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Ion Homeostasis and Stress-Related Gene Activity in *Lemna minor* During Salt Stress

Ahmed Y. Lafta Hzaa[1], **Najim M. Aziz** [2] **and Ahmed Zaer Resan**[3]

[1,2]Department of Marine Biology, Marine Science Centre, University of Basrah, Basrah, Iraq.
[3]Department of Horticulture and Landscape Design, College of Agriculture, University of Basrah, Basrah, Iraq.

[1]E-mail: ahmed.lafta@uobasrah.edu.iq

Abstract. Salinity stress greatly affects the ion balance and alters the expression of important stress-related genes in Lemna minor, a model aquatic plant. Exposure to higher NaCl concentrations (50–200 mM) resulted in a significant increase in Na^+, reaching 465.4% at a concentration of 200 mM, compared to the control sample. In contrast, K^+ levels peaked at 50 mM (5.176 mg/g DW) and then sharply decreased at higher salinities, showing an initial compensatory response that faileds with prolonged stress. As a result, the Na^+/K^+ ratio increased significantly from 0.24 in the control to 1.73 at 200 mM indicating a serious ionic imbalance. Gene expression analysis showed clear patterns: the NHX gene, which is responsible for vacuolar Na^+ storage, was upregulated 256 times at 200 mM NaCl, indicating activation of compartmentalization under high salinity. In contrast, the K^+ transporter (K Trans) gene was progressively downregulated (as low as 7% of the control), suggesting that Na^+ competition hinders K^+ uptake. The aquaporin gene TIP4 showed peak expression (22.4-fold) at 100 mM NaCl, emphasizing its role in regulating water transport during moderate stress. The transcription factor MYB was significantly induced at 100 mM (16-fold) but suppressed at 200 mM (0.03-fold), indicating its involvement in early stress signaling that decreases under severe conditions. These results show that Lemna minor uses a complex molecular and physiological response to salinity, involving ion regulation, osmotic adjustment, and transcriptional control, with critical thresholds influencing adaptive success.

Keywords. Salt Stress, Na^+/K^+ ratio, Gene expression, *Lemna minor*.

1. Introduction

Environmental stress plays a pivotal role in applied sciences by, limiting the growth potential of crops and plants. Globally, plants face various forms of abiotic stress because of harsh environmental conditions, with salinity being the most common. [1]. Salt stress causes metabolic and physiological problems in plants, affecting growth, productivity, development, and quality. This is particularly true in arid and semi-arid regions, where inadequate irrigation and seawater intrusion exacerbate the problem. [2].

Aquatic plants have diverse potential, for example, as nutritional components for the development of new functional foods and food products, are viewed as a potential food source, *Lemna minor* L. is a

useful model organism for ecological and physiological studies because of their rapid growth, small genome, ease of cultivation, and sensitivity to environmental stress. [3;4].

Its capacity to flourish in nutrient-rich freshwater makes it especially susceptible to salinity changes caused by human activities such as agricultural runoff and coastal aquaculture [5]. Although it is ecologically and biotechnologically important, the molecular and physiological processes that explain L. minor's reaction to salinity stress are still not well understood. The build-up of soluble salts, mainly NaCl, disrupts ion balance, causes osmotic stress, and results in oxidative damage, which ultimately hinders metabolic functions and decreases biomass accumulation [6]. Maintaining the cellular ion balance, especially the cytosolic Na^+/K^+ ratio, is crucial for plant salinity tolerance, as high Na^+ levels can affect K-dependent enzyme activities and membrane potential [7].

Maintaining a low cytosolic Na^+/K^+ ratio is a key feature of salt-tolerant plants. This is done through the coordinated regulation of ion transporters like SOS1 (Salt Overly Sensitive 1), Na^+/H^+ exchangers (NHX), and (High-Affinity K^+ transporters (HKT) [8]. Plants utilize a complex array of ion transporters to maintain a delicate balance between sodium and potassium ions within the cell, an essential balance which determines the efficiency of growth and continued development. For example, in Arabidopsis the protein SOS1 acts as a sodium /hydrogens transporter at the plasma membrane, expelling sodium ions from root cells and, preventing their harmful accumulation in the cytoplasm. In parallel, NHX proteins and sodium/hydrogen transporters that trap sodium within vacuoles, limiting their toxic effects on cellular components. This integration of external expulsion and internal isolation constitutes a tight defense mechanism, enabling the plant to adapt to saline environments and reduce ionic pressure which may hinder its vital functions. [9;10;11].

Despite several recent studies on aquatic plants demonstrating that similar genes are involved in ion balance, their precise functions when exposed to salt stress in Lemna minor are still unknown. [12].

At the genetic level, plants respond to environmental challenges by modifying their gene expression to produce proteins and substances that aid them in adapting to environmental conditions. Plants can develop mechanisms to resist or tolerate these conditions through gene expression, which is a vital response that enables them to produce protective proteins or accumulate beneficial molecules. Differentiation in gene expression patterns is crucial for the growth of plant phenotypes. [13].

Different salinity levels led to the analysis of gene expression on several duckweed strains in a study conducted by [14]. Under salt stress conditions, the Qassim strain exhibited a significant increase in the expression of NHX, BZIP, ST, and KTrans genes . This highlights the importance of these genes in helping this strain cope with salt stress.

The fundamentals of salinity tolerance in aquatic plants are paramount. To clarify this, it is necessary to understand the mechanisms that regulate ion balance and gene expression efficiency in lentil plants grown under varying salinity levels, and to highlight the ability of freshwater ecosystems to adapt to the challenges of increasing salinity [15]. This highlights the importance of using lentils in phytoremediation efforts and in sustainable biomass production under salt stress.

Our study aimeds to reveal the effect of salt stress on the growth of *Limna minor*, by measuring the ionic balance and the ratio between Na^+/K^+, and evaluate the efficiency of gene expression in detecting genes associated with stress. Thise study provides important information about the range of salt concentrations at which plants can survive under climate change conditions, and identifies some molecular pathways associated with ion regulation and stress adaptation. These findings contribute to opening new research avenues for understanding the mechanisms of physiological adaptation in plants, enabling the exploration of their defense strategies in the face of salt stress and helping to develop sustainable environmental management plans.

2. Materials and Methods

Fresh plant biomass was collected from the local freshwater bodies. Is located in the Abu Al-Khaseeb region of Basrah, Iraq. The experiment was conducted in the Aquatic Plants Laboratory within the Marine Biology Department at the Marine Science Center of the University of Basrah, in March 2024. The plants were rinsed with water, cultivated, and allowed to reproduce at a temperature of 25°C under 12 h of light each day.

Duckweed plants with a weight of 10 g/L^{-1} were moved into 5-liter plastic containers that had various levels of salt (control, 50, 100, 125, 150, 175 and 200 mM) NaCl, with three replicates for each treatment. After two weeks, the leaves of the plants were gathered, and data were recorded based on the following criteria, following the method described below for each criterion:
Method for Digesting Plant Tissues to Measure sodium and potassium ions.[16].
One gram of plant tissue powder was weighed and placed into 25 mL Pyrex tubes. The mouths of the tubes were then covered with glass stoppers. To each sample, 3 mL of nitric acid (HNO_3) and hydrochloric acid (HCl) were added. The tubes were gently shaken, sealed again with glass stoppers, and left overnight (for approximately 24 h) under vacuum. The tubes were then placed in a water bath for approximately an hour to accelerate digestion. After removal from the water bath, 2–3 mL of distilled water (D.W.) was added to each tube. The tubes were then placed on a hot plate at approximately 70°C until the volume was reduced to approximately 2 mL .Finally, the solution was diluted with distilled water to the desired volume for analysis. Sodium (Na+) and potassium (K+) contents were performed using an iCAP™ PRO ICP-OES device from Thermo Fisher Scientific.

2.1. RNA Extraction and cDNA Production
Fifty milligrams of each sample from the seven treatments were measured, quickly frozen in liquid nitrogen, and stored at -80°C. The GENEzol™ TriRNA Pure Kit was uesd to extract total RNA following the manufacturer's guidelines. RNA concentration was assessed using a Quantus™ Fluorometer E6150, and RNA quality was verified on a 1.3% agarose gel. The RNA was subsequently stored at -80°C for future use.
The Syntol OT-1 Reverse Transcription Reagent Kit was used to conert RNA into cDNA. 2 µL of was mixed with other components, such as random primers and oligonucleotides, heated to 65°C for 5 min, and then cooled on ice. Next, reverse transcriptase was added to convert the RNA into cDNA at 42°C for 1 h. The resulting cDNA was then diluted with sterile water and stored at -20°C.
To study gene expression in *Lemna minor*. Five genes associated with salt stress are listed in Table 1.

Table 1. Salt stress-related genes used in this study.

Gene Name	Sequence 5'–3'	Description	Reference
NHX2-F NHX2-R	ATGCAGGGTTCCAGGTCAAG AGACCGAATCTGTAGCGGC	Na^+/H^+ antiporter	JZ905382
K^+ TRANSP-F K^+ TRANSP-R	CAAGAAGGACACGAGAGGG GACGTGCTTGACGTACATGG	Potassium transporter	JZ981156
TIP4-F TIP4-R	GGAACTGGACGGATCACTGG GGCGAACGAAGACTTCAACG	Aquaporin TIP4-1	JZ546376
MYB306-F MYB306-R	ACCGAGCAGGAGGAGAAGATC TGCTTGGCCATCTCTATGTC	Myb-related protein	JZ982222
ACTIN-F ACTIN-R	GGCTACTCCTTCACCACCAC GCTCGTAGGTCTTCTCGACG	Beta-actin	-

2.2. Real-Time Polymerase Chain Reaction (qPCR)
To measure the gene expression levels of selected genes, a reaction (qPCR) was performed, based on a previously described method [17]. Each amplification reaction was prepared in a total volume of 20 µl, consisting of 2 µl of complementary DNA template (cDNA), 1 µl of each gene starter, 10 µl of the main mixture with QIAGEN's SYBR Green dye, and 6 µl of deionized distilled water. A CHAI Open QPCR (USA) device was used to perform the reactions and three replicates were used for each sample, according to the company manufacturer's protocol.
The reaction was carried out according to the following program: first activation for 5 min at 50 °C, after which the initial decomposition phase began at 95 °C for 3 min. Then 50 amplification cycles consisting of: decomposition at 95 °C for 30 seconds، primer ligation at 55-58 °C for 30 seconds, and chain extension at 72 °C for 30 seconds. Gene expression analysis of the target genes was performed on plant samples exposed to a saline solution for a duration of 72 h. The Actin gene was used as an internal reference to normalize the gene expression data. Relative changes in transcript levels were

calculated using the 2^(-ΔΔCT) method, which allows for the relative quantitative estimation of gene expression compared to the control sample and the reference gene. [18].

2.3. Statistical Analysis

Data from each experiment are shown as mean ± standard error of the mean (SEM) based on three replicates. Statistical analyses were conducted using Genestat version 19 with a one-way analysis of variance (ANOVA), using the LSD test at a probability level of 0.05. [19].

3. Results and Discussion

3.1. The Content of Na+, K+ and the Na+/K+ Ratio

The data in Figure 1 indicate that higher salinity levels lead to a significant increase in sodium (Na^+) absorption in the leaves of duckweed plants. Even at 50 mM, the sodium level was over 3.4 times the baseline. The peak increase occurred at 200 mM, where the sodium concentration reached 465.4% of the baseline, or approximately 4.65 times. There was a minor drop in the concentration at 125 mM compared to 100 mM (from 404.5% to 359.5%), but it remained well above the baseline. The values then steadily increase with increasing salinity, showing that the plant's sodium uptake response intensifieds as salt stress increaseds. In contrast, the 50 mM treatment showed the highest K^+ level at 5.1764 mg/g, which was a 66.1% increase compared to the control. This change is abrupt and is seen as an unexpected reaction, possibly caused by a temporary boost in potassium movement to the leaves as an initial response to stress. As the salinity increase from 100 mM and higher, there was a sharp and ongoing drop in potassium levels. At 175 mM, it fell to 62.9% of the original value. A slight increase was observed at 200 mM (65.6%), but remained significantly lower than that of the control treatment.
Regarding the Na^+/K^+ ratio, the control treatment showed a ratio of 0.2439, indicating that potassium was more then four times higher than sodium, which demonstrates a natural ionic balance crucial for plant health. However, as salinity increased from 50 to 200 mM, there was a significant rise starting from 50 mM (207.7%), ultimately reaching 709.7% at 200 mM, where sodium was more than seven times higher than potassium.

Recently, duckweed (Lemna minor) has garnered increased attention owing to its potential as a source of animal feed and bioenergy, its ability to remediate plant pollution, and its diversity [20;21]. However, salt stress is an environmental factor that negatively affects the growth and development of duckweed, as salts accumulate in their cells to maintain ionic balance and osmotic pressures, leading to the accumulation of toxic substances, increased oxidative stress, and damage to cell membranes. These negative effects lead to decreased growth and photosynthetic efficiency [22 ;23]. The data in Figure 1 revealed that salt stress significantly disrupteds the ionic balance within the tissues of *Lemna minor*. This disorder manifests itself in a high Na+ concentration versus a sharp decrease in K+ levels, which leads to a high Na+/K+ ratio. These transformations constitute extremely important biophysiological indicators for assessing the response of plants to saline conditions [24].
High salinity concentrations led to a significant increase in sodium ion accumulation in duckweed leaves, with the 200 mM treatment recording a 4.65-fold increase compared to the control treatment. This increased accumulation indicates that there may be an increase in sodium ion absorption through sodium-specific channels or transporters during salt stress, a trait observed in many plants under salt stress conditions [25,26].

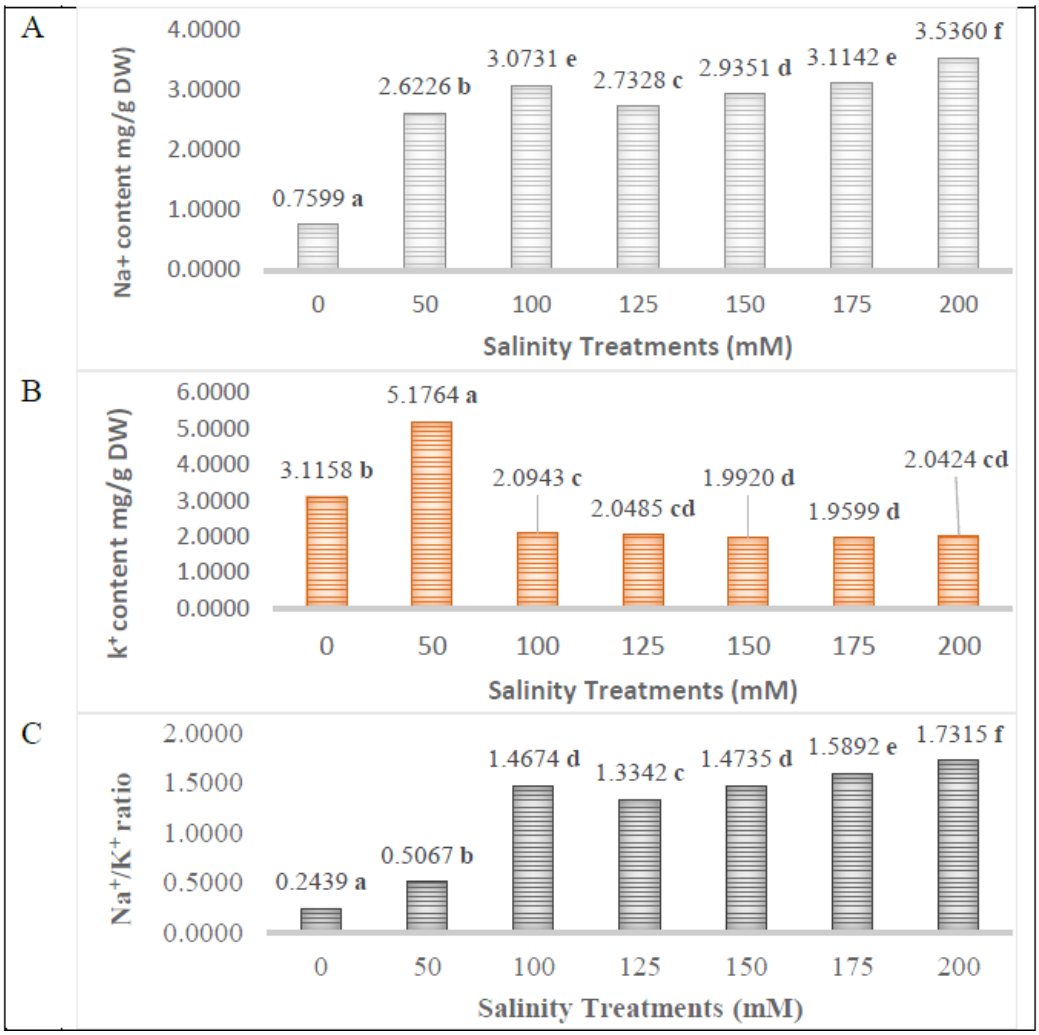

Figure 1. The content of Na+ (A) and K+ ions (B), as well as the Na+/K+ ratio (C), in duckweed exposed to various salinity levels. The values are presented as means ± SD from three replicates for each concentration. Different letters signify statistically significant differences at the 5% level.

The saturation of plant tissues with sodium ions may lead to instability of the cell membrane, which negatively affects the efficiency of photosynthesis and the function of enzymes, therefore, the tissue deteriorates under salt stress. [27]. However, it was observed that potassium ions increased when treated at a concentration of 50 mM, reaching 66% compared to the control treatment. This increase may be an initial response to mitigate the effect of salt shock, after which a gradual decrease in potassium levels was observed, reaching a concentration of 100 mM, recording the lowest value at a concentration of 175 mM, by less than a third of its original levels. This decrease in potassium levels may be due to a decrease in the ability of cells to selectively absorb potassium, which competes with sodium. This may be due to a decrease in the efficiency of the enzyme systems responsible for regulating the ionic balance, as the accumulation of sodium disrupts the activity of some membrane transporters, which weakens the ability of the plant to maintain its metabolic stability under salinity conditions. In addition, increased salt stress increases the loss of potassium from plant cells through transpiration channels, and thus, its concentration in the cells decreases, which negatively affects the physiological functions of the plant. [28,29].

The results revealed that the efficiency of K^+ regulation within Lemna minor plant tissues is negatively affected when salinity levels rise, and this weakens the plant's ability to maintain stable potassium levels, a common feature observed in many salt-sensitive plant species. [30]. One of the most

important basic parameters, that is a sensitive indicator for assessing the physiological response of plants under salinity pressure, is the Na+ / K+ ratio. In this study, a significant increase in this ratio was observed from 0.24 in the control treatment to 1.73 in the treatment exposed to a concentration of 200 mM, an increase of more than sevenfold compared to the control. This ionic shift reflects a fundamental imbalance in the Ionic homeostasis mechanism, since excess Na+ interferes with the transfer of K+ through ion channels and cocarriers, impairing the ability to maintain the cellular membrane voltage necessary for vital cell functions. Therefore, such an increase indicates a breakdown in the functional ionic balance which is a prerequisite for survival under conditions of increased salinity. [31,32].

Several studies have demonstrated that a plant's ability to maintain a low Na^+/K^+ ratio is a critical factor in salt stress tolerance, as a high ratio is directly associated with reduced photosynthetic efficiency, root growth, and biomass production. Thus, the ability to maintain a Na^+/K^+ ratio of less than 0.5 is classified as a reliable physiological indicator for determining salinity tolerance in different agricultural species.[33].

3.2. Gene Expression of Four Selected Stress-Related genes in Salt-Tolerant Duckweed

The expression levels of four genes associated with salt stress (NHX, K Trans, TIP, and MYB) were monitored during the vegetative growth phase under salt stress for 72 h (Fig. 2). Most stress-related genes showed higher expression levels than the control group, suggesting that these genes play a role in the response plants to salt.

Analysis of gene expression of the Na^+/H^+ antiporter (NHX) gene in duckweed at various salt concentrations (0–200 mM NaCl) showed distinct differences in expression. The results indicated that low to medium salt treatments (50–150 mM) did not significantly increase gene expression; in fact, the expression levels were similar to or even lower than of the control (NHX-1). This suggesteds that the cells did not need to activate the vacuolar sodium pumping mechanism at these concentrations. The observed decrease or stability in expression could be due to the salt levels not being high enough to cause severe ionic stress, which would require a strong molecular response. In contrast, treatments with 175 and 200 mM, NaCl resulted in a notable increase in gene expression, achieving 63-fold and 256-fold increases respectively, compared to the control. This indicateds a robust defensive response from plants under high-salinity conditions. Such a significant increase in expression signifies a key adaptive strategy that helps sequester sodium ions in cell vacuoles (compartmentalization), thereby minimizing their harmful effects in the cytoplasm and preserving the ionic balance (Na^+/K^+) essential for crucial functions.

Gene expression analysis of the potassium transporter gene (K^+ transporter) in duckweed showed a significant response to salt stress caused by NaCl. When compared to unstressed conditions (0 mM NaCl), gene expression steadily decreased as the salt concentration increased. At 50 mM, the expression was 57.6% of the control level, and at 100 mM and 125 mM, it decreased to 41.4% and 43.3%, respectively. Beyond 125 mM, the decrease became more rapid, with expression levels dropping to 21.4% at 150 mM, 10.8% at 175 mM, and 7.0% at 200 mM after 72 h of exposure. This gradual yet sharp decline in gene expression highlights the significant negative impact of salinity on the regulation of K transport genes in this aquatic plant.

The expression level of TIP4- (aquaporin) was measured. The findings indicated that gene expression increased significantly as the NaCl concentration rose, peaking at 100 mM NaCl (22.4-fold) compared to the control. Expression also increased at 50 mM (3.96-fold), was lower at 125 mM (11.4-fold), and then gradually increase to approximately 15.0–14.5-fold at 175–200 mM. These findings show that TIP4-1 reacts nonlinearly to salt stress, with the highest expression at 100 mM, followed by a stable but weaker response at higher concentrations.

These findings indicated that the expression of MYB genes was much higher at concentrations of 50, 100, and 125 mM compared to the control, with relative expression values of approximately 3.2, 16.0, and 12.0, respectively. The results showed that these genes were activated at low to moderate salt levels. However, at higher salt concentrations, gene expression decreased significantly, with values of 0.55, 0.12, and 0.03 recorded at 150, 175, and 200 mM, respectively. This indicates that gene expression was inhibited under severe stress.

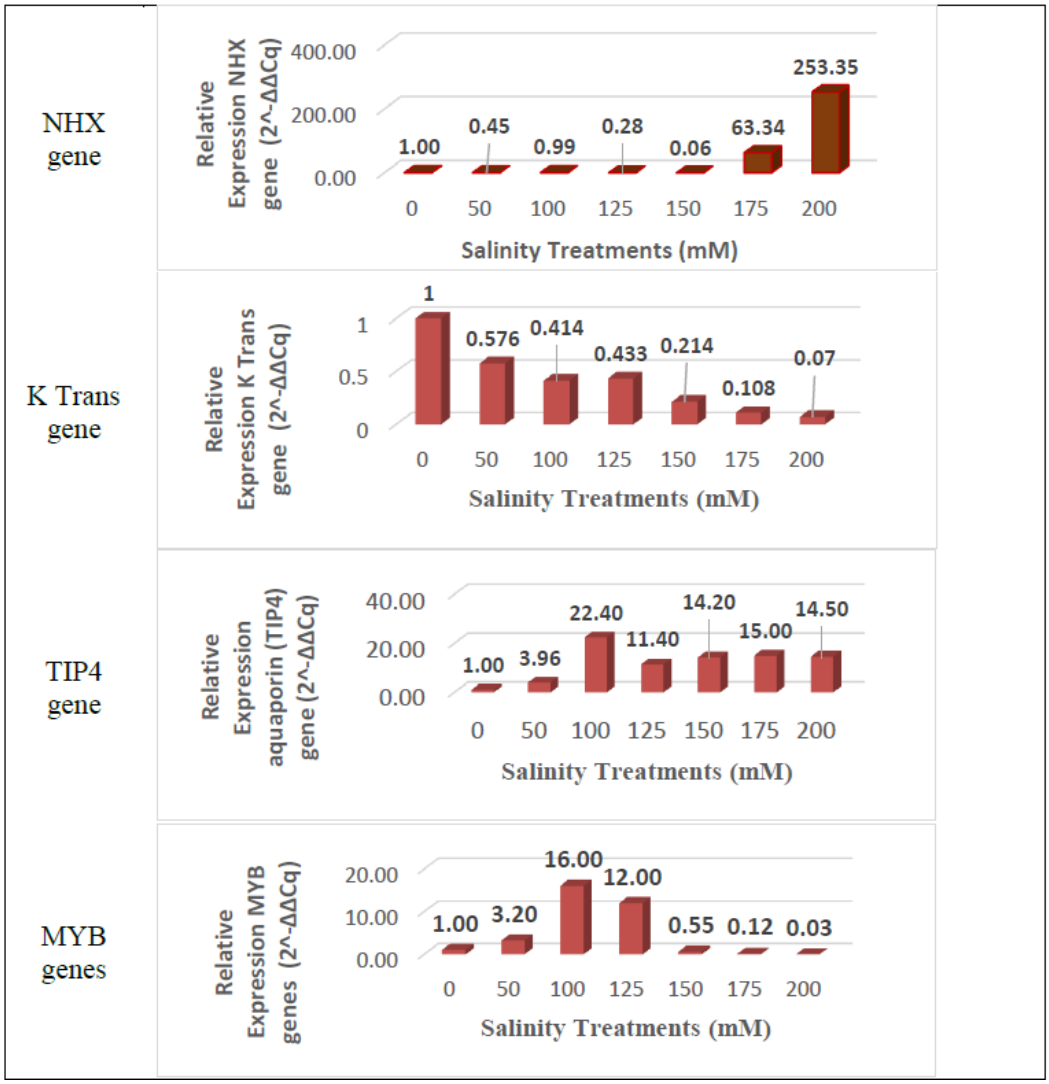

Figure 2. The relative expression of four genes related to salt stress in duckweed across seven different salt concentrations.

In summary, gene expression analysis showed that NHX plays a key role in the defense against sodium, especially at high concentrations. Meanwhile, TIP4 aids in water adaptation, peaking at 100 mM. MYB acts as a driver for the responses of other genes, but its activity ceases at 200 mM. The K Trans gene exhibiteds a steady decrease, indicating competitive effects between Na^+ and K^+.

In *Lemna minor*, analysis of gene expression under salinity stress revealed distinct activation patterns of NHX1, K-trans, TIP4, and MYB genes, indicating the effectiveness of the plant in using different strategies to cope with salinity stress.

Concerning the NHX (Na^+/H^+ antiporter) gene, expression levels did not show significant changes at low and moderate NaCl concentrations (50–150 mM), but increased dramatically at high concentrations (175–200 mM) by 63- and 256-fold respectively, compared to the control. This indicates that activation of the Na^+ pumping mechanism into the vacuole is a later strategy that occurs when the ionic stress threshold is surpassed, aiming to decrease cytoplasmic Na^+ buildup and maintain the ionic balance (Na^+/K^+) essential for biological functions.[34;35].

The potassium transporter (K-trans) gene showed a steady decline as the NaCl concentration increased, from 57.6% at 50 mM to 7% at 200 mM. This trend illustrates the dual impact of salinity: Na^+ competes with K^+ for uptake and transport sites, and the gene is downregulated during acute

stress, which hinders the ability ot the plant to maination a stable K^+/Na^+ ratio and worsens metabolic damage [35]. This finding aligns with detailed studies of ion transport-related genes, confirming that the regulation of K^+ transporters is a key indicator of salt sensitivity and tolerance. [36].
The TIP4-1 (aquaporin) gene exhibited a nonlinear response; its expression rose sharply at 100 mM (22.4-fold), decreased at 125 mM, and then stabilized at intermediate levels (approximately 14–15-fold) at 175–200 mM. This indicates that aquaporins play a role in restoring cellular water balance during osmotic stress. Their activity increases with moderate salinity to enhance the water flow [37]. But at higher concentrations, other factors (like rapid gating through phosphorylation/channel closure or protein degradation due to ionic stress) can affect and alter phenotypic expression or channel activity. This accounts for the peak at 100 Mm, followed by a stable yet lower response at 175–200 mM. Gating and vesicular traffic control mechanisms may clarify the speed and extent of changes in water permeability [38;39].
MYB expression was significantly increased in at low and intermediate concentrations (3.2–16-fold at 50–100 mM), suggesting that they act as early regulators that trigger defense responses, such as regulating gene expression and antioxidant pathways, however, their levels dropped sharply at high concentrations (0.55 at 150 mM and down to 0.03 at 200 mM), indicating transcriptional inhibition or even cell collapse, which leads to reduced expression, thus, MYB may play a catalytic role at levels that allow cells to respond, but it is inhibited under severe stress[35;40].
Overall, these findings show a two-level defense strategy in *Lemna minor*. Early activation of regulatory genes (MYB and TIP) helps manage moderate salinity, whereas high levels of NHX are triggered at high salinity as a last resort to maintain ionic balance, and a slow decrease in K-trans indicates sensitivity to salt stress.

Conclusion

This study highlights the relationship between ion regulation and gene expression in *Lemna minor*. This shows how different concentrations lead to molecular strategies that determine salt tolerance, emphasizing its potential in phytoremediation and stress biology studies.

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