Genomic and Functional Characterization of Vacuolar NHX (Na+/H+) Transporter Genes in *Zea mays* Cultivars under Saline Stress Across Tissues

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ABSTRACT: The vacuolar Na^+/H^+ exchanger (NHX) family is essential for maintaining ion homeostasis and pH regulation in *Zea mays*, particularly under salt stress conditions. This study investigated the expression dynamics of NHX genes in maize leaves and roots across 12 time points (0–168 hours) using publicly available transcriptomic datasets. Among the family members, four genes—*ZmNHX2*, *ZmNHX5*, *ZmNHX6*, and *ZmNHX13*—exhibited notable expression levels (RPKM > 1). Overall, expression was higher in leaves compared to roots. In leaves, significant upregulation was observed at 48, 72, 120, and 168 hours, whereas in roots, increased expression was limited to 120 and 168 hours. These findings indicate that these NHX genes may play a crucial role in alleviating sodium toxicity and enhancing salt stress tolerance in maize. These findings underscore the importance of considering temporal dynamics in genetic manipulation strategies to optimize salt tolerance across different developmental phases of the plant.

KEYWORDS: Salt tolerance, Na^+/H^+ exchange, maize.

Caracterização genômica e funcional de genes de transportadores vacuolares NHX (Na+/H+) em cultivares de *Zea mays* em tecidos sob estresse salino

RESUMO: A família de trocadores vacuolares Na^+/H^+ (NHX) desempenha um papel fundamental na homeostase iônica e regulação do pH em *Zea mays*, especialmente sob estresse salino. Este estudo analisou a expressão de genes NHX em folhas e raízes de milho submetidas ao estresse salino em 12 pontos temporais (0–168 h), utilizando dados transcriptômicos públicos. Dentre os membros da família, quatro genes (*ZmNHX2*, *ZmNHX5*, *ZmNHX6*, and

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ZmNHX13) apresentaram expressão significativa (RPKM > 1). A expressão foi geralmente maior em folhas do que em raízes. Nas folhas, observou-se aumento significativo aos 48, 72, 120 e 168 h, enquanto nas raízes, aumentos ocorreram apenas aos 120 e 168 h. Os resultados indicam um possível papel desses genes na atenuação da toxicidade por sódio e na melhoria da tolerância ao sal em milho. Essas descobertas ressaltam a importância de considerar a dinâmica temporal em estratégias de manipulação genética para otimizar a tolerância ao sal em diferentes fases de desenvolvimento da planta.

PALAVRAS-CHAVE: expressão gênica, salinidade, milho

INTRODUCTION

Salinity is one of the most damaging abiotic stresses affecting plant development, growth, and productivity worldwide. It is responsible for an estimated 70% reduction in the yields of major crops such as wheat, maize, rice, and barley (Acquaah, 2007), and contributes to significant agricultural losses, estimated at US$ 27.3 billion per year globally (Chaurasia et al., 2022). The expansion of saline soils is intensifying due to global climate change, including decreased rainfall and rising temperatures (Corwin, 2021). By 2050, more than 50% of arable land is expected to be affected by salinization (Jamil et al., 2011).

As sessile organisms, plants are constantly exposed to biotic and abiotic stresses such as high soil salinity. High NaCl concentrations can lead to disruptions in plant metabolism, damage to the redox system, pH alterations, photosynthesis limitations, and ultimately, plant growth inhibition (Farooq et al., 2015). To survive in saline environments, plants have developed several adaptive mechanisms, including the compartmentalization of sodium ions (Na^+) to maintain ionic homeostasis (Waheed et al., 2024). Na^+ homeostasis is primarily regulated by Na^+/H^+ antiporters located on the tonoplast (NHX family), which sequester Na^+ into vacuoles to reduce its toxic effects in the cytosol. The sequestration of Na+ into the vacuole is a key and energy-efficient strategy, as it not only reduces sodium toxicity in the cytosol but also contributes to osmotic homeostasis maintenance (Zhu, 2003). Overexpression of NHX genes has been shown to increase Na^+ sequestration and improve the K^+/Na^+ ratio in the cytosol, enhancing salinity tolerance in transgenic plants (Yarra et al., 2012; Zhang et al., 2012).

Maize (*Zea mays*), a glycophyte, is particularly sensitive to salt stress. A key adaptive trait in glycophytes is shoot Na^+ exclusion (Pitann et al., 2013). In maize, previous studies have identified the expression of 13 NHX genes across various tissues under salt stress (Kong et al., 2021). Further research has detected *ZmNHX2* gene expression in both shoots and roots of seedlings under NaCl stress, suggesting its role in vacuolar Na+ sequestration (Huanca-Mamani et al., 2018). The increased expression of the *ZmNNHX7* gene was also identified in maize plants exposed to salt stress (Bosnic et al., 2018). This plant appears to be an advantageous model for in silico studies of gene expression due to the large volume of transcriptomic data available in public databases.

In this study, we reanalyzed 14 NHX genes in maize (*Zea mays*) using publicly available transcriptomic data to investigate their expression profiles at different time points (0–168 h). Our objective was to determine the timing and which of these genes exhibit transcriptional responses to salinity, in order to understand the mechanisms underlying maize tolerance to this type of stress. By contributing this knowledge, we aim to support the genetic improvement of crops in saline soils

MATERIALS AND METHODS

Homologous NHX proteins from *Arabidopsis thaliana* were used in tBLASTn searches to retrieve genes (RefSeq representative genome) and cDNAs (Refseq RNA) of maize from GenBank databases. Expression data were obtained from BioProject PRJNA670840, which includes transcriptomic data from leaves and roots of maize cultivar Jing724 under salt stress (100 mM NaCl) across 12 time-intervals (0, 0.5, 1, 2, 4, 8, 12, 24, 48, 72, 120 and 168 h) with three biological replicates per sample. NHX gene expression analysis in the transcriptomic data was conducted in three steps: (1) read mapping using the Magic-BLAST software (Boratyn et al., 2019); (2) quantification of mapped reads using HTSeq (Anders & Huber, 2015); and (3) normalization of read counts across all samples. After read quantification, normalization between samples was performed using the RPKM (Reads Per Kilobase of transcript per Million mapped reads) method (Mortazavi et al., 2008), according to the following equation: RPKM = (number of mapped reads × 10^9) / (number of sequences in each dataset × number of nucleotides in each cDNA). Log_2 Fold Change was also calculated to compare gene expression between the treatment and control groups for the studied genes.

RESULTS AND DISCUSSION

The NHX gene family plays an important role in maintaining Na+ homeostasis in plant cells under salt stress conditions. In this study, 14 NHX genes were identified in the *Zea mays* genome using NCBI databases. Among these, we further characterized the genes expressed under salt stress conditions. Previous genome-wide analyses identified 11 and 13 NHX genes in *Zea mays*, respectively (Amaal & Mohammed, 2024; Kong et al., 2021), suggesting that differences in methodology or database versions may account for the variation in gene counts.

For the gene expression analysis, RNA-seq data from BioProject PRJNA670840 were used to evaluate the expression of 14 ZmNHX genes in maize roots and leaves under salt stress at multiple time points. Expression was quantified as the sum of normalized counts for all NHX genes (Figure 1).

Table 1 - Information of maize NHX genes identified in this study

Gene name	Gene model	Chromosomal location	DS (pb)	Protein length (aa)
ZmNHX2	*Zm*00001d024832	Chr10: 90134129–90,141,743	1680	559
ZmNHX5	*Zm*00001d022504	Chr7:179047417–179,052,457	1620	539
ZmNHX6	*Zm*00001d048732	Chr4: 4469601–4,475,393	1641	546
ZmNHX13	*Zm*00001d025052	Chr10: 101802712–101,807,654	1563	520

Overall, total ZmNHX expression was higher in leaves than in roots across most time points. In leaves, increased expression was observed at 48, 72, 120, and 168 hours after salt treatment. In contrast, root expression showed a delayed response, with notable increases only at 120 and 168 hours (Figure 1). The activation of NHX in leaves may facilitate Na+ sequestration within vacuoles, thereby protecting the photosynthetic machinery before salt reaches toxic levels (Bassil & Blumwald, 2014).

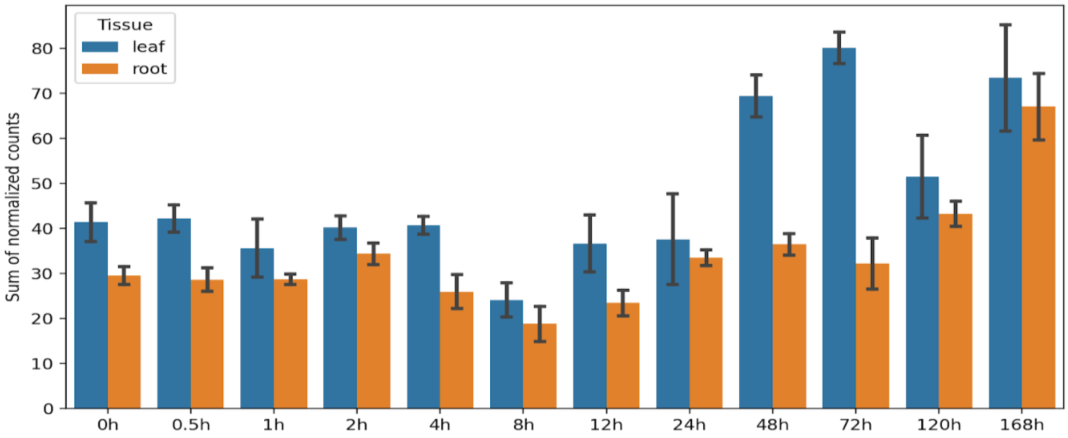


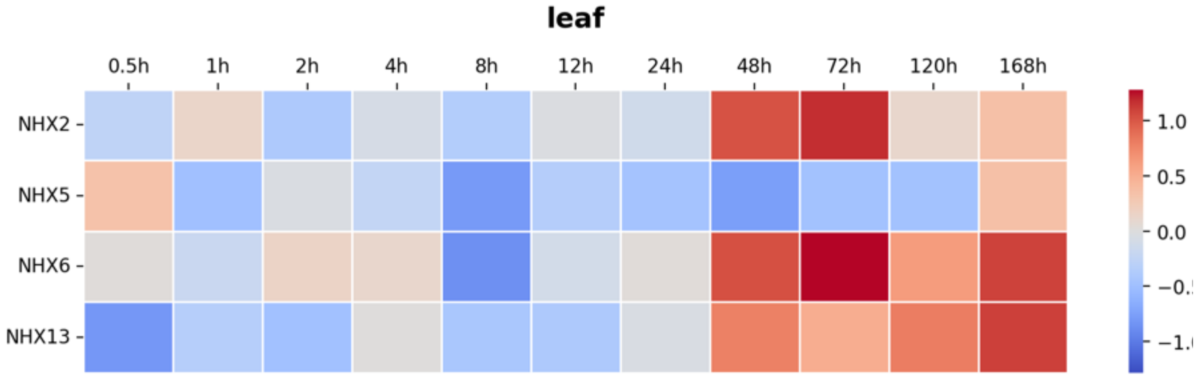
Figure 1. Total gene expression of NHX in maize leaves and roots at different time points under salt stress.

Among the 14 ZmNHX genes, four (*ZmNHX2*, *ZmNHX5*, *ZmNHX6*, and *ZmNHX13*) contributed most to the overall expression, showing consistently high levels (RPKM > 1) across samples (data not shown). Differential expression analysis using $\log_2$ fold change (FC) was performed for these four genes, comparing expression at each time point with the 0 h control under salt stress conditions (Figure 2). Particularly, *ZmNHX5* was upregulated ($\log_2$ FC ≥ 1) at 2, 24, 48, 120, and 168 hours in roots; *ZmNHX6* was upregulated in leaves at 48, 72, and 168 hours, and, in roots, it was downregulated (log2 FC ≤ 1) at 8 and 12 hours; *ZmNHX2* was upregulated in leaves at 48 and 72 hours; and *ZmNHX13* was upregulated at 168 hours in both tissues.

Previous study detected that the expression of three genes (*ZmNHX2*, *ZmNHX5*, and *ZmNHX6*) was consistent with our findings (Table 1) at different time points (0 h, 1 h, 2 h, 4 h, and 24 h) under salt stress (100 mM NaCl) in leaves, whereas in roots, *ZmNHX5* was up-regulated after 4 h (Kong et al., 2021).

In Miscanthus sinensis, expression of the NHX1 gene was shown to be upregulated in leaves 24 hours after NaCl treatment (Sun et al., 2021). Similarly, in *Zea mays*, *ZmNHX2* expression increased under salt stress in both shoots and roots, suggesting its involvement in vacuolar Na^+ sequestration (Huanca-Mamani et al., 2018).

Na^+ exclusion from leaves is a key adaptation to preserve vital metabolic processes such as photosynthesis. Supporting this, Rizk et al. (2024) observed that Na^+ sequestration mediated by NHX transporters occurred in the salt-tolerant maize cultivar SC168, but was absent in the salt-sensitive SC180. SC168 accumulated more Na^+ in roots and less in leaves, while SC180 showed the opposite pattern, highlighting the role of roots in Na^+ compartmentalization and protection of photosynthetic activity. Altogether, these findings suggest that NHX gene activity contributes to the differential salt stress responses observed among maize cultivars.



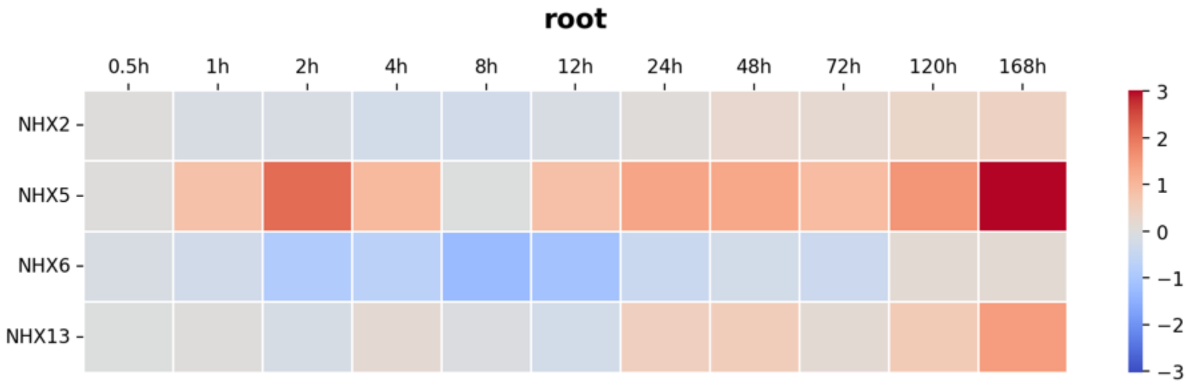


Figura 2. Heatmap of Log2 Fold Change comparing gene expression under salt stress to control in leaves and roots.

CONCLUSIONS

The increased expression of ZmNHX genes at later stages of salt stress indicates their potential role in the adaptive response to salinity in both maize roots and leaves. These findings highlight the functional diversification of NHX genes in maize and emphasize the need to incorporate temporal and spatial expression profiles when designing salt-tolerant maize varieties through breeding or genetic engineering. Further research should explore the specific mechanisms by which these genes regulate ion homeostasis to enhance crop resilience under saline conditions.

The differentially expressed genes identified in this study support the involvement of NHX transporters in the molecular mechanisms of salt stress tolerance. These genes represent promising targets for future research on isoform-specific functions and for genetic improvement strategies aiming to enhance salt tolerance in maize.

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