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In vitro assessment of salt secretion and its correlation with transporter gene expression in zoysiagrass (*Zoysia* spp.)

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Abstract

Soil salinity is a major threat to global agriculture. The recretohalophytic turfgrass species *Zoysia matrella* (L.) Merr. and *Zoysia japonica* Steud. possess unique salt glands that enable them to secrete excess salt. Understanding the molecular mechanisms underlying salt secretion could enable the introduction of salt secretion in other grasses, thereby improving their salt tolerance. In this study, we developed an *in vitro* leaf assay to investigate salt secretion patterns and the expression of key sodium/potassium transporters, including HKTs and endosomal NHXs. The present study revealed that *Z. matrella* consistently exhibited a higher salt secretion rate than *Z. japonica*. In both species, salt secretion increased with rising salinity, peaking at 300 mM NaCl. Beyond this threshold, further increases in salinity did not significantly enhance secretion, suggesting a maximum capacity for the salt glands. Interestingly, at 900 mM NaCl, salt secretion was completely inhibited in *Z. japonica*, whereas *Z. matrella* maintained a low level of secretion. Among the *HKT* genes, *HKT1;4* was the most highly expressed in the leaves of both species, and its expression was negatively correlated with salt secretion. Additionally, *NHX6* expression in *Z. matrella* was negatively associated with secretion, a pattern not observed in *Z. japonica*. The contrasting expression patterns of endosomal *NHX* genes may contribute to the differential salt tolerance between the two species. The *in vitro* leaf assay developed in this study provides an efficient tool for evaluating salt secretion in breeding programs. The present findings offer valuable insights into salt secretion and tolerance mechanisms in zoysiagrass, paving the way for the development of new salt-tolerant varieties.

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Introduction

Salinity stress is a growing global threat to agriculture, impacting an estimated 1,381 million hectares of land, representing 10.7% of the total land area[1]. Agricultural practices, such as overirrigation, the use of reclaimed water, and excessive fertilizer application, have exacerbated this issue, leading to salinity stress in approximately 10%–33% of irrigated farmland worldwide[1–4]. Most crop species are salt-sensitive glycophytes that experience significant yield losses due to the detrimental effects of excessive sodium (Na^+) on growth and development[5,6]. In contrast, salt-tolerance halophytes, comprising only 1% of plant species[7], can thrive in Na^+ concentrations exceeding 200 mM. Halophytes have evolved diverse strategies to combat salinity, including Na^+ compartmentalization within vacuoles (euhalophytes), sequestration in less-sensitive tissues (salt excluders), and active secretion through specialized salt glands (recretohalophytes). Understanding the mechanisms underlying halophyte salt tolerance offers valuable insights for improving crop resilience and restoring salt-affected lands.

Zoysiagrass (*Zoysia* spp.) is a halophyte belonging to the subfamily Chloridoideae of the family Poaceae[8]. It originated from the coastline and islands of the west Pacific and Indian Ocean and was introduced to the US as a lawn grass in 1892[9,10]. Two major species, *Z. matrella* and *Z. japonica*, are widely grown as turfgrass in the southern US. Both species are recretohalophytes with active salt glands located on the adaxial surface of leaf blades[11,12]. In *Z. matrella* and *Z. japonica*, salt glands are located on the adaxial surface of the leaf blades, arranged in rows between the stomata[12,13]. These glands exhibit a distinctive bicellular structure, featuring a partitioning membrane in the basal cell and a cuticular cavity atop the cap cell, both of which are crucial for salt secretion[13,14]. Research has demonstrated that *Z. matrella* varieties exhibit greater salt tolerance than *Z. japonica* varieties, exhibiting lower leaf firing rates, higher leaf and shoot dry weights, and maintaining lower internal Na^+ content under salinity stress[9,12,15]. *Z. matrella* has a higher salt gland density on the leaf surface and can secrete substantially more salt than *Z. japonica*[12,16]. However, the molecular mechanisms underlying salt secretion through salt glands remain poorly understood. In addition to salt secretion, other mechanisms associated with salt tolerance in zoysiagrass have been observed, including Na^+ accumulation, sequestration from root stele, and exclusion from root maturation zone[16,17]. This suggests that zoysiagrass incorporates multiple strategies to achieve high salinity tolerance. It is important to note that *in vivo* experiments using whole plants or runners, as employed in the abovementioned research, may not accurately reflect the stress levels experienced by leaf blade and salt glands because other tolerance mechanisms may act as barriers, preventing Na^+ from reaching the salt glands.

The uptake, transport, and secretion of Na^+ in plants rely on a variety of transmembrane transporters[18–20], including high-affinity K^+ transporters (HKTs) and Na^+/H^+ antiporters (NHXs). HKTs, located on the plasma membrane, transport Na^+ or K^+ from the apoplast into the cytoplasm[21,22]. HKTs are classified into two groups based on their sequence phylogeny and ion selectivity. Class I HKTs exclusively transport Na^+ and are found in both dicots and monocots, while Class II HKTs are Na^+ and K^+ symporters and are found only in

monocots. Class I HKTs play significant roles in salt tolerance in glycophytes through their involvement in Na^+ redistribution processes. For example, AtHKT1;1 in *Arabidopsis thaliana* and OsHKT1;5 in *Oryza sativa* are expressed in xylem parenchyma and phloem cells, where they retrieve Na^+ from the xylem sap and load it into the phloem for transport to less-sensitive tissues such as roots and leaf sheaths[23–25]. Class II HKTs also contribute to salt tolerance by mediating Na^+ uptakes and maintaining K^+ and Na^+ homeostasis under salt stress.

NHXs represent another important group of Na^+ transporters involved in salt stress responses. These transporters transport Na^+ or K^+ out of the cytosol in exchange for H^+. Based on their subcellular localization and transport direction, NHXs are categorized into three groups: (1) Vacuolar NHXs: located on the tonoplast and vacuole membranes, these transporters sequester Na^+ into vacuoles, contributing to osmotic adjustment and turgor maintenance for cell expansion and plant growth under saline conditions[26]; (2) Endosomal NHXs: located on endomembrane systems such as the endoplasmic reticulum (ER), Golgi apparatus, trans-Golgi network (TGN), and prevacuolar compartment (PVC), endosomal NHXs play important roles in intracellular vesicular trafficking, pH regulation, and ion homeostasis[27–29]. They are likely involved in salt secretion[30]; (3) Plasma-membrane NHXs: located on the plasma membrane, these transporters, also known as Salt Overly Sensitive 1 (SOS1), extrude cytosolic Na^+ into the apoplast[31]. The SOS1-mediated salt tolerance mechanism is widely conserved across various plant species, including glycophytes and halophytes.

To study salt secretion and the associated gene expression of *HKT* and endosomal *NHX* genes in zoysiagrass under varying salinity stress, we developed an *in vitro* salt secretion assay using leaf blade explants. We selected 'Diamond' (*Z. matrella*) and 'Meyer' (*Z. japonica*) as high and low salt-tolerant varieties, respectively, based on prior greenhouse and field evaluations[11,12,32]. We found that both varieties reach maximum salt secretion at 300 mM NaCl. Diamond exhibited significantly higher secretion capacity, consistent with its greater salt tolerance. While *HKT1;4* genes were upregulated in both varieties under high salinity conditions (where salt secretion was inhibited), endosomal *NHX* gene expression was induced by salinity stress only in Diamond. This research provides valuable insights into the molecular mechanisms underlying salt tolerance in zoysiagrass.

Material and methods

Plant material and *in vitro* salt secretion assay

Zoysiagrass plants (*Z. matrella* cv. Diamond and *Z. japonica* cv. Meyer) were maintained in a greenhouse at the Texas A&M AgriLife Research and Extension Center at Dallas, TX, USA. Rhizomes were transplanted to 4-inch square pots containing potting mix (SunGro, Agawam, MA, USA) and fertilized with 30-10-10 (N-P-K) fertilizer (Miracle-Gro, Marysville, OH, USA). After establishing plants with at least three fully extended leaves, leaf blades were collected and cut into 10-mm-long segments. Segments from a leaf blade were considered as biological replicates. Surface contaminants were removed using nuclease-free water, and the segments were air dried for approximately 30 s. Segments were then immediately placed on 1% agar solidified media with the abaxial leaf surface in contact with the media. The media contains 1X Murashige & Skoog Basal Salt Mixtures with Vitamins (Sigma-Aldrich, St. Louis, MO, USA) and supplied with NaCl at the following concentrations: 0, 100, 200, 300, 600, and 1,000 mM. Following incubation at 25 °C with a 16:9 day/night cycle for 12, 24, and 48 h, the leaf segments were removed from the medium for analysis.

Salt secretion capacity was evaluated after 24 and 48 h of salt exposure. Leaf segments were carefully adhered to sticky microscopic slides with the adaxial surface facing upward and fully extended without disturbing. Salt was crystallized on the leaf surface at room temperature and then immediately imaged under a stereo microscope (Carl Zeiss AG, Oberkochen, Germany) at 10× magnification. To quantify salt secretion, a 'salt index' were calculated for each segment: the area covered by salt crystals (measured using Image J[33]) was divided by the sample area. For each treatment, 9–12 leaf segments were evaluated, and two-tailed t-tests were performed between the two varieties.

Identification of HKTs and NHXs from genome assemblies of *Z. matrella* and *Z. japonica*

To identify *HKT* and *NHX* genes in *Z. matrella*, we first utilized the genome assembly and annotation of *Z. matrella* cv Diamond. These sequences were then used as queries in BLASTp searches against the *Zoysia* Genome Database[34] to identify homologous genes in *Z. japonica* cv. Nagirizaki (r1.1). For comparative analysis, we also retrieved *HKT* and *NHX* sequences from *Oryza sativa* Japonica Group (IRGSP-1.0) via Ensembl Plants, ensuring completeness by cross-referencing with the genome annotation. Further, *HKT* genes from *Triticum aestivum* (wheat) and *NHX* genes from *Zea mays* (maize) were included in the analysis, based on data from previous studies[35,36]. To validate the function of the identified genes, we performed protein domain analysis using InterProScan[37]. *HKT* candidates were confirmed based on the presence of the 'Cation transporter' (IPR003445) or 'TrkH Potassium Transport' (IPR051143) protein family memberships, and the 'monoatomic cation transmembrane transporter activity' (GO:0008324) Gene Ontology term. Similarly, *NHX* candidates were validated through the presence of the 'Cation/H^+ exchanger, CPA1 family' (IPR018422) protein family membership and the 'antiporter activity' (GO:0015297) GO term. Phylogenic analysis of *HKT* and *NHX* genes was performed using Molecular Evolutionary Genetics Analysis (MEGA-11)[38]. TBtools-II[39] was used to investigate the syntenic relationships of *NHX* and *HKT* genes between *Z. matrella* and *O. sativa* genome assemblies.

RNA extraction and gene expression analysis

We used quantitative reverse transcription PCR (qRT-PCR) to quantify *HKT* gene expression in the leaf blade of zoysiagrass, including 2 *HKT1;3* (*ZmdHKT1;3-A* and *B*), 2 *HKT1;4* (*ZmdHKT1;4-A* and *B*), 1 *HKT2;1* (*ZmdHKT2;1*), 2 *HKT2;2* (*ZmdHKT2;2-A* and *B*), and 1 *HKT2;4* (*ZmdHKT2;4*) from *Z. matrella* and 2 *HKT1;4* (*ZjnHKT1;4-A* and *B*), 1 *HKT2;1* (*ZjnHKT2;1*), 1 *HKT2;2* (*ZjnHKT2;2*), and 1 *HKT2;4* (*ZjnHKT2;4*) from *Z. japonica*. Vascular *NHX* genes, including 1 *NHX5* (*ZmdNHX5*), and 2 *NHX6* (*ZmdNHX6-A* and *B*) from *Z. matrella*, and 1 *NHX6* (*ZjnNHX6*) from *Z. japonica*, were also analyzed. The extra leaf segments used in the *in vitro* salt secretion assay were harvested at 12, 24, and 48 h after salt treatment and used for total RNA extraction using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocol. Leaf segments were flash-frozen in liquid nitrogen and cryoground into a fine powder using a HG-600 Geno/Grinder (Cole-Parmer, Vernon Hills, IL, USA) at 1,750 strokes per minute for three cycles of one minute each. RNA was further purified using the Quick RNA Miniprep Kit (ZYMO Research, Irving, CA, USA), including on-column DNase I (ZYMO) digestion to eliminate genomic DNA contamination. The cDNA synthesis was performed with the SuperScript IV First-Strand Synthesis Kit (Thermo Fisher, Waltham, MA, USA) according to the manufacturer's instructions. The resulting cDNA was diluted 1:1 with water and used for qRT-PCR analysis with an Applied Biosystems StepOne Real-Time PCR System (Thermo Fisher, Waltham, MA, USA). The qRT-PCR was performed using iTaq Universal SYBR Green Supermix

(Bio-Rad Laboratories, Hercules, CA, USA) with the following cycling conditions: 95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min, and a final melt curve analysis from 60–95 °C to confirm amplification specificity. Gene-specific primers were designed using Primer 3[40] based on coding DNA sequences and validated by PCR (Supplementary Table S1). Three biological replications were incorporated for each treatment, and each contained three technical replications. Relative expression levels were calculated using the $2^{-\Delta Ct}$ method[41] with a putative zoysia polyubiquitin gene serving as an internal reference control (Supplementary Table S1). Samples treated with 0 mM NaCl were used as the control group. Statistical analysis, including ANOVA and multiple comparison tests with Tukey HSD adjustment under each NaCl concentration, was conducted using R software.

Results

Salt secretion of *Z. matrella* and *Z. japonica* under different salinity levels

In vitro salt secretion assays revealed sustained salt secretion in both *Z. matrella* and *Z. japonica* for at least 48 h. Liquid droplets, containing secreted salts, appeared on the adaxial leaf surfaces of both species, with *Z. matrella* exhibiting visible droplets as early as 2 h after exposure, while *Z. japonica* showed droplets at 12 h. Salt crystallization from these droplets was observed at both 24 and 48 h (Fig. 1a). Control samples treated without Na^+ also exhibited a few salt crystals secreted, likely due to the continuation of secretion of pre-existing internal Na^+ before the experiment.

Salt secretion rates measured at 24 and 48 h after treatments exhibited similar patterns across the different levels of NaCl treatment. Measurements collected at both 24 and 48 h after treatments showed the rate of salt secretion in both species increased with salinity levels from 0 to 300 mM NaCl, reaching a maximum rate between 300 and 600 mM. This suggests that the salt glands in both species have similar sensitivities to salinity stress and that Na^+ levels become excessive at 600 mM. Above 600 mM, secretion rates declined dramatically in both species, with *Z. japonica* showing almost complete inhibition at or above 900 mM. However, *Z. matrella* maintained a low level of secretion even at the highest salinity level (1,000 mM) (Fig. 1b, c). Throughout the salinity range tested, *Z. matrella* consistently exhibited a significantly higher salt secretion rate than *Z. japonica*.

HKT and *NHX* genes in *Z. matrella* and *Z. japonica*

The *Z. matrella* cv. Diamond genome harbors 10 potential *HKT* genes distributed across chromosomes LG5, LG8, LG11, LG12, LG19, and LG20, as well as two unanchored contigs (Contig1247 and Contig1355). A tandem duplication of the *HKT1;4* gene was identified on chromosome LG12. In contrast, the *Z. japonica* genome contains eight potential *HKT* genes, including tandem duplications of *HKT2;1/2;2*. Phylogenetic analysis of 37 *HKT* genes from four monocot species and *Arabidopsis thaliana* clearly distinguishes Class I and Class II *HKTs*. Following the established nomenclature system for *HKTs*[42] and considering the rice genome's resemblance to the ancestral form of the grass genome[43,44], we assigned names to the zoysiagrass *HKT* genes based on sequence homology with *OsHKT* genes. A letter identifier was added to differentiate genes within the same group. This resulted in the designation of 2 *HKT1;3* (*ZmdHKT1;3-A* and *B*), 3 *HKT1;4* (*ZmdHKT1;3-A*, *B*, and *C*), 1 *HKT1;5* (*ZmdHKT1;5*), 3 *HKT2;1* (*ZmdHKT2;1-A*, *B*, and *C*), and 1 *HKT2;4* (*ZmdHKT2;4*) in *Z. matrella*, and 2 *HKT1;4* (*ZjnHKT1;4-A* and *B*), 1 *HKT1;5* (*ZjnHKT1;5*), 1 *HKT2;4* (*ZjnHKT2;4*), and 4 *HKT2;1* (*ZjnHKT2;1-A*, *B*, *C*, and *D*) in *Z. japonica* (Table 1, Fig. 2a).

Sixteen potential *NHXs* were identified in *Z. matrella*, located on chromosomes LG3, LG4, LG10, LG13, LG15, LG17, LG18, LG19, and LG20, and five unanchored contigs. *Z. japonica* has nine *NHX* genes. Phylogenetic analysis of *NHXs* revealed two monophyletic sister groups: plasma membrane (*SOS1*) and endosome *NHXs*. The majority of the remaining *NHXs* are paraphyletic vacuolar *NHXs*. Based on sequence homology, we identified three and two plasma membrane *NHXs*, three and one endosomal *NHXs*, and 11 and seven vacuolar *NHXs* in *Z. matrella* and *Z. japonica*, respectively (Fig. 2b). Similar to the *HKTs*, we adopted a nomenclature system for the *NHX* genes based on sequence homology to *OsNHX* genes (Table 1).

Z. matrella is an allotetraploid species with 2n = 4x = 40 chromosomes[45]. Following the genome duplication event, the duplicated genes are not equally retained throughout the genome, and this process is accompanied by significant chromosomal rearrangements, leading to a restructured genome where the duplicated genes are not always located in the same positions as the original copies. Genome synteny comparisons between *Z. matrella* and rice revealed collinearity of some *HKT* and *NHX* genes (Fig. 3). While duplicated copies of *HKT1;4*, *NHX1*, and *NHX3*[44] are conserved, only one copy of *HKT2;1*, *HKT2;4*, *NHX2*, *NHX4*, and *SOS1* has been retained. In some instances, collinearity is less clear, such as *ZmdHKT1;5* on LG5 and *OsHKT1;5* on chromosome Os01. Additionally, *ZmdNHX5* lacks a rice counterpart on Os08, suggesting chromosomal rearrangements and potential subfunctionalization after the genome duplication event.

Expression dynamics of HKTs and NHXs in *Z. matrella* and *Z. japonica* under different salinity stress levels

qRT-PCR analysis revealed that *HKT1;4* genes were the most highly expressed *HKTs* in leaf blades of both *Z. japonica* and *Z. matrella*. In contrast, expression of class II *HKTs* in the leaf blades was much lower (Fig. 4; Supplementary Fig. S1). Both species exhibited similar *HKT1;4* expression patterns in response to salinity stress at various time points (Fig. 4a, b, e, & f). At 12 h, *HKT1;4* genes were expressed at significantly lower levels in leaf segments treated with 0 and 300 mM NaCl than in those treated with 600 and 1,000 mM NaCl. These differences were also observed at 24 and 48 h. The *HKT2;4* is the highest expressed class II *HKT* gene in leaf blades of both species and showed similar expression patterns in response to salinity stress in both species. At 12 h, the expression levels of *HKT2;4* were upregulated with the increasing NaCl concentration. However, at 24 and 48 h, the difference between treatments became less significant.

Gene expression analysis of endosomal *NHX* genes revealed distinct responses between the two species. *Z. matrella* possessed three endosomal *NHX* genes with similar expression dynamics (Fig. 4d; Supplementary Fig. S1e & f), exhibiting an overall upregulation with increasing salinity stress. At 12 h, no significant difference in expression was observed between 0 and 300 mM treatment groups, and between 600 and 1,000 mM treatment groups. At 24 and 48 h, the gene expression showed gradually increased expression levels with increasing stress levels. In contrast, *Z. japonica* showed no significant differences in endosomal *NHX* gene expression between different salinity stress treatments (Fig. 4h).

Discussion

Salt secretion capacity in zoysiagrass

The Poaceae family exhibits a wide range of salinity tolerance across species. While salt gland-like structures are present in most species (with the exception of Pooideae), only 15 species within the Chloridoideae subfamily have demonstrated active salt

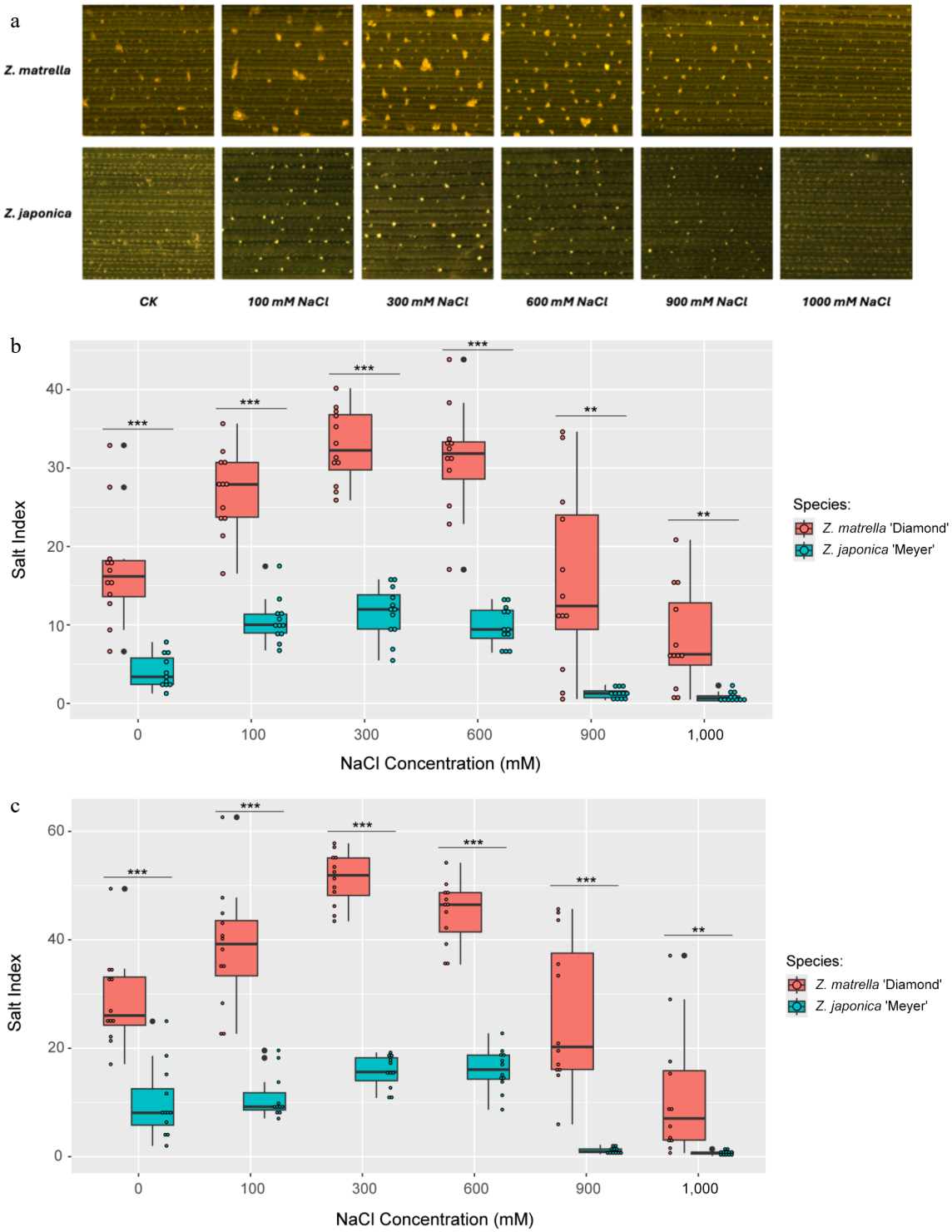

Fig. 1 Salt secretion in *Z. matrella* and *Z. japonica* under *in vitro* conditions with ambient NaCl. (a) Adaxial leaf surface after 24 h of salt treatment, illustrating the quantity of salt secreted. Boxplots comparing salt secretion rates per unit area for the two species measured at (b) 24 h, and (c) 48 h of salt treatment. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

excretion[14,46]. Elucidating the molecular mechanisms underlying salt secretion could enable the reintroduction or enhancement of this trait in grasses that have lost this ability, thereby improving their tolerance to saline environments and bolstering their resilience to salt stress.

Zoysiagrass employs multiple strategies to achieve high salinity tolerance, among which salt secretion is considered a primary mechanism. Traditional *in vivo* experiments, such as growing plants hydroponically or in potting mix with added salt, have been widely used to evaluate salt tolerance in zoysiagrass[12,15,17]. However, these methods have limitations when studying salt secretion. While they mimic field conditions, they do not directly expose salt glands to salinity stress. Instead, salt must first be absorbed by the roots and transported through the plant, with only a fraction ultimately reaching the salt glands for secretion. During this process, Na^+ can gradually accumulate in shoots, as observed in *Z. matrella* and *Z. japonica* under hydroponic salinity stress[47]. In contrast, *in vitro* leaf assays offer a more direct and efficient approach to assessing salt

Table 1. Summary of *HKT* and *NHX* genes in *Zoysia matrella* and *Zoysia japonica*.

Transporter	Species	Gene ID	Gene name	Type	Chr/contig	Start	End	Protein
High-affinity K^+ transporters (HKTs)	*Zoysia matrella*	evm.TU.chrLG5.503	*ZmdHKT1;5*	Class I HKT	LG5	1E+07	1E+07	399
		evm.TU.chrLG8.161	*ZmdHKT1;3-A*		LG8	1E+06	1E+06	694
		evm.TU.chrLG11.785	*ZmdHKT1;4-A*		LG11	2E+07	2E+07	856
		evm.TU.chrLG12.814	*ZmdHKT1;4-B*		LG12	1E+07	1E+07	966
		evm.TU.chrLG12.815	*ZmdHKT1;4-C*		LG12	1E+07	1E+07	613
		evm.TU.contig_1355.5	*ZmdHKT1;3-B*		Contig1355	47032	60625	1369
		evm.TU.chrLG19.1489	*ZmdHKT2;1*	Class II HKT	LG19	3E+07	3E+07	447
		evm.TU.chrLG20.855	*ZmdHKT2;2-A*		LG20	2E+07	2E+07	541/487[1]
		evm.TU.chrLG20.1433	*ZmdHKT2;4*		LG20	2E+07	2E+07	519
		evm.TU.contig_1247.6	*ZmdHKT2;2-B*		Contig1247	40514	42319	541
	Zoysia japonica	Zjn_sc00004.1.g09630	*ZjnHKT1;4-A*	Class I HKT	Zjn_sc00004.1	420993	4E+06	925
		Zjn_sc00011.1.g08720	*ZjnHKT1;5*		Zjn_sc00011.1	5E+06	5E+06	479
		Zjn_sc00023.1.g02580	*ZjnHKT1;4-B*		Zjn_sc00023.1	1E+06	1E+06	672
		Zjn_sc00008.1.g01340	*ZjnHKT2;4*	Class II HKT	Zjn_sc00008.1	560759	566679	876
		Zjn_sc00068.1.g02380	*ZjnHKT2;1*		Zjn_sc00068.1	1E+06	1E+06	420
		Zjn_sc00068.1.g02390	*ZjnHKT2;2-C*		Zjn_sc00068.1	1E+06	1E+06	253
		Zjn_sc00107.1.g01070	*ZjnHKT2;2-A*		Zjn_sc00107.1	564336	566416	436
		Zjn_sc00107.1.g01080	*ZjnHKT2;2-B*		Zjn_sc00107.1	570341	571277	290
Na^+/K^+ antiporters (NHXs)	*Zoysia matrella*	evm.TU.chrLG13.696	*ZmdNHX5*	Endosomal NHX	LG13	2E+07	2E+07	265
		evm.TU.contig_183.15	*ZmdNHX6-A*		Contig183	324272	333257	553
		evm.TU.contig_992.2	*ZmdNHX6-B*		Contig992	27765	37237	530
		evm.TU.chrLG15.666	*ZmdSOS1-A*	Plasma membrane NHX	LG15	1E+07	1E+07	1144/1046[1]
		evm.TU.chrLG20.731	*ZmdSOS1-B*		LG20	1E+07	1E+07	1154/1057[1]
		evm.TU.contig_1213.8	*ZmdSOS1-C*		Contig1213	59774	68887	975
		evm.TU.chrLG3.849	*ZmdNHX1-A*	Vacuolar NHX	LG3	2E+07	2E+07	540/391[1]
		evm.TU.chrLG4.846	*ZmdNHX1-B*		LG4	2E+07	2E+07	429/401[3]
		evm.TU.chrLG10.558	*ZmdNHX2*		LG10	1E+07	1E+07	544
		evm.TU.chrLG17.148	*ZmdNHX3-A*		LG17	2E+06	2E+06	811
		evm.TU.chrLG17.295	*ZmdNHX3-B*		LG17	6E+06	6E+06	392
		evm.TU.chrLG18.192	*ZmdNHX3-C*		LG18	2E+06	2E+06	583
		evm.TU.chrLG19.386	*ZmdNHX4-A*		LG19	9E+06	9E+06	440
		evm.TU.chrLG19.393	*ZmdNHX4-B*		LG19	9E+06	9E+06	523
		evm.TU.contig_738.3	*ZmdNHX4-C*		Contig738	15126	19095	524/448/395/444[4]
		evm.TU.contig_1250.10	*ZmdNHX1-D*		Contig1250	58727	63951	540/391/458[2]
		evm.TU.contig_1736.2	*ZmdNHX1-C*		Contig1736	7	4443	429
	Zoysia japonica	Zjn_sc00085.1.g00950	*ZjnNHX6*	Endosomal NHX	Zjn_sc00085.1	966404	976204	634
		Zjn_sc00035.1.g02750	*ZjnSOS1-B*	Plasma membrane NHX	Zjn_sc00035.1	3E+06	3E+06	1057
		Zjn_sc00105.1.g00050	*ZjnSOS1-A*		Zjn_sc00105.1	32266	47024	1073
		Zjn_sc00001.1.g01510	*ZjnNHX-1A*	Vacuolar NHX	Zjn_sc00001.1	634926	639121	540
		Zjn_sc00016.1.g04350	*ZjnNHX3-B*		Zjn_sc00016.1	3E+06	3E+06	525
		Zjn_sc00044.1.g04210	*ZjnNHX1-B*		Zjn_sc00044.1	2E+06	2E+06	546
		Zjn_sc00096.1.g00980	*ZjnNHX2*		Zjn_sc00096.1	445565	450176	452
		Zjn_sc00146.1.g00380	*ZjnNHX4*		Zjn_sc00146.1	294475	297777	245
		Zjn_sc00166.1.g00230	*ZjnNHX3-A*		Zjn_sc00166.1	148408	157386	507

[1]Two isoforms; [2]three isoforms; [3]five isoforms: one isoform of 429 aa and four isoforms of 401 aa; [4]six isoforms: three isoforms of 395 aa, one isoform of 528 aa, one isoform of 448 aa, one isoform of 444 aa.

secretion. By directly exposing leaf explants to salt, Na^+ rapidly reaches the cells via diffusion, enabling a precise and efficient measurement of salt secretion. This direct method offers a valuable tool for rapidly assessing salt secretion capacity, which is particularly useful in breeding programs aiming to enhance salinity tolerance.

Previous studies in *Z. japonica* have demonstrated a direct correlation between salt tolerance and the amount of Na^+ secreted through salt glands[17]. However, the relationship between gland density and secretion capacity is not always straightforward[12,16]. For instance, *Z. japonica*, despite having a lower salt gland density than *Z. matrella*, doesn't necessarily translate to reduced salt secretion[12,16]. This highlights the need to investigate whether salt glands from different species and varieties respond similarly to salinity stress. Our study revealed distinct differences in salt secretion capacity between *Z. japonica* and *Z. matrella*. *Z. matrella* consistently exhibited a higher salt secretion rate than *Z. japonica*. In both species, salt secretion increased in response to increasing salinity stress, reaching a peak at 300 mM NaCl. Beyond this concentration, further increases in salinity did not significantly enhance secretion, indicating that the salt glands had reached their maximum capacity. Interestingly, at 900 mM NaCl and above, salt secretion was completely inhibited in *Z. japonica*, whereas *Z. matrella* maintained a low but measurable level of secretion. These findings suggest that different capacities for salt secretion between the two species contribute to their differing response to extreme saline conditions.

Salt tolerance mechanism and the role of *HKTs* and endosomal *NHXs* in zoysiagrass

To date, the salt secretion mechanism remains largely unknown. Recent proposed models[14,30,46] suggest that basal cells within salt

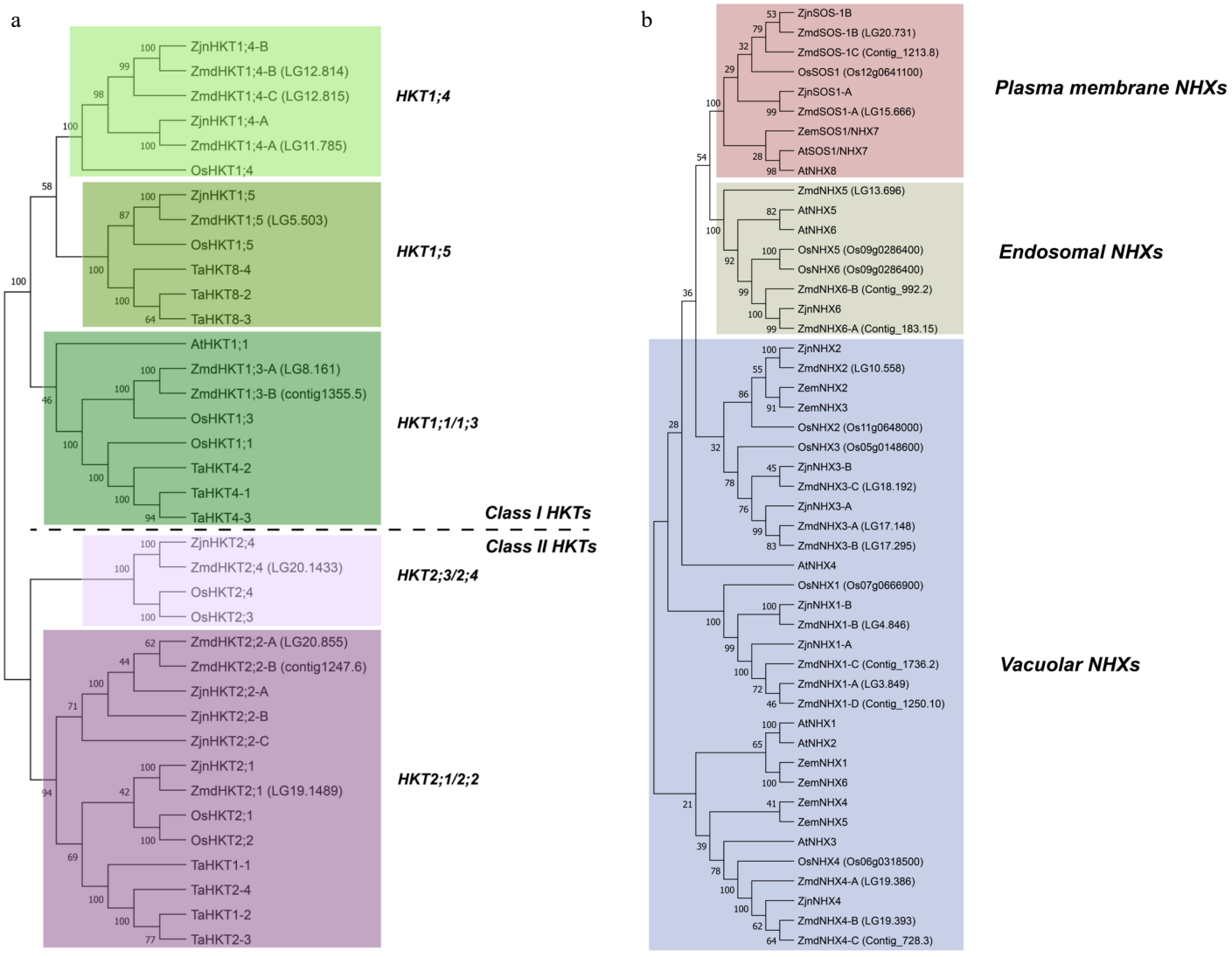

Fig. 2 Phylogenetic analysis of (a) *HKT*, and (b) *NHX* gene families. Protein sequences were aligned using the MUSCLE alignment tool, and Neighbor-Joining (NJ) phylogenetic trees were constructed with 1,000 bootstrap replicates using MEGA-11 software. The sequences used in the analysis are listed in Supplementary Tables S2 and S3. Species abbreviations: Zmd, *Zoysia matrella* cv Diamond; Zjn, *Zoysia japonica* cv Nagirizaki; At, *Arabidopsis thaliana*; Os, *Oryza sativa*; Ta, *Triticum aestivum*; Zem, *Zea mays*.

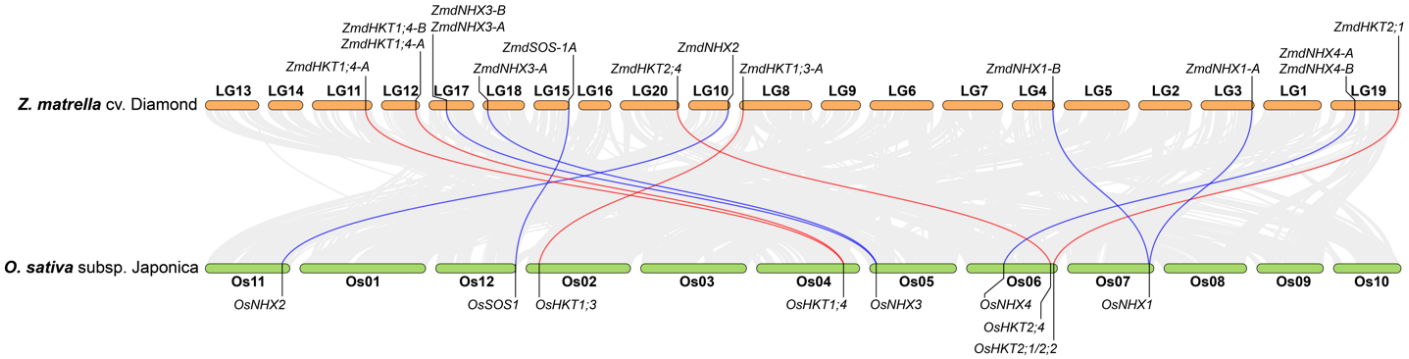

Fig. 3 Genome collinearity analysis between *Z. matrella* cv. Diamond and *O. sativa* subsp. japonica, highlighting the relationships of *HKT* and *NHX* genes between the two species. Genes located on unanchored contigs in *Z. matrella* were excluded from the analysis.

glands take up Na^+ from surrounding mesophyll cells and apoplast, potentially through plasmodesmata, and concentrate them in intracellular vesicles. These vesicles are then transported to cap cells for secretion into the atmosphere *via* exocytosis. Sodium transporters like HKTs, non-selective cation channels (NSCCs), cyclic-nucleotide-gated cation channels (CNGCs), and endosomal NHXs are likely involved in this process. In addition to salt secretion, zoysiagrass also employs other strategies for salt tolerance, including sequestration of Na^+ in vacuoles, increased potassium (K^+) uptake, and compartmentalization of Na^+ in less sensitive tissues[17]. Transporters such as HKTs and NHXs also play important roles in these processes.

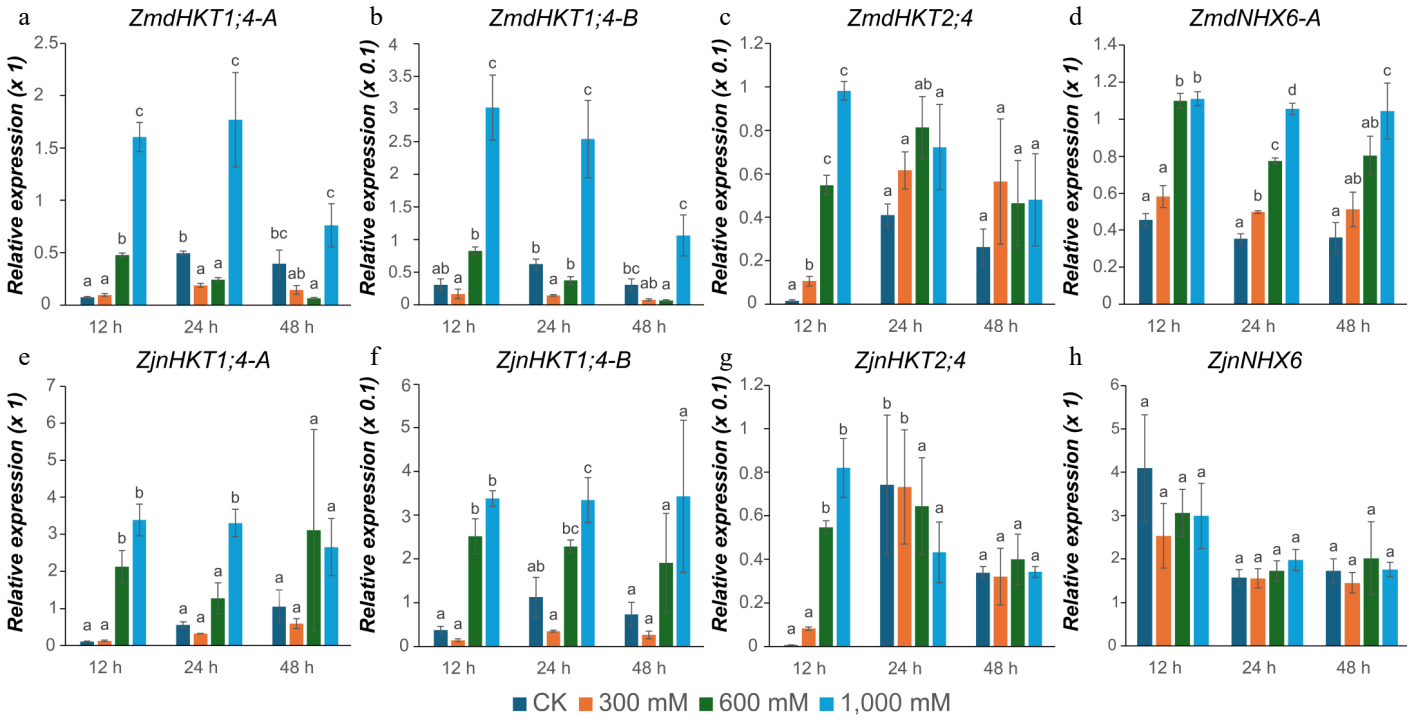

Fig. 4 Dynamics of relative expression levels of *HKT* and *NHX* genes in zoysia leaf blades under *in vitro* conditions treated with varying NaCl concentrations. (a)–(d) Expression profiles of *Z. matrella* genes: *ZmdHKT1;4-A*, *ZmdHKT1;4-B*, *ZmdHKT2;4*, and *ZmdNHX6-D*. (e), (f) Expression profiles of *Z. japonica* genes: *ZjnHKT1;4-A*, *ZjnHKT1;4-B*, *ZjnHKT2;4*, and *ZjnNHX6*. Statistical differences among NaCl treatments were analyzed using Tukey's HSD test ($p < 0.05$).

Zoysiagrass is an allotetraploid species and rice serves as a suitable reference for comparative analysis due to its conserved genome structure of the grass ancestor[43,44]. Comparative genomic analysis between zoysiagrass and rice revealed post-polyploidization genome rearrangements, including differential retention and translocation of *HKT* and *NHX* genes. We identified *HKT* and *NHX* gene families in *Z. matrella* and *Z. japonica* and detected gene duplications derived from whole-genome duplication and tandem duplication events. While the number of *HKT* genes are comparable across species (*Z. matrella*: 10, *Z. japonica*: eight, *O. sativa*: eight), zoysiagrass possess significantly more *NHX* genes (*Z. matrella*: 17, *Z. japonica*: nine, *O. sativa*: six), suggesting an expansion of this gene family in zoysiagrass. Such gene duplications provide the raw material for the evolution of novel gene functions, which may contribute to the enhanced salt tolerance of zoysiagrass. Our gene expression analysis, coupled with *in vitro* salt secretion assays, provided intriguing insights. Only *HKT1;4* genes were highly expressed in zoysiagrass leaf blades, and their expression levels were upregulated under high salinity conditions, such as 600 mM (12 h) and 1,000 mM (24, 48 h) (Fig. 4). This regulation, however, showed a negative correlation with salt secretion observed under the same experiment conditions. This suggests that HKT1;4 transporters in zoysiagrass may play roles in multiple pathways in salinity stress response and potentially contribute to Na^+ accumulation or compartmentation. These pathways may serve as alternative pathways and only be activated when salt secretion is impaired under extreme saline conditions.

Previous studies showed overexpression of endosomal *NHXs* increased salt tolerance in glycophytes like *A. thaliana*, which lack salt secretion capacities[28,48]. Our study showed a significant increase in the expression of the endosomal *NHX* gene in *Z. matrella*, particularly at high salinity levels when salt secretion is impaired. In contrast, such significant upregulation of the endosomal *NHX* gene was not observed in *Z. japonica*. This differential expression pattern suggests that increased Na^+ sequestration within the endomembrane system may be a pronounced salt tolerance strategy in *Z. matrella* under high salinity. The contrasting expression patterns of endosomal *NHX* genes likely contribute to the observed differences in salt tolerance between these two *Zoysia* species. Future research should focus on fully elucidating the precise functional roles of these transporters in the comprehensive salt tolerance mechanisms of zoysiagrass.

Conclusions

To isolate salt secretion from other salt tolerant mechanisms and efficiently evaluate salt secretion capacity across germplasm accessions, we developed an *in vitro* salt secretion assay. Using this assay, we compared salt secretion in *Z. matrella* and *Z. japonica*. Our study revealed that *Z. matrella* consistently exhibited a higher salt secretion rate than *Z. japonica*. Salt glands in both species exhibited similar levels of sensitivity to salinity stress, with their secretion peaking at 300 mM NaCl. Based on our findings, we propose an updated model for salt secretion in zoysiagrass (Fig. 5), expanding on the model by Lu et al.[30]. Under low salinity stress (internal Na^+ < 300 mM), Na^+ is transported to leaf blades and secreted by salt glands. Under higher salinity stress, *HKT1;4* is upregulated, which intercepts Na^+ from the tapoplast for storage or loading into the vascular system (not shown in the figure). In *Z. matrella*, the upregulation of endosomal *NHXs* facilitates the loading of excess Na^+ into the endomembrane system. This sequestered Na^+ could then be directed to several potential destinations: salt glands (I), vacuoles (II), or even excreted back into the apoplast (III).

Author contributions

The authors confirm their contributions to the paper as follows: research activities coordinating: Yu Q; experiments design: Yu Q,

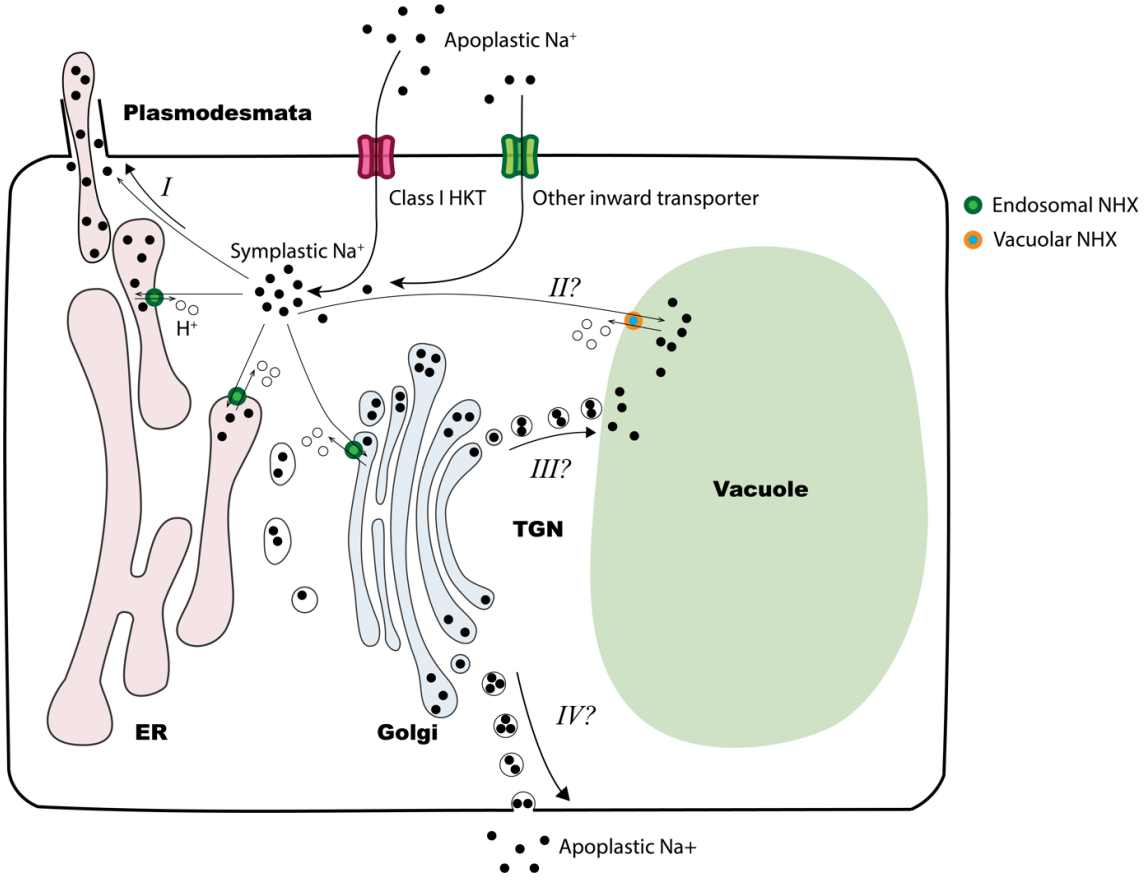

Fig. 5 Proposed model of zoysiagrass response to salinity. Under salinity stress, apoplastic Na^+ enters leaf cell through class I HKT transporters or other inward transporters. At high salinity stress levels, the cytosol may act as a temporary sink for Na^+. The accumulated Na^+ can be loaded to vascular system (not depicted in the figure), transported into endomembrane system via endosomal NHX transporters, or sequestered into the vacuole by vacuolar NHXs (option II). Excess Na^+ within endomembrane system may be transported to salt glands *via* plasmodesmata (option I), to the vacuole (option III) or excreted back into the apoplast (option IV). In *Z. matrella*, the upregulation of endosomal *NHXs* under high levels of salinity stress suggests that options III and IV may serve as alternative responses to salt stress.

Zhao Z; experiments conducting and data analysis: Zhao Z, Lyu H, Xu Y, Wang F; plant materials providing: Chandra A; draft the manuscript: Zhao Z; manuscript revision: Yu Q. All authors reviewed the results and approved the final version of the manuscript.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

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Conflict of interest

The authors declare that they have no conflict of interest.

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