Levin et al., 2010) were performed using whole seedlings grown at different salt concentrations (0, 350, 500, and 800 mM NaCl), as described previously (T. Wang et al., 2017). These libraries were sequenced on the PacBio RSII platform (Iso-seq) and the HiSeq 2500 sequencing platform (RNA-seq, paired-ends 125 bp).

**Bioinformatics analysis**

*Spartina* full-length non-chimeric (FLNC) transcripts were identified from PacBio SMRT reads using the PacBio's SMRT Analysis pipeline (v2.3.0) (Gordon et al., 2015), and were further corrected by proovread (Hackl, Hedrich, Schultz, & Forster, 2014) using Illumina RNA-seq data. These FLNC transcripts were clustered into

unique transcript clusters by a two-rounds clustering incorporating CD-HIT-EST (v4.6) (Fu, Niu, Zhu, Wu, & Li, 2012) and GMAP (v2016-06-09) (T. D. Wu & Watanabe, 2005), and the longest isoform was defined as the unigene for each cluster.

The 5′ UTR, open reading frame (ORF), and 3′ UTR of each FLNC transcript were obtained using TransDecode (v3.0.1, https://transdecoder.github.io/). Functional annotations were generated using Blast2GO (blastx 2.2.26+) (Conesa et al., 2005) with the National Center for Biotechnology Information's (NCBI) non-redundant database (e-value < 1e-5). Transcript datasets of *Oryza sativa* (IRSGP1.0) and *Arabidopsis thaliana* (TAIR10) were downloaded from EnsemblPlants (release34, http://plants.ensembl.org). All isoforms of *Spartina* were aligned to these datasets using blastn (e-value < 1e-5).

Gene ontology (GO) enrichment analysis (p-value < 0.05) was performed by BiNGO (Maere, Heymans, & Kuiper, 2005). Non-coding RNAs (ncRNAs) were predicted using CPAT (coding score < 0.298) (L. Wang et al., 2013) and the plant non-coding RNA database (PNRD) (e-value < 1e-5) (Yi, Zhang, Ling, Xu, & Su, 2015). Transcription factors were identified using the Plant Transcription Factor

Database (PTFD, v4.0) with default parameters (Jin et al., 2017).

**Co-expression network analysis and alternative splicing**

Differentially expressed (DE) unigenes were identified from RNA-seq data by RSEM (v1.2.25) combined with bowtie2 (v2.2.9) and EBSeq (v1.20.0) (Leng et al., 2013; B. Li & Dewey, 2011) based on the following criteria: false discovery rate (FDR)<0.05, and |fold change|≥2. All DE genes were used to construct the co-expression network by WGCNA (v1.66) (Langfelder & Horvath, 2008). The GO enrichment analysis of genes in each module was conducted by topGO (V2.32.0)(Alexa, Rahnenfuhrer, & Lengauer, 2006). Hub genes from the salt stress network were identified using Cytoscape plugin cytoHubba and ranked according to the maximal clique centrality (MCC) scores (Chin et al., 2014). AS events were identified using AStrap (Ji et al., 2018) and differentially expressed alternative splicing events (DEAS) were also characterized by using RNA-seq data (see Supplemental Methods).

## Experimental validation

RT-PCR and qRT-PCR experiments were conducted as described in a previous study (T. Wang et al., 2017). Primers used are listed in Table S1. Results from RT-PCR were visualized in 1% agarose gels. Coding sequences of *SaAF2* and *SaHSP70-I* (without stop codons) were cloned into modified pCambia3301 constructs (*pACT2::gene-GFP*), and transformed into the gene slicing suppression *rdr6-11* Arabidopsis mutants (Peragine, Yoshikawa, Wu, Albrecht, & Poethig, 2004). Two independent $T_3$ transgenic lines were grown on plates containing half-strength Murashige and Skoog medium (1/2 MS) for 10 days under different salinity stress conditions (0, 120, and 150 mM NaCl). The primary root length was measured using ImageJ (Schneider, Rasband, & Eliceiri, 2012).

## Availability and accession numbers

Datasets from Illumina HiSeq 2500 and PacBio SMRT sequencing have been deposited at the NCBI website under the Bioproject accession number PRJNA413596. The SAPacBio website was built to query and visualize full-length isoforms, DE

226 genes and AS in *Spartina* (http://plantpolya.org/SAPacBio/).

227 **RESULTS**

228 **Characterization of the full-length transcriptome in *Spartina alterniflora***

229 We used PacBio SMRT sequencing to obtain full-length reference transcripts of
230 high-confidence in *Spartina* to avoid potential bias attributed to its complex
231 aneu-hexaploid genome (2n = 6x = 62) (Bedre, Mangu, Srivastava, Sanchez, &
232 Baisakh, 2016). An overview of the experimental procedure is illustrated in Fig. 1.
233 Briefly, 6-week-old whole *Spartina* plants were treated with 0, 350, 500, and 800 mM
234 NaCl for 24 hours to simulate non-salt stressed conditions, low, medium, and high
235 salt-stressed conditions. Total RNA was isolated from each salt stress condition and
236 mixed equally to prepare three different size-selected libraries (1-2 kb, 2-3 kb,
237 and > 3 kb). Seven cells were sequenced on the PacBio RSII real-time (RT)
238 sequencing platform. The length distribution of reads of inserts (ROIs) in each library
239 is consistent with the selected size (Fig. **2a** and Table **S2**).

240 After the quality control by SMRT analysis (Gordon et al., 2015) and further
241 correction with ~250 millions Illumina RNA-seq reads by proovread (Hackl et al.,

2014), a total of 179,194 FLNC reads (including a poly(A) tail, 5′ UTR and 3′ UTR) were obtained, 90,587 of which are non-redundant FLNC reads (Table **S2**). Over 90% of FLNC transcripts are ORF-containing transcripts with an average coding sequence (CDS) length of 1601 bp. These FLNC transcripts cover a wide variety of functional annotations such as catalytic activity, metabolic processes, and transporter activity (Fig. **S1**). These transcripts were further grouped into 24,670 unique transcript clusters (unigenes). Among them, 55.72% contained multiple isoforms (Fig. **2b**), the percentage of which is comparable to that from previous studies (M. Wang et al., 2018; T. Wang et al., 2017). The number of isoforms in each cluster is correlated with their expression; single isoform-containing clusters tend to have lower expression levels than clusters with multiple isoforms (Fig. **S2**). Additionally, 3494 ncRNAs (Table **S3**) were identified. Furthermore, we used the plant transcription factor database (Jin *et al.*, 2017) to identify transcription factors (TFs) in *Spartina*. In total, 4997 transcripts from 1323 unigenes were identified as encoding functional domains of TF families (Tables **S4-5**).

We further compared FLNC transcripts from PacBio with those obtained from

RNA-seq data (Bedre et al., 2016). The average length of FLNC transcripts (2405 bp) is significantly longer than that from RNA-seq data (324 bp) (Bedre et al., 2016). Over 73% of RNA-seq contigs can be mapped to FLNC transcripts, while 64,755 FLNC transcripts are newly discovered and not present in RNA-seq data (Fig. **2c**). Eight FLNC transcripts were randomly selected and all were further validated by RT-PCR (Fig. **S3**). Overall, our PacBio FLNC transcripts provide qualified full-length reference sequences for comprehensive profiling of the *Spartina* transcriptome.

**Genome-wide comparison of *Spartina* full-length transcriptome to other species**

As *Spartina* is a monocot halophyte (Subudhi & Baisakh, 2011), we examined whether its transcripts are homologous to other plants. To this end, FLNC transcripts of *Spartina* were mapped to Arabidopsis and rice. Less than 9% of transcripts were mapped to Arabidopsis, while over 80% are highly similar to transcripts in rice (Fig. **2d**). These results indicated that *Spartina* is highly homologous to the monocotyledonous-crop rice, and thus has the potential to provide genetic resources for engineering salt-tolerant rice. To understand the potential difference between

*Spartina* and other plant species, we conducted a comparison of GO term percentages that was previously used to compare *Arabidopsis thaliana* and *Thellungiella salsuginea* (H. J. Wu et al., 2012). GO terms were mapped to 19,093 unigenes in *Spartina* using Blast2GO (Conesa et al., 2005), and were assigned to 30,821 annotated genes in *Arabidopsis thaliana* and 30,241 annotated genes in *Oryza sativa* using AgriGO (Tian et al., 2017). Detailed analysis revealed that the proportion of genes in the "membrane" (35.8% for *Spartina*, 27% for Arabidopsis, 9.5% for rice, with Fisher's exact test p-value=1.94e-95 for the comparison between *Spartina* and Arabidopsis, and p-value=0 for *Spartina* and rice), "localization" (13.4% for *Spartina*, 8.8% for Arabidopsis, 7.3% for rice, with Fisher's test p-value=4.70e-56 for *Spartina* and Arabidopsis, and p-value=6.18e-108 for *Spartina* and rice) and "transporter activity" (6.6% for *Spartina*, 4.6% for Arabidopsis, 4.8% for rice, with Fisher's test p-value=2.06e-20 for *Spartina* and Arabidopsis, and p-value=7.19e-17 for *Spartina* and rice) categories were significantly increased in *Spartina* compared to both rice and Arabidopsis (**Figs. 3c-d**). As transporters are mainly membrane proteins and modulate cellular localization, the increasing percentage of transporter coding genes

might contribute to the high salt tolerance phenotype in *Spartina*. Furthermore, 1577 *Spartina* transcripts differ from transcripts in other plant species, suggesting that these transcripts might be specifically expressed in *Spartina* and play unique roles in salt adaptation (**Table S6**).

**Genome-wide dynamics of gene expression profile under salt stress in *Spartina***

To identify salt-responsive transcripts and explore salt tolerance mechanisms in *Spartina*, twelve RNA-seq libraries were constructed from plants grown under four salt stress gradients with three biological replicates. RNA-seq samples from the same conditions were clustered together, indicating the high reproducibility of our RNA-seq data (Fig. **S4**). A total of 3875 unigenes are differentially expressed (FDR < 0.05 and $|\log_2 FC| \geq 1$) among the salt-gradient experiments (Table **S7**). Moreover, 5108 FLNC transcripts, corresponding to 3375 unigenes, are differentially expressed between salinity stresses and control conditions. The number of DE transcripts increases with the salinity gradient (Fig. **S5a**). Moreover, the number of up-regulated transcripts is greater than that of down-regulated transcripts, particularly under high

306 salt stress (Fig. **S5b-c**). The same trend was observed for DE unigenes (Figs. **4a**,
307 **S5d-f**), indicating that salt stress significantly affects the expression of *Spartina*
308 unigenes.

309 Next, high salt stress associated unigenes (DE unigenes between 800 mM NaCl
310 and control) were used to identify enriched pathways. Up-regulated unigenes are
311 enriched in pathways including ion transport, response to stress, and amino acid
312 metabolic processes (Fig. **4b**), while photosynthesis and translation are significantly
313 enriched in down-regulated unigenes (Fig. **4c**). Importantly, these pathways are
314 crucial components of salt stress-responsive mechanisms in plants (Deinlein et al.,
315 2014; Munns & Tester, 2008; Soni et al., 2015), suggesting that high salt stress
316 greatly affects the gene expression of salt-responsive pathways in *Spartina*.

317 To validate high salinity stress-associated unigenes, 15 DE unigenes were
318 randomly selected for qRT-PCR experiments. The relative transcript levels measured
319 by qRT-PCR and RNA-seq correlated significantly (Pearson correlation = 0.713,
320 p-value = 3.908e-08, Fig. **4d**). In addition, 10 DE unigenes were randomly selected
321 for RT-PCR experiments and results confirmed the gene expression pattern from

RNA-seq (Fig. **S6**).

**Co-expression network analysis reveals regulatory hub genes under high salt stress**

To unveil the interrelationships among high-salt-stress responsive genes, we performed the weighted gene co-expression networks analysis (WGCNA) (Liu et al., 2019) with all 3875 DE unigenes from varying degrees of salt stress (Table **S7**). The co-expression network was built and DE unigenes were classified into five modules with hierarchical clustering. Different colors (blue, brown, green, turquoise, yellow) were used to represent distinct modules.

The largest module turquoise contains 2380 DE unigenes, showing an increasing expression trend especially under high salt stress (800 mM NaCl) (Table **S8**). We next performed gene ontology analysis to decipher potential functions of DE unigenes in the turquoise module. These genes are significantly enriched in “cellular homeostasis”, “chemical homeostasis” and “carbohydrate homeostasis” (Table **S8**). Specially, we observed that ion homeostasis processes such as metal ion homeostasis, cellular ion

338 homeostasis, cellular metal ion homeostasis and transition metal ion homeostasis are
339 enriched in DE genes of the turquoise module (Table **S8**). Consistently, transporters
340 response to these homeostasis processes are also over-represented, which is further
341 supported by the enrichment of "plasma membrane" under cellular components
342 category and "transporter activity" under molecular function category (Table **S8**).
343 These results suggested that transporters are involved in response to high salt stress.

344 The second largest module (blue) contained 1261 DE unigenes and showed a
345 decreasing expression trends along with salt stress gradient, which was opposite with
346 the turquoise module (Table **S8**). DE genes of the blue module are mainly enriched in
347 "translation" and "photosynthesis" (Table **S8**). The module brown (97 DE genes) was
348 induced under salt stress of 350 mM and 500 mM NaCl and is enriched in "small
349 molecule metabolic process", "methylation" and "amino acid metabolic process"
350 (Table **S8**).

351 To uncover potential key regulators under high salt stress, we identified top-ten
352 hub genes from each module. Several genes related to environmental stress regulation
353 were identified (Fig. **4e-f**). In the turquoise up-regulation module (Fig. **4e**), *OST1*

(*Cluster17805*, also known as *SnRK2.6* or *SnRK2E*) encodes an ABA activated protein kinase, playing vital roles in the ionic osmotic stress by regulating the stomatal closure (Boudsocq, Barbier-Brygoo, & Lauriere, 2004; Fujii et al., 2011). *CHIA* (*Cluster32181*) encodes a chitinase, which was reported not expressed at normal conditions but exclusively expressed under environmental stresses especially salt and wound stress in Arabidopsis (Takenaka, Nakano, Tamoi, Sakuda, & Fukamizo, 2009). *CIPK10* (*Cluster17512*, also known as *PKS2, SIP1,* and *SnRK3.8*) encodes a CBL-like protein kinase which was expressed higher in roots and showed increased expression under 200 mM NaCl treatment (Guo, Halfter, Ishitani, & Zhu, 2001). In the blue down-regulation module (Fig. **4f**), *SUS4* (*Cluster1314*) encodes sucrose synthase 4, which was involved in response to hypoxia stress (Bieniawska et al., 2007). Therefore, these newly discovered hub genes might play crucial roles in the high salt stress associated regulatory network of *Spartina*.

**Complex gene expression dynamics of transporters and associated protein kinases**

Genome-wide comparison analysis and co-expression network analysis showed that ion transporters play important roles in high salt tolerance of *Spartina* (Fig. **3** and Table **S8**). To further explore the function of ion transporters, we investigated the gene expression dynamics of sodium and potassium transporters under high salt stress. Gene expression levels of each ion transporter for sodium and potassium transport and related protein kinases are listed in Table **S9**. In general, under high salt stress, most of the sodium and potassium transporters showed gene expression variation under high salt stress.

Genes involved in SOS pathways including $Na^+/H^+$ antiporter *SaSOS1* (*Cluster1424*) and the $Na^+$-$K^+$ homeostasis modulator *SaSOS2* (*Cluster19661*) were significantly induced under high salt stress. HKT1 restricts long-distance transport of $Na^+$ from root to leave and therefore has antagonistic functions with SOS1 (J. K. Zhu, 2016). However, *SaHKT1* is not induced under high salt stress at the transcription level (Table **S9**). In *Spartina*, seven *SaAHAs* were identified and *SaAHA2* *(Cluster4145)* showed a significant up-regulation under high salt stress (Table **S9**). These results indicated that SaAHA2 might be involved in the high salt tolerance of

386 *Spartina*.

387 SOS2 represents a large number of similar protein kinases, which include 10
388 SnRK2 and 25 SnRK3 in Arabidopsis (J. K. Zhu, 2016). In *Spartina*,
389 *SaSnRK2.10/SnRK2B (Cluster27491)* and *SaOST1/SnRK2.6/SRK2E (Cluster17805)*
390 from SnRK2 family are significantly up-regulated under high salt stress (Table **S9**).
391 However, *SaASK1/SnRK2.4/SRK2A (Cluster27812)* is not induced under high salt
392 stress (Table **S9**). Ten SnRK3s showed differential expression in salt stress by the
393 co-expression network analysis. Eight of them including *SaSnRK3.8/CIPK10,*
394 *SaSnRK3.9/CIPK12 (Cluster4217), SaSnRK3.11/SOS2 (Cluster19661),*
395 *SaSnRK3.14/CIPK6/SIP3 (Cluster23013), SaSnRK3.16/CIPK1 (Cluster23029),*
396 *SaSnRK3.22/CIPK11/SIP4 (Cluster25001), SaSnRK3.24/CIPK5 (Cluster21691)* and
397 *SaSnRK3.25/CIPK25 (Cluster23892)* are significantly up-regulated under high salt
398 stress (Table **S9**). In addition, the expression of *SaSnRK3.12/CIPK9 (Cluster4692)* is
399 increased under mild salt stress but decreased under high salt stress (Table **S9**).
400 However, *SaSnRK3.3/CIPK4* (*Cluster28689*) is the only down-regulated *SnRK3s*
401 under high salt stress (Table **S9**).

Ion transporters from Shaker family are responsible for potassium uptake and transport (Barragan et al., 2012; Lacombe et al., 2000). In *Spartina*, *SaAKT1* did not show any significant expression change under high salt stress, while *SaAKT2* showed a significant down-regulation (Table **S9**). The inward $K^+$ channel *SaKAT2* (*Cluster24848*) was significantly up-regulated under high salt stress, while *SaKAT1* (*Cluster13882*) did not have significant change (Table **S9**). The $K^+$ transport related *SaSKOR* (*Cluster12966*) gene was also up-regulated under high salt stress (Table **S9**). We also found that the expression of tonoplast localized $K^+/H^+$ antiporter *SaNHX2 (Cluster17097)* was dramatically decreased at lower salt stress condition, while significant up-regulation was observed under high salt stress (Table **S9**).

**High salt stress induces global alternative splicing in *Spartina***

To investigate whether high salt stress affects AS in *Spartina*, 14,058 non-redundant AS events corresponding to 20,431 transcripts were identified using our bioinformatics pipeline. Approximately 39% (5328/13,748) of unigenes with multiple isoforms are associated with AS events. Nearly half (2857/5328) of

AS-containing unigenes have more than two associated AS events (Fig. **5a**). The distribution of different types of AS in *Spartina* is comparable to that of other species (Reddy, Marquez, Kalyna, & Barta, 2013; Shen et al., 2014). Intron retention (IR) is the major AS type (56%). Most IR-AS events (93%) contain the canonical GT-AG splice site; the percentage of AS using alternative acceptor sites (AltA) is two times of that of alternative donor sites (AltD) (Fig. **5b**).

Importantly, 1154 AS events corresponding to 1627 transcripts are associated with salinity stress in *Spartina*, accounting for ~11% (578/5328) of AS-containing unigenes. The number of AS events is increased with growing salinity stress (Fig. **5c**), suggesting that high salt stress induces AS events in *Spartina*. In particular, IR remains the major AS type associated with salt stress (Fig. **5c**), and 233 IR events were identified in response to high salt stress (Table **S10**). According to the GO analysis, these AS events mainly affect photosynthesis-related unigenes (Fig. **S7**). Six IR events were selected for validation (Fig. **5d**). Levels of transcripts associated with these IR events were indeed increased under high salt stress when examined by both RT-PCR and qRT-PCR (Fig. **5e, f**). Overall, these results suggest that AS responds to

high salt stress in *Spartina.*

**Functional validation of *Spartina* salt-tolerant unigenes in Arabidopsis**

To test whether high salt stress responsive transcripts could be candidate gene resources for engineering salt-tolerant plants, we constitutively overexpressed heat shock protein coding gene- *SaHSP70-I (Cluster1653-042)* and NAC transcription factor- *SaAF2 (Cluster30510-002)* in Arabidopsis. Two independent $T_3$ generation transgenic lines of overexpressed *SaHSP70-I* were grown at 120 and 150 mM NaCl. They showed better salt tolerance and had longer primary roots than control plants under salt stress (Fig. **5a-b**). Similarly, the salt tolerance analysis in Arabidopsis showed that plants with overexpressed *SaAF2* also exhibited increased salt tolerance (Fig. **5c-d**), indicating that *AF2* is a salt-tolerant candidate gene. The identification of many salt-responsive transcripts in *Spartina* and their functional validation in Arabidopsis further demonstrate the potential of using *Spartina* genes to engineer salt-tolerant plants.

## DISCUSSION

### Overview of the *Spartina alterniflora* full-length transcriptome

Salt stress is one of the most important environmental stresses in crop growth and production. High salt stress severely delays crop growth and significantly reduces its yield. *Spartina alterniflora* is a highly salt-tolerant monocotyledonous halophyte that belongs to the *Poaceae* family. In this study, we performed comprehensive transcriptome analysis in *Spartina* exposed to different salt stresses using the hybrids of SMRT full-length transcriptome sequencing and RNA-seq. A total of 90,587 non-redundant full-length transcripts, 24,670 unigenes, 3494 ncRNAs, 1323 TFs and 1577 specific transcripts in *Spartina* were identified (Tables. **S3-6**). More importantly, the sequence similarity analysis showed that approximately 80% of the full-length transcripts in *Spartina* could be aligned to rice transcripts (Fig. **2d**), indicating that *Spartina* is evolutionarily similar to rice. As *Spartina* is very similar to rice from evolutionary aspects (Fig. **2d**), we also attempted to apply the *Spartina* unigenes for engineering salt-tolerance plants. We over-expressed two *Spartina* salt

stress-responsive genes *SaHSP70-I* and *SaAF2* in Arabidopsis and observed that their transgenic lines confer salt-tolerance phenotypes (Fig. **6**). Previous studies showed that the over-expression of *Spartina* vacuolar ATPase subunit c1 (*SaVHAc1*) and actin-depolymerizing factor (*SaADF2*) genes in rice promoted the salt tolerance of transgenic rice (Baisakh et al., 2012; Sengupta et al., 2019). Therefore, these results indicate that *Spartina* could be an ideal species for engineering salt tolerance in crops. Overall, the full-length transcriptome of *Spartina* potentially provides valuable resources for improving the salt tolerance of plants.

**Gene expression dynamics of ion transporters under high salt stress**

Genome-wide analysis showed that *Spartina* has a higher proportion of transporter related genes compared to rice and Arabidopsis (Fig. **3**) and the co-expression network analysis also found that ion transporters are significantly enriched in the high salt stress associated module (Table **S8**). These results indicate that ion transporters may play a key role in the high salt tolerance of *Spartina*.

The most well known ion transporter under salt stress is SOS1 (Salt Overlay

Sensitive 1), a $Na^+/H^+$ antiporter that is used to extrude $Na^+$ from root cells into the soil and load $Na^+$ into the xylem for long-distance transportation from roots to leaves via transpiration systems (Yang & Guo, 2018; Y. Zhang et al., 2018; J. K. Zhu, 2002, 2016). Another important ion transporter is HKT1, which plays important roles in long-distance $Na^+$ transport and has an antagonistic functions with SOS1 to restrict long-distance transport of $Na^+$ from root to leave (J. K. Zhu, 2016). It is proposed that plants can activate *SOS1* under mild salt stress to transfer $Na^+$ from root to leave, while HKT1 is activated under high salt stress to limit the transport of $Na^+$ from roots because $Na^+$ accumulation in leaves exceeds its storage capacity (J. K. Zhu, 2016). However, in *Spartina*, we showed that the expression level of *SaSOS1* is significantly higher under high salt stress, while no significant gene expression change was observed for *SaHKT1* (Table **S9**). These results suggest that the long-distance transport of $Na^+$ from root to leave may continue to be active under high salt stress in *Spartina*. One of the explanations is that leaves of *Spartina* have salt glands (Nestler, 1977; Subudhi & Baisakh, 2011) and can secrete salt, so that high expression of *SaHKT1* may not be required to shut down $Na^+$ transport compared to other plants

(Jaime-Perez et al., 2017; M. Zhang et al., 2018).

It has been reported that the $Na^+/K^+$ ratio is very important in plant salt tolerance, and salt stress increases $Na^+$ but leads to loss of $K^+$ (G. Chen et al., 2015; Y. Zhang et al., 2018; X. Zhu et al., 2018). In general, *Spartina* maintained a higher K+ accumulation under high salt stress via the active gene expression dynamics of *SaAKT2*, *SaKAT2*, *SaSKOR* and *SaNHX2*. It is suggested that AKT1 is a potassium transporter under lower $K^+$ conditions for uptake of $K^+$, while AKT2 is one $K^+$ efflux carrier under higher $K^+$ conditions (Y. Zhang et al., 2018). In *Spartina*, the reduction in expression of *SaAKT2* but no change of *SaAKT1* (Table **S9**) may limit the $K^+$ efflux under high salt stress and the results supported the physiologic observation that *Spartina* has a higher $K^+$ accumulation level (Hester et al., 2001; Subudhi & Baisakh, 2011). Moreover, the down-regulation of *SaAKT2* may also reduce the $Na^+$ uptake root cells initially through the $K^+$ channel via *SaAKT2* (Salvador-Recatala, 2016). KAT2 and KAT1 have been reported to form inward $K^+$ channels in the form of heterodimers and KAT2 can also form active homotrimeric channels in the plasma membrane (Nieves-Cordones et al., 2014). We found *SaKAT2* rather than *SaKAT1* is

514 significantly up-regulated under high salt stress (Table **S9**), suggesting that *SaKAT2*
515 may play a role in $K^+$ uptake and maintain a higher $K^+$ level through its homotrimeric
516 channels in *Spartina* under high salt stress. SKOR is involved in delivering of $K^+$
517 from stelar cells of roots to xylem toward the shoot (Gaymard et al., 1998). *SaSKOR*
518 is also up-regulated under high salt stress (Table **S9**), indicating higher $K^+$ transport
519 from root to leaf under high salt stress. More importantly, the result is consistent with
520 the observation that the lower $Na^+$ / $K^+$ ratio is present in *Spartina* leaves (Bradley &
521 Morris, 1991; Smart & Barko, 1980). Higher $Na^+/K^+$ ratio is considered to be the
522 major physiological reason for high salt tolerance of *Spartina* (Subudhi & Baisakh,
523 2011). The vacuolar is the largest dynamics reservoir of $K^+$ in plant cells and the
524 tonoplast localized $K^+/H^+$ antiporter, NHX1 and NHX2, are reported to mediate the
525 $K^+$ uptake from cytosol to vacuolar (Barragan et al., 2012). We found that *SaNHX2*
526 *(Cluster17097)* showed significant up-regulation under high salt stress (Table S9),
527 indicating that *SaNHX2* may involve in the $K^+$ reservoir in *Spartina* (Hester et al.,
528 2001).

529 Overall, these results indicate that the reduction of $Na^+$ exclusion, the activation of

530 $Na^+$ long-distant transport to secret them through salt gland and the higher $K^+$
531 accumulation under high salt stress may be several important high salt tolerance
532 mechanisms in *Spartina*.
533
534 **Protein kinase plays hub roles in the salt stress regulatory network**
535 Protein phosphorylation plays important regulatory roles in plants in response to
536 environmental stresses at the post-translational level (Y. Ding et al., 2015). The
537 CBL-interacting protein kinase SOS2 involves in the SOS regulatory pathway to
538 phosphorylate and activate the $Na^+/H^+$ antiporter SOS1 (J. K. Zhu, 2016). In *Spartina*,
539 the expression level of *SaSOS2* (*Cluster19661*) is significantly increased under high
540 salt stress (Table **S9**), indicating that *SaSOS2* is involved in high salt tolerance of
541 *Spartina*.
542 Moreover, three SnRK2s and 16 SnRK3s were identified in *Spartina* (Table **S9**).
543 Among them, two of *SnRK2s* and ten of *SnRK3s* showed significantly gene
544 expression dynamics under high salt stress (Table **S9**). In particular, *SaOST1* from
545 *SnRK2s* and *SaCIPK10* from *SnRK3s* belong to the top ten hub genes in the high salt

546 stress associated up-regulation modules (Fig. **4e**). The OST1 is triggered by ABA
547 signaling under stress to phosphorylate the ABREs-binding proteins and activate the
548 expression of stress responsive genes (Furihata et al., 2006). For example, the OST1
549 interacts and phosphorylates the ICE1 from C-repeat binding factors dependent
550 pathways under cold stress to stable their proteins (Y. Ding et al., 2015). However,
551 how OST1 involves in the high salt stress remains elusive. *SaOST1* is present in the
552 top ten hub genes under high salt stress (Fig. **4e**), suggesting that OST1 may also play
553 crucial roles in the high salt tolerance of *Spartina* via post-translational regulation of
554 salt stress related genes. CIPKs from many species including rice, maize and wheat
555 have been reported to play important roles in salt tolerance and the overexpression of
556 *CIPKs* significant improved salt tolerance of transgenic plants (X. Chen et al., 2014;
557 Deng et al., 2013; Xiang, Huang, & Xiong, 2007). However, it is unexplored whether
558 CIPKs play regulatory roles in salt tolerance. In this study, we showed that *SaCIPK10*
559 is among the top ten hubs genes of high salt stress associated up-regulation modules
560 in *Spartina* (Fig. **4e**). These results suggest that SaCIPK10 may play regulatory roles
561 in high salt tolerance. Interestingly, three of the top ten hub genes in the high salt

562 stress associated down-regulation module are *leucine-rich repeat receptor kinases*
563 *(LRRs)* (Fig. **4f**), indicating that LRRs may also involve in the regulation of high salt
564 tolerance.

565 In summary, over one fourth of the hub genes from high salt stress associated
566 regulator network are protein kinases from SnRKs or LRRs families, indicating that
567 kinases may play important regulatory roles in high salt tolerance of *Spartina*.

568

569 **The transcriptional and post-transcriptional regulation of photosynthesis related**
570 **genes in *Spartina***

571 Photosynthesis is impaired under salt stress and it has been reported that *Spartina*
572 saturates photosynthesis at relatively low $CO_2$ level (Bedre et al., 2016). At the
573 molecular level, we showed that photosynthesis genes, especially those associated
574 with photosynthetic pigment content and the performance of photosystem II
575 (Brzezowski, Richter, & Grimm, 2015; Chaves, Flexas, & Pinheiro, 2009), are also
576 down-regulated under high salt stress (Fig. 4c), indicating that photosynthesis is
577 regulated at transcriptional level.

578 AS is one post-transcriptional regulatory mechanism used by plants to respond to
579 different environmental stresses (Deng & Cao, 2017; F. Ding et al., 2014; Feng et al.,
580 2015). In this study, transcriptome-wide analysis indicated that high salt stress affects
581 global AS (Fig. **5**, Table **S10**). In particular, high salt stress induces a higher number
582 of AS events along salinity stress gradients (Fig. **5c**). In addition, the percentage of
583 salt stress-associated AS genes (11%) is very close to that found in cotton and
584 Arabidopsis (10%) (F. Ding et al., 2014; J. Zhang et al., 2014). Although salt stress
585 increased IR events in several key salt-responsive genes in Arabidopsis (F. Ding et al.,
586 2014; Feng et al., 2015), we did not observe this phenomenon in *Spartina* (Fig. **S7**).
587 However, interestingly, photosynthesis-related genes are significantly enriched in the
588 high salt stress associated AS, indicating extensive regulation of this process (Fig, **S7**).
589 Overall, our results suggest that photosynthesis is highly regulated at both
590 transcriptional and post-transcriptional level in *Spartina* under high salt stress.

591 In summary, our results provide a glimpse of the high salt tolerance mechanism
592 of *Spartina alterniflora*. In addition, the full-length reference sequences, splice
593 isoforms, transcription factors, ncRNAs and high salt stress associated hub genes in

*Spartina* identified in this study will provide useful information for future research on halophytes and engineering salt tolerant plants.

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## Author contributions

L.M. conceived the ideas. L.M., X.W., G.J., and C.L. designed experiments. T.W., W.W., and F.L. performed the experiments. X.W., W.Y., S.L., S.Z., T.W. and Q.L. contributed to data analysis and L.M. wrote the manuscript.

## Figure legends

**Fig. 1** Overview of experimental and bioinformatics procedures in this study.

**Fig. 2** Characterization of the full-length transcriptome of *Spartina alterniflora*. (**a**) Distribution of reads of insert (ROI) lengths in each size-selected library. (**b**) Distribution of unique transcript clusters supported by different numbers of isoforms. (**c**) Comparison of transcripts identified from RNA-seq and PacBio data. Contigs from *de novo* assembly of RNA-seq data (Bedre *et al.*, 2016) were mapped to PacBio full-length transcripts (FLs), and vice versa. nContigs: the number of *de novo*-assembled contigs from RNA-seq data; nQ: the number of contigs mapped to PacBio FLs; pQ: the percentage of contigs mapped to FLs; nS: the number of FLs mapped to contigs; pS: the percentage of FLs mapped to contigs. (**d**) Sequence similarity analysis of *Spartina* FLNC transcripts compared to rice and Arabidopsis

using BLAST (e-value < 1e-5).

**Fig. 3** Comparison of gene ontology category percentage among *Spartina alterniflora*, rice and Arabidopsis. Blast2GO results of annotated genes or unigenes from these three species were mapped to categories in the second level of GO terms. The p-value was calculated using Fisher's exact test and double stars represent p-value below 0.01, while a single star represents p-value between 0.01 and 0.05.

**Fig. 4** Identification of salt stress related transcripts and regulatory hub genes. (**a**) Number of differentially expressed (DE) genes in 24 hours under different NaCl stress conditions compared to control conditions. FC: fold change; up-regulated: FDR < 0.05 and $\log_2$FC ≥ 1; down-regulated: FDR < 0.05 and $\log_2$FC ≤ –1. (**b-c**) Gene ontology enrichment of high salt stress associated unigenes. (**b**) GO results for up-regulated unigenes; (**c**) GO results for down-regulated unigenes. (**d**) Plot of relative expression values of 15 DE genes from qRT-PCR and RNA-seq experiments. Each point denotes the $\log_2$(fold change) of expression levels between the respective treated condition and the control condition. The Pearson correlation is 0.713 and the p-value is 3.908e-08. (e) Top ten hub genes in the turquoise module of salt stress

642 regulatory network. Hub genes were ranked according to the maximal clique
643 centrality (MCC) scores with colors indicating MCC scores from high (red) to low
644 (white). (f) Top ten hub genes in the blue module of the salt stress regulatory network.
645
646 **Fig. 5** Alternative splicing (AS) and high salt stress-associated AS in *Spartina*. (**a**)
647 Distribution of unique transcript clusters supported by different numbers of AS events.
648 (**b**) Proportion of different types of AS in *Spartina*. (**c**) Number of differentially
649 expressed AS (DEAS) events associated with salinity stress. (**d**) Schematic showing
650 the strategy used to validate intron-retention (IR) AS events. Primers were designed to
651 specifically detect the expression of IR-containing transcripts by qRT-PCR under
652 high salt stress, and a pair of primers flanking the IR region was used to validate the
653 AS events by RT-PCR. (**e**) Relative expression of IR-containing transcripts measured
654 by qRT-PCR. (**f**) Expression of IR transcripts and normal transcripts (NT) by
655 RT-PCR.
656 **Fig. 6** Functional validation of salt-responsive transcripts. (**a**) The salt tolerance
657 phenotype of *SaHSP70-I* transgenic lines in Arabidopsis: The $T_3$ generation seedlings

658 of *SaHSP70-I* overexpression lines were grown on half-strength Murashige and
659 Skoog medium after 10 days of salinity stress treatment. (b) Overexpression of
660 *SaHSP70-I* confers better salt tolerance in Arabidopsis transgenic line. The primary
661 root lengths (n > 20) were measured with ImageJ. (**c**) The salt tolerance phenotype of
662 the *SaAF2* transgenic lines in Arabidopsis. (**d**) Overexpression of *SaAF2* improved
663 salt tolerance of Arabidopsis transgenic line (n > 20).
664

665 **SUPPORTING INFORMATION**

666 **Supplemental Figures**

667 **Fig. S1** Gene ontology analysis of unigenes in *Spartina alterniflora.*

668 **Fig. S2** Clusters with multiple isoforms showed higher expression levels than those
669 with a single isoform.

670 **Fig. S3** RT-PCR validation of randomly selected transcripts in *Spartina alterniflora*.

671 **Fig. S4** Heatmap showing the Pearson correlation of unigene expression levels in
672 different samples and repeated experiments**.**

673 **Fig. S5** Differentially expressed transcripts and unigenes in different conditions.

674 **Fig. S6** RT-PCR experimental validation of salt-responsive unigenes.

675 **Fig. S7** GO enrichment analysis using transcripts involved in differentially expressed

676 alternative splicing events.

677

678 **Supplemental Tables**

679 **Table S1.** List of primers used in this study.

680 **Table S2.** Summary of PacBio single-molecule long-read sequencing data.

681 **Table S3.** List of non-coding RNAs in *Spartina alterniflora*.

682 **Table S4.** List of transcription factor coding transcripts in *Spartina alterniflora*.

683 **Table S5.** Summary of transcription factor families in *Spartina alterniflora.*

684 **Table S6.** List of *Spartina*-specific transcripts.

685 **Table S7.** List of salt stress-responsive unigenes in *Spartina alterniflora*.

686 **Table S8.** Selective modules with diverse patterns in salt gradient experiment

687 **Table S9.** List of ion transporters and related protein kinases

688 **Table S10.** List of high salt stress associated intron retention events.

689

690

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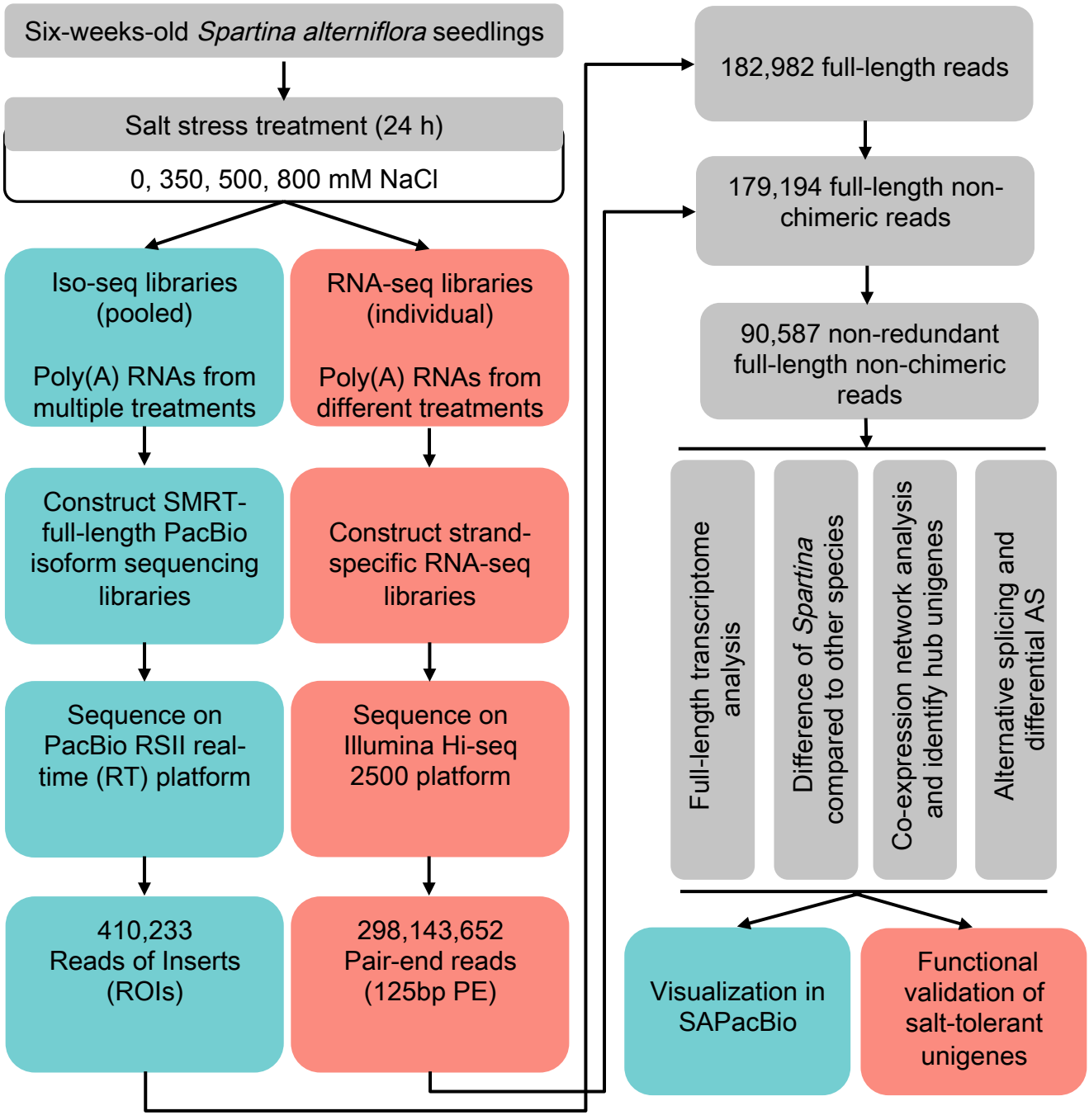
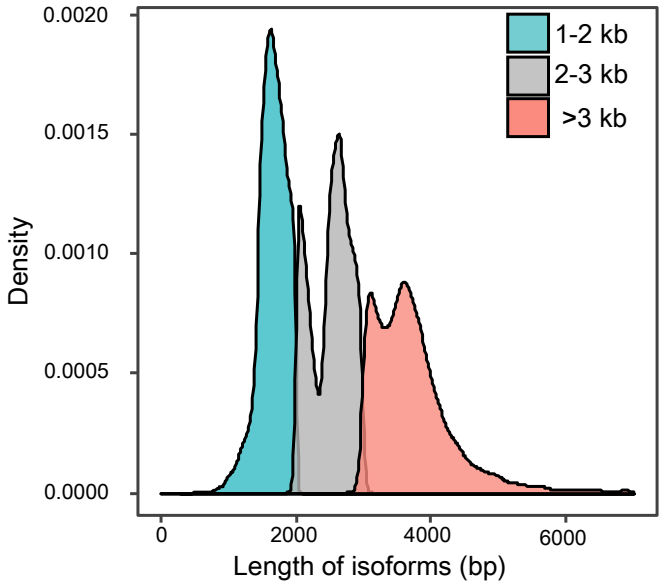
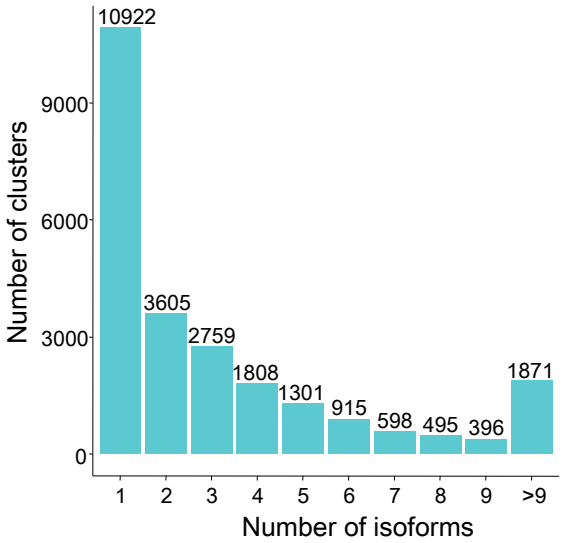



**Fig. 1** Overview of experimental and bioinformatics procedures in this study.

(a)




(b)




(c)

| | nContigs | nQ | pQ | FL number | nS | pS |
|---|---|---|---|---|---|---|
| Leaf control | 27,262 | 23,614 | 86.62% | 90,587 | 17,192 | 18.98% |
| Leaf salt | 23,906 | 21,018 | 87.92% | 90,587 | 15,708 | 17.34% |
| Root control | 15,216 | 5,493 | 36.10% | 90,587 | 2,726 | 3.01% |
| Root salt | 5,139 | 2,141 | 41.66% | 90,587 | 957 | 1.06% |
| Total | 71,249 | 52,062 | 73.07% | 90,587 | 25832 | 28.52% |

(d)

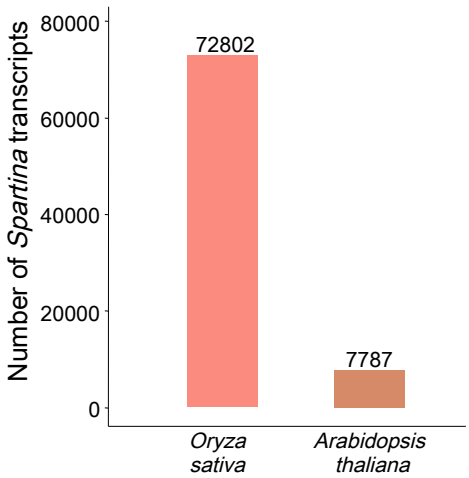



**Fig. 2** Characterization of the full-length transcriptome of *Spartina alterniflora*.

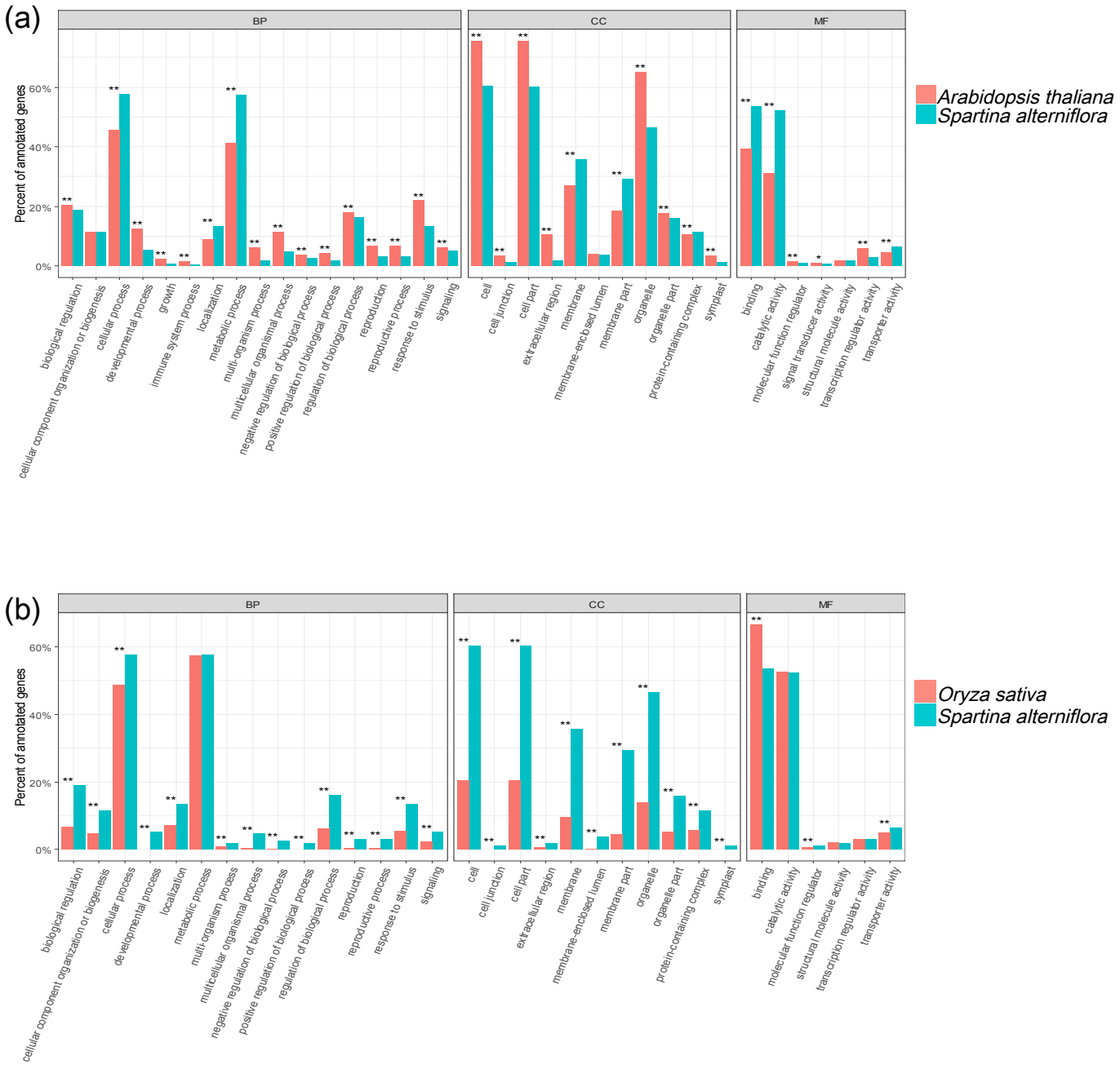



**Fig. 3** Comparison of gene ontology category percentage among *Spartina alterniflora*, rice and Arabidopsis.

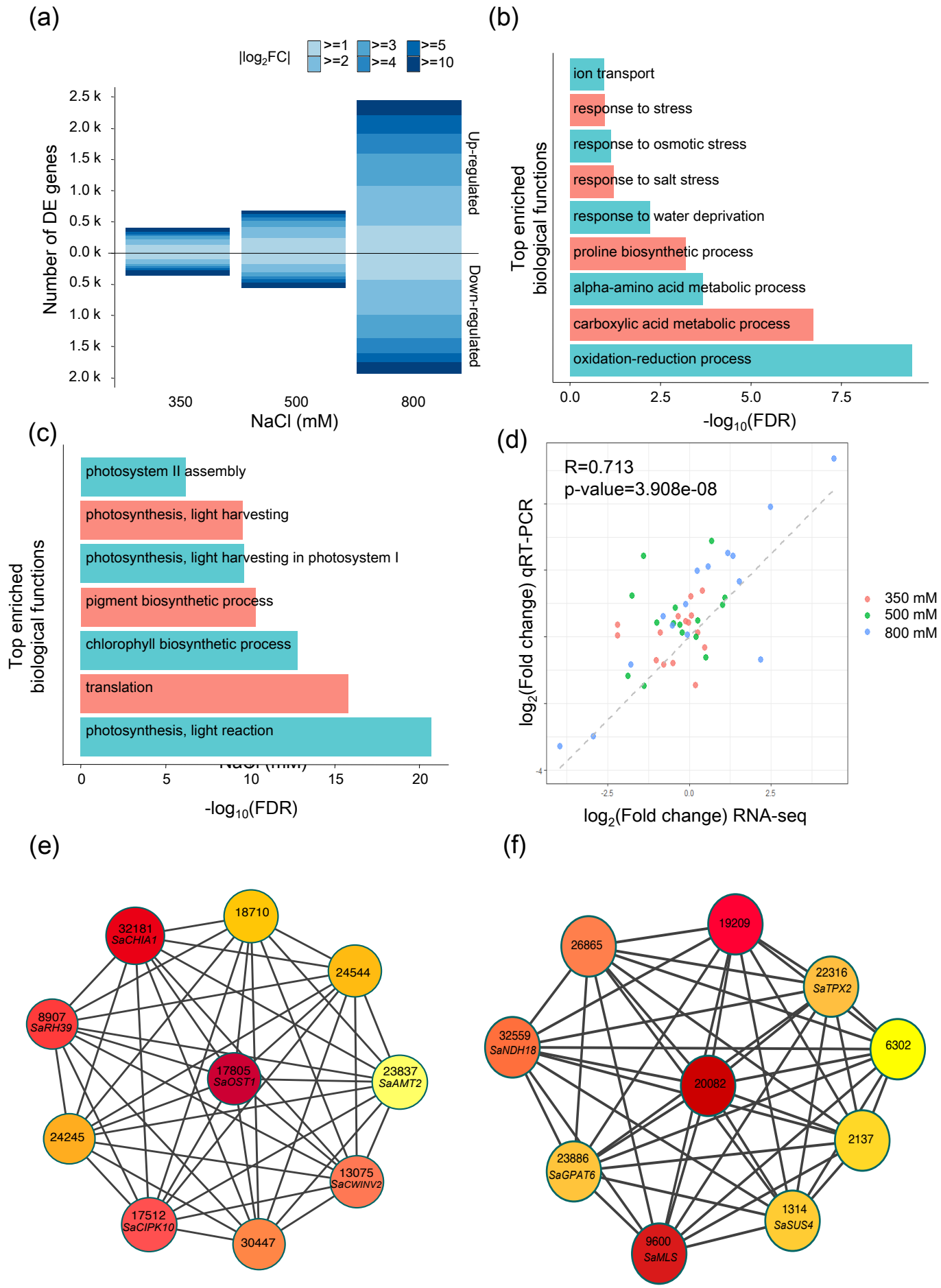



**Fig. 4** Identification of salt stress related transcripts and regulatory hub genes.

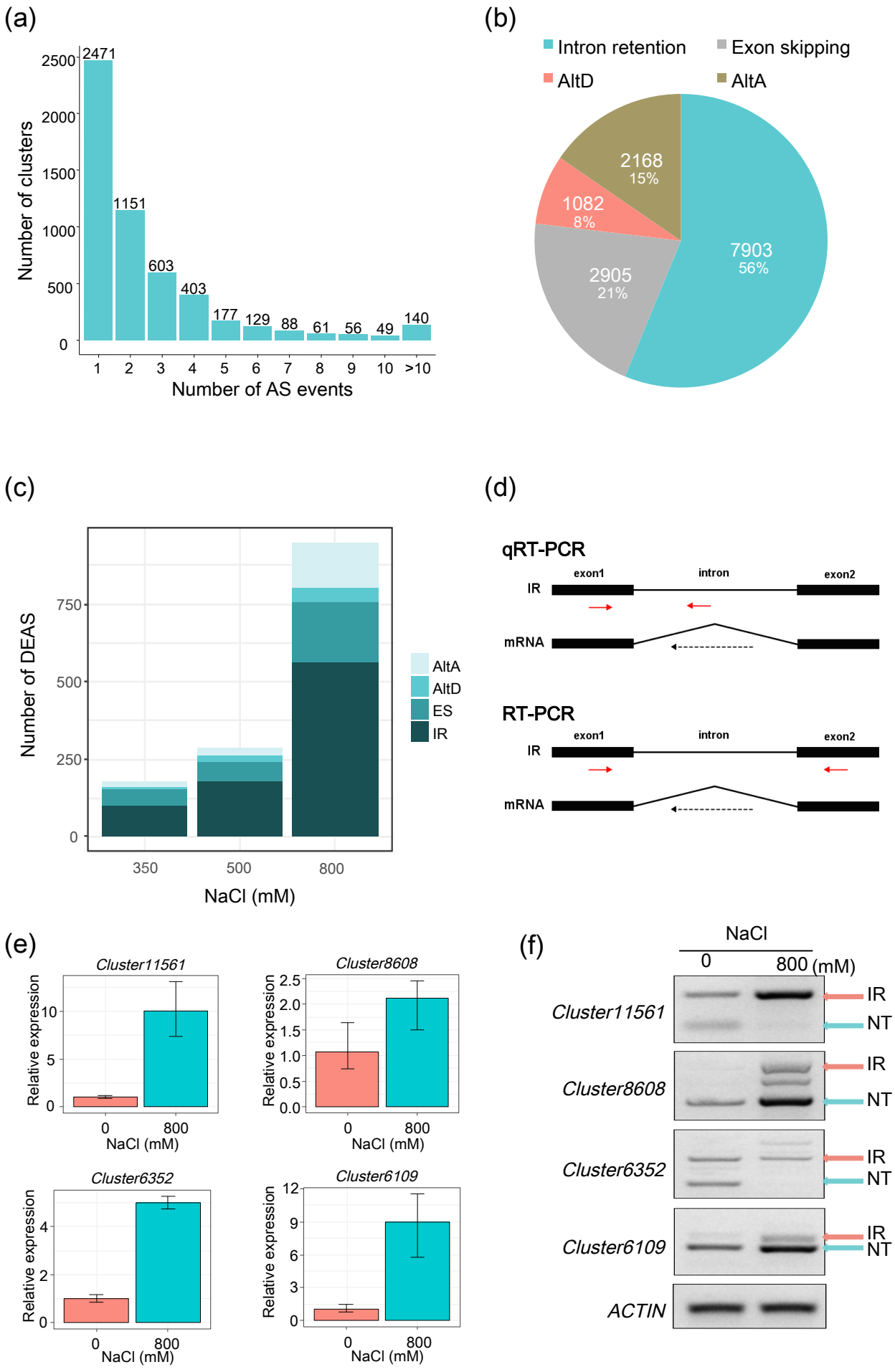



**Fig. 5** Alternative splicing (AS) and high salt stress-associated AS in *Spartina*.

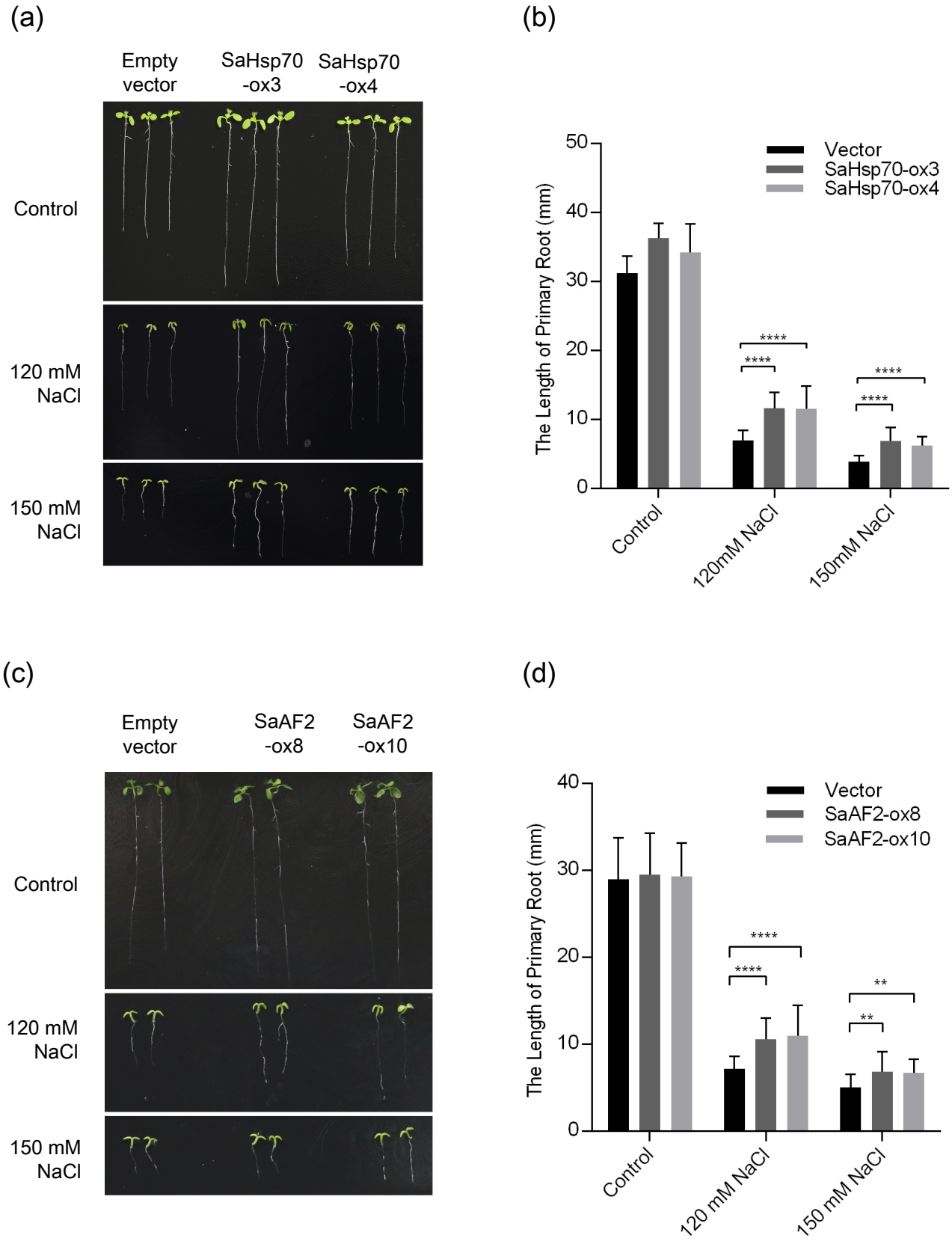



**Fig. 6** Functional validation of salt-responsive transcripts.