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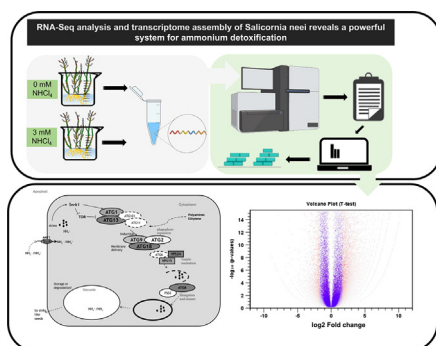
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## Research Article

RNA-seq analysis and transcriptome assembly of *Salicornia neei* reveals a powerful system for ammonium detoxificationMónica Díaz-Silva<sup>a</sup>, Jonathan Maldonado<sup>b,c</sup>, Pamela Veloso<sup>a</sup>, Nicol Delgado<sup>a</sup>, Herman Silva<sup>b</sup>, José A. Gallardo<sup>a,\*</sup><sup>a</sup>Laboratorio de Genética y Genómica Aplicada, Escuela de Ciencias del Mar, Pontificia Universidad Católica de Valparaíso, Valparaíso, Chile<sup>b</sup>Laboratorio de Genómica Funcional & Bioinformática, Facultad de Ciencias Agronómicas, Universidad de Chile, Av. Santa Rosa 11315, 8820808 La Pintana, Santiago, Chile<sup>c</sup>Laboratorio de Multiómica Vegetal y Bioinformática, Departamento de Biología, Facultad de Química y Biología, Universidad de Santiago de Chile, Santiago, Chile

## GRAPHICAL ABSTRACT



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## ABSTRACT

**Background:** *Salicornia neei* is a halophyte plant that has been proposed for use in the phytoremediation of the saline wastewater generated by land-based aquaculture. To identify the molecular mechanisms related to ammonium response, we analyzed the transcriptome of *S. neei* in response to growth in saline water containing 3 mM ammonium.

**Results:** The RNA sequencing generated a total of 14,680,108 paired-end reads from the control and stressed conditions. De novo assembly using the CLC Genomic Workbench produced 86,020 transcripts and a reference transcriptome with an N50 of 683 base pair.

A total of 45,327 genes were annotated, representing 51.2% of the contig predicted from de novo assembly. As regards differentially expressed genes, a total of 9,140 genes were differentially expressed in response to ammonium in saline water; of these, 7,396 could be annotated against functional databases. The upregulated genes were mainly involved in cell wall biosynthesis, transmembrane transport and antiporter activities, including biological Kyoto Encyclopedia of Genes and Genomes, pathways linked to the biosynthesis of secondary metabolites, plant hormone signal transduction, autophagy, and nitrogen metabolism. In addition, a set of 72 genes was directly involved in ammonium metabolism, including glutamine synthetase 1, glutamate synthase 1, and ferredoxin-dependent glutamate synthase chloroplastic.

**Abbreviations:** DEGs, differentially expressed genes; EggNOG, orthology predictions and functional annotation; Fd-GOGAT, ferredoxin-dependent glutamate synthase chloroplastic; FDR, false discovery rate; GDH, glutamate dehydrogenase; GO, gene ontology; GOGAT, glutamate synthase; GS, glutamine synthetase;  $I_{max}$ , maximum rate; KEGG, Kyoto Encyclopedia of Genes and Genomes;  $K_m$ , half-saturation constant; NR database, no redundant database; Pas, polyamines.

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Immersive  
 Nitrogen metabolism  
 Phytoremediation  
 RNAseq  
*Salicornia neei*  
 Saline wastewater  
 Succulent hydrohalophyte  
 Transcriptome assembly

**Conclusions:** Our results support the hypothesis that an ammonium detoxification system mediated by glutamine and glutamate synthase was activated in *S. neei* when exposed to ammonium and saline water. The present transcriptome profiling method could be useful when investigating the response of halophyte plants to saline wastewater from land-based aquaculture.

**This manuscript includes an interactive 360 degree video**, supplementary to the materials and methods section. To view the video correctly, it is necessary to scroll through the screen to navigate across the laboratory where you will find 6 interactive points. For an immersive experience a head-mounted display can be used. **Please, visit this URL:** [http://ejbiotechnology.info/public/360view/2022/VTPGALLARDO\\_1v3/index.htm](http://ejbiotechnology.info/public/360view/2022/VTPGALLARDO_1v3/index.htm).

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## 1. Introduction

*Salicornia neei* has been proposed for the treatment of saline wastewater produced by land-based aquaculture effluents in South America [1]. *S. neei* is a herbaceous succulent hydrohalophyte that is abundantly distributed throughout much of the western coast-line of the South Pacific [2]. Similar to other related species from Europe and North America, *S. neei* has attracted great interest due to its potential use as a leafy green food, because it contains significant amounts of nutrients and functional metabolites [3,4]. Its potential to germinate and be cultivated in different salinity gradients and nitrogen concentrations has recently been evaluated in land-based white shrimp (*Litopenaeus vannamei*) farming systems [5,6] and constructed wetlands (CWs) [1].

Land-based monoculture production systems use a large amount of artificial food rich in nitrogenous compounds, which are not fully utilized and may contaminate the culture water [7] or the environment if they are discarded without treatment. Although the accumulation of these compounds is variable and depends on both the species and the water treatment system involved [8], studies have shown that 133 kg of nitrogen (N) is released into the environment for each ton of harvested product [9]. The main nitrogenated compounds that are generated as waste are ammonia ( $\text{NH}_3$ ) and ammonium ( $\text{NH}_4^+$ ), which generally emerge as the final products of protein metabolism [10]. These compounds are found in equilibrium depending on the pH, temperature [11], and salinity [12]. The accumulation of ammonia and ammonium and other derived products, namely, nitrate ( $\text{NO}_3^-$ ) and nitrite ( $\text{NO}_2^-$ ), deteriorates water quality [13,14,15] and can generate eutrophication processes [16,17,18,19,20] and even encourage the development of diseases in cultivated organisms [21]. Therefore, taking advantage of nitrogenated compounds through the implementation of integrated marine aquaculture systems with halophyte plants has been proposed as a more sustainable alternative for the future development of land-based marine aquaculture [8,22].

Nitrate and ammonium, the main forms of inorganic N that plants absorb, act as nutrients and signals affecting the growth and metabolism of the plant [23]. In general, the preference of plants for nitrate or ammonium depends on both the genotype and environmental variables such as soil pH and the availability of other nutrients [24].

Ammonium is a nutrient with qualities of rapid absorption but excessive accumulation in tissues; in plants, high concentrations can lead to symptoms of toxicity if it is the only source of N [25]. However, the addition of ammonium and nitrate together can be favorable for the plant, with a synergistic action [26]. In ammonium-tolerant plants, such as of the *Salicornia* genus, the  $\text{NH}_4^+$  assimilation activity is higher compared to glycophytes.

Ammonium stimulates its assimilation via the upregulation of several key enzymes involved in ammonium metabolism [27,28]. After direct uptake or conversion from  $\text{NH}_3$ ,  $\text{NH}_4^+$  is assimilated to glutamine and glutamate via the activity of glutamine synthetase (GS), glutamate synthase (GOGAT), and glutamate dehydrogenase (GDH). The products of these pathways are required for the biosynthesis of other nitrogenous compounds [29]. These enzymes are part of two distinct pathways, the GS/GOGAT and GDH pathways. GS assimilates ammonium by catalyzing the amination of glutamate to form glutamine, while GOGAT catalyzes the reductive transfer of an amide-amino group from glutamine to 2-oxoglutarate, with the production of glutamate. GDH catalyzes the reversible amination of 2-oxoglutarate and ammonium to form glutamate [30].

In most glycophyte plants, highly saline soils disrupt metabolism, mainly due to decreased N uptake, altered activities of nitrate ( $\text{NO}_3^-$ ), and ammonium- ( $\text{NH}_4^+$ ) assimilating enzymes, inducing changes in amino acid synthesis and increasing the activity of hydrolyzing enzymes such as RNase, DNase, and protease, which lead to macromolecule degradation [31]. However, halophytic plants have the enzymatic potential to synthesize GS, GOGAT, and GDH in order to assimilate ammonium under saline conditions [32]. These enzymes have been demonstrated to be powerful regulators of gene expression, and may be involved in diverse stress responsiveness [33]. For example, many halophytic plants accumulate large amounts of soluble nitrogenous compounds that are involved in salt tolerance, such as proline and the proline analog 4-hydroxy-N-methyl proline, glycine betaine, pinitol, myoinositol, mannitol, sorbitol, O-methylmucoinositol, and polyamines (PAs) [32,34,35].

To identify the molecular mechanisms of ammonium response, we conducted a transcriptomic analysis of *S. neei* in response to ammonium in saline water. It was expected that *S. neei* would have a gene response profile typical of halophyte plants when exposed to abiotic stressors of both ammonium and salinity.

## 2. Materials and methods

### 2.1. Plant material collection

*S. neei* wild plants were collected from the “Pullally” salt marsh (Papudo, Chile; latitude, 32°24'50"S; longitude, 71°23'35.01"W) (Fig. 1) and transported to the facilities of Marine Farms Inc. (Laguna Verde, Chile, latitude 33°6'36"S; longitude 71°40'48"O) for propagation using cuttings. Stem cuttings were placed in an aquaponic cultivation system until they developed roots. Then, 180 rooted cuttings of *S. neei* were obtained from Marine Farms Inc. and transported to controlled growth chambers at the Pontificia Universidad Católica de Valparaíso (Valparaíso, Chile, latitude



Fig. 1. *Salicornia neei* wild plants from “Pullally” salt marsh (Papudo, Chile; latitude, 32°24'50”S; longitude, 71°23'35.01”W).

33°1'21”S, longitude 71°37'57”W). The cuttings were acclimated for 7 d in hydroponic culture pots using UV-filtered seawater (600 mM NaCl) to remove suspended particles and microorganisms. The cuttings were reared in a 12/12 h (day/night) photoperiod cycle at a temperature of 20°C and constant oxygenation, until being used in ammonium removal biokinetics or RNA-seq experiments. No extra nitrogen source was added to the filtered seawater during the acclimatization period, which had a basal concentration of  $28 \pm 6.5 \mu\text{M L}^{-1}$  of ammonium.

## 2.2. Michaelis–Menten ammonium removal biokinetics

A total of sixty cuttings were used to characterize the Michaelis–Menten ammonium removal biokinetics. They were randomly distributed in five treatments that consisted of filtered seawater solutions with the addition of ammonium chloride ( $\text{NH}_4\text{Cl}$ ) at the following concentrations: 0, 1, 2, 3, and 4 mM. Each treatment was set up in 500 mL flasks in which 4 cuttings with an average weight of  $21.8 \pm 9.9$  g were placed (Fig. S1). For each treatment, a flask without plants was also used as a control or blank. Ammonium removal was evaluated every 60 min for a period of 5 h. At each time point, two 1.5 mL samples were taken from the treatments and control, and stored in Eppendorf tubes for subsequent analysis. The Nessler method [36] was used for the determination of ammonium, using a HACH DR2800 spectrophotometer at 425 nm. The ammonium uptake rate was expressed in  $\mu\text{M NH}_4\text{Cl g}^{-1} \text{ DW h}^{-1}$ . After uptake, the root fresh weights were also recorded. The regressive relationship between uptake rate and ammonium concentration in the external solution was illustrated with the Michaelis–Menten [37] equation, as follows:

$$I = \frac{I_{\max}[C]}{K_m + [C]}$$

where  $I$  is uptake rate,  $I_{\max}$  is the maximum uptake rate,  $K_m$  is the half-saturation constant (Michaelis–Menten constant), and  $C$  is the ion concentration in solution. Michaelis–Menten curves were fitted using RStudio 7.0.

## 2.3. RNA-seq experiment and RNA extraction

A total of 24 cuttings were transferred to six plastic culture pots (four cuttings per pot and three biological replicates) containing 500 mL of filtered seawater with 0 or 3 mM  $\text{NH}_4\text{Cl}$ . After 2.5 h, the cuttings were harvested for analysis. Approximately 1 g of collected stem tissue was extracted from each cutting, immediately frozen in liquid nitrogen, and stored at  $-80^\circ\text{C}$  until RNA extraction (Fig. S2). Total RNA extraction was performed on three biological

samples at 0 and 3 mM ammonium. RNA extractions were performed following the previously described pine tree extraction method [38] used in combination with  $\beta$ -mercaptoethanol [39] and TRIzol (Life Technologies, Corp., Carlsbad, USA) according to the manufacturer’s protocol. To homogenize the sample, it was ground in a mortar and pestle in the presence of liquid nitrogen and subsequently heated at  $65^\circ\text{C}$  for 5 min in the presence of 1 mL pine tree buffer (PTB). Then, 20  $\mu\text{L}$   $\beta$ -mercaptoethanol was added to achieve greater sample purification, and the TRIzol steps were carried out according to the manufacturer’s protocol. Each sample was eluted in 60  $\mu\text{L}$  of DEPC water and subjected to quantification by fluorometry using a Nanodrop (Biotek). Then, 5  $\mu\text{g}$  of each sample was treated with RNase-free DNase I (Thermo Fischer Science) to remove residual genomic DNA, and precipitated with 3 M NaAc pH 5.2 and 100% EtOH. Finally, integrity was evaluated by electrophoresis in a 1% agarose gel, as described in [40].

## 2.4. Library construction, deep sequencing, and de novo transcriptome assembly

Library construction and deep sequencing of *S. neei* following treatment with 0 or 3 mM  $\text{NH}_4\text{Cl}$  were performed at Macrogen (Inc. Seoul, South Korea) using the Solexa HiSeq2000 platform with the Truseq mRNA library previously constructed for paired-end applications, according to Macrogen’s protocol. For de novo transcriptome assembly, the raw data were cleaned of adaptor sequences, while low-quality reads ( $Q$ -value  $\leq 20$ ), reads with poly-N segments (reads containing more than 50% unknown bases) and short reads (less than 50 base pairs (bp)) were removed. The sequence quality algorithm contained in the CLC Genomics Workbench version 8.0 (<https://www.clcbio.com>) was used for this purpose. These results were processed using the scaffolding contig algorithm in the de novo assembly function of the CLC Genomics Workbench with default parameters. Single pools of 3 samples of 0 mM  $\text{NH}_4\text{Cl}$  and 3 samples of 3 mM  $\text{NH}_4\text{Cl}$  were mapped separately against the assembled set of contigs using the RNA-seq tool of the CLC Genomics Workbench with the following parameters: similarity = 0.9; length fraction = 0.6; maximum mismatches = 2; unspecific match limit = 10. Paired reads were counted as 2, and paired-end distances were set as 101 bp.

## 2.5. Functional annotation

To identify the putative biological function, all assembled contigs were searched against public protein databases using the BLASTx algorithm of BLAST version 2.6.0+ [41]; the searched databases included the NCBI non-redundant (NR) protein database



with an  $E$ -value cut-off of  $10^{-5}$ , the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database with an  $E$ -value of  $10^{-5}$ , and EggNOG (orthology predictions and functional annotation) with an  $E$ -value of  $10^{-10}$ . Furthermore, to consolidate the information, all contigs were assigned to Gene Ontology (GO) categories using the Blast2Go version 5.2 [42] software package and an  $E$ -value cut-off of  $10^{-5}$ . Finally, because the genus *Salicornia* is poorly represented in the protein databases, contigs were also aligned against the nucleotide sequences of *Salicornia* available from the NCBI database using BLASTn with an  $E$  value of  $10^{-5}$ .

## 2.6. Differentially expressed genes (DEGs)

To determine the differentially expressed genes in *S. neei* under the two nutritional conditions, RNA-seq analysis was performed using the CLC Genomics Workbench program version 8.0 (<https://www.clcbio.com>). The relative transcript levels were defined as the number of reads that uniquely mapped to a gene. The expression levels were compared using a Z-Test [43,44,45,46] with 0 mM  $\text{NH}_4\text{Cl}$  as the reference. This test compares counts by considering the proportions that make up the total sum of counts in each sample, correcting the data for sample size. For visual inspection, the original expression values were  $\log_{10}$ -transformed and then normalized using the quantile method that best fits the results [44]. As a significant threshold for the selection of genes with differential expression, a false discovery rate (FDR)  $p$ -value  $< 0.001$  and an absolute fold change (FC) of 2.0 were adopted. Finally, GO enrichment analyses were performed separately on genes up-regulated in the 0 and 3 mM  $\text{NH}_4\text{Cl}$  treatments to investigate the differences in the ammonium response mechanism. For details see Supplementary 360° interactive video available at [http://ejbiotechnology.info/public/360view/2022/VTPGALLARDO\\_1v3/index.htm](http://ejbiotechnology.info/public/360view/2022/VTPGALLARDO_1v3/index.htm).

## 3. Results

### 3.1. Kinetics of ammonium removal

The parameters of the  $\text{NH}_4\text{-N}$  uptake kinetics have not been previously determined for *S. neei*. The ammonium uptake rate increased with increasing ammonium concentration in the external solution, and tended toward saturation in the range of 3–4 mM (Fig. 2). Using regression analysis, it was found that the kinetic characteristics of ammonium uptake by the test plant could be illustrated using the Michaelis–Menten equation at a significance level of  $p < 0.001$ . The kinetic parameters for ammonium uptake were estimated as a maximum rate ( $I_{\text{max}}$ ) of  $7.07 \pm 0.27$

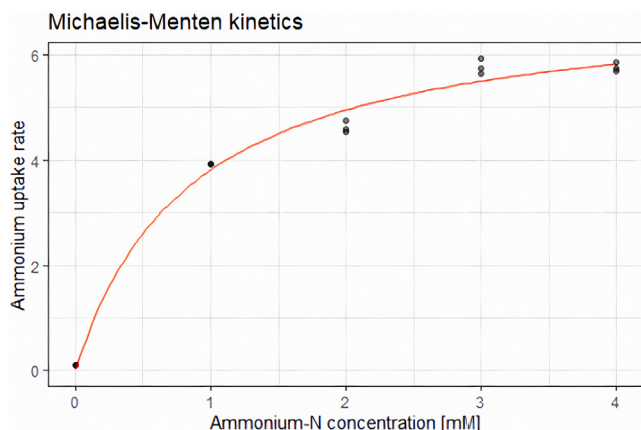


Fig. 2. *Salicornia neei* ammonium uptake rate as a function of ammonium concentration.

and a half-saturation constant ( $K_m$ ) of  $0.85 \pm 0.12$ . Ammonium removal was significantly different from zero in all treatments (Table S1).

### 3.2. Transcriptomic sequencing, de novo assembly, and data availability

The transcriptomic analysis of pooled samples resulted in a total of 147,935,752 clean reads; the comprehensive reads were assembled into contigs using paired-end reads, resulting in 86,020 contigs with an average length of 586 bp (Table 1 and Table 2). The raw sequencing data were deposited in the NCBI Short Read Archive (SRA) database under accession code SRR9694999, and they have been assigned the BioProject accession number PRJNA554118.

### 3.3. Overview of sequences annotation and differentially expressed genes (DEGs)

The functional annotation process of the contigs using the NR database determined the presence of 86,020 contigs, most of which were assigned to *Spinacia oleracea* and *Chenopodium quinoa*. Most sequence homologies (Table 3) were found via comparisons against the NCBI–NR database (45,327 contigs), followed by GO (37,784 contigs) and EggNOG (20,047 contigs). Furthermore, 32,609 contigs were compared locally against the nucleotide sequences of *Salicornia* sp. Thus, a total of 13,906 new sequences from *S. neei* were found that did not match with those of other databases (Fig. S3). Based on the *S. neei* global transcriptome (86,020 contigs), 9,140 contigs were differentially expressed in their response to ammonium in saline water (7,040 up-regulated and 2,100 down-regulated), but only 7,396 could be annotated against the NR, GO, EggNOG and KEGG databases. Moreover, 588 DEGs were unique to the ammonium treatment. This was judged based on the abundance of transcription in the control, which was too low.

### 3.4. Function annotation and classification

A total of 150,944 GO terms were assigned to 37,784 (43.9%) of the analyzed sequences; in multiple cases, several terms were assigned to the same sequence (mean = 4). Of the GO functional groups, 11 were assigned to biological processes, 10 to molecular functions, and 7 to cellular components. The top five functional groups were organic substance metabolic process (18,160), intracellular anatomical structure (18,042), cellular metabolic process (17,526), primary metabolic process (17,076), and organelle (15,242) (Fig. 3). GO terms retrieved for the DEGs of *S. neei* were subjected to a functional enrichment analysis, obtaining 17 enriched terms belonging to three main categories (BP, MF, and

Table 1  
Reads trimming and mapping stats.

		Library- 0 mM- $\text{NH}_4\text{Cl}$	Library- 3 mM- $\text{NH}_4\text{Cl}$
Quality and adapter trimming	Number of reads	71,793,586	74,986,522
	Avg length (bp)	97.7	97.6
	Number of reads after trim	71,695,733	74,879,764
Reads mapping to de novo assembled <i>S. neei</i> transcriptome	Reads mapped in pairs	51,430,428	51,493,796
	Reads mapped in broken pairs	7,007,230	9,483,298
	Reads not mapped	13,161,064	13,796,746
	Total	71,598,722	74,773,840

**Table 2**  
Summary of RNA-seq and de novo sequence assembly for *Salicornia neei*.

	Count	Average length (bp)	Total bases
Total Reads	147,935,752	96.32	14,248,757,629
Matched	120,663,553	96.32	11,622,050,087
Not matched	27,272,199	96.31	2,626,707,542
Contigs	129,008	586	75,674,621
Reads in pairs	102,737,332	153.3	
Broken paired reads	16,680,278	96.26	

**Table 3**  
Summary of annotations of assembled *Salicornia neei* contigs.

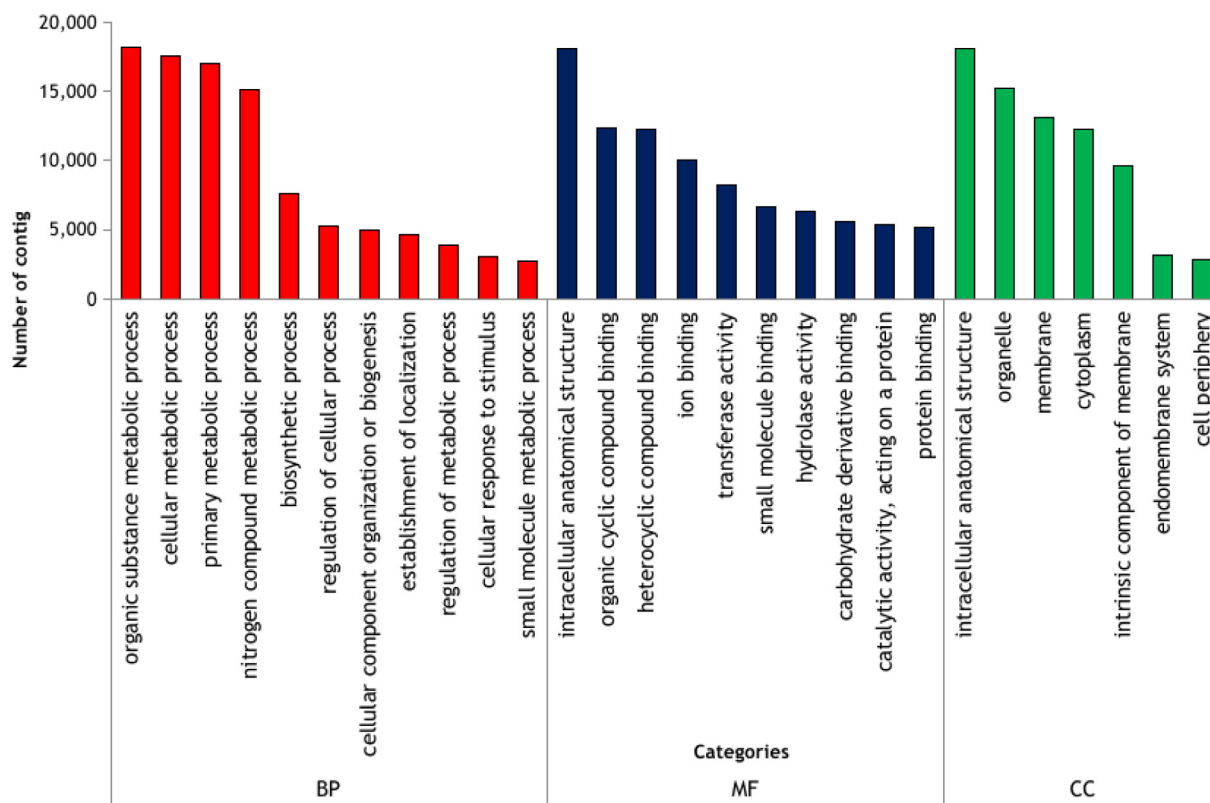
	Number of contigs annotated	Percentage of contigs annotated
NCBI (NR)	45,327	52.6
NCBI (NT)	32,609	37.9
Salicornia)		
GO	37,784	43.9
KEGG	13,604	15.8
EggNOG	20,047	23.3

GO: Gene Ontology; KEGG: Encyclopedia of Genes and Genomes.

CC) (Fig. 4). The enrichment proportion in the *S. neei* test set corresponds to the double or triple proportion of those found in the reference set: Xyloglucan metabolic process (0.3% in test set vs. 0.05% in reference set), beta-glucan metabolic process (0.72% in test set vs. 0.25% in reference set), beta-glucan biosynthetic process (0.63% in test set vs. 0.22% in reference set), cell wall organization biogenesis (1.25% in test set vs. 0.36% in reference set), sterol metabolic process (0.35% in test set vs. 0.10% in reference set), steroid metabolic process (0.46% in test set vs. 0.14% in reference set), glucosyltransferase activity (1.0% in test set vs. 0.37% in reference set),

ion channel activity (0.7% in test set vs. 0.3% in reference set), and extracellular region (1.3% in test set vs. 0.69% in reference set) (Fig. 4).

Several DEGs were induced in response to ammonium treatment, mostly related to the maintenance of homeostasis. Out of the 91 DEGs related to nitrogen metabolism, 72 were up-regulated, including the following: glutamine synthetase (GS), 22-fold; oxoglutarate dehydrogenase (2-OGD), 2.34-fold; ferredoxin-dependent glutamate synthase chloroplastic (Fd-GOGAT), 4.29-fold; glutamate synthase 1 (NADH) chloroplastic isoform X1 (GLT1), 6.74-fold and ammonium transporter (AMT1), 2.39-fold (Table S2). Phytohormone biosynthesis was also up-regulated; here, we found genes related to ethylene biosynthesis (S-adenosylmethionine synthetase (S-AdoMet) (20.82-fold), S-adenosyl-L-methionine (SAM) (4.8-fold), 1-aminocyclopropane-1-carboxylate synthase (ACC) (13.96-fold)) and abscisic acid biosynthesis (zeaxanthin epoxidase (ZEP) (2-fold), violaxanthin de-epoxidase, chloroplastic (VDE1) (2.56-fold) and xanthoxin dehydrogenase (ABA2) (2-fold)). We also found many genes closely related to polyamine biosynthesis and autophagy, such as arginine decarboxylase-1 (ADC1), 2.36-fold; s-adenosylmethionine decarboxylase proenzyme (SAMDC1), 2.25-fold; polyamine oxidase-1 (PAO-1), 17.56-fold; autophagy-related protein 1 (ATG1), 2-fold; autophagy-related protein 13 (ATG13), 2.11-fold; autophagy-related protein 9 (ATG9), 5.89-fold; autophagy-related protein 2 (ATG2), 3.91-fold; and autophagy-related protein 18, 2.57-fold. Genes responsible for vesicle nucleation, such as vacuolar protein sorting 34 (VPS34) (3.26-fold) and vacuolar protein sorting 15 (VPS15) (2.0-fold), were also found up-regulated. Finally, genes responsible for the elongation and closure vacuolar protein sorting 8 (ATG8) were up-regulated 2.0-fold, and phosphatidylserine decarboxylase proenzyme 2 (PSD2) was up-regulated 2.0-fold;



**Fig. 3.** Histogram of Gene Ontology (GO) classification of 37,784 *S. neei* transcripts assigned to three main categories: biological process (BP), molecular function (MF) and cellular component (CC).

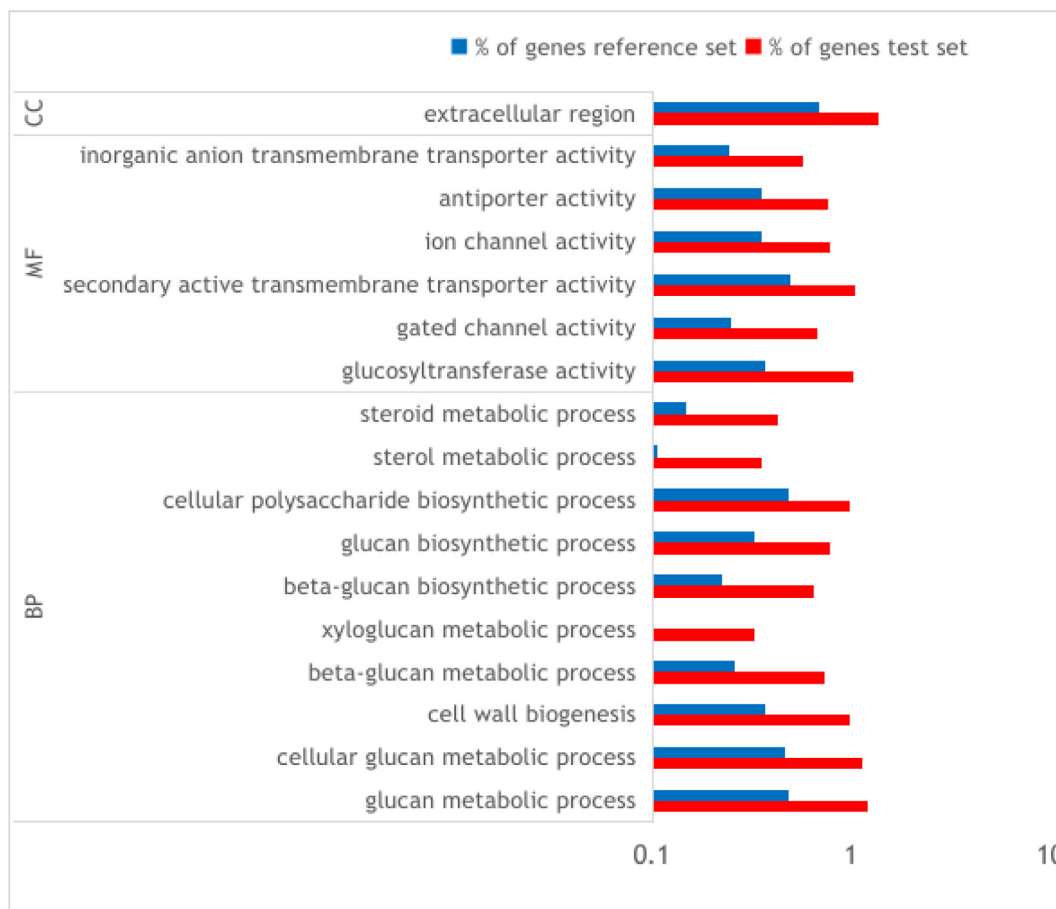


Fig. 4. Gene Ontology enrichment analysis of differentially expressed genes in *S. neei* transcripts upregulated in test set vs upregulated genes in reference set.

**Table 4**  
Summary of distribution of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in the *S. neei* transcriptome.

	KEGG Pathway	N° Contigs
Biosynthesis of secondary metabolite	map01110	297
Biosynthesis of antibiotics	map01130	95
Amino acid biosynthesis	Map01230	71
Carbon metabolism	map01200	61
Starch and sucrose metabolism	map00500	55
Plant hormone signal transduction	map04075	53
Phenylpropanoid biosynthesis	map00940	52
Cysteine and methionine metabolism	map00270	34
Arginine and proline metabolism	map00330	28
Nitrogen metabolism	map00910	25
Purine metabolism	Map00230	21
Autophagy - other	map04136	20

both of these catalyze the formation of phosphatidylethanolamine (PE).

DEGs were almost mapped to the metabolic pathway in KEGG; 1,206 DEGs were mapped to 167 KEGGs. Among the 167 pathways, the largest pathway was the biosynthesis of secondary metabolites (296 up-regulated genes), followed by ubiquitin-mediated proteolysis (55 up-regulated genes) and plant hormone signal transduction (48 up-regulated genes). DEGs were mostly mapped to autophagy (other (20 up-regulated genes) and nitrogen metabolism (14 up-regulated genes)) (Table 4). Finally, to identify the proteins distributed in eukaryotic orthologous groups, and in clusters of orthologous groups and non-supervised orthologous groups, the annotated contigs were mapped to the annotations of the corre-

sponding orthologous groups in the EggNOG database. A total of 20,047 (23.3%) sequences were divided into 25 categories and specific functional groups were identified (Fig. 5). In the 25 EggNOG categories, the largest proportion of contigs belong to the cluster of “function unknown” (9,012; 44.9%), followed by “O: posttranslational modification, protein turnover, chaperones” (2,948; 14.7%) and “T: signal transduction mechanisms” (3,031; 15.11%). Other important groups closely related to ammonium nutrition response and stress response were “E: amino acid transport and metabolism” (1,391; 6.9%), “Q: Secondary metabolites biosynthesis, transport, and catabolism” (883; 4.4%), “M: cell wall/membrane/envelope biogenesis” (486; 2.42%), and “V: defense mechanisms” (259; 1.2%). Only a small number of contigs were assigned to “W: extracellular structures” (6; 0.02%) or “Y: nuclear structure” (14; 0.006%). For details see Supplementary 360° interactive video available at [http://ejbiotechnology.info/public/360view/2022/VTPGALLARDO\\_1v3/index.htm](http://ejbiotechnology.info/public/360view/2022/VTPGALLARDO_1v3/index.htm).

#### 4. Discussion

The use of halophyte plants to treat wastewater highly loaded with nitrogen compounds derived from the metabolism of aquatic organisms has been proposed to increase the sustainability of land-based aquaculture. However, the molecular mechanisms by which these plants can absorb and use these compounds in highly saline environments are poorly understood [20,47]. This work used transcriptome analysis of the halophyte plant *S. neei* to investigate the possible pathways that are implicated in their response to ammonium nutrition. We considered possible ammonium homeostasis

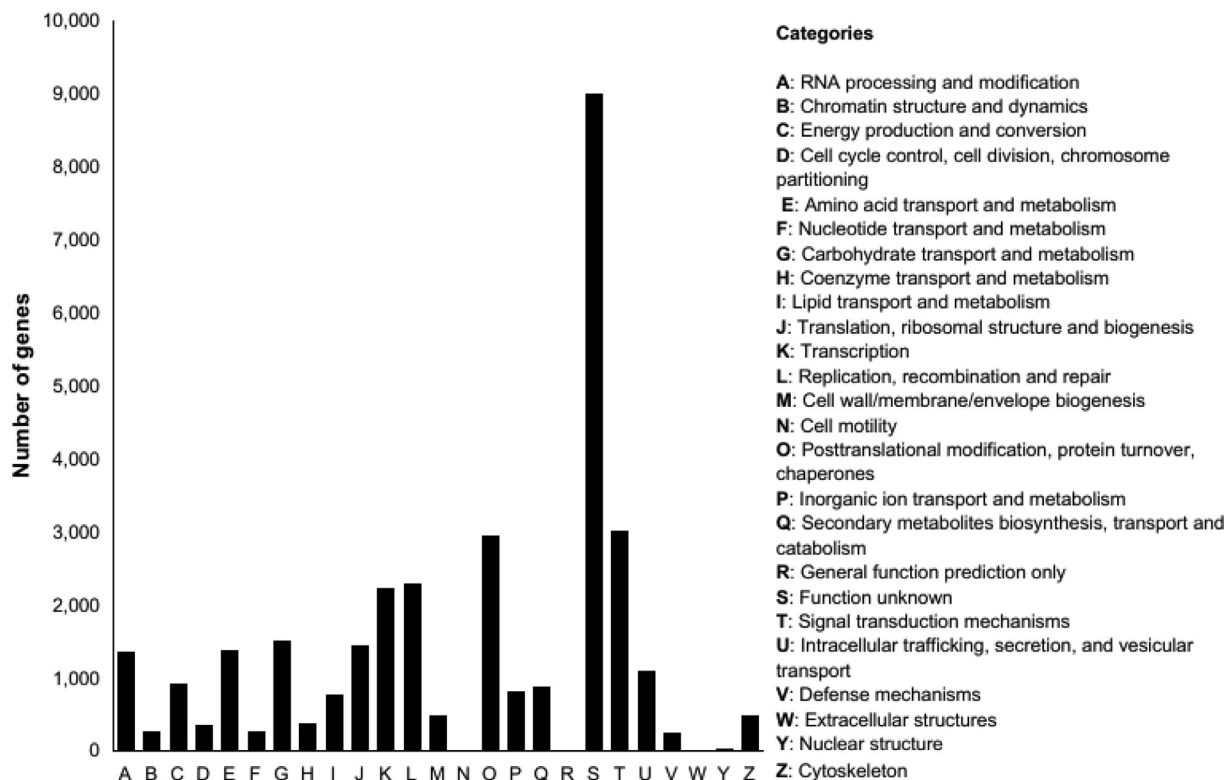


Fig. 5. Functional annotation of contigs identified in *Salicornia neei* transcriptome. EggNOG classification analysis.

through ammonium metabolism and the encapsulation of ammonium in vacuoles to avoid increasing the acidity in the apoplast. We studied the kinetics of ammonium absorption in concentration gradients to demonstrate the activity of HATS (high-affinity transport systems) (AMT1), which are mainly related to the entry of ammonium into the cell, and therefore the disappearance of ammonium in the substrate.

The  $\text{NH}_4$  uptake kinetics of *S. neei* plants incubated at different concentrations fitted the Michaelis–Menten model well, up to  $4 \text{ mM NH}_4 \text{ L}^{-1}$  (Fig. 2), indicating the plant's HATS activity at the tested concentrations. Quinta et al. [7] found a good fit for the Michaelis–Menten model up to  $2 \text{ mM NH}_4 \text{ L}^{-1}$  in *S. europaeae*, and suggested that HATS (high-affinity transport systems) are responsible for N uptake in that plant. In this study, *S. neei* displayed higher ammonium absorption compared to *S. europaeae* [7], demonstrating a better capacity for use in aquaculture wastewater.

The Michaelis–Menten equations obtained with the tested ammonium concentrations ( $1\text{--}4 \text{ mM NH}_4\text{Cl}$ ) describe changes in ammonium absorption according to the amount of ammonia supplied. It was found that *S. neei* had a high affinity for the substrate ( $K_m = 0.85 \pm 0.12 \text{ mM NH}_4 \text{ L}^{-1}$ ), indicating that this plant can perform well in substrates with high concentrations of ammonia. In addition, for aquaculture wastewater with concentrations equal to or greater than  $4 \text{ mM NH}_4 \text{ L}^{-1}$  and up to  $7.07 \pm 0.27 \text{ mM NH}_4 \text{ g}^{-1} \text{ FW h}^{-1}$ , it is believed that the uptake rate does not increase because the plant blocks AMTs at higher concentrations. Concerning the kinetic characteristics of N uptake in plants, it has been proposed that species with higher maximum uptake rates might be better suited to cleaning up wastewater with high nutrient concentrations [48].

The cell wall plays an important role in stress perception by facilitating the activation of signaling pathways and remodeling growth strategies in response to stresses [49]. This structure con-

stitutes the first line of defense against biotic and abiotic environmental influences through wall reinforcement via callose deposition [50]. In this study, an enrichment analysis of DEGs under ammonium nutrition revealed that a set of overexpressed genes related to secondary cell wall materials could contribute to the maintenance of cell wall structure and the functional properties of *S. neei*. Some studies have reported that genes related to cell wall maintenance are significantly enriched in treatments that induce biotic or abiotic stress [51]. This is very interesting, as it has been shown that the cell wall structure is reassembled through the biosynthesis of cell wall polymers, which allows it to adapt to stress conditions [49,52,53]. Wang et al. [53] demonstrated that cell wall modifications were highly active in response to salt stress in cotton. The most common adaptations of the cell wall to stress include (i) increased levels of xyloglucan endotransglucosylase/hydrolase (XTH) and expansin proteins, associated with an increase in the degree of rhamnogalacturonan I branching, which maintains cell wall plasticity, and (ii) increased cell wall thickening through the reinforcement of the secondary wall with hemicellulose and lignin deposition [49,54]. It was proposed that xyloglucan regulation by expansins could improve the efficiency of nutrient uptake. Several types of expansins respond to deficiencies in different nutrients, including nitrogen, phosphorus, potassium, and iron [55]. In this study, we found up-regulated genes related to the xyloglucan metabolic process, cellular–glucan metabolic process categories, which can regulate several physiological plant responses through cell wall remodeling in *S. neei*. Also, we found up-regulated genes related to sterol metabolic processes and steroid metabolic processes, which play crucial roles in various physiological and biochemical processes during development and stress resistance in plants (Fig. 4).

KEGG analysis showed that DEGs were most enriched in the biosynthesis of secondary metabolites (Table 4). This intracellular



physiological defense response helps plants resist environmental stresses, indicating that they may likely play an important role in ammonium stress response, even though the shoot only receives  $\text{NH}_4^+$  stress signals from the root indirectly [56]. Some studies indicate that the accumulation of secondary metabolites is highly dependent on environmental factors such as light, temperature, soil water, soil fertility, and salinity [57]. Another pathway observed in high-ammonium conditions was plant hormone signal transduction (Table 4); the abundance of DEGs related to this pathway is in agreement with the increased expression of the ethylene-responsive transcription factor and the ABA responsive element binding factor. Plant hormones play critical roles in response to various adverse biotic and abiotic environmental stresses (drought, heat, soil salinity, tropospheric ozone and excess UV radiation) [58], but have not been reported in ammonium stress. The genes involved in transcription, consistent with the role of phytohormones as triggers of signal transduction cascades, are key components in responses to abiotic stress. We investigated the common and specific responses of *S. neei* shoots to ammonium stress through up-regulated or down-regulated DEGs, with a special focus on the role of phytohormones, and we found abscisic acid (ABA) and ethylene to be essential plant hormones regulating abiotic and biotic stresses [59,60]. According to previous studies on some halophytes species, under high-salinity conditions, ethylene production is increased in the leaves and roots [61]. Similarly, it was observed that ABA and ET signaling pathways were increased under ammonium treatment in rice and maize [23,56], suggesting that ET and ABA biosynthesis might be increased in response to stress. It is well known that these pathways interact among themselves at various nodes, such as hormone-responsive transcription factors, to regulate plant defense responses [62,63,64]. Among the

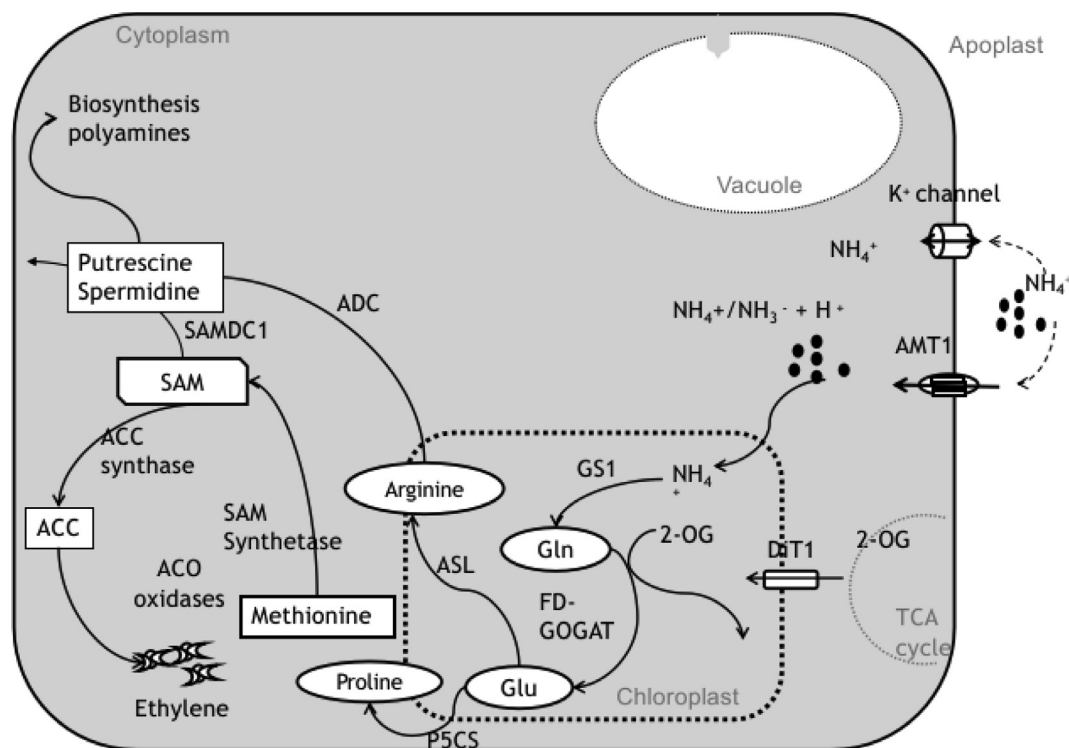
hormone pathways identified, ABA and ethylene biosynthesis genes were up-regulated under ammonium treatment in *S. neei*. In our study, ABA was found in one pathway (plant hormone signal transduction) and ethylene was found in three pathways (biosynthesis of secondary metabolite, plant hormone signal transduction) in the top 10 KEGG table (Table 4 and Table 5, and Table S2). We found that S-AdoMet, SAM and ACC (direct precursor of the plant hormone ethylene) [65] (Fig. 6) are centrally involved in ethylene biosynthesis, and ZEP, VDE1 and ABA2 are involved in ABA biosynthesis. In addition, we found the key ABA responsive element binding factor and ethylene-responsive transcription factor. Therefore, it seems that the transduction pathway, regulated by ABA and ethylene, plays an important role in the stress response of *S. neei* to  $\text{NH}_4^+$ .

The biosynthesis pathways of polyamines (PAs) and ethylene are interrelated, with SAM as a common precursor that can be used to form ACC, the precursor of ethylene that is active in the conversion of PAs [66]. Their physiological functions are distinct and at times antagonistic [67], but both have been identified as important signaling molecules involved in stress tolerance [68]. In this study, overexpressed genes related to the metabolic pathways of polyamines were found (Table 5, Fig. 6, and Table S2). According to Navin et al. [69], putrescine, spermidine, and spermine biosynthesis are also involved in the amelioration of drought, salinity, cold, and heat stresses. Polyamines counter stress by binding with nucleic acids, proteins, and phospholipids to stabilize their structures in response to diverse abiotic stress conditions [69]. Bouchereau et al. [70] stated that  $\text{NH}_4^+$  nutrition is associated with significant changes in the free polyamine content in the shoots or roots of plants, which could be key to the protection of a stressed cell. We found three genes, PAO1, PAO2

**Table 5**  
Genes involved in ammonium homeostasis and phytohormone biosynthesis in *Salicornia neei*.

Classification	NCBI Accession	Differentially expressed protein-coding genes	Role	Log2-fold change
genes related to nitrogen metabolism	GHTP01016006.1	glutamine synthetase (GLN1)	Plays an essential role in the metabolism of nitrogen by catalyzing the condensation of glutamate and ammonia to form glutamine	22.0
	GHTP01002667.1	ammonium transporter 3 member 1-like (AMT3.1)	Involved in ammonium transport	7.75
	GHTP01006207.1	pyrroline-5-carboxylate synthetase (P5CS)	Plays a key role in proline biosynthesis, leading to osmoregulation in plants	5.68
	GHTP01009881.1	glutamate synthase 1 [NADH] chloroplastic isoform X1 (GS)	Catalyzes reactions that convert $\alpha$ -ketoglutarate and ammonia to glutamate, which is then used in a wide variety of biosynthetic reactions	5.19
	GHTP01037737.1	ferredoxin-dependent glutamate synthase chloroplastic (Fd-GOGAT)	Has a primary role in photosynthetic tissues	4.29
genes related to polyamines	GHTP01000190.1	high affinity nitrate transporter 2.5-like (NRT2.5)	Might be involved in the transfer of nitrate from stored pools to cytoplasm	4.18
	GHTP01019462.1	arginine decarboxylase (ADC)	Important enzyme responsible for putrescine biosynthesis	2.36
	GHTP01012076.1	S-adenosylmethionine decarboxylase (SAM)	Key enzyme in higher polyamine (PA) biosynthesis	2.25
	GHTP01012076.1	adenosylmethionine decarboxylase proenzyme (SAMDC1)	Plays an essential regulatory role in the polyamine biosynthetic pathway	2.25
genes related to ethylene biosynthesis	GHTP01019486.1	polyamine oxidase 1 (PAO1)	Involved in the production of hydrogen peroxide in response to salt and cold stresses	2.04
	GHTP01022642.1	ethylene-responsive transcription factor like (ERF109)	May be involved in the regulation of gene expression by stress factors and by components of stress signal transduction pathways	25.0
	GHTP01023420.1	ethylene-insensitive protein 2 isoform X1 (EIN2)	Might take part in multiple hormones signaling pathways and in response to pathogens as demonstrated by recent genetic and biochemical studies	2.39
genes related to ABA biosynthesis	GHTP01045464.1	heat stress transcription factor A-3 (HsfA3)	Conferring salt tolerance	4.93
	GHTP01059070.1	ABA responsive element binding factor	Transcription factors that regulate expression of target genes involved in plant tolerance to drought, high salinity, and osmotic stress	4.2
	GHTP01022623.1	dehydration-responsive element-binding protein (DREB2A)	Plays a critical role in plant development and abiotic stress-mediated gene expression	2.05





**Fig. 6.** Putative model of ammonium metabolism in *Salicornia neei*, polyamines and ethylene biosynthesis, according to the major DEGs that were modulated by  $\text{NH}_4^+$ : ammonium transporter 1 (AMT1), glutamine synthetase 1 (GS1), ferredoxin-dependent glutamate synthase (Fd-GOGAT), glutamate (Glu), glutamine (Gln), pyrroline-5-carboxylate synthetase (P5CS), 2-oxoglutarate (2-OG), dicarboxylate transporter 1 (DiT1), arginine decarboxylase (ADC), S-adenosylmethionine decarboxylase (SAMDC1), tricarboxylic acid cycle (TCA), and potassium  $\text{K}^+$  channel, ACO, ACC. Figure adapted from Ma et al. [30] and Chen et al. [60].

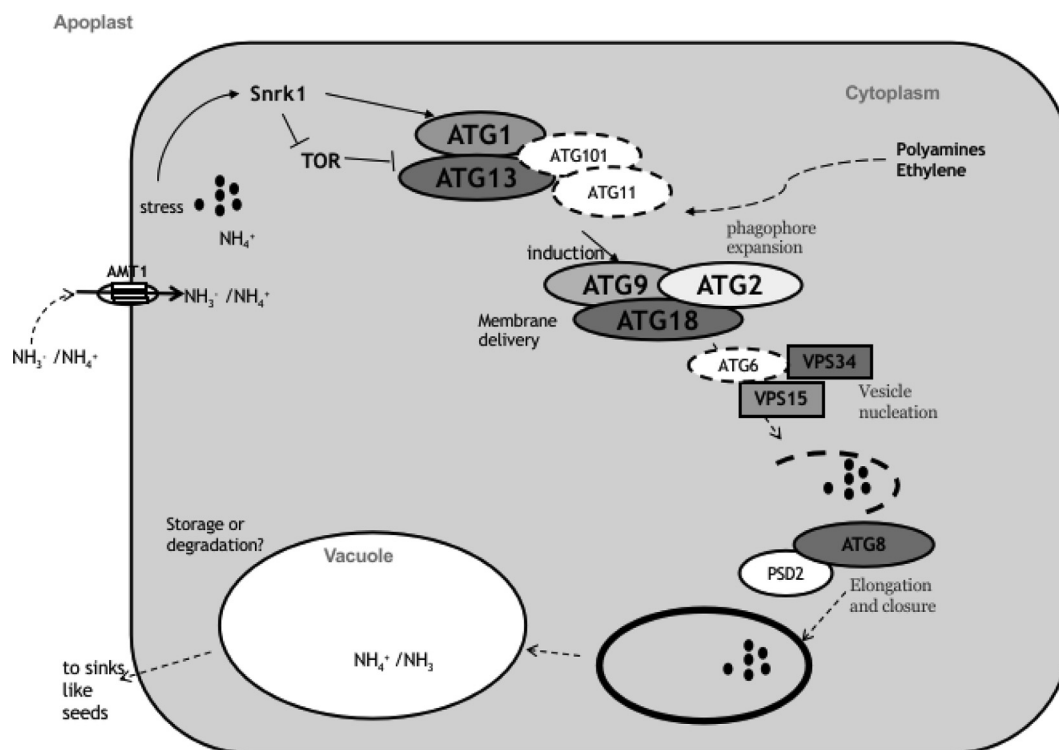
and PAO4, encoding polyamine oxidase (PAO), which is an enzyme with distinct physiological roles that is responsible for polyamine catabolism. In plants, increasing evidence suggests that PAO genes play essential roles in the abiotic and biotic stress response, as some PAOs catalyze the reverse reaction of PA synthesis via the PA back-conversion pathway [71]. Tavladoraki et al. [72] identified PAO1 in *Arabidopsis* as an enzyme that possesses back-conversion capacity, responsible for the conversion of Spm to Spd. Similarly, Moschou et al. [73] identified that, in *Arabidopsis*, PAO1 and PAO4 were able to convert Spm to Spd, and PAO2 and PAO3 catalyzed the production of Spd from Spm before producing Put. This result suggests the involvement of PAO genes in stress responses, and their probable implications for PA homeostasis in *S. neei*.

Considering the possible abiotic stress induced by ammonium-based nutrition, we looked for processes activated in the plant that help to avoid ammonium accumulation or to mitigate its effects. Thus, sets of 72 up-regulated and 19 down-regulated genes related to nitrogen metabolism were found to be modulated by  $\text{NH}_4^+$  at salinity concentrations close to 600 mM NaCl in the shoots. Among the main genes recognized in the metabolism of  $\text{NH}_4^+$  in plants, we propose a possible route based on several up-regulated genes, such as GS, GLT-1, 2-oxoglutarate dehydrogenase (2-OGD), ferredoxin-dependent glutamate synthase chloroplast (Fd-GOGAT), and glutamate synthase 1 (NADH) chloroplast isoform X1 (GLT1) (Table 5, Fig. 6 and Table S2). Similar results have been published concerning the halophyte plant *Salicornia europaea*, which showed high GS and GDH activity at high salinity concentrations [30]. Further, Ma et al. [30] noted that *Salicornia* plants fed with ammonia also require salinity of 200 mM NaCl or more in the substrate, conditions that can presumably stimulate the detoxification mechanisms generated by stress [74]. This could explain the ability of

halophyte plants to thrive in environments where salinity is high and ammonia is a common nutrient. It has previously been revealed that some wetland plants and other marine species that grow in terrestrial habitats where  $\text{NH}_4^+$  prevails over  $\text{NO}_3^-$  have a special preference for ammonia [75].

In plants, diverse abiotic stresses have been shown to induce autophagy, including nutrient starvation and oxidative stress, improving plant resistance [76]. The energy sensors SNF-related kinase 1 (SnRK1) and target of rapamycin (TOR) control autophagy not only under energy deficiency, but also under diverse stress conditions [77]. Signorelli et al. [77] have suggested that the accumulation of GABA, PA, ethylene and ABA under stress conditions can indirectly control autophagy as well, by different pathways.

Studies have theorized that additional cytosolic nutrients can be maintained by compartmentalizing them at sites where they are not metabolized, such as the vacuole [78,79]. This direct encapsulation of nitrogen is possible through specialized autophagic vesicles that subsequently fuse with the vacuole for proteolysis and hydrolysis [80]. Moreover, materials such as nutrients can be remobilized to new growing organs and sinks, such as seeds. In *Arabidopsis*, it was observed that autophagy can regulate the abiotic stress caused by the excessive uptake of toxic ions; researchers reported that the level of autophagy peaks within 30 min after salt stress, and then settles into a new homeostasis, but such an induction is absent in defective mutants in autophagy [81]. In this transcriptome, we found a series of autophagy-related genes (ATGs) that are responsible for the initiation and formation of autophagosomes, such as the following: 1) the ATG1/ATG13 kinase complex that initiates autophagosome formation; 2) ATG9 forms a complex with accessory proteins ATG2 and ATG18, which promotes phagophore expansion and has been described as the only integral complex necessary for the formation of the autophagosomal membrane



**Fig. 7.** Putative schematic diagram of the autophagy process in *Salicornia neei*. Energy sensors SNF-related kinase 1 (SnRK1), target of rapamycin (TOR), autophagy-related protein 1 (ATG1), autophagy-related protein 11 (ATG11), autophagy-related protein 101 (ATG101), autophagy-related protein 13 (ATG13), autophagy-related protein 9 (ATG9), autophagy-related protein 2 (ATG2), autophagy-related protein 18, vacuolar protein sorting 34 (VPS34), vacuolar protein sorting 15 (VPS15), autophagy-related protein 6 (ATG6), protein sorting 8 (ATG8), phosphatidyserine decarboxylase proenzyme 2 (PSD2) (2.0-fold). In dotted oval ATG not observed. Figure adapted from Chen et al. [80].

[82]; 3) a complex for vesicle nucleation; 4) the ATG8/PE conjugates, which are located in both the inner and the outer autophagosome membranes, aid phagophore expansion and vesicle closure to form autophagosomes, and recognize autophagic cargoes through ATG8-interacting proteins [83]. These genes related to autophagy are included in the autophagy pathway and others (Table 3). This vacuolar complex is probably induced to avoid apoplastic acidification, which generates stress and leaf senescence [28,84,85] (Fig. 7).

## 5. Conclusions

The results suggest that the ammonium detoxification system in *S. neei* is mediated by a wide variety of up-regulated genes that are associated with the maintenance of ammonium homeostasis through the activation of glutamine and glutamate synthetase, accompanied by the biosynthesis of the phytohormones and polyamines involved in the protection of important protein structures under stress conditions.

In *S. neei*, the vacuolar complex was probably created to avoid the apoplastic acidification induced by ammonium nutrition.

Cell wall biosynthesis may correspond to the stress response induced by excess ammonia, taking into account the fact that several studies addressing stress response in plants have reported the biosynthesis of cell wall structures.

The kinetics of ammonium uptake by *Salicornia neei* under ammonium nutrition have been characterized using the Michaelis–Menten equation. Our results support the hypothesis that *S. neei* is very effective in removing ammonium at concentrations <4 mM, compared to other species used in aquaculture wastewater [7,86]. The maximum rates ( $I_{max}$ ) and the half-saturation constants ( $K_m$ ) for ammonium uptake were identified, demonstrating that *S. neei* can be used to treat effluents with ammonium pollutants. *Salicornia neei* showed a high affinity for the substrate, which can be

used to decontaminate waters with high nutrient loads derived from marine aquaculture.

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## Conflict of interests

All authors declare that there are no present or potential conflicts of interest between the authors and other people or organizations that could inappropriately bias their work.

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## Data availability

In this repository you will find the data and codes to partially reproduce the research carried out on the manuscript. [https://github.com/GenomicsLaboratory/ReproducibleResearch/tree/main/Diaz\\_et al\\_2021](https://github.com/GenomicsLaboratory/ReproducibleResearch/tree/main/Diaz_et al_2021).

## Supplementary material

<https://doi.org/10.1016/j.ejbt.2022.05.003>.

## References

- [1] Diaz MR, Araneda J, Osses A, et al. Efficiency of *Salicornia neei* to treat aquaculture effluent from a hypersaline and artificial wetland. *Agriculture* 2020;10(12):621. <https://doi.org/10.3390/agriculture10120621>.
- [2] Alonso MA, Crespo MB. Taxonomic and nomenclatural notes on South American taxa of *Sarcocornia* (Chenopodiaceae). *Ann Bot Fenn* 2008;45(4):241–54. <https://doi.org/10.5735/085.045.0401>.
- [3] Riquelme J, Olaeta JA, Galvez L, et al. Nutritional and functional characterization of wild and cultivated *Sarcocornia neei* grown in Chile. *Cienc Inv Agr* 2016;43(2):283–93. <https://doi.org/10.4067/S0718-16202016000200011>.
- [4] Alonso MF, Orellana C, Valdes S, et al. Effect of salinity on the germination of *Sarcocornia neei* (Chenopodiaceae) from two contrasting habitats. *Seed Sci Technol* 2010;45(1):252–8. <https://doi.org/10.15258/sst.2017.45.1.20>.
- [5] Beyer C, Gómez S, Lara G, et al. *Sarcocornia neei*: A novel halophyte species for bioremediation of marine aquaculture wastewater and production diversification in integrated systems. *Aquaculture* 2021;543:737961. <https://doi.org/10.1016/j.aquaculture.2021.736971>.
- [6] de Souza MM, Mendes CR, Doncato KB, et al. Growth, phenolics, photosynthetic pigments, and antioxidant response of two new genotypes of sea asparagus (*Salicornia neei* Lag.) to salinity under greenhouse and field conditions. *Agriculture* 2018;8(7):115. <https://doi.org/10.3390/agriculture8070115>.
- [7] Quinta R, Santos R, Thomas DN, et al. Growth and nitrogen uptake by *Salicornia europaea* and *Aster tripolium* in nutrient conditions typical of aquaculture wastewater. *Chemosphere* 2015;120:414–21. <https://doi.org/10.1016/j.chemosphere.2014.08.017>. PMID: 25216470.
- [8] Turcios AE, Papenbrock J. Sustainable treatment of aquaculture effluents-what can we learn from the past for the future? *Sustainability* 2014;6(2):836–56. <https://doi.org/10.3390/su6020836>.
- [9] Islam MS. Nitrogen and phosphorus budget in coastal and marine cage aquaculture and impacts of effluent loading on ecosystem: review and analysis towards model development. *Mar Pollut Bull* 2015;50(1):48–61. <https://doi.org/10.1016/j.marpolbul.2004.08.008>. PMID: 15664033.
- [10] Maillard VM, Boardman GD, Nyland JE, et al. Water quality and sludge characterization at raceway-system trout farms. *Aquacult Eng* 2005;33(4):271–84. <https://doi.org/10.1016/j.aquaeng.2005.02.006>.
- [11] Bisogni J, Timmons M. Control of pH in closed cycle aquaculture systems. *Dev Aquacult Fish Sci* 1994;27:235–45.
- [12] Bower CE, Bidwell JP. Ionization of ammonia in seawater: effects of temperature, pH, and salinity. *J Fish Res Board Can* 1978;35(4):1012–6. <https://doi.org/10.1139/f78-165>.
- [13] Carbalreira C, De Orte MR, Viana IG, et al. Implementation of a minimal set of biological tests to assess the ecotoxic effects of effluents from land-based marine fish farms. *Ecotoxicol Environ Saf* 2012;78:148–61. <https://doi.org/10.1016/j.ecoenv.2011.11.022>. PMID: 22137361.
- [14] Playchoom C, Pungrasmi W, Powtongsook S. Effect of carbon sources and carbon/nitrogen ratio on nitrate removal in aquaculture denitrification tank. *International conference on biology, environment and chemistry, Singapore* 2011;1:307–311.
- [15] Shpigel M, Ben-Ezra D, Shauli L, et al. Constructed wetland with *Salicornia* as a biofilter for mariculture effluents. *Aquaculture* 2013;412–413:52–63. <https://doi.org/10.1016/j.aquaculture.2013.06.038>.
- [16] Brown JJ, Glenn EP, Fitzsimmons KM, et al. Halophytes for the treatment of saline aquaculture effluent. *Aquaculture* 1999;175(3–4):255–68. [https://doi.org/10.1016/S0044-8486\(99\)00084-8](https://doi.org/10.1016/S0044-8486(99)00084-8).
- [17] Cao L, Wang W, Yang Y, et al. Environmental impact of aquaculture and countermeasures to aquaculture pollution in China. *Environ Sci Pollut Res* 2007;14(7):452–62. <https://doi.org/10.1065/espr2007.05.426>. PMID: 18062476.
- [18] Cloern JE. Our evolving conceptual model of the coastal eutrophication problem. *Mar Ecol Prog* 2001;210:223–53. <https://doi.org/10.3354/meps210223>.
- [19] Holmer M, Wildish D, Hargrave B. Organic enrichment from marine finfish aquaculture and effects on sediment biogeochemical processes. In: Hargrave BT, editor. *Environmental effects of marine finfish aquaculture. Handbook of environmental chemistry*. Berlin, Heidelberg: Springer; 2005. p. 181–206. <https://doi.org/10.1007/b136010>.
- [20] Hu Z, Lee JW, Chandran K, et al. Effect of plant species on nitrogen recovery in aquaponics. *Bioresour Technol* 2015;188:92–8. <https://doi.org/10.1016/j.biortech.2015.01.013>. PMID: 25650140.
- [21] Meade JW. Allowable ammonia for fish culture. *Prog Fish-Cult* 1985;47(3):135–45.
- [22] Waller U, Buhmann AK, Ernst A, et al. Integrated multi-trophic aquaculture in a zero-exchange recirculation aquaculture system for marine fish and hydroponic halophyte production. *Aquacult Int* 2015;23(6):1473–89. <https://doi.org/10.1007/s10499-015-9898-3>.
- [23] Ravazzolo L, Trevisan S, Forestan C, et al. Nitrate and ammonium affect the overall maize response to nitrogen availability by triggering specific and common transcriptional signatures in roots. *Int J Mol Sci* 2020;21(2):686. <https://doi.org/10.3390/ijms21020686>. PMID: 31968691.
- [24] Coleto I, Bejarano I, Marín-Peña AJ, et al. *Arabidopsis thaliana* transcription factors *MYB28* and *MYB29* shape ammonium stress responses by regulating Fe homeostasis. *New Phytol* 2021;229(2):1021–35. <https://doi.org/10.1111/nph.16918>. PMID: 32901916.
- [25] Britto DT, Kronzucker HJ. NH<sub>4</sub><sup>+</sup> toxicity in higher plants: a critical review. *J Plant Physiol* 2002;159(6):567–84. <https://doi.org/10.1078/0176-1617-0774>.
- [26] Tang ZH, Liu YJ, Guo XR, et al. The combined effects of salinity and nitrogen forms on *Catharanthus roseus*: The role of internal ammonium and free amino acids during salt stress. *J Soil Sci Plant* 2011;174(1):135–44. <https://doi.org/10.1002/jipln.200900354>.
- [27] Cruz C, Bio AFM, Dominguez-Valdivia MD, et al. How does glutamine synthetase activity determine plant tolerance to ammonium? *Planta* 2006;223(5):1068–80. <https://doi.org/10.1007/s00425-005-0155-2>. PMID: 16292661.
- [28] Liu Y, von Wiren N. Ammonium as a signal for physiological and morphological responses in plants. *J Exp Bot* 2017;68(10):2581–92. <https://doi.org/10.1093/jxb/erx086>. PMID: 28369490.
- [29] Meng XQ, Zhou J, Sui N. Mechanisms of salt tolerance in halophytes: current understanding and recent advances. *Open Life Sci* 2018;13(1):149–54. <https://doi.org/10.1515/biol-2018-0020>. PMID: 33817080.
- [30] Ma JB, Cirillo V, Zhang DY, et al. Regulation of ammonium cellular levels is an important adaptive trait for the euhalophytic behavior of *Salicornia europaea*. *Plants* 2020;9(2):257. <https://doi.org/10.3390/plants9020257>. PMID: 32079337.
- [31] Ashraf M, Shahzad SM, Imtiaz M, et al. Salinity effects on nitrogen metabolism in plants - focusing on the activities of nitrogen metabolizing enzymes: A review. *J Plant Nutr* 2018;41(8):1065–81. <https://doi.org/10.1080/01904167.2018.1431670>.
- [32] Stewart GR, Rhodes D. Nitrogen-metabolism of halophytes III. Enzymes of ammonia assimilation. *New Phytol* 1978;80(2):307–16. <https://doi.org/10.1111/j.1469-8137.1978.tb01563.x>.
- [33] Gutiérrez RA, Stokes TL, Thum K, et al. Systems approach identifies an organic nitrogen-responsive gene network that is regulated by the master clock control gene *CCA1*. *PNAS* 2008;105(12):4939–44. <https://doi.org/10.1073/pnas.080021105>. PMID: 18344319.
- [34] Yokoiishi T, Tanimoto S. Seed-germination of the halophyte *Suaeda japonica* under salt stress. *J Plant Res* 1994;107(1088):385–8. <https://doi.org/10.1007/BF02344061>.
- [35] Kumari A, Das P, Parida AK, et al. Proteomics, metabolomics, and ionomics perspectives of salinity tolerance in halophytes. *Front Plant Sci* 2015;6:537. <https://doi.org/10.3389/fpls.2015.00537>.
- [36] Crosby NT. Determination of ammonia by the Nessler method in waters containing hydrazine. *Analyst* 1968;93(1107):406–8. <https://doi.org/10.1039/an9689300406>.
- [37] Adamowicz S, Le Bot J. Trends in modelling nitrate uptake. *Acta Horticult* 1999;507:231–40. <https://doi.org/10.17660/ActaHortic.1999.507.27>.
- [38] Chang S, Puryear J, Cairney JA. A simple and efficient method for isolating RNA from pine trees. *Plant Mol Biol Report* 1993;11:113–6. <https://doi.org/10.1007/BF02670468>.
- [39] Lal L, Sahoo R, Gupta R, et al. RNA isolation from high phenolic tea leaves and buds. *Plant Mol Biol Report* 2001;19(2):181. <https://doi.org/10.1007/BF02727161>.
- [40] Meisel L, Fonseca B, González S, et al. A rapid and efficient method for purifying high quality total RNA from peaches (*Prunus persica*) for functional genomics analyses. *Biol Res* 2005;38(1):83–8. <https://doi.org/10.4067/S0716-97602005000100010>. PMID: 15977413.
- [41] Camacho C, Coulouris G, Avagyan V, et al. BLAST+: architecture and applications. *BMC Bioinf* 2009;10:421. <https://doi.org/10.1186/1471-2105-10-421>. PMID: 20003500.
- [42] Conesa A, Götz S, García-Gómez JM, et al. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* 2005;21(18):3674–6. <https://doi.org/10.1093/bioinformatics/bti610>. PMID: 16081474.
- [43] Kal AJ, van Zonneveld AJ, Benes V, et al. Dynamics of gene expression revealed by comparison of serial analysis of gene expression transcript profiles from yeast grown on two different carbon sources. *Mol Biol Cell* 1999;10(6):1859–72. <https://doi.org/10.1091/mbc.10.6.1859>. PMID: 10359602.
- [44] Bolstad BM, Irizarry RA, Astrand M, et al. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* 2003;19(2):185–93. <https://doi.org/10.1093/bioinformatics/bt9.2.185>. PMID: 12538238.
- [45] Morales A, Zurita-Silva A, Maldonado J, et al. Transcriptional responses of chilean quinoa (*Chenopodium quinoa* Willd.) under water deficit conditions uncovers ABA-independent expression patterns. *Front Plant Sci* 2017;8:216. <https://doi.org/10.3389/fpls.2017.00216> PMID: 28337209.
- [46] Ramos-Ruiz R, Poirot E, Flores-Mosquera M. GABA, a non-protein amino acid ubiquitous in food matrices. *Cogent Food Agric* 2018;4(1):1534323. <https://doi.org/10.1080/23311932.2018.1534323>.
- [47] Hu JN, Liu JN, Fan JL, et al. Effect of salinity on the performance of constructed wetlands treating mariculture wastewater with different halophytes and its molecular biological mechanism. *Desalin Water Treat* 2017;99:255–65. <https://doi.org/10.5004/dwt.2017.21702>.
- [48] Wang L, Yao WJ, Sun Y, et al. Association of transcription factor *WRKY56* gene from *Populus simonii* x *P. nigra* with salt tolerance in *Arabidopsis thaliana*. *PeerJ* 2019;7:e7291. <https://doi.org/10.7717/peerj.7291>. PMID: 31328047.
- [49] Le Gall H, Philippe F, Domon J-M, et al. Cell wall metabolism in response to abiotic stress. *Plants* 2015;4(1):112–66. <https://doi.org/10.3390/plants4010112>. PMID: 27135320.



- [50] Kesten C, Menna A, Sánchez-Rodríguez C. Regulation of cellulose synthesis in response to stress. *Curr Opin in Plant Biol* 2017;40(1):106–13. <https://doi.org/10.1016/j.pbi.2017.08.010>. PMID: 28892802.
- [51] Tenhaken R. Cell wall remodeling under abiotic stress. *Front Plant Sci* 2015;5:771. <https://doi.org/10.3389/fpls.2014.00771>. PMID: 25709610.
- [52] Walia H, Wilson C, Condamine P, et al. Comparative transcriptional profiling of two contrasting rice genotypes under salinity stress during the vegetative growth stage. *Plant Physiol* 2005;139(2):822–35. <https://doi.org/10.1104/pp.105.065961>. PMID: 16183841.
- [53] Wang DL, Lu XK, Chen XG, et al. Temporal salt stress-induced transcriptome alterations and regulatory mechanisms revealed by PacBio long-reads RNA sequencing in *Gossypium hirsutum*. *BMC Genomics* 2020;21(1):838. <https://doi.org/10.1186/s12864-020-07260-z>. PMID: 33246403.
- [54] Jiao PP, Wu ZH, Wang X, et al. Short-term transcriptomic responses of *Populus euphratica* roots and leaves to drought stress. *J For Res* 2021;32:841–53. <https://doi.org/10.1007/s11676-020-01123-9>.
- [55] Li XX, Zhao J, Walk TC, et al. Characterization of soybean beta-expansin genes and their expression responses to symbiosis, nutrient deficiency, and hormone treatment. *Appl Microbiol Biotechnol* 2014;98(6):2805–17. <https://doi.org/10.1007/s00253-013-5240-z>. PMID: 24113821.
- [56] Sun L, Di DW, Li GJ, et al. Spatio-temporal dynamics in global rice gene expression (*Oryza sativa* L.) in response to high ammonium stress. *J Plant Physiol* 2017;212:94–104. <https://doi.org/10.1016/j.jplph.2017.02.006> PMID: 28282528.
- [57] Jan R, Asaf S, Numan M, et al. Plant secondary metabolite biosynthesis and transcriptional regulation in response to biotic and abiotic stress conditions. *Agronomy* 2021;11(5):968. <https://doi.org/10.3390/agronomy11050968>.
- [58] Zhao ZR, Kou MZ, Zhong R, et al. Transcriptome analysis revealed plant hormone biosynthesis and response pathway modification by *Epichloe gansuensis* in *Achnatherum inebrians* under different soil moisture availability. *J Fungi* 2021;7(8):640. <https://doi.org/10.3390/jof7080640>. PMID: 34436179.
- [59] Binder BM. Ethylene signaling in plants. *J Biol Chem* 2020;295(22):7710–25. <https://doi.org/10.1074/jbc.REV120.010854>. PMID: 32332098.
- [60] Chen K, Li GJ, Bressan RA, et al. Abscisic acid dynamics, signaling, and functions in plants. *J Integr Plant Biol* 2020;62(1):25–54. <https://doi.org/10.1111/jipb.12899> PMID: 31850654.
- [61] Ellouzi H, Hamed KB, Hernandez I, et al. A comparative study of the early osmotic, ionic, redox and hormonal signaling response in leaves and roots of two halophytes and a glycophyte to salinity. *Planta* 2014;240(6):1299–317. <https://doi.org/10.1007/s00425-014-2154-7>. PMID: 25156490.
- [62] Verma V, Ravindran P, Kumar PP. Plant hormone-mediated regulation of stress responses. *BMC Plant Biol* 2016;6:86. <https://doi.org/10.1186/s12870-016-0771-y> PMID: 27079791.
- [63] Li ZF, Zhang LX, Yu YW, et al. The ethylene response factor AtERF11 that is transcriptionally modulated by the bZIP transcription factor HY5 is a crucial repressor for ethylene biosynthesis in *Arabidopsis*. *Plant J* 2011;68(1):88–99. <https://doi.org/10.1111/j.1365-3113.2011.04670.x>. PMID: 21645149.
- [64] Madhavan S, Chrominski A, Smith BN. Effect of ethylene on stomatal opening in tomato and carnation leaves. *Plant Cell Physiol* 1983;24(3):569–72. <https://doi.org/10.1093/oxfordjournals.pcp.a076550>.
- [65] Polko JK, Kieber JJ. 1-Aminocyclopropane 1-carboxylic acid and its emerging role as an ethylene-independent growth regulator. *Front Plant Sci* 2019;10:1602. <https://doi.org/10.3389/fpls.2019.01602>. PMID: 31921251.
- [66] Zapata PJ, Serrano M, Garcia-Legaz MF, et al. Short term effect of salt shock on ethylene and polyamines depends on plant salt sensitivity. *Front Plant Sci* 2017;8:855. <https://doi.org/10.3389/fpls.2017.00855>. PMID: 28588603.
- [67] Pandey S, Ranade SA, Nagar PK, et al. Role of polyamines and ethylene as modulators of plant senescence. *J Biosci* 2000;25(3):291–9. <https://doi.org/10.1007/BF02703938>. PMID: 11022232.
- [68] Gupta K, Dey A, Gupta B. Plant polyamines in abiotic stress responses. *Acta Physiol Plant* 2013;35(7):2015–36. <https://doi.org/10.1007/s11738-013-1239-4>.
- [69] Kumar N, Mallick S. Ameliorative Mechanisms of polyamines against abiotic stress in the rice plants. In: Hasanuzzaman M, Fujita M, Nahar K, et al. (eds) *Advances in rice research for abiotic stress tolerance*, Woodhead Publishing, 2019;35:725–735. <https://doi.org/10.1016/B978-0-12-814332-2.00035-6> PMID: PMC6678468.
- [70] Bouchereau A, Aziz A, Larher F, et al. Polyamines and environmental challenges: recent development. *Plant Sci* 1999;140(2):103–25. [https://doi.org/10.1016/S0168-9452\(98\)00218-0](https://doi.org/10.1016/S0168-9452(98)00218-0).
- [71] Liu Q, Nishibori N, Imai I, et al. Response of polyamine pools in marine phytoplankton to nutrient limitation and variation in temperature and salinity. *Mar Ecol Prog Ser* 2016;544:93–105. <https://doi.org/10.3354/meps11583>.
- [72] Tavladoraki P, Rossi MN, Sacchi G, et al. Heterologous expression and biochemical characterization of a polyamine oxidase from *Arabidopsis* involved in polyamine back conversion. *Plant Physiol* 2006;141(4):1519–32. <https://doi.org/10.1104/pp.106.080911>. PMID: 16778015.
- [73] Moschou PN, Pascalidis KA, Roubelakis-Angelakis KA. Plant polyamine catabolism: The state of the art. *Plant Signal Behav* 2008;3(12):1061–6. <https://doi.org/10.4161/psb.3.12.7172>. PMID: 19513239.
- [74] Flowers TJ, Colmer TD. Plant salt tolerance: adaptations in halophytes. *Annals Bot* 2015;115(3):327–31. <https://doi.org/10.1093/aob/mcu267>. PMID: 25844430.
- [75] Thursby GB, Harlin MM. Interaction of leaves and roots of *Ruppia maritima* in the uptake of phosphate, ammonia and nitrate. *Mar Biol* 1984;83(1):61–7. <https://doi.org/10.1007/BF00393086>.
- [76] Liu YM, Xiong Y, Bassham DC. Autophagy is required for tolerance of drought and salt stress in plants. *Autophagy* 2009;5(7):954–63. <https://doi.org/10.4161/auto.5.7.9290>. PMID: 19587533.
- [77] Signorelli S, Tarkowski LP, Van den Ende, et al. Linking autophagy to abiotic and biotic stress responses. *Trends Plant Sci* 2019;24(5):413–430. <https://doi.org/10.1016/j.tplants.2019.02.001> PMID: 30824355.
- [78] Howitt SM, Udvardi MK. Structure, function and regulation of ammonium transporters in plants. *Biochim Biophys Acta Biomembr* 2000;1465(1–2):152–70. [https://doi.org/10.1016/S0005-2736\(00\)00136-X](https://doi.org/10.1016/S0005-2736(00)00136-X).
- [79] Martinoia E, Maeshima M, Neuhaus HE. Vacuolar transporters and their essential role in plant metabolism. *J Exp Bot* 2007;58(1):83–102. <https://doi.org/10.1093/jxb/erl183>. PMID: 17110589.
- [80] Chen QW, Shinozaki D, Luo J, et al. Autophagy and nutrients management in plants. *Cells* 2019;8(11):1426. <https://doi.org/10.3390/cells8111426>. PMID: 31726766.
- [81] Luo LM, Zhang PP, Zhu RH, et al. Autophagy is rapidly induced by salt stress and is required for salt tolerance in *Arabidopsis*. *Front Plant Sci* 2017;8:1559. <https://doi.org/10.3389/fpls.2017.01459>. PMID: 28878796.
- [82] He CC, Klionsky DJ. Atg9 trafficking in autophagy-related pathways. *Autophagy* 2007;3(3):271–4. <https://doi.org/10.4161/auto.3912>. PMID: 17329962.
- [83] Johansen T, Lamark T. Selective autophagy mediated by autophagic adapter proteins. *Autophagy* 2011;7(3):279–96. <https://doi.org/10.4161/auto.7.3.14487>. PMID: 21189453.
- [84] Marino D, Moran JF. Can ammonium stress be positive for plant performance? *Front Plant Sci* 2019;10:1103. <https://doi.org/10.3389/fpls.2019.01103>. PMID: 31608080.
- [85] Patterson K, Cakmak T, Cooper A, et al. Distinct signalling pathways and transcriptome response signatures differentiate ammonium- and nitrate-supplied plants. *Plant Cell Environ* 2010;33(9):1486–501. <https://doi.org/10.1111/j.1365-3040.2010.02158.x>. PMID: 20444219.
- [86] Yao F, Sun J, Tang C, et al. Kinetics of ammonium, nitrate and phosphate uptake by candidate plants used in constructed wetlands. *Procedia Environ Sci* 2011;10(part B):1854–61. <https://doi.org/10.1016/j.proenv.2011.09.290>.