# The role of $Na^+/H^+$ antiporter activity in leaf cells of newly developed maize (*Zea mays* L.) hybrids for sodium inclusion and salt resistance

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*Submitted by*

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**To my Father in spirit whom I always remember**

**And my dear mother for her love**

**And to my wife who helped me to finish this work**

**And finally to my kids**

**Ahmed, Moustafa and Salma**

**I wish them a good life in future Inshallah**

## List of Abbreviation

| | |
|---|---|
| ΔF | Decrease in fluorescence |
| A | Ampere |
| AAS | Atomic absorption spectrophotometer |
| ABA | Abscisic Acid |
| ACMA | 9-amino-6-chloro-2-methoxyacridine |
| bp | Base pair |
| BSA | Bovine serim albumin |
| CAM | Crassulacean acid metabolism |
| cm | Centimeter |
| CNGC | Cyclic nucleotide-gated channel |
| DEPC | Diethylepyrocarbonate |
| dS | dezi Siemens |
| DTT | Dithiothreitol |
| DW | Dry weight |
| e.g. | Exempli gratia (for example) |
| EC | Electric conductivity |
| g | Gram |
| GLR | Glutamate receptor |
| h | Hour |
| HAK | High-affinity potassium transporter |
| HKG | House keeping gene |
| HKT | High-affinity potassium transporter |
| KUP | Potassium uptake permease |
| LCT | Low-affinity cation transporter |
| M | Molar |
| mg | Milligram |
| min | Minute |
| mL | Millilitre |
| mM | Millimolar |
| mm | Millimetre |
| NaCl | Sodium Chloride |
| NaExIL | Sodium Excluding Inbredline |
| NHX | Sodium proton antiporter |
| nm | Nanometre |
| nmol | Nanomol |
| NTC | No template control |
| P | Probability value |
| PCR | Polymerase chain reaction |
| $P_i$ | Inorganic phosphorus |
| r | Pearson product-moment correlation coefficient |

| | |
|---|---|
| ROS | Reactive oxygen species |
| rpm | Rounds per minute |
| RT-PCR | Real time-Polymerase chain reaction |
| s | Second |
| SDS | Sodium dodecyl sulphate |
| SE | Standard error |
| SWS | Südwestsaat |
| UV | Ultra violett |
| VIC | Voltage-independent channel |
| µg | Microgram |
| µL | Microlitre |
| µM | micromolar |

# 1 Introduction

Salinity affects 6% of the world's total land area i.e. approximately 800 million hectares, and about 3 ha of arable land are lost due to salinity in each minute (FAO 2008). Accumulation of soluble salts results in salinization of soils, thus hinders plant growth and leads to reduction of world food production (Munns 2005). Soil salinity is measured in terms of electric conductivity (EC), and soils with an EC > 4 dS $m^{-1}$, which is equivalent to 40 mM NaCl, are considered as saline. Maize is one of the major cereal crops and is considered as moderately salt-sensitive (Rhoades et al. 1992). In order to increase salt resistance of maize and other higher plants, we need to understand the physiological mechanisms responsible for reduction in plant growth under salt stress.

## 1.1 Two-phase model for inhibition of plant growth under salt stress

According to Munns (1993), growth reduction in plants occurs in two phases (Fig. 1). In the first phase, high concentrations of NaCl in the soil hinder plants to take up water and lead to stunted shoot growth, small dark-green leaves, decreased photosynthesis, respiratory changes, and loss of cellular integrity. In the second phase, specific ion toxicity causes biochemical perturbations, tissue necrosis (Eker et al. 2006), hampers growth, finally leads to plant death (Cheeseman 1988; Tuteja 2007). The growth reduction is regulated by root signals (e.g. ABA) in the first phase (Shahzad pers. comm.) whereas in the second phase a lower photosynthetic capacity of the plant causes further growth reduction (Munns and Tester 2008) due to salt accumulation to toxic levels in transpiring leaves. These toxic levels are determined by the ability of the plant to sequester their ions from the cytoplasm of the leaf cells into the vacuoles, which is an important strategy to reduce ion toxicity in the cytoplasm (Flowers and Hajibagheri 2001). In contrast, when plants are not able to sequester $Na^+$ ions into leaf vacuoles, the concentration of $Na^+$ will increase in the leaf cytoplasm, causing negative effects on leaf growth and impairing leaf-cell metabolism, e.g. enzyme function (Flowers et al. 1977).

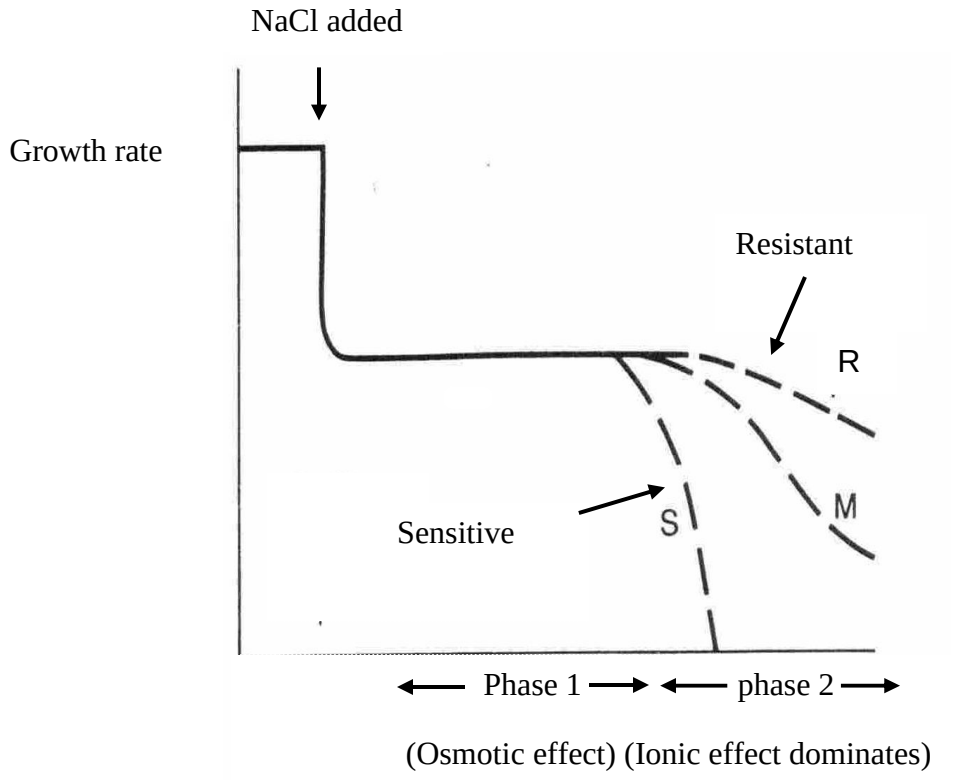


**Figure 1:** The biphasic model describes a two-phase growth response of plants treated with NaCl. **(S)** salt-sensitive, **(M)** moderate salt-resistant and **(R)** salt-resistant maize genotypes.

In order to avoid salt-induced growth reduction and toxicity symptoms in both phases, halophytic plants have evolved mechanisms to adapt to high salinity levels. During the last years, progress has been made in introducing salt resistance into plants ( Zhang and Blumwald 2001; Møller et al. 2009; Schubert et al. 2009). Two different approaches were adopted: 1) the transgenic manipulation of crops by altering the expression levels of genes or by incorporating alien genes and 2) the combination of various strategies of salt-resistance by classical breeding. Improvement of the salt-sensitive maize would be of considerable value and screening methods are immediately needed to determine salt-resistant genotypes (Carpici et al. 2010). To improve salt resistance especially in the second phase of salt stress, Schubert et al. (2009) established salt-resistant SR maize hybrids in which all three resistance mechanisms were combined to various degrees. After selfing of the efficiently $Na^+$-excluding maize hybrid Pioneer 3906, those individuals of the F2 generation were selected for ongoing selfing and recurrent selections which

showed improved $Na^+$ exclusion by the root surface combined with low $Na^+$ root-to-shoot translocation. The emerging homogeneous inbred line NaExIl demonstrated significantly reduced $Na^+$ uptake and root-to-shoot translocation, but showed only poor osmotic resistance. To overcome this problem, NaExIl was crossed with different inbred lines showing osmotic resistance. The newly developed salt-resistant maize hybrids showed not only improved growth in the first phase of salt stress but also high performance in the second phase (Schubert et al. 2009). For example, salt-resistant SR 03 can grow at NaCl concentration up to 200 mM while Pioneer 3906 is moderately salt-sensitive and shows severe growth reduction at 100 mM NaCl.

### 1.2 Physiological adaptations to salinity

Salt resistance is defined as the ability of plant to maintain growth and normal metabolism under salt stress Yeo (1983). The responses of plants to salinity are divided into two main mechanisms namely avoidance and tolerance mechanisms as reported by Levitt (1980). Mechanisms for salt resistance can be attributed to a number of strategies (Gorham 1995); (I) limited $Na^+$ uptake, (II) reduction of $Na^+$ concentration in cytoplasm in order to prevent toxic levels in the transpiring leaves.

#### 1.2.1 Osmotic stress resistance

In general, growth reduction in the first phase of salt stress is a strategy to grow and it occurs in a genotypically dependent manner (Munns and Tester 2008). Due to a lower water potential, plant cells transiently loose water but regain their original turgor owing to osmotic adjustment. These changes in plant growth are similar to drought stress responses (Munns 2002b). Furthermore, there are evidences that the inhibition of proton pumping of the plasma membrane $H^+$-ATPase (Pitann et al. 2009b; Zörb et al. 2005b; Hatzig et al. 2010) and thus a reduced apoplastic acidification (Pitann et al. 2009a) cause a reduction in shoot growth of maize. However, it was also shown that the growth of the resistant genotype SR 03 and salt-resistant sugar beet (Wakeel et al. 2010) was also significantly reduced even though they maintained a low apoplastic pH, a premise for acid-growth (Hager et al. 1991). Therefore, additional factors may contribute to

limited cell-wall extensibility (Cramer and Bowman 1991; Hatzig et al. 2010; Pitann et al. 2009a; Wakeel et al. 2010).

Osmotic resistance of the cell is achieved by compatible solutes such as proline, saccharides, glycine-betaine, and glycerol. They are accumulated in the cytosol of plant cells, decrease the osmotic potential (Chinnusamy et al. 2005), and balance the increased osmolality of the apoplast or the vacuole thereby avoiding dehydration of the cytoplasm (Aziz and Khan 2003; Ashraf and Harris 2004). These compatible solutes also help maintaining protein membrane structure and thus protect biochemical reactions from inorganic ion damage by detoxification of reactive oxygen species (ROS) (Skopelitis et al. 2006). In this context, a positive correlation between salt resistance and the accumulation of osmoprotectants in maize plants was found (Saneoka et al. 1995).

### 1.2.2 Ion toxicity avoidance

Excessive $Na^+$ concentration in the plant shoot accounts for specific ion toxicity effects (Levitt 1980). Chlorotic and necrotic symptoms on the tips and margins of the older leaves have been observed in many plant species (Eker et al. 2006; Munns 2002a). These symptoms are due to continuous accumulation of toxic $Na^+$ inside the plant tissues leading to growth reduction (Mühling and Läuchli 2002) and a reduced yield (Schubert et al. 2009). Ion toxicity can contribute to plant-growth reduction not only in the second phase but also during the first phase of salt stress (Sümer et al. 2004). In contrast to salt-sensitive genotypes, salt-resistant ones show only slight toxicity symptoms and better shoot growth. Severe plant damage occurs when high cytoplasmic $Na^+$ concentrations resulted in displacement of $K^+$ causing a reduction in enzyme activation (Anil et al. 2007), and disturbance of stomatal aperture (Slabu et al. 2009) and chloroplast function (Marschner and Mix 1973). Fricke et al. (2004) observed that in some salt-resistant plants stomata remain closed to ameliorate tissue dehydration by reducing water loss, limiting the accumulation of toxic ions inside plant tissues (Veselov et al. 2008). There are some strategies commonly used by plants to maintain optimal $K^+/Na^+$ ratios in the cytosol (Tester and Davenport 2003), which include regulation of $K^+$ uptake and/or minimizing $Na^+$ entry, efflux of $Na^+$ from the cell, and utilization of $Na^+$ for osmotic adjustment. Plant salt resistance has three strategies to prevent $Na^+$ concentration in leaves: (1) $Na^+$ exclusion by the root, (2) $Na^+$ exclusion from the shoot and (3) $Na^+$ compartmention in leaf vacuoles.

### 1.2.2.1 $Na^+$ exclusion by the root

$Na^+$ exclusion from the roots ensures that $Na^+$ does not accumulate to toxic levels inside plant cells. If plant species are efficient in $Na^+$ exclusion at the root surface they can resist high salt concentrations outside the cell. This strategy includes the following mechanisms: (a) restricted $Na^+$ influx, (b) $Na^+$ efflux from the root.

**(a) Restricted $Na^+$ influx**

High $Na^+$ concentration in the soil and the negative voltage in cytosol of plant cell (-140 mV) will favor a passive influx of $Na^+$ into the cytosol of root cortical cells (Cheeseman 1982). In this case, $Na^+$ enters the root cell through various ion channels and other transporters in the plasma membrane (Tester and Davenport 2003). Sodium exclusion by the root surface minimizes $Na^+$entry into the root (Blumwald et al. 2000) and reduces the capacity of $Na^+$-mediating channels in the plasma membrane (Munns 2002a), thus, becoming more resistant (Schubert and Läuchli 1990) (Fig. 2A).

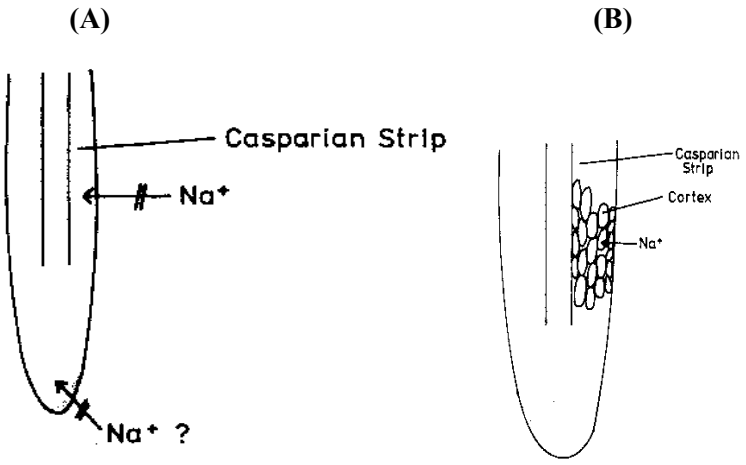


**Figure 2A:** $Na^+$ exclusion by the root surface **(A),** $Na^+$ inclusion in the cortical root vacuoles **(B)** (Schubert and Läuchli 1990).

As mentioned by Apse and Blumwald (2007), high-affinity transport for both $Na^+$ and $K^+$ is mediated by high-affinity $K^+$ transporters (*HKT*) which rapidly saturate the system. There are also other candidate channels such as cyclic nucleotide-gated channels (CNGC) (Leng et al. 2002; Gobert et al. 2006), glutamate-activated channels (*GLR*) (Cheffings 2001; Qi et al. 2006)

and low-affinity cation transporters (*LCT*1) for $Na^+$ transport. These channels play an important role in increasing cation influx and are hypersensitive to $Na^+$ in yeast cells. This mechanism is a non-saturating low affinity transport system (Schachtman et al. 1997; Amtmann et al. 2001). On the other hand, Platten et al. (2006) and Grabov (2007) suggested that there is a potential transporter mediating the influx of $Na^+$ through non-selective cation channels which are encoded by the *HKT*, *KUP* and *HAK* gene families.

**(b) $Na^+$ efflux and $Na^+$ inclusion in root vacuoles**

Efflux of $Na^+$ from plant roots is an important mechanism to improve salt resistance by minimizing $Na^+$ concentration in the root cells. In order to protect the cytosol from toxic $Na^+$ effects, sequester $Na^+$ ions into apoplast by a plasma membrane-associated $Na^+/H^+$-antiporter or into root vacuoles by a tonoplast-associated $Na^+/H^+$-antiporter (Amtmann and Sanders 1999; Blumwald and Poole 1985) plays an impotent role. In the case of plasma membrane-associated $Na^+/H^+$-antiporter, a primary active P-ATPase uses the energy of ATP hydrolysis to pump $H^+$ out of the cell generating an electrochemical $H^+$ gradient. This generated proton motive force energizer the secondary active $Na^+/H^+$ antiport to transport excess $Na^+$ out of the cytosol, thereby reducing its toxic effects inside the cytosol. The primary active V-ATPase and V-PPase (pyrophosphatase) also energize the tonoplast for secondary active transport of $Na^+$ into the vacuole by $Na^+/H^+$ antiport (Yamaguchi and Blumwald 2005; Gaxiola et al. 2002), and lead to salt resistance in the plants, (Fig. 2B). Compartmentation of $Na^+$ in root vacuoles as was shown for *Arabidopsis* (Apse et al. 1999; Pardo et al. 2006; Sottosanto et al. 2007) is achieved by tonoplast $Na^+/H^+$-antiporters which belong to the $Na^+/H^+$ exchanger (NHX) family, which are driven by a proton gradient generated by vacuolar $H^+$-ATPases and pyrophosphatases (Blumwald and Poole 1985; Yamaguchi and Blumwald 2005). A direct relation between salt resistance and $Na^+$ sequestration was already confirmed for *Arabidopsis*, sugar beet, sunflower and potato, in which relative transcription of *NHX* was generally increased in salt-resistant plant genotypes (Apse et al. 1999; Ballesteros et al. 1997; Blumwald and Poole 1985; Queiros et al. 2009). Likewise, transcript levels of the tonoplast $Na^+/H^+$ antiporters in maize (*ZmNHX*) have been investigated in roots of an $Na^+$-excluding inbred line (NaExIL) (Zörb et al. 2005a; Zörb et al. 2001). These authors reported that relative transcription of *ZmNHX* in roots was positively related to increasing root $Na^+$ concentrations, thus limiting $Na^+$ transport to the root xylem of maize under increasing levels of salinity and excluding $Na^+$ from the shoot. The $Na^+$ efflux at

root level not only protects the cell from toxic effects of $Na^+$ but also plays an important role for decreased $Na^+$ translocation to the shoot.

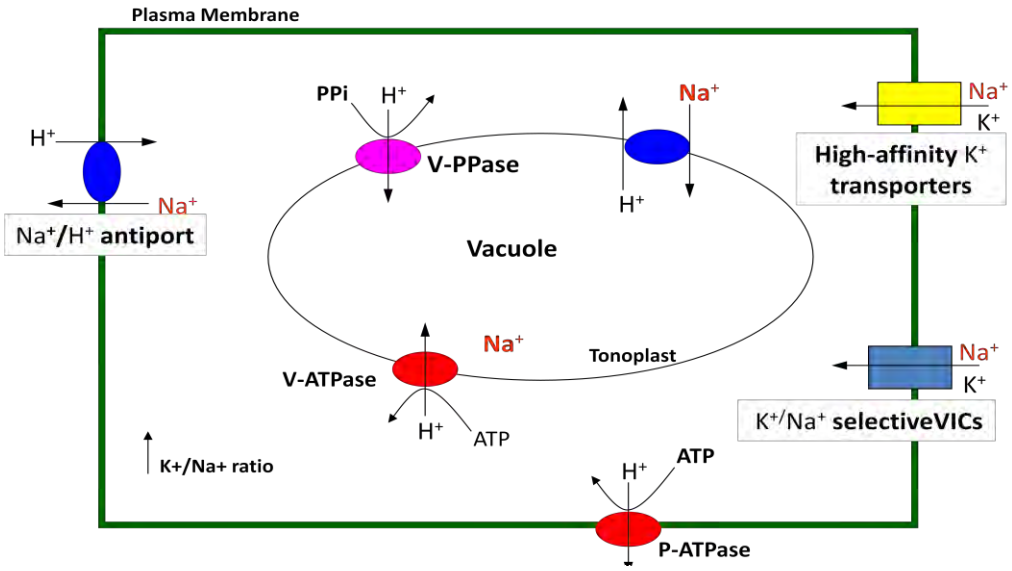


**Figure 2B:** Developing salt-resistance crop plants: challenges and opportunities; $Na^+$ inclusion strategy. Adapted from Mansour et al. (2003)

### 1.2.2.2 $Na^+$ exclusion from the shoot

In most plants, the leaf blade is the most sensitive part, and develops toxicity symptoms when $Na^+$ is translocated to the shoot via xylem. Therefore all mechanisms which lead to $Na^+$ exclusion from the shoot contribute to salt resistance (Munns 2005). Sodium translocation depends much on the influx into the root cortex and efflux back into the rhizosphere. While passive entry of $Na^+$ is restricted by strong discrimination between $K^+$ and $Na^+$ at the plasma membrane by ion-specific channels (Fulgenzi et al. 2008; Mäser et al. 2002), active efflux via plasma membrane $Na^+/H^+$ antiporters has been shown for higher plants (Tester and Davenport 2003). Accordingly, Schubert and Läuchli (1990) were able to show the active efflux at the root surface of maize, but both the more efficiently $Na^+$-excluding genotype Pioneer 3906 and the less efficiently $Na^+$-excluding genotype XL75 showed similar efflux rates. It was suggested that

xylem parenchyma cells play an important role in $Na^+$-compartmentation in roots (Yeo et al. 1977). Retrieval of $Na^+$ from xylem into xylem parenchyma cells (Fig. 3) is suggested to be mediated by members of the HKT family (Davenport et al. 2007), followed by an immediate sequestration into the vacuoles by $Na^+/H^+$-antiporters. However, findings by Neubert et al. (2005) for maize indicate that sequestration into the root cortex vacuoles may predominantly cause exclusion from the shoot due to reduced $Na^+$ translocation to shoot.

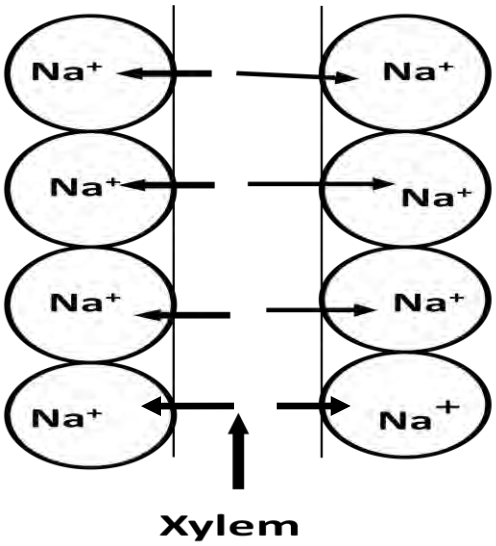


**Figure 3:** $Na^+$ re-absorption from the xylem by xylem parenchyma cells reduces $Na^+$ transport to the shoot according to Läuchli (1984).

Some plant species have special salt glands on the leaf surface in order to avoid toxicity. Halophytic plants thus excrete excessive salts transported to the leaves (Drennan and Pammenter 1982). Excretion of $Na^+$ from leaves through salt glands has been investigated by several researchers (Lüttge 1971; Fahn 1979; Liphschitz and Waisel 1982). This mechanism helps plants to maintain a steady state of salt balance in leaves (Flowers and Yeo 1986; Ball, 1988) and allows them to grow under high soil salinity levels for a long time period (Munns et al. 2006). Salt glands were also shown on the leaf of *Diplachne fusca* (L.) and *Avicennia marina.* Thus excreted salts are finally removed by the action of wind and water (Rains and Epstein 1967; Warwick and Halloran 1992).

### 1.2.2.3 $Na^+$ compartmentation in leaf vacuoles

Tissue tolerance is achieved by sequestration of $Na^+$ from the cytoplasm into the leaf vacuoles in which $Na^+$ concentrations of up to 200 mM do not affect cell function (Tester and Davenport 2003). In plant shoots, NHX proteins were shown to be directly associated with the sequestration of $Na^+$ into leaf cell vacuoles (Apse et al. 1999; Sottosanto et al. 2007; Xia et al. 2002; Zhang and Blumwald 2001). Some plant species such as barely, wheat and rice showed an improved salt resistance at the vegetative stage (Mass and Haffman 1977; Rawson et al. 1988). In addition, Ohta et al. (2002) reported that transgenic rice plants showed increased salt resistance under saline conditions, (300 mM NaCl). This improvement of cereal plants was due to the introduction of new genes, by crossing with new donor germplasm or by transformation with single genes (Munns et al. 2006). Recently, Qiao et al. (2010) showed that vacuolar $Na^+/H^+$ antiporters of the transgenic plants have enhanced salt resistance by increasing $Na^+$ sequestration, thereby avoiding $Na^+$-specific toxicity. The following digram explains the salt resistance mechanisms which were found to be related with an ability to transport and sequester $Na^+$ into leaf cell vacuoles of salt-resistant plants which induced tonoplast $Na^+/H^+$ antiporter to maintain cellular ion homeostasis, pH regulation, osmotic adjustment in cytosol, and finally salt resistance (Fig. 4).

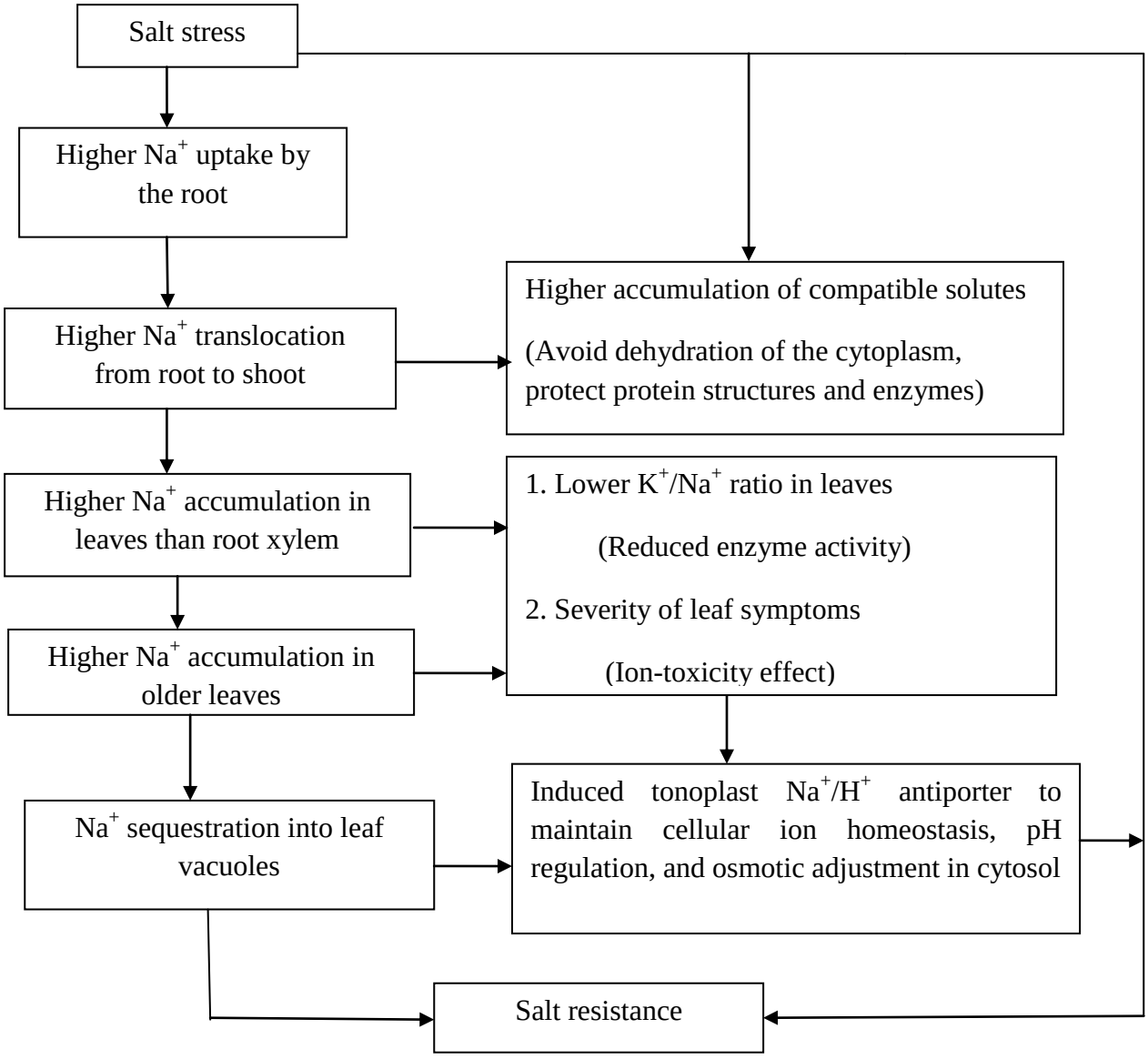


**Figure 4:** The possible biochemical and physiological mechanisms of salt tissue resistance in maize SR hybrids

### 1.3 Objectives

Despite some investigations about the role of *NHX* in salt resistance, there is still a lack of knowledge about the exact contribution of $Na^+/H^+$-antiport in the salt resistance of maize. This study targets the differences in tissue tolerance of different maize SR hybrids in the second phase of salt stress. To clarify the role of tonoplast-associated $Na^+/H^+$ antiporters in salt resistance, the effect of salt stress on transcription levels of shoot vacuolar *NHX* are determined. The objectives of this study can be divided into following four groups:

1. To study the severity of leaf symptoms, relative reduction in shoot dry weight and low shoot $Na^+$ concentration to identify salt-resistant maize hybrids in the second phase of salt stress.

2. To investigate the role of $Na^+$ exclusion at the root surface and $Na^+$ exclusion from the shoot in contributing to salt resistance of maize in the second phase of salt stress.

3. To examine the transcription of tonoplast $Na^+/H^+$ antiporters in shoots of salt-resistant maize genotypes (SR 03 and SR 05) and thier capacity to facilitate $Na^+$ inclusion in the leaf vacuoles of shoot cells.

4. To study the effect of salinity on $Na^+/H^+$ antiport activity in tonoplast vesicles isolated from leaves of salt-resistant maize genotypes under salt stress.

### 1.4 Hypotheses

It was hypothesized that:

SR maize hybrids show better shoot growth performance than salt-sensitive ones in the second phase of salt stress. Moreover, based on the severity of leaf symptoms, maize genotypes can be classified into salt resistant and salt-sensitive.

$Na^+$ exclusion at the root surface and from the shoot contribute to the salt resistance of maize hybrids (SR 03 and SR 05).

Salt-resistant maize hybrids show a higher transcription of tonoplast $Na^+$ /$H^+$ antiporters in shoots, thereby maintaining low $Na^+$ concentration in the cytoplasm of leaf cells.

Salt stress increases the activity of tonoplast $Na^+/H^+$ antiporters in leaves of salt-resistant maize genotypes SR 03 and SR 05 and thus increases $Na^+$ transport from the cytoplasm to the vacuoles

## 2. Material and Methods

### 2.1 Development of salt-resistant maize hybrids

Salt-resistant maize hybrids were developed by crossing of an $Na^+$-excluding maize inbred line (NaExIL) with osmotically resistant (SWS) maize inbred lines. Maize hybrids referred to as SR hybrids (salt-resistant), were developed in the Institute of Plant Nutrition, Justus Liebig University Giessen, Germany (Schubert et al. 2009). The breeding scheme of the SR hybrids is given in Fig. 5.

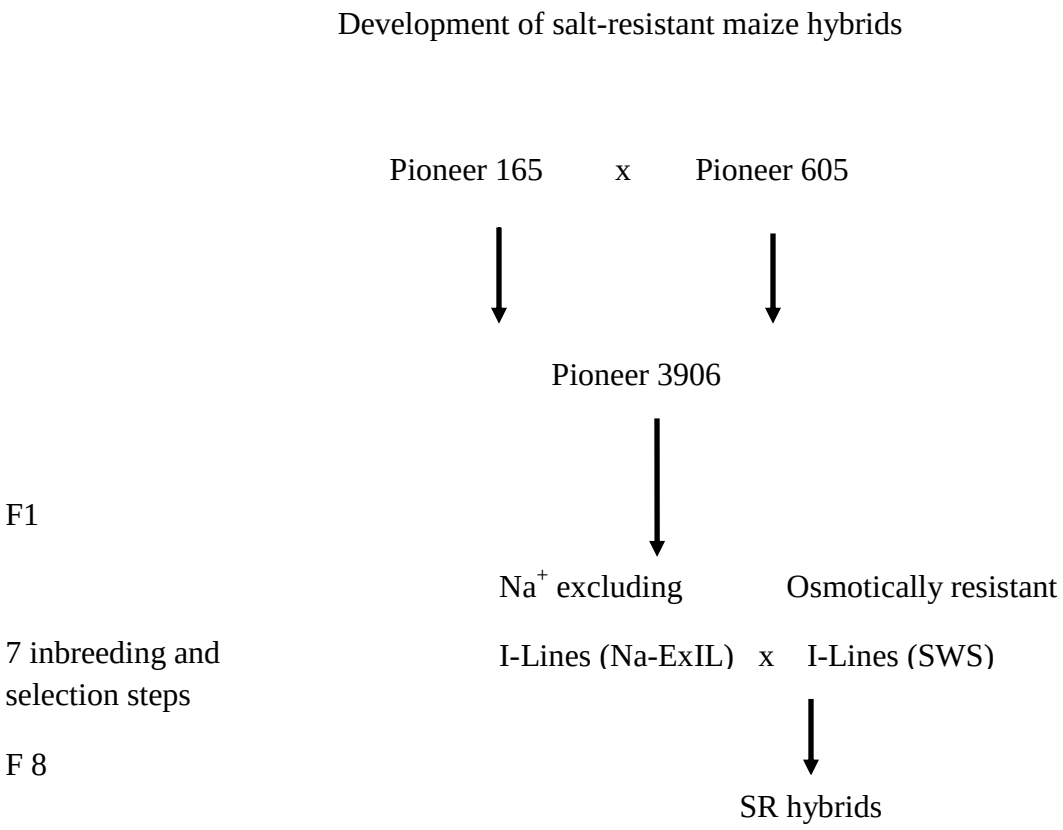


**Figure 5**: Scheme showing the development of maize SR hybrids according to Schubert et al. (2009). Pioneer 3906 is considered as moderately salt-resistant, it was developed by crossing of Pioneer 165 (efficient $Na^+$ exclusion at the root surface) and Pioneer 605 (efficient $Na^+$ exclusion from the shoot). By self-crossing of Pioneer 3906 and seven inbreeding and selection steps, the excluding $Na^+$ inbred line were developed. SR hybrids were obtained by crossing NaExIL with osmotically resistant inbred lines.

In this study, ten maize genotypes were screened for their resistance against ion toxicity (severe leaf symptoms on the older leaf) in the second phase of salt stress under control (1 mM NaCl) and 200 mM NaCl for 26 d in hydroponic solutions. Genotype Across 8023 which is considered as a salt-sensitive maize cultivar was used as a control because it showed inefficient $Na^+$ exclusion in the first phase of salt stress, (Fortmeier and Schubert, 1995). On the other hand, Pioneer 3906 is a moderately salt-resistant maize genotype that efficient good $Na^+$ exclusion in the first phase of salt stress (Schubert and Läuchli, 1986).

## 2.2 Growth conditions and plant cultivations

### 2.2.1 Screening of SR hybrids for ion toxicity

Ten maize genotypes were screened for their ability to resist 200 mM NaCl. Plants were carried out in the vegetation hall at the experimental station of the Institute of Plant Nutrition, Justus Liebig University Giessen, Germany. Plants were grown under control (1mM NaCl) and salt stress (200 mM NaCl) under open-air conditions during the day. At night they were transferred into the vegetation hall. The free surfaces of the pots were covered with a black Aluminum foil in order to inhibit the growth of green algae and minimize heating of the nutrient solution by sunlight. The experiment was set up in a completely randomized design and pots were randomized every 2 d. For plant cultivation, seeds of ten maize genotypes (Across 8023, Pioneer 3906 and eight newly developed maize hybrids) were soaked in aerated 1 mM $CaSO_4$ solution for 7 d at 25°C in the dark between two layers of filter paper moistened with 1 mM $CaSO_4$. After 7 d homogenously grown seedlings were transferred to 8.0 L plastic pots containing ¼ strength nutrient solution. The concentration of the nutrient solution was increased to ½ and full strength after 2 d and 4 d, respectively.

**Table 1:** The full-strength nutrient solution had the following compositions:

| Macronutrients | Concentrations (mM) | Micronutrients | Concentrations (µM) |
|---|---|---|---|
| $Ca(NO_3)_2$ | 2.5 | $H_3BO_4$ | 1.0 |
| $K_2SO_4$ | 1.0 | $MnSO_4$ | 0.2 |
| $KH_2PO_4$ | 0.2 | $ZnSO_4$ | 0.5 |
| $MgSO_4$ | 0.6 | $CuSO_4$ | 0.3 |
| $CaCl_2$ | 5.0 | $(NH4)_6Mo_7O_{24}$ | 0.005 |
| | | Fe-EDTA | 200 |

To avoid nutrient depletion, nutrient solutions were changed every 2 d. Sodium chloride was added in 25 mM increments daily until a final concentration of 200 mM was reached. Control plants were grown with 1 mM NaCl. Each treatment was run in four replicates.

### 2.2.2 Plant harvest and analysis

Plants were harvested after final concentration of NaCl treatment applied and also after appearance of necrotic spots on the older leaves. The maize plants were in the second phase of salt stress, because $Na^+$ toxicity symptoms were observed as necrotic spots on the old leaves. For analysis, plants were separated into shoots and roots. After determination of fresh weights, plant shoots were put into small bags and oven-dried at 80°C. After 48 h, shoot dry weights were recorded. For determination of fresh weights, roots of maize genotypes were washed twice with 1 mM $CaSO_4$ solution, rinsed with distilled water, blotted dry, and root fresh weight was recorded. Finally, the dried plant material was ground to pass a 1 mm sieve and the dry powder was stored for further analyses.

Beside shoot and root fresh and dry weights, leaf length was chosen to screen the SR hybrids for their level of salinity-induced osmotic stress resistance. Leaf length was recorded as distance from leaf blade base to leaf blade tip. Then, the relative reduction of leaf length was calculated (% of control). Also, leaf symptoms (number of necrotic spots on the older leaves)

were accounted. These necrotic spots were noticed first on the tips and margins of the older leaf plants.

### 2.2.3 Parameters of $Na^+$ exclusion:

$Na^+$ uptake by roots was calculated as ratio of total plant $Na^+$ content and root dry weight. This parameter served to characterize $Na^+$ exclusion at the root surface. $Na^+$ translocation from root to shoot was calculated as ratio of shoot $Na^+$ content and root $Na^+$ content. It is the parameter that describes $Na^+$ exclusion from shoots.

## 2.3 Chemical analysis (cation analysis):

For cation analysis, about 200 mg of dried plant material (shoots and roots) were weighed and used for determination of $Na^+$, $K^+$, and $Ca^{2+}$ ion concentrations. Plant material was dry-ashed at 550°C over night in a forced-air oven, and after cooling digested in 5 M $HNO_3$. The digested plant material was heated prior to boiling. Then all material was filtered into 50 mL volumetric flasks through white ribbon filter (Schleicher & Schuell, Germany). Flasks were filled up to volume (50 mL) with double-distilled water and analyzed for cation concentrations. Concentrations of $Na^+$, $K^+$, and $Ca^{2+}$, were measured by atomic-absorption spectrophotometry (Spectr*AA* 220 FS, Varinan, Mulgrare, Victoria, Australia).

## 2.4 Identification of plant salt resistance:

Four maize (*Zea mays* L.) genotypes from the first experiment were selected for further investigation. Two of the genotypes (SR 03 and SR 05) were recorded as salt- resistant, because these two genotypes showed low $Na^+$ concentration in the shoot and lowest number of necrotic spots per leaf. The other two genotypes (Across 8023 and SR 20) were recorded as salt-sensitive, (they showed high number of necrotic spots per leaf). Cultivation of plants was conducted as described for the first experiment (see chapter 2.2.1). Salt treatment was started after reaching full-strength nutrient solution and was increased by 25 mM NaCl increments every 2 d to the final salt concentration of 200 mM NaCl. Control nutrient solution contained 1 mM NaCl. The experiment was carried out under controlled growth conditions in a growth chamber with a light

intensity of 500 µmol $m^{-2}$ $s^{-1}$ for 16 h $day^{-1}$ during the day period and 8 h at 18°C during the dark period at a relative air humidity of 60%. The pots were randomized every 2 d. Each treatment was run in triplicate with four plants per replicate.

### 2.4.1 Plant harvest and cation analysis in single leaf blades:

Plants were harvested 26 d after the beginning of plant cultivation. Single leaf blades were separated from the leaf sheath of all maize genotypes and then divided into young and old leaves. Fresh and dry weights of single leaf blades were measured. Samples were ground to pass 1 mm sieve. For cation analysis, concentrations of $Na^+$, $K^+$ and $Ca^{2+}$ were measured using atomic-absorption spectrophotometry (Spectr*AA* 220 FS, Varinan, Mulgrare, Victoria, Australia).

### 2.5 Collection of plant material for molecular methods:

For molecular analysis, immediately after plant harvesting root and single non-necrotic leaf blades of four maize genotypes were numbered, separated, rinsed with 1 mM $CaSO_4$ and distilled water, blotted dry and immediately frozen in liquid nitrogen for storage at –80ºC.

### 2.5.1 Isolation and purification of total RNA from roots and single leaf blades

The frozen roots and single non-necrotic leaf blades were crushed in liquid nitrogen using a pre-cooled mortar and pestle. Total RNA was isolated from the crushed material with phenol-chloroform according to a modified method (Cox and Goldberg 1988). About 200 mg of homogenized plant material were mixed with 1 ml RNA extraction buffer (lysis buffer) that facilitates cell lysis and the inhibition of RNase by vigorously vortexing. The lysis buffer for isolation of total RNA had the following composition:

100 mM Tris-HCl (pH 8.6)

2% Lausrolyl-sarcosine

25 mM EDTA pH 8

25 mM EGTA pH 8

100 mM β-mercaptoethanol
5 mM DTT

1% SDS

Note: β-mercaptoethanol was always freshly added before use.

The organic phase and total RNA were separated by 10 min centrifugation at 13 000 rpm. Denatured proteins, DNA, and cell wall components were collected in the lower organic phase while RNA was only present in the upper aqueous phase. The upper phase was collected, 60% (v/v) isopropanol, and 10% (v/v) 3 M Na-acetate (pH 5.2) were added to precipitate RNAs overnight at -20°C. The obtained pellet of RNAs was washed with 10 mL of ice-cold ethanol 70% (v/v) and by centrifugation 10 min (13 000 rpm) at 4°C. Finally, the solution was then stored in liquid nitrogen at -80°C for further investigations. Fig. 6 explains the isolation and purification methods for total RNA from roots and shoots.

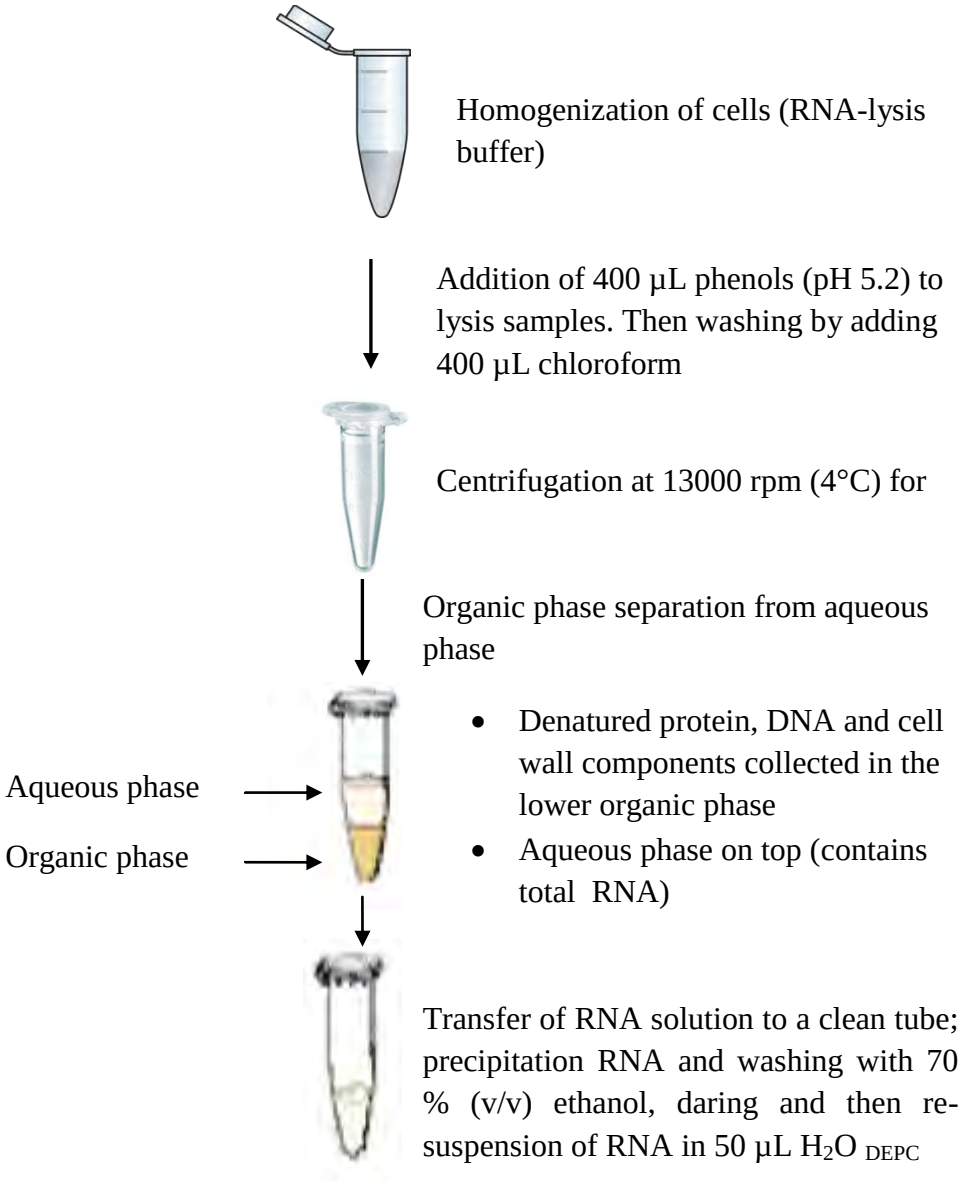


**Figure 6:** Scheme showing the purification procedure of total RNA isolation.

To prevent RNA degradation by ribonucleases (RNases), the purification procedure must be carried out quickly and samples were kept at 4°C during processing. Many sources of contaminating RNase are known such as microorganisms in the air, solutions, water supply and bacterial cultures. It recommended wearing gloves when handling any reagents or reaction vessels.

### 2.5.2 Estimating RNA quality and quantity with spectrophotometry:

A spectrophotometer (Cary 4, Varian) was used to assess the concentration and purity of total RNA samples. Absorbance was determined at 260nm (RNA concentration) and 280 nm (protein concentration). Pure RNA exhibited a ratio of A260/A280 within the range of 1.8 - 2.0. If there is contamination with protein or phenol, the A260/A280 ratio will be significantly less than the values given above, and accurate quantification of the amount of nucleic acid will not be possible. Total RNA concentration was calculated using the formula:

$$A_{260} \text{ X dilution X } 40 = [\text{RNA}]\ \mu g/mL$$

### 2.5.3 Messenger RNA isolation:

Total RNA consists of a complex mixture of polynucleotide chains (rRNA, tRNA, mRNA, and other small RNAs) that vary in functions and lengths. To purify mRNA from total RNA, paramagnetic particle technique was used following the instructions of the manufacturer. Whereas mRNA contains a poly A-tail, which binds to Dynabeads of oligo (dT) $_{25\ \text{matrixes}}$, the other RNA can be washed away, because it lacks a poly A- tail. The following diagram explains the purification methods for mRNA from other RNAs (Fig. 7).

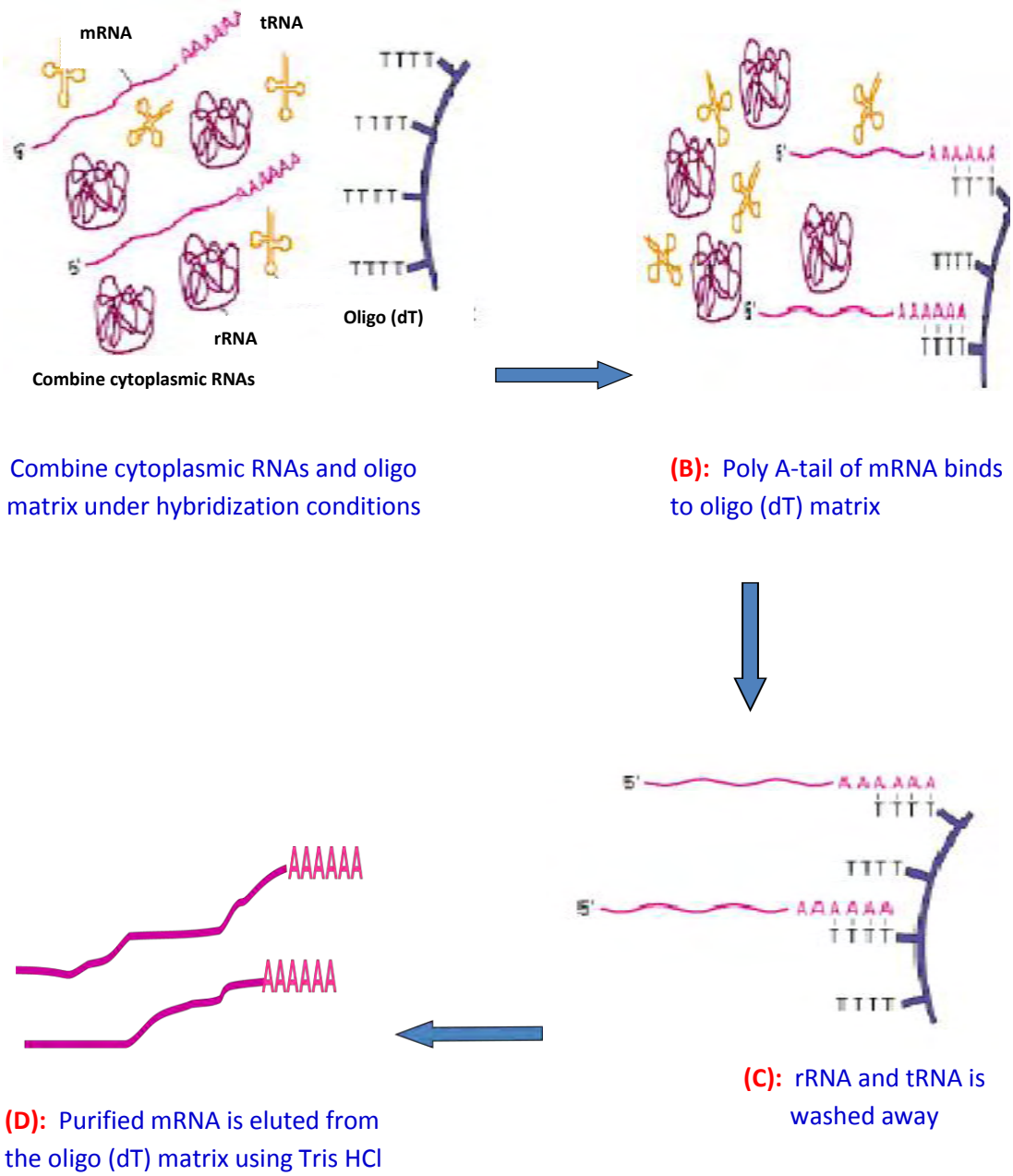


**Figure 7:** Purification of mRNA from total RNA using the oligo (dT) matrix (A). Whereas the poly A-tail of mRNA binds to oligo (dT) matrix (B) the other RNAs lacking a poly A-tail are washed away (C). Then purified mRNA is eluted from the oligo (dT) matrix using Tris-HCl (pH 7.5) as in (D).

In order to prevent the contamination of mRNA, beads were resuspended in100 µL $H_2O_{DEPC}$, incubated for 2 min at 65°C and then washed with 50 µL binding buffer. Beads were washed twice with 100 µl reconditioning solution for 2 min at 65°C and then kept in storage buffer at 4°C.

**Table 2:** Reagent used for mRNA isolation and purification:

| **Reagent** | **Substrate** |
|---|---|
| Binding buffer | 20 mM Tris-HCl (pH 7.5) |
| | 1 M LiCl |
| | 2 mM EDTA |
| Washing buffer | 10 mM Tris-HCl (pH 7.5) |
| | 0.15 M LiCl |
| | 1.0 mM EDTA |
| Elution reagent | 10 mM Tris-HCl (pH 7.5) |
| Reconditioning solution | 0.1 M NaOH |
| Storage buffer | 250 mM Tris-HCl (pH 7.5) |
| | 20 mM EDTA |
| | 0.1% Tween20 |
| | 0.2% $NaN_3$ |

About 37 µg total RNA were adjusted to 50 µL with $H_2O_{DEPC}$, denaturized for 2 min at 65ºC and incubated on ice for 2 min. For hybridization of poly A-tail mRNA to the beads, 50% beads/binding buffer added to 50% total RNA samples and the mixing took place by continuously shaking the samples for 5 min at the room temperature. To separate the beads, the tube was placed on the magnet until the solution was clear and the supernatant was removed. The beads were washed twice by resuspending in 100 µL washing buffer using the magnet. After that the beads were resuspended in 10 µL 10 mM Tris-HCl (pH 7.5), incubated for 2 min

at 70ºC, magnetized and the eluted mRNA was transferred to a new RNase-free tube. mRNA samples were either used as template for subsequent first-strand cDNA synthesized or shock-frozen and stored at -80ºC.

### 2.5.4 First-strand cDNA synthesis:

First-strand cDNA was synthesized using 1 µg mRNA following the manufacturer's instructions in Super Script TM$^{-II}$ First-Strand Synthesis System for RT-PCR (Invitrogen, Germany). This mRNA could be used as template for reverse transcriptase to synthesise a complementary DNA (cDNA) (Fig. 8). Compared with DNA, cDNA has no introns or non-transcriptable regions, which can directly reflect the activity of gene expression. All reactions were run on a T-Gradient Thermocycler (Biometra, Germany) 0.5 µl Oligo (dT) $_{12\text{-}18}$ primer (500µg/ml), 0.5 µl dNTP Mix (10mM each), and 2.0 µl (1 µg) mRNA were denaturised for 5 min at 65ºC and quick chilled on ice. Tube contents were collected by brief centrifugation at 4ºC and 4.5 µl of the following reaction mix were gently mixed with the RNA assay containing the following.

**Table 3:** The composition of the RNA assay mixture

| Master mix | Volume |
|---|---|
| First strand buffer (pH 8.3) | 1.0 µL |
| 25 mM $MgCl_2$ | 2.0 µL |
| 0.1 M DTT | 1.0 µL |
| RNaseOUT$^{TM}$ (40 units/µl) | 0.5 µL |

The mixture was incubated for 2 min at 42ºC and 0.5 µl SuperScript$^{TM}$ II Reverse Transcriptase (50 units) was added and mixed by pipetting gently up and down to start the reverse transcription. After incubation at 42ºC for 50 min the reverse transcriptase was stopped by heating at 70ºC for 15 min. To remove remaining RNA complementary to the cDNA, 0.5 µl (1 unit) of *E.coli* RNase H was added and incubated at 37ºC for 20 min. The synthesized cDNA

was diluted (1:20) with autoclaved, bi-distilled water, making it possible to be used as a template for amplification in PCR and RT-PCR reactions or stored at -20ºC.

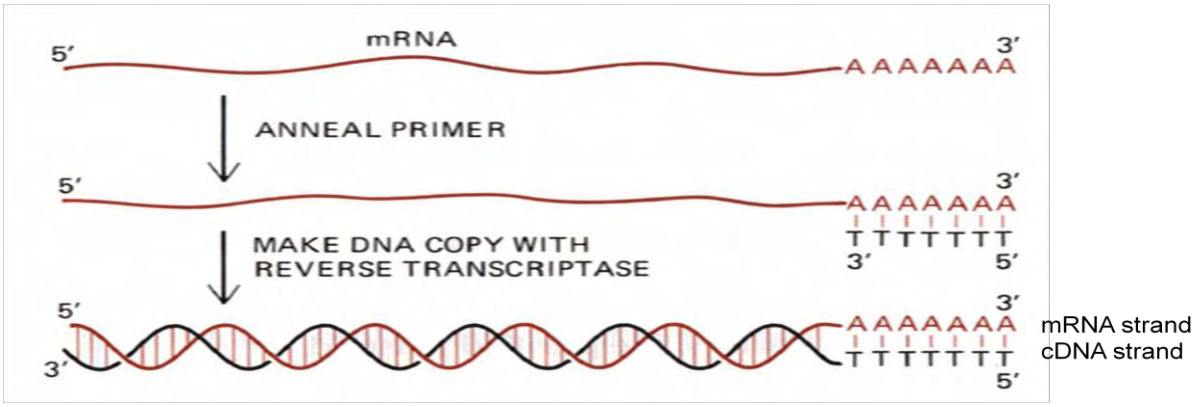


**Figure 8:** mRNA template is copied into cDNA via reverse transcription. The synthesized cDNA strand is *complementary* to the mRNA template

### 2.5.5 PCR amplification of cDNA:

PCR (polymerase chain reaction) is a powerful tool to find new genes and to amplify cDNA synthesis. All reactions were run on a T-Gradient Thermocycler (Biometra, Germany). The following primers were used in the PCR:

First primer is actin which used as a house keeping gene; consist of **s *ZmActin*-580bp** (5'-GAG CTC CGT GTT TCG CCT GA-3') and ***as ZmActin*-752bp** (5'-CAG TTG TTC GCC CAC TAG CG3'). And annealing temperature for primer is 60°C.

The second primer is *ZmNHX*, which consists of **s *ZmNHX*-249** (5'-CAT HTA YCT NYT NCC NCC NAT HAT HTT CAA TGC-3') and **as *ZmNHX*-542** (5'-CCY TCN CCG AAT ACN AGA CTG TA-3'). Annealing temperature for primer is 54°C

Whereas: **s** means sense, and **as** means anti-sense; letters: **H** (A, C, T), **N** (A, G, C, T), **Y** (C, T)

**Table 4:** The Master Mix of PCR reactions had the following composition:

| Master mix | Volumes |
|---|---|
| 10x PCR buffer (containing $MgCl_2$ 15mM) | 1.0 µL |
| 25 mM $MgCl_2$ | 0.4 µL |
| dNTPs (10 mM) | 0.2 µL |
| primer pair (100 p mol/µL) | 0.2 µL |
| sterile H2O | 6.1 µL |
| Taq DNA polymerase (5 units/µL) | 0.1 mL |

The PCR program was designed as in the following diagram:

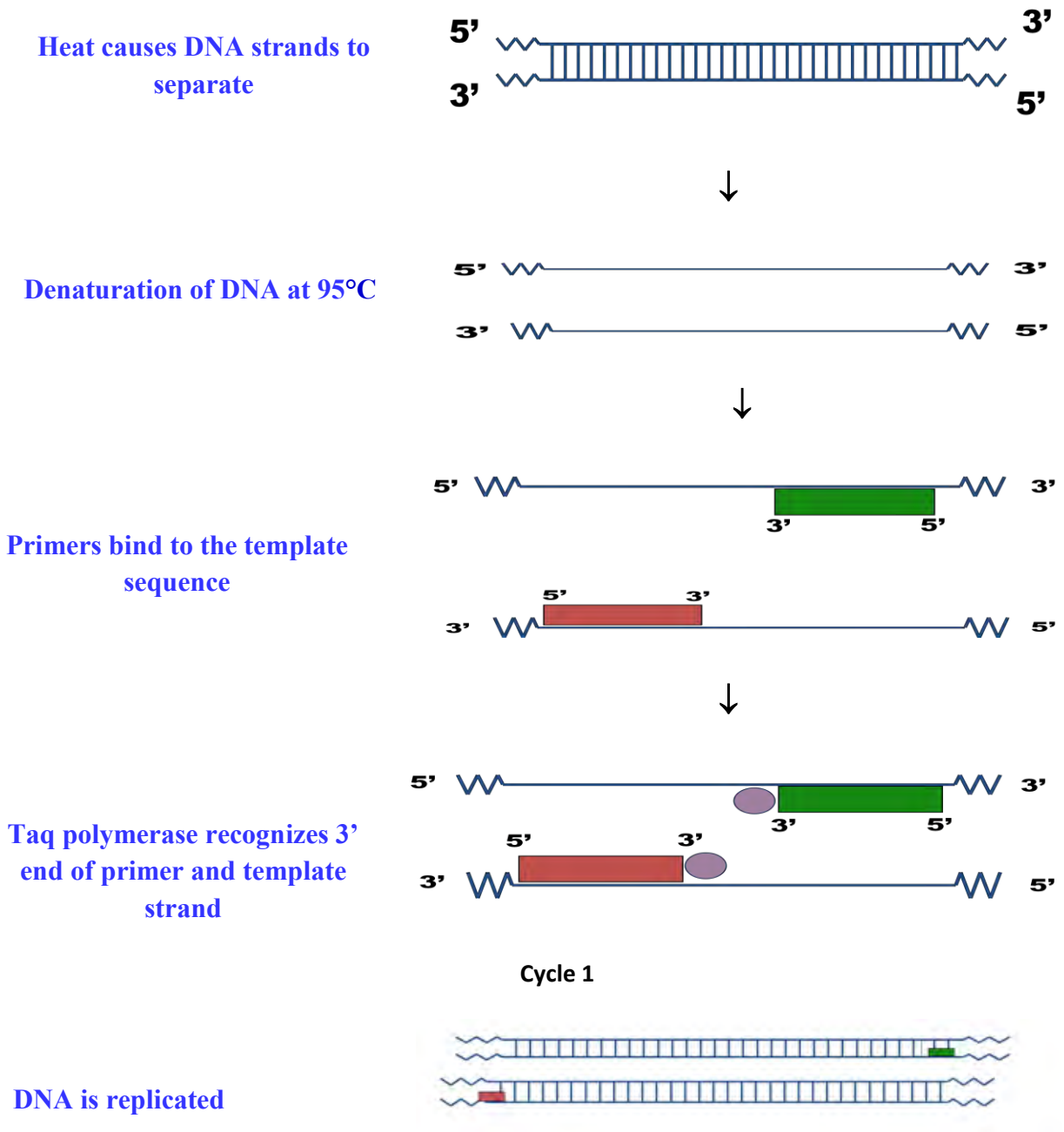


**Figure 9:** The cycling protocol of PCR consisted of 35-40 cycles, each including denaturation at 95ºC for 3 min, annealing primer at primer pair-specific temperatures ( *ZmActin* 60ºC, and *ZmNHX* 54ºC ) for 20 s, extension step at 72ºC for 20 s and Final elongation at 72ºC for 3 min.

### 2.5.6 DNA-Gel Electrophoresis:

The PCR products were separated on 1% agarose gel.

**Table 5:** The contents of solutions used for agarose gel electrophoresis is given below:

| Reagents | Substance |
|---|---|
| 1x TBE running buffer | 400 mM Tris-Borat<br>10 mM EDTA (Ph 8.0) |
| X.B. loading buffer<br>(in 1 x TBE running buffer) | 0.25 % [w/v] Xylene cyanol<br>0.25% [w/v] Bromophenol blue<br>0.25% [w/v] Glycerol |
| DNA marker | 1 µg/µL DAN ladder (invitrogen)<br>1µg/µL X.B. loading buffer |

The 1% agarose gel was prepared by using 1g agarose powder, completed to 100 mL/ (mg) with 1 x TBE running buffer. The mixture was cooked in microwave for 3 min at 600 W until agarose was melted. After the agarose solution cooled down to 60ºC, 6 µL ethidium bromides were added and mixed gently. The solution was solidified at room temperature. After solidification, the comb was removed and the solidified agarose gel was transferred into electrophoresis chamber and covered with1 x TBE running buffer. A10 µL PCR products were mixed with 3 µL of X.B. loading buffer, which mixed gently. From the mixed samples, 11 µL was pipetted into the sample wells on the gel electrophoresis. After 1 h, for 120 V and 50 A current was applied, the labeled DNA fragments were visualized by UV-graphy (LTF Labortechnik, Germany).The size of separated DNA strands was determined by comparison of their relative position to DNA strands with known size of an included DNA marker (Invitrogen, Germany).

### 2.5.7 Real-time PCR protocols:

Real-time PCR assays were performed on the Rotor-Gene 2000/3000. Real-Time Amplification Thermal Cycling System following Zörb et al. (2005). In contrast to the traditional end-point analysis of amplification products via gel electrophoresis, this method depends on the relationship between the amount of starting target DNA and the amount of amplification product during the exponential phase of a cycling programme.

RT-PCR was done using poly $A^+$- mRNA-based cDNA templates extracted from roots and single leaf blades were used as templates for real-time PCR. The reaction mixtures in a final volume of 10 µL contained cDNA (1:20).

**Table 6:** The composition of the master mix for cDNA synthesis as the following:

| Master mix | Volume |
|---|---|
| Sterile water | 2.70 µL |
| Absolute$^{TM}$ QPCR SYBR Green Mix (ABgene, UK) | 5µL |
| Primer pair (100 p mol/ µL each; Carl Roth, Germany) | 0.3µL |
| cDNA (1:20) | 2.0 µL |

The real-time PCR reaction was initiated with an activation of the Hot Start taq polymerase at 95ºC for 15 min. The cycling protocol of real-time PCR consisted of 35-40 cycles, each including 1) denaturation at 94ºC for 30 s, 2) annealing primer at primer pair-specific temperatures ( *ZmActin* 60ºC, and *ZmNHX* 54ºC ) for 30 s and 3) extension step at 72ºC for 30 s. After every elongation step, the fluorescence of SYBR Green was acquired at 470nm. As intercalating dyes bind nonspecifically to any double-stranded DNA, a melting curve analysis of amplification products was allowed for differentiation at the end of the run. Finally, a melting curve was run from 72 to 99ºC. The melting curve was prepared using SYBR Green fluorescence of obtained PCR-sequences detected no hairpin or loop formation. Single specific bands of the amplification products were checked via DNA gel electrophoresis. Negative controls with no templates (NTC) were carried out with each run.

### 2.5.8 Relative quantification of the real-time PCR data:

The relative transcription of the tonoplast $Na^+/H^+$ antiporters at mRNA level was quantified following the relative quantification method with two standard curves. This method includes a house-keeping gene as an internal control and measures the expression level of the gene of interest (in this case vacuolar $Na^+/H^+$ antiporters, *ZmNHX*) with reference to the internal control. The expression of the house-keeping gene (HKG) was not affected by the treatments. In this study, Actin was used as a house-keeping gene according to Zörb et al (2005). The threshold was set at a level where specific fluoresces of the sample became significantly higher than the background florescence and where the rate of amplification was exponential. The number of PCR cycles it took for a sample to reach the threshold level, yielded the corresponding $C_T$ value. The $C_T$ value is negatively correlated to the template concentration in the cDNA sample. Each sample was separately analyzed with actin as well as tonoplast $Na^+/ H^+$ antiporters. In each case, standard curves were generated from dilution series of a single DNA sample. The values of the expression in each sample relative to the standard curve were calculated. The resulting data were normalized by dividing the value of the expression of the respective *ZmNHX* of a sample by the value of expression of the HKG. Relative expression values are means of three biological replication ± SE. these normalized values or relative transcription in the two genotypes (SR 03 and SR 05) were compared to other two genotypes (Across 8023 and SR 20). The relative transcription values of tonoplast *ZmNHX* in all tested genotypes were arbitrarily set to 100%, and relative transcription values were expressed as present of respective actin values. No unit is given to this value. The relative expression ratio of a target gene calculation is based on *E* and $C_T$ values, reported by Pfaffl (2001).

$$\text{Ratio} = \frac{(^{E}_{\text{target}})^{\Delta C_{T\,\text{target}}}(\text{control-sample})}{(^{E}_{\text{ref}})^{\Delta C_{T\,\text{ref}}}(\text{control-sample})}$$

Whereas, $\Delta C_T$ values were calculated by comparing $C_T$ values of actin and $C_T$ values of *Zm NHX* derived from the same cDNA templates. $E_{target}$ is the RT-PCR efficiency of target gene transcript; $E_{ref}$ is the RT-PCR efficiency of reference gene transcript.

## 2.6 The effect of salt stress on tonoplast membrane $H^+$-ATPase activity of two maize genotypes

### 2.6.1 Isolation of tonoplast membrane vesicles

Tonoplast vesicles were isolated from young leaves of control and salt-treated plants of maize hybrids AMADEO (KWS, Kleinwanzleben), SR 03 and SR 05 using differential centrifugation and sucrose gradients (Queirós et al. 2009) with slight modifications. The commercial hybrid AMADEO was used for establishing the tonoplast isolation method. After achieving satisfactory purity of tonoplast vesicles, SR 03 was cultivated without and with salt stress for tonoplast isolation. A second experiment with SR 03 was not performed since the seed material for this hybrid was limited. Instead, SR 05 which also showed enhanced transcription of NHX antiporters was investigated. After harvesting, young leaves were cut and washed three times with deionized water. About 100 g fresh leaves were ground in 200 mL of ice-cold extraction buffer.

**Table 7:** The extraction buffer for isolation of tonoplast vesicles had the following composition:

| Chemical materials | Concentrations |
|---|---|
| Glycerol | 10% (v/v) |
| EDTA adjusted to pH 8.0 | 5 mM |
| BSA (bovine serum albumin) | 0.13% (w/v) |
| Tris-HCl buffer (pH 8.0) | 0.1 M |
| KCl | 150 mM |
| DTT (dithiothreitol) | 3.3 mM |
| PMSF (phenylmethylsulfonyl fluoride) | 1 mM |
| PVP (PVP-40, 40 KD) polyvinylpyrrolidone | 0.5% (w/v) |

All procedures for buffer preparation and separation of tonoplast membrane vesicles were conducted at 4°C. To remove cell debris, the homogenized plant materials were filtered through four layers of cheesecloth (Calbiochem-Novabiochem, San Diego). The supernatant was collected and centrifuged at 10,000 *g* for 10 min. The supernatant was centrifuged again at 10,000 *g* for 10 min. Pellets were discarded and the supernatants were again centrifuged at 100,000 *g* for 40 min to pellet the microsomal membranes. After the supernatant was aspirated,

the microsomal pellet was resuspended gently in a small volume of ice-cold buffer. The resuspension buffer contained 10% (v/v) glycerol, 1 mM DTT, 1 mM EDTA (pH 7.6), and 10 mM TRIS-HCl adjusted to pH 7.6. The resulting supernatant was then layered on top of discontinuous sucrose each consisting of 15 mL 46%, 12 mL 25% and 9 mL 10% (w/v) sucrose solution and centrifuged at 80,000 *g* for 3 h and 30 min in a swinging bucket rotor (Sorvall AH 629 rotor, 36 mL, Du Pont Company, Wilmington, Delaware). The gradient solutions contained the following substances: 10/25/46% (w/w) sucrose, 10 mM Tris-HCl buffer adjusted to pH 7.6, 1 mM DTT and 1 mM EDTA.

The tonoplast-enriched fraction was collected from the 10/25% sucrose interface using a Pasteur pipette, was diluted three times in ice-cold water and was centrifuged at 100,000 *g* for 40 min. The resulting pellet was re-suspended in a medium containing 10 mM Tris-HCl adjusted to pH 7.6, 10% (v/v) glycerol, 1 mM DTT, and 1 mM EDTA. The vesicles were either used immediately or frozen in liquid $N_2$ and stored at 80°C.

Protein concentrations were determined by the method of Bradford (1976), using bovine serum albumin as a standard. The Bradford reagent was composed of 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol and 8.5% (w/v) phosphoric acid. 20 µL resuspended membrane protein were mixed and completed to 2.5 mL with Bradford reagent. After 40 min incubation at room temperature, protein was determined using a spectrophotometer (Varian, Cary 4 Bio UV-Visible Spectrophotometer) at 595 nm.

### 2.6.2 Proton-pumping activity

The proton-pumping activity across tonoplast vesicles was measured as the initial rate of fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine (ACMA), modified from Façanha and de-Meis (1998). Fluorescence quenching was monitored in infinite F200PRO (TECAN) at 25°C using a plate reader with excitation at 415 nm and emission at 485 nm, with a slid width of 5 nm. The assay of V-$H^+$-ATPase was performed in100 µL of a buffer containing 6.6 µg protein of tonoplast vesicles, 100 mM KCl, 2.5 mM Mg-ATP-BTP (mixture of 5 mM $MgSO_4$ and 10 mM $K_2$-ATP, adjusted to pH 7.0 with BTP), 2 µM ACMA. 50 mM NaCl were added into the assay to initiate $Na^+/H^+$ antiporter activity. The reaction medium was completed to

100 µL by adding deionized water. The assay medium was mixed gently and placed in a dark chamber of the plate reader after stirring for 5 min before start of the fluorescence reading. The reactions were initiated by the addition of 2.5 mM Mg-ATP-BTP and quenching of fluorescence proceeded until a steady-state (constant level) was achieved. The pH gradient was established to a constant level in 20 min after the initiation of proton pumping. The initial rate of ACMA fluorescence quenching of proton pumping activity (relative change of fluorescence per min) was determined after 100 s. Also, maximum pH gradient (% of initial fluorescence) was calculated by taking a slope of initial pump activity during the first 100 s against time. Furthermore, ΔF means the decrease of the fluorescence (ΔF/Fi x 100) and initial rate calculated as (ΔF/Fi x 100 $min^{-1}$), unit according to Qiu et al. (2004).

### 2.6.3 Determination of the purity and V-$H^+$-ATPase hydrolytic activity of tonoplast membrane

Hydrolytic activity of $H^+$-ATPase was determined by measuring the release of $P_i$ from hydrolysis of ATP in the leaves of maize plants cultivated under control and salt stress (200 mM NaCl). The purity of the tonoplast preparations was estimated by using specific inhibitors. The inhibitors for ATPases of tonoplast, mitochondria, and plasma membranes were nitrate (50 mM $KNO_3$), azide (0.5 mM $NaN_3$), and vanadate (0.3 mM; $Na_3VO_4$), respectively. $Na^+$-molybdate (1 mM) was used to assess the presence of unspecific acid phosphatases. The ATPase activity was sensitive to nitrate (50 mM $KNO_3$) and was strongly inhibited by about 80-85%. However, the ATPase activity of all membrane fractions isolated from control and salt-resistant plants was insensitive to azide, vanadate and molybdate. The reaction was started by the addition of 3 µg of the sample protein to a reaction mixture (final volume 0.5 mL).

**The reaction mixture contained:**

5 mM disodium-ATP
5 mM $MgSO_4$
100 mM KCl
0.02% (v/v) Triton X-100
50 mM MOPS-TRIS adjusted to pH (7.2)
50 mM $KNO_3$

0.5 mM $NaN_3$

0.3 mM; $Na_3VO_4$

1mM $Na^+$-Molybdate

After 30 min incubation at 30°C, the reaction was stopped with the addition of 1 mL of stopping reagent. The stopping reagent contained; 2% (v/v) $H_2SO_4$, 5% (w/v) SDS, and 0.7% (w/v) ($(NH_4)_2MoO_4$).

Immediately after addition of 1 mL of stopping reagent, 100 µL of 10 % (w/v) ascorbic acid were added. After 15 min, 1.45 mL of arsenite citrate regent was added to prevent further color development due to non-enzymatic hydrolysis of ATP under acidic conditions as reported by Baginski et al. (1967). The arsenite citrate regent had the following compositions; 2% [w/v] sodium citrate, 2% [w/v] sodium *m*-arsenite, 2% [w/v] glacial acetic acid. After 30 min at 30°C, the absorbance at 820 nm was measured using a spectrophotometer (Varian, Cary 4 Bio UV-Visible Spectrophotometer).

**2.7 Statistical analysis**:

Within the framework of this study, data are means of at least three biological replications was investigated. Variation among the biological replications was characterized by standard errors. The coefficient of correlation (r) was used for studying correlation between variables (Snedecor 1956). One way ANOVA was conducted to analyze the data for variance using SPSS 13.0 computer software. Multiple comparisons separating means in homogenous subgroups were done using the post hoc Tukey test ($p \leq 5\%$, $p \leq 1\%$).

## 3 Results

The results of this study can be divided into the following four main parts:

- Screening of SR hybrids for ion toxicity
- Effect of different salinity levels on $Na^+$ exclusion at the root surface and $Na^+$ exclusion from the shoot
- Contribution of $Na^+$ inclusion into leaf vacuoles to salt resistance of newly developed maize hybrids in the second phase of salt stress.
- Effect of salinity and $Na^+$ on $H^+$-ATPase activity in tonoplast vesicles isolated from control and salt-treated maize genotypes

### 3.1 Screening of SR hybrids for ion toxicity

In order to demonstrate resistance to ion toxicity during the second phase of salt stress, various newly developed maize hybrids were compared regarding shoot growth, $Na^+$ accumulation and number of necrotic leaf spots.

#### 3.1.1. Effect of salt stress on plant growth parameters in the first phase of salt stress

Maize genotypes were affected by salinity stress to varying degrees. While the salt-sensitive genotype Across 8023 showed the highest reduction in shoot growth, salt-resistant SR 03 showed relatively less reduction of growth under a high salinity level (200 mM NaCl) (Fig. 10). A similar effect of salt stress was found for leaf growth. The leaf length was lowest in Across 8023 with only 22.5 cm under 200 mM NaCl compared to all other genotypes tested which showed a larger leaf length (Fig. 11A). Accordingly, Across 8023 had the highest relative reduction in leaf length with approximately 19% (Fig. 11B). Similarly, Pioneer 3906 which is classified as medium salt-resistant showed a large reduction under salt stress. On the other hand, the minimum reduction of leaf length was recorded in four SR hybrids (SR 03, SR 05, SR 13 and SR 16) which is in line with their higher absolute leaf length (Fig. 11A). The remaining maize hybrids, SR 09, SR 12, SR 20 and SR 21 showed a medium relative reduction of leaf lengths in a range of approximately 8.0% to 11.0% (Fig. 11B).

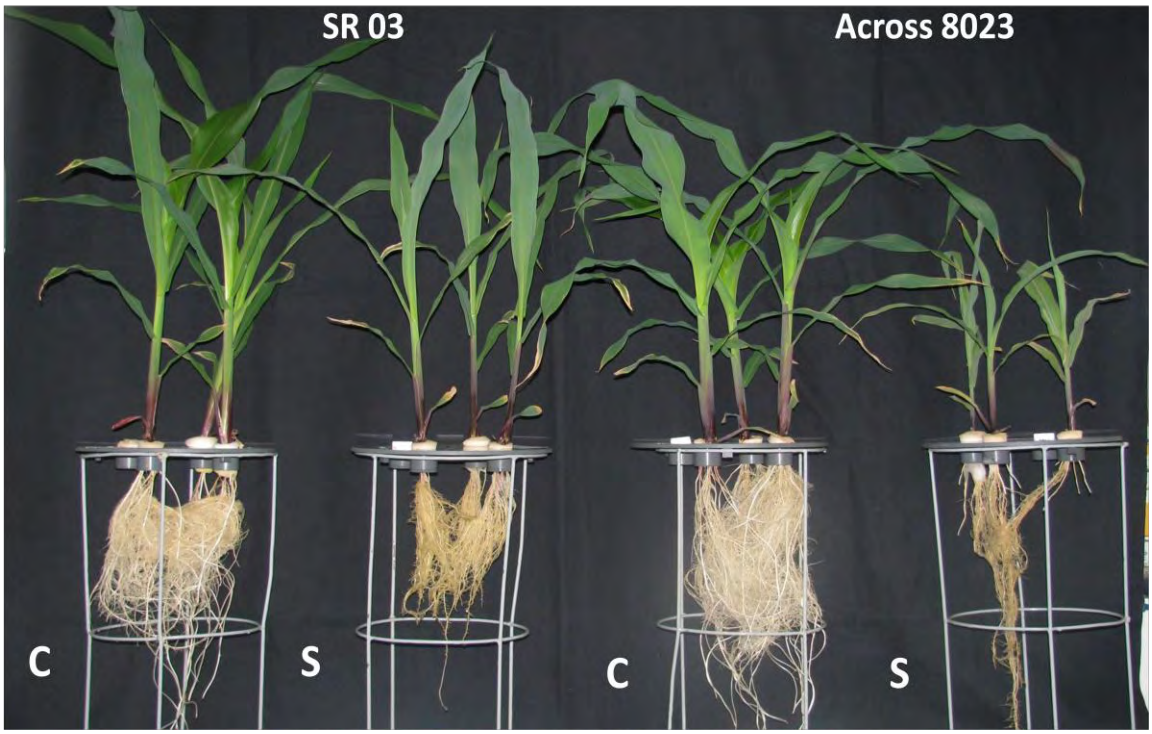


**Figure 10**: Growth of maize plants (*Zea mays* L., SR 03 and Across 8023) cultivated under control (1 mM NaCl) and saline conditions (200 mM NaCl). Plants were harvested 26 d after the beginning of plant cultivation.

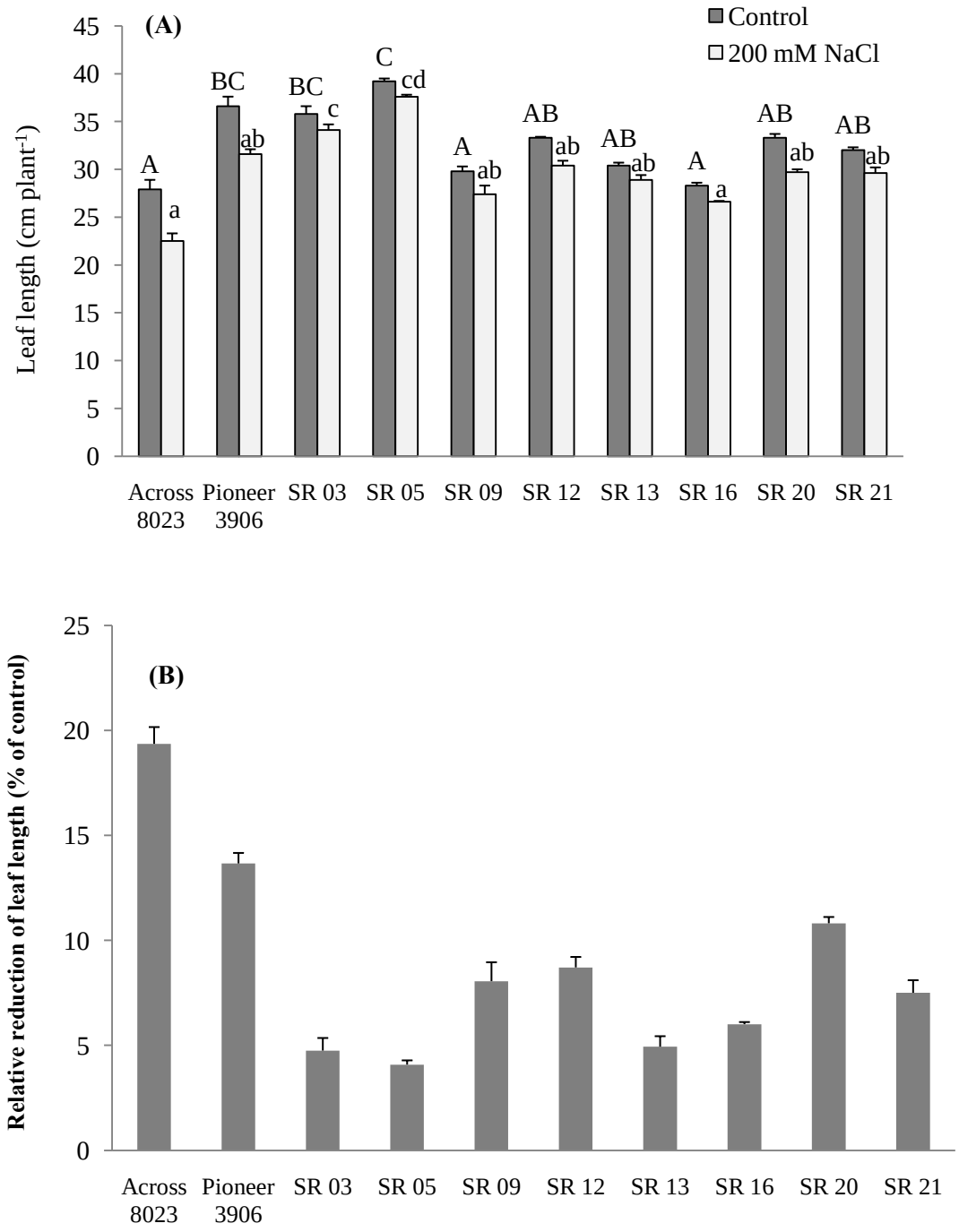


**Figure 11:** Leaf growth of various maize genotypes as affected by salinity. **(A)** Absolute leaf lengths under control (1 mM NaCl) and saline conditions (200 mM NaCl). Data are means of four replicates ± SE. **(B)** Relative reduction of leaf length compared to control (control = 100 %). Significant differences ($P \leq 5\%$) between treatments and genotypes are indicated by different letters.

Beside leaf length, shoot and root fresh weights were considered as the most important parameters to select and classify maize genotypes as salt-resistant and salt-sensitive particularly in the first phase of salt stress. As shown in Figures 12A, all newly developed SR hybrids showed higher shoot fresh weights compared to Across 8023 in terms of absolute shoot fresh weight. In this context, the highest relative reduction of shoot fresh weight under high salinity (200 mM NaCl) was recorded for SR 16 and Across 8023 with approximately 71%, while the lowest relative reduction was found for SR 03, SR 05 and SR 21(63%) as shown in Figure 12B. The remaining SR hybrids showed medium reduction of shoot fresh weight. SR 03, SR 05 and SR 21 also maintained higher absolute root fresh weights compared to Across 8023 and other SR hybrids (Fig. 13A). In contrast, SR 03, SR 05 and SR 21 showed the lowest relative reduction of root fresh weight under high levels of salinity with 58%, 35% and 59%, respectively and differed from all the other tested maize genotypes (Fig. 13B). On the other hand, the two genotypes Across 8023 and SR 16 showed the highest relative reduction of about 66% of root fresh weight, while the SR hybrids SR 09, SR 12, SR 13 and SR 20 showed a relative reduction of root fresh weight in a range between 61% to 65%. Based on absolute and relative reduction of shoot and root fresh weight, SR 03 and SR 05 were classified as the most salt-resistant maize hybrids.

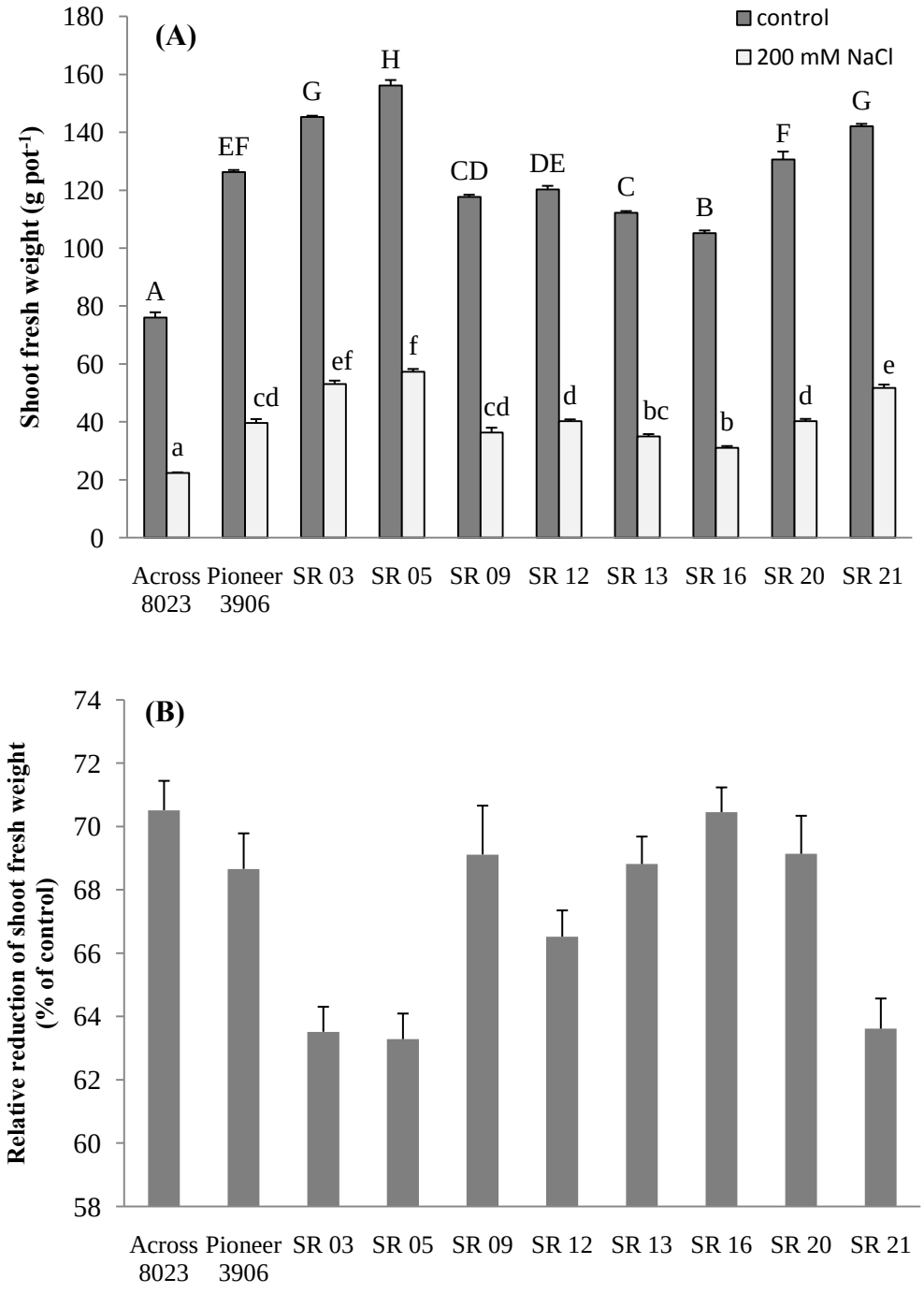


**Figure 12:** Absolute shoot fresh weight **(A)** and relative reduction of shoot fresh weight **(B)** of various maize genotypes under control (1 mM NaCl) and saline (200 mM NaCl) conditions. Data are means of four replicates ± SE. Significant differences ($P \leq 5\%$) between treatments and genotypes are indicated by different letters.

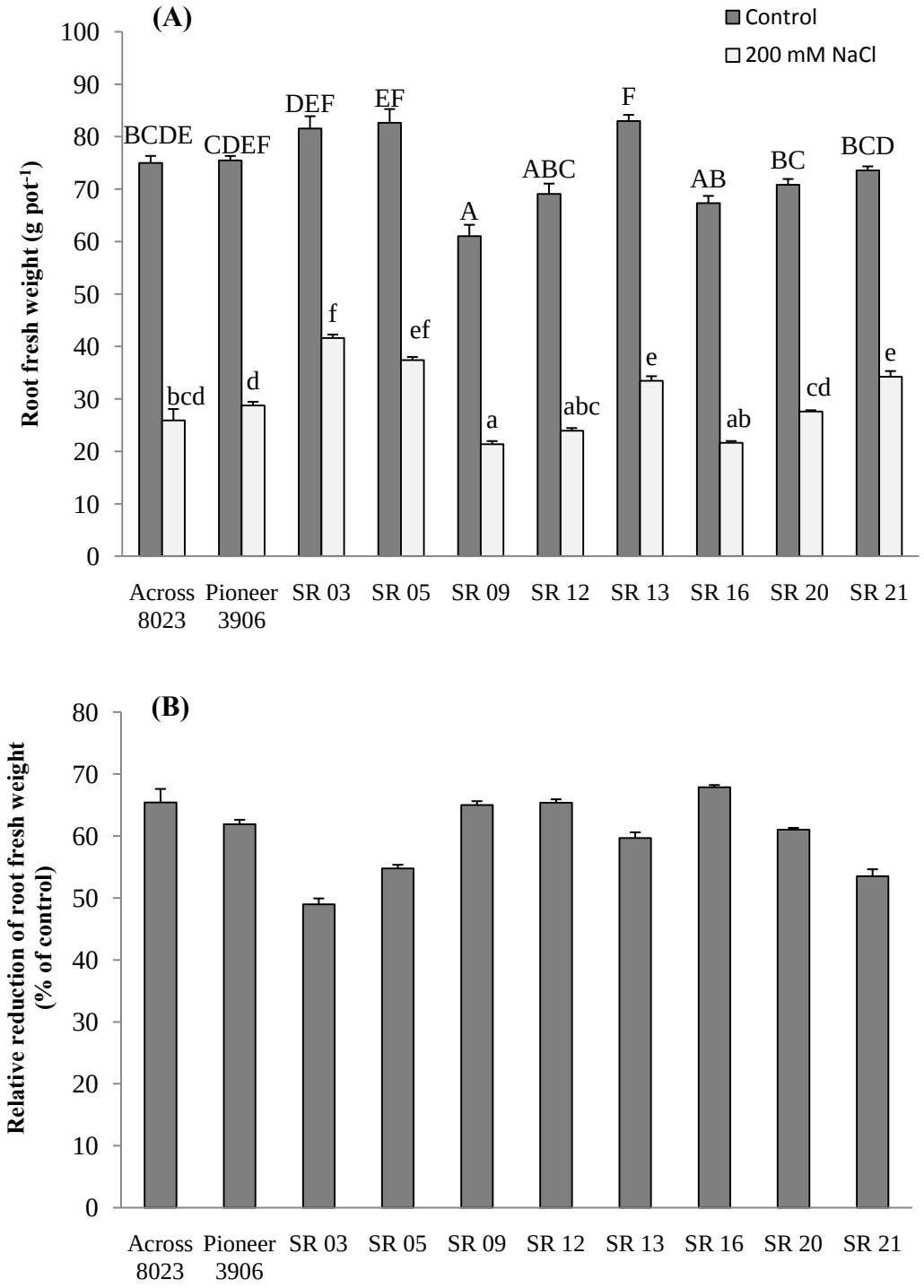


**Figure 13:** Absolute root fresh weight **(A)** and relative reduction of root fresh weight of various maize genotypes as influenced by salt treatment. Data are means of four replicates ± SE. **(B)** (control =100). Significant differences ($P \leq 5\%$) between treatments and genotypes are indicated by different letters.

On a dry matter basis, shoot and root growth parameters of all genotypes were negatively affected by the high level of salinity (Fig. 14 and 15). The highest reduction in shoot dry weight caused by salinity was about 72% in Across 8023 and 62% in Pioneer 3906, respectively (Fig. 14B). In contrast, the genotypes SR 03 and SR 05 showed the lowest relative reduction in shoot dry weight with 30% and 35%, respectively (Fig. 14B). The other SR hybrids showed a medium absolute shoot dry weight, while the relative reduction ranged between 42% in SR 21 and 53% in SR09. The results for root dry weight were similar to the shoot dry weight (Fig. 15A). SR 05 and SR 03 showed the lowest relative reduction with 33% and 37%, respectively, as compared to Across 8023 and Pioneer 3906 which exhibited a higher relative reduction of 48% and 50%, respectively, (Fig. 15B). In contrast, some SR hybrids showed the highest relative reduction of root dry weight, e.g. SR 16 with a maximum value of 58% (Fig. 15B).

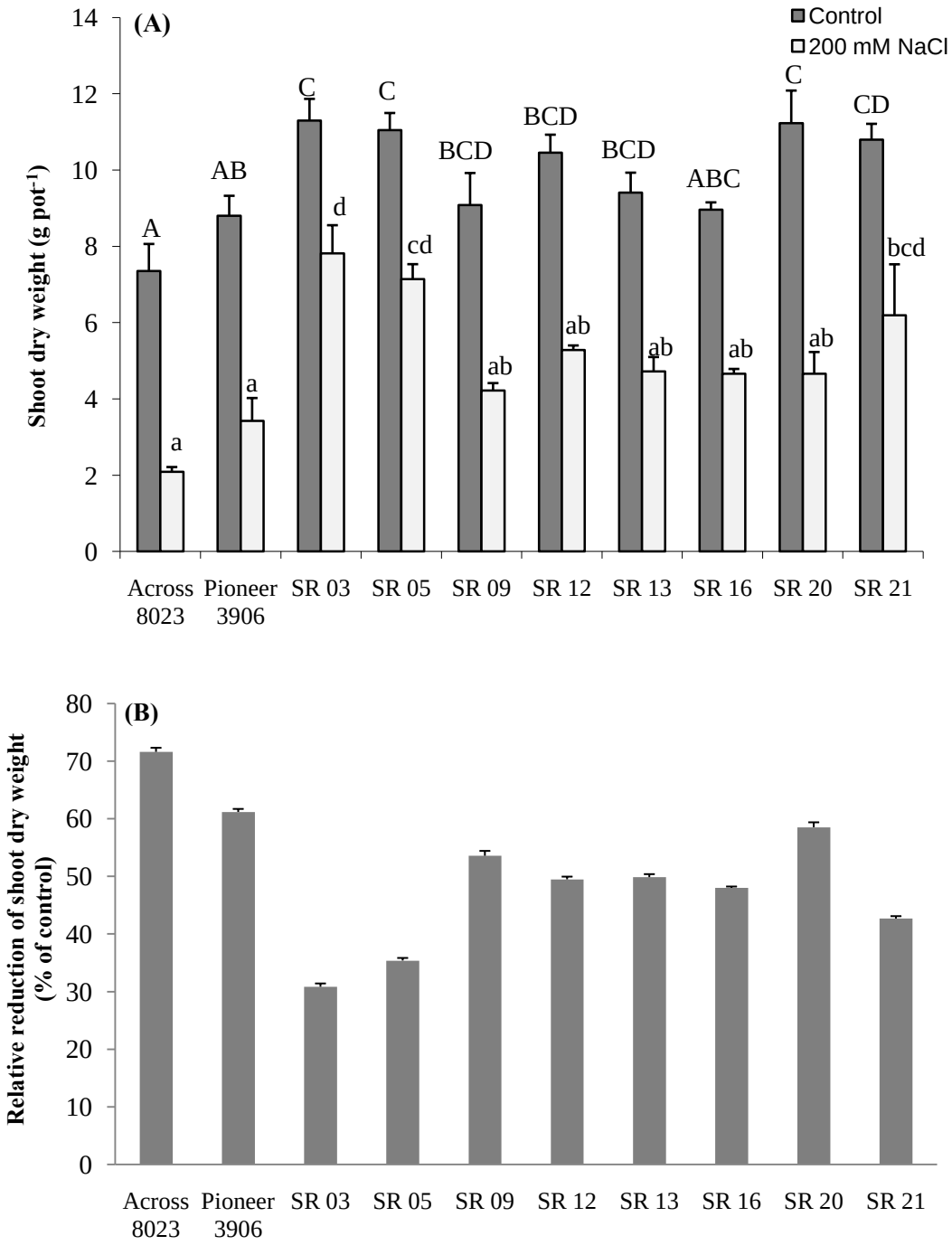


**Figure 14: (A)** Absolute shoot dry weight of various maize genotypes under control (1 mM NaCl) and saline (200 mM NaCl) conditions. Data are means of four replicates ± SE. Plants were harvested 26 d after the beginning of plant cultivation. **(B)** Relative shoot dry weight reduction compared to control (control = 100 %). Significant differences ($P \leq 5\%$) between treatments and genotypes are indicated by different letters.

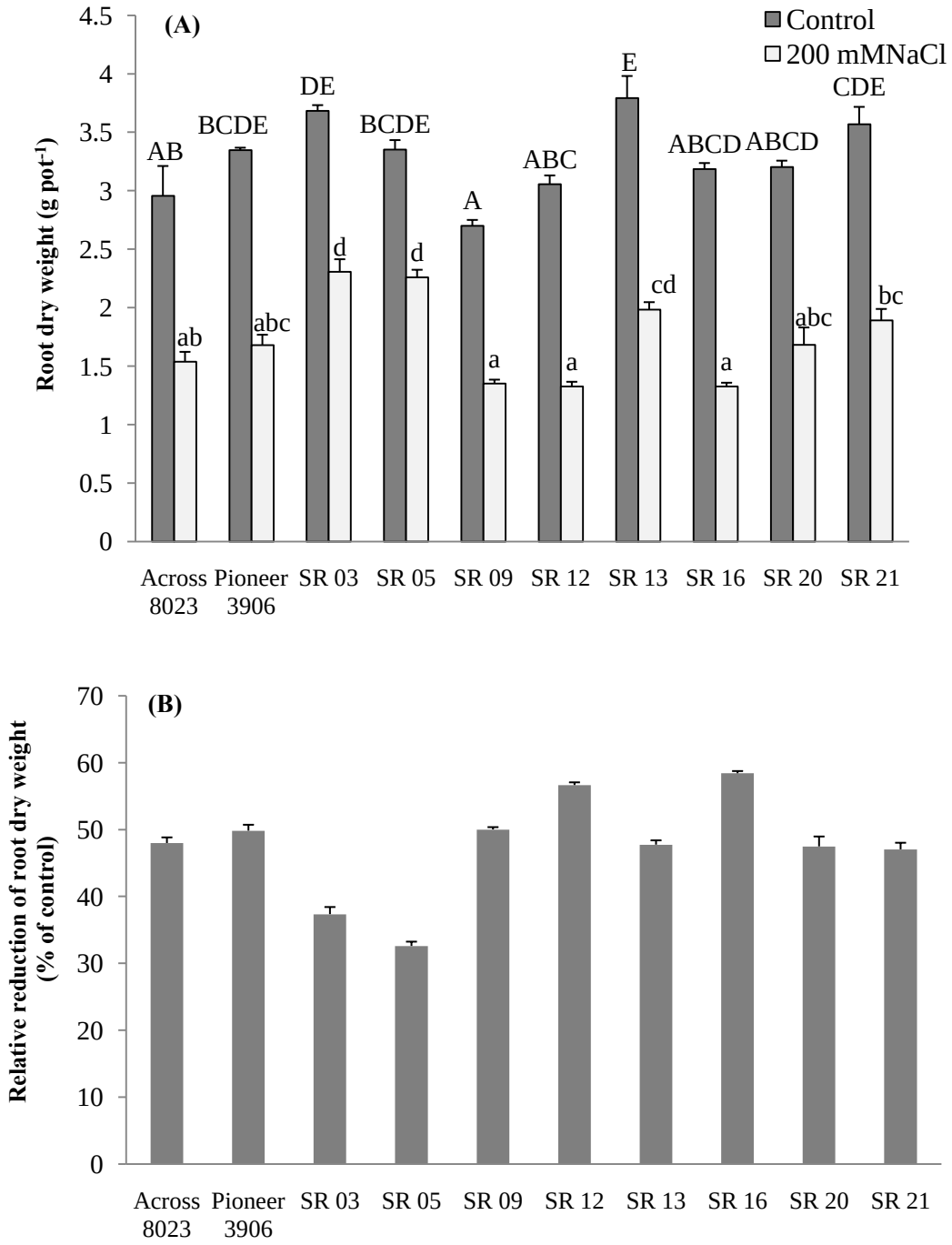


**Figure 15: (A)** Absolute root dry weight of various maize genotypes under control (1 mM NaCl) and saline (200 mM NaCl) conditions. Data are means of four replicates ± SE. Plants were harvested 26 d after the beginning of plant cultivation. **(B)** Relative root dry weight compared to control (control = 100%). Significant differences ($P \leq 5\%$) between treatments and genotypes are indicated by different letters.

### 3.1.2 $Na^+$ accumulation in roots and shoots under salt stress

When high a salinity level (200 mM NaCl) was applied to the growth medium it caused significantly increased shoot and root $Na^+$ concentrations in all tested maize genotypes (Fig. 16). In terms of $Na^+$ concentration, newly developed SR hybrids showed a significant variation in both shoot and root $Na^+$ concentration compared to Across 8023. Among the genotypes, SR 03, SR 05, SR 09, SR 12, SR 16, SR 20 and SR 21 had low shoot $Na^+$ concentrations in a range between 9 and 14 mg $Na^+$ $g^{-1}$ DW, respectively, while Pioneer 3906 (15 mg $Na^+$ $g^{-1}$ DW) and SR 13 (17 mg $Na^+$ $g^{-1}$ DW) showed medium values of shoot $Na^+$ concentration. Salt-sensitive Across 8023 showed the highest shoot $Na^+$ concentration of 27 mg $Na^+$ $g^{-1}$ DW (Fig. 16). On the other hand, SR 03 exhibited a lower $Na^+$ concentration in the roots (22 mg $Na^+$ $g^{-1}$ DW) compared to Across 8023 and SR 13, which had the highest $Na^+$ concentration with 45 mg $Na^+$ $g^{-1}$ DW and 40 mg $Na^+$ $g^{-1}$ DW, respectively. Moderate root $Na^+$ concentrations with an average value of 25 mg $Na^+$ $g^{-1}$ DW (Fig. 16) were found in the remaining genotypes Pioneer 3906, SR 05, SR 12, SR 16, SR 20 and SR 21.

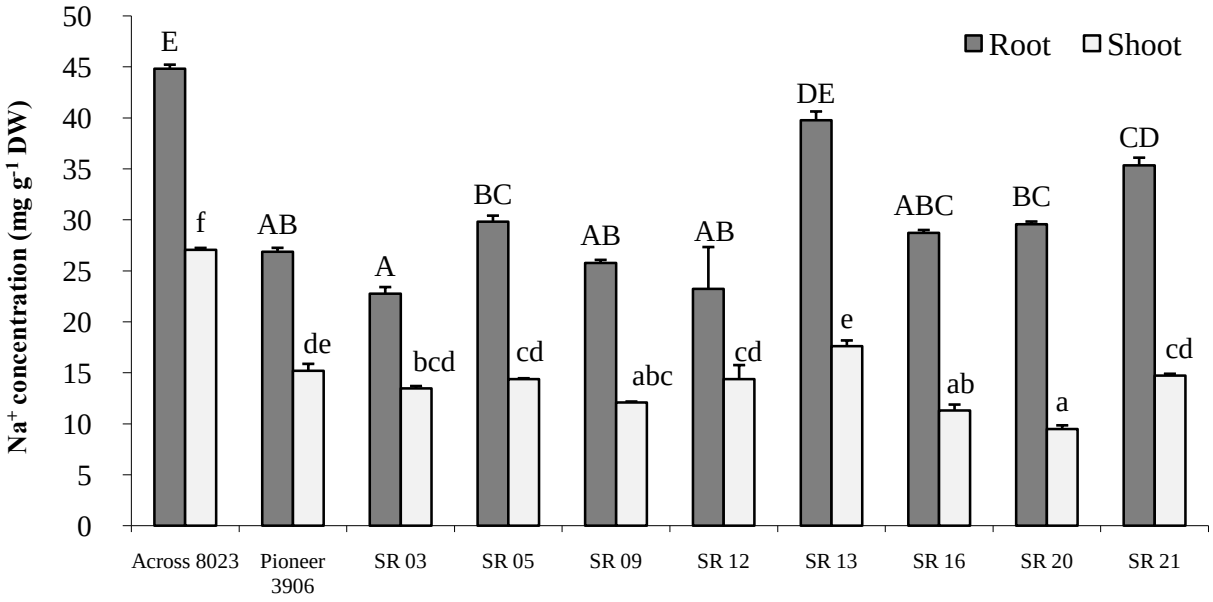


**Figure 16:** Sodium concentration in the root and shoot dry weights of various maize genotypes under control (1 mM NaCl) and salinity treatment (200 mM NaCl). Data are means of four replicates ± SE. Maize plants were harvested 26 d after the beginning of plant cultivation. Significant differences ($P \leq 5\%$) between treatments and genotypes are indicated by different letters.

### 3.1.3 $Na^+$ uptake and $Na^+$ translocation from root to shoot

As shown in Figure 17A, the highest level of $Na^+$ uptake (110.21 mg $Na^+$ $g^{-1}$ dry weight) was recorded in the root of Across 8023 followed by SR 13 (82. 3 mg $g^{-1}$ dry weight), SR 05 (77. 14 mg $Na^+$ $g^{-1}$ dry weight) and SR 21 (76. 47 mg $Na^+$ $g^{-1}$ dry weight). In contrast, the lowest $Na^+$ uptake was recorded in the root of SR 16 (57, 57 mg $Na^+$ $g^{-1}$ dry weight) followed by SR 20 (57. 92 mg $Na^+$ $g^{-1}$ dry weight). Moderate levels of $Na^+$ uptake were detected in the roots of the other four maize genotypes (Fig. 17A). $Na^+$ translocation from root to shoot of various maize genotypes grown under 200 mM NaCl ranged between 0. 96 and 1.92 with the highest level recorded in SR 03 and SR 12 and the lowest level of $Na^+$ translocation was detected in SR 20 and SR 16. The other maize genotypes exhibited moderate levels of $Na^+$ translocation from root to shoot ranging from 1.62 to 1.16 (Fig. 17B).

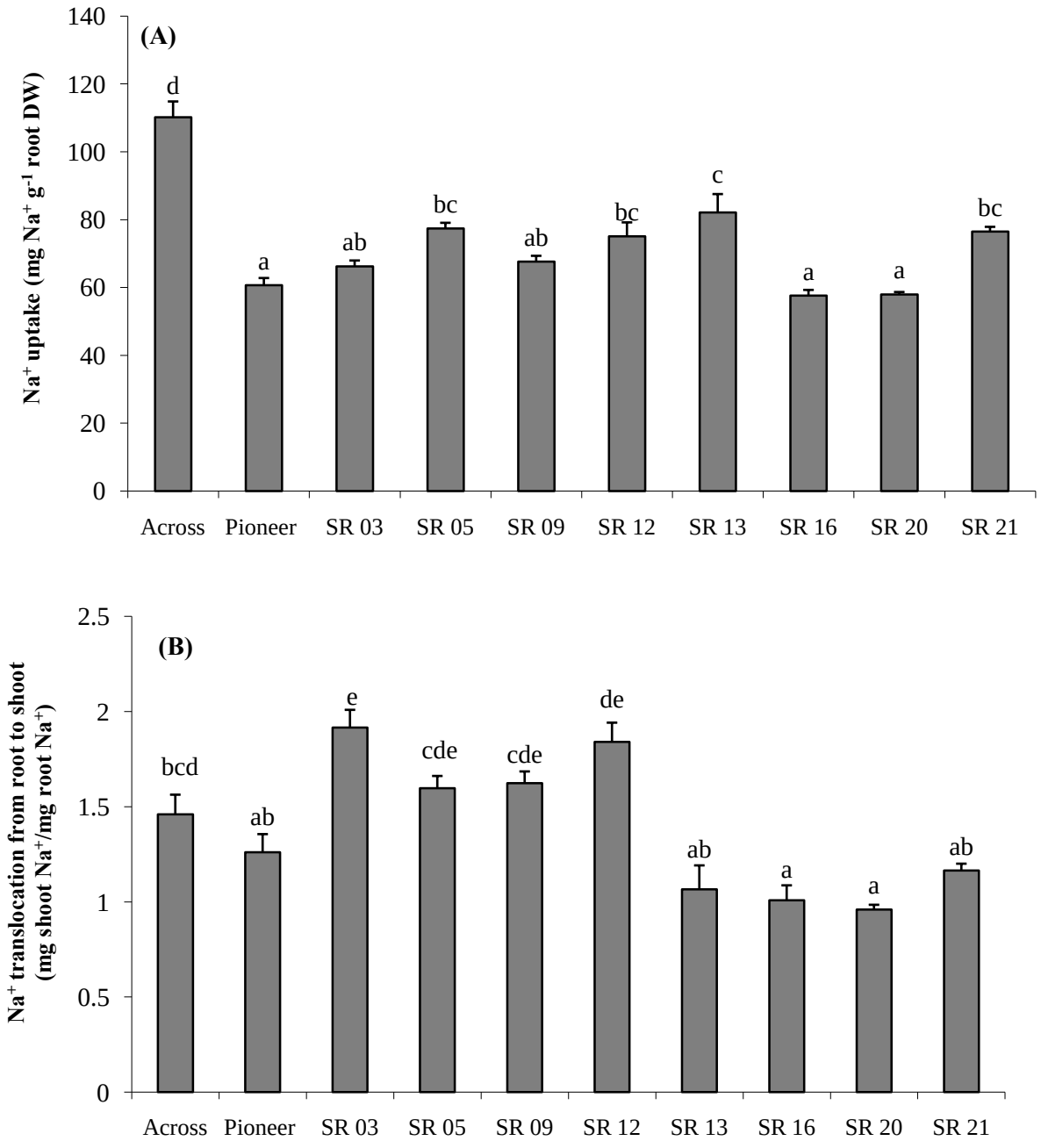


**Figure 17: (A)** Sodium uptake at root level (plant $Na^+$ content/ root dry weight) and **(B)** sodium translocation from root to shoot of various maize genotypes under control (1 mM NaCl) and salinity conditions (200 mM NaCl). Data are means of four replicates ± SE. Maize hybrids were harvested 26 d after the beginning of plant cultivation. Significant differences ($P \leq 5\%$) between treatments and genotypes are indicated by different letters.

### 3.1.4 Relationship between $Na^+$ concentration in shoots and number of necrotic spots per leaf

The second phase of salt stress is defined as the appearance of ion toxicity symptoms. A large variation among maize genotypes in their resistance to salinity was observed on the basis of severity of leaf symptoms (Fig. 18). Salt stress (200 mM)-induced leaf symptoms were observed as necrotic spots on the older leaves in all tested genotypes (Fig. 18). Among the maize genotypes, SR 03 and SR 05 showed a lower number of necrotic spots on the older leaves compared to other genotypes which showed very severe leaf symptoms (Across 8023, SR 13, SR 16, SR 20, SR 21 and Pioneer 3906). On the same basis, SR 09 and SR 12 showed a moderate level of $Na^+$ toxicity. The results show that the salt-resistant genotypes SR 03 and SR 05 had low $Na^+$ concentrations in their shoot (Fig. 19) and also showed a lower number of necrotic spots on the older leaves (Fig. 19). On the other hand, the salt-sensitive genotypes Across 8023 and also the hybrid SR 20 showed the highest number of necrotic spots per leaf (11.75 and 10.50). SR 20 showed the lowest shoot $Na^+$ concentration compared to the salt-sensitive Across 8023 which showed the highest shoot $Na^+$ concentration (Fig. 19). The other maize genotypes exhibited medium $Na^+$ concentration in the shoot but a high number of necrotic spots per leaf at the same time. The correlations between $Na^+$ concentration and number of necrotic leaf spots clearly shows that there is no relation between $Na^+$ concentrations in shoots and the number of necrotic spots per leaf. Based solely on the severity of leaf symptoms, SR 03 and SR 05 could be identified as plants which are salt-resistant in the second phase of salt stress, while SR20 and Across 8023 were classified as salt-sensitive.

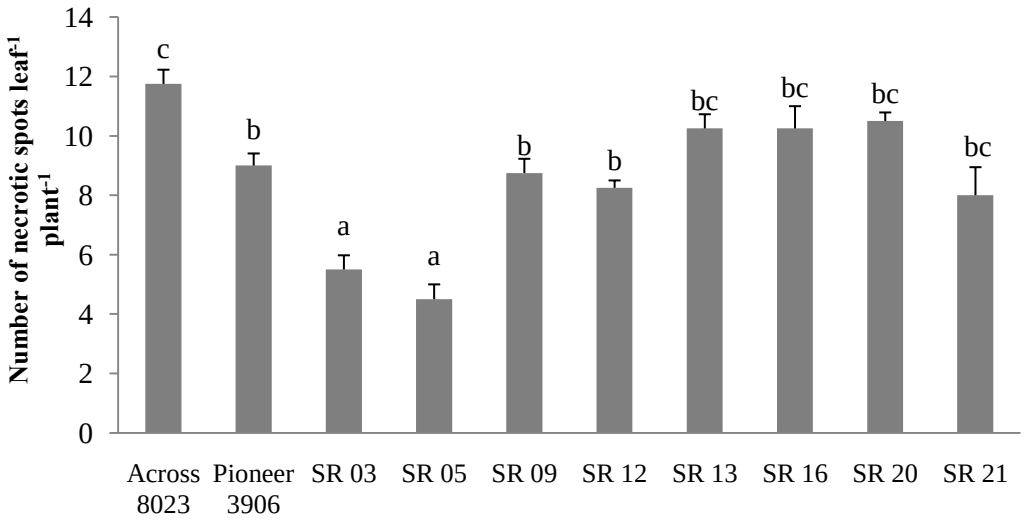


**Figure 18:** Number of necrotic spots per leaf of various maize genotypes as affected by salinity treatment (200 mM NaCl). Data are means of four replications ± SE. Plants were harvested after 26 d when necrotic symptoms appeared on old leaves of plants grown under salinity. Significant differences ($P \leq 5\%$) between treatments and genotypes are indicated by different letters.

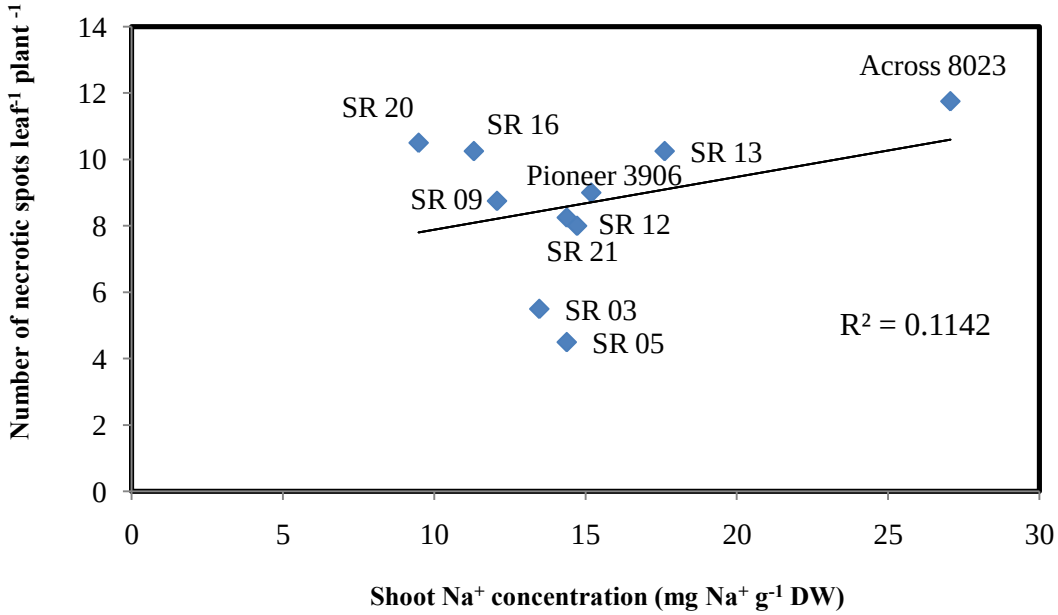


**Figure 19:** Correlation between shoot $Na^+$ concentration and number of necrotic spots per leaf of various maize genotypes in the second phase of salt stress (200 mM NaCl). Data are means of four replicates ± SE.

### 3.1.5 Ion concentrations in shoots and roots of maize hybrids

High salinity levels (200 mM NaCl) in the nutrient solution decreased the concentrations of $K^+$ and $Ca^{2+}$ in shoots of all genotypes (Fig. 20 and 21). However, plants never faced $K^+$ deficiency according to Bergmann (1993) (Fig. 20A). Under salt stress (200 mM NaCl), the salt-resistant and salt-sensitive maize genotypes did not differ in terms of $K^+$ shoot concentration. In comparison, the decrease in $K^+$ concentration of all tested maize genotypes was more pronounced in the roots. Among the genotypes, SR 03, SR 05, SR16 and SR 20 showed a higher root $K^+$ concentration than the other maize genotypes (Fig. 20B). Similar to $K^+$ concentration, the shoot $Ca^{2+}$ concentration in all tested genotypes was above the low critical value (3 mg $Ca^{2+}$ $g^{-1}$ shoot DW) (Fig. 21A). Figure 21B shows that similar results were found in the roots for all tested maize genotypes. The $Ca^{2+}$ concentration was within the two critical range of 3 mg and 10 mg $Ca^{2+}$ $g^{-1}$ shoot DW, respectively.

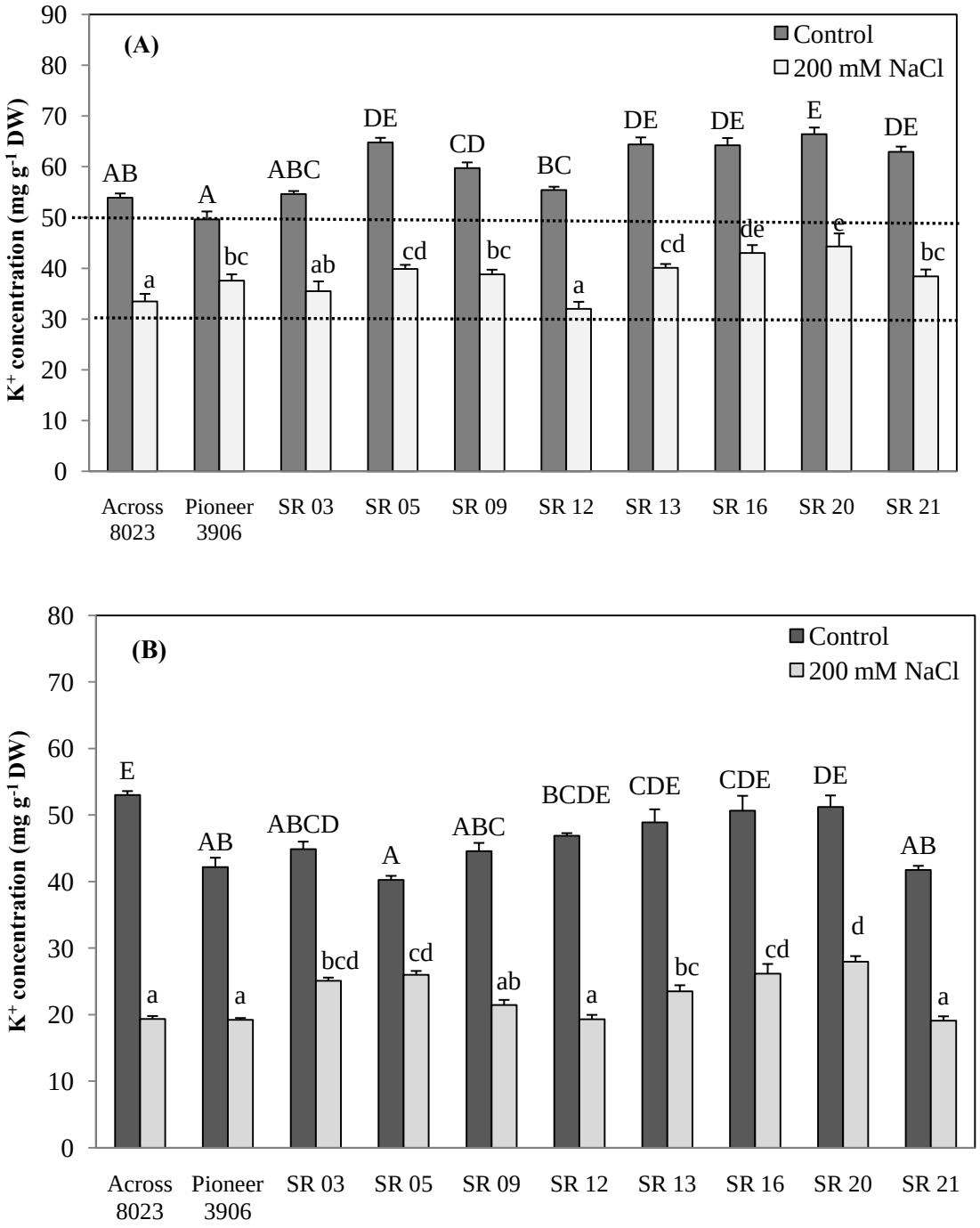


**Figure 20**: Potassium concentrations in shoots **(A)** and in roots **(B)** of various maize genotypes under control (1 mM NaCl) and salinity (200 mM NaCl). Data are means four replicates ± SE. The low critical value for $K^+$ (30 mg $K^+$ $g^{-1}$ DW) and high critical value of maize shoots (50 mg $K^+$ $g^{-1}$ DW) are indicated according to Bergmann (1993). Significant differences ($P \leq 5\%$) between treatments and genotypes are indicated by different letters.

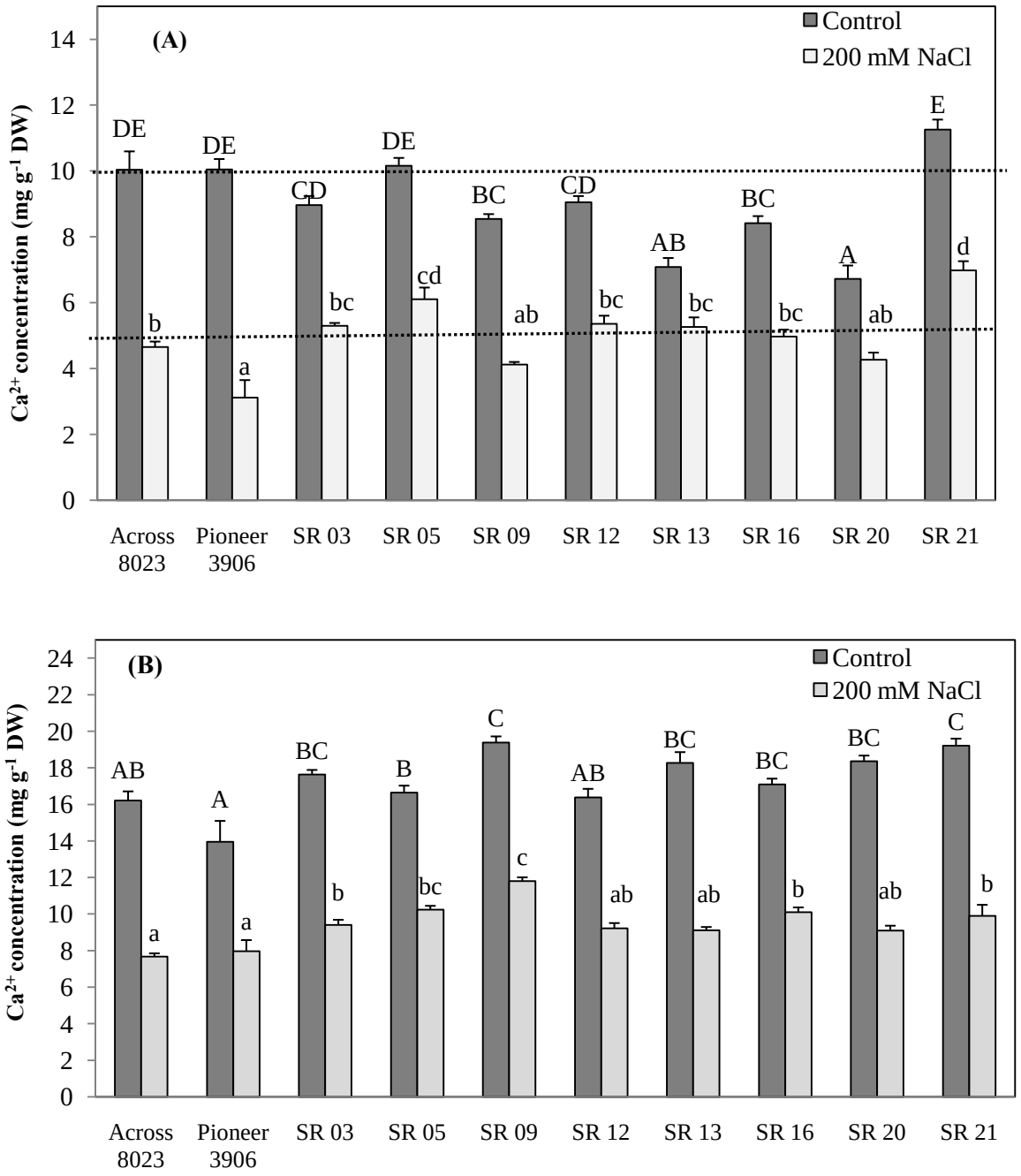


**Figure 21:** Calcium concentrations in shoots **(A)** and in roots **(B)** of various maize genotypes under control (1 mM NaCl) and salinity (200 mM NaCl). Data are means four replicates ± SE. The low critical value for $Ca^{2+}$concentration (3 mg $Ca^{2+}$ $g^{-1}$ DW) and high critical value of maize shoots (10 mg $Ca^{2+}$ $g^{-1}$ DW) are indicated according to Bergmann (1993). Significant differences ($P \leq 5\%$) between treatments and genotypes are indicated by different letters.

## 3.2 Effect of different salinity levels on $Na^+$ exclusion at the root surface and $Na^+$ exclusion from the shoot

### 3.2.1 Plant growth parameters

In order to determine the effect of salt stress on plant growth in the second phase of salt stress, growth parameters were measured. Leaf length was much more decreased by salt stress (200 mM NaCl) in Across and SR 20 compared to SR 03 and SR 05, which showed lowest reduction (Fig. 22). The other growth parameters such as shoot and root fresh and dry weights were also decreased by salt stress. The highest relative reduction of shoot fresh and dry weights caused by NaCl treatment (200 mM NaCl) was observed for Across 8023, being significantly higher compared to SR 03, which showed the lowest reduction (Fig. 23). These reductions were more pronounced under high salinity treatment (200 mM NaCl) compared to lower salinity levels (50 mM NaCl) with a maximum decrease found in Across 8023 (77%) while SR 03 showed only a decrease by 53% (Fig. 24). Similar results were found for root fresh and dry weights, whereas the highest reduction of root fresh weight was observed for Across 8023 (60%) while the lowest reduction was recorded for SR 05 under high salinity level (Fig. 24A). Accordingly, all SR hybrids showed low relative reduction of root dry weight under low salt treatment (50 mM NaCl); for Across 8023 this parameter was significantly increased. On the other hand, high salinity (200 mM NaCl) led to high reduction of root dry weight in all tested maize genotypes which ranged from 53% for SR 05 up to 63% for Across 8023 (Fig . 24B).

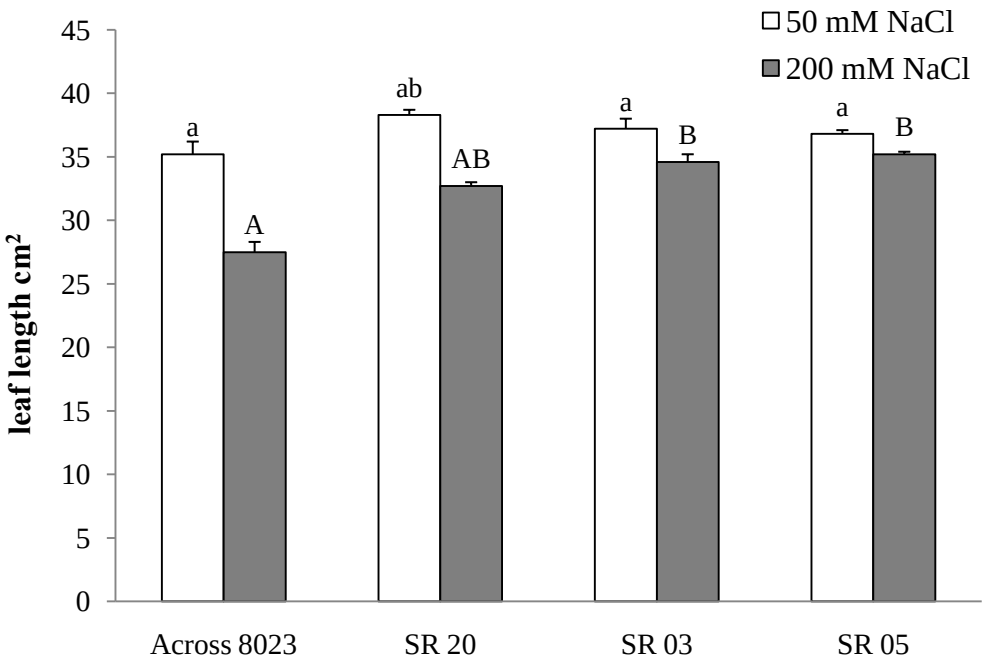


**Figure 22:** Leaf length (cm) of four maize genotypes as affected by low (50 mM NaCl) and high salinity treatment (200 mM NaCl). The results represent means of three replicates ± SE. Significant differences ($P \leq 5\%$) between treatments are indicated by different letters. Plants were harvested 26 d after the beginning of plant cultivation.

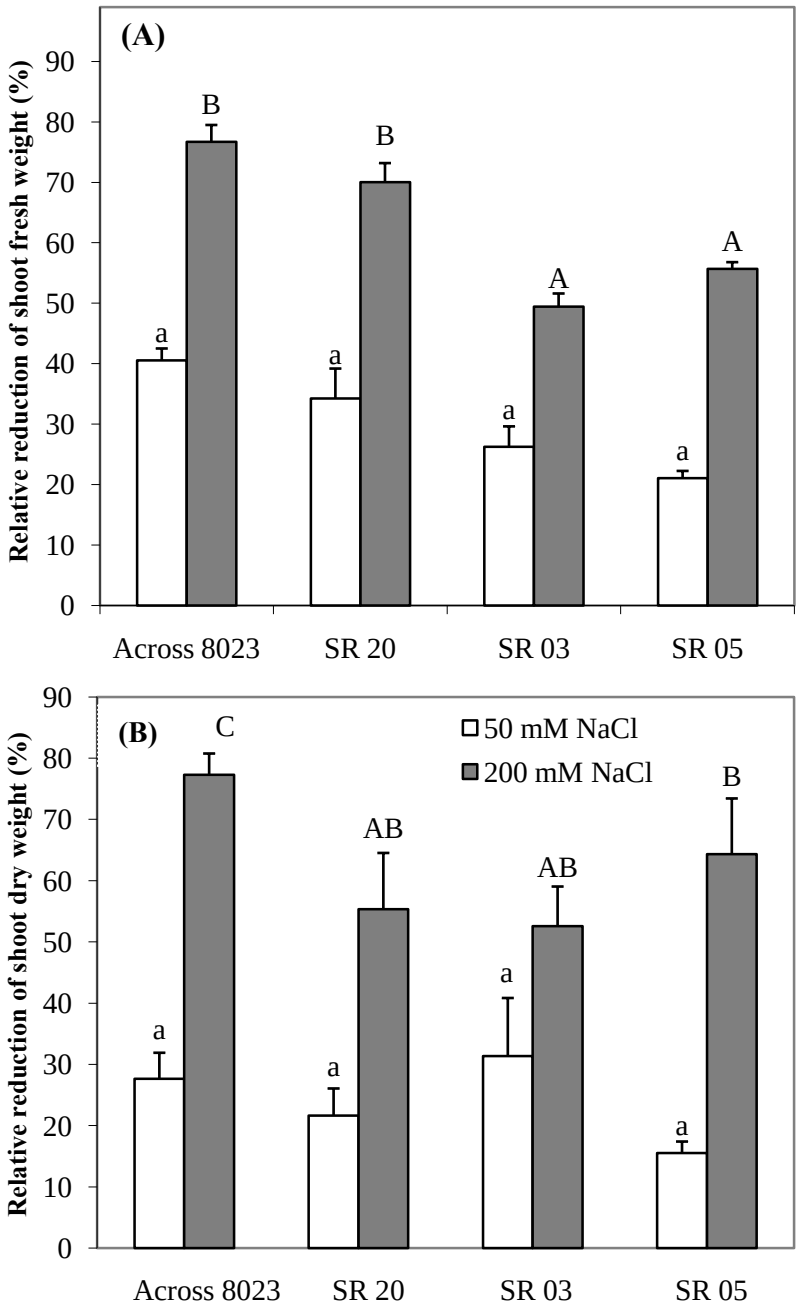


**Figure 23:** Relative reduction of **(A)** shoot fresh weight and **(B)** shoot dry weight of maize genotypes as affected by low (50 mM NaCl) and high salinity treatment (200 mM NaCl). The results represent means ± SE of three replicates. Significant differences (P ≤ 5%) between treatments are indicated by different letters. Plants were harvested 26 d after the beginning of plant cultivation.

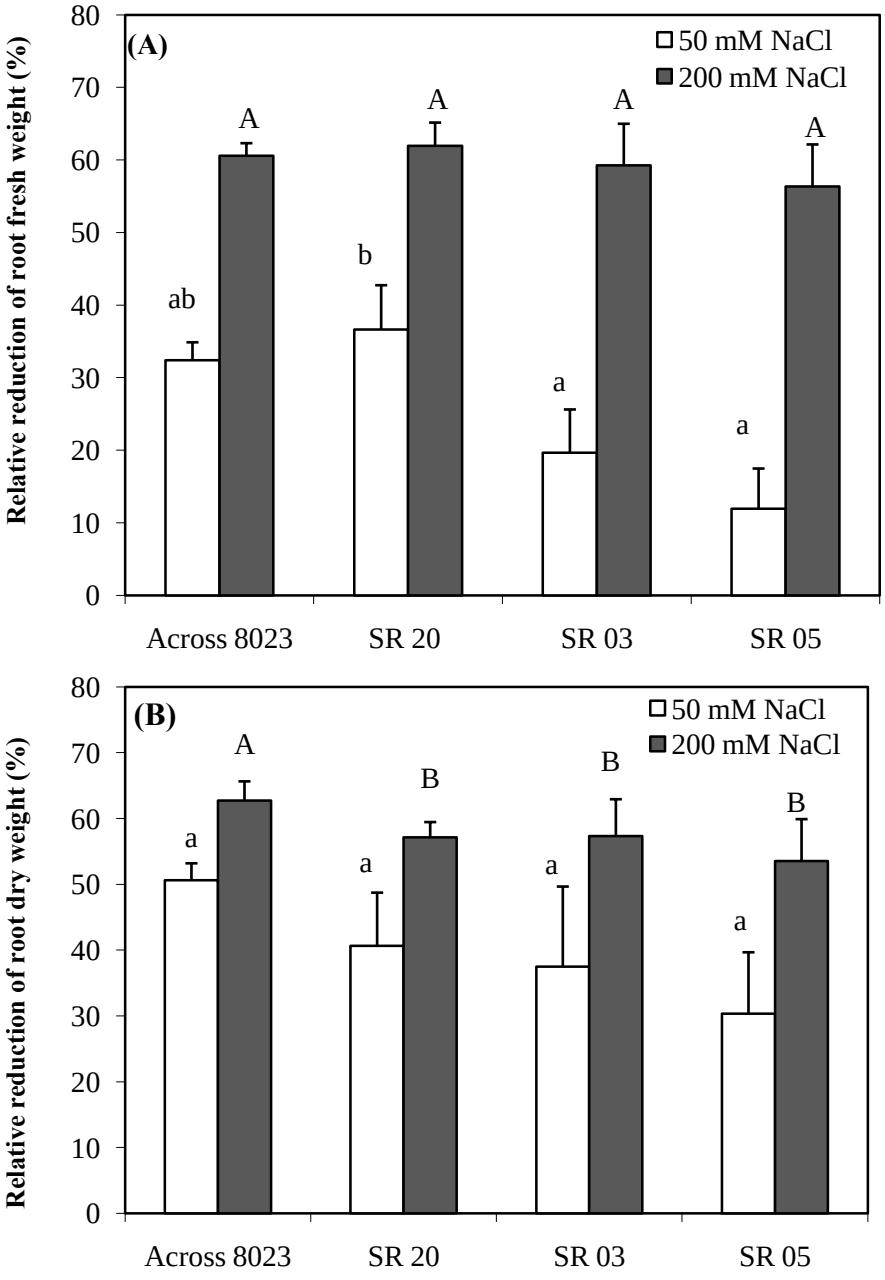


**Figure 24:** Relative reduction of **(A)** root fresh weight and **(B)** root dry weight of maize genotypes as affected by low (50 mM NaCl) and high salinity treatment (200 mM NaCl). The results represent means ± SE of three replicates. Significant differences ($P \leq 5\%$) between treatments are indicated by different letters. Plants were harvested 26 d after the beginning of plant cultivation.

### 3.2.2 $Na^+$ accumulation in shoots and roots under salt stress

After application of 200 mM NaCl, increased $Na^+$ concentrations in shoots and roots of all tested maize genotypes were recorded. However, $Na^+$ concentration was higher in Across 8023 under low and high NaCl treatment with 13 and 30 mg Na $g^{-1}$ DW, respectively (Tab. 8). On the other hand, all SR-hybrids exhibited lower $Na^+$ concentration which ranged from 11 mg $Na^+$ $g^{-1}$ DW in SR 20 up to 21 mg $Na^+$ $g^{-1}$ DW in SR 05 under a high salinity level. Under low salinity levels (50 mM NaCl) no significant differences among the tested SR hybrids were determined. Similar results were found in roots, in which Across 8023 showed highest $Na^+$ concentrations, while for SR hybrids $Na^+$ concentrations ranged between 32 mg $Na^+$ $g^{-1}$ DW in SR 20 and 44 mg $Na^+$ $g^{-1}$ DW in SR 05 (Tab. 8).

**Table 8:** Effect of salt treatment in nutrient solution on the sodium concentration in shoots and roots of various maize genotypes under two salinity levels (50 mM and 200 mM NaCl). Data are means ± SE (n = 3). Plants were harvested 26 d after the beginning of plant cultivation. Significant differences (P ≤ 5%) between treatments are indicated by different letters.

| | Shoot | | Root | |
|---|---|---|---|---|
| | 50 mM | 200 mM | 50 mM | 200 mM |
| Across 8023 | 13.21 ± 0.8c | 30.25 ± 3.3D | 26.11 ±1.2 b | 45.66 ± 0.3C |
| SR 20 | 2.15 ± 0.6 a | 11.09 ± 1.2 A | 19.06 ± 1.3a | 31.99 ± 1.1A |
| SR 03 | 5.48 ± 0.2b | 14.38 ± 1.9AB | 22.94 ± 0.8 ab | 39.33 ± 1.6 B |
| SR 05 | 3.87 ± 0.8ab | 20.86 ± 0.8 C | 23.25± 0.7ab | 45.59 ± 1.2 B |

### 3.2.3 Correlation between $Na^+$ concentrations in shoots and shoots fresh weights and the number of necrotic leaves

High salinity treatment (200 mM NaCl) resulted in necrotic patches on the older leaves and also caused a reduction of shoot fresh weight in all tested genotypes. However, SR 03 and SR 05 had a lower number of necrotic spots per leaf and at the same time exhibited lower reduction of shoot fresh weight (Fig. 25). The other two genotypes (Across 8023 and SR 20) exhibited a higher number of necrotic spots per leaf and they had a higher reduction of shoot fresh weight. However, a poor relationship ($R^2 = 0.21$) was found between shoot $Na^+$ concentration and shoot fresh weight (Fig. 25A). A slightly better correlation ($R^2 = 0.35$) was found between shoot $Na^+$ concentration and severity of leaf symptoms (Fig. 25B). Based on the results of the growth parameter and the number of necrotic spots per leaf, the genotypes SR 03 and SR 05 were classified as salt-resistant whereas the genotypes Across 8023 and SR 20 were referred to as salt-sensitive.

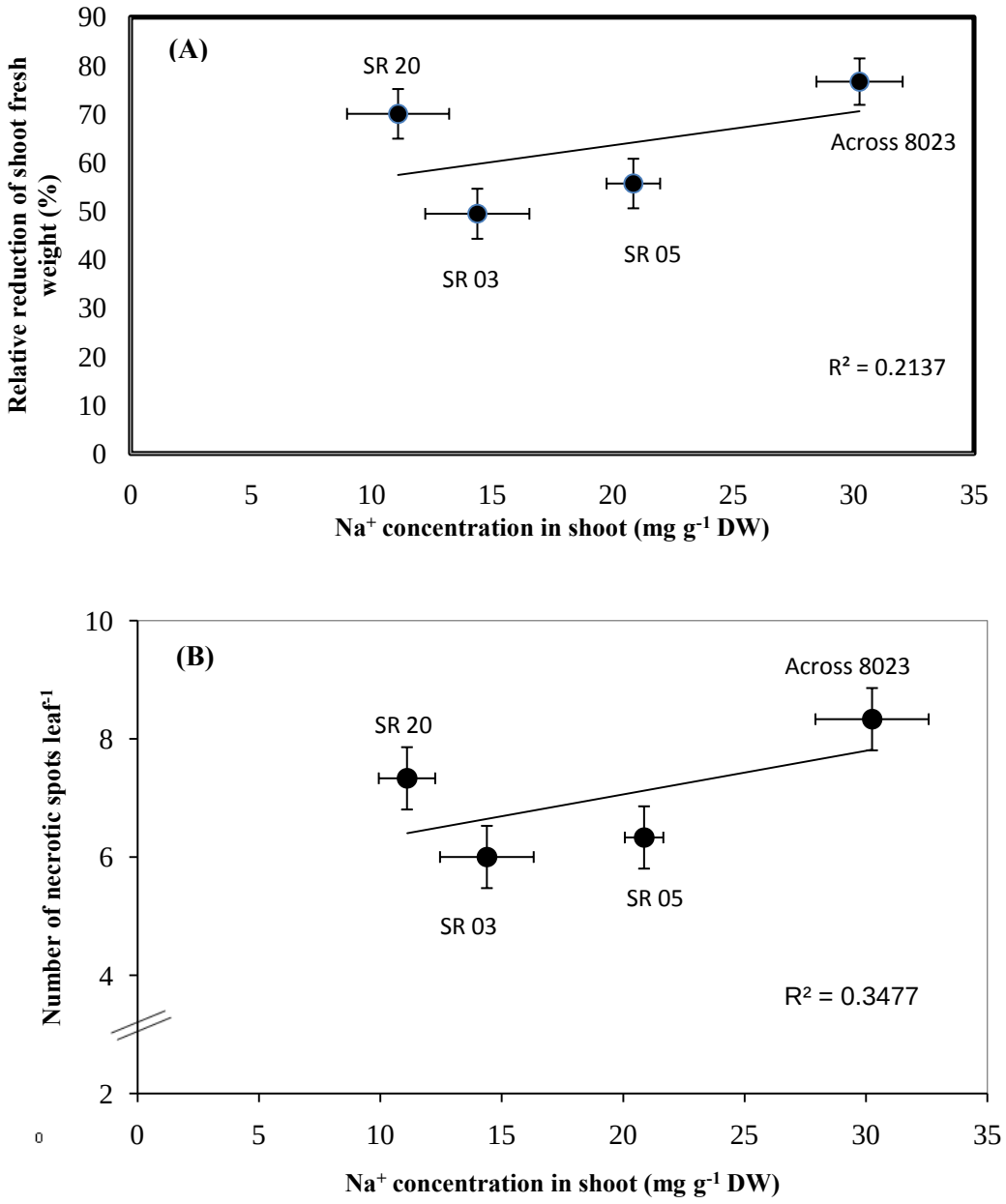


**Figure 25:** Correlation between shoot $Na^+$ concentration (mg $g^{-1}$ DW) and shoot fresh weight (A) and number of necrotic spots per leaf per plant (B) of four different maize hybrids under high salinity (200 mM NaCl). The results represent means ± SE of the three replicates. Plants were harvested 26 d after the beginning of plant cultivation.

### 3.2.4 $Na^+$ uptake and $Na^+$ translocation from root to shoot under salt stress

$Na^+$ uptake by the roots and translocation of $Na^+$ from root to shoot was studied in the second phase of salt stress under low and high salt treatment (50 mM and 200 mM NaCl) (Fig. 26A and B). In contrast to low salinity, a high salinity treatment led to significantly increased $Na^+$ uptake and $Na^+$ translocation in all tested maize genotypes. Higher $Na^+$ uptake and translocation under low salt treatment (50 mM NaCl) was observed in Across 8023 compared to all SR hybrids which did not differ. On the other hand, under high salinity (200 mM NaCl) the second salt-sensitive maize hybrid SR 20 showed the lowest $Na^+$ uptake by the roots (53.18 mg $g^{-1}$ DW) and also the lowest $Na^+$ translocation to the shoot (0.69) (Fig. 26), as compared to the other genotypes, in which both parameters were significantly increased.

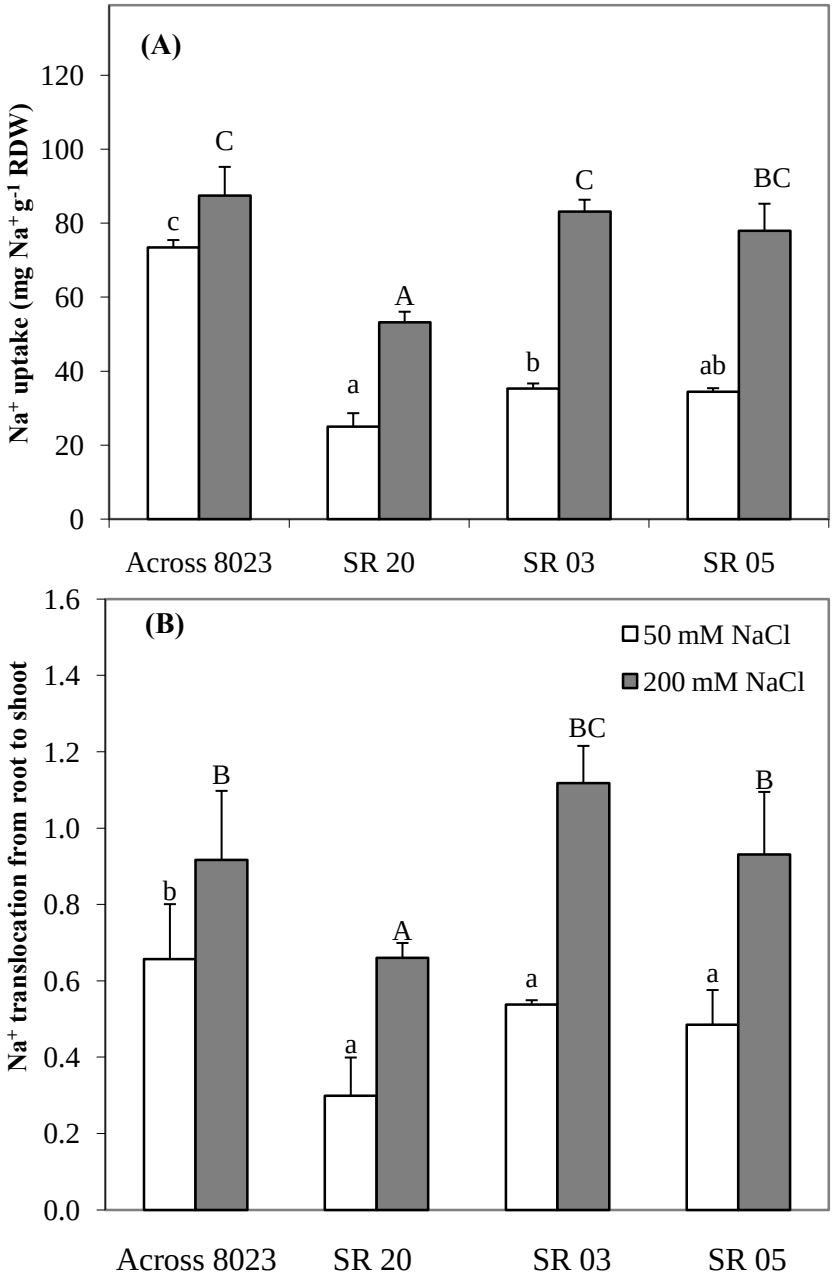


**Figure 26:** Effect of two salinity levels (50 and 200 mM NaCl) in the nutrient solution on $Na^+$ uptake at root surface **(A)** and $Na^+$ translocation to the shoot **(B)** of various maize hybrids. The results represent means ± SE of the three replicates. Significant differences ($P \leq 5\%$) between treatments are indicated by different letters. Plants were harvested 26 d after the beginning of plant cultivation.

### 3.2.5 Cation analysis in shoots and roots:

Cation concentrations, i.e. $K^+$ and $Ca^{2+}$ in shoots and roots of the four tested maize genotypes under low and high salinity treatment (50 mM and 200 mM NaCl) were studied. The salt-sensitive maize hybrid SR 20 showed the highest $K^+$ shoot concentration, while in Across 8023 only 33 mg $g^{-1}$ DW were detected (Tab. 9). On the other hand, the two salt-resistant SR 03 and SR 05 showed $K^+$ concentrations in shoot of 41 mg $g^{-1}$ DW and 43 mg $g^{-1}$ DW, respectively. Root $K^+$ concentrations were lower in the two maize hybrids SR 03 and SR 05 ranging between 18 mg $g^{-1}$ DW and 27 mg $g^{-1}$ DW, respectively, while Across 8023 showed the highest concentration of $K^+$ in roots (Tab. 9). $Ca^{2+}$ concentrations in shoots and roots were comparable for all maize genotypes. A higher $Ca^{2+}$ concentration was observed in shoot of SR 05 (8 mg $Ca^{2+}$ g DW)$^{-1}$ compared to SR 20 which exhibited the lowest $Ca^{2+}$ concentrations (5 mg $Ca^{2+}$ g DW)$^{-1}$. Average values were observed in both Across 8023 and SR 03 (6 mg $Ca^{2+}$ g DW)$^{-1}$. For root $Ca^{2+}$ concentration, there were no significant differences among all SR hybrids. In contrast, Across 8023 exhibited lower root $Ca^{2+}$ concentration (8 mg $Ca^{2+}$ g DW)$^{-1}$ only under high salinity level (Tab. 9).

**Table 9:** Effect of salt stress on $K^+$ and $Ca^{2+}$ concentrations in shoots and roots of four maize genotypes under two salinity levels (50 mM and 200 mM NaCl). Data are means of three replicates ± SE. Plants were harvested 26 d after the beginning of plant cultivation. The critical values for $K^+$, $Ca^{2+}$ and $Mg^{2+}$ are 30, 3 and 2.5 mg $g^{-1}$ DW, respectively, according to Bergmann (1993).

| | | | $K^+$ | $Ca^{2+}$ |
|---|---|---|---|---|
| Across 8023 | Shoot | 1 mM | 56.3 ± 1.8 | 11.10 ± 0.7 |
| | | 50 mM | 50.3 ± 2.8 | 7.30 ± 0.8 |
| | | 200 mM | 33.3 ± 3.0 | 5.87 ± 0.6 |
| | Root | 1 mM | 43.7 ± 4.5 | 20.1 ± 0.8 |
| | | 50 mM | 31.9 ± 3.1 | 11.5 ± 0.7 |
| | | 200 mM | 27.9 ± 2.4 | 7.84 ± 0.6 |
| SR 20 | Shoot | 1 mM | 46.6 ± 0.7 | 7.57 ± 0.3 |
| | | 50 mM | 51.1 ± 2.6 | 5.20 ± 0.5 |
| | | 200 mM | 52.6 ± 0.9 | 4.89 ± 0.01 |
| | Root | 1 mM | 16.7 ± 0.8 | 21.7 ± 0.3 |
| | | 50 mM | 24.1 ± 1.4 | 11.7 ± 1.0 |
| | | 200 mM | 22.4 ± 1.1 | 10.4 ± 0.1 |
| SR 03 | Shoot | 1 mM | 39.7 ± 4.3 | 8.67 ± 0.2 |
| | | 50 mM | 44.2 ± 1.7 | 7.72 ± 0.3 |
| | | 200 mM | 41.1 ± 0.7 | 5.71 ± 0.9 |
| | Root | 1 mM | 20.3 ± 2.2 | 17.8 ± 1.3 |
| | | 50 mM | 24.7 ± 1.2 | 10.9 ± 0.01 |
| | | 200 mM | 21.3 ± 1.4 | 9.02 ± 0.4 |
| SR 05 | Shoot | 1 mM | 40.2 ± 2.6 | 8.67 ± 0.6 |
| | | 50 mM | 43.5 ± 1.6 | 6.84 ±0.4 |
| | | 200 mM | 43.1 ± 2.3 | 8.04 ± 0.8 |
| | Root | 1 mM | 23.9 ± 0.6 | 14.9 ± 0.2 |
| | | 50 mM | 18.2 ± 2.4 | 12.0 ± 0.4 |
| | | 200 mM | 18.5 ± 1.5 | 10.6 ± 0.2 |

## 3.3 Contribution of $Na^+$ inclusion in leaf vacuoles to salt resistance of newly developed maize hybrids in the second phase of salt stress.

### 3.3.1 Effect of salt stress on plant growth in the second phase of salt stress

In order to study the clear effect of high salinity (200 mM NaCl) on leaf growth, single leaf blade fresh and dry weights of all maize genotypes were measured (Fig. 27). Under high salinity (200 mM NaCl), the fresh weights of single leaf blades were reduced in Across 8023 and SR 20 (Fig. 27A,B and D) as compared to SR 03 which had higher fresh weights of single leaf blades by about 6 g $leaf^{-1}$ (Fig. 27C). However, under low salinity level (50 mM NaCl), the reduction of fresh weight of single leaf blades was similar for all maize genotypes. Dry weights of single leaf blades were also decreased by the two salinity levels (50 mM NaCl and 200 mM NaCl). Similar to fresh weight of leaf blades, 50 mM NaCl also showed the same effect on dry weights of single leaf blades of all maize genotypes. However, under 200 mM NaCl all tested maize genotypes showed lower reduction in dry weight of single leaf blades, indicating that salinity stress had a greater effect on fresh weight (Fig. 28).

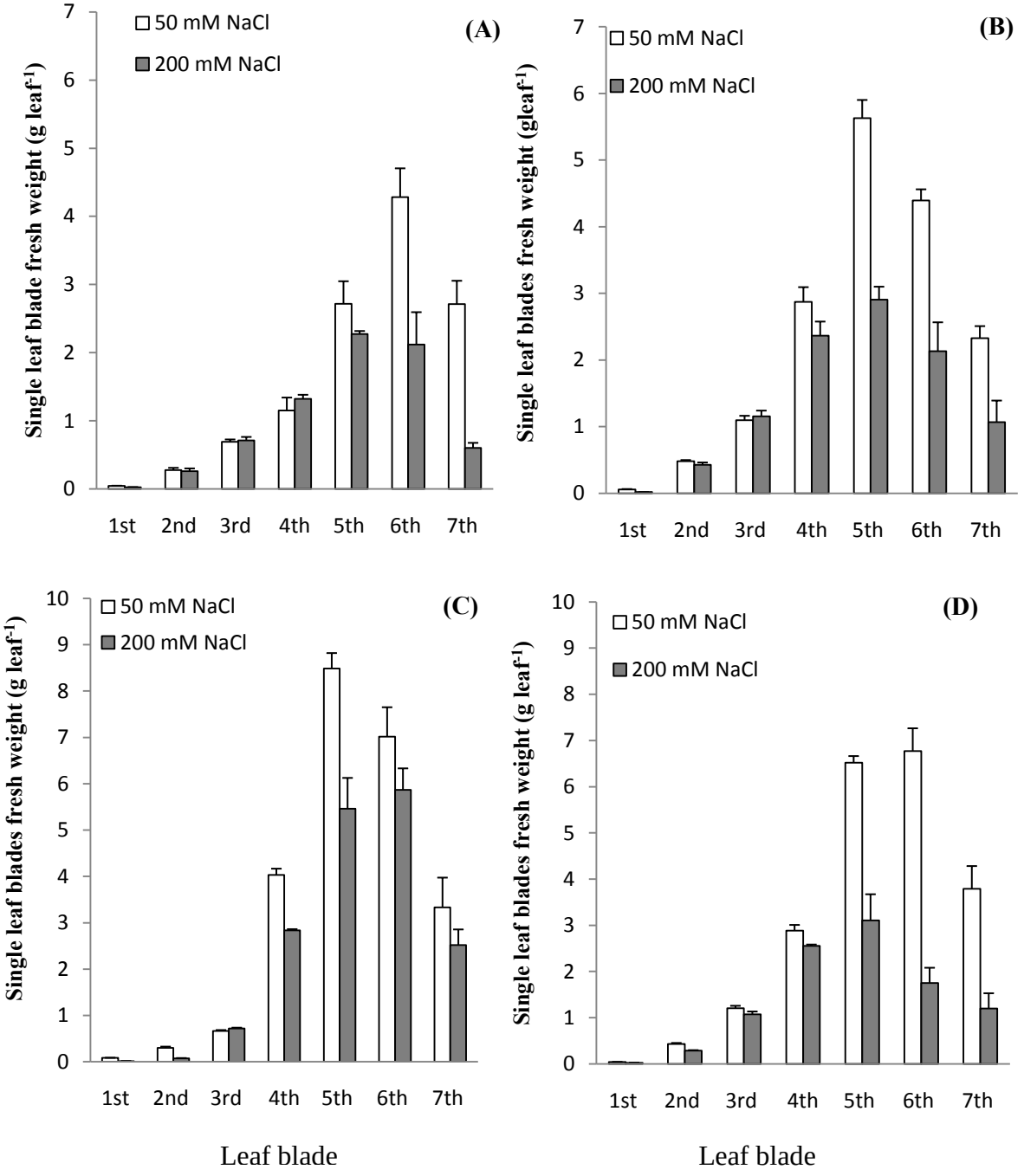


**Figure 27**: Fresh weight of single leaf blades of Across 8023 **(A),** SR 20 **(B)**, SR 03 **(C)** and SR 05 **(D)** at two salinity level (50 mM and 200 mM NaCl) in the second phase of salt stress. Data are means of three replicates ± SE.

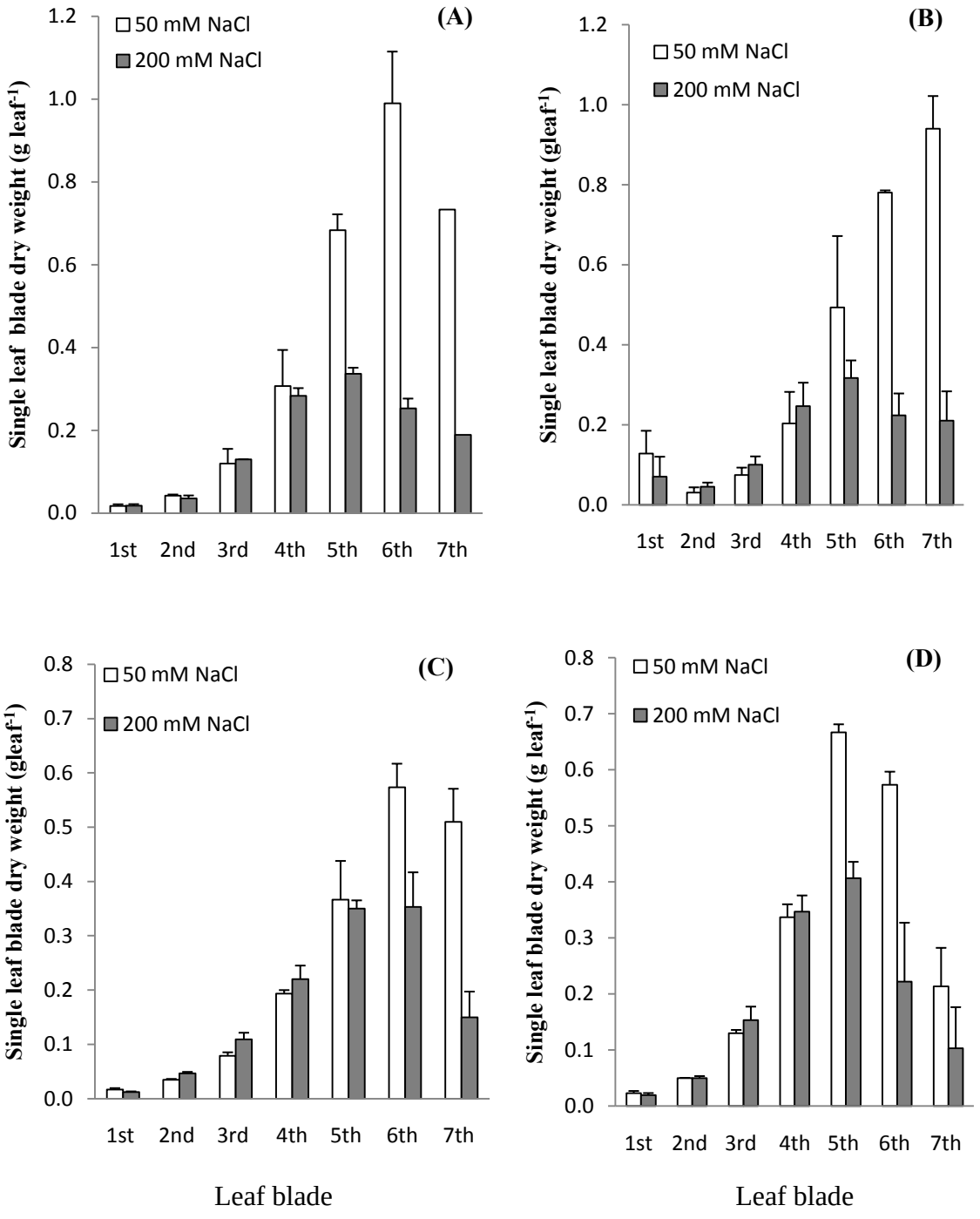


**Figure 28:** Dry weight of single leaf blades of Across 8023 **(A)**, SR 20 **(B)**, SR 03 **(C)** and SR 05 **(D)** at two salinity levels (50 mM and 200 mM NaCl) in the second phase of salt stress. Data are means of three replicates ± SE.

### 3.3.2 Effect of salt stress on $Na^+$ accumulation in maize leaf blades

Treatments with 200 mM NaCl showed a significant increase in $Na^+$ concentrations in single leaf blades of all maize genotypes. The salt-resistant hybrids (SR 03 and SR 05) thereby showed lower leaf $Na^+$ concentration compared to Across 8023, which had highest leaf $Na^+$ concentration (Fig. 29). On the other hand, the salt-sensitive maize hybrid SR 20 showed lowest leaf $Na^+$ concentration compared to all maize genotypes tested and showed significantly higher sensitivity to salt stress in the second phase.

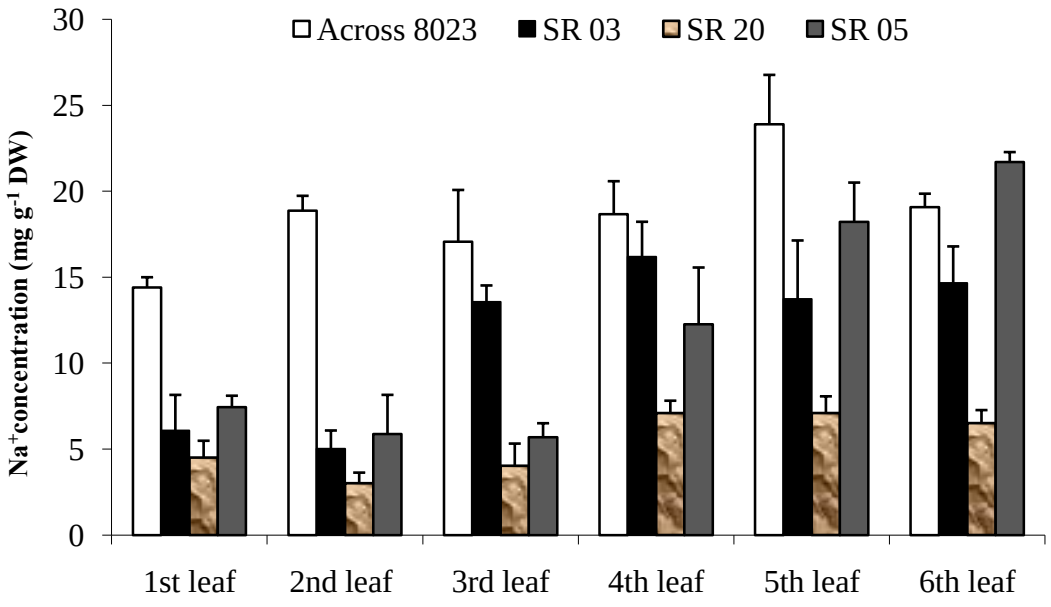


**Figure 29:** Effects of salinity (200 mM NaCl) on $Na^+$ concentration in single leaf blades of four maize hybrids. Data are means of three replicates ± SE.

In order to investigate the potential of salt-resistant genotypes to retain more $Na^+$ within the leaf sheath, $Na^+$ concentrations in shoot and in single leaf blades were studied. The salt-sensitive maize genotypes Across 8023 and SR 20 showed lower leaf blade $Na^+$ concentrations (Fig. 30A and B) compared to $Na^+$ concentration in shoot. On the other hand, single leaf blades and shoot showed no significant difference in $Na^+$ concentration under high salinity treatments in both salt-resistant maize hybrids SR 03 and SR 05 (Fig 30C). However, SR 03 and SR 05 showed no difference between complete shoot $Na^+$ concentration and $Na^+$ concentration in single leaf blades (Fig. 30C and D).

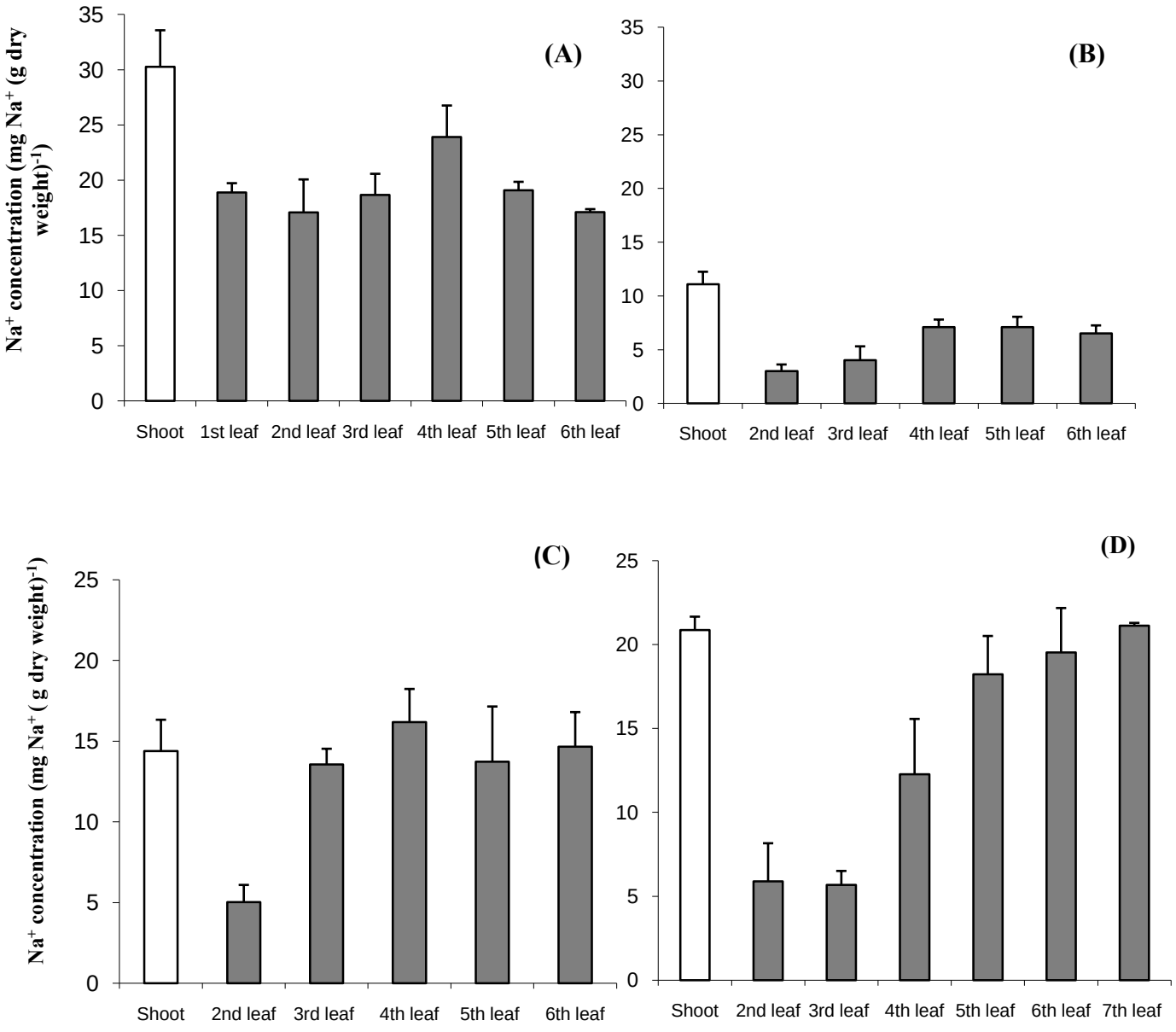


**Figure 30:** $Na^+$ concentration in shoot and single leaf blade dry weight of Across 8023 **(A)**, SR 20 **(B)**, SR 03 **(C)** and SR 05 **(D)** at 200 mM NaCl. Data are means of three indepentant replicates ± SE.

### 3.3.3. Relative transcription of tonoplast $Na^+/H^+$ antiporters in roots and leaves

In the second phase of salt stress, the relative transcription of tonoplast $Na^+/H^+$ antiporters was studied in roots and single non-necrotic leaf blades using real-time PCR. The two genotypes SR 03 and SR 05 showed higher relative transcription levels of tonoplast $Na^+/H^+$ antiporters in the $4^{th}$ and $6^{th}$ leaf blades under high salt stress (200 mM NaCl) compared to Across 8023 and SR 20 (Fig. 31A and B). However, all maize genotypes showed only a slight change in transcription levels of the tonoplast $Na^+/H^+$ antiporters (*ZmNHX*) in the $4^{th}$ and $6^{th}$ leaf under 50 mM NaCl treatment. In roots, relative transcription was affected by high salinity and resulted in small change in *ZmNHX* for both genotypes (SR 03 and SR 05) (Fig. 31C). The high salinity level led to a decrease in relative transcription level of $Na^+/H^+$ antiporters for Across 8023 in both leaf blades and roots (Fig. 31C). However, for SR 20, the relative transcription was about seven-fold higher in roots after 200 mM NaCl and it was about two-fold after 50 mM NaCl (Fig. 31C). Corresponding to the salt resistance level, Across 8023 showed lowest transcription in the leaves and roots (weak density of bands) and SR 20 showed lowest transcription in the leaves but at the same time showed increased transcription level in root under high salinity treatment, while in SR 03 and SR 05 salt stress led to an increase of the relative transcription of tonoplast $Na^+/H^+$ antiporters in leaves as represented by strong density of bands (Fig. 32).

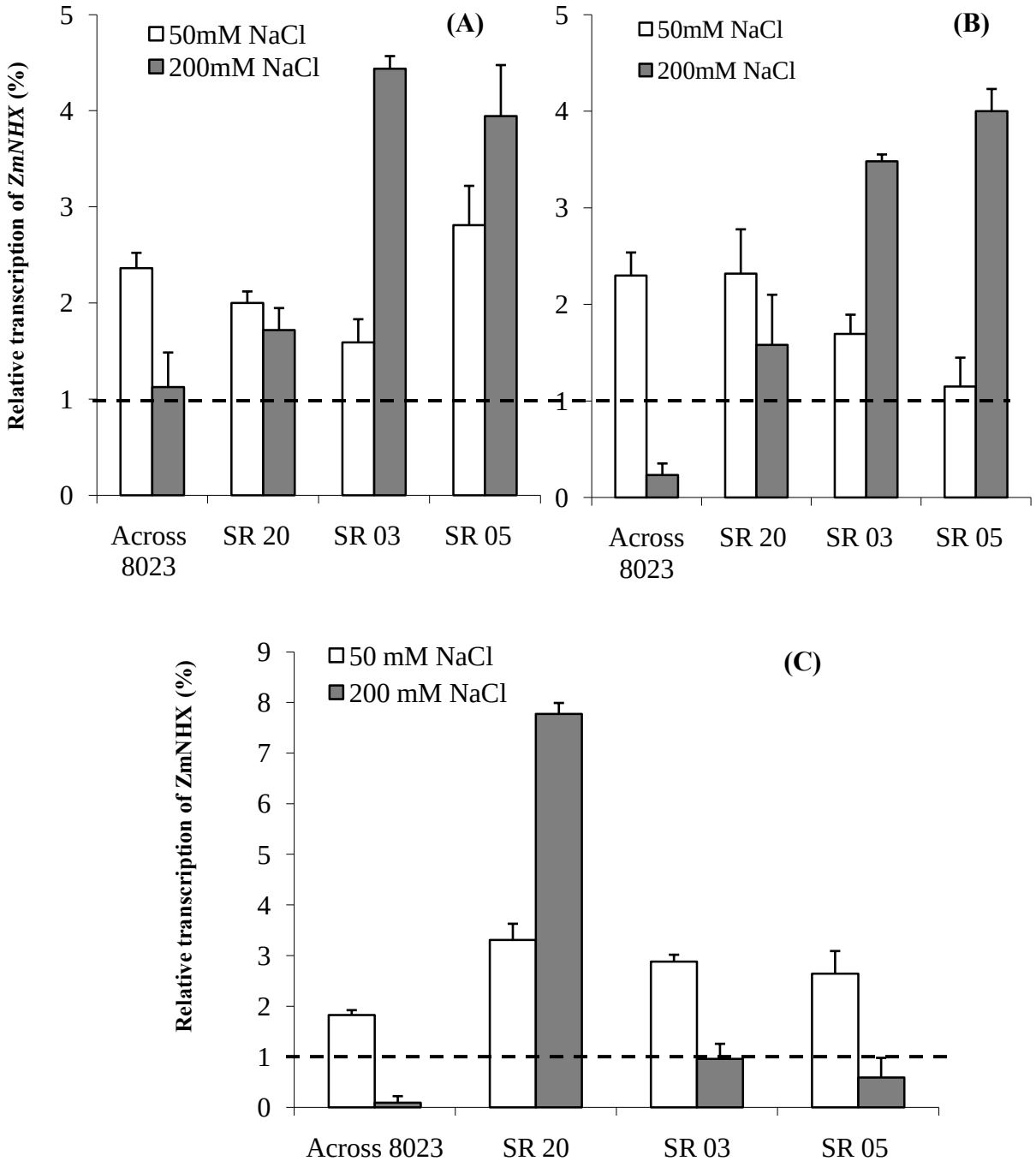


**Figure 31:** Relative transcription of tonoplast $Na^+/H^+$ antiporters (*ZmNHX*) in **(A)** 4th leaf, **(B)** 6th leaf, and **(C)** roots of four maize genotypes at two salinity levels (50 mM and 200 mM NaCl). Values represent means of three replicates ± SE. The transcription level of control plants was set to value of 1.0. The change in transcription level above 1.0 is considered as up-regulation and that below 1.0 as down-regulation.

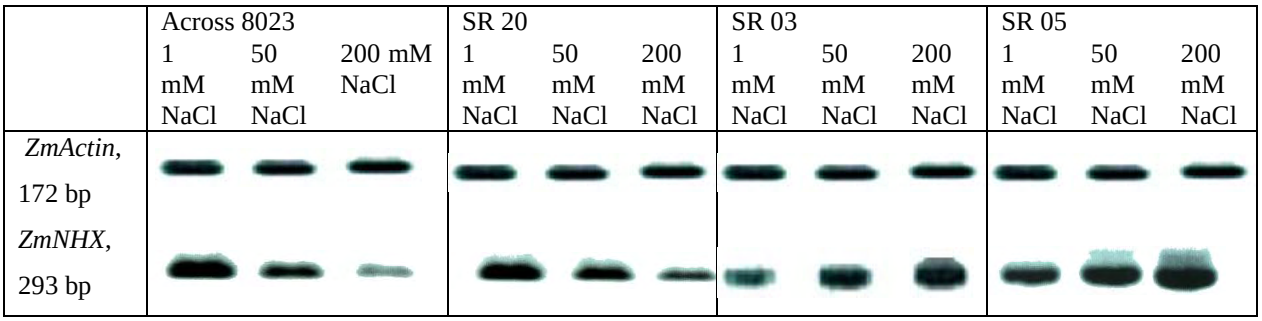


**Figure 32:** Effect of increasing salt treatment on the transcription levels of *ZmNHX* in leaves of four different maize genotypes. *ZmActin* was used as housekeeping gene. Gel images were obtained after staining with ethidium bromide.

## 3.4 Effects of salinity and $Na^+$ on proton transport of tonoplast vesicles isolated from control and salt-treated maize genotypes

### 3.4.1 Effects of various inhibitors on $H^+$-ATPase hydrolytic activity

The tonoplast fraction was obtained from leaves of maize hybrid SR 05 by a combination of differential and sucrose density gradient centrifugation. To measure if the isolated membrane fractions consisted of only tonoplast, ATPase activities of all cellular membranes were measured using specific inhibitors. These inhibitors used for ATPases of tonoplast, mitochondria, and plasma membranes were nitrate, azide, and vanadate, respectively. Molybdate was used to assess the presence of unspecific acid phosphatases. As shown in Fig. 33A and B, 50 mM $KNO_3$ strongly inhibited the activities of ATPases in membranes isolated form control and salt-treated plants of SR 05 by 79% and 85%, respectively. The ATPase activity of all membrane fractions isolated from control and salt-resistant plants showed very low sensitivity to azide and vanadate (Fig. 33A and B). Moreover, the membrane fractions of control and salt-treated plants showed a negligible sensitivity to molybdate. These results indicate that the tonoplast vesicles were free from other membranes.

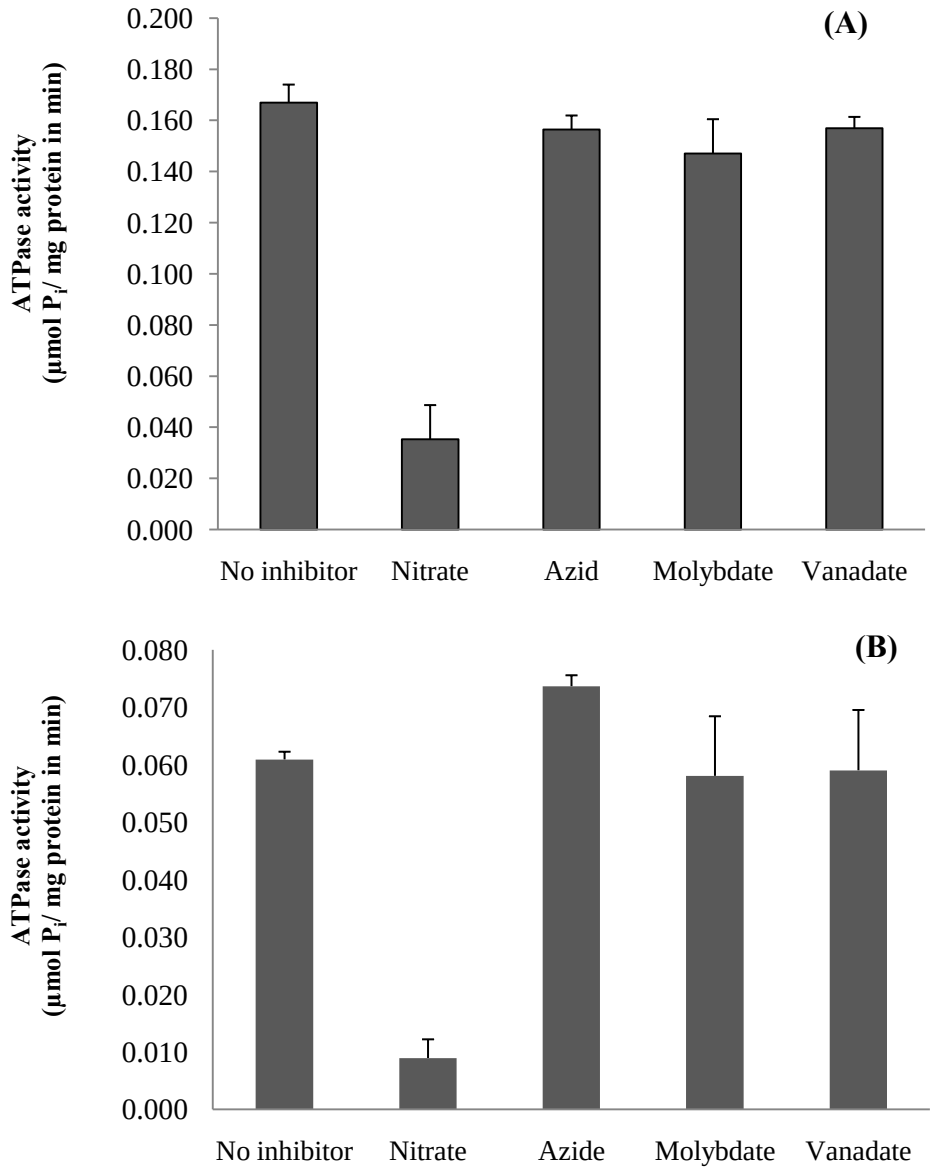


**Figure 33:** Purity of tonoplast membrane vesicles of salt-resistant maize hybrid SR 05 from control **(A)**, and salt treatment **(B)**. Plants were cultivated in a complete nutrient solution plus 1 mM NaCl or 200 mM NaCl. Specific inhibitors were used as markers of tonoplast (nitrate), mitochondrial (azide), unspecific phosphate (molybdate) and plasma membrane (vanadate) origins. Plants were harvested 26 d after the beginning of plant cultivation.

As 50 mM $KNO_3$ was used to determine the purity of tonoplast vesicles isolated from maize hybrids SR 03 and SR 05, it was also tested to determine proton-pumping activity of tonoplast ATPase. As already shown in Fig. 34, in the absence of $KNO_3$ the addition of ATP to the assay medium resulting in fluorescence quenching of ACMA, and thus proton pumping activity was observed. However, in the presence of nitrate with adding ATP as source of energy to the assay medium, the fluorescence quenching of ACMA was not determined.

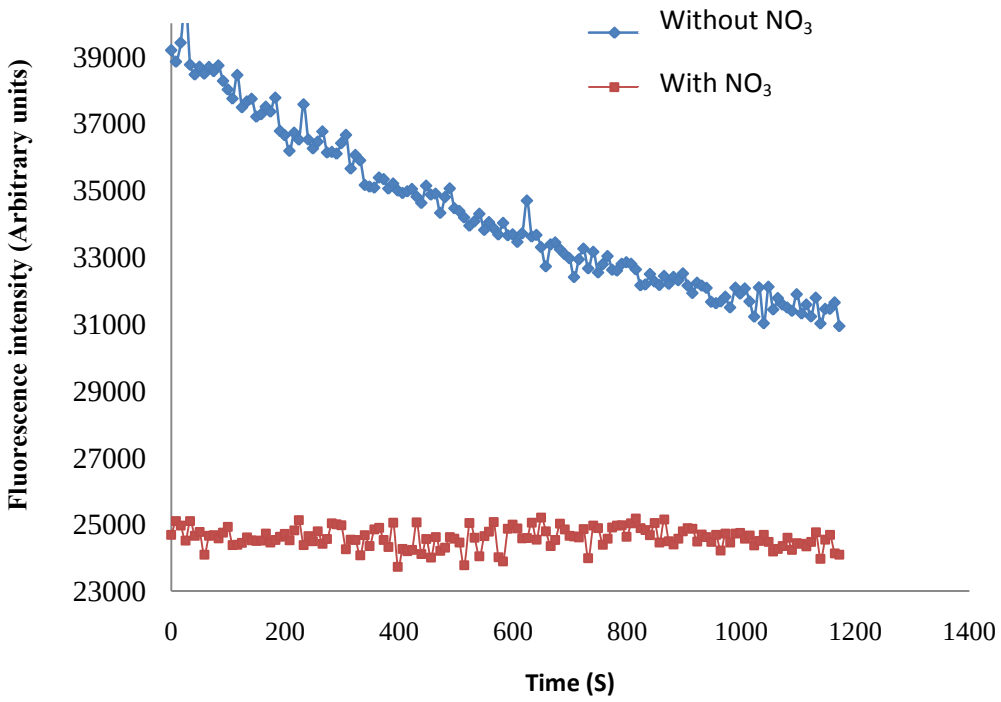


**Figure 34:** Effect of nitrate on the fluorescence quenching of maize tonoplast vesicles. The tonoplast membrane reaction was generated by ATP. Vesicles were already at the start of the assay. 50 mM $KNO_3$ was also added in the begging in the assay medium.

### 3.4.2 Effect of salt stress on the tonoplast ATPase hydrolytic activity

Hydrolytic activity of $H^+$-ATPase was measured in vesicles from leaves of maize hybrid SR 05, cultivated under control and salt stress by measuring the release of $P_i$ after hydrolysis of ATP. The hydrolytic activity was 167 nmol $P_i$ $mg^{-1}$ $min^{-1}$ in control plants and 61 nmol $P_i$ $mg^{-1}$ $min^{-1}$ in salt-treated plants (Tab. 10).

**Table 10:** Effect of salt stress on the hydrolytic activity of the tonoplast $H^+$-ATPase from leaves of maize hybrid SR 05, cultivated for 26 d in hydroponics using two treatments (1mM NaCl and 200 mM NaCl). The hydrolytic activity was measured as $P_i$ release in nmol $mg^{-1}$ $min^{-1}$ (n = 2).

| Treatments | Maize hybrid (SR 05) |
|---|---|
| Control (1 mM NaCl) | 167 ± 7.05 |
| Salt (200 mM NaCl) | 61 ± 1.33 |

### 3.4.3 Stimulation of proton pumping activity by malate and chloride

The initial rates of ACMA-fluorescence quenching (measure of the initial rate of vesicle acidification; Queirós et al. 2009) were greatly dependent on the anion present. Malate and chloride were used to stimulate the $H^+$ pumping activity for the maize hybrid SR 05. To see whether malate or chloride modify the $H^+$-ATPase activity, 100 mM K-malate and 100 mM KCl were added to the assay medium. Malate supports a higher rate of acidification in tonoplast vesicles of CAM plants compared to chloride (White and Smith, 1989). However, the level of stimulation was higher for chloride than malate in tonoplast membrane vesicles isolated from maize plants (Fig. 35).

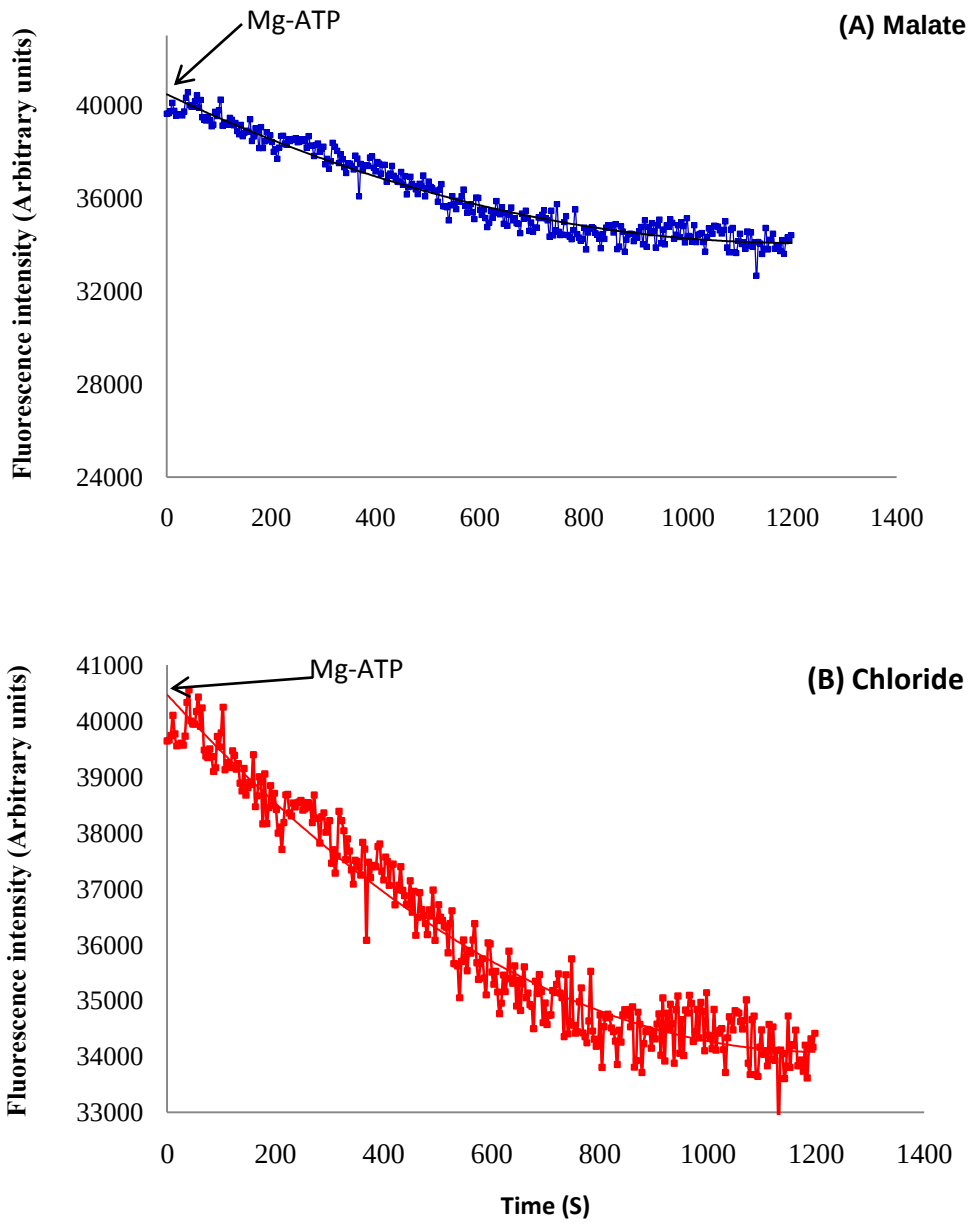


**Figure 35:** Proton pumping activity by tonoplast vesicles of maize hybrid SR 05 was monitored by the quenching of fluorescence in the presence of 100 mM K-malate **(A)** and 100 mM KCl **(B)**. Tonoplast vesicles were isolated from the leaves of maize hybrids cultivated at 1 mM NaCl and salinity stress (200 mM NaCl).

### 3.4.4 Measurement of $Na^+$ transport in tonoplast vesicles of SR 05

$Na^+$ is transported across tonoplast membranes by exchange for $H^+$ ions. $Na^+$ is driven by the transmembrane pH gradient as a secondary active process involving $Na^+/H^+$ antiporters. For $Na^+$ transport assays, tonoplast vesicles were isolated from leaves of the salt-resistant maize genotypes grown in the presence of 1 mM NaCl or 200 mM NaCl. Proton-pumping activity of tonoplast ATPase was measured as fluorescence quenching of ACMA (Fig. 36).

In this study, $H^+$ pumping by $H^+$-ATPase in tonoplast vesicles was compared in the presence and absence of $Na^+$ in the assay medium. *In vitro* addition of $Na^+$ to pumping assays decreased the initial rate in two of three replicates by 57% and 59%, but for the third replicate the decrease was only 19% (Fig. 37A). Thus, the overall effect was not significant. However, $Na^+$ addition to pumping assays significantly decreased the pH gradient established by tonoplast vesicles from salt-treated plants (Fig. 37B). This strongly indicates that $Na^+/H^+$ antiporters were operating in vesicles from salt-treated plants. Relative reductions in initial rate of control and salt-treated plants were 18% and 45% and those for pH gradient were 38% and 51%, respectively (Fig. 38).

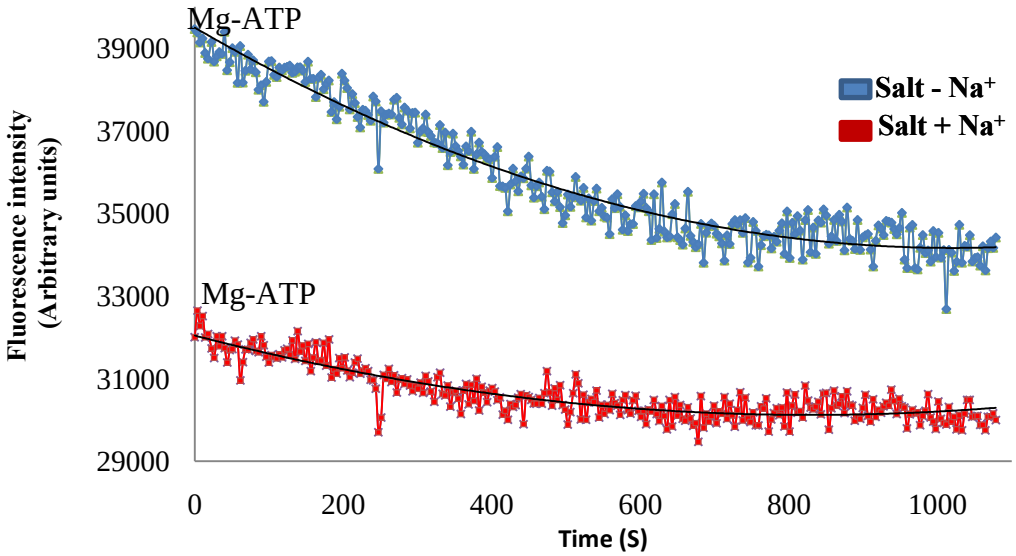


**Figure 36:** Effect of 50 mM $Na^+$ on $H^+$ pumping of the tonoplast vesicles isolated from leaves of salt-treated plants SR 05 cultivated under saline conditions (200 mM NaCl). Initial rate and pH gradient were monitored as a decrease in fluorescence quenching of ACMA. Initial rate was measured after the addition of Mg-ATP and change in fluorescence within 100 s.

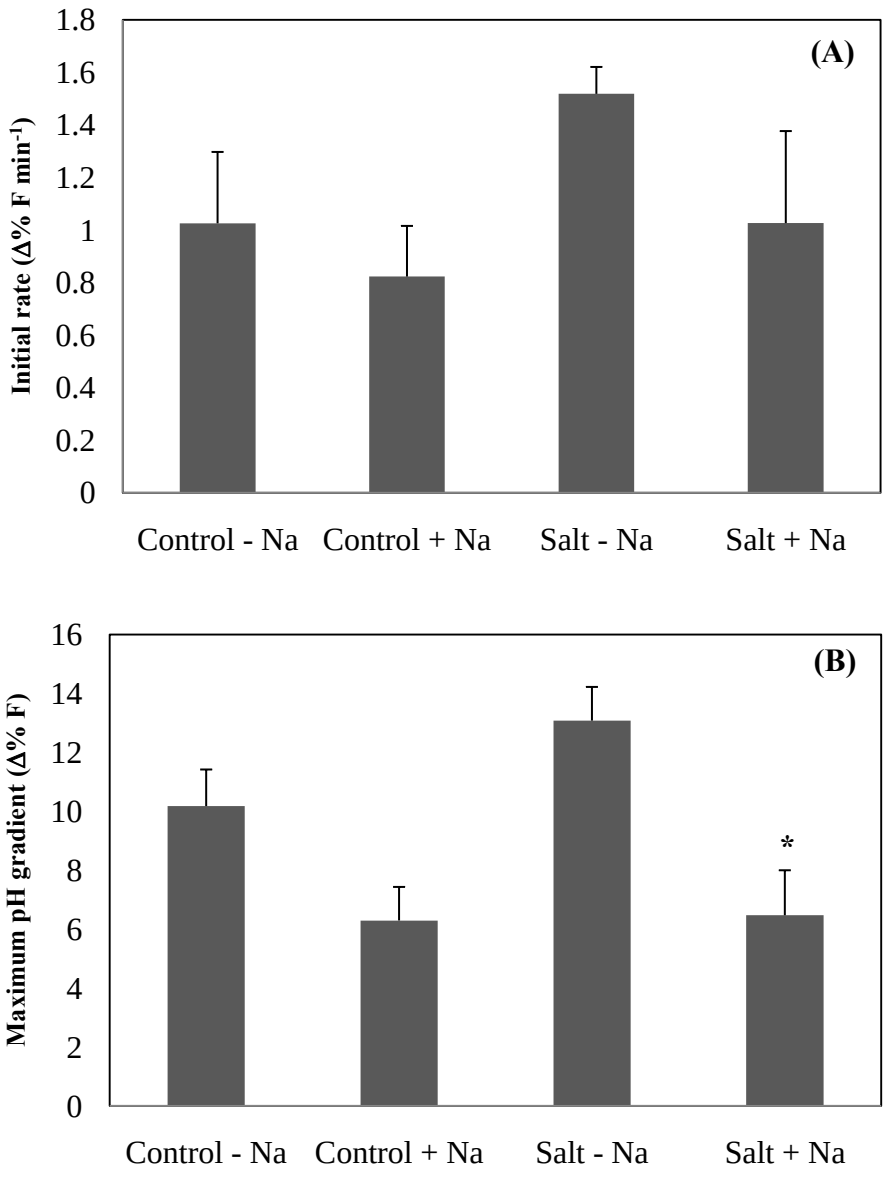


**Figure 37**: Proton pumping activity in tonoplast vesicles isolated from leaves of SR 05 grown under control (1 mM NaCl) and saline conditions (200 mM NaCl). The accumulation of protons inside the vesicles was determined by measuring the fluorescence quenching of ACMA (Queriós et al. 2009). **(A)** Initial rates of proton pumping from control and salt-treated plants without and with $Na^+$ in the assay medium (relative change of fluorescence per min). **(B)** Maximum pH gradient (% of initial fluorescence) in the absence and in presence of 50 mM $Na^+$ in the assay medium. ΔF means the decrease of fluorescence (ΔF/Fi x 100) and initial rate calculated as (ΔF/Fi x 100 $min^{-1}$), unit according to Qiu et al. (2004). Significant effect of $Na^+$ in the assay indicated by * ($p \leq 5\%$). Data are means ± SE (n = 3).

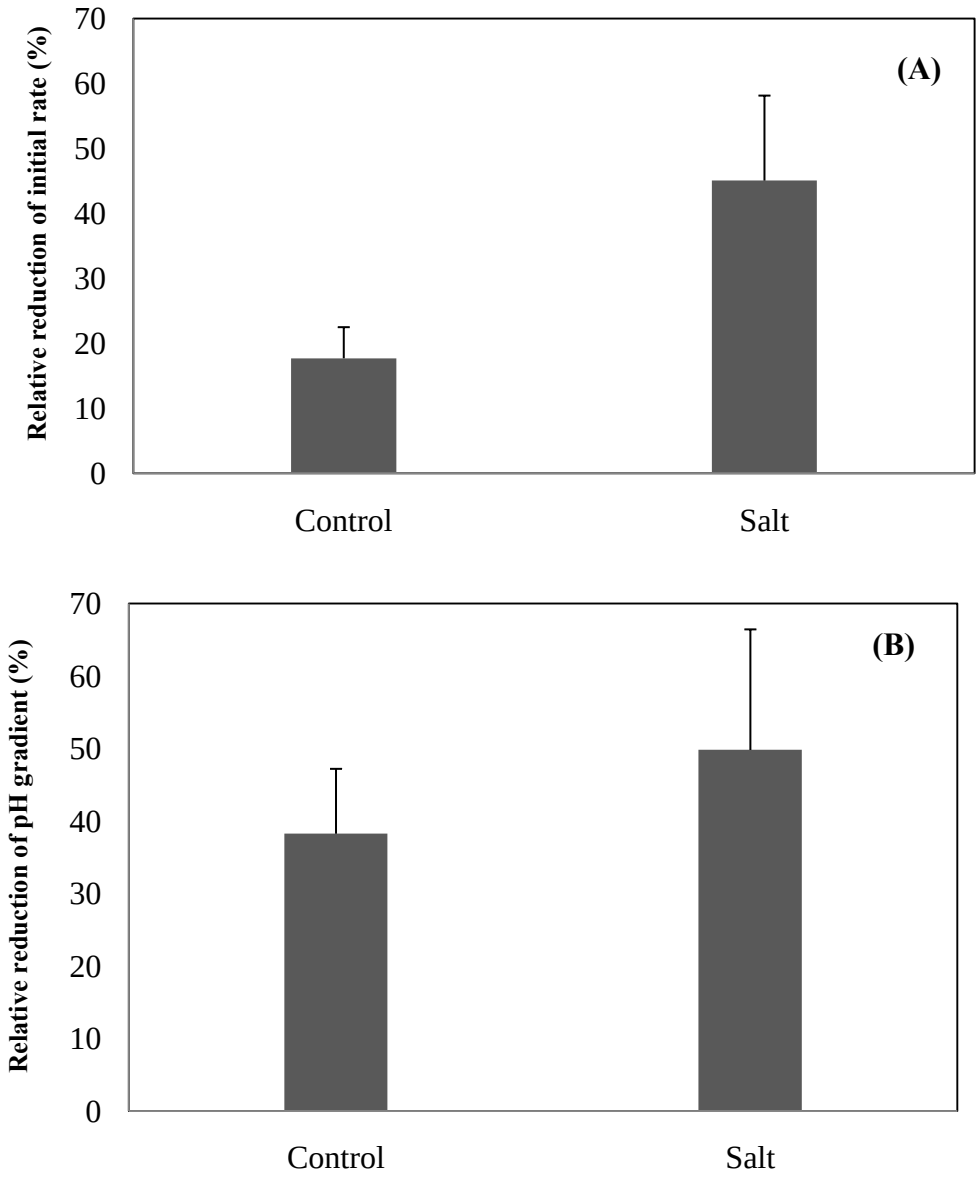


**Figure 38: (A)** Relative reduction of the initial rate of pumping activity and **(B)** relative reduction of the pH gradient by 50 mM $Na^+$ added to the assay medium. Plants were harvested 26 d after the beginning of plant cultivation. SR 05 was cultivated under control (1 mM NaCl) and saline conditions (200 mM NaCl).

# 4. Discussion

## 4.1 Screening of maize genotypes during the second phase of salt stress

The first aim of this study was to screen ten different maize genotypes (Across 8023, Pioneer 3906 and eight newly developed maize SR hybrids) for salt resistance during the second phase of salt stress. Little is known about the newly developed maize hybrids (SR) and their ability to exclude $Na^+$ from root and shoot and to sequester $Na^+$ into leaf vacuoles especially in the second phase of salt stress. These maize hybrids (SR) were generated from crossings between osmotically resistant inbred lines (SWS) and an efficiently $Na^+$-excluding inbred line (NaExIL) (Schubert et al. 2009). In this study, the level of salt resistance in response to ion toxicity stress in these maize genotypes was investigated with different parameters: (1) leaf length, (2) shoot growth (fresh and dry weights; Neto et al. 2004), (3) the number of necrotic spots per leaf (Lenis et al. 2011) and (4) shoot $Na^+$ concentration. These parameters are suggested to be suitable to identify individuals with desirable traits which can be used for breeding of salt-resistant cultivars. Accordingly, leaf length was used as an indicator for salt resistance during the first phase of salt stress (Schubert et al. 2009) and the severity of leaf symptoms was used to determine the resistance during the second phase of salt stress (Eker et al. 2006). In our experiments plant growth was reduced in both phases, which could be due to either osmotic effects or ion toxicity. This finding is in line with those of Sümer et al. (2004), who found that a simple separation of the two phases is not possible, since ion toxicity could also occur during the first phase; just as well as osmotic stress persists in the second phase. Nevertheless, salinity resistance can be identified on the basis of genotypic differences in shoot growth under saline conditions (Munns and James 2003).

Our results show that leaf length of all SR hybrids showed a lower relative reduction compared to Across 8023 and Pioneer 3906. Among SR hybrids, SR 03, SR 05, SR 13, and SR 16 exhibited the lowest relative reduction of leaf length in the first phase of salt stress (Fig. 11). Similar results were obtained by Hatzig et al. (2010) and Neubert and Shahzad (pers. comm.) who found that leaf length in SR 03 was less affected under salt stress compared to Pioneer 3906. These results indicate that the observed reduction in leaf length was mainly due to the osmotic aspect of salt stress and not related to ion-specific effects (Cramer 1992; Rahnama et al. 2010).

### 4.1.1 Identification of salt resistance based on shoot growth parameters: Genotypic variation

Plant growth reduction is a parameter which is used to describe the first phase as well as the second phase. Genotypic differences already occur in the first phase due to different osmotic resistance. In SR 03 and SR 05, shoot fresh weight was reduced by approximately 63% when cultivated at 200 mM NaCl (Fig. 12). On the other hand, for other maize hybrids such as Lector (Geilfus et al. 2010) and Black Mexican Sweet corn (Pesqueira et al. 2003) a salt treatment of only 100 mM NaCl was high enough to reduce shoot fresh weight by 65% and 81%. This is in line with results of Neubert (pers. comm.) who showed that SR 03 and SR 05 had more vigorous early vegetative growth compared to Pioneer 3906 treated with 150 mM NaCl. This indicates a markedly greater osmotic resistance of the two maize hybrids SR 03 and SR 05 in the first phase, in which only high salt concentrations led to severe growth limitations. In accordance with our results, numerous studies provided evidence that SR 03 showed an improvement of salt-resistance due to the maintenance of proton pumping and cell-wall acidification in comparison to more salt-sensitive maize genotypes ( Pitann et al. 2009b; Hatzig et al. 2010).

Long-term application of NaCl reduced the shoot growth of all maize genotypes tested, though it was again lowest in SR 03 and SR 05 (Fig. 10). At the same time, the appearance of toxicity symptoms on the older leaves of salt-treated plants, typical for the second phase of salt stress, indicated that shoot growth retardation was also due to ion toxicity and not osmotic stress as described by the biphasic model of growth reduction (Munns 1993). The negative effects of ion toxicity caused a further growth reduction due to the formation of severe leaf symptoms, which makes it possible to clearly distinguish between salt-resistant and salt-sensitive genotypes (Eker et al. 2006) especially during the early vegetative stages (Carpici et al. 2010). The same differentiation between salt-resistant and salt-sensitive genotypes was found in previous under saline conditions (Fortmeier and Schubert 1995). Additionally, Saboora and Kiarostami (2006) reported a large reduction in biomass production in short-term experiments with only little genotypic differences, which might be explained in terms of osmotic stress, they could confirm strong genotypic differences in long-term experiments.

But not only shoot fresh weights are adversely affected by high salinity. Also shoot dry weights were significantly reduced by toxicity stress especially in Across 8023, Pioneer 3906 and SR 20,

while there was only a low reduction in SR 03 and SR 05 (Fig. 14). Comparison of all genotypes supported the evidence that SR 03 and SR 05 are relatively salt stress-resistant (Fig. 14). This conclusion is supported by other studies which show that shoot dry weight production is positively correlated with salt resistance, e.g. in *Brassica juncea* and also in maize (Joshi et al. 2010). It has been suggested that the reduction of shoot growth could be a useful parameter to classify plants as resistant and sensitive (Cicek and Cakirlar 2002; Ashraf et al. 2003; Neto et al. 2004; Carpici et al. 2010). Based on our results, the hybrids SR 03 and SR 05 were selected for their improved shoot growth.

### 4.1.2 Severity of leaf symptoms and shoot $Na^+$ concentration in the second phase of salt stress

In order to differentiate between maize genotypes we selected additional parameters, and hypothesized that a low level of leaf symptoms combined with a low shoot $Na^+$ concentration can be used to identify salt-resistant plants in the second phase of salt stress. To make sure that plants reached the second phase of salt stress, all maize genotypes were cultivated again and harvested when some of them exhibited severe symptoms of $Na^+$ toxicity. The observed symptoms such as necrosis on the tips and margins of older leaves (Fig. 18 and 19) may be due to $Na^+$ accumulation which finally resulted in disorders of protein synthesis and enzyme activation (Tester and Davenport 2003). The hybrids SR 03 and SR 05 showed a lower number of necrotic spots on the older leaves compared to the other genotypes, especially Across 8023 and SR 20. Similar observations were reported by Eker et al. (2006) who found a significant difference between maize varieties based on the severity of leaf symptoms.

Furthermore, results in this work showed that $Na^+$ concentration was higher in the root compared to the shoot (Fig. 16) which has also been observed for other maize genotypes (Alberico and Cramer 1993; Erdei and Taleisnik 1993; Neto and Tabosa 2000). Less severe leaf symptoms combined with low shoot $Na^+$ concentration (Munns et al. 2006; Schubert et al. 2009) can be considered as strong parameters to characterize salt resistance (Eker et al. 2006). In this context Saqib et al. (2005a; 2005b) demonstrated for wheat that low $Na^+$ concentration in the shoot was correlated with higher shoot fresh and dry weight in the more salt-resistant genotype SARC-1. Other studies on *Arabidopsis* and plant species within the family of *Brassicaceae* did not show a

correlation between shoot $Na^+$ and salt resistance (He and Cramer 1993; Huang and Redman 1995; Møller and Tester 2007). In contrast to these results, Schachtman et al. (1989) found that salt-sensitive wheat genotypes had lower shoot $Na^+$ concentration than the more resistant ones. This is in line with results of this study in which a low shoot $Na^+$ concentration is also not correlated with a lower number of necrotic spots per leaf. Particularly, the salt-sensitive SR 20 showed a significant reduction in shoot $Na^+$ concentration, but exhibited a higher number of necrotic spots per leaf as compared to SR 03 and SR 05. Similar findings were reported by Neubert (pers. comm.) who found that low shoot $Na^+$ concentration was not correlated with salt resistance of maize. Alberico and Cramer (1993) suggested that resistance to salinity in maize is not related to shoot $Na^+$ concentration itself but to the ability of plant cells to maintain low $Na^+$ in the cytoplasm by compartmentation into vacuoles. From this diversity in stress resistance under different shoot $Na^+$ levels Kao et al. (2006) derived that there is a large genetic variation among genotypes to toxicity stress of $Na^+$ inside the plant.

Since shoot $Na^+$ concentration did not help in separating salt-resistant from salt-sensitive maize genotypes, a classification was conducted by using the severity of leaf symptoms. Based on this, SR 03 and SR 05 could be identified as plants which were salt-resistant in the second phase of salt stress, while SR 20 and Across 8023 were classified as salt-sensitive.

### 4.2 Strategies of $Na^+$ exclusion from the shoot for salt resistance in the second phase

Based on the hypothesis that $Na^+$ exclusion at the root surface and from the shoot contribute to the salt resistance of maize hybrids, four maize genotypes (Across 8023, SR 20, SR 03 and SR 05) were selected from the previous experiments to study the strategies to reduce $Na^+$ accumulation in leaves under salt stress (Fig. 26 and see also Fig. 17). Earlier studies on maize have shown that genotypes which have a capacity to exclude $Na^+$ from the shoot showed better growth (Fortmeier and Schubert 1995).

Salt-resistant plant species have been found to possess the ability to avoid toxicity stress in the cytoplasm of plant cells. Avoidance of toxicity can be achieved by minimizing $Na^+$ uptake by the root and transport to the shoot (Tester and Davenport 2003; Shavrukov et al. 2009). These different strategies of $Na^+$ exclusion from root and from shoot contributed to a delayed onset of $Na^+$ toxicity in the second phase of salt stress (Møller and Tester 2007; Schubert et al. 2009).

Schachtman et al. (1989) also suggested that salt-resistant species have a higher capability to exclude $Na^+$ from the shoot compared to salt-sensitive species.

These findings could not be confirmed in our study, where it was found that even though SR 20 had the lowest shoot $Na^+$ concentration (Tab. 8), it exhibited poor shoot growth (Fig. 22, 23 and 24) and a high number of necrotic spots per leaf (Fig. 25B and see also Fig. 18 and 19). It seems that SR 20 is indeed efficient in $Na^+$ exclusion at the root surface which is reflected by low $Na^+$ concentrations in the roots especially under low salinity levels, but fails to resist salt stress. In contrast, under high salinity (200 mM NaCl) SR 03 and SR 05 showed a relatively high $Na^+$ translocation from root to shoot (Fig. 26B and see also Fig. 17B), but a lower appearance of necrotic spots per leaf (Fig. 25 and see also Fig. 18 and 19) and a better shoot growth performance than was obvious in Across 8023 and SR 20. This is in line with Shahzad (pers. comm.) who described that SR 03 showed a lower $Na^+$ exclusion from root and from shoot under lower salinity levels (100 mM NaCl). Despite a higher $Na^+$ translocation, it can be assumed that SR 03 and SR 05 were able to improve shoot growth by protecting the leaf tissues and especially the cytoplasm from toxic $Na^+$ levels. Accordingly, Cramer et al. (1994) found that salt-resistant maize genotypes translocated $Na^+$ to leaves at a higher rate than salt-sensitive genotypes. Thus, it was concluded that resistance to salinity is not only related to the ability to exclude toxic ions from the shoot. As was already suggested by Munns et al. (2006), salt resistance can also be observed under high $Na^+$ translocation rates due to increased sequestration of $Na^+$ which in turn improves tissue tolerance, a strategy which may also account for SR 03 and SR 05 (Fig. 26).

From this work it can be concluded that salt resistance is not necessarily related to $Na^+$ exclusion from the root and shoot.

**Salt resistance and ionic homeostasis in maize plants**

Regarding the ion-specific effects of salinity, the $K^+/Na^+$ ratio is another adequate parameter to characterize salt resistance because plant cells have to maintain low cytoplasmic $Na^+$ concentrations and concurrent high concentrations of macronutrients such as potassium. Potassium is involved in many physiological processes such as enzymatic activation and osmotic regulation (Borowitzka 1981). In the context of salt resistance, Niu et al. (1995) and Tiwari et al.

(2010) reported that low $Na^+$ concentrations and high $K^+$ concentrations in the shoot are necessary to promote $Na^+$ export from the cytosol into the apoplast or to sequester $Na^+$ into the vacuoles, thus contributing to tissue tolerance. These results are in agreement with the results of Arzani (2008); Dvorak et al. (1994); Munns et al. (2000) who found a significant relation between salt resistance and shoot $K^+/Na^+$ ratio in some plant species such as bread wheat and durum wheat grown under 150 mM NaCl. Furthermore, Meneguzzo et al. (2000); Santa-Maria and Epstein (2001) showed that toxicity stress caused a reduction of the $K^+/Na^+$ ratio which was accompanied by decreased shoot growth.

When plants are grown in typical NaCl-dominated saline environments in nature, it comes to an accumulation of $Na^+$ in the cytosol resulting in a high $Na^+/K^+$ ratio. This alteration finally disrupts enzymatic functions that are usually achieved in cells (Munns et al. 2006). Therefore, it is very important for cells to maintain a low $Na^+/K^+$ ratio in the cytosol under salt stress (Maathuis and Aontmann 1999). The hybrids SR 03, SR 05 and SR 20 showed higher $K^+$ concentrations in the shoots under salinity as compared to Across 8023 (Tab. 9; see also Fig. 20). Even though $Na^+$ translocation was increased in SR 03 and SR 05, both genotypes were able to maintain an adequate $K^+/Na^+$ ratio, which contributes to ion homeostasis within the leaf cell cytoplasm and thus better growth performance.

Calcium ($Ca^{2+}$) plays an important role in plant nutrition. Besides other functions, calcium is involved in the maintenance of high growth rates under salt stress (Marschner 1995) possibly by increasing the selectivity of $K^+/Na^+$ uptake by roots (Cramer et al. 1987; Cramer 2002; Taster and Davenport 2003; Bolat et al. 2006). Several reports show a significant role of calcium ($Ca^{2+}$) in improving salt resistance of plants. As already mentioned by Dabuxilatu and Ikeda (2005), an additional supply of $Ca^{2+}$ to salt-treated soybean and cucumber improved the salt resistance by reducing the $Na^+$ uptake and transport of $Na^+$ from root to shoot. It also acts as secondary messenger and is involved in environmental signaling e.g. using abscisic acid (ABA) in response to stresses such as salinity (Lee et al. 2004). In the present study, salinity decreased $Ca^{2+}$ concentrations in all plants (Tab. 9; see also Fig. 21) due to ion competition, ion interaction and an increase in ionic strength that reduced the activity of $Ca^{2+}$. Even though the concentration of $Ca^{2+}$ was decreased by increasing $Na^+$, as was already reported elsewhere (Akram et al. 2010;

Ashraf et al. 2006; Eker et al. 2006; Garg and Gupta 1997; Liu et al. 2006; Mühling and Läuchli 2002), no deficiency symptoms were observed.

From these results it is concluded that $K^+/Na^+$ ratio was used as adequate parameter to characterize salt resistance in plants because salt-resistant SR03 and SR 05 showed higher salt resistance and at the same time higher shoot $K^+$ concentration compared to salt sensitive Across 8023.

The retention of $Na^+$ in the leaf sheaths could be another strategy to explain the higher salt resistance of SR 03 and SR 05. In order to investigate if this strategy protects the leaf blades from toxic effects of high salinity, the total shoot $Na^+$ concentration and $Na^+$ concentration in single leaf blades were compared. It was shown that Across 8023 and SR 20 had a lower $Na^+$ concentration in leaf blades as compared to the total shoot (Fig. 30A and B). Although these genotypes are able to retain more $Na^+$ in the leaf sheaths and thus reduce $Na^+$ accumulation in the leaf blades, they are not able to avoid $Na^+$ toxicity. On the other hand, SR 03 and SR 05 exhibited no differences between $Na^+$ concentration in the leaf blades and total shoots (Fig. 30C and D), and also less toxicity symptoms were observed on leaves (Fig. 25) and also showed better growth parameters (Fig. 27 and 28). These results are in agreement with those of Fortmeier (1996); Davenport et al. (2005) who found that salt-resistant maize and wheat genotypes had the ability to retain more $Na^+$ in the leaf sheaths, which led to reduced $Na^+$ accumulation in the leaf blades. Since reduction in $Na^+$ translocation and retention of $Na^+$ in the leaf sheath cells were not the reason for improved salt resistance in SR 03 and SR 05, sequestration of $Na^+$ into shoot cell-vacuoles may play an important role in salt resistance (Bernstein 1975).

### 4.3 Strategies of $Na^+$ inclusion: Transcription level of tonoplast $Na^+/H^+$ antiporter and salt resistance

In order to test the hypothesis that salt-resistant maize hybrids show a higher transcription of tonoplast $Na^+/H^+$ antiporters in shoots, thereby maintaining low $Na^+$ concentration in the cytoplasm of leaf cells, Across 8023, SR 20, SR 03 and SR 05 were studied under 50 mM NaCl and 200 mM NaCl. Intracellular ion homeostasis is a key factor for salinity resistance in plants (Niu et al. 1993) and a typical strategy to avoid $Na^+$ accumulation in the cytoplasm is

compartmentation of excessive $Na^+$ in vacuoles (Wyn Jones 1981; Zhu 2003; Munns 2005) mediated by tonoplast-associated $Na^+/H^+$ antiporters (*NHX*) (Mimura et al. 2003; Zhang and Blumwald 2001). A direct relation between salt resistance and $Na^+$ sequestration mediated by *NHX* was confirmed in cell cultures (Queiros et al. 2009) and for various plant species in the context of $Na^+$ exclusion from the shoot (Apse et al. 1999; Ballesteros et al. 1997; Neubert et al. 2005). Relative transcription of *ZmNHX* in roots was eight-fold higher in SR 20 compared with other genotypes (Fig. 31C). These findings are also in agreement with results of Zörb et al. (2005a), who found that an $Na^+$-excluding maize inbred line showed an up-regulation of *ZmNHX* in roots when grown under high NaCl concentrations. Furthermore, increased transcription of *ZmNHX* presumably increased the tonoplast $Na^+/H^+$ antiport in roots of SR 20 and thus reduced $Na^+$ transport from root to shoot. Our results suggest that the up-regulation of *ZmNHX* enables an increased inclusion of $Na^+$ into root-cell-vacuoles. In contrast, the role of *NHX* in sequestration of $Na^+$ in leaf vacuoles was long only described for *Arabidopsis* (Blumwald and Poole 1985). Although the involvement of leaf vacuolar *NHX* in salt resistance was investigated in transgenic wheat (Xue et al. 2004) and tomato (Zhang and Blumwald 2001), only little progress was made in understanding inherited tissue tolerance during the last few years. Only for rice (Chen et al. 2007) it was shown that relative transcription of *NHX* was increased in salt-resistant plant genotypes.

Our results support the hypothesis that transcript levels of tonoplast $Na^+/H^+$ antiporters in shoots of salt-resistant genotypes are significantly higher than those of salt-sensitive genotypes. The *ZmNHX* transcript levels in leaves of SR 03 and SR 05 were four-fold up-regulated compared to those of Across 8023 and SR 20 after high salt treatment (Fig. 31A and B). Neubert (pres. comm.) also reported that the transcription level of *ZmNHX* in shoots of salt-resistant SR 03 was higher compared to that of salt-sensitive ones. Similar results were obtained for wheat (Saqib et al. 2005), and the authors suggested that the higher transcript level of tonoplast-associated $Na^+/H^+$ antiporters in shoots of the salt-resistant wheat cultivar may contribute to better growth, which was not the case in the salt-sensitive wheat cultivar. Several studies provided evidence that transcription of tonoplast $Na^+/H^+$ antiporters in shoot of various species was positively correlated to the salt resistance of the shoot (Apse et al. 1999; Fukuda et al. 2004; Brini et al. 2007).

As tonoplast $Na^+/H^+$ antiporters function to exclude $Na^+$ from the cell cytoplasm, it is assumed that the higher transcript level of *ZmNHX* in shoots of SR 03 and SR 05 delays the appearance of $Na^+$ toxicity in older leaves due to the maintenance of an adequate $K^+/Na^+$ ratio. The transgenic tobacco plants had better growth and they also retained more $Na^+$ ions in the vacuoles, (Uddin et al. 2008). Maize genotypes Across 8023 and SR 20 showed progressive necrosis, reduced leaf extension and inhibited plant growth when cultivated under salt stress. Contrarily, salt-resistant SR 03 and SR 05 were not affected and showed increased tonoplast $Na^+/H^+$ antiporter activity and $Na^+$ accumulation in the vacuoles as well (Fig. 32). These results are in agreement with the results obtained by Apse et al. (1999); Brini et al. (2007) and Uddin et al. (2008). Previous findings by other researchers and the present results with maize SR hybrids, support the suggestion that higher growth and vigor could possibly be achieved due to a high transcription level of $Na^+/H^+$ antiporters of these plants which leads to enhance $Na^+$ compartmentation into vacuoles (Blumwald et al. 2000). Therefore, it is possible that SR03 and SR 05 were more salt-resistant than Across 8023 and SR 20 because they were more efficient in excluding $Na^+$ from leaf cell cytoplasm. Thus, tonoplast $Na^+/H^+$ antiporters could function in contributing to salinity resistance of maize hybrids by delayed toxicity due to accumulation of $Na^+$ ions in the vacuoles.

### 4.4 Strategies of $Na^+$ inclusion: Tonoplast $H^+$-ATPase activity

This study was conducted to test the hypothesis that salt stress increases the activity of tonoplast $Na^+/H^+$ antiporters in leaves of salt-resistant maize genotype SR 05 and thus increases $Na^+$ transport from the cytoplasm to the vacuoles. As was indicated by the previous results, the transcription level of *ZmNHX* was increased under salt stress in leaves of SR 03 and SR 05. It is assumed that this increase may result in increased antiporter activity and thus contributes to salt resistance. Furthermore, increased transcription of *ZmNHX* presumably increased the tonoplast $Na^+/H^+$ antiport in roots of SR 20 and reduced the sodium transport from root to shoot. Studying the activity of the $Na^+/H^+$ antiport and $H^+$-ATPase hydrolytic and proton pumping activities in tonoplast vesicles isolated from salt-resistant maize genotypes under control and 200 mM NaCl stress could give further insights into the strategy of salt resistance in maize genotypes. In order to investigate the $H^+$-ATPase activity of membrane vesicles, it is important to isolate pure tonoplast fractions. In the present study, isolated membrane fractions showed a sensitivity to nitrate of about 79% under control and 85% under saline conditions in SR 05 (Fig. 33A and B).

In contrast, sensitivity to azide and vanadate was very low. Moreover, the membrane fractions of control and salt-treated plants showed a negligible sensitivity to molybdate. This indicates that the tonoplast membrane fraction was free from other ATPase contaminations.

In the present study, the purity of tonoplast membranes isolated from SR 05 was at a similar level to those reported for preparation from potato (Queirós et al. 2009), tobacco (Gao et al. 2006), corn coleptile (Façanha and de Meis 1998) and from rice cultured cells (Kasamo 1988). The purity determined in this investigation was apparently higher than observed for tonoplast isolated from *Plantago* species in which nitrate inhibited the tonoplast of these plants by about 50% (Staal et al. 1991). The low purity in this tonoplast membrane probably reflects the presence of vacuolar membranes that are deteriorating or being degraded (Suzuki and Kasamo 1993). It is concluded that the high purity of the tonoplast obtained from maize hybrids described herein is suitable for investigation of the effect of salt stress on the proton pumping activity of ATPase enzyme.

### 4.4.1 Effects of nitrate on proton pumping activity

As nitrate (50 mM $KNO_3$) was tested to determine the purity of tonoplast membrane vesicles isolated from maize hybrid SR 03 and SR 05, it was also used to determine proton pumping activity of the tonoplast ATPase. Giannini and Briskin (1987); White and Smith (1989) reported that nitrate can be used to inhibit ATP-dependent proton transport which is often used to quantity transport in tonoplast vesicles. The results in this study showed that in the absence of nitrate the addition of ATP as the source of energy to the assay medium resulted in fluorescence quenching of ACMA, which showed proton pumping activity. On the other hand, in the presence of nitrate after adding ATP to the assay medium, fluorescence quenching was not observed, indicating that nitrate inhibited the $H^+$-ATPase activity completely (Fig. 34). Since Churchill and Sze (1984) and Mandala and Taiz (1985) found that proton pumping activity was completely inhibited by nitrate. It is concluded that proton pumping activity shown in this study indeed originated from the tonoplast.

### 4.4.2 The activity of tonoplast $H^+$-ATPase and $H^+$-PPase

The electrogenic tonoplast $H^+$-ATPase and $H^+$-PPase are major components of the vacuolar membrane of plant cells (Maeshima 2001). All plant species from which tonoplast membranes

were studied exhibit $H^+$-PPase activity in addition to $H^+$-ATPase activity (Müller et al. 1996). To elucidate if the proton-pumping activity is generated by ATPase in leaves of plants under salt stress, we tested first the isolated tonoplast membrane vesicles of salt-resistant maize hybrid SR 05 in the presence of ATP. The results show that ATP application to the assay medium as a source of energy caused fluorescence quenching of ACMA. In order to evaluate proton-pumping activity independent of ATPase, $PP_i$ was added instead of ATP as a substrate. ATP and $PP_i$ were separately added to the assay medium because ATPase is inhibited by $PP_i$. Similarly, Zhang et al. (2004) reported that the increase of $H^+$-PPase activity rether than $H^+$-ATPase in barley roots could be due to inhibition of ATPase by $PP_i$. In the current study, $H^+$-PPase generated the initial rate and pH gradient similar to those observed for the $H^+$-ATPase (data not shown). This is in agreement with previous studies by Johannes and Felle (1989) and Sarafian and Poole (1989) who found that tonoplast $H^+$-ATPase can generate a pH gradient of similar or greater magnitude than the $H^+$-PPase. Furthermore, Suzuki and Kasamo (1993) observed that the ATPase activity is more suitable than PPase activity because PPase activity decreases rapidly as the plant ages. On the other hand, in some plant species such as pumpkin cotyledons Suzuki and Kasamo (1993), pumpkin seed (Maeshima et al. 1994) and maize seeds (Façanha and de Meis 1998), $H^+$-PPase activity was three-fold higher in early developmental stages than the ATPase activity. However, this was changed few days after germination, while in the same time the concentration of $PP_i$ was decreased, indicating that $PP_i$ is a key energy source during the germination stages of plant cells. Based on the results of Carystinos et al. (1995); Darley et al. (1995); Kabata and Ktobus (2001) who reported that under stress condition the level of cellular ATP is severely reduced, PPase may play a key role in generating a transmembrane electrochemical $H^+$ gradient. Rea and Poole (1993) observed that pyrophosphatase was more stable than ATPase under stress conditions. In contrast to these observations, Zhang et al. (2004) reported that $Na^+$ is an inhibitor of $H^+$-PPase, indicating that the tonoplast $H^+$-PPase activity was more sensitive to NaCl than ATPase activity. Tonoplast $H^+$-ATPase pumping activity was increased in salt-treated plants of tobacco, salt-treated roots of barley, mung bean and sunflower as well as in cowpea seedlings cultivated under salt stress (Reuveni et al. 1990; Matsumoto and Chung 1988; Nakamura et al. 1992; Ballesteros et al. 1997; Otoch et al. 2001). In accordance with these data, our results showed that addition of $Na^+$ to the pumping assay significantly decreased the pH gradient in vesicles of salt-treated plants by 51% (n = 3). This strongly indicates that $Na^+/H^+$ antiporters were operating in vesicles

from salt-treated plants. In control plants, addition of $Na^+$ to the pumping assay decreased the pH gradient by 38%, which was not significant. One explanation could be that $H^+$-PPase takes over the function of $H^+$-ATPase during salt treatment.

### 4.4.3 Stimulation of $H^+$-ATPase activity by anions and cations

The initial rates of ACMA-fluorescence quenching (measure of the initial rate of vesicle acidification, Queirós et al. 2009) were largely dependent on the anion present. In order to study whether malate or chloride change the $H^+$-ATPase activity, 100 mM K-malate and 100 mM KCl were added to the assay medium. In this study, it was shown that the level of stimulation was higher for chloride compared to malate in tonoplast vesicles isolated from maize plants (Fig. 35). In contrast, Struve and Lüttge (1987); White and Smith (1989) reported that with malate the rate of acidification in tonoplast vesicles was higher compared to chloride. Malate stimulation of the tonoplast ATPase was not ford for maize plants (Fig. 35).

$H^+$-ATPase of *Chara* vacuolar membranes was stimulated by KCl (Takeshige and Hager 1988). It was estimated that the stimulatory effect of $Cl^-$ was attributed to a direct effect on the enzyme protein by 75%, while only 25% are caused by a dissipation of the membrane potential (Churchill and Sze 1984). The acidification of tonoplast vesicles was observed in the presence of KCl but not in the presence of NaCl (Hirata et al. 2000; Wang et al. 1986), indicating that potassium chloride was stimulating the $H^+$-PPase as well as $H^+$-ATPase activity. This is in contrast to a study of Kabata and Ktobus (2001) who found no effects by chloride on tonoplast $H^+$-PPase activity isolated from roots of cucumber when treated with KCl and NaCl. However, Wang et al. (1986) reported that in the presence of KCl but not NaCl $H^+$-PPase activity was markedly enhanced.

### 4.4.4 Tonoplast $H^+$-ATPase activity and transcription level of *ZmNHX*

In order to elucidate the transcription level of the *ZmNHX* in maize genotypes SR 05 grown under control and salt stress treatments, a specific primer as reported by Zörb et al. (2005) was used for RT-PCR analysis. We were able to detect primer-specific amplification for *NHX* using RT-PCR in SR 03 and SR 05 under control and salt treatments. It was investigated whether $Na^+/H^+$ antiporter activity was present in tonoplast vesicles isolated from maize genotype SR 05.

ATP was added to tonoplast vesicles to build up an electrochemical $H^+$ gradient by $H^+$-ATPase. $Na^+$ inclusion in the vacuoles through a $Na^+/H^+$ antiporter is energized by the pH gradient established by tonoplast $H^+$-ATPase (Zhang and Blumwald 2001). These findings are in agreement with those of Silva et al. (2009) who found that a decrease in tonoplast $H^+$-ATPase activity has been observed for Oliv plants grown in the absence of $Na^+$, which is associated with a reduced $Na^+/H^+$ antiport activity. Tonoplast $H^+$-ATPase has been shown to be involved in plant adaptation to high salinities particularly in halophytes. An increased $H^+$-ATPase may be required to sequester $Na^+$ ions into the vacuoles in order to protect the cytoplasm from toxic effects.

As protons efflux from the tonoplast vesicles was accelerated by the addition of $Na^+$, the influx of $Na^+$ into the tonoplast vesicles was accelerated by a pH gradient generated by $H^+$-ATPase and $H^+$-PPase. This indicates that $Na^+/H^+$ antiporters functioned as $Na^+$ transporter in the tonoplast membranes of rice plants (Fukuda et al. 1988). Our results showed that the ATP-dependent $H^+$ transport across tonoplast membranes was determined in the presence and absence of $Na^+$ in the assay medium (Fig. 36). These two assays showed significant difference of the maximum pH gradient between vesicles and assay medium. While the initial rate of fluorescence quenching generated by $H^+$-ATPase has already been used to demonstrate the antiporter activity (Uddin et al. 2008), in the present study evidence for antiporter activity comes from measurements of pH gradients. Earlier studies on *Chara* have shown that the $H^+$ translocating enzyme could be inhibited due to an increased $Na^+$ concentration in the cytoplasm with a decrease in cytoplasmic $K^+$ concentration (Takeshige and Hager 1988). Inhibition of the $H^+$-translocating enzyme might cause a decrease in the pH gradient across the tonoplast membrane vesicles and result in inhibition of the transport of ions into the vacuoles. As shown in Figure 37 and 38, good evidence for tonoplast $Na^+/H^+$ antiporters was found in SR 05 after salt stress. These results are in agreement with the results of Staal et al. (1991) who found that when $Na^+$ was added to the assay medium of *P. maritime* plants control and salt-treated plants, the pH gradient showed large decrease by about 50% only in salt-treated plants, indicating the presence of tonoplast $Na^+/H^+$ antiporters. Similarly, Fukuda et al. (1998) found that the antiporter activity was increased in roots of barley plants by addition of 100 mM NaCl. The activation of tonoplast $H^+$-ATPase drives $Na^+$ into the vacuole by tonoplast $Na^+/H^+$ antiporters (Apes et al.1999), which was found

in the salt-resistant cultivar "Tanyin 2" during the initial exposure to NaCl (Zhang et al. 2004). SR 05 showed higher transcription levels of *ZmNHX* under saline condition. This up-regulation of the transcription levels of tonoplast $Na^+/H^+$ antiporters probably facilitated the demonstration of antiporter activity in this investigation (Hasegawa et al. 2000). This shows that the antiporter activity of SR 05 was stimulated by adding NaCl in the assay medium and indicated its higher capacity of $Na^+$ accumulation in the vacuoles. In order to prevent the backflux of $Na^+$ to the cytoplasm after having been sequestered to the vacuoles by tonoplast $Na^+/H^+$ antiporters, $K^+$ and $Na^+$ channels have to be closed as already tested by patch clamp studies on *Plantago* species (Maathuis and Prins 1990). The activity of $Na^+/H^+$ antiporters is one important factor to limit the $Na^+$ accumulation in the cytosol of plant cells and for determination of the salt resistance in plants (Uddin et al. 2008). The evidence for $Na^+/H^+$ antiporter activity support the hypothesis that the increased transcription of *ZmNHX* can enhance the accumulation of $Na^+$ ions into the vacuoles.

Maize genotypes SR 03 and SR 05 are salt-resistant as they showed lower toxicity symptoms in older leaves as compared to Across 8023 and SR 20. Although low toxicity symptoms were observed, SR 03 and SR 05 showed higher $Na^+$ root uptake and also higher $Na^+$ translocation to the shoot. This shows that $Na^+$ exclusion from root and shoot is not alone responsible for salt resistance of maize plants in the second phase of salt stress. Additional strategies such as retaining more $Na^+$ into leaf sheaths and $Na^+$ sequestration in leaf vacuoles limit $Na^+$ concentration in the cytosol of leaf cells. For SR 03 and SR 05 accumulation of $Na^+$ in the vacuoles avoids the toxic effects of $Na^+$ ions in the cytoplasm thus increasing leaf tissue tolerance to salt stress.

## 5. Summary

Screening of plant crops is necessary to identify salt-resistant germplasm for breeding programs to evolve salt-resistant and high-yielding crop genotypes. For this reason, ten different maize genotypes (Across 8023, Pioneer 3906 and eight newly developed maize SR hybrids) were screened for salt resistance during the second phase of salt stress. In order to investigate the role of tonoplast $Na^+/H^+$ antiporters (*ZmNHX*) in salt-stress resistance three of the newly developed maize SR hybrids (SR 03, SR 05 and SR 20) and cv. Across 8023 were selected for further studies under low (50 mM NaCl) and high salinity level (200 mM NaCl). Resistance to $Na^+$ toxicity was monitored in terms of shoot growth and number of necrotic spots per leaf. SR 03 and SR 05 showed better shoot growth and a lower number of necrotic spots per leaf as compared to SR 20 and Across 8023. Based on these results, SR 03 and SR 05 were classified as salt-resistant, and SR 20 and Across 8023 as salt-sensitive genotypes. The relative transcription of the tonoplast $Na^+/H^+$ antiporters (*ZmNHX*) in shoots and roots was quantified in all genotypes under two levels of salt stress, 50 mM and 200 mM NaCl. At 200 mM NaCl, the salt-resistant SR 03 and SR 05 showed a significant up-regulation of *ZmNHX* in leaves compared with the salt-sensitive Across 8023 and SR 20. Relative transcription of *ZmNHX* in roots was only increased in SR 20 compared to other genotypes. Furthermore, the activity of $Na^+/H^+$ antiporters was increased in the salt-treated plants of SR 05 but not in control plants. The salt stress-induced increase in transcription of *ZmNHX* may lead to enhanced tonoplast $Na^+/H^+$ antiport activity in leaves of SR 05. Hence, sequestration of $Na^+$ into the leaf vacuoles contributes to salt resistance in these genotypes by protecting the cytoplasm from detrimental effects of $Na^+$.

From these results it is concluded that:

- Based on the growth parameters and the number of necrotic spots, SR 03 and SR 05 are classified as salt-resistant while Across 8023 and SR 20 are referred to as salt-sensitive. However, there is no relationship between $Na^+$ concentrations in shoot and number of necrotic leaves. This means that shoot $Na^+$ concentrations are not suitable to identify salt resistance in the second phase of salt stress.

- The better growth performance of SR 03 and SR 05 under high salt stress was not related to $Na^+$ exclusion from the shoot, but was accompanied by relatively higher $Na^+$ translocation and higher $Na^+$ concentrations in the shoot.

- Lower appearance of toxicity symptoms on the leaves of salt-resistant SR 03 and SR 05 together with their improved shoot growth were accompanied by the up-regulation *ZmNHX*. Thus, salt resistance in these maize genotypes correlated positively with the relative transcription of tonoplast $Na^+/H^+$ antiporters in leaves under different salinity levels (50 mM and 200 mM) but not in roots.

- Tonoplast $Na^+/ H^+$ antiporter activity improved $Na^+$ inclusion into leaf vacuoles of salt-resistant maize genotype SR 05, thus preventing toxic effects of $Na^+$ in the cytoplasm, increasing leaf tissue tolerance to salt stress. This indicates that salt-treated plants have significantly increased capacity for $Na^+$ sequestration by increasing the activity of $Na^+/H^+$ antiport. This increased activity of $Na^+/H^+$ antiporter only occurred in salt-treated SR 05 but not in control plants. Thus, the increased transcription level of *NHX* together with a higher activity contributed to salt resistance of SR 05.

## 6. Zusammenfassung

Da Salzstress weltweit immer noch ein sehr großes Problem für die landwirtschaftliche Produktion darstellt, ist es von Bedeutung, diejenigen Kulturpflanzen und Genotypen zu identifizieren, welche sich durch eine verbesserte Salzresistenz auszeichnen. Dieses genetische Potential kann dann genutzt werden, um salzresistente und ertragsreiche Kulturarten zu entwickeln. Aus diesem Grund, wurden in dieser Arbeit zehn verschiedene Maisgenotypen (Across 8023, Pioneer 3906, sowie acht neu entwickelte, salzresistente (SR) Hybride) auf ihre Salzresistenz in der zweiten Phase von Salzstress untersucht und klassifiziert.

Von besonderem Interesse war die Rolle der Tonoplasten-$Na^+/H^+$-Antiporter (NHX) in der Ausbildung von Salzresistenz. Hierzu wurden drei der neu entwickelten SR-Hybride, die sich im Screening als salzresistenter herausgestellt haben, sowie der empfindliche Genotyp Across 8023 unter geringem (50 mM NaCl) und hohem Salzstress (200 mM NaCl) kultiviert und untersucht. Hierbei dienten zunächst das Sprosswachstum und die Anzahl nekrotischer Flecken am Blatt als Parameter zur Charakterisierung von $Na^+$-Toxizität. Unter hohem Salzstress zeigte sich, dass SR03 und SR 05 insgesamt ein besseres Wachstum aufwiesen bei gleichzeitig weniger nekrotischen Blattveränderungen als SR 20 und Across 8023. Basierend auf diesen Ergebnissen wurden SR 03 und SR 05 als salzresistent, SR20 und Across 8023 dagegen als salzempfindlich eingestuft.

Im Weiteren wurde dann die relative Transkription des Tonoplasten- $Na^+/H^+$-Antiporter *ZmNHX* und dessen Beteiligung an des Salzresistenz untersucht. So zeigte sich unter hohem Salzstress (200 mM NaCl) eine signifikante Hochregulierung von *ZmNHX* im Spross von SR 03 und SR 05, sowie eine signifikante Hochregulierung von *ZmNHX* in der Wurzel von SR 20. Im Fall von SR 20 war die erhöhte relative Transkription jedoch nicht ausreichend, um die Salzresistenz maßgeblich zu verbessern. Jedoch sagt die Menge an Transkripten allein noch nichts über Salzresistenz aus, sondern vielmehr muss auch die Aktivität der Antiporter auf Proteinebene mit berücksichtigt werden. In diesem Zusammenhang zeigte sich, dass im Spross von SR 05 die Aktivität von NHX analog der relativen Transkription ebenfalls unter Salzstress erhöht war, jedoch unter Kontrollbedingungen keine Veränderung stattfand. Somit scheint in SR 05 das positive Zusammenwirken der erhöhten Transkription und Aktivität des Antiporters ursächlich

für die verbesserte Salzresistenz zu sein, da hierdurch verstärkt $Na^+$ in der Vakuole kompartimentiert wird.

Insgesamt können aus dieser Arbeit folgende Schlüsse gezogen werden:

- Basierend auf den Wachstumsparametern und der nekrotischen Blattveränderungen wurden SR 03 und SR 05 als salzresistent, SR 20 und Across 8023 als salzempfindlich eingestuft. Hierbei steht die $Na^+$-Konzentration im Spross selbst in keinem Zusammenhang mit der Ausbildung der Blattnekrosen, so dass dieser Parameter nicht geeignet ist, Salzresistenz zu beschreiben.

- Das verbesserte Wachstum von SR 03 und SR 05 unter hohem Salzstress ist nicht bedingt durch eine $Na^+$ Exklusion aus dem Spross, da hier eine erhöhte $Na^+$ Translokation sogar zu erhöhten $Na^+$-Konzentrationen im Blatt führte.

- Salzresistenz bei SR 03 und SR 05 ist auf $Na^+$-Inklusion zurückzuführen. Durch die Hochregulierung des $Na^+/H^+$-Antiporter-Transkripts *ZmNHX* kommt es im Spross zu einer verbesserten Gewebetoleranz.

- Gleichzeitig mit der erhöhten relativen Transkription von *ZmNHX* im Spross von SR 05 konnte auch eine erhöhte Antiporter-Aktivität unter Salzstress gemessen werden, welche zu einer verstärkten $Na^+$-Kompartimentierung in der Vakuole führt.

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## Erklärung

„Ich erkläre: Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig- Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.“

**Gießen**, July 21, 2011 **Abdel-Kareem Mohamed**

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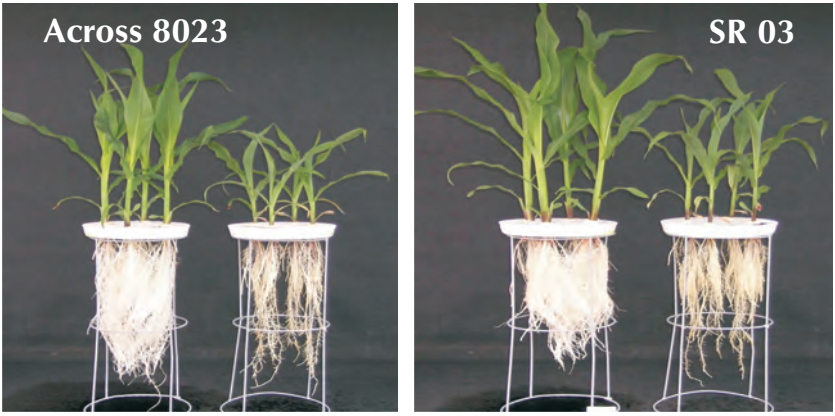


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