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Regulation of Na^+ and K^+ Transport and Oxidative Stress Mitigation Reveal Differential Salt Tolerance of Two Egyptian Maize (*Zea mays* L.) Hybrids at the Seedling Stage

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Abstract

Maize is sensitive to salinity stress, which has adverse effects on plant growth and yield. In this study, two Egyptian white maize hybrids, single cross 131 (SC131) and single cross 132 (SC132), were exposed to 100 mM NaCl stress for 12 days in a hydroponic culture and physiological and biochemical parameters, and gene expression for some Na^+ and K^+ transporters were evaluated. The results revealed that the total dry weight of SC131 was greater than that of SC132. The root growth was also better for SC131 than SC132. The Na^+ concentration in leaves and stems, and H_2O_2 were significantly lower in SC131 than in SC132, while the proline concentration was higher in the leaf of SC131 than in SC132 under salt stress. Moreover, catalase and ascorbate peroxidase activities increased with salinity in SC131 leaves compared to catalase and GR in SC132. Salt stress slightly induced the expression of *ZmHKT1;5* (for Na^+ exclusion) in SC132, but repressed it in SC131. On the other hand, the expression of *ZmHKT2* gene (for K^+ exclusion) was highly induced by salt stress in SC132, but was not detected in SC131. The salt increased the expression of *ZmNHX1* (for vacuolar Na^+ sequestration) in SC132, but repressed it in SC131. Taken together, these results suggest that the maize hybrid SC131 is more tolerant to salinity than SC132, thanks to a more efficient leaf Na^+ exclusion mechanism, that is yet to be investigated, and to a better antioxidant defense system. Thus, SC131 could constitute a new hybrid worthy of consideration in maize breeding programs for enhanced productivity on salt-affected soils.

Keywords Antioxidant enzyme · Na^+ exclusion · Reactive oxygen species · Salt stress · *ZmHKT2*

Introduction

Maize (*Zea mays* L.) is an important cereal crop (Adhikari et al. 2016) that has many critical economic uses (AbdElgawad et al. 2016). World maize production should be doubled by 2050 to meet the consumption of an increasing population (Gondim et al. 2010), but maize production is being affected by several abiotic stresses (Köşkeroğlu and Tuna 2010). The occurrence of elevated levels of salts (especially NaCl) in the soil (salinity) constitutes a common environmental factor that significantly decreases maize yield (Hanks et al. 1978). Maize is a glycophytic plant and appears to be sensitive to salt stress (Farooq et al. 2015; Maas et al. 1983). Since sensitivity or tolerance varies even among individuals of same hybrid, it is imperative to ascertain how maize complies with salinity stress to develop salt-tolerant maize hybrids.

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Soils are considered salt affected, when the dissolvable salts and commutable sodium exceed in the root zone (Farooq et al. 2015). More than 0.4 billion hectares of land worldwide are affected by salinity and sodicity, respectively (Munns 2005). Acute salinity stress leads to grave damage to many cellular and physiological processes such as photosynthesis, nutrient uptake, water absorption, root growth, and cellular metabolism, all of which cause yield reduction (Chuamnakthong et al. 2019; Mekawy et al. 2015; Pardo 2010).

Osmosis and ion toxicity are two phases of salinity effect on plants (Munns 1993; Munns and Tester 2008). The osmotic phase represents the decrease in environmental water potential that reduces plant growth (Fortmeier and Schubert 1995; Munns and Tester 2008), while, the detrimental rise in Na^+ within the plant (ion toxicity) could impair cellular Na^+/K^+ homeostasis (Fricke et al. 2004; Zhang et al. 2018). Therefore, maintaining normal cytosolic Na^+/K^+ ratio with low accumulation of Na^+ in the cell cytoplasm plays a fundamental role in sustained plant salt tolerance during growth stages (Assaha et al. 2015; Munns and Tester 2008; Zhang et al. 2016). In plants, K^+ is more important than Na^+, whereas K^+ is substantial for the enhancement of membrane potential and turgor pressure, stimulation of enzymes, organizing of osmotic pressure, stomatal movement, and tropisms (Golldack et al. 2003; Pitann et al. 2013). The uncontrolled production of reactive oxygen species (ROS), such as superoxide radicals (O_2^-), hydroxyl radicals (OH^-), singlet oxygen ($^1O_2$), and concomitantly hydrogen peroxide (H_2O_2) occurs due to salinity stress, and leads to perturbations in cellular structures of mitochondria and chloroplasts, and causes oxidative damage in nucleic acids and proteins (AbdElgawad et al. 2016; Gondim et al. 2010; Köşkeroğlu and Tuna 2010).

Different mechanisms have been adapted by plants to cope with salinity stress damages. These include strategies that help alleviating ion toxicity such as, ion exclusion/sequestration, and osmotic stress such as buildup of compatible solutes (for example proline), allowing a balanced cellular pressure potential (Bohnert and Gensen 1996). Proline accumulation is a common feature of various genotypes under salt stress and it is beneficial to stressed plants as an important osmoprotectant, as well as ROS scavenger, although in some plants its accumulation is not correlated with salt tolerance (Lv et al. 2015).

Many genes encode different types of transporters and/or channels involved in the control of transmembrane Na^+ and K^+ fluxes in plants (Assaha et al. 2017; Wangsawang et al. 2018). These transporters are responsible for the exclusion of Na^+ from leaves, such as members of the plasma membrane-bound high-affinity K^+ transporters (HKTs), including the *Arabidopsis thaliana* HKT (AtHKT1;1), and its ortholog in maize (ZmHKT1), which mediate root–shoot Na^+ translocation by retrieving Na^+ from the xylem sap to the surrounding parenchyma cells (Munns et al. 2012; Ren et al. 2005; Zhang et al. 2018). There are many alleles for *HKT* genes which are associated with high levels of salt stress tolerance in maize and used in breeding for salt-tolerant hybrids and varieties (Jiang et al. 2018).

Salt tolerance is associated not only with the expression of HKT family genes but also with the activity of Na^+/H^+ exchanger (NHX) family that is localized in the vacuolar membrane (Pitann et al. 2013; Venema et al. 2002). It has been shown that high levels of Na^+ concentration in maize roots are positively related to the transcription of *ZmNHX* that sequestrates Na^+ from the cytoplasm into vacuoles, and consequently limits delivery of Na^+ to shoot via the xylem, leading to Na^+ exclusion from shoots (Zörb et al. 2005). Also, the expression of *NHX* genes in leaves enhances salt tolerance by sequestrating Na^+ into vacuoles and avoiding harmful effects such as reduced enzyme activity (Carden et al. 2003; Pitann et al. 2013).

Furthermore, the antioxidant enzyme system is one of the most important defense systems that protects plants from the harmful effects of salinity stress (Adhikari et al. 2016; Assaha et al. 2016; Mekawy et al. 2018a, b). In this system, superoxide dismutase (SOD) dissociates O_2^- to H_2O_2, which is either directly scavenged by catalase (CAT) or is used as substrate in other antioxidant enzyme pathways involving ascorbate peroxidase (APX), guaiacol peroxidase (GPX), and glutathione reductase (GR), thereby preventing it from amassing to toxic levels (Arora et al. 2008; Gondim et al. 2013).

In Egypt, salinity is a deleterious threat that significantly decreases maize production, especially in new reclaimed lands. In this context, the present study aimed to study the effects of salinity stress on growth, physiological adaptation, and the role of HKT and NHX transporters in salt resistance in two Egyptian maize hybrids.

Materials and Methods

Genotypes and Growth Conditions

Maize (*Zea mays* L.) kernels of two Egyptian white single crosses (SC131 and SC132) were acquired from the Maize Department, Field Crops Research Institute, Agricultural Research Center, Sakha Station, Egypt. These genotypes were selected from the germplasm collections based on their agronomic importance. The experiment was performed in the greenhouse of the Laboratory of Plant Nutritional Physiology, Faculty of Integrated Science for life, Hiroshima University, Japan under ambient conditions (25 °C/18 °C day/night temperature and 12/12-h light/dark cycle). Maize seeds were surface sterilized with a 2% sodium hypochlorite

(NaClO) solution for 30 min, which were then completely washed with distilled water. Seeds were posteriorly germinated on petri dishes in an incubator for 48 h at 28 °C. After germination, the seedlings were transplanted into soil in plastic pots (3 plants per pot) and kept for 5 days. Then the seedlings were later moved to a modified half-strength Hoagland nutrient solution in two separate 100 L containers (24 plants per container). The solution contained 1 mM KH_2PO_4, 2 mM $NH_4H_2PO_4$, 2 mM $MgSO_4{\cdot}7H_2O$, 6 mM KNO_3, 4 mM $Ca(NO_3)_2{\cdot}4H_2O$, 9.145 µM $MnCl_2{\cdot}4H_2O$, 3.52 µM $CuSO_4{\cdot}5H_2O$, 46.256 µM H_3BO_3, 0.496 µM $Na_2MoO_4{\cdot}2H_2O$, 0.765 µM $ZnSO_4{\cdot}7H_2O$, and 136.85 µM Fe-EDTA. After 4 days, the solution in both containers was changed to a full-strength; the first one was used as a control (0 mM NaCl) and the second one was supplied with 100 mM NaCl (salinity). The salt treatment was given gradually (50, and 100) at an interval of 4 days to avert membrane damage from osmotic shock (Shavrukov 2013), and the start of the salt (NaCl) treatment was considered from when the desired concentration of 100 mM NaCl was added to the salt-treated plants. The medium was subjected to constantly aeration using pumps and renewed every 4 days to avoid nutrients depletion, and the pH was checked daily to maintain the range of 5.5–6.0. The experiment lasted for 12 days after adding 100 mM NaCl.

Growth Traits Measurements

After 12 days of experimentation, the length (cm) of root and shoot was measured. Also, total dry weight of whole plant (shoot + root) was weighed after oven-drying for 72 h at 70 °C.

Determination of Na^+ and K^+ Concentrations

A flame photometer (ANA 135, Tokyo Photoelectric, Tokyo, Japan) was used to determine Na^+ and K^+ concentrations in leaves, stems, and roots as previously described (Mekawy et al. 2015). Briefly, dried samples (10 mg) were gently shaken in 0.1 N HCl for 24 h, and the concentrations of Na^+ and K^+ were calculated using curves generated from Na^+ and K^+ standard solutions.

Measurement of Hydrogen Peroxide (H_2O_2) Concentration

The H_2O_2 concentration was measured using Xylenol Orange (FOX) (Rhee et al. 2010). Briefly, liquid nitrogen was used to homogenize 0.5 g of leaf tissue with 4 mL of cold acetone followed by centrifugation at 8000*g* for 15 min at 4 °C. Then, 0.1 mL of the aqueous portion was transferred to a new tube containing FOX reagent, and incubated for 1 h at room temperature. Then the absorbance of the mixture was recorded at 560 nm. The H_2O_2 concentration in the extracts was then calculated based on the standard curve generated from known concentrations of H_2O_2.

Determination of Free Proline Concentration

Proline concentration was measured as previously described (Bates et al. 1973), using 0.5 g of leaf tissues. Briefly, the leaf tissue was homogenized with sulfosalicylic acid and then, 2 mL of the extracts was mixed with 2 mL of acid-ninhydrin in a glass tube and incubated at 100 °C in a water bath for 1 h. The samples were cooled down and kept in ice water, to which 4 mL of toluene was added and the mixture vigorously shaken to mix. Finally, the absorbance of the chromophore layer was recorded at 560 nm. Then standard concentrations of L-proline were used to calculate the proline concentration in the extracts.

Antioxidant Enzyme Activity

The activities of antioxidant enzymes were determined with reference to protein concentration in the extracts of enzymes which were determined by the Protein Assay kit with bovine serum albumin as the standard, and all the enzyme activities were expressed per amount of protein. Frozen tissues of leaves were homogenized using a mortar and pestle with liquid nitrogen, after which 5 mL of ice-cold extraction buffer [25 mM potassium phosphate, 0.5 mM EDTA, 1 mM ascorbic acid, 2% (w/v) PVPP (polyvinylpolypyrrolidone), pH 7.8] was added. After extraction, homogenized samples were centrifuged at 15,000*g* for 15 min at 4 °C and the supernatant was transferred to a new tube to determine antioxidant enzyme activities. Then the activities of catalase (CAT) (EC 1.11.1.6), ascorbate peroxidase (APX) (EC 1.11.1.11), soluble peroxidase (sPOD) (EC 1.11.1.7), glutathione reductase (GR) (EC 1.6.4.2), and superoxide dismutase (SOD) (EC: 1.15.1.1) were assessed as described previously (Beauchamp and Fridovich 1971; Chance and Maehly 1955; Takagi and Yamada 2013). For CAT activity, a decrease in H_2O_2 was monitored at 240 nm and the activity of CAT calculated as mmol H_2O_2 consumed per min. Oxidation of ascorbate was measured at 290 nm and its concentration estimated using the extinction coefficient of 2.8 mM^{-1} cm^{-1}. A unit of APX was quantified as µmol ascorbate oxidized per min. For sPOD, the enzyme activity was evaluated using the extinction coefficient (26.6 mM^{-1} cm^{-1}) for tetraguaiacol. A unit of sPOD activity was defined as mmol tetraguaiacol formed per min. To determine the activity of GR, the concentration of oxidized NADPH was calculated using the extinction coefficient (6.22 mM^{-1} cm^{-1}) and one unit GR activity defined as µmol NADPH oxidized per min. To detect SOD activity, 1.5 mL of the assay mixture (50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 µM

nitro blue tetrazolium (NBT), 2 µM riboflavin, and 3.3% enzyme extract) was prepared and illuminated for 15 min, while control samples were incubated in the dark. Then the absorbance of both set of samples was recorded at 560 nm. A unit of SOD was quantified and expressed as the amount of enzyme needed to produce a 50% inhibition of the NBT photo-reduction rate.

Gene Expression Analysis

Total RNA was extracted from the leaves and roots of control and stressed plants using total RNA Extraction Kit (Plant), following the manufacturer's instructions. Then DNaseI-treated RNA (1 µg) was reverse-transcribed to cDNA using a ReverTra Ace qPCR RT kit. Quantitative polymerase chain reaction (qPCR) was performed as previously described (Ueda et al. 2013), using a Thunderbird SYBR qPCR Mix and an ABI Step One Plus system. The quantitative RT-PCR was conducted for 40 cycles as follows: an initial denaturation at 95 °C for 1 min, followed by the denaturation at 95 °C for 15 s and extension at 60 °C for 60 s. The $2^{-\Delta\Delta CT}$ method was used to compare the expression level of *ZmHKT1;5, ZmHKT2* (root) and *ZmNHX1* (leaf and root) to that of *actin* (reference gene, whose expression did not significantly change: less than twofold change among samples) (Jain et al. 2006; Livak and Schmittgen 2001). A gene was considered repressed if the fold-change value is less than 1. The primers used in this expression analysis are given in Table 1. The qPCR outputs were analyzed in two ways: a melting curve analysis was conducted by increasing the temperature from 60 °C to 95 °C in order to detect the separation of double strand DNA; and by agarose gel electrophoresis.

Statistical Analysis

All collected data in this study were subjected to one-way analysis of variance (ANOVA) using the SPSS statistics package, version 22, and the means ($n=4$) were separated using Duncan's multiple range test at $p<0.05$.

Results

Growth Traits

Salinity stress decreased markedly the shoot length in SC131 and SC132 by 12.0% and 13.0%, respectively, compared with control (Fig. 1a). For SC131, the root length increased by 15.0%. On the contrary for SC132, root length decreased significantly by 27.0% compared to the control (Fig. 1b). A remarkable decrease in total dry weights of SC132 by 36.0% compared to 13.0% of SC131 in comparison with the controls, by salt treatment (Fig. 1c).

Table 1 Primers used for quantitative real-time RT-PCR

Genes	Forward primer (5´-3´)	Reverse primer (5´-3´)	References
ZmHKT1;5	TCAACTTCAGCGTCCTCAACA	GAATCCCACGTTGCCATACG	Jiang et al. (2018)
ZmHKT2	GCTCAACTTCTCCACGTTCA	CACCACCCAGAGAAGCTGTA	Cao et al. (2019)
ZmNHX1	ATGCAGGGTTCCAAGTGAAG	AATATTGCCCCAAGTGCAAG	Jiang et al. (2017)
ZmActin	GACCTCACCGACCACCTAATG	CTGAACCTTTCTGACCCAATG	Jiang et al. (2017)

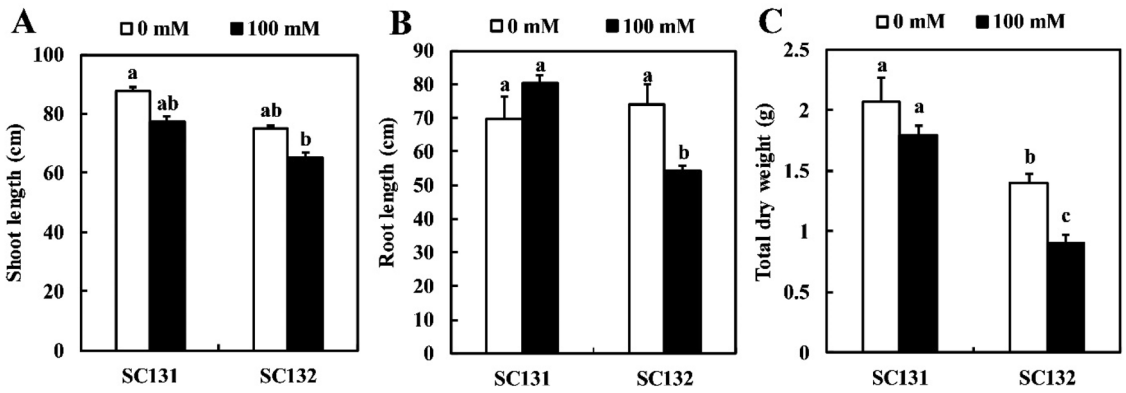

Fig. 1 **a** Shoot length, **b** Root length, and **c** Total dry weight of the maize hybrids SC131 and SC132 grown under control and salinity stress (100 mM NaCl) conditions for 12 days. Data represent the means of four replicates ± SE. The different letters indicate significant differences ($P<0.05$)

Na^+ and K^+ Concentrations

Data for the concentration of Na^+ and K^+, and the Na^+/K^+ ratio in root, stem, and leaf of the maize hybrids SC131 and SC132, under control and salt stress conditions are presented in Table 2. Salinity stress dramatically increased Na^+ concentration in the three examined parts of the two hybrids. The data showed that Na^+ concentration increased significantly in the leaf and stem of SC132 by 59% and 46% compared to that in SC131, respectively, under salt treatment. In roots, high Na^+ concentrations with no significant difference between the two hybrids was observed. The K^+ concentration was significantly decreased in all plant organs examined when the two hybrids were treated with 100 mM NaCl. However, this reduction in K^+ concentration was much severe in SC132. In leaves, compared to control conditions, the concentration of K^+ decreased by 26% in SC131 compared to that in SC132 (48%). The concentration of K^+ decreased more significantly in the stem of SC132 (24.5%), than in SC131 (7.5%). In roots, the concentration of K^+ significantly decreased in both hybrids, but the decrease was more marked in SC132 (64%) than in SC131 (49%). Hence, a higher Na^+/K^+ ratio in the leaves and stems of SC132 compared to those in SC131 under salt treatment was observed, while in root the Na^+/K^+ ratio did not significantly change in both hybrids.

Hydrogen Peroxide Concentrations

Under salt stress, H_2O_2 accumulates in plant tissues as one of the primary ROS. The H_2O_2 concentration was measured in leaves of both hybrids (Fig. 2). By salt treatment, H_2O_2 concentration markedly increased in SC132 (twofold higher) as compared to controls, while a slight non-significant increase of H_2O_2 concentration was observed for SC131 (Fig. 2).

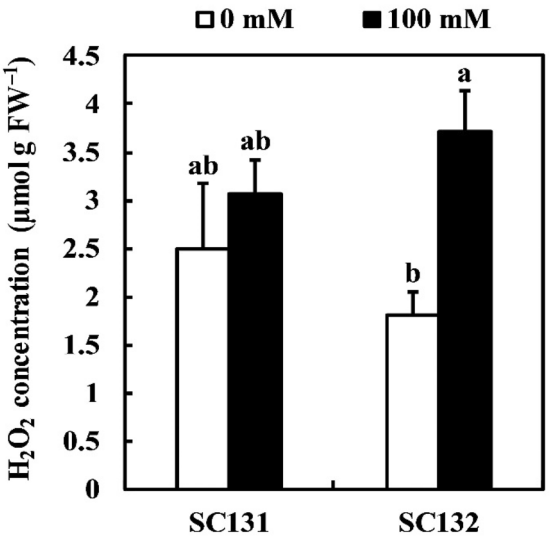

Fig. 2 Leaf H_2O_2 concentration of the maize hybrids SC131 and SC132 grown under control and salinity stress (100 mM NaCl) conditions for 12 days. Data represent the means of four replicates ± SE. The different letters indicate significant differences ($P < 0.05$)

Proline Concentrations

Proline concentration was determined in the leaves of both hybrids under control and salt treatments (Fig. 3). The results showed that 100 mM NaCl induced a significant increase in proline concentration (2.6-fold higher) in the leaves of SC131 as compared to those in SC132 (Fig. 3).

ROS Scavenging Enzymes

Leaf CAT activity increased appreciably for SC131and SC132 by 30% and 24%, respectively, in salt-stressed plants compared to those in control plants (Fig. 4a). Also, the salinity treatment markedly induced the APX activity in leaves of SC131 (45%) more than in SC132 (25%) as compared to controls (Fig. 4b). Leaf POD, GR, and SOD activities displayed a reduction in SC131 leaves (16, 16, and 29%,

Table 2 Na^+, K^+ concentrations, and Na^+/K^+ ratio in leaf, stem, and root of the maize hybrids SC131 and SC132 under control and salt stress conditions

Organ	Variety	Na^+ concentration (mg gDW^{-1})		K^+ concentration (mg gDW^{-1})		Na^+/K^+ ratio	
		Control	Salt stress	Control	Salt stress	Control	Salt stress
Leaf	SC131	0.5 ± 0.1^{c}	13.0 ± 3.2^{b}	104.0 ± 28.8^{a}	77.0 ± 4.3^{a}	0.005 ± 0.002^{c}	0.17 ± 0.031^{b}
	SC132	0.2 ± 0.0^{c}	31.8 ± 3.3^{a}	122.2 ± 20.5^{a}	63.2 ± 6.5^{a}	0.002 ± 0.0^{c}	0.5 ± 0.045^{a}
Stem	SC131	0.3 ± 0.2^{c}	31.1 ± 1.3^{b}	49.7 ± 5.2^{b}	46.0 ± 5.5^{b}	0.006 ± 0.002^{c}	0.67 ± 0.046^{b}
	SC132	0.1 ± 0.0^{c}	57.8 ± 5.5^{a}	81.6 ± 8.5^{a}	61.5 ± 10.2^{ab}	0.00122 ± 0.0^{c}	0.94 ± 0.059^{a}
Root	SC131	1.8 ± 0.1^{b}	108.2 ± 25.2^{a}	60.5 ± 19.0^{ab}	30.9 ± 4.7^{b}	0.03 ± 0.011^{b}	3.5 ± 0.46^{a}
	SC132	1.3 ± 0.5^{b}	112.6 ± 21.7^{a}	83.2 ± 25.2^{a}	30.2 ± 6.5^{b}	0.016 ± 0.008^{b}	3.7 ± 0.26^{a}

Data represent the means of four replicates ± SE. The different letters indicate significant differences ($P < 0.05$)

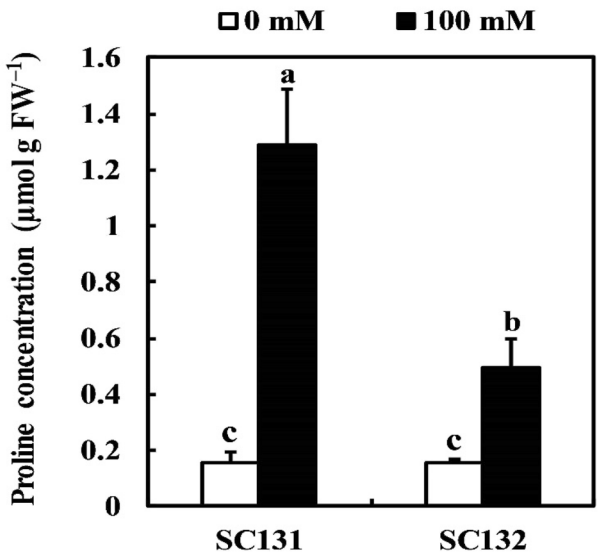

Fig. 3 Leaf proline concentration of the maize hybrids SC131 and SC132 grown under control and salinity stress (100 mM NaCl) conditions for 12 days. Data represent the means of four replicates ± SE. The different letters indicate significant differences ($P < 0.05$)

respectively), while showed increased activities in SC132 leaves (13, 35, and 23%, respectively) compared to controls (Fig. 4c–e).

Gene Expression Analysis

Expression of both *HKT* transporter and *NHX* antiporter genes was investigated to ascertain how these transporters are involved in salinity tolerance in maize. The quantitative RT-PCR analyses indicated that the expression of *ZmHKT1;5* was not significantly affected by salt stress in both SC131 and SC132 (Fig. 5a). Since there exist nucleotide variations in the *HKT* genes at positions A134G and A511G, which are known to affect the properties of Na^+ and K^+ transportation, and hence salt tolerance in maize, the nucleotide sequence of the coding region of the *ZmHKT1;5* gene was examined in both SC131 and SC132. The DNA sequencing analysis revealed that these two varieties have no nucleotide variations at positions 134 and 511, and both varieties had an adenine (A) at positions 134 and 511 of the *ZmHKT1;5* gene.

To assess the contribution of ZmHKT2 in Na^+ and K^+ accumulation in the leaf of maize, expression of the *ZmHKT2* gene in roots was examined. *ZmHKT2* was highly induced in SC132 by 5.6-folds, while its transcripts were not detected in SC131 (Fig. 5b). Data revealed that the expression of *ZmHKT2* gene (5.6-folds) was higher than that of *ZmHKT1;5* (1.7-folds) in the roots of SC132 (Fig. 5a, b), which also has major implications in the increase in Na^+/K^+ ratio in leaves (Table 2). Also, the expression of *ZmNHX1* was analyzed in the leaves and roots of both SC131 and SC132 hybrids (Fig. 5c, d), and it was found to be induced by salinity stress in the leaves of SC132 by 2.4-folds (Fig. 5c). Conversely, the expression of *ZmNHX1* was repressed (< onefold expression) in the leaves and roots of SC131 (Fig. 5c, d).

Discussion

In the present study, two commercial Egyptian maize hybrids (SC131 and SC132) were used to illuminate their response to salinity stress via various physiological, biochemical, and transcriptional analyses. The two hybrids portrayed varying responses under short-term salt stress (100 mM NaCl for 12 days), with SC131 being more tolerant than SC132 in terms of dry matter accumulation. There are several salt-tolerant strategies employed by plants to adapt and grow on salty soils, such as root and shoot Na^+ exclusion (Munns et al. 2020b; Munns and Tester 2008), osmolyte accumulation for osmotic adjustment (Munns et al. 2020a), enhanced ROS scavenging by antioxidant enzymes and non-enzymes (Gill and Tuteja 2010), and vacuolar Na^+ sequestration (Munns et al. 2020a). Thus, it is possible that SC131 exhibited one or more of these adaptive strategies to out-grow SC132 under salt stress. Therefore, in the current study, measurements were carried out to ascertain the components of the stress tolerance exhibited by the SC131 hybrid.

Generally, upon introduction of salt in a growth environment, there is a buildup of ions, especially Na^+ in the soil, which tends to disrupt water uptake due to difference in osmotic potential between root cells and the external soil solution, and an increasing tendency of Na^+ uptake down an electrochemical gradient. Faced with this influx of Na^+, root cells need to rapidly extrude the excess Na^+ in favor of water uptake, such that only a very minimal amount will reach the leaf tissues via the xylem (Munns et al. 2020b). In addition to this rapid root Na^+ extrusion, retrieval of Na^+ from the xylem into neighboring xylem parenchyma cells, and Na^+ recirculation via the phloem from leaf to root, both mediated by HKT, and its eventual extrusion to soil mediated by SOS1 (Assaha et al. 2017; Munns and Tester 2008), are also very important in avoiding excessive Na^+ accumulation in the leaf. These collectively contribute to shoot Na^+ exclusion, which stands out as one of the most critical mechanisms involved in salt stress adaptation, especially in glycophytes under salt stress (Assaha et al. 2017). In the present study, both hybrids showed reduced Na^+ concentrations in the leaves and higher concentrations in the stems and roots characteristic of leaf Na^+ exclusion. However, the two hybrids exhibited marked differences in Na^+ concentration in the leaves, with SC132 having more than twice that of SC131 (Table 2). This result shows clearly

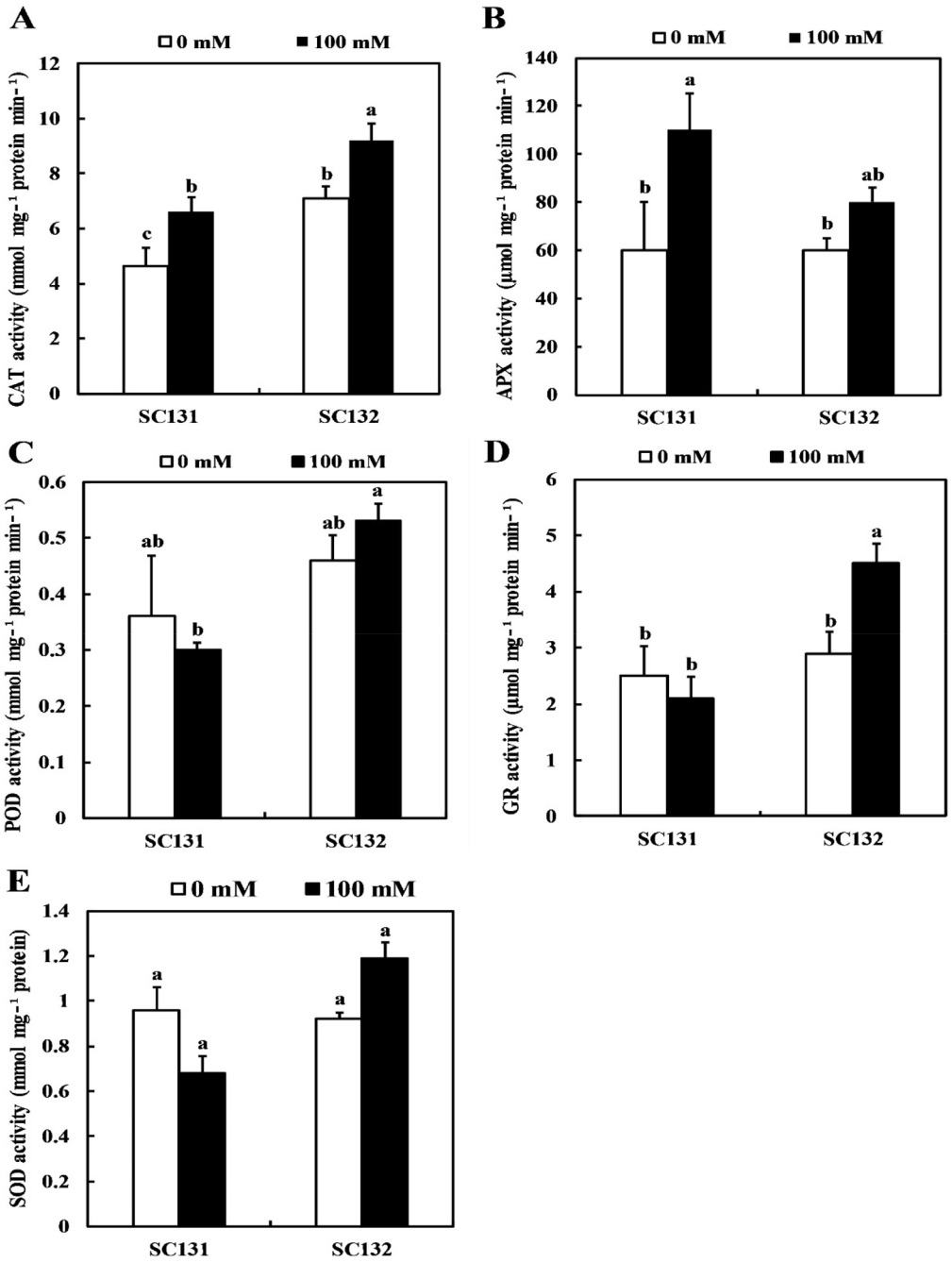

Fig. 4 Activity of the antioxidant enzymes, **a** Catalase (CAT), **b** Ascorbate peroxidase (APX), **c** Peroxidase (POD), **d** Glutathione reductase (GR), and **e** Superoxide dismutase (SOD) in the leaves of the maize hybrids SC131 and SC132 grown under control and salinity stress (100 mM NaCl) conditions for 12 days. Data represent the means of four replicates ± SE. The different letters indicate significant differences ($P < 0.05$)

that SC131 is more efficient at excluding Na^+ from the leaf than SC132. Na^+ exclusion from the leaf blade often correlates with enhanced salt stress tolerance and brought about by HKT, such as OsHKT1;5 in rice (Mekawy et al. 2015; Suzuki et al. 2016), SsHKT in *Solanum scabrum* (Assaha et al. 2015), HvHKT1;5 in barley (van Bezouw et al. 2019), and TaHKT1;4/5 in wheat (Munns et al. 2012). In maize, the *Zea mays* ZmHKT1;5 is known to be important for salt stress adaptation, by limiting shoot Na^+ concentrations, thereby improving the Na^+/K^+ ratio (Jiang et al. 2018). This ZmHKT1;5 had 2 SNPs (A134G and A511G), which were found to be linked to differential salinity tolerance levels in maize varieties. In addition, ZmHKT1;5 in transgenic tobacco not only improved the Na^+/K^+ ratio, but also

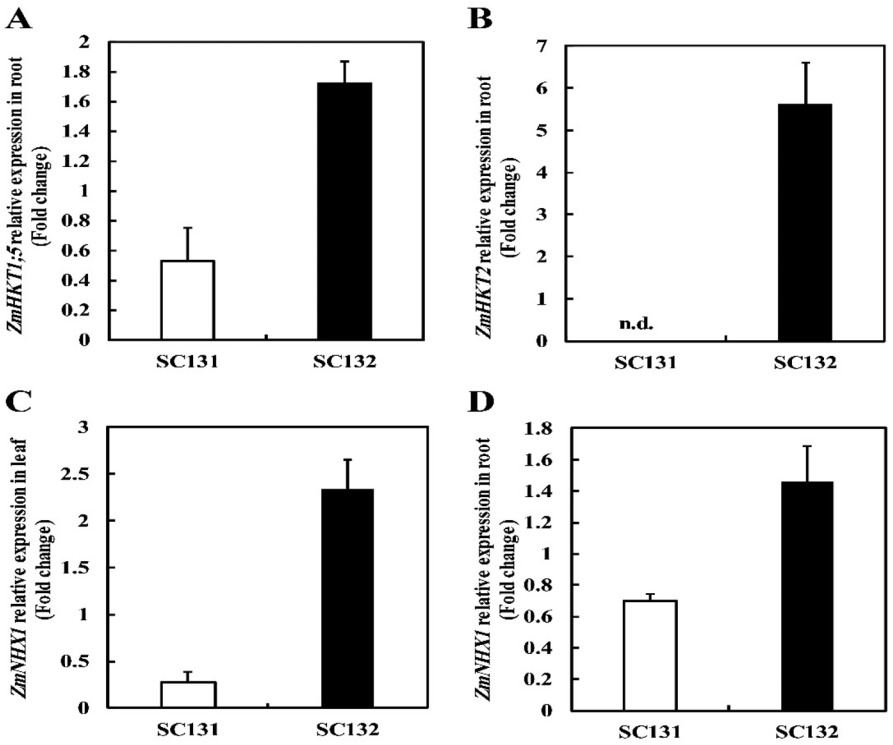

Fig. 5 Relative expression of the genes encoding Na^+ transporter proteins, **a** *ZmHKT1;5* in roots, **b** *ZmHKT2* in roots, **c** *ZmNHX1* in leaves, and **d** *ZmNHX1* in roots of the maize hybrids SC131 and SC132 grown under control and salinity stress (100 mM NaCl) conditions for 12 days. Data represent the means of two independent experiments ± SE, (n.d.) not detected

alleviated oxidative stress in the plants, indicating diverse roles of the HKT in stress tolerance of maize. In the present study, *ZmHKT1;5* was induced in SC132, but not in SC131, suggesting that it is involved in shoot Na^+ exclusion in SC132, but not in SC131 and that other transporters may be involved in SC131, which need to be investigated, especially as the Na^+/K^+ ratio was better for SC131 (0.17) than SC132 (0.5). Also, the *ZmHKT1;5* gene in the present study had no SNPs, suggesting that it may not be involved in salt stress tolerance in these hybrids, and therefore there is need for characterization of Na^+ transporters involved in Na^+ exclusion in these maize hybrids. It is however, clear that differential Na^+ exclusion in the leaf blade contributed to the differential tolerance in the two hybrids.

This difference in leaf Na^+ concentration may be attributed to differential expression of *HKT2* in root (Fig. 5b), where SC132 showed marked expression (> 5-folds), whereas it was not detected in SC131. Class II HKTs (HKT2) mostly favor K^+ transport over Na^+ under low Na^+ concentrations, due to the GGGG motive of the selective filter pore domain, as opposed to SGGG in HKT1, which favors Na^+ over K^+ transport (Hauser and Horie 2010). In rice, for example, OsHKT2;1 intervenes in root Na^+ influx and consequently reduced tolerance to salt stress (Garciadeblás et al. 2003; Mekawy et al. 2015). Thus, repression of such HKTs under salt stress may confer salt stress tolerance (Assaha et al. 2017). In addition, ZmHKT2 was recently (Cao et al. 2019) shown to be a K^+-selective transporter, with Na^+ transport activity only under conditions of 100 mM NaCl and much lower K^+ concentrations. The ZmHKT2 in the study by Cao et al. (2019) was localized to the plasma membrane of xylem parenchyma cells of the stele and responsible for K^+ retrieval from the xylem, with reduced K^+ delivery to the shoot, and hence reduced tolerance to salt stress. Furthermore, in the same study, *ZmHKT2* knockout mutants were more tolerant than wild type plants, with better shoot K^+ concentration than wild-type plants. In the current study, *ZmHKT2* expression was found only in the root of SC132, with higher Na^+ and lesser K^+ concentration in the leaves (Table 2) than SC131, in which the gene was not detected (Fig. 5b). Thus, it is possible that ZmHKT2 is responsible for reduced leaf K^+ concentration, and enhanced Na^+ uptake in the root and later more translocation to the shoot, leading to reduced tolerance compared to SC131. Thus, the repressed expression of a xylem K^+ retrieving and Na^+ influx transporter genes or their absence would constitute an important salinity tolerance mechanism in SC131.

Once Na^+ gets to the leaf blade and is not immediately taken care of, especially excess cytosolic Na^+, gross physiological perturbations can occur leading to growth inhibition under salt stress. Thus, cytosolic Na^+/K^+ balance under salt stress is central to maintaining optimal physiological functions and hence enhanced salt tolerance (Anschütz et al. 2014; Assaha et al. 2017). To achieve this balance, plants sequester the excess Na^+ into vacuoles and use it for low-energy osmotic adjustment (Munns et al. 2020a). It is widely believed that NHX1 and NHX2 are primarily responsible for vacuolar Na^+ sequestration under elevated salinity

(Nieves-Cordones et al. 2016). The enhanced expression of NHX under conditions of high salinity should therefore correspond to salt stress tolerance and repression to susceptibility (Assaha et al. 2017). Data on NHX expression analysis and function in maize are very scanty and variable. Zörb et al. (2005) studied the expression of NHXs in maize hybrids and found that ZmNHX1,2 and 6 expressions were root specific, and coincided with an increase in NaCl concentration in the root medium of up to 13-fold increase in expression under 100 mM NaCl in hydroponic culture. This result indicates that vacuolar Na^+ sequestration in the leaf of maize may not be operational, but is fully functional in the root. In another study, Pitann et al. (2013) showed that the difference in salt tolerance between maize hybrids owed to differences in expression of NHX in leaf and roots, where the tolerant hybrids had higher expressions in both tissues, with enhanced shoot Na^+ exclusion driven by root vacuolar Na^+ sequestration, and Na^+ sequestration in the leaves. Additionally, a recent study (Mekawy et al. 2015) showed that under salt stress conditions (50 mM NaCl for two weeks), the rice gene *OsNHX1* expression was induced in the leaf of the sensitive rice cultivar (Sakha 102), which had high Na^+ concentrations, but repressed in the tolerant one (Egyptian Yasmine), which had lower Na^+ concentrations, suggesting that the expression of *OsNHX1* in these cultivars would be mainly a symptomatic response to elevated Na^+ levels. In the current study, the expression of NHX1 was detected in both leaf and root tissues, contrary to the observations of Zörb et al. (2005). However, the expression was upregulated in the root (1.5-fold) and leaf (twofold) in SC132, it was repressed in both tissues of SC131. In comparison with previous expression data for NHX1 (13-fold) expression under 100 mM NaCl, (Zörb et al. 2005), the data on the expression levels in the present study may not be sufficient to impart significant stress tolerance in the SC132 hybrid. This further suggests that vacuolar Na^+ compartmentation may not be an essential salt tolerance trait in these two hybrids.

Aside tissue Na^+ toxicity, overproduction and accumulation of reactive oxygen species (ROS) under salt stress is an important stress effector, which inflicts tremendous damages to plants, by for example damaging proteins, nucleic acids, and biomembranes (Gill and Tuteja 2010). Hydrogen peroxide (H_2O_2), a product of superoxide anion dismutation is a direct indicator of ROS production in plants under salt stress and its accumulation is often associated with salt stress susceptibility (Esfandiari and Gohari 2017). In the present study, the H_2O_2 concentration was twofold higher than control levels in SC132, but did not significantly change in SC131. This clearly suggests that SC132 likely suffered more oxidative stress damage under salt stress in the leaf than SC131. This susceptibility may be attributed to a poor ROS scavenging system, as the activities of the antioxidant enzymes POD, APX, and SOD remained unaltered, while that of CAT and GR although increased (Fig. 4), but appeared insufficient to abate the situation. A similar trend was observed for SC131, but only CAT and APX were enhanced, which can offer more protection as they directly use H_2O_2 as substrate in their scavenging pathways (Gill and Tuteja 2010; Mekawy et al. 2020). Moreover, SC131 might have benefited from enhanced proline concentration (eightfold increase with respect to control, Fig. 3) to scavenge more ROS. The ROS scavenging role of proline under salt stress is well established (Banu et al. 2009; Smirnoff and Cumbes 1989; Vass and Rehman 2018). Obviously, SC132 lacked this capacity as the leaf proline concentration did not significantly increase.

Besides its role in ROS detoxification, proline accumulation under salt stress has been more associated with osmoregulation (Munns and Tester 2008). The need to maintain water uptake for normal metabolic functions is imperative to withstand salt stress, where Na^+ accumulation in the soil and within the plant generates more negative potentials that prevent water uptake. Therefore, plants synthesize organic solutes such as proline to balance this potential and facilitate water uptake. For this reason, proline accumulation under conditions of high salinity is closely linked to salt stress tolerance (Mekawy et al. 2020; Munns and Tester 2008). However, in some sensitive plants proline accumulation becomes symptom of the stress rather than adaptive factor (Lv et al. 2015). In the present study, proline concentration markedly increased in the tolerant hybrid (SC131), but remained unaltered in the sensitive one (SC132), indicating that it is required for salt stress adaptation in SC131, but not in SC132.

In conclusion, the difference in tolerance between two maize hybrids SC131 (tolerant) and SC132 (sensitive), subjected to 100 mM NaCl stress in a hydroponic culture, rests on two main strategies: **1**. Regulation of Na^+ and K^+ translocation to the shoot, which is weaker in SC132, owing to the expression of a potential xylem K^+ retrieving and Na^+ influx transporter gene (*ZmHKT2*) in the root, and the absence of this transporter in the root of SC131. However, further studies are needed to fully understand the mechanisms underlying shoot Na^+ exclusion in these two hybrids. **2**. Enhanced ROS detoxification in SC131 as revealed by unaltered H_2O_2 concentration, coupled with an increase (eightfold) in proline concentration that is a well-known ROS scavenger. These ROS protective measures were more or less absent in SC132, thus exposing it to the detrimental effects of high salinity. Hence, the maize hybrid SC131 could become a new candidate to consider in maize breeding programs for sustainable maize production under salt-affected soil conditions.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interests.

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