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The Role of Jasmonic Acid (JA) and Abscisic Acid (ABA) in Salt Resistance of Maize (*Zea mays* L.)

A thesis submitted for the requirement of the doctoral degree in agriculture from Faculty of Agricultural and Nutritional Sciences, Home Economics and Environmental Management Justus Liebig University Giessen

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To my parents for their endless efforts for their children and my life partner

for her absolute faith in me and finally to my son Abdullah,

wishing him a bright future

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1 Introduction

1.1 Salt stress and plant growth

Salinity is a major abiotic stresses, which limits crop production worldwide. Salinity is the accumulation of dissolved salts in soil, which degrades the soil and reduces its crop productivity. These dissolved salts are a mixture of cations (Na^+, Mg^+, Ca^{2+} and Mg^{2+}) and anions (Cl^-, SO_4^{2-} and HCO_3^-). Rock weathering, poor irrigation management, rainfall and high evapo-transpiration in the arid and semi-arid areas are the major causes of salinity (Rengasamy, 2006). About 830 million ha of the land area area affected by high salinity (Martinez-Beltran and Manzur, 2005), of which 45 million ha are irrigated (Pitman and Läuchli, 2002).

Salinity mainly affects shoot growth and also to lesser extent root growth. Shoot growth mainly depends on the production and expansion of leaf cells. It has been shown that expansion of leaf cells is drastically reduced by salt stress. Salt stress inhibits the rate of leaf expansion in two phases, osmotic stress and ion toxicity (Fig. 1; Munns, 1993). During the first phase of salt stress, salts accumulate in the soil and reduce the water potential of soil solution and make it difficult for the roots to take up water. Rates of leaf expansion in result are sharply decreased and then continue at lower rates later on. Shoot growth is further reduced in the second phase of salt stress, when salts accumulate to higher concentrations in the leaf tissues and become toxic. High concentrations of toxic ions restrict cytoplasmic enzyme activities or dehydrate the cells on their accumulation in apoplast.

Plants have evolved various strategies to survive under the saline conditions. Plants are classified as salt-sensitive (glycophytes) or salt-resistant (halophytes) depending on their ability to cope with salinity. Salt-resistant plants restrict the accumulation of toxic ions in sensitive tissues by excluding them and/or by sequestering them in old tissues and in vacuoles. However, inhibition of leaf growth during the first phase of salt stress is mainly caused by osmotic problems rather than ion toxicity. During the first phase of salt stress, toxic ions such as Na^+ and Cl^- do not accumulate to higher extents in leaves and cause no injury. According to Munns (2002) genotypes with varying capabilities to accumulate sodium show similar growth inhibition during the first phase of salt stress and show differences in growth performance only over a longer period of salt stress.

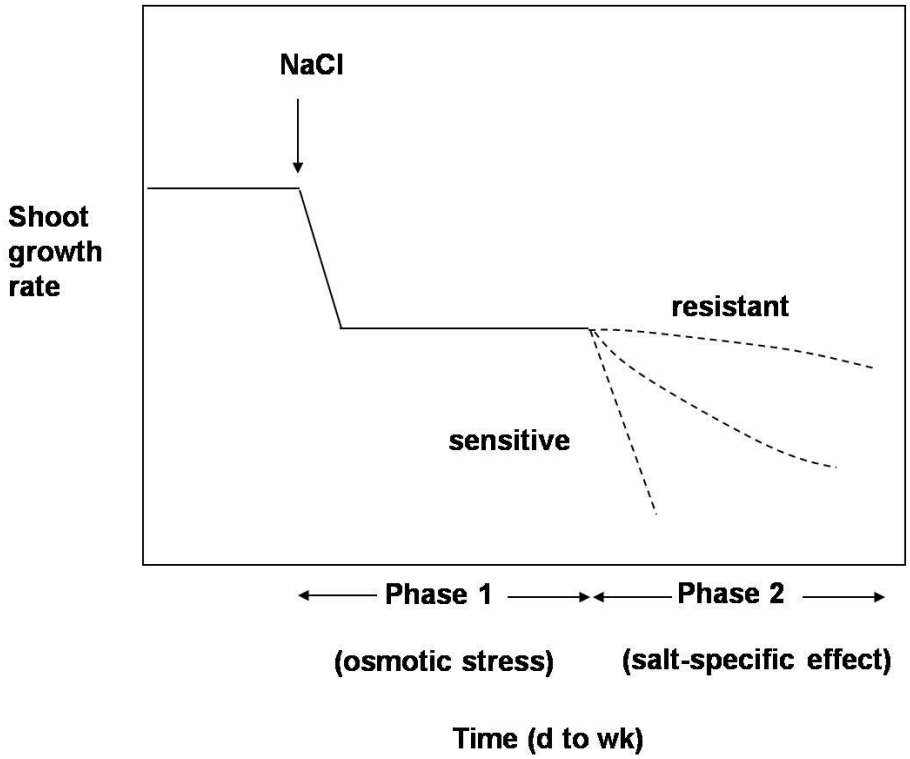

Figure 1: Model of two-phase growth reduction by salt stress of genotypes differing in salt resistance (Munns, 1993).

Maize (*Zea mays* L.) is a salt-sensitive crop (Maas and Hoffman, 1997) and shows a similar two phase growth inhibition under salt stress. For example, the growth differences in two-maize genotypes with differences in Na^+ accumulation were apparent only after 8 weeks of 100 mM NaCl stress (Fortmeier and Schubert, 1995). The reduced Na^+ accumulation in leaves of salt-resistant Pioneer 3906 was the result of two better strategies of Na^+ exclusion; low uptake of Na^+ at the root surface and lower root-shoot translocation of Na^+. To further improve the avoidance of Na^+-toxicity by Na^+ exclusion, Schubert *et al*. (2009) developed an efficient Na^+-excluding inbred line (NaExll) by self-crossing and recurrent selection of Pioneer 3906 for seven generations. To combine the traits of Na^+ exclusion and osmotic resistance in SR hybrids, NaExll was crossed with inbred lines exhibiting resistance during the first phase of salt stress. SR hybrids showed an improved growth during the first phase of salt stress (De Costa *et al*., 2007; Pitann *et al*., 2009) and are valuable tools to understand the physiological basis of salt resistance.

1.2 Control of leaf growth during the first phase of salt stress by root-sourced hormonal signals

It is generally assumed that during the first phase of salt stress/drought, a decline in leaf water relations determines the rate of leaf expansion (Kramer, 1988; Hsiao *et al*., 1998; Munns *et al*., 2000) but reduction in growth often occurs without any decrease in shoot turgor (Passioura, 1988; Gowing *et al*., 1990). It has been proposed by Davies *et al*. (1990), that root may sense the reduced availability of water and transfer this information to shoot, which in turn effects the physiology and growth of leaves. Further evidence of leaf growth control by root-sourced chemicals has been provided by Gowing *et al*. (1990) in split root experiments. The half of the roots of apple plants was allowed to dry and the other half was kept fully watered. Leaf expansion was reduced without any change in leaf water relations and excision of the dried roots removed the inhibitory effect on leaf expansion. These results indicated that leaf growth was reduced due to chemical signals coming from the roots and this inhibitory effect was removed when the source of the signal production (dried roots) was excised.

The contradiction over the inconsistency of the results regarding growth control by turgor or root-sourced signals may be explained on the basis of time-scale (Munns *et al*., 2000). In response to salt stress, leaf expansion in first hours declined sharply due to a transient decrease in shoot turgor. Maintenance of turgor by pressurizing the roots resumed the transient reduction in growth, but failed to maintain the growth over a longer period of time. They supposed that the growth reduction over a period of days is probably controlled by hormonal signals. Regulation of shoot growth is a complex mechanism; it is not clear whether the plant responses under salt stress are controlled by a single hormone or by a combination of various signaling components. Moreover, plant responses to hormonal signals depend on the amount, transport, and the presence of receptors or other components of the signal transduction (Davies, 2004).

1.3 Jasmonic acid

Phytohormones play critical roles in regulating plant responses to various stresses. Jasmonates are naturally occurring plant oxylipins and important members of their family include jasmonic acid (JA), methyl jasmonate (Me-JA), and jasmonyl-isolucine (JA-Ile).

They are involved in various physiological processes of plant development such as seed germination (Nojavan-Asghari and Ishizawa, 1998), root growth (Staswick *et al*., 1992), tuberization (Saniewski *et al*., 1998) and senescence (Ueda and Kato, 1998). Jasmonates are also involved in plant defense responses against wounding by insects and pathogens attack (Wasternack and Parthier, 1997). Levels of jasmonic acid and its metabolites increase transiently in plant tissues in response to wounding and pathogens (Reymond and Farmer, 1998; Zhang and Turner, 2008). Stress-induced jasmonates as well as their exogenous application affect the expression of genes (Devoto and Turner, 2003; Lorenzo and Solano, 2005) and induce stress or pathogen-related proteins (Moons *et al*., 1997).

JA-induced plant responses have been well investigated for biotic stresses; however its role in plant responses to salt stress is not well understood. The levels of JA are also increased in plant tissues in response to salt and water stress (Creelman and Mullet, 1995; Moons *et al*., 1997; Pedranzani *et al*., 2003; Tani *et al*., 2008). The increased levels of JA under drought or salt stress are consistent with the induction of genes for JA synthesis (Kiribuchi *et al*., 2005; Tani *et al*., 2008) indicating the involvement of JA in signal transduction.

Stress-induced JA promotes the expression of defense genes (Devoto and Turner, 2003; Lorenzo and Solano, 2005). However, many experiments have also demonstrated their role as an inhibitor of plant growth (Yamane *et al*., 1980; Ueda and Kato, 1982). Jasmonic acid inhibits plant growth after stress-induced endogenous accumulation and also when applied exogenously (Kim *et al*., 2009). Modification in endogenous JA is often consistent with the growth inhibition in response to wounding (Yan *et al*., 2007; Zhang *et al*., 2008) and herbivore attack (Moore *et al*., 2003). Apart from inhibition of vegetative growth (Cipollini, 2005), JA also reduces grain yield in rice under drought stress (Kim *et al*., 2009).

1.4 Abscisic acid

The plant hormone abscisic acid regulates a number of physiological processes such as induction of seed dormancy, inhibition of growth, stimulation of stomatal closure, and provides the protection under various stresses. Upon exposure to salt stress, concentrations of ABA are increased in roots and are transported to leaves through xylem stream. ABA transported to shoots is synthesized mostly in root tips (Zhang *et al*., 1997; Hartung *et al*.,

2002). The proof that ABA is transported from root to shoot has been provided by Davies *et al*. (1990) who showed that drying a part of the root system in a split root experiment increased the ABA in roots. They found the closure of stomata shortly after an increased ABA in roots. Accumulation of ABA in plants in response to salt/drought stress and similar effects of applied ABA on plant growth and gene induction as exerted by water stress suggest the involvement of ABA in signaling (Zhu, 2003).

ABA is considered as a negative regulator of leaf growth in response to water stress. This view point is supported by the observations that inhibition of leaf growth coincides with the increased endogenous ABA in leaf tissues of stressed plants (Cramer et al., 1998) and that the effects of endogenous ABA on leaf growth are similar to the effects of exogenous ABA supplied to the root medium (Saab *et al*., 1990; Zhang and Davies, 1990). Furthermore, the negative effects of ABA under water stress conditions can be alleviated by either inhibiting the ABA synthesis with fluridone (Hoffmann-benning and Kende, 1992) or removing the source of ABA production (roots, Gowing *et al*., 1990) and that of exogenous ABA under well watered conditions by removing its supply (Cramer *et al*., 1998). Similar effects were observed in leaves of many plant species including barley, soybean, sunflower, and maize (Zhang and Davies, 1990; Ben Haj Salah and Tardieu, 1997; Creelman *et al*., 1990; Dodd and Davies, 1996; Cramer and Quarrie, 2002).

Genetic manipulations of ABA synthesis in plants demonstrate contradictory roles of ABA in controlling the leaf growth under water stress. ABA-deficient maize seedlings show better shoot growth than the control plants under water stress, defining ABA as an inhibitor of growth (Sharp *et al*., 1994). In contrast, ABA has also been shown to maintain or promote the leaf growth in *Arabidopsis* and tomato under water stress (Cramer, 2002; Sharp *et al*., 2000). Similarly, maize genotypes show variable correlations between ABA concentrations and the extent of shoot-growth inhibition under salt stress (Cramer and Quarrie, 2002, De Costa *et al*., 2007). It has been suggested that the variation in ABA responses may probably be because of the different experimental conditions, the type of plant species or the developmental status of plants, used in these studies (Tardieu *et al*., 2010, Wilkinson and Davies, 2010).

1.5 Cell-wall acidification and leaf growth

H^+-ATPase localized in plasmalemma is a master enzyme which regulates various physiological processes such as ion transport, cell growth, stomatal regulation etc. As a transporter it hydrolyzes ATP, uses this energy to pump protons into the apoplast and maintains the gradients for pH and membrane potential across the plasmalemma. In addition to its involvement in various transport mechanisms, H^+-ATPase is also proposed to regulate the expansion growth of plant cells. According to the acid growth theory (Hager, 2003), acidification of the apoplast is a necessary requirement for the loosening of cell wall and cell growth. The theory of acid growth is supported by the observation that the plant hormone auxin stimulates the H^+-ATPase, increases the acidification of apoplast and thus increases the cell elongation of oat coleoptiles (Rayle and Cleland, 1992; Hager, 2003). Acidification of apoplast induces loosening of cell walls by stimulating the cleavage of bonds between wall polymers and by activating the wall enzymes related to cell-wall loosening (Cosgrove, 2000). Inhibition of leaf growth under water stress has been shown to be related to a decrease in leaf apoplast pH (Van Volkenburgh and Boyer, 1985; Wilkinson and Davies, 1998). Likewise, decrease in cell-wall acidification in leaves during the first phase of salt stress has been shown to inhibit leaf growth of the salt-sensitive maize genotype Pioneer 3906 (Pitann *et al*., 2009).

The reduced cell-wall acidification was caused by a decrease in proton pumping by plasmalemma H^+-ATPase. However, hydrolytic activity and total enzyme density in plasma membrane were not affected by salt stress (Zörb *et al*., 2005). In comparison to salt-sensitive Pioneer 3906, proton pumping by H^+-ATPase in leaves of salt-resistant SR 03 was not affected during the first phase of salt stress (Pitann *et al*., 2009). These studies proposed that the lower cell wall acidification in the salt-sensitive genotype was caused by a decrease in the proton-pumping efficiency of H^+-ATPase. The activity of plasmalemma H^+-ATPase is controlled by an auto-inhibitory domain at the C-terminus (Palmgren *et al*., 1991) and modifications in this domain can affect the pumping efficiency of the enzyme (H^+/ATP coupling).

Plsma membrane H^+-ATPase belongs to a multigene family and comprises several isoforms. Until recently 9-12 isforms of PM H^+-ATPase have been identified in various plant species. Isoforms related to nutrient transport and cell growth are widely expressed in most plant parts

(Arango *et al*., 2003; Gaxiola *et al*., 2007). Isoforms present in a single cell or tissue type may have different enzyme kinetics and may exhibit different H^+/ATP coupling ratios (Luo *et al*., 1999). Therefore, the changes in H^+-ATPase activity during the first phase of salt stress may also result from the expression of different isoforms. The role of plasmalemma H^+-ATPase in various transport and growth-related functions has been extensively studied. However little is known about the internal signals regulating the enzyme at biochemical and genetic levels.

The aim of the present study was to investigate the roles of hormones in controlling the leaf growth of maize genotypes during the first phase of salt stress. The following hypotheses were chosen to test in this study:

1. Salt stress stimulates the accumulation of JA in maize genotypes that is involved in the root-to-shoot signaling during the first phase of salt stress.

2. Inhibition of maize leaf growth during the first phase of salt stress is controlled by root-born signals ABA and/or JA.

3. Salt stress-induced decrease in plasmalemma H^+-ATPase proton pumping and decrease in acidification of apoplast in leaves of Pioneer 3906 are controlled by plant hormones. Hormonal signals inhibit proton pumping by affecting the expression of isoforms of H^+-ATPase with different enzyme kinetics.

4. Variation of maize genotypes in cell-wall acidification and salt resistance depends on the type of hormone signal and sensitivity to this signal.

2 Materials and methods

2.1 Effect of salt stress during the first phase on the accumulation of jasmonic acid in maize genotypes

2.1.1 Plant cultivation

To find out a possible role of jasmonic acid (JA) in salt resistance during the first phase of salt stress, two maize (*Zea mays* L.) genotypes, salt-sensitive Across 8023 and a relatively resistant hybrid SR 03 (Schubert and Zörb, 2005; Schubert et al. 2009) were used in this experiment. Seeds were soaked in aerated 1 mM $CaSO_4$ for 12 h and then placed between two layers of moistened filter paper at 25°C. At d 7, seedlings were transferred to quarter strength nutrient solution in 4.5 L plastic containers (four plants per container). After 2 and 4 d of seedlings transfer, the nutrient solution concentration was increased to half and full strength, respectively. The composition of the nutrient solution at full concentration is given in Tab. 1. Plants were grown in a growth chamber for 18 d under controlled conditions. The day/night temperature was 26°C/18°C under a 16 h photoperiod with a light intensity of 150 W m^{-2} (Philips Master HPI-T Plus, 400 W). The relative humidity was about 50%.

Table 1: Composition of the full-strength nutrient solution.

Nutrient	Final concentration	Substrate
N	5.0 mM	$Ca(NO_3)_2$
P	0.2 mM	KH_2PO_4
K	1.2 mM	K_2SO_4
		KH_2PO_4
Ca	7.5 mM	$Ca(NO_3)_2$
		$CaCl_2$
Mg	0.6 mM	$MgSO_4$
Fe	0.2 mM	Fe-EDTA
B	1.0 µM	H_3BO_3
Mn	2.0 µM	$MnSO_4$
Zn	0.5 µM	$ZnSO_4$
Cu	0.3 µM	$CuSO_4$
Mo	0.005 µM	$(NH_4)_6Mo_7O_{24}$

2.1.2 Application of stress treatments

The stress treatments were started when the full nutrient concentration was applied. The salt-treated plants were gradually adapted to a maximum stress of 100 mM NaCl with 25 mM increments over a period of 48 h. Polyethylene glycol (PEG) with a molecular weight of 6000 does not penetrate into cells or apoplast (Hohl and Schopfer, 1991) and can be used as an external osmoticum to induce osmotic stress. For PEG-induced osmotic stress, PEG was added to the nutrient medium in four increments, in such a way that both the NaCl and PEG treatments had the same solute potential at each increment. The solute potential was measured by means of an osmometer (freezing point technique). There was a linear decrease in the solute potential with increasing NaCl or PEG in the nutrient solution (Tab. 2). The solute potential of both treatments at full intensity was -0.49 MPa. The corresponding control was supplied with 1 mM NaCl, and each treatment was run in four replicates.

Table 2: Effect of NaCl or PEG on the solute potential (Ψ_s) of the nutrient solution.

	PEG (gL^{-1})	NaCl (mM)	Ψ_s (MPa)
1	71	25	-0.18 ± 0.00
2	100	50	-0.28 ± 0.00
3	119	75	-0.38 ± 0.01
4	133	100	-0.49 ± 0.01

2.1.3 Plant harvest

Plants were harvested 36 h after the application of full concentration of 100 mM NaCl separating the shoot from the root, and fresh weights were measured. Plant material for jasmonic acid analysis was homogenized by grinding the tissue in liquid nitrogen with mortar and pestle. Ground material was stored at -80°C.

2.1.4 Jasmonic acid measurements

The analysis of jasmonic acid followed the previously described protocol of Müller *et al*. (2002). Root (300 mg) and shoot (1 g) tissue samples (n = 4) were extracted with pre-warmed (60°C) methanol (1 mL each), supplemented with 200 pmol and 50 pmol, respectively, of $[^{13}C]_2$-JA (internal standard) and incubated under shaking for 3 min at 60°C. Incubation under shaking was continued for 1 h at 25°C. The cell-free supernatants were taken in separate vials and then vacuum-dried for subsequent gas chromatography-tandem mass spectrometry analysis. The dried sample residues were dissolved in 30 µL methanol and 200 µL of diethyl ether were added to it. The particle-free samples were then applied to microscale aminopropyl solid-phase extraction-cartridges. The cartridge was washed with 250 µL of $CHCl_3$: 2-propanol = 2 : 1 (v/v). The hormone-containing fraction was then eluted with 400 µL of acidified diethyl ether (2% acetic acid (v/v)). The eluates were dried, re-dissolved in 20 µL methanol, treated with 100 µL ethereal diazomethane, and afterwards transferred to auto sampler vials (Chromacol 05-CTV (A) 116; Fisher Scientific, Schwerte, Germany). The remaining solvent and excessive diazomethane were removed in a gentle stream of nitrogen, and samples were dissolved in 15 µL chloroform. To analyze the jasmonate content, 1 µL aliquot of each sample was injected into the GC-MS system. All spectra were recorded on a Varian Saturn 2000 ion-trap mass spectrometer connected to a Varian CP-3800 gas chromatograph (Varian, Walnut Creek, CA, USA), equipped with a ZB-50 fused silica capillary column (Phenomenex, Torrance, CA, USA). The mass spectrometer was used in CI-MRM mode with methanol as the reactant gas and positive ion detection. The setting for derivatized endogenous JA was chosen as follows: MeJA m/z = 225 $[M+H]^+$, 0.50 V. A second channel analyzing the methylated $[^{13}C]_2$-JA standard used identical excitation amplitude for the following parent ion: $[^{13}C]_2$-MeJA m/z = 227 $[M+H]^+$. The amount of endogenous compound was calculated from the signal ratio of the unlabeled over the stable isotope-containing mass fragment observed in both analyzing channels.

2.2 Effect of abscisic acid and jasmonic acid on shoot extension of maize seedlings

To investigate the effect of hormones on the extension growth of maize shoots, 6 d old maize seedlings were cultivated in quarter-strength nutrient solution. 30 µM JA (JA-2500, Sigma Aldrich) and 10 µM ABA (2-cis, 4-trans-Abscisic acid, Sigma Aldrich) dissolved in ethanol were supplied in the nutrient medium. The control seedlings were also supplied with equal concentration of ethanol. In order to confirm that the hormones applied in nutrient medium are easily taken up by roots and could influence the shoot growth, a separate set of seedlings with roots excised (1 cm from the root tip) was also grown in parallel to the experiment. Extension in shoot growth was measured daily and seedlings were harvested 3 d after the start of treatment. After harvest, root and shoot fresh weights were determined. To investigate the growth sensitivity of maize genotypes to exogenous JA, 6 d old seedlings of Across 8023 and SR 03 were cultivated in quarter-strength nutrient solution. Jasmonic acid dissolved in ethanol was supplied in nutrient solution at four levels (0, 5, 25, and 50 µM).

2.3 Effect of jasmonic acid on Na^+ homeostasis

In this experiment it was investigated whether exogenous application of JA may increase the salt resistance during the first phase in two maize genotypes, the salt-sensitive genotype Across 8023 and the resistant hybrid SR 03. Plants were grown for 18 d under controlled growth conditions, as described previously. The plants were supplied with following treatments:

Control	1 mM NaCl
NaCl	100 mM NaCl
JA	1 mM NaCl + 30 µM JA
JA + NaCl	100 mM NaCl + 30 µM JA

Two days after reaching full-strength nutrient solution, 30 µM JA and 25 mM NaCl were applied in the nutrient solution. NaCl concentration was increased up to a final concentration of 100 mM by 25 mM steps at 12 h intervals. Control and JA-treated plants were additionally supplied with 1 mM NaCl during the stress period.

2.3.1 Plant harvest and cation analysis

Plants were harvested 36 h after the application of 100 mM NaCl, root and shoot dry weights were measured. After grinding, the oven-dried plant material was used for analysis of K^+, Na^+, Ca^{2+} and Mg^{2+} concentrations. The ion concentrations were measured by means of an atomic absorption spectrophotometer (SpectrAA 220 FS, Varian; Mulgrave, Victoria, Australia). Na^+ uptake at the root surface and Na^+ translocation from root to shoot were calculated as:

Na^+ uptake at the root surface = Total plant Na^+ content / Total root dry weight

Na^+ translocation from root to shoot = Shoot Na^+ content / Root Na^+ content

2.4 Effect of abscisic acid, jasmonic acid and salt stress on leaf expansion and leaf apoplast pH

2.4.1 Plant material

To investigate the role of hormones in controlling leaf expansion and leaf apoplast acidification during the first phase of salt stress, two maize genotypes Pioneer 3906 and SR 03 were compared in this study. Pioneer 3906 is an F1 hybrid: a combination of two inbred lines, Pioneer 165 and Pioneer 605. Selfing of Pioneer 3906 for seven generations resulted in homozygous inbred line (NaExll), which has a strong capability to exclude Na^+ at the root surface and from the shoot but exhibit poor shoot growth under the first phase of salt stress. SR 03 was developed by incorporating the traits of osmotic resistance after crossing the NaExll with an osmotically resistant inbred line (Schubert *et al*., 2003).

2.4.2 Plant cultivation

Maize genotypes were cultivated for 24 d in nutrient solution as described previously. NaCl treatment was started when the full nutrient concentration was achieved with 25 mM daily increments. Salt-treated plants were supplied with 100 mM NaCl for 8 d before harvest. For hormone treatment, plants were supplied with 10 µM ABA and 50 µM JA in the nutrient medium for 3 d before harvest. Control and hormone-treated plants were also supplied with 1 mM NaCl throughout the stress period. To determine the effect of salt stress and hormones on

leaf expansion, leaf area was determined by measuring lengths and widths of individual leaves with a ruler. Plants were harvested on d 24 and shoot fresh weights were determined.

2.4.3 Measurement of leaf apoplast pH with fluorescent ratio-imaging

The second-youngest leaves from all treatments were selected to measure the leaf apoplast pH. The excised leaves were cut in segments of about 6 cm and after washing with deionized water were placed in a 60 mL plastic syringe. Leaf apoplast was infiltrated with 50 µM fluorescein isothiocyanate (FITC)-dextran (MW = 10,000, Sigma-Aldrich) through vacuum-infiltration (Pitann *et al*., 2009). For complete infiltration of apoplast and to remove air bubbles, the procedure was repeated thrice. To remove the adhering dye, the infiltrated leaves were washed again with deionized water. The leaves were cut into segments of approximately 4 cm^2, and the segments were placed upside down between an object plate and cover slip.

The ratio-imaging device used for this study was an inverse microscope (Leica DM IRB, Solms, Germany) connected to a highly sensitive CCD camera (CoolSNAP, Photometrics, Tucson, Arizona, USA) and coupled to a computer. Data acquisition and calculation of images were carried out with the Meta Fluor® imaging system (Visitron, Puchheim, Germany) using the program Meta Series (Vers. 6.2). Applying the dual excitation technique (Mühling *et al*., 1995; Mühling and Läuchli, 2000), the adaxial side of the leaf was excited at 490 nm: 440 nm by using the 20× objective (Leica pH 1; 20× / 0.40). The fluorescence of the whole image, resulting from the intercellular spaces (apoplast) of the intact leaf epidermis, was detected and used for the ratio imaging. Ratios were converted to pH values by *in vivo* calibration for pH (Mühling *et al*., 1995). For *in vivo* calibration, strong buffers (MES) with pH ranging from 5 to 7 were vacuum-infiltrated in leaves and values of ratio images were measured.

2.5 Effect of salt stress and abscisic acid on plasma membrane H^+-ATPase activity of two maize genotypes

2.5.1 Plasma membrane isolation

To investigate the plasmalemma H^+-ATPase activity in salt-sensitive and salt-resistant maize genotypes under 100 mM NaCl and 10 µM ABA treatments, plasma membrane was isolated

from young leaves using two-phase partitioning according to Yan *et al*. (2002). Segments of leaves from the leaf elongation zone were cut and washed three times with chilled, de-ionized water. The midribs of the leaves were removed and 60 g of leaf material were ground in ice-cold homogenization buffer (with a ratio of 4 mL buffer/g plant material). The homogenate mixture was filtered through two layers of Miracloth (Calbiochem-Novabiochem, San Diego) and was centrifuged in a swinging bucket rotor (AH 629 rotor, 36 mL, Sorvall Products, Newtown, CT) at 11,500 *g* for 10 min at 0°C. The supernatants were centrifuged again and pelleted at 87,000 *g* for 35 min. The microsomal pellets were re-suspended in phosphate buffer.

The **homogenization buffer** contained:
250 mM sucrose
10% (v/v) glycerol
0.5% (w/v) bovine serum albumin
250 mM KI
2 mM EGTA
2 mM dithiothreitol
1 mM phenylmethylsulfonyl fluoride
5 mM 2-mercaptoethanol
50 mM 1,3-bis (tris[hydroxymethyl]-methylamino) propane (BTP) adjusted to pH 7.8 with MES

Phosphate buffer consisted of:
250 mM sucrose
3 mM KCl
5 mM KH_2PO_4, adjusted to pH 7.8

The plasma membrane from microsomal membranes was fractionated with two-phase partitioning in aqueous dextran T-500 and polyethylene glycol 3350 (PEG) according to the method of Larsson (1985). To prepare 32 g of phase system, stock solutions of polymers (20% (w/w) dextran T-500 and 40% (w/w) PEG 3350) were weighed and diluted to 6.1% (w/w, each polymer) with phosphate buffer. The concentration of the dextran stock solution was measured by optical rotation (Larsson, 1985). Six grams of microsomal suspension were

added to the upper phase of each start tube (polymers diluted to 26 g). The tubes after sealing with Parafilm (American National Can, Greenwich, CT) were mixed by inversion (30 times). Phase separation of plasma membrane was carried out by centrifugation at 4°C and 720 g for 23 min. The plasmalemma-enriched upper phase was carefully removed and was centrifuged again in two identical separation steps, afterwards. The centrifugation times for washing steps were 15 and 10 min, respectively. The upper phases collected after the third step were diluted with phosphate buffer and centrifuged at 134,600 g for 40 min. The pellets were washed with re-suspension buffer and were pelleted again. The pellets were re-suspended in resuspension buffer (containing 1 mM DTT, pH 7.8), divided into aliquots, and immediately stored in liquid nitrogen.

The **resuspension buffer** contained:
250 mM sucrose
3 mM KCl
5 mM BTP/MES, pH 7.8

Quantification of protein concentration was carried out according to the method of Bradford (1976), using bovine serum albumin (Sigma) as a standard. The principle of the method is based on the observation that the absorbance maximum of Coomassie Brilliant Blue G-250 after binding with protein shifts from 465 nm to 595 nm. 20 µL re-suspended membrane protein were mixed with 2.375 mL reagent and incubated for 40 min at room temperature. The absorption was measured at 595 nm by means of a spectrophotometer.

Bradford reagent was composed of:
0.01% (w/v) Coomassie Brilliant Blue G-250
4.7% (w/v) ethanol
8.5% (w/v) phosphoric acid.

2.5.2 Measurement of ATPase activity

Activity of plasmalemma H^+-ATPase was determined by measuring the amount of inorganic phosphate released by ATPase through ATP hydrolysis in a defined period of time. Purity of the membrane fraction was estimated by measuring the inhibitor-sensitive ATPase activity of

various membranes. Vanadate (0.1 mM), nitrate (50 mM) and azide (1 mM) were used to inhibit the activities of plasmalemma, tonoplast, and mitochondrial ATPases, respectively. 1 mM molybdate was used to measure the phosphate release due to activity of unspecific acid phosphatases. Hydrolytic activity of plasmalemma H^+-ATPase was assayed by incubating 3 µg of membrane protein at 30°C in 0.5 mL of reaction mixture containing:

30 mM BTP/MES

5 mM $MgSO_4$

50 mM KCl

50 mM KNO_3

1 mM Na_2MoO_4

1 mM NaN_3

0.02% (w/v) Brij 58

5 mM disodium-ATP

ADP released from ATP hydrolysis can inhibit the ATPase activity in the assay medium. To avoid the ADP formation and decrease in ATP, an ATP-regenerating system (5 mM PEP and 5 units of pyruvate kinase) was also included in all assays. The reaction was stopped after 30 min with 1 mL of stopping reagent [2% (v/v) conc. H_2SO_4, 5% (w/v) SDS, and 0.7% (w/v) $(NH_4)_2MoO_4$] followed immediately by 100 µL of 10% (w/v) ascorbic acid. To prevent the phosphate release by ATP hydrolysis under acidic conditions (Baginski *et al*., 1967), 1.45 mL of arsenite citrate reagent (2% [w/v] sodium citrate, 2% [w/v] sodium m-arsenite, and 2% [w/v] glacial acetic acid) was added after 15 min. Color development was completed after 30 min, and ΔA_{820} was measured by means of a spectrophotometer (Varian, Cary 4 Bio UV-Visible Spectrophotometer). ATPase activity was calculated as phosphate liberated in excess of a boiled-membrane control. To determine the direct effect of ABA on the hydrolytic activity of ATPase, ABA at various concentrations (0.0, 0.01, 0.1, 1.0, and 10 µM) was added in the reaction medium.

2.5.3 Measurement of proton-pumping activity

The pumping activity of plasmalemma H^+-ATPase in *inside-out* vesicles (Fig. 2) was measured as the quenching of A_{492} by acridine orange (AO) using a spectrophotometer (Carry 4 Bio, Varian Australia Pty Ltd., Mulgrave, Victoria, Australia).

The assay mixture contained:

5 mM BTP/MES (pH 6.5)

7.5 µM AO

100 mM KCl

0.05% (w/v) Brij 58

0.5 mM EGTA (adjusted to pH 6.5 with BTP)

1 mM NaN_3

1 mM Na_2MoO_4

50 mM KNO_3

50 µg of membrane protein in a final volume of 1.5 mL

Brij 58 was used to create *inside-out* vesicles (Johansson et al., 1995). After equilibration of the membrane vesicles with the reaction medium (about 15 min), the reaction was initiated by the addition of 5 mM Mg-ATP (mixture of $MgSO_4$ and Na_2-ATP, adjusted to pH 6.5 with BTP). The reaction temperature was 25°C. Change in absorbance for the first 1 min and at the equilibrium was calculated as initial rate (IR) of active proton pumping and maximum pH gradient, respectively. Maximum quenching was achieved 70 min after initiation of the H^+-pumping. At equilibrium, net H^+ transport across the plasma membrane was zero and H^+ influx due to active pumping and passive H^+ efflux reached equilibrium. To determine passive H^+ transport, Na_3VO_4 (500 µM) was added after pH gradients of plasma-membrane vesicles had reached identical levels. The established pH gradient was completely collapsed by 5 mM gramicidine. To measure the effect of *in vitro* ABA on proton pumping, ABA (0.0, 0.1, and 10 µM) dissolved in ethanol was added in the assay mixture. The final concentration of ethanol in all assays was about 0.003%.

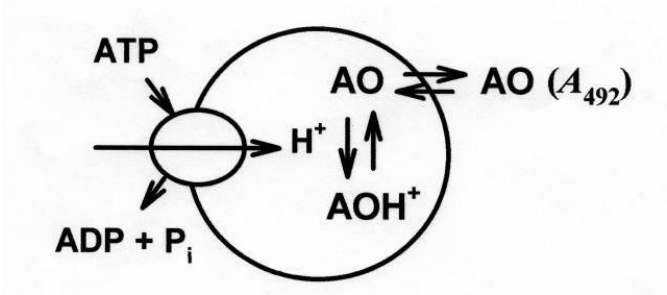

Figure 2: Measurement of proton pumping by H^+-ATPase in *inside-out* plasma membrane vesicles using acidine orange (AO; Bennet and Spanswick, 1983).

2.6 Gel electrophoresis and immune-detection of plasma membrane H^+-ATPase

Plasma membrane proteins were separated on the basis of their molecular weight using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970). Membrane proteins were separated on a discontinuous SDS-polyacrylamide gel (6% (w/v) acrylamide stacking gel and 10% (w/v) acrylamide separating gel). The separation gel was first poured between two glass plates (10 cm x 8 cm) and after polymerization the stacking gel was layered over the separation gel. Membrane vesicles (3 µg membrane proteins) were solubilized in SDS-loading buffer and shaken for 30 min at room temperature (22°C).

The **stacking gel** contained the following reagents:
1.25 mL H_2O bidest
0.625 mL 0.5 M Tris-HCl, pH 6.8; 0.4% SDS
0.5 mL Acrylamid solution
10 µL 10% APS (w/v)
10 µL TEMED

The **separation gel** contained the following reagents:
1.5 mL H_2O bidest
1.5 mL 1.5 M Tris-HCl, pH 8.8; 0.4% SDS
3 mL Acrylamid solution
50 µL 10% APS (w/v)
5 µL TEMED

The **SDS-loading buffer** was composed of:
0.125 mM Tris-HCl (pH 7.4)
10% (w/v) SDS
10% (v/v) glycerol
0.2 M dithiothreitol
0.002% (w/v) bromocresol blue
5 mM phenylmethylsulfonyl fluoride
0.05% (w/v) trasylol

For western blotting, SDS-PAGE was incubated in blotting buffer for 15 min at room temperature. After incubation, samples were loaded on the SDS-polyacrylamide gels (submerged in running buffer) and the gels were electrophorized at 25 mA for about 1h.

For the identification of plasmalemma H^+-ATPase on the linear acrylamide gel, all proteins were transferred to PVDF membrane filters (0.2 µm, Pall Specialty Materials, Port Washington, NY). 15 min after the incubation of gel and PVDF membrane in blotting buffer, the transfer of proteins took place under the following conditions: 1 h tension of 110 mA. After incubating in a blocking buffer (5% milk powder in TBS buffer) for 2 h, the membranes were washed several times with TBS-T buffer. For identification of the plasma membrane H^+-ATPase, the membranes with plasma membrane proteins were incubated for 2 h at room temperature with a polyclonal antibody (Dr. Michael G. Palmgren, Royal Veterinary and Agricultural University, Copenhagen) specific for the central part of plant H^+-ATPase (amino acids 340-650 of AHA2) diluted in PBS buffer (1 : 3000, v/v). After washing in TBS-T, the membranes were incubated for 2 h at room temperature with a 1 : 30000 (v/v) diluted secondary antibody (alkaline phosphatase-conjugated anti-rabbit IgG, Sigma). After several washing steps in TBS-T and TBS buffers, membranes were incubated for 5 min in AP buffer and Western Blots were developed using a buffer containing the substrates BCIP and NBT. For quantification of plasma membrane H^+-ATPase, the blots were scanned, and the H^+-ATPase immuno-reactive bands were quantified densitometrically (software TINA, Raytest Isotopenmessgeräte, Straubenhardt, Germany).
The ingredients of the buffers were as follows:

Blotting buffer
0.025 M tris base, pH 8.0
0.192 M glycin
10 % methanol
TBS buffer
1 mM Tris-HCl, pH 8.0
15 mM NaCl

TBS-T-buffer
0.1% Tween 20 in TBS-buffer

PBS-Puffer

0.14 M NaCl

2.7 mM KCl

1.8 mM Na2HPO4

20.3 mM KH2PO4

pH 7.4 with NaOH

AP-buffer

100 mM Tris-HCl, pH 9.5

100 mM NaCl

5 mM MgCl2

Developing buffer

66 µL NBT (100 mg NBT in 1.9 mL 70% (v/v) Dimethylformamid)

32 µL BCIP (100 mg BCIP in 1.9 mL Dimethylformamid)

in 10 mL AP-buffer

2.7 Effect of salt stress and abscisic acid on the mRNA transcription of plasmamembrane H^+-ATPase isoforms

2.7.1 RNA isolation

Total RNA was isolated from young leaves of Pioneer 3906 and SR 03 cultivated under salt stress and ABA treatments as described above. Segments of young leaves from the leaf elongation zone were cut and immediately shock-frozen in liquid nitrogen. Frozen tissues were ground in liquid nitrogen using pre-cooled mortar and pestle and stored at -80^{o}C.

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the method of Chomczynski and Sacchi (1987). TRIzol reagent is a mono-phasic solution of phenol and guanidine isothiocyanate. 100 mg tissue was homogenized with 1 mL reagent by vigorously vortexing. For complete dissociation of nucleoprotein complexes, homogenized samples were incubated for 5 min at room temperature. After adding 0.2 mL chloroform, samples were incubated at room temperature for 3 min and centrifuged at 4°C (12000 g) for 15 min. After centrifugation, the sample mixture was separated into an upper colorless aqueous phase containing RNA, an inter phase and a lower phenol-chloroform phase. For RNA precipitation, 0.5 mL of upper aqueous phase was mixed with 0.5 mL of isopropanol and incubated for 10 min at room temperature. RNA was subsequently pelleted by centrifugation for 10 min at 4°C (12000 g), washed with 75% ethanol and pelleted again by centrifugation for 5 min at 4°C (7500 g). RNA pellets were air-dried and dissolved in DEPC-water. The dissolved RNAs were then incubated at 60°C for 10 min and aliquoted at -80°C.

2.7.2 Quantification of RNA

RNA was quantified using a NanoDrop spectrophotometer (ND 1000, Thermo Scientific). RNA has its maximum absorption at 260 nm and an OD_{260} of 1.0 is equivalent to about 40 µg/ml of RNA. The ratio of absorbance at 260 nm and 280 nm (OD_{260}/OD_{280}) was used to assess the purity of RNA samples. Pure RNA samples have OD_{260}/OD_{280} of 2.0 or more than 2.0. A ratio below than 2.0 indicates that the sample is contaminated with protein or phenol and cannot be used for downstream applications.

2.7.3 Determination of RNA integrity

To check the integrity of RNA and contamination from genomic DNA, total RNA was run on 1% agarose gel electrophoresis and stained with ethedium bromide. Integrity of mRNA is very important for cDNA synthesis. Total RNA comprises only 1-2% of mRNA, which is not detectable on agarose gel. A good quality RNA shows two distinct bands of 18 S and 28 S ribosomal RNA. The sharpness of the ribosomal RNA bands provides a rough indication of whether the mRNA is degraded.

To prepare 1% agarose gel, 0.1 g of agarose was mixed with 100 mL of TBE buffer. To dissolve agarose, the mixture was heated in a microwave oven for 3 min. When the solution was cooled down to 80°C, 6 µL of ethidium bromide were mixed in the solution and poured in to a mold. 3 µg of total RNA was mixed with loading dye and loaded into slots of gel submerged in TBE buffer. The gel was electrophorised at 120 V for 1 h and photographed using UV. TBE buffer contained 400 mM Tris-Borate and 10 mM EDTA (pH 8.0), dissolved in bi-destilled water.

2.7.4 Synthesis of cDNA

cDNA from total RNA was synthesized following the manufacturer's instructions in Verso cDNA kit (Thermo Scientific). 5 µg total RNA was filled up with DEPC water to 10 µL volume and incubated at 70°C for 5 min to remove any RNA secondary structures. After incubation, the RNA samples were immediately placed on ice for 5 min and 10 µL of the following reaction mixture were added:

4 µL 5X cDNA synthesis buffer
2 µL dNTP mix
1 µL RNA primer (Oligo-dT)
1 µL RT enhancer
1 µL Verso enzyme mix
1 µL DEPC water

RT enhancer was included to remove the contaminating DNA and to degrade the double-stranded DNA during reverse transcription. To start reverse transcription, the mixture (20 µL) was incubated at 42°C for 40 min. After reverse transcription, the cDNA was heated at 95°C

for 2 min to inactivate the RT enhancer and reverse transcriptase. The newly synthesized cDNA was then aliquoted at -20°C.

2.7.5 Real-time PCR analysis of H^+ATPase isoforms

To quantify mRNA expression of the H^+ATPase and its isoforms, cDNA from leaves of two maize genotypes was used as a template for real-time PCR. Polymerase chain reaction (PCR) is the method which makes an exponential amplification of DNA. In real-time PCR (qPCR), use of fluorescent dye SYBR Green (which binds with double stranded DNA) provides an opportunity to precisely quantify small amounts of DNA. Primers for the reference and target genes as reported by Zörb *et al*. (2005) and Santi *et al*. (2003) are summarized below:

Actin	Forward Primer	GAGCTCCGTGTTTCGCCTGA	J0238
172 bp	Reverse Primer	CAGTTGTTCGCCCACTAGCG	J0238
MHA1	Forward Primer	TTTGGAAGTTTGACTTCCCA	U09989
215 bp	Reverse Primer	AAGAAGTCGGTCTTGTACGC	U09989
MHA2	Forward Primer	AAGACCTTCGGAAAGGAGAGA	X85805
	Reverse Primer	AAGACGGGTACCCAACCATA	X85805
MHA3	Forward Primer	GAGAACAAGACCGCCTTCAC	AJ441084.1
436 bp	Reverse Primer	AAGACGGGTACCCAACCATA	AJ441084.1
MHA4	Forward Primer	TCTGGCTCTACAGCATCGTG	AJ539534
230 bp	Reverse Primer	CTTGTCGTGGAACAGCGTGC	AJ539534
MHAfam	Forward Primer	ATCGTCAGCCAGGCTCTGAT	*
231 bp	Reverse Primer	CGAAGCGGATGAAGAACTTG	

*designed on the basis of homologue regions of all possible *MHA* isoforms (family-specific primers) of maize

SYBR Green mix from Sigma was used in all assays. Real-time PCR assays for all specific genes were performed on the Rotor-Gene 2000/3000 Real-Time Amplification Thermal Cycling System. For 10 µL of each reaction, the following components were mixed:

5 µL SYBR Green Mix

0.2 µL primer pair (10 µM)

2.8 µL sterile water

2 µL diluted cDNA (1 : 10)

The program was designed as follows:

Cycle 1 Initial denaturation at 94°C for 2 min

Cycle 2-35 Denaturation at 94°C for 15 s

Primer specific annealing at 50-60°C for 30 s

Primer elongation at 72°C for 30 s

Cycle 36 Melt curve analysis at 72-99°C

Quantification of the gene expression followed the equation of Pfaffl (2001). It does not quantify the absolute amounts of the transcript but a relative change in the target gene in comparison to a reference gene. The ratio of relative expression gives the physiological changes in gene expression. Actin was used as a reference gene, which is supposed not to be influenced by any treatment. The following equation shows Pfaffl´s model of relative expression.

$$\text{ratio} = \frac{(E_{target})^{\Delta Ct\ target\ (control-treated)}}{(E_{ref})^{\Delta Ct\ ref\ (control-treated)}}$$

Where,

ΔCt_{target} = difference in the Ct values for the target gene between control and treated samples

ΔCt_{ref} = difference in the Ct values for the reference gene between control and treated samples

E_{target} = Real-time PCR amplification efficiency of the target gene.

E_{ref} = Real-time PCR amplification efficiency of the reference gene.

Real-time PCR amplification efficiencies ($E= 10^{[-1/slope]}$) were calculated by making a series of dilution of cDNA (1: 5, 1: 10, 1: 20, 1: 40, 1: 80). Specificities of the primers for target and reference genes were confirmed by making the melt curve analysis.

2.8 Statistical analysis

To determine significant differences among treatments for various parameters, data were subjected to analysis of variance (ANOVA) using SigmaPlot 11 and t-test using Microsoft Excel 2007. Means of the treatments that exhibited significant differences were separated using the least significant difference (LSD) test. For all analyses, a P-value of less than 5% was interpreted as statistically significant.

2.9 List of chemicals

ABA (2-cis, 4-trans-abscisic acid), Sigma Aldrich

Agar (Agar Agar Kobe I): Serva 11392

Ammonium-Molybdat ($(NH_4)_6Mo_7O_{24}$) : 82.3 % MoO_3; Sigma

AO = Acridin Orange (3.6-Bis [Dimethylamin] Acridin-Base): ca. 95% Farbstoffgehalt; Sigma

L(+)-Ascorbinsäure: mid. 99.7% Reinheit (idodometrisch); Merck

Brij 58 (Polyoxyethylen-20-cetylether): Sigma

BSA (Bovines Serumalbumin): fettsäurefrei, ≥ 96% Albumin; Sigma

BTP (1,3-Bis[tris(hydroxymethyl)methylamino]propan: mind. 99% Reinheit (Titration); Sigma

Coomassie Brilliant Blue G-250: Calbiochem Corp., La Jolla

Dextran T 500: durchschnittliches Molekulargewicht = 485 000 g/mol; Sigma

Dikaliumhydrogenphosphat (K_2HPO_4): p.a.; Merck

DTT (DL-Dithiothreitol): 99% Reinheit (Titration); Sigma

EGTA (Ethylenglycol-bis(ß-aminoethylether)N,N,N`,N`-Tetraessigsäure): 97% Reinheit; Sigma

EDTA

Glycerin: 99% Reinheit; Sigma

Gramicidin D: von Bacillus brevis, 1080 µg Gramicidin mg^{-1}; Sigma

JA (Jasmonic acid, JA-2500), Sigma

Kaliumchlorid (KCl): p.a.; Fluka

Kaliumdihydrogenphosphat (KH_2PO_4): p.a.; Fluka

Kaliumjodid (KJ): ≥ 99.5% Reinheit (argentometrische Titration); Fluka

Kaliumnitrat (KNO_3): p.a.; Merck

Kaliumsulfat (K_2SO_4): p.a.; Fluka

2-Mercaptoethanol: mind. 99% Reinheit (GC); Serva

MES (2-[N-Morpholin]ethansulfonsäure): freie Säure, mind. 99.5% Reinheit (Titration).; Serva

Magnesiumsulfat ($MgSO_4$): p.a.; Fluka

Na_2ATP (adenosin 5´-Triphosphat, Na_2Salz): 98% Reinheit; Merck

Natriumazid (NaN_3): p.a.; Merck

Natriumcitrat-Dihydrat: p.a.; Merck

Natrium-Metaarsenit ($NaAsO_2$): mind.99% Reinheit; Sigma

Natriummolybdat (Na_2MoO_4): p.a.; Merck

Natrium-Orthovanadat (Na_3VO_4): mind. 95% Reinheit; Sigma

PEG 3350 (Polyethylenglycol): durchschnittliches Molekulargewicht = 3350 g/mol; Sigma

PEG 6000 (Polyethylenglycol): durchschnittliches Molekulargewicht =

6000 g mol-1; Merck

PEP (Phosphoenolpyruvat): Boehringer Mannheim GmbH

PMSF (Phenylmethylsulfonylfluorid): > 99% Reinheit(GC) ; Sigma

Pyruvat-Kinase: 1000 U; Sigma

D(+)-Saccharose: für biochemische & mikrobiologische Zwecke; Merck

SDS (Natriumdodecylsulfat): 99% Reinheit; Sigma

3 Results

3.1 Changes in endogenous levels of jasmonic acid in maize genotypes during the first phase of salt stress

3.1.1 Shoot growth during the first phase of salt stress

The effects of NaCl and PEG stress on shoot and root fresh weights of maize genotypes are described in Tab. 3. Short-term osmotic stress (-0.5 MPa) mainly affected the shoot growth and leaves appeared dark-green. No visual symptoms of Na^+ toxicity were found in salt-treated plants of both genotypes. Shoot fresh weights were significantly reduced for Across 8023 after the application of 100 mM NaCl at full concentration for 1.5 d. Similarly, an equimolar PEG-stress caused a significant depression in shoot growth of Across 8023. In contrast, shoot growth of relatively resistant SR 03 was not significantly affected by NaCl and PEG treatments. Although NaCl caused a slight reduction in shoot growth of SR 03, comparison of both genotypes (Fig. 3) revealed that SR 03 produced significantly more relative shoot fresh weights than Across 8023 under both stress treatments. The relative shoot fresh weights attained by Across 8023 under NaCl and PEG treatments were 73% and 80% and by SR 03 were 84% and 93%, respectively. NaCl and PEG did not significantly affect the root growth in both genotypes. No significant differences between the effects of NaCl and PEG treatments on shoot and root fresh weights were observed in both genotypes.

Table 3: Effect of NaCl and PEG at identical solute potential (-0.5 MPa) on shoot and root fresh weights (g $plant^{-1}$) of maize genotypes Across 8023 and SR 03. Plants were treated for 1.5 d with control (1 mM NaCl), PEG (133 g PEG L^{-1}), and NaCl (100 mM NaCl) treatments. The values are means ± SE of four replicates. Significant ($p \leq 5\%$) differences between treatments within each genotype are indicated with different letters (capital letters for SR 03).

		Control	PEG	NaCl
Shoot	Across 8023	10.35 ± 0.7 a	8.24 ± 0.6 b	7.49 ± 0.2 b
	SR 03	8.31 ± 0.5 A	7.67 ± 0.4 A	6.90 ± 0.2 A
Root	Across 8023	5.61 ± 0.4 a	4.50 ± 0.4 a	4.63 ± 0.4 a
	SR 03	3.37 ± 0.3 A	3.44 ± 0.1 A	3.22 ± 0.1 A

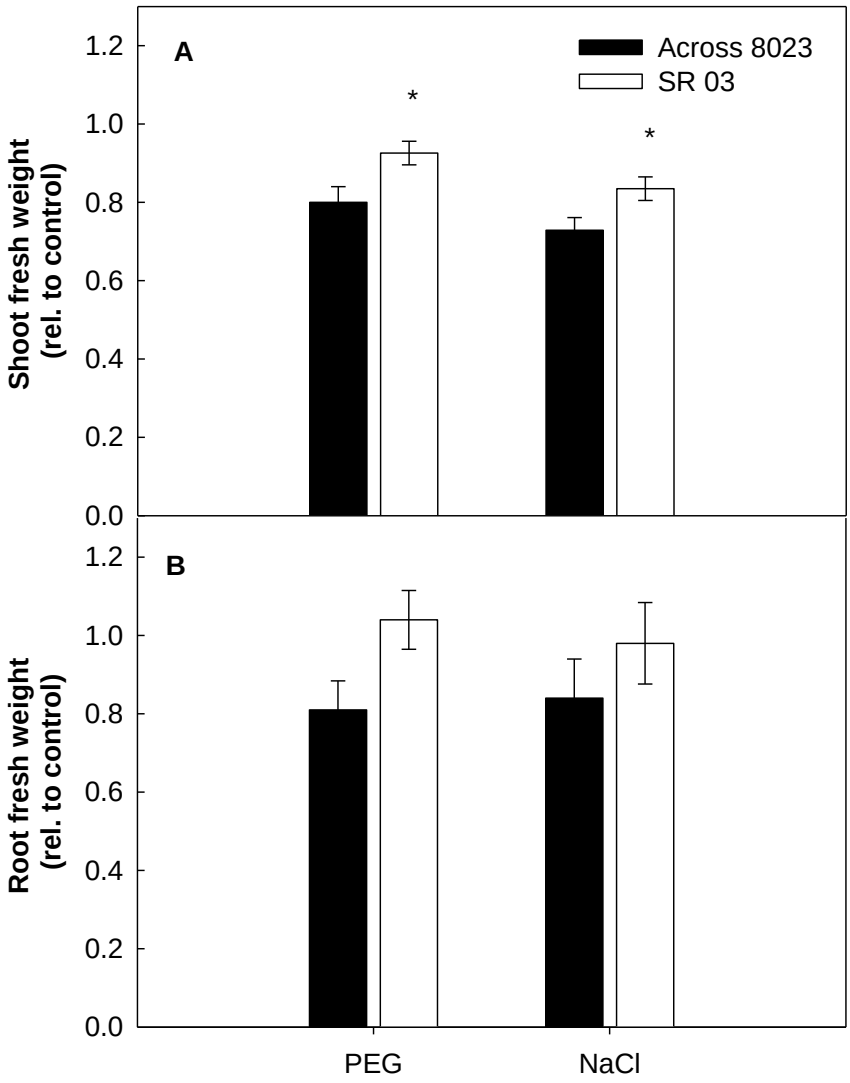

Figure 3: Effect of NaCl and PEG at same solute potential on relative shoot (A) and root (B) fresh weights of Across 8023 and SR 03. Data represent the reduction of fresh weights by stress treatments relative to control (1 mM NaCl). The values are means of four replicates ± SE. Significant ($p \leq 5\%$) differences between genotypes are indicated by an *asterisk*.

3.1.2 Shoot turgor during the first phase of salt stress

Salt stress significantly reduced the shoot water and osmotic potentials (data not shown) of both genotypes. However, the shoot turgor that was calculated as the difference between shoot water and osmotic potentials was not significantly affected by salt stress (Fig. 4). No significant differences were observed for shoot water relations b etween both genotypes

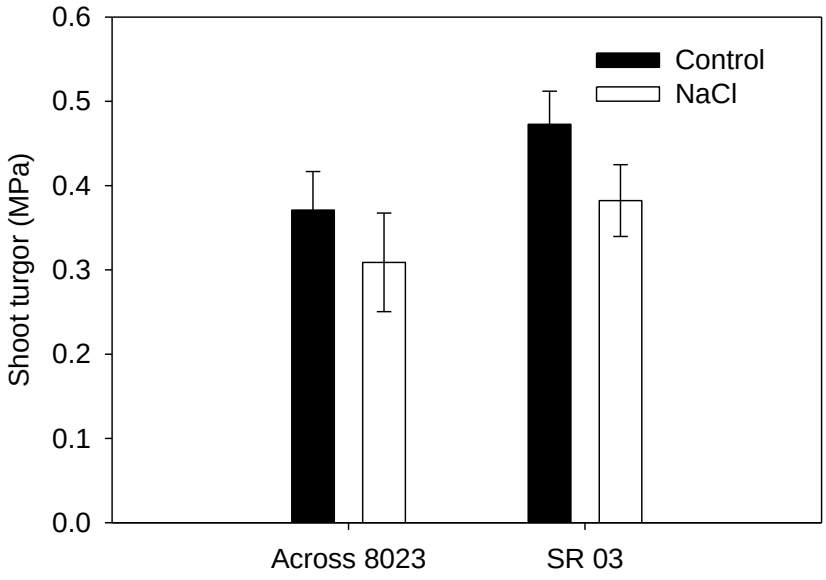

Figure 4: Effect of salt stress on the shoot turgor of two maize genotypes. Vertical bars are means ± SE of four replicates. No significant ($p \geq 5\%$) differences between control and salt stress were observed within each genotype.

3.1.3 Concentrations of jasmonic acid

In order to study the changes in JA in two maize genotypes with differences in salt resistance during the first phase of salt stress and to find out whether salt-induced JA is induced by osmotic or ionic stress, we compared the concentrations of JA in plant tissues under 100 mM NaCl or PEG at equal solute potential (-0.5 MPa). Both genotypes produced considerable amounts of basal JA in root and shoot tissues. Basal levels of JA were 5-7 folds higher in the roots than shoot tissues of both genotypes.

The salt-resistant SR 03 had higher basal levels of JA in roots than the salt-sensitive Across 8023 (Fig. 6). Endogenous levels of JA were significantly increased in the root tissues of Across 8023 by salt stress during the first phase, in comparison to corresponding control. Similarly, an equiosmotic PEG treatment also increased the JA in the root tissues of salt-sensitive Across 8023. Relative to non-stressed plants, root JA was increased by 67% and 83% under PEG and NaCl treatments, respectively. On the other hand, concentrations of JA were not altered in root tissues of salt-resistant SR 03 under both stress treatments. JA levels were not changed in shoot tissues of both genotypes by either stress treatments (Fig. 5).

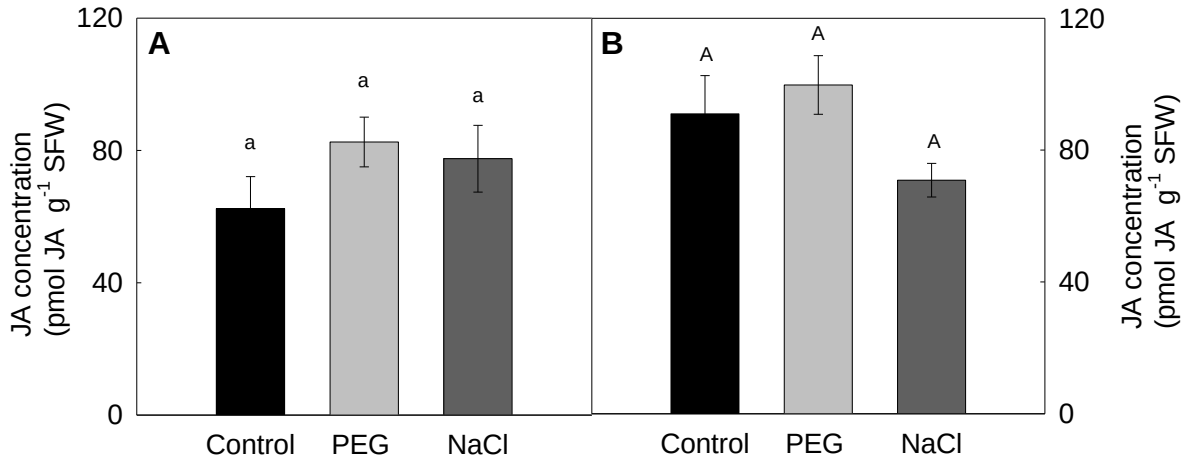

Figure 5: Effect of NaCl and PEG at identical solute potential on shoot jasmonic acid (JA) concentrations of Across 8023 (A) and SR 03 (B). Vertical bars are means ± SE of four replicates. No significant ($p \leq 5\%$) differences between control and stress treatments were observed within each genotype.

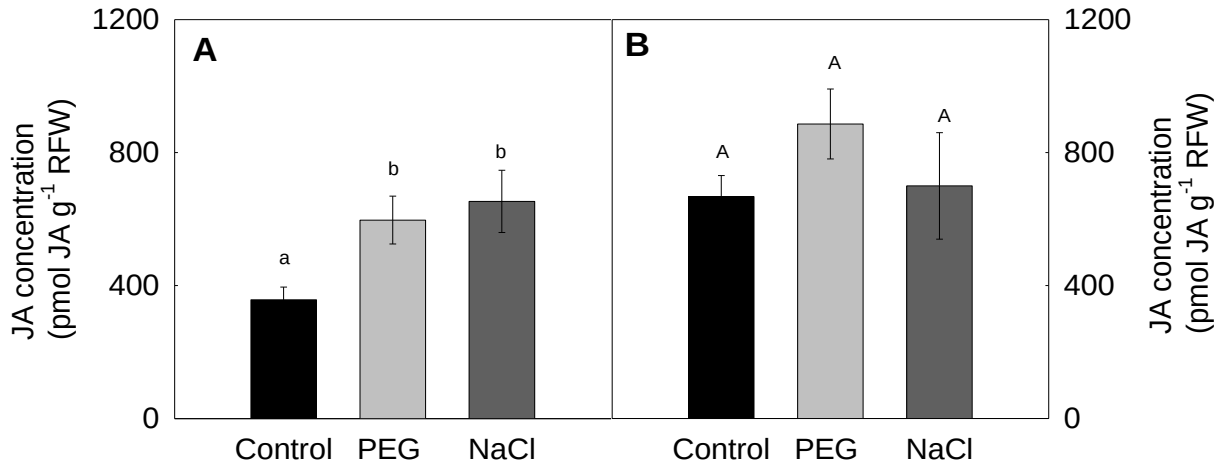

Figure 6: Effect of NaCl and PEG at identical solute potential on root jasmonic acid (JA) concentrations of Across 8023 (A) and SR 03 (B). Vertical bars are means ± SE of four replicates. Significant ($p \leq 5\%$) differences between control and stress treatments within each genotype are indicated as different letters.

3.2 Effect of exogenous jasmonic acid and abscisic acid on maize seedlings growth

In this study we investigated whether exogenous application of JA or ABA in the nutrient medium could influence shoot growth of maize seedlings. Fig. 7 shows the effect of JA and ABA on the growth of maize seedlings. Shoot length was measured over a period of 3 d with the start of treatment. The results show that JA supplied in the nutrient medium significantly reduced shoot extension. The reduction in shoot extension by JA was apparent 2 d after the treatment (Fig. 8), and maximum shoot length achieved was reduced by 25% in comparison to control. Similarly, JA also reduced the rate of shoot extension and root and shoot fresh weights of seedlings (Tab. 4).

Control JA ABA Control (Ex.) JA (Ex.) ABA (Ex.)

Figure 7: Effect of JA or ABA on the growth of maize seedlings. 6 d old seedlings were grown with or without 30 µM JA or 10 µM ABA for 3 d. In order to test whether hormones can be taken up by the roots, a separate set of seedlings with roots excised 1 cm from root tip (Ex.) was also grown under the same treatments.

No significant differences in shoot growth of control plants between excised or intact roots were observed. A similar extent of growth inhibition by ABA or JA was found in maize seedlings with excised roots, when compared to ABA or JA-treated seedlings with intact roots, respectively (Fig. 8). These results show that both JA and ABA can be easily taken up by roots and inhibit shoot growth, when supplied in nutrient solution.

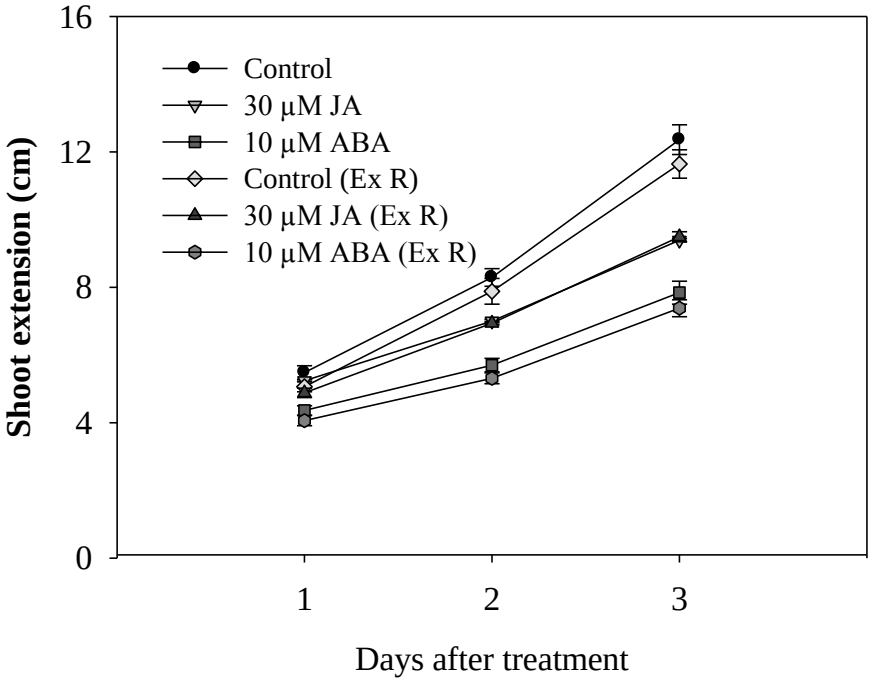

Figure 8: Effect of exogenous application of ABA or JA on shoot extension of maize seedlings. Maize seedlings with intact or excised roots were supplied with or without ABA or JA in nutrient medium. Data points are means ± SE of four replicates.

Table 4: Effect of JA or ABA on various growth parameters of maize seedlings. 6 d old seedlings were grown with or without 30 µM JA or 10 µM ABA. The values represent means ± SE of four replicates.

	Rate of shoot extension	**Shoot fresh weight**	**Root fresh weight**	**Root length**
	cm d^{-1}	g	g	cm
Control	4.1 ± 0.2	1.2 ± 0.2	1.1 ± 0.1	30 ± 0.4
30 µM JA	2.4 ± 0.1	0.8 ± 0.2	0.6 ± 0.0	19 ± 0.4
10 µM ABA	2.1 ± 0.2	0.6 ± 0.2	0.6 ± 0.1	22 ± 0.4
Control (Ex.R)	3.8 ± 0.2	1.0 ± 0.2	1.1 ± 0.0	17 ± 0.2
30 µM JA (Ex.R)	2.6 ± 0.2	0.8 ± 0.2	0.5 ± 0.0	16 ± 0.3
10 µM ABA (Ex.R)	2.1 ± 0.2	0.6 ± 0.2	0.6 ± 0.0	16 ± 0.4

Because JA inhibits shoot growth, we tested whether salt-sensitive (Across 8023) and salt-resistant (SR 03) maize genotypes differ in sensitivity to JA. We compared the effect of physiological concentrations of JA (0, 5, 25, and 50 μM) on the growth of 6 d old seedlings of both genotypes. No visible symptoms of senescence or chlorosis were observed. Seedling growth was significantly reduced in a dose-dependent manner with the application of JA in nutrient medium. The data for the effect of JA on the rate of shoot extension growth are given in Fig. 9, which shows that reduction in the rate of shoot extension was increased by increasing the concentration of JA in the nutrient solution. Shoot and root fresh weights were also reduced in a similar way (data not shown). The pattern of shoot growth inhibition by increasing JA concentrations in both maize genotypes was not significantly different, which indicates that salt-sensitive and salt-resistant maize genotypes are equally sensitive to JA.

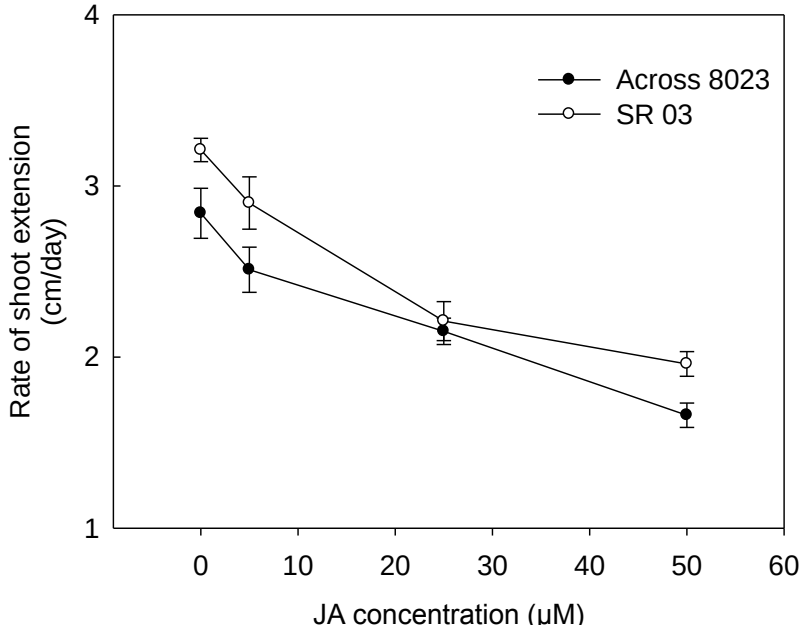

Figure 9: Comparison of rate of shoot extension of Across 8023 and SR 03 under various JA concentrations. 6 d old seedlings of both genotypes were maintained at four levels of JA (0, 5, 25 and 50 μM) for 4 d. Data points are means ± SE of four replicates.

3.3 Effect of exogenous jasmonic acid on ion homeostasis of salt-stressed maize genotypes

In this study, we investigated whether pretreatment with jasmonic acid could ameliorate the deleterious effects of salt stress during the first phase in two maize genotypes. Plants were treated with either salt stress or in combination of JA and salt stress. Exogenous application of JA did not alleviate the salt stress-induced inhibition of shoot growth in both maize genotypes (data not shown).

Analysis of the ion concentrations showed that salt-resistant SR 03 accumulated significantly lower shoot [Na^+] as compared to the salt-sensitive Across 8023 (Tab. 5). The lower shoot [Na^+] in SR 03 was the result of both low Na^+ uptake at the root surface and lower root-to-shoot translocation of Na^+. As shown in Fig. 10, SR 03 showed significantly lower Na^+ uptake (27 mg Na^+ g^{-1} root DM) as compared to Across 8023 (41 mg Na^+ g^{-1} root DM) under 100 mM NaCl. Similarly a lower translocation of Na^+ from root to shoot was found in salt-treated SR 03 as compared to salt-treated Across 8023.

Table 5: Effect of salt stress and JA plus salt stress on the Na^+, K^+, Mg^{2+}, and Ca^{2+} concentrations of Across 8023 and SR03. Salt-treated plants were gradually adapted to 100 mM NaCl in 48 h, and supplied with or without 30 µM JA. Values are means ± SE of four replicates.

		Shoot				Root			
		Na^+	K^+	Mg^{2+}	Ca^{2+}	Na^+	K^+	Mg^{2+}	Ca^{2+}
Across 8023	**Control**	-	44.0	2.0	9.4	-	38.1	5.7	17.6
	NaCl	9.6	35.7	1.8	7.5	19.5	25.7	4.7	11.0
	JA + NaCl	6.5	33.9	1.4	5.7	8.1	14.6	3.2	11.7
SR 03	**Control**	-	42.9	1.9	10.8	-	46.4	5.3	13.7
	NaCl	4.7	31.5	1.7	7.1	17.5	23.5	4.1	8.6
	JA + NaCl	2.8	34.9	1.3	5.8	4.8	19.6	3.4	9.8

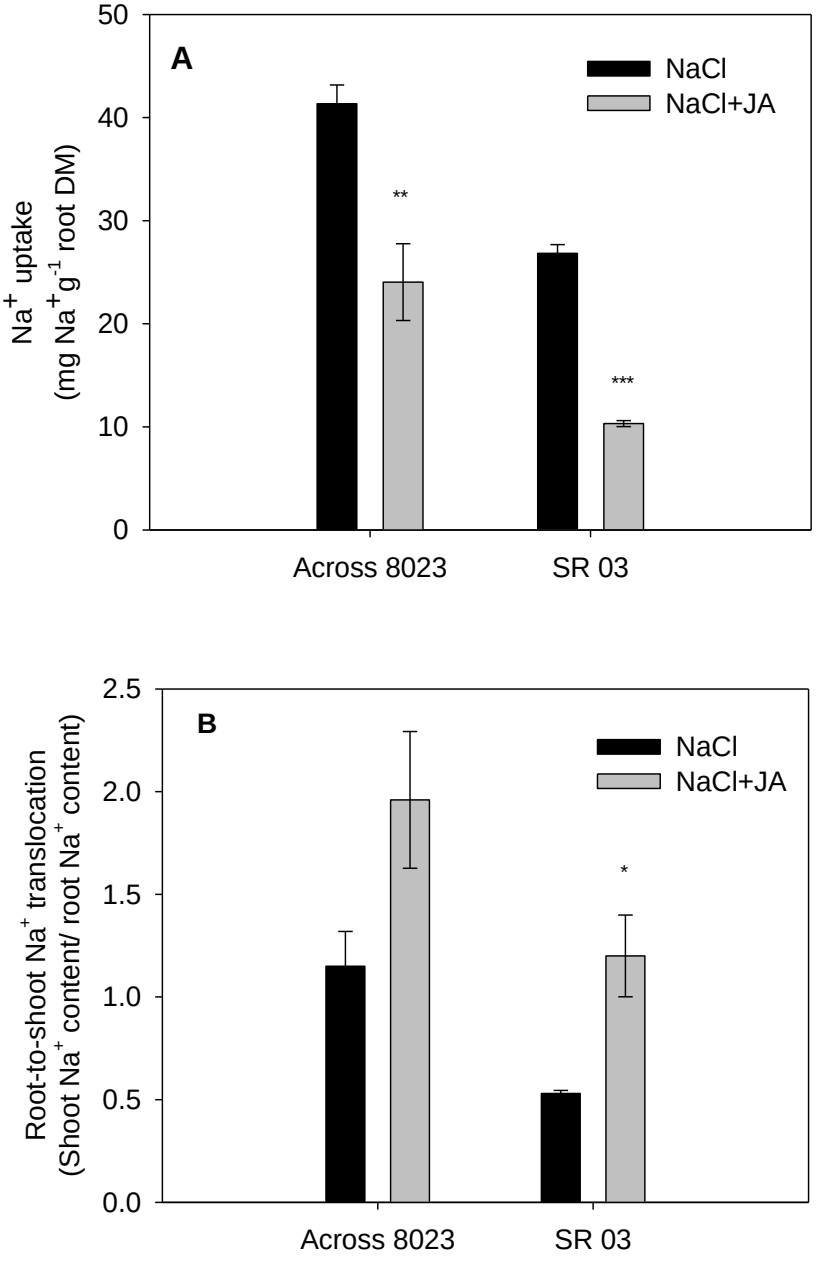

Figure 10: Effect of salt stress and JA plus salt stress on Na^+ uptake (A) and root-to-shoot Na^+ translocation (B) of two maize genotypes. The plants were treated with 100 mM NaCl and in combination with 30 µM JA. Sodium uptake at the root surface was determined as ratio of total plant Na^+ content to root dry matter. To calculate root-to-shoot translocation of Na^+, total shoot Na^+ content was divided by total root Na^+ content. Vertical bars are means ± SE of four replicates. Significant ($p \leq 5\%$) differences between stress treatments within each genotype are indicated by an *asterisk*.

Pretreatment with JA of salt-stressed plants significantly reduced the Na^+ uptake at the root surface in both maize genotypes, when compared to the plants grown under salt stress alone. However, root-to-shoot Na^+ translocation was increased after the application of JA to salt-stressed plants. As a result, JA pretreatment to salt-stressed plants decreased the Na^+ concentrations in plant tissues (Tab. 5). Our results indicate that JA application improves Na^+ exclusion either by decreasing passive influx or by increasing the active efflux of Na^+.

In comparison to control, the concentrations of K^+ and Ca^{2+} were decreased by salt treatment in both root and shoot tissues. Mg^{2+} concentrations were not significantly affected by salt stress in both genotypes. Application of JA to the salt-treated plants did not further affect the $[Mg^{2+}]$ and $[Ca^{2+}]$ in plant tissues as compared to salt-treated plants, whereas in comparison to salt treatment, a slight decrease in $[K^+]$ was found when JA was applied in combination with salt stress. According to Bergmann (1992), the plants contained sufficient concentrations of K^+, Mg^{2+} and Ca^{2+} under stress treatments.

3.4. Changes in cell-wall acidification and leaf growth during the first phase of salt stress and their relationship with abscisic acid and jasmonic acid

Acidification of cell wall in leaves of salt-sensitive maize genotype Pioneer 3906 was found decreased during the first phase of salt stress but not affected in a relatively resistant SR 03 (Pitann *et al*., 2009). Maize genotypes varying in salt resistance also showed a differential accumulation of ABA and JA. Since both hormones inhibit shoot extension in maize seedlings, the goal of this investigation was to study whether the differential responses of maize genotypes in shoot growth and cell-wall acidification to salt stress are controlled by hormonal signals.

3.4.1 Effect of salt stress and hormones abscisic acid and jasmonic acid on shoot growth

In the first phase of salt stress, maize plants showed stunted shoot growth with dark-green leaves. No clear visible symptoms of sodium toxicity were observed in salt-stressed plants of both genotypes. Shoot fresh weights of both genotypes were significantly reduced by salt stress during the first phase (Tab. 6). Comparison of genotypes showed that SR 03 produced more relative shoot fresh weights than Pioneer 3906 under salt stress. Likewise, application of 10 μM ABA also significantly reduced the shoot fresh weights. ABA-induced growth

inhibition was less pronounced in SR 03 as compared to Pioneer 3906. However, the two genotypes differing in growth response to salt stress and ABA responded similarly to the application of jasmonic acid (50 µM JA) and significantly reduced the shoot fresh weights by about 24%.

3.4.2 Sensitivity in leaf expansion of maize genotypes to salt and abscisic acid stress treatments

Salt stress and ABA treatments had no effect on the expansion of old leaves of both genotypes, but caused a rapid decline in the expansion of young leaves of Pioneer 3906. Fig. 11 shows the comparison of expansion growth for leaf 5 to 8 of Pioneer 3906 and SR 03 under salt stress and ABA treatments. Reduction in leaf growth by salt stress was only apparent from leaf 5 onward. In comparison to control, maximum expansion of leaf six and seven were reduced by 44% and 69% in Pioneer 3906, respectively. The leaf 8 did not emerge in the salt-treated plants of Pioneer 3906.

Table 6: Effect of salt stress and stress hormones on shoot fresh weights (g plant^{-1}) and relative shoot fresh weights (compared to controls) of Pioneer 3906 and SR 03. Plants were treated with 1 mM NaCl (Control), 100 mM NaCl for 8 d (NaCl), and 50 µM JA or 10 µM ABA for 3 d. Values are means ± SE of four replicates. Significant differences ($p \leq 5\%$) between treatments within each genotype are indicated by different letters.

	Shoot fresh weight g plant^{-1}		**Relative shoot fresh weight %**	
	Pioneer 3906	SR03	Pioneer 3906	SR03
Control	65.42 ± 2.06 A	66.17 ± 2.04 a	-	-
NaCl	28.37 ± 1.80 C	36.02 ± 0.80 d	43.37	54.44
JA	49.44 ± 1.93 B	51.08 ± 3.8 c	75.58	77.19
ABA	51.70 ± 0.55 B	58.84 ± 1.67 b	79.04	88.92

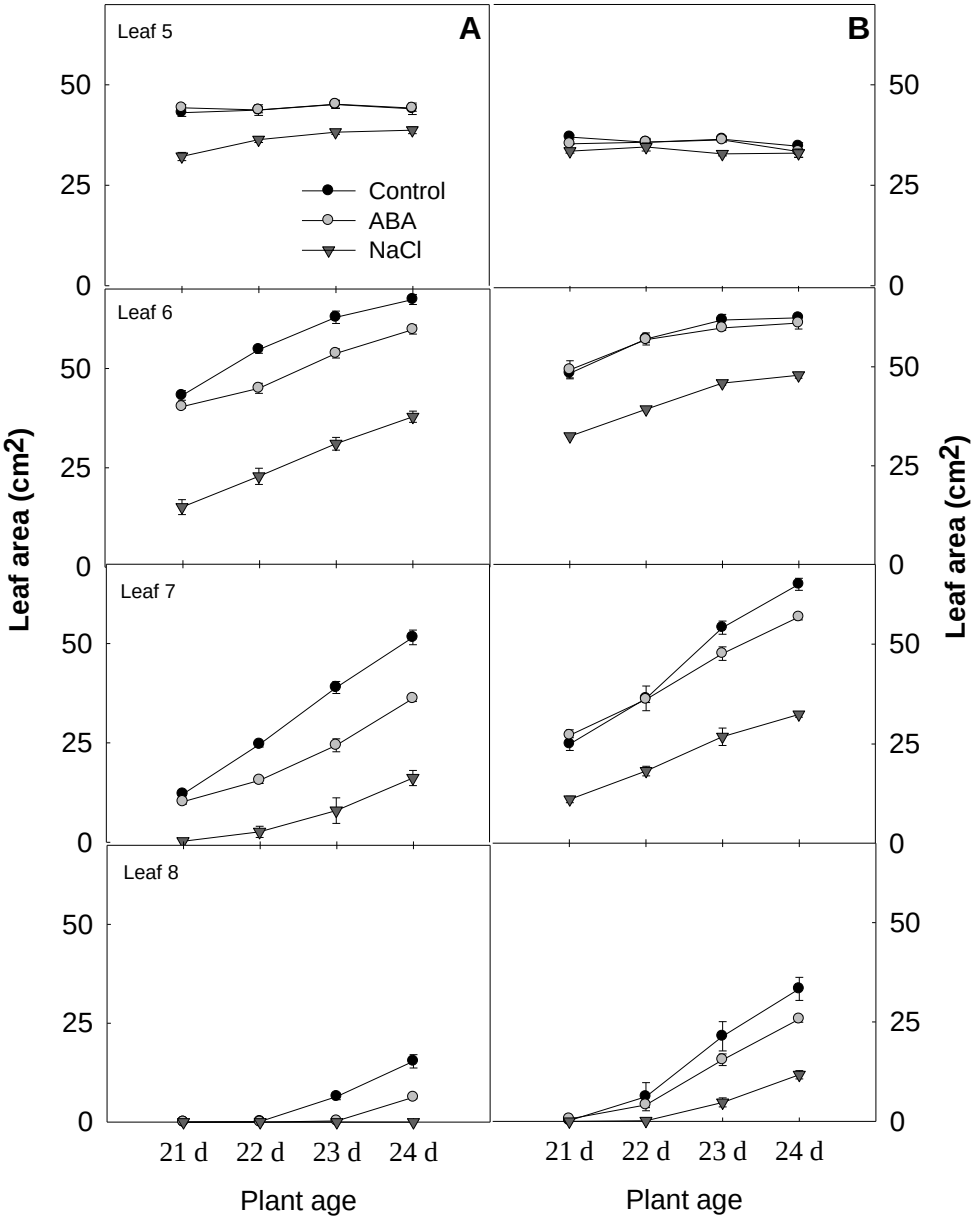

Figure 11: Effect of salt stress and ABA on leaf area expansion of Pioneer 3906 (A) and SR 03 (B). Salt stress was applied as 100 mM NaCl for 8 d, and for ABA treatment, 10 µM ABA were supplied in the nutrient medium for 3 d. Data represent the daily leaf area measurements of 5th to 8th leaves, starting with the application of ABA (d 21). The values are means ± SE of four independent experiments.

In contrast, leaf growth inhibition by salt stress was less prominent in SR 03. On d 24, salt stress-induced relative reduction in area of leaf 6 and 7 of SR 03 plants was 23% and 50%, respectively. Supply of ABA in nutrient medium inhibited the growth of only young leaves, identical to the first phase of salt stress. ABA predominantly reduced the growth of leaves 6 to 8 in Pioneer 3906 by 11%, 30% and 59%, respectively. In contrast, ABA-induced inhibition of growth in SR 03 was only obvious in leaves 7 and 8. In comparison to control, ABA reduced the area of leaves 7 and 8 by 13% and 23%, respectively. There were clear genotypic differences for ABA-induced growth inhibition, with Pioneer 3906 the more sensitive as compared to SR 03.

3.4.3 Salt stress and abscisic acid-induced changes in leaf apoplast pH

The leaf apoplast pH was measured by recording the fluorescence of pH-sensitive dye, infiltrated in leaves. To measure the apoplastic pH values, the 2nd youngest leaf from all treatments was selected, because stress treatments mainly affected the growth of young leaves (Fig. 11). Use of the ratio-imaging technique provides the opportunity to estimate the cell wall pH in intact leaves with high temporal and spatial resolution (Hoffmann *et al*., 1992; Pitann *et al*., 2009). Fluorescent images showed that the dye was accumulated selectively in the leaf apoplast (data not shown). In comparison to control, a significant decrease in acidification of leaf apoplast of 0.2 units was observed in salt-treated plants of Pioneer 3906 (Fig. 13). In contrast, no variation in apoplast pH was found for salt-resistant SR 03 under control and salt stress conditions.

Plants of both genotypes grown in the presence of 50 µM JA, showed no variation in pH values of leaf apoplast (Fig. 12) when compared to control plants. On the other hand, application of 10 µM ABA in the root medium alkalinized the leaf apoplast of Pioneer 3906, and caused an increase of 0.2 units in pH values in comparison to control. In contrast, SR 03 plants showed an opposite response to the application of ABA with a decrease in apoplast pH of almost 0.25 units as compared to control plants (Fig. 13).

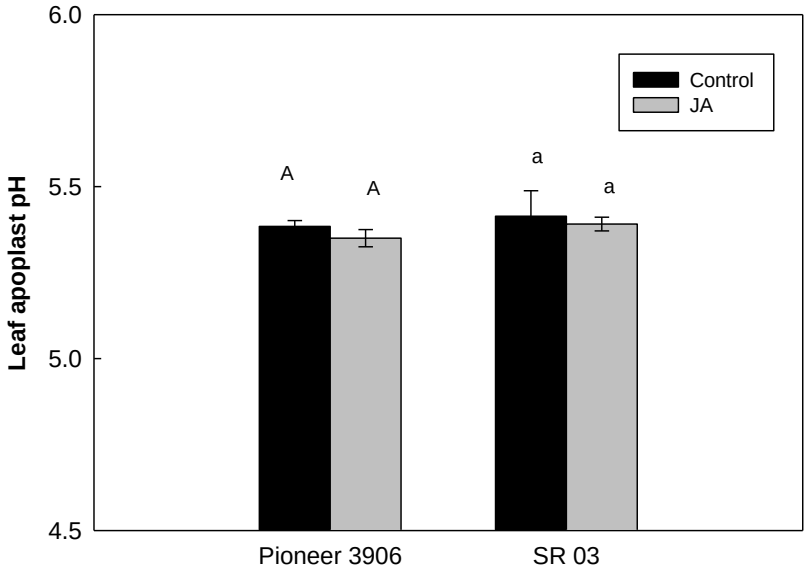

Figure 12: Effect of JA on the apoplast pH of the 2nd youngest leaf of Pioneer 3906 and SR 03. Plants were supplied with or without 50 µM JA in the nutrient medium for 3 d before the pH measurements. Vertical bars represent means ± SE of four independent experiments.

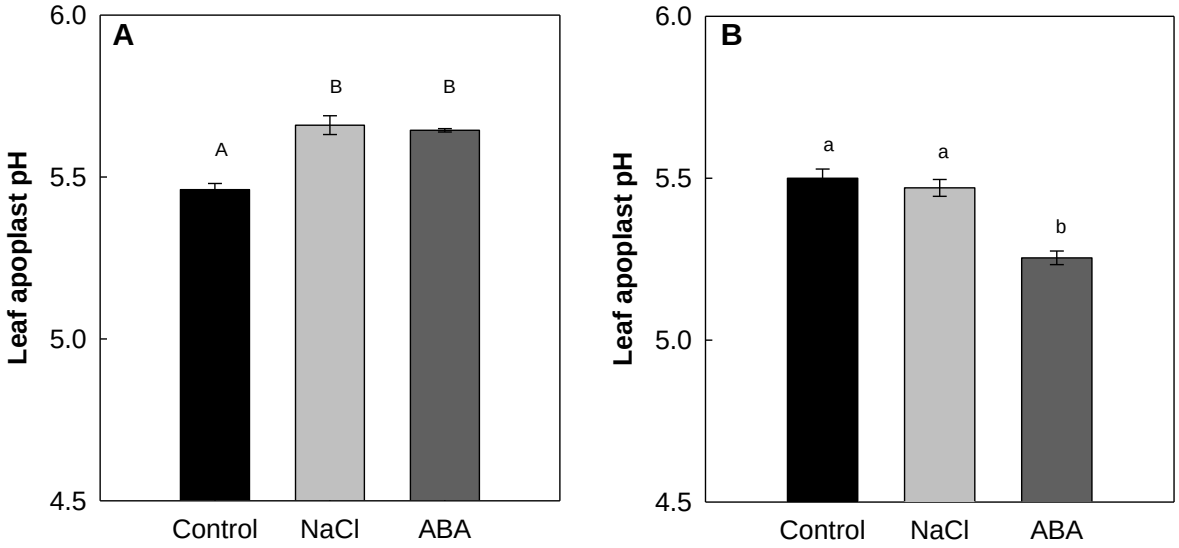

Figure 13: Effect of salt stress and ABA on the apoplast pH of the 2nd youngest leaf of Pioneer 3906 (A) and SR 03 (B). Salt stress was applied as 100 mM NaCl for 8 d, and for ABA treatment, 10 µM ABA were supplied in the nutrient medium for 3 d. pH of leaf apoplast was assessed using fluorescent microscopy, after infiltrating the leaves with 50 µM FITC-dextran. Vertical bars represent means ± SE of four independent experiments. Significant ($p \leq 5\%$) differences between control and stress treatments within each genotype are indicated by different letters.

3.5 Effect of salt stress and abscisic acid on the activity of leaf plasmalemma H^+-ATPase

In order to investigate whether the changes in cell-wall acidification are caused by modifications in H^+-ATPase activity, plasma membranes were isolated from young leaves of two maize genotypes. Plants were cultivated for 24 d and treated with 1 mM NaCl, 100 mM NaCl for 8 d and 10 µM ABA for 3 d before harvest.

3.5.1 Purity of plasmalemma

To avoid an overestimation of the enzyme activity through contamination by other phosphatases, we determined the inhibitor-sensitive ATPase hydrolytic activity. The inhibitor-sensitive ATPase hydrolytic activity of each membrane fraction was calculated by subtracting the ATPase hydrolytic activity in the presence of specific inhibitors from the activity of control (ATPase activity in the absence of inhibitors). ATPase-specific activities (percentage relative to control) in membrane vesicles of plants grown under different treatments are presented in Fig. 14. The ATPase activity of all membrane fractions showed a negligible sensitivity to azide, an inhibitor for the mitochondrial ATPases. The nitrate-sensitive activity of the tonoplast ATPases was slightly increased due to the presence of extra potassium in the form of KNO_3. Overall, all the membrane fractions showed almost 87% sensitivity to vanadate. The membrane fractions of control and salt-treated plants of Pioneer 3906 showed a slight sensitivity to molybdate, which indicates the presence of unspecific acid phosphatases. Similarly, a slight sensitivity to molybdate was also observed in the control and ABA-treated plants of SR 03. Therefore, in all assays of ATPase activity, 1 mM molybdate, 50 mM nitrate, and 1 mM azide were included.

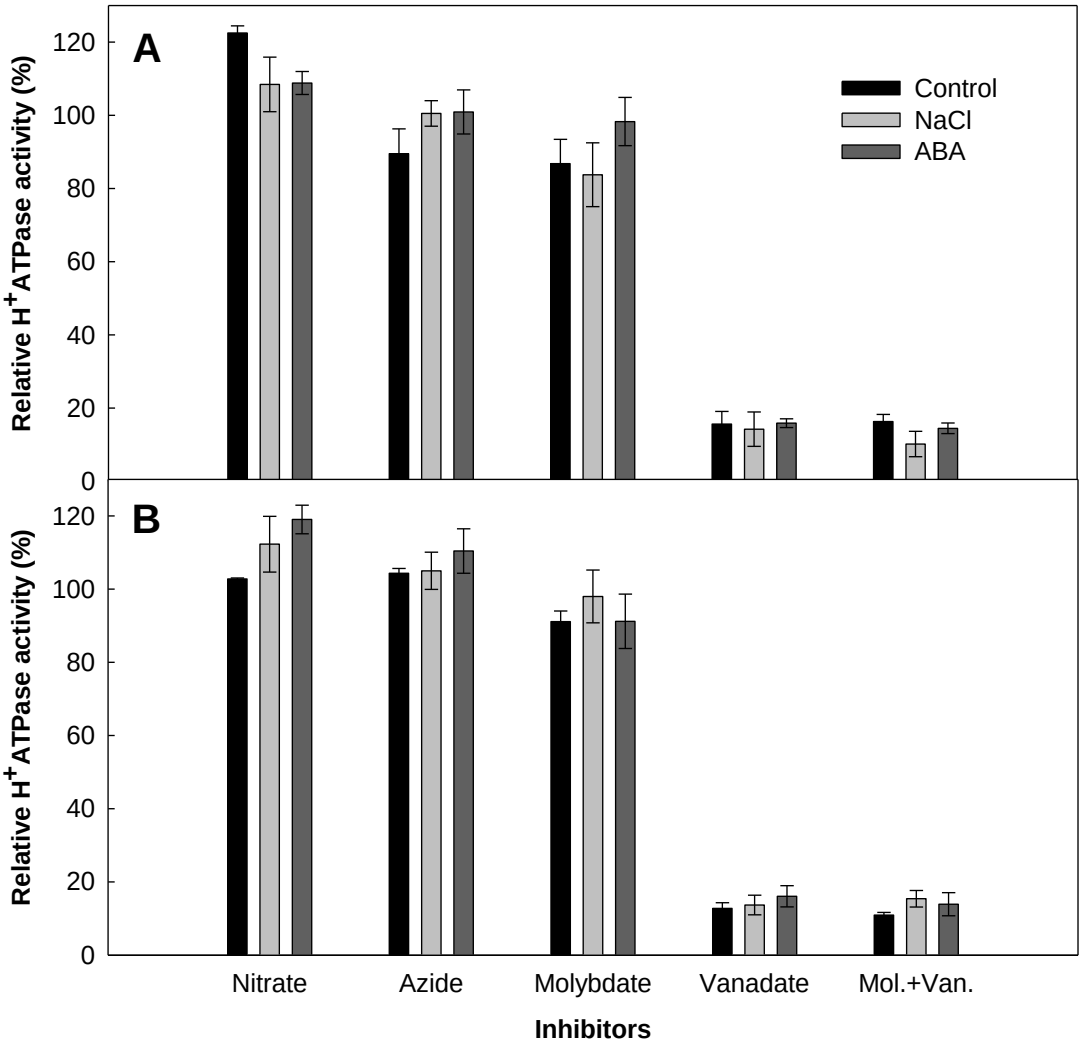

Figure 14: Inhibitor-sensitive ATPase hydrolytic activity associated with plasma membranes of maize genotypes Pioneer 3906 (A) and SR 03 (B). Membranes were isolated from leaves of 24 d old plants treated with 1 mM NaCl (Control) and 100 mM NaCl (NaCl) for 8 d, and with 10 µM ABA (ABA) for 3 d. Assays were conducted at 30°C. The inhibitor-sensitive activity was calculated by subtracting the ATPase hydrolytic activity in the presence of inhibitor from the activity of the control. The values represent means ± SE of three independent experiments.

3.5.2 Effect of abscisic acid and salt stress on the plasmalemma ATPase hydrolytic activity

The hydrolytic activity of H^+ ATPase in membrane vesicles of both genotypes was not significantly affected by salt stress in comparison to control (Tab. 7). The ATPase hydrolytic activity of the membranes isolated from the leaves of ABA-treated plants of Pioneer 3906 was

also not significantly different from that of control plants. On the contrary, plasma membranes of ABA-treated SR 03 plants showed significantly lower ATPase hydrolytic activity when compared to the membranes of control plants of SR 03.

Table 7: H^+ ATPase hydrolytic activity (µmol Pi mg^{-1} min^{-1}) in membrane vesicles isolated from Pioneer 3906 and SR 03 cultivated under control, 100 mM NaCl and 10 µM ABA treatments. Values are means ± SE of four independent experiments. Significant ($p \leq 5\%$) differences between control and stress treatments within each genotype are indicated by different letters.

	Pioneer 3906	**SR 03**
Control	0.39±0.06 A	0.42±0.05 a
NaCl	0.50±0.07 A	0.39±0.03 a
ABA	0.36±0.03 A	0.26±0.04 b

3.5.3 Effect of abscisic acid and salt stress on the plasmalemma ATPase pumping-activity

Proton-pumping activity of plasmalemma vesicles was measured as a decrease in the absorbance (A_{492}) of acridine orange (Fig. 15). H^+ pumping after the addition of Mg-ATP was initially very rapid, which then reached a constant level after 80 min. Compared with control plants, the membrane vesicles isolated from the salt-treated plants of both genotypes showed an identical proton-pumping activity (Fig. 16). The absorbance quenching of AO in membrane vesicles from control plants of Pioneer 3906 was more rapid at the start and attained higher level as compared to ABA-treated plants. On the other hand, absorbance quenching of AO in vesicles of control and ABA-treated plants of SR 03 was not significantly different (Fig. 15).

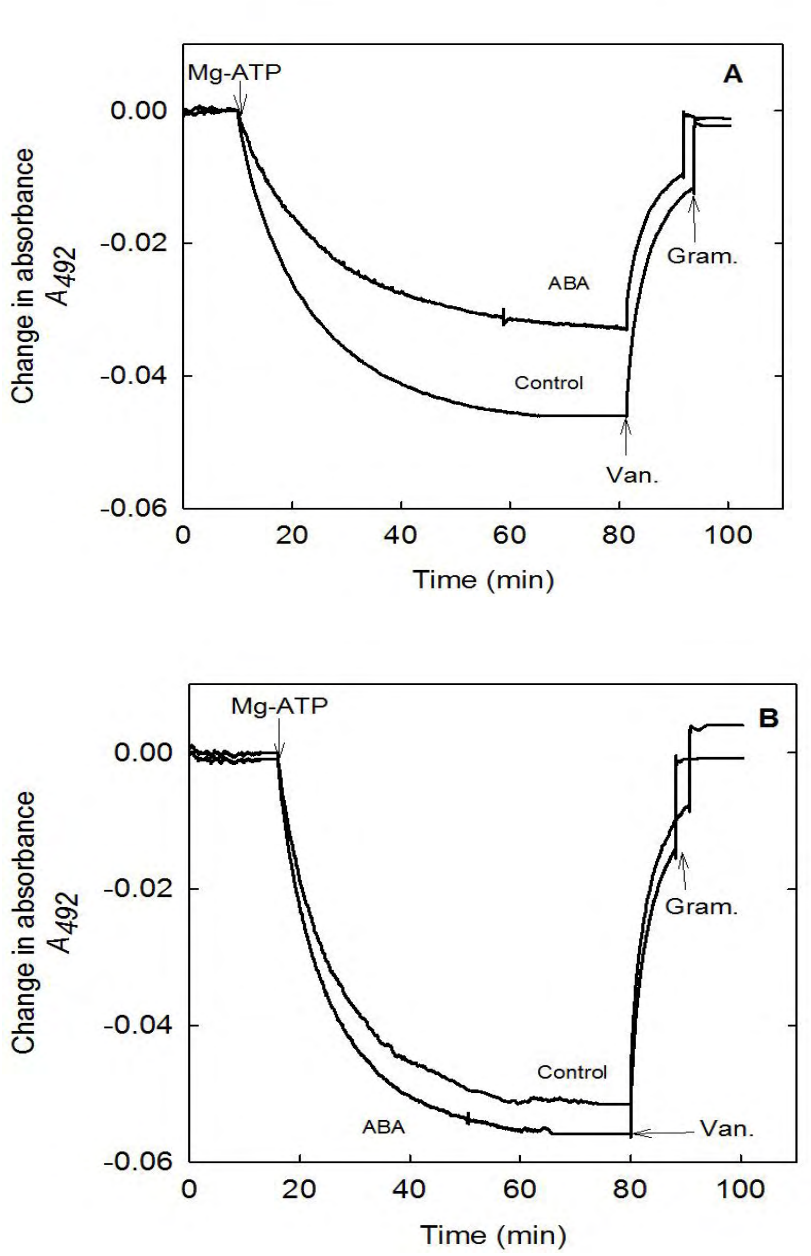

Figure 15: Effect of ABA on active proton transport by plasmalemma H^+-ATPase and passive leakage of protons from membrane vesicles isolated from leaves of Pioneer 3906 (A) and SR 03 (B). Formation of pH gradient in *inside-out* membrane vesicles was monitored as a decrease in A_{492} of AO. Intravesicular acidification was initiated by the addition of Mg-ATP and change in absorbance for 1st min and at equilibrium was calculated as initial rate of active proton pumping and maximum pH gradient, respectively. Pumping activity was stopped by the addition of vanadate and passive efflux of protons from membrane vesicles was compared at identical pH gradients. The established pH gradient was completely collapsed by the addition of gramicidin.

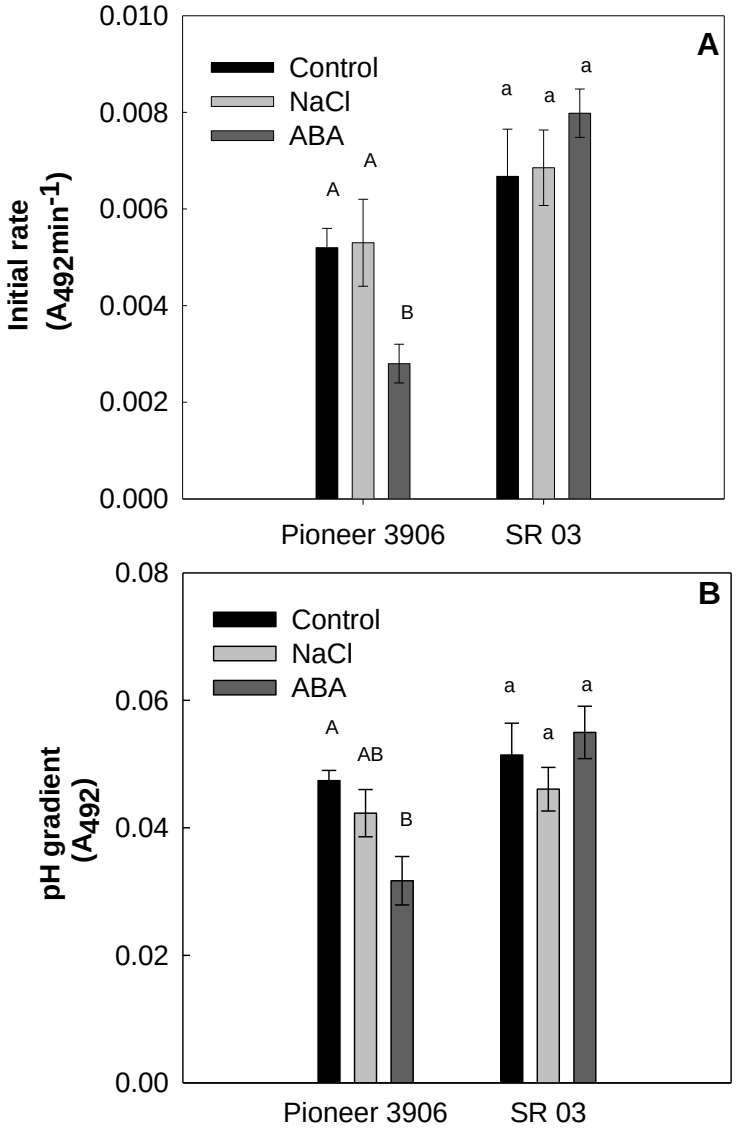

Figure 16: Effect of salinity and ABA on the initial rate of active proton pumping (A) and maximum pH gradient (B) established by plasma membrane H^+-ATPase. Plasmalemma was isolated from leaves of two maize genotypes (Pioneer 3906 and SR 03) cultivated under 100 mM NaCl for 8 d and under 10 µM ABA for 3 d. Assays with 50 µg protein were performed at 25°C. Vertical bars represent means ± SE of four independent experiments. Significant ($p \leq 5\%$) differences between control and stress treatments within each genotype are indicated by different letters.

Pumping activity of plasmalemma H^+-ATPase was determined as the initial rate and maximum pH gradient (Fig. 16). The initial rate and maximum pH gradient in membrane vesicles of both genotypes grown under control and saline conditions were not significantly different. The initial rate of proton pumping in vesicles of ABA-treated Pioneer 3906 was significantly reduced by 46% in comparison to control. Furthermore, the pH gradient established by plasmalemma vesicles of ABA-treated Pioneer 3906 was reduced by 33%. In contrast, no significant difference in the initial rate of active pumping and pH gradient among control and ABA-treated SR 03 was observed.

To determine passive efflux of H^+ from membrane vesicles, H^+ pumping was stopped by adding vanadate which caused a rapid recovery in absorbance. Since the passive efflux of proton depends on the pH gradient; a comparison between the treatments within each genotype was made at the same pH gradient. The results show that passive H^+ efflux in plasmalemma vesicles was not affected in salt and ABA-treated plants of both genotypes (Fig. 17, A). The resting pH gradient was completely collapsed by gramicidin.

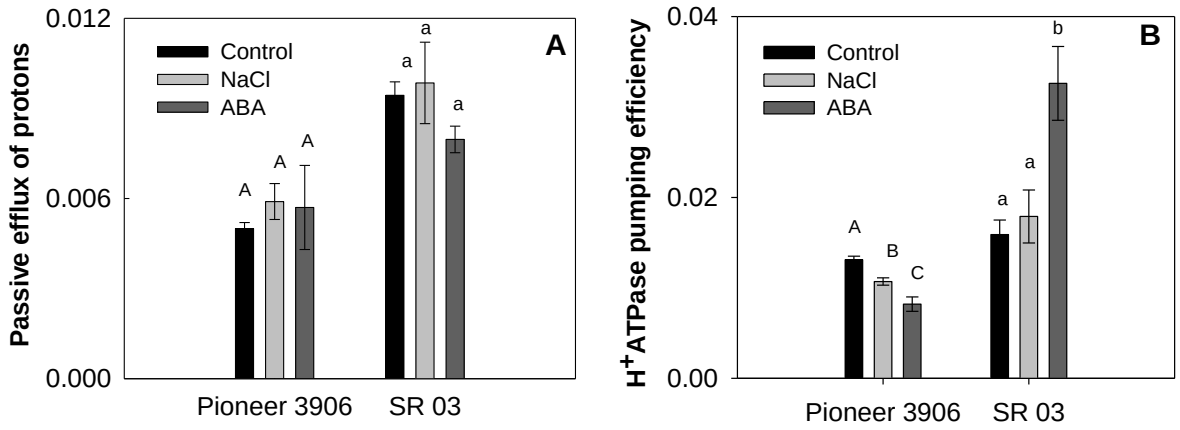

Figure 17: Effect of salinity and ABA on passive leakage of protons from membrane vesicles (A) and proton pumping efficiency (B) of plasma membrane H^+-ATPase. Plasmalemma was isolated from leaves of two maize genotypes (Pioneer 3906 and SR 03) cultivated under 100 mM NaCl for 8 d and under 10 µM ABA for 3 d. Assays with 50 µg protein were performed at 25°C. Vertical bars represent means ± SE of four independent experiments. Significant ($p \leq 5\%$) differences between control and stress treatments within each genotype are indicated by different letters.

Proton pumping efficiency of H^+ATPase was calculated as ratio of initial rate of active proton pumping to hydrolytic activity. This ratio reflects the decrease in absorbance of AO due to number of protons pumped by H^+ATPase per unit of ATP utilized. Pumping efficiency of vesicles isolated from ABA and salt-treated plants of Pioneer 3906 was significantly reduced in comparison to control (Fig. 17, B). On the other hand, in comparison to control, pumping efficiency of plasmalemma H^+-ATPase of SR 03 was not affected by salt stress, but increased by a factor of 2 under ABA treatment.

3.5.4 Effect of *in-vitro* abscisic acid treatment on H^+ATPase hydrolytic and pumping activity

We showed that *in-vivo* application of ABA in the nutrient medium of plants inhibited the H^+ATPase pumping in the salt-sensitive maize genotype and hydrolytic activity in the salt-resistant genotype. In order to elucidate a direct effect of ABA on hydrolytic and pumping activity of H^+ATPase, assays were performed in the presence of ABA at various concentrations. The results show that *in vitro* application of ABA had no significant influence on ATPase hydrolytic activity in membrane vesicles of all treatments (Fig. 18).

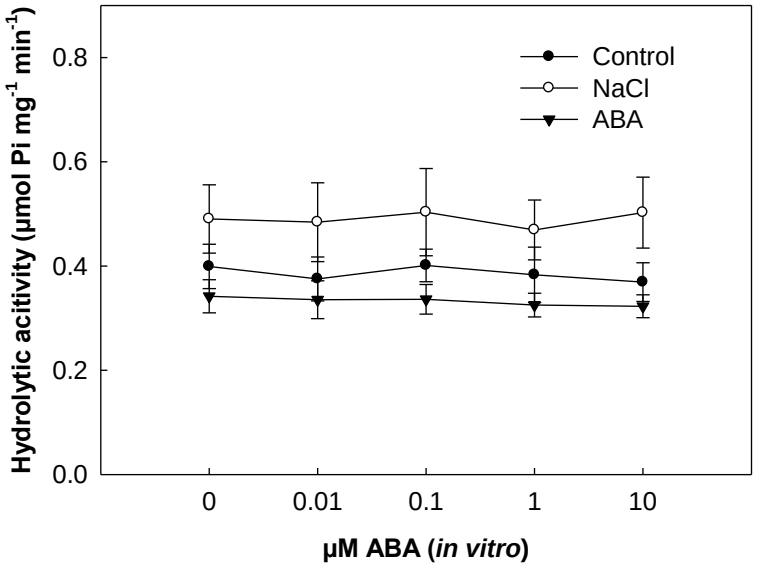

Figure 18: Effect of *in vitro* application of increasing concentrations of ABA on H^+ATPase hydrolytic activity in membrane vesicles of Pioneer 3906 treated either with 1 mM NaCl, 100 mM NaCl or 10 µM ABA. ABA dissolved in ethanol at various concentrations was included in the assay medium and assays were performed at 30°C. Values represent means ± SE of four independent replicates.

Effects of *in vitro* application of ABA on the initial rate of active proton pumping and pH gradient developed by H^+ATPase in membrane vesicles of different treatments are shown in Tab. 8. Addition of ABA in the assay medium did not affect the proton extrusion by plasmalemma H^+-ATPase, which indicates that ABA does not directly inhibit H^+ ATPase activity.

Table 8: Effect of *in vitro* application ABA on the initial rate of active proton pumping (A) and pH gradient (B) established by H^+ATPase in membrane vesicles of Pioneer 3906 and SR 03 cultivated under different treatments. ABA dissolved in ethanol at various concentrations, was included in pumping assays and assays were performed at 25°C. Values represent means ± SE of four independent replicates.

A	**Pioneer 3906**			**SR03**		
	Control	NaCl	ABA	Control	NaCl	ABA
0 µM ABA	0.005±0.000	0.005±0.001	0.003±0.002	0.007±0.001	0.007±0.001	0.008±0.000
0.01 µM ABA	0.006±0.000	0.006±0.000	0.003±0.000	0.007±0.001	0.006±0.000	0.009±0.001
10 µM ABA	0.006±0.001	0.005±0.001	0.003±0.001	0.007±0.002	0.006±0.001	0.008±0.000

B	**Pioneer 3906**			**SR03**		
	Control	NaCl	ABA	Control	NaCl	ABA
0 µM ABA	0.047±0.002	0.042±0.004	0.031± 0.004	0.051±0.005	0.046±0.003	0.055±0.004
0.01 µM ABA	0.046±0.000	0.045±0.005	0.031±0.004	0.051±0.005	0.047±0.003	0.058±0.006
10 µM ABA	0.047±0.002	0.044±0.004	0.031±0.003	0.053±0.005	0.045±0.004	0.056±0.004

3.5.5 Effect of abscisic acid and salt stress on plasmalemma H^+-ATPase enzyme concentration in membrane vesicles isolated from mize leaves

Plasma membrane proteins were separated by SDS-PAGE on 10% acrylamide gel using western blot. A polyclonal antibody raised against central part of the plasmalemma H^+-ATPase was used to detect the differences in enzyme concentration. For a quantitative comparison, the intensity and area of signals was carried out by setting control as 100% in four independent experiments. In comparison to control plants, the band intensities of the plasma membrane H^+-ATPase (Fig. 19) in the membranes of salt and ABA-treated plants of both genotypes were not significantly different.

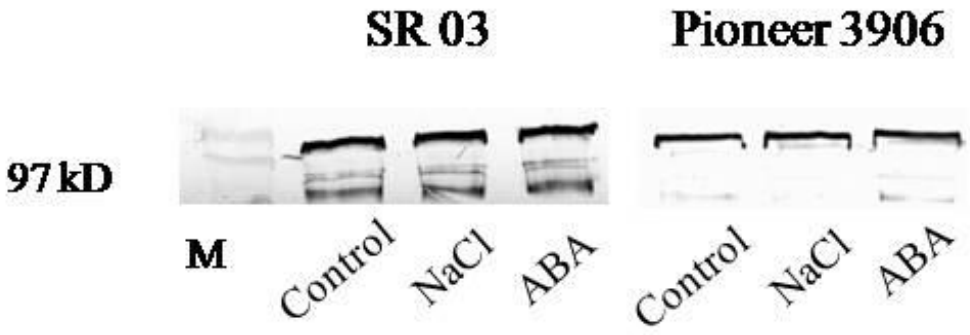

Figure 19: Immuno-detection of plasma membrane H^+-ATPase (97 kD) by Western-blotting. M abbreviates for standard marker of a known molecular mass. For separation of plasmalemma proteins, membrane vesicles (3 μg membrane proteins) were loaded onto polyacrylamide gel. For western-blot analysis, after separation on the gel the membrane proteins were transferred to polyvinylidene difluoride (PVDF) membrane filter. Membrane blots were incubated with a polyclonal antibody raised against the central portion of AHA2 (amino acids 340-650) and visualized with a secondary antibody (alkaline phosphatase-conjugated anti-rabbit IgG, Sigma).

3.6 Effect of salt stress and abscisic acid on the relative mRNA transcription of H^+ATPase isoforms

To investigate the changes in relative transcription of plasmalemma H^+-ATPase isoforms by salt stress and ABA in maize genotypes of varying salt resistance, isoform-specific primers for *MHA1*, *MHA2*, *MHA3*, and *MHA4* were used in RT-PCR analysis. In addition, to investigate the changes in transcription of all isoforms of H^+ATPase in total, family-specific primers were also used. The total RNA was extracted from young leaves of plants treated with 1 mM NaCl, 100 mM NaCl for 8 d or with 10 µM ABA for 3 d. The mRNA was reversely transcribed to cDNA. The gene expression of different isoforms was quantified by means of real-time PCR. The expression of mRNA for control treatment was defined as 1 and mRNA expression of salt stress and ABA-treatments were compared with that of control and defined as relative expression.

Primer-specific amplifications were confirmed with melting-curve analysis and were further verified by running the products on 1% agarose gel after RT-PCR. The amplification of *MHA2* could not be detected in this study. Melting-curves for actin and H^+ATPase isoforms are shown in Fig. 20. There were single-peak melting curves for actin, *MHA1*, *MHA3* and *MHAfam*, indicating the amplification of single gene products that were further confirmed using gel-electrophoresis as shown in Fig. 20 F. *MHA4* showed a double-constant peak irrespective of the treatments and genotypes that may indicate the contamination or the amplification of more than one product due to un specificity of the primers. However, a single distinct band for *MHA4* can be seen on the gel that clearly means that the primers were highly specific for the gene of interest and that the nature of amplified product itself was responsible for double-peak signal. Similarly, no product was amplified in the ntc (no template control; Fig. 20), excluding the possibility of contamination. This double peak persisted even when different annealing temperatures, different primer or cDNA concentrations were tested (data not shown). It has been suggested by *Weis et al*. (2010) that the annealing of sense/sense or antisense/antisense primer strands or the formation of hairpins could be responsible for the observation. These products/hairpins may have lower melting temperatures than the antisense/sense hybrids and may have contributed to the second peak. As the presence of double peak in melting-curve analysis would compromise the accuracy of results, the data for *MHA4* were not subjected to further analysis.

RT-PCR analysis showed that the relative transcripts of *MHA1* were not significantly changed by either salt stress or ABA in both genotypes (Fig. 21). However, in comparison to control plants the mRNA transcript levels of isoform *MHA3* were significantly reduced by salt stress (72%) and similarly by ABA (76%) in salt-sensitive Pioneer 3906. Furthermore, salt stress and ABA treatments also reduced the transcription of *MHAfam* by 50% in Pioneer 3906. On the other hand, both salt stress and ABA treatments did not significantly change the relative mRNA transcription of *MHA3* and *MHAfam* in SR 03 (Fig. 21).

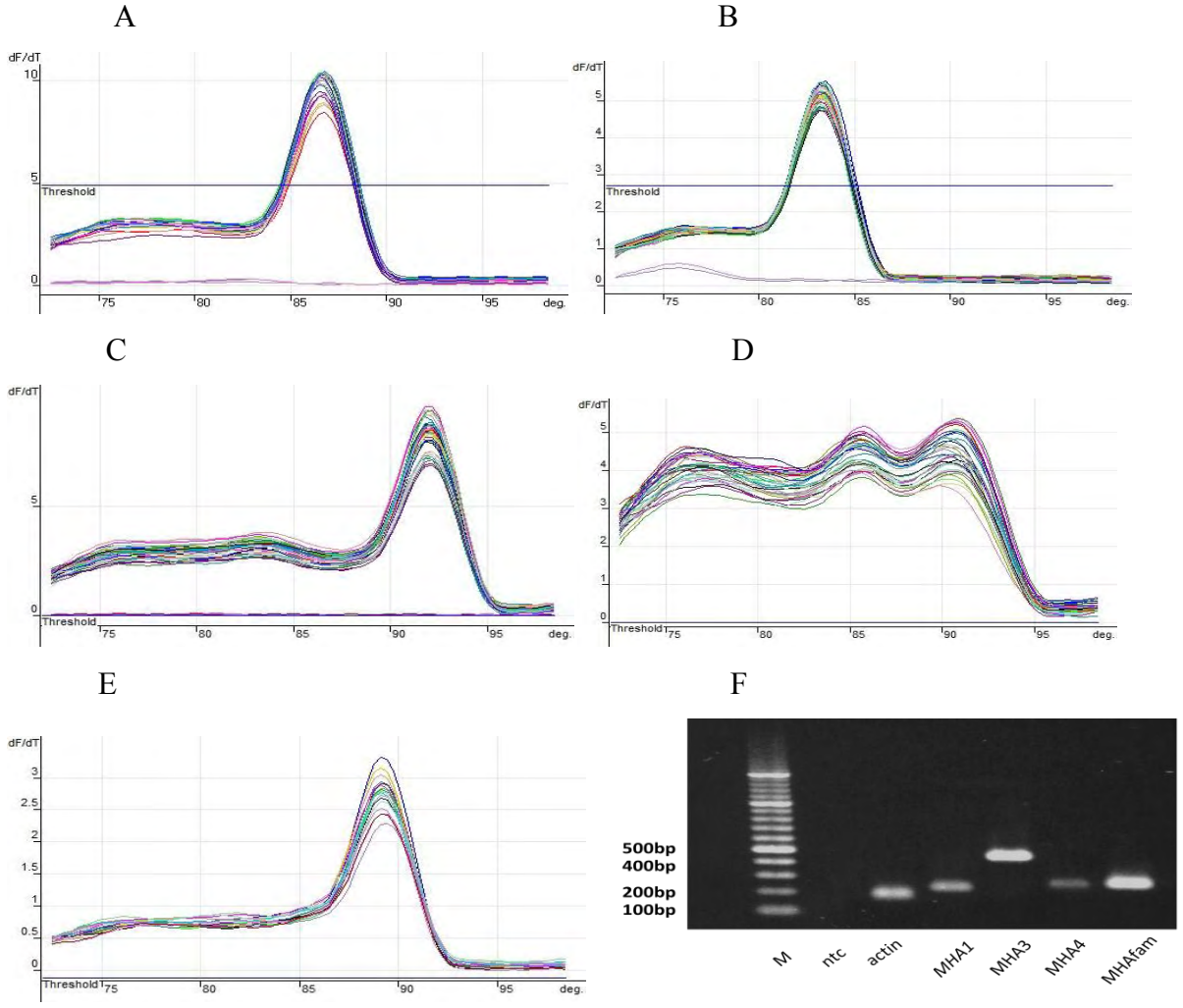

Figure 20: Representative melting-curve analysis for the amplicons of (A) *actin*, (B) *MHA1*, (C) *MHA3*, (D) *MHA4*, and (E) *MHAfam*. After real-time PCR, the reactions were subjected to melting by gradually increasing the temperature from 72-99°C. Gel-electrophoresis of RT-PCR reactions showed single bands for all amplicons (F), confirming the absence of any non-specific amplicon.

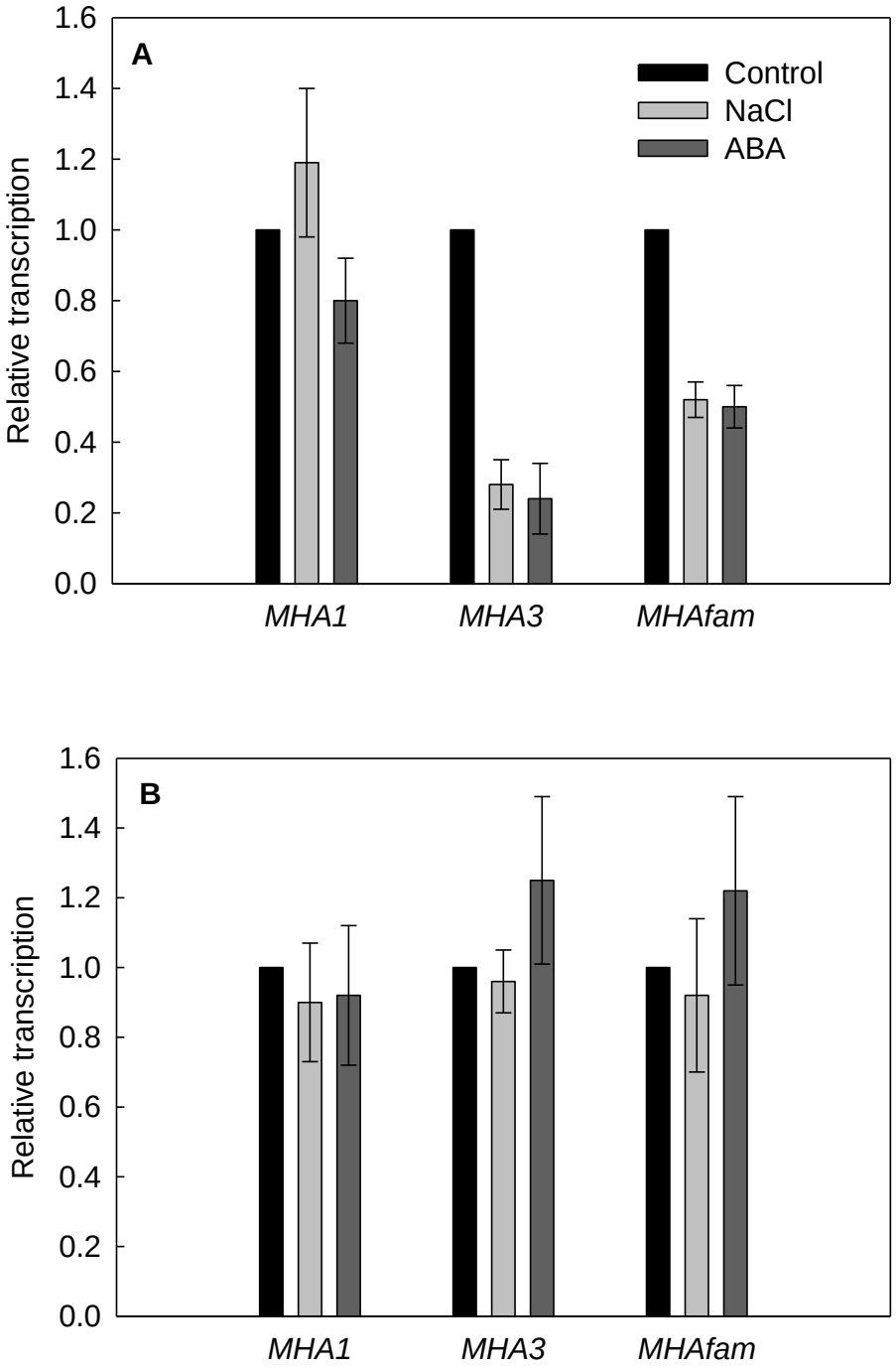

Figure 21: Relative transcription of plasma membrane H^+-ATPase isoforms *MHA1*, *MHA3*, and *MHAfam* in Pioneer 3906 (A) and SR 03 (B). The total RNA was extracted from young leaves of plants treated with 1 mM NaCl, 100 mM NaCl for 8 d or with 10 µM ABA for 3 d. To evaluate the expression of PM H^+-ATPase isoforms and *actin* (internal standard), real-time PCR with specific primers for each gene was performed. Values represent means ± SE of four independent experiments.

4 Discussion

4.1 Jasmonic acid is involved in salt-stress signaling and maize genotypes varying in salt resistance during the first phase of salt stress also differ in jasmonic acid accumulation.

The aim of this study was to compare the relative changes in shoot growth of two maize genotypes during the first phase of salt stress and to identify the possible relationship between JA levels and salt resistance. The results show that shoot growth of maize genotypes was more sensitive than root growth during the first phase of salt stress. Short-term application of NaCl or PEG for 36 h at identical solute potential (-0.5 MPa) inhibited the shoot growth to a similar extent (Tab. 3). These results, together with the observation of absence of any toxicity symptoms on leaves of salt-treated plants, indicate that shoot growth was mainly reduced by osmotic stress. This is in agreement with the bi-phasic model of salt stress (Munns, 1993) that growth during the first phase is predominately inhibited by osmotic stress rather than sodium toxicity. Although slight effects of Na^+ toxicity on leaf expansion by 100 mM NaCl treatment have been shown in maize genotype cv. Pioneer 3906, no significant differences were observed in shoot biomass between NaCl and PEG treatments for 9 d (Sümer *et al*., 2004). Shoot fresh weights were significantly reduced by osmotic stress in Across 8023 but only slightly in SR 03 (Tab. 3). Comparison of genotypes showed that SR 03 was able to maintain better shoot growth than Across 8023 during the first phase of salt stress which indicates that SR 03 is a relatively osmotic-resistant genotype (Fig. 3). Growth differences among maize genotypes of varying salt resistance range only between 10-15% (De Costa *et al*., 2007; Pitann *et al*., 2009), which supports the two-phase model of salt stress that genotypic variations for growth responses during the first phase of salt stress are small.

Leaf growth can possibly be reduced due to reduced uptake of water and a decrease in shoot turgor pressure. Many experiments have confirmed that inhibition of leaf growth often occurs without any change in leaf water relations (Passioura, 1988; Gowing *et al*., 1990). In the present study, application of 100 mM NaCl significantly decreased shoot water and solute potentials (data not shown) but did not affect shoot turgor in both genotypes (Fig. 4), which indicates that the growth differences observed in maize genotypes were not the result of variation in shoot turgor maintenance. Our results are consistent with the observations of De

Costa *et al*. (2007) who showed that the shoot turgor among maize genotypes (irrespective of resistance) was not disturbed in the first phase of salt stress. Munns *et al*. (2000) demonstrated that turgor-induced inhibition of growth, if any, is only transient and recovers within hours. This recovery leads to a new steady low growth rate, which is proposed to be controlled by hormonal signals.

Little is known about the involvement of jasmonic acid (JA) in salt stress-signaling. JA is a potent signal compound that accumulates rapidly and transiently when plants are under biotic and abiotic stress (Lehmann *et al*., 1995) and is involved in a variety of physiological mechanisms (Balbi and Devoto, 2008). In this study, the changes in JA levels in response to osmotic stress in two maize genotypes differing in salt resistance were investigated. Our results show that osmotic stress significantly increased JA levels in root tissues of the salt-sensitive maize genotype Across 8023 (Fig. 5). Both NaCl and PEG at identical solute potential (-0.5 MPa) were effective in JA induction in Across 8023, which indicates that the response was due to osmotic stress. A transient increase of JA in dehydrated barley leaves (Creelman and Mullet, 1995), or after exposure of tomato (Pedranzani *et al*., 2003) and rice (Moons *et al*., 1997) to salinity, and under drought stress of rice (Tani *et al*., 2008) indicates that JA may function as a signal in response to water stress (Balbi and Devoto, 2008). Induction of JA-induced proteins is also well correlated with the endogenous accumulation of JA in barley leaves subjected to osmotic stress (Lehmann *et al*., 1995). These results support the hypothesis that JA signaling is involved in mediating the plant responses during the first phase of salt stress.

On the other hand, root JA concentrations in SR 03 were not affected by osmotic stress, which suggests that the signal transduction in this salt-resistant maize genotype is different from the sensitive one. It may also be possible that JA signaling in SR 03 is switched off through degradation or inactivation by conjugation (Wasternack *et al.,* 1998). The genotypic differences have also been reported for tomato cultivars, where salt stress-induced accumulation of JA was more pronounced in the salt-sensitive cultivar (Pedranzani *et al*., 2003). Similarly, differential accumulation of JA in two genotypes of *Pinus pinaster* Ait. under water and cold stress (Pedranzani *et al*., 2007) suggests that different species and tissue types vary in their responses. Therefore, it can be proposed that differential sensitivity of maize genotypes to osmotic and salt stress can be the result of different patterns of JA

accumulation (Andrade *et al*., 2005). The increased concentrations of root JA in Across 8023 are probably caused by *de novo* synthesis in root tissues. Occurrence of some of the enzymes for JA biosynthesis in roots indicates that roots are able to synthesize JA (Stenzel *et al*., 2008; Hause *et al*., 2000). Changes in JA in roots of other plant species under salt stress such as in tomato (Abdala *et al*., 2003) and rice (Moons *et al*., 1997) have also been found.

The concentrations of JA in shoot tissues of both genotypes were not affected by NaCl and osmotic stress (Fig. 4), which indicates that JA is not directly involved in long-distance signaling of root to shoot. However, the possibilities that jasmonic acid may be involved in root-shoot signaling through its conjugated forms or through a cross talk with other hormones cannot be excluded. The bioactive members of the jasmonate family such as OPDA, MeJA and JA-Ile are also involved in signaling (Wasternack *et al*., 1998) and concentrations of these metabolites are also increased by osmotic stress (Kramell *et al*., 1995). Future analysis of JA metabolites may clarify the nature of the JA signal in root-to-shoot communication of salt stress. Many genes expressed in response to stress are induced by JA alone or in combination with other plant hormones. For example, Lehmann *et al*. (1995) showed that JA and ABA induced similar patterns of proteins in barley leaves under osmotic stress. They suggested that endogenous ABA and JA may interact in signaling and mediation of plant responses to osmotic stress. Induction of the osmotin protein in tobacoo seedlings under osmotic stress is the result of synergistic interaction between JA and ethylene (Xu *et al*., 1994).

Increased levels of JA in the roots of salt-sensitive Across 8023 suggest that JA may increase the sensitivity of plants to salt stress and may inhibit shoot growth. The hypothesis is further supported by the evidence that mutants defective in JA biosynthesis or in JA signaling showed less growth inhibition in response to wounding (Yan *et al*., 2007; Zhang and Turner *et al*., 2008). The results presented in this study show that exogenous application of JA significantly reduced the shoot extension of maize seedlings (Fig. 6, 7). Comparison of the maize seedlings grown in the presence of JA with excised or intact roots confirmed that JA supplied to roots is taken up by roots and can affect shoot growth physiology. These results are in agreement with the studies that also showed that exogenous JA inhibits shoot growth (Yamane *et al*., 1980; Dathe *et al*., 1981; Ueda and Kato, 1982; Cipollini, 2005).

In order to investigate the sensitivity in terms of shoot extension (responsiveness) of a salt-sensitive (Across 8023) and a salt-resistant genotype (SR 03) to JA, seedlings were supplied with increasing concentrations of JA in nutrient medium. Application of JA to roots significantly reduced the rate of shoot extension in a dose-dependent manner. Both genotypes showed a similar pattern of growth inhibition (Fig. 8), which indicates that both genotypes are sensitive to JA. Keeping in view the inhibition of shoot growth by exogenous JA, it is suggested that the reduction in shoot growth of salt-sensitive Across 8023 during the first phase of salt stress may also be caused by endogenous JA. Although, during the first phase of salt stress, increased JA in roots coincided with the reduction of shoot growth of Across 8023 and comparingly better shoot growth of SR 03 also correlated with unaltered JA, the link between root and shoot signaling remaines obscure.

The reduction in shoot growth during the first phase of salt stress is mainly caused by a decrease in cell-wall extensibility and cell elongation. The wall extensibility can be affected by changes in cell turgor, wall-loosening through wall acidification and by changes in the synthesis of wall components (detailed discussion in later chapters). It is assumed that JA-induced inhibition of maize shoot extension in the first phase of salt stress may be due to the effects of JA on these processes. However, shoot turgor remains stable under salt stress and therefore JA does not seem to affect shoot turgor. Furthermore, measurements of leaf-apoplast pH in two maize genotypes after the application of JA for 3 d showed that JA does not affect cell-wall acidification. Our results support the findings of Ueda *et al*. (1994), who showed that the inhibition of auxin-induced elongation of oat coleoptiles by JA is not caused by changes in cell osmoregualtion or by affecting the auxin-induced wall loosening. They found that JA affects sugar metabolism and reduces the synthesis of cell wall polysaccharides.

During the first phase of salt stress Na^+ does not accumulate to toxic levels in plant tissues that could inhibit growth (Munns, 2005) and genotypes with higher capability of Na^+ exclusion exhibit better growth during the second phase of salt stress (Fortmeier and Schubert, 1995). However, better exclusion would help the resistant genotypes to delay the onset of the second phase of salt stress as compared to the sensitive genotypes. The results presented in this study show that the higher Na^+ exclusion from the shoots of SR 03 was due to lower uptake of Na^+ at the root surface and lower root-to-shoot translocation of Na^+ (Fig. 9). These results are in agreement with the observations of Schubert *et al*. (2009) who

described that these two different strategies of Na^+ exclusion are an important trait of the SR hybrids that helps them to avoid Na^+ toxicity in the second phase of salt stress.

Our results further show that treatment with JA of salt-stressed plants decreased Na^+ uptake at the root surface and hence improved Na^+ exclusion from the shoots of both maize genotypes (Fig. 9). Although the shoot Na^+ concentrations in comparison to NaCl were low in NaCl + JA treatment, shoot growth was not improved by JA. These results confirm that shoot Na^+ did not reach toxic levels during the short period of salt stress. However, JA treatment increased the root-to-shoot Na^+ translocation, which indicates that the lower shoot Na^+ in JA + NaCl plants was mainly due to increased Na^+ exclusion at the root surface. Na^+ exclusion at the root surface can be controlled by passive influx through selective cation channels (Tester and Davenport, 2003) or by active efflux of Na^+ from root cells. The active efflux of Na^+ through plasmalemma-localized Na^+/H^+ antiporters has been reported for many plant species (Blumwald *et al*., 2000; Zhu, 2003), a similar mechanism in maize has not been identified. It can therefore be proposed that JA mainly affects the passive influx of Na^+ at the plasma membrane of root cells. A similar response to JA has also been observed in barley (Walia *et al*., 2007) where JA-pretreatment significantly increased the Na^+ exclusion 7 and 14 d after reaching the full stress treatment. These results suggest that the mechanism of Na^+ exclusion starts much earlier in the first phase of salt stress and is mediated by osmotic stress-induced JA.

4.2 Inhibition of leaf growth during the first phase of salt stress is controlled by abscisic and salt resistance in maize genotypes is determined by their sensitivity to abscisic acid.

Several studies have suggested that shoot growth inhibition during the first phase of salt stress and under drought is controlled by root-sourced hormonal signals (Passioura, 1988; Saab and Sharp, 1989; Davies *et al*., 1990; Saab *et al*., 1990; Zhang and Davies, 1990; Munns and Sharp, 1993; Cramer *et al*., 1998). However, the exact mechanism of growth inhibition by root-sourced hormone signals is not known. Salt stress changes the concentrations of stress hormones such as ABA, JA and ethylene. It is likely to assume that shoot growth during the first phase of salt stress may be controlled by more than one hormone signal or by a crosstalk between different signaling pathways.

Leaf expansion is very sensitive to salt stress (Dale, 1988) and leaf area and shoot fresh weights are suitable parameters to identify salt resistance in maize genotypes during the first phase of salt stress (Schubert *et al*., 2009). Our results show that the application of 100 mM NaCl for 8 d strongly reduced the expansion of young developing leaves of both genotypes (Fig. 10). Reduction in leaf area was more pronounced in Poineer 3906 as compared to SR 03 indicating that the latter is a relatively resistant genotype. Similarly, SR 03 produced more shoot fresh weight as compared to Pioneer 3906 (Tab. 6). These results are in agreement with the findings of De Costa *et al*. (2007) and Pitann *et al*. (2009). They demonstrated that shoot osmotic adjustment and maintenance of cell-wall acidification contribute to the resistance of SR 03 during the first phase of salt stress.

Salt and water stress stimulate the biosynthesis and availability of ABA in root tissues (Wilkinson and Davies, 2002) which is then translocated to shoot tissues through xylem and acts as a signal (Hartung *et al*., 2002). The concept of growth control by ABA is based on the observations that the decrease in leaf elongation is strongly correlated with the increase in endogenous ABA under water-deficit conditions and that ABA applied exogenously has also similar effects on leaf growth as drought or salt stress (Zhang and Davies, 1990). Patterns of gene expression in roots and leaf sheath of rice in response to drought, salinity and ABA treatments to roots also indicate that ABA is involved in chemical signaling between root and shoot under water stress (Claes *et al*., 1990).

To elucidate if inhibition of leaf expansion is caused by increased concentrations of ABA in leaves of plants under salt stress, we first tested maize seedlings growth in the presence of ABA. The results show that ABA application to roots can strongly inhibit shoot extension (Fig. 6), which indicates that ABA is a negative regulator of leaf growth. The reduction in leaf expansion is often inversely correlated with the ABA produced internally under salt stress or applied externally under well watered conditions (Cramer *et al*., 1994; 1998). In contradiction to the growth inhibitory role of ABA under drought or salt stress (Cramer *et al*., 1998; Dodd and Davies, 1996), ABA has also been shown to resume or promote leaf growth during water stress. Fricke *et al*. (2004) suggested that the inhibition of leaf elongation immediately after the imposition of 100 mM NaCl stress is resumed through increased concentrations of ABA which have caused the transpiration to decrease and the xylem water potential to rise. The initial growth recovery due to turgor maintenance and osmotic adjustment may be mediated by ABA, but do not explain the steady state lower growth rates over periods of days after salt stress (Munns *et al*., 2000). Moreover, ABA may promote shoot growth under water stress conditions by limiting the ethylene production in leaves which otherwise suppresses growth (Sharp and LeNoble, 2002). The broad spectrum of the actions of ABA ranges from gene expression and enzyme synthesis at the cell level to stomatal conductance at the organ level and to the root-shoot signaling of growth control at the whole-plant level (Tardieu *et al*., 2010). Many of the actions are exerted by ABA alone or by an interaction with other signals such as pH or by an interaction with hormones such as ethylene. Keeping in mind the involvement of ABA in multiple physiological processes, a contradiction of the growth inhibitory or growth promoting effect of ABA can be expected (Tardieu *et al*., 2010).

In an experiment with maize genotypes differing in salt resistance, Cramer and Quarrie (2002) observed that the genotypes producing less ABA under salt stress showed more growth inhibition to salt stress and to ABA applied in the root medium. They suggested that increased sensitivity of some maize genotypes to salt stress is related to ABA sensitivity. In a previous study, it was shown that the salt-resistant SR hybrids accumulated more ABA in leaf tissues than the salt-sensitive Pioneer 3906 during the first phase of salt stress (De Costa *et al*., 2007). These results may indicate that the lower amounts of ABA in salt-sensitive genotypes were insufficient to suppress the extra ethylene which is inhibitory to shoot growth under salt stress. However, growth inhibition by ABA under non-stress conditions (Cramer and Quarrie, 2002) when ethylene levels are low, do not support this notion.

The differential inhibition of leaf extension growth by increased ABA under salt stress may be due to a change in sensitivity to ABA. To test the hypothesis that the differences in leaf growth in maize genotypes during the first phase of salt stress are related to ABA sensitivity, the effects of ABA on the leaf growth of two genotypes were compared. The results show that the application of ABA inhibited the expansion of young growing leaves, similar to the effects of the first phase of salt stress. Further, the ABA-induced reduction in leaf growth was less prominent in SR 03 as compared to Pioneer 3906 (Fig. 10). These results altogether suggest that leaf growth inhibition during the first phase of salt stress is controlled by ABA and the differential sensitivity to ABA determines the resistance of maize genotypes. The sensitivity to ABA among the genotypes may be due to a modification in the signal transduction pathway or the presence of receptors.

4.3 Abscisic acid inhibits proton pumping of plasmalemma H^+-ATPase by down-regulating the efficient isoform and causes a decrease of cell-wall acidification in leaves of a salt-sensitive maize genotype during the first phase of salt stress.

The aim of this work was to identify the nature of the signal that differentially regulates the apoplast acidification in maize genotypes during the first phase of salt stress. The results presented here show that the imposition of salt stress reduced the apoplast acidification in growing leaves of Pioneer 3906 (Fig. 12). Several environmental factors such as drought (Van Volkenburgh and Boyer, 1985) and cold stress (Kumon and Suda *et al*., 1985) alter cell-wall acidification in plants. A decrease in cell-wall acidification is often observed in leaves of plants under water stress (Hartung *et al*., 1988; Wilkinson and Davies, 2008). In contrast, Neves-Piestun and Bernstein (2001) were not able to detect changes in leaf apoplast pH in salt-stressed maize.

In comparison to Pioneer 3906, leaf-apoplast pH of SR 03 was not affected by salt stress and was identical to that of control plants (Fig. 12). Similarly, leaf-apoplast pH in leaves of moderately salt-resistant sugar beet was remained unaltered during the first phase of salt stress (Wakeel *et al*., 2010). These results suggest that the decrease in the rate of leaf cell-wall acidification is not a general plant response under salt stress and is variable among different plant genotypes. Sharp and Davies (2009) have recently compared the effects of drought stress on sap/apoplast pH reported in literature with the changes in apoplast pH in 22 perennial species under drought stress. They observed that water stress-induced alterations in leaf-apoplast pH is not universal and found that the regulation of alkalinization depends on the natural ability of plant species.

The results reported here confirm the findings of Pitann *et al*. (2009) that salt stress differently affects the cell-wall acidification in maize genotypes, but the signaling mechanisms that control this process were not known. Moreover, it was also not clear how modifications in that signaling pathway cause differential acidification of leaf apoplast in maize genotypes differing in salt resistance. It has already been demonstrated that leaf growth under salt stress is controlled by hormonal signals and maize genotypes differ in accumulation of the stress

hormone ABA (De Costa *et al*., 2007). It was therefore proposed that the acidification of leaf apoplast in response to salt stress is controlled by these hormonal signals.

Growing leaves of Pioneer 3906 responded to the addition of ABA in the root medium, which prevented leaf-apoplast acidification (Fig. 12). This ABA effect on cell-wall acidification is similar to that of salt stress which suggests that ABA-signaling is involved in the alteration of leaf apoplastic pH in Pioneer 3906 under salt stress. ABA-induced inhibition of wall acidification has also been reported in isolated protoplasts of rape leaves (Schubert and Matzke, 1985) and in rice leaves (Chen and Kao, 1988). Furthermore, the blue light-dependent apoplast acidification of leaf cells of pea (Desiree den Os *et al*., 2007) and of guard cells of *Arabidopsis* and *Vicia faba* (Goh *et al*., 1996; Brault *et al*., 2004) is also inhibited by ABA-signaling. In contrast, ABA-induced inhibition of leaf apoplast acidification was not observed in SR 03 (Fig. 12), which coincides with the maintenance of wall acidification during the first phase of salt stress. Instead, ABA treatment increased the wall acidification in leaves of SR 03. These results suggest that ABA controls the salt stress-induced acidification of cell walls and that modification in ABA signaling enables SR 03 to maintain a low leaf apoplast pH under salt stress.

Leaf apoplast is a thin film and contains various inorganic and organic ions which may also influence the pH (Felle *et al*., 2005). An increase in leaf-apoplast pH by applying NaCl or ABA to roots can also occur, which is transient in nature (few minutes) and is caused by changes in ion distribution (Felle *et al*., 2005). These short term changes in apoplast pH are not related to drought or salt-adapted plants (Wilkinson and Davies, 2008; Pitann *et al*., 2009) and should be distinguished from long-term stress responses. Salt stress-induced decrease in wall acidification in Pioneer 3906 was associated with the reduction in proton pumping by plasmalemma H^+-ATPase (Zörb *et al*., 2005; Pitann *et al*., 2009; Hatzig *et al*., 2010). Inhibition of PM H^+-ATPase by using vanadate also results in lower wall acidification (Jia and Davies, 2007). It was therefore hypothesized that ABA-induced lack of leaf-apoplast acidification may also be caused by the inhibition of H^+-ATPase activity.

To investigate the H^+-ATPase activity in membrane vesicles, it is important to isolate pure plasma membrane fractions. In the current study, membranes isolated from Pioneer 3906 and

SR 03 plants grown under control and stress treatments showed high sensitivity to vanadate (almost 87%, Fig. 13) and complete insensitivity to azide and nitrate. This indicates that the plasma membrane fractions were free of mitochondrial and tonoplast membranes. However, ATP hydrolytic activity in membrane fractions of control and salt-treated plants of Pioneer 3906 showed a slight sensitivity to molybdate (Fig. 13), which indicates the presence of unspecific acid phosphatases (Widell and Larsson, 1990). This suggests that the isolated membrane fractions can be considered as plasma membranes with little or no contamination of acid phosphatases. Addition of molybdate in ATPase assays to suppress the phosphatase-related activity excludes the contamination effect on H^+-ATPase activity.

Plasmalemma H^+-ATPase has been shown to be involved in plant adaptations to high salinities particularly in halophytes. These studies preferentially investigated the enzyme activities in roots (Braun *et al*., 1986; Janicka-Russak *et al*., 2007) or callus cultures (Niu *et al*., 1993) and showed increased *in vivo* hydrolytic and pumping activities. Since these cells come into direct contact with the dissolved salts in external medium, an increased H^+-ATPase activity may be required to exclude the toxic Na^+ ions. Na^+ efflux out of cells through Na^+/H^+ antiporters is energized by the pH gradient established by plasmalemma H^+-ATPase (Palmgren, 2001). A decrease in root plasmalemma H^+-ATPase activity has been observed for glycophytes such as wheat associated with a reduced Na^+/H^+ antiport activity (Mansour *et al*., 2000). Such an H^+/Na^+ antiport activity could not be proved so far in the plasmalemma of maize. Similarly, plasmalemma H^+-ATPase activity in root tissues of maize (cv. Pioneer 3906) was also found not affected by salt stress (Fortmeier, 2000 and Schubert, 1990).

Recent investigations have demonstrated that proton pumping by plasmalemma H^+-ATPase in young expanding leaves of Pioneer 3906 is significantly reduced by salt stress (Zörb *et al*., 2005; Pitann *et al*., 2009; Hatzig *et al*., 2010). Both the initial rate of active proton pumping and maximum pH gradient were reduced by 45%. These findings support the conclusions of Bogoslavsky and Neumann (1998) who suggested that growth inhibition in expanding leaves of maize under water stress is caused by an inactivation of plasmalemma H^+-ATPase. These results are in agreement with the salt stress-induced decrease in cell-wall acidification observed in the current study. Despite reduced proton pumping, the ATP hydrolysis as well as total enzyme density in the membrane fraction were not significantly affected by salt stress (Zörb *et al*., 2005). The authors found that the decrease in proton extrusion into the leaf

apoplast is well correlated with a decrease in H^+-ATPase proton pumping *in vitro* but not with the hydrolytic activity and total enzyme amount, suggesting a decreased H^+/ATP coupling ratio. Proton pumping in membrane vesicles of the relatively resistant SR 03 was not changed by salt stress (Fig. 15). These results together with the observation that cell-wall acidification remained unaltered in salt-stressed SR 03 suggest an adaptation mechanism in the resistant genotype. A similar adaptive response has also been demonstrated for the moderate halophyte sugar beet (Wakeel *et al*., 2010) where plasmalemma H^+-ATPase hydrolytic and pumping activities in young expanding leaves were not affected by salt stress.

Results of the present study are consistent with previous findings that salt stress induces a lack of leaf-apoplast acidification by inhibiting the H^+-ATPase proton pumping in a salt-sensitive maize genotype. Salt stress was not able to cause such effects in the salt-resistant maize genotypes and in sugar beet indicating a change in the signaling pathway that controls the H^+-ATPase activity. Therefore, identification of the components that target the pump itself or of the signaling pathway that controls its activity is necessary to gain further understanding of the mechanisms of salt-induced differential acidification in maize genotypes. In this context, our results indicate that salt stress-induced changes in cell-wall acidification are controlled by ABA.

In comparison to control, the proton pumping in vesicles of ABA-treated plants of Pioneer 3906 (Fig. 14A) was significantly reduced. Inhibition of the proton pumping by ABA was only partial and the maximum inhibition of initial rate and pH gradient was 46% and 33%, respectively (Fig. 15). This indicates the incomplete nature of ABA-induced inhibition of H^+-ATPase activity. ABA-induced inhibition of proton pumping is consistent with the ABA-induced inhibition of apoplast acidification which indicates that apoplast pH was increased mainly by decreasing the proton release. In addition, there was no difference between control and ABA-treated plants of Pioneer 3906 in the passive efflux of protons (Fig. 16A) indicating that the lower pH gradient was not due to an increase in membrane permeability. A similar inhibition of proton pumping by ABA treatment could not be observed in membrane vesicles of salt-resistant SR 03 suggesting an ABA insensitivity of the enzyme in this maize genotype. In contrast to Pioneer 3906, proton pumping in the membrane vesicles of ABA-treated plants of salt-resistant SR 03 was slightly increased, but was not significantly different from that of control plants (Fig. 14B). These results are consistent with the maintenance of proton

pumping in vesicles and leaf cell-wall acidification of salt-treated plants of SR 03 indicating a modification in ABA signaling that controls the enzyme activity.

The question arises by which mechanism ABA inhibits H^+-ATPase proton pumping in Pioneer 3906. The lower proton-pumping activity of the plasmalemma H^+-ATPase observed in the leaves of the salt-sensitive maize genotype under ABA and salt treatments may be due to a lower gene expression, lower protein synthesis or because of a modification in the functionality of the enzyme (Sze *et al*., 1999). It is well known that H^+-ATPase activity at the enzyme level is regulated by an auto-inhibitory domain at its C-terminal region (Palmgren et al., 1991). Displacement of the auto-inhibitory domain upon phosphorylation and the subsequent binding of the 14-3-3 proteins activate the H^+-ATPase (Fuglsang *et al*., 1999). A change in phosphorylation of the enzyme by stress treatments such as Al (Shen *et al*., 2005) or P-deficiency (Yan *et al*., 2002) affects proton pumping. ABA has been shown to interact with blue-light signaling and inhibits proton pumping in plasma membranes of guard cells (Goh *et al*., 1996; Brault *et al*., 2004). ABA inhibits proton pumping and causes medium alkalinization in *Arabidopsis* suspension cells by decreasing the phosphorylation of H^+-ATPase (Zhang *et al*., 2004). Our results show that ABA-induced inhibition of proton pumping was accompanied by unchanged ATPase hydrolytic activity, suggesting that the phosphorylation was not limited and therefore not responsible for lower proton transport in membrane vesicles of Pioneer 3906.

Inhibition of blue-light dependent H^+-ATPase activity in guard cells involves ABA-induced changes in cytosolic Ca^{+2} (Brault *et al*., 2004) and hydrogen peroxide levels (Zhang *et al*., 2004). Although ABA treatment decreases the blue light-dependent proton pumping activity by 60% in guard cells of *Vicia faba in vivo*, the *in vitro* enzyme activity remained unchanged in microsomal vesicles isolated from guard cells (Goh *et al*., 1996). In contrast, it was shown that ABA treatment inhibited the proton pumping by H^+-ATPase both *in vivo* and *in vitro*. Furthermore, ABA does not directly inhibit the pump. This is evident from the fact that *in vitro* addition of ABA in the assay medium did not affect ATPase proton pumping (Tab. 8) and hydrolytic activity (Fig. 17). Therefore, the possibility that ABA-induced changes in components of the signaling pathway at physiological level can account for the inhibition of pumping in the current study can be ruled out. These results suggest that the ABA-induced inhibition of proton pumping in Pioneer 3906 is not regulated at the enzyme level.

Immuno-detection of H^+-ATPase protein density in isolated membrane fractions from Pioneer 3906 show that salt stress and ABA treatments did not affect the quantity of total enzyme (Fig. 19), which is consistent with unchanged ATP hydrolysis but in contradiction with lower proton pumping. A decrease in H^+-ATPase pumping efficiency under ABA and salt stress (Fig. 16) confirms the reduced coupling ratio between ATP hydrolysis and proton transport. This raises the question of how this may occur. A possible explanation might be that this ABA-induced low H^+/ATP ratio is due to a modification at the auto-inhibitory domain of the H^+-ATPase protein. The native plasmalemma H^+-ATPases occur in a partially uncoupled state. A post-translational modification is needed to fully activate the pump activity (Palmgren *et al*., 1994). Permanent removal of the auto-inhibitory domain through mutation (Gevaudant *et al*., 2007; Merlot *et al*., 2007) would increase the H^+/ATP coupling ratio that is characterized as increased pumping activity relative to hydrolytic activity. It may be proposed that ABA indirectly targets this auto-inhibitory domain and increases its binding with the C-terminus of the enzyme and hence alter the H^+/ATP coupling ratio in Pioneer 3906. A modification in the binding of the auto-inhibitory domain at the C-terminus of yeast H^+ATPases has also been observed in plasma membranes isolated after glucose treatment (Serrano *et al*., 1983; Portillo *et al*., 1989).

On the other hand, ABA treatment increased the H^+/ATP coupling ratio in SR 03 (Fig. 16B). However, the total enzyme protein in membrane fractions of SR 03 was not affected by ABA (Fig. 19). These results suggest that a similar mechanism of ABA-induced decrease in H^+/ATP coupling observed in Pioneer 3906 does not exist in SR 03. Since the plasma membrane H^+-ATPase belongs to a multi-gene family and comprises several isoforms and data for enzyme activity in membrane vesicles may reflect the contribution of several isoforms, a change in enzyme activity as a whole may not reflect the true picture of regulation (Arango *et al*., 2003).

These isoforms may differ in their expression and have often been shown to have different enzyme kinetics (Morsomme and Boutry, 2000). For example, isoform *AHA3* has a different turnover rate for ATP hydrolysis and different sensitivity to vanadate as compared to *AHA1* and *AHA2* in Arabidopsis (Morsomme and Boutry, 2000). The expression of two isoforms of tobacoo *PMA2* and *PMA4* in yeast revealed functional differences in their activity. Yeast expressing PMA4 was able to grow at pH as low as 4.0 and showed higher proton pumping as

compared to the yeast expressing *PMA2* (Luo *et al*., 1999). It has been suggested by Arango *et al*. (2003) that *PMA4* may be less controlled by its auto-inhibitory domain and hence shows a higher H^+/ATP ratio. So an alternative hypothesis that ABA differently regulates the expression of isoforms with different H^+/ATP ratios in both genotypes can be suggested.

Plant plasmalemma H^+-ATPase is encoded by multiple genes and to date genome sequences of more than twelve isogenes have been identified. Expression of different isoforms can vary according to tissue or cell type and by developmental stage or environmental stimuli (Michelet and Boutry, 1995; Moriau *et al*., 1999; Santi *et al*., 2003, Arango *et al*., 2003). Although various external signals modulate the enzyme activity under *in vivo* and *in vitro* conditions, regulation of the enzyme at the level of translation or transcription is very small (Gaxiola *et al*., 2007). Regulation of H^+-ATPase at transcription level is affected by treatments such as aluminum toxicity (Shen *et al*., 2005), iron deficiency (Santi *et al*., 2005), sugar (Mito *et al*., 1996), ABA, and salt stress (Janicka-Russack *et al*., 2007). A single cell type may contain several isoforms (Gaxiola *et al*., 2007), which may differ in their expression and enzyme kinetics. Until now only four isoforms of PM H^+-ATPase have been reported in maize: *MHA1-4* (Jin and Bennetzen, 1994; Frias *et al*., 1996; Santi *et al*., 2003). In the gene bank published isoforms *MHA1* and *MHA2* represent full DNA and/or mRNA sequences, whereas *MHA3* and *MHA4* have only been partially identified. *MHA1* is only weakly expressed in roots and other tissues (Jin and Bennetzen, 1994), whereas *MHA2* is highly expressed in roots (Frias *et al*., 1996). According to the nucleotide sequences, *MHA1* is placed in subfamily I and the other three belong to subfamily II (Santi *et al*., 2003; Morsomme and Boutry, 2000).

The isoforms of plasmalemma H^+-ATPase have not been completely sequenced, while the possibility of presence of other isoforms of the plasmalemma H^+-ATPase can only be assumed. In *Oryza sativa* and *Arabidopsis thaliana*, genome sequences have confirmed the presence of 10 to 11 different isoforms of the H^+-ATPase, respectively (Baxter *et al*., 2003). The presence of so many isoforms can be explained by the fact that plants require the expression of the enzyme with different qualities and quantities in different tissues (Baxter *et al*., 2003) and need their tight regulation under different developmental or environmental conditions.

To investigate the transcription of isoforms of the plasmalemma H^+-ATPase in maize genotypes Pioneer 3906 and SR 03 grown under control, salt stress and ABA treatments, isoform-specific primers for *MHA1-4* (as reported by Santi *et al*., 2003; Zörb *et al*., 2005) were used for RT-PCR analysis. It was possible to detect primer-specific amplification only for *MHA1*, *MHA3* and *MHAfam* using RT-PCR in both genotypes under all treatments. However, the gene-specific expression of *MHA2* in leaves was not detected, which indicates that it may not be present in the genotypes tested in this study. This assumption is supported by the findings of Santi *et al*. (2003) who were also not able to observe the expression of *MHA2*. In this work, beside isoform-specific primers we also used family-specific primers for H^+ATPas. While using family-specific primers, which should cover the transcription for the entire multi-gene family of the plasmalemma H^+-ATPase, it may not be neglected that there cannot be a universal family-specific primer. The specificity of each isoform of the proton pump to that family-specific primers can be variable in maize genotypes and conditions used in this study and thus provide only a rough estimation of the transcription of these isoforms. The unknown isoforms, if there are any which have not been yet identified in maize, possibly cannot be measured with this family-specific primer.

The data show that the transcription of *MHA1* was not changed in leaves of Pioneer 3906 by ABA and salt treatments (Fig. 21A). On the other hand, transcripts of *MHA3* were significantly reduced by both treatments (Fig. 18A). The down-regulation of *MHA3* coincided with low proton pumping (Fig. 15, 16) and reduced acidification (Fig. 13) of the leaf apoplast in Pioneer 3906 treated with ABA and salt stress. As *MHA3* was highly expressed in comparison to *MHA1* under control conditions (Fig. 20), its down-regulation caused a significant decrease in proton pumping. An identical down-regulation of *MHAfam* also indicates that the *MHA3* is a major isoform in generating the pH gradients across the plasmalemma. In contrast to the down-regulation of *MHA3*, the total amount of enzyme protein was comparable in isolated membranes of control and treated plants (Fig. 19), which indicates the presence of another unknown isoform whose transcripts were increased. These results clearly show that the salt stress-induced ABA acts as a signal and targets the H^+-ATPase transcription. Different isoforms show an overlap expression in plant cells that makes difficult to functionally characterize the single isoform (Arango *et al*., 2003). Furthermore, the over-expression or silencing of an isoform is compensated with down or up-regulation of the enzyme, respectively (Duby and Bounty, 2009). It is concluded that *MHA3* is an efficient

isoform with a high H^+/ATP coupling ratio and its down-regulation was compensated with the up-regulation of an inefficient isoform in leaves of salt-sensitive Pioneer 3906.

On the contrary, expression of *MHA3* and *MHAfam* did not respond to salt treatment and remained unchanged in salt-resistant SR 03 (Fig. 21B). This is also well correlated with the maintenance of cell-wall acidification and *in vitro* proton pumping in that genotype. Analysis of H^+-ATPase isoforms in leaves of SR 03 show that ABA treatment slightly increased the transcripts of *MHAfam* and *MHA3* in comparison to control treatment (Fig. 21B), though the differences were not statistically significant. However, the transcription of *MHA1* was not affected by ABA treatment. The ABA-induced increase in *MHA3* transcription explains the higher rates of wall acidification and higher H^+-pumping efficiency in membrane vesicles of SR 03, on one hand and confirms our proposition that *MHA3* is an efficient isoform, on the other. Since the activities of individual isoforms could not be studied in the current work, the possibility of an indirect ABA-induced modification at the auto-inhibitory domain of *MHA3* that would change the H^+/ATP coupling ratio, can not be excluded. Furthermore, insensitivity of *MHA1* transcription to ABA-induced inhibition in both maize genotypes indicates the implication of distinct signaling pathways for the regulation of these isoforms. ABA has been shown to inactivate *AHA1* in guard cells and cause closure of stomata under drought (Merlot *et al*., 2007). Partial inhibition of the pumping activity by ABA suggests that only certain isoforms respond to ABA and others remain insensitive (Merlot *et al*., 2007). It is therefore concluded that *MHA3* is insensitive to ABA-induced inhibition in SR 03 and plays a key role in the maintenance of leaf cell-wall acidification and contributes to the resistance of SR 03 during the first phase of salt stress.

Plants need to adjust H^+-ATPase activities through tight regulation (Gaxiola *et al*., 2007) to cope with constantly changing environmental conditions. The exact signaling pathway that controls the expression of H^+-ATPase under various physiological and environmental conditions is not clear yet (Duby and Boutry, 2009). The H^+-ATPase is a major protein of the plasmalemma and has various physiological functions that may require more than one regulatory mechanism. The search for ABA receptors and other components of signal transduction has been attempted in numerous studies. Multiple candidate proteins were reported that complicate the understanding of the mechanism (Klingler *et al*., 2010; Umezawa *et al*., 2010). However, recent findings of a novel family of soluble ABA receptors

PYR/PYL/RCAR (Ma *et al*., 2009; Park *et al*., 2009) and the identification of their downstream components PP2C and SnRK2 (Umezawa *et al*., 2010) present a simple core model for ABA perception and signal transduction (Fig. 22).

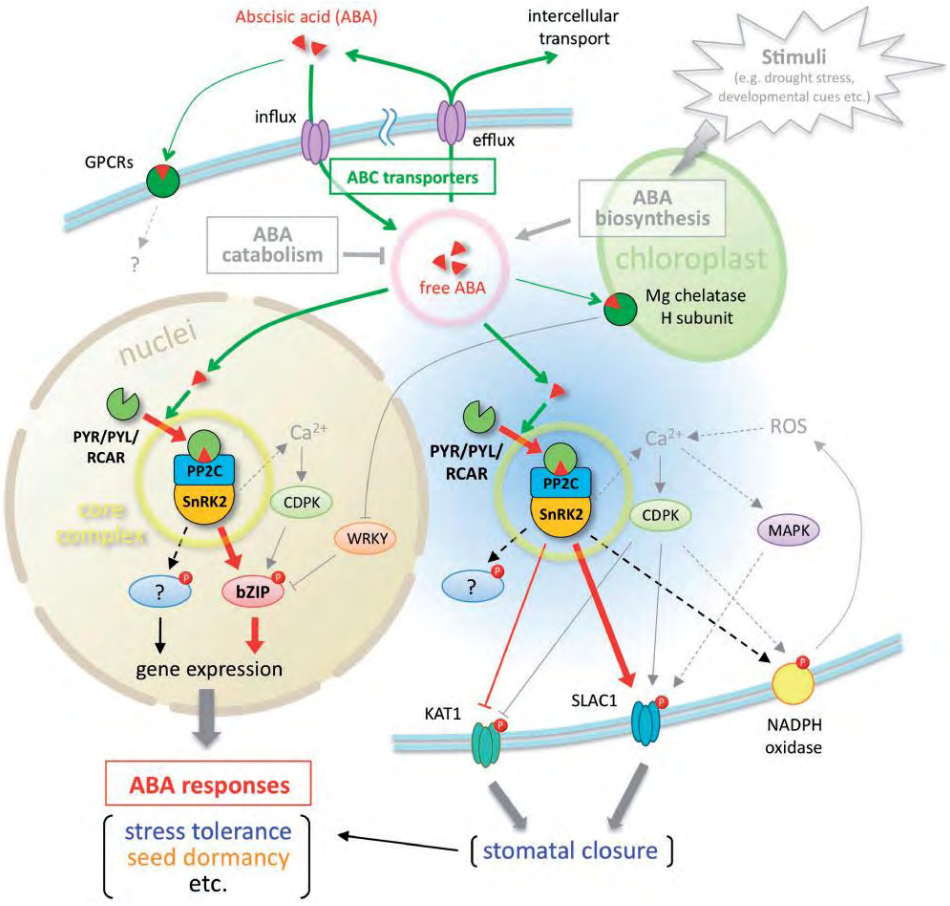

Figure 22: Schematic model for ABA transport, perception and signal transduction (Umezawa *et al*., 2010)

According to this model, the signaling complex can work both in cytosol and nucleus to regulate physiological and transcriptional responses. Screening of several proteins from *Arabidopsis* showed that H^+-ATPase (AHA1/OST2) is one of the downstream target proteins for this signaling complex (Nishimura *et al*., 2010). Moreover, presence of many members of PYR/PYL/RCAR, PP2C, and SnRK2 could give multiple potential combinations for transcriptional regulation (Umezawa *et al*., 2010). These results support our conclusions that *MHA3* is a key target of inhibition by ABA signaling in salt-sensitive Pioneer 3906 during the first phase of salt stress. Modification in the signaling pathway may have caused insensitivity of *MHA3* to ABA-induced inhibition in SR 03.

4.4 Role of cell-wall acidification in salt resistance of maize genotypes during the first phase of salt stress and its relationship with abscisic acid

Inhibition of cell elongation by salt stress is primarily caused by a decrease in cell-wall extensibility, which depends on the loosening of existing cell wall and the synthesis and incorporation of new cell-wall components. According to the acid-growth theory (Hager, 2003), protons secreted by H^+-ATPase decrease the apoplast pH and increase growth. Acidification of the apoplast stimulates the loosening of ionic or covalent bonds between wall polymers and loosens the cell walls. Alterations in cell-wall pH can also affect the activity of cell-wall loosening proteins such as expansins (McQueen-Mason and Cosgrove 1994).

The relationship between leaf growth and wall acidification can be concluded from the studies of auxin-stimulated elongation growth of coleoptiles of oat, maize (Hager, 2003), and pea (Jacobs and Ray, 1976). The growth regulator auxin increases the acidification of cell walls by increasing the ATPase-pumping activity and stimulates the elongation growth. Furthermore, induction of cell elongation by the application of fusicoccin is also associated with increased H^+-ATPase activity (Peters and Felle, 1991; Felle, 1998). It has been suggested that the substances and conditions that increase plasmalemma H^+-ATPase activity also increase elongation growth (Hager, 2003). Conversely, the reduction in H^+-ATPase activity and wall acidification would result in the reduction of elongation growth.

Wilkinson and Davies (2008) demonstrated that growth reduction and stomatal closure were correlated with the application of alkaline buffers (pH 6.4 -7.0) to leaves, similar to responses under stress conditions. A decrease in cell-wall acidification is often observed in leaves of plants under water stress (Hartung *et al*., 1988; Wilkinson and Davies, 1998) and is associated with growth inhibition (Van Volkenburgh and Boyer, 1985). As effects of salt stress during the first phase are almost identical to drought stress, the inhibition of leaf expansion in salt-stressed Pioneer 3906 may also be caused by a decrease in the acidification of leaf apoplast. These findings are consistent with the conclusions of Pitann *et al*. (2009) that the salt stress-induced lack of leaf-apoplast acidification is a one cause of growth reduction in salt-sensitive Pioneer 3906. Moreover, inhibition of leaf expansion in salt-sensitive Pioneer 3906 by ABA also correlates with the ABA-induced decrease in cell-wall acidification. These results lead to

the conclusion that ABA controls the expansion of leaves during the first phase of salt stress by regulating the plasmalemma H^+-ATPase activity.

In comparison to Pioneer 3906, maintenance of H^+-ATPase pumping activity and cell-wall acidification in salt-resistant SR 03 under salt stress suggests that the leaf expansion of this genotype is not limited by cell-wall acidification and it gives an advantage over Pioneer 3906 to show better growth under salt stress. These results demonstrate a role of H^+-ATPase in salt resistance of maize. A role of H^+-ATPase in salt resistance has also been described in transgenic studies. Constitutive activation of *PMA4* in transgenic tobacco plants (C-terminus truncated, ΔPMA4) resulted in increased proton pumping and increased rates of cell-wall acidification relative to untransformed plants (Gevaudant *et al*., 2007). The ΔPMA4 plants grown under normal growth conditions exhibited abnormal growth due to increased rates of cell expansion. Furthermore, better growth performance under saline conditions by plants of ΔPMA4 suggests the involvement of H^+-ATPase in salt resistance. The hypothesis is further supported by the findings of Vitart *et al*. (2001) that disruption of *AHA4* in an Arabidopsis mutant caused a 50% decrease in the transcripts of *AHA4* and increased the sensitivity to salt stress.

Results presented in this study show that in comparison to salt-sensitive Pioneer 3906, cell-wall acidification in leaves of SR 03 is not inhibited by ABA and is therefore responsible for the salt resistance of SR 03 during the first phase of salt stress. Despite maintenance of cell-wall acidification, leaf expansion of SR 03 was still strongly reduced by ABA and salt stress. Additional factors such as cell-wall loosening proteins and the synthesis of cell-wall components have been suggested to limit the extension growth of SR 03 under salt stress (Schubert *et al*., 2009). However, it is not clear whether these factors are also controlled by ABA. ABA may decrease the wall extensibility by affecting the physical properties of cell walls (Van Volkenburgh and Davies, 1983; Kutschera and Schopfer, 1986) and by reducing the hemicellulose deposition in cell walls (Wakabayashi *et al*., 1989; Wakabayashi *et al*., 1991).

5 Summary

Salt stress is a major constraint to crop production. The expansion of young growing leaves is arrested by osmotic problems faced by roots in the first phase of salt stress. The leaf growth of salt-sensitive maize genotype Pioneer 3906 has been shown to be limited due to reduced plasmalemma H^+-ATPase proton pumping into the leaf apoplast. This leads to a lower acidification of the cell wall, which according to the acid growth theory decreases the cell-wall extensibility and cell elongation. However cell-wall acidification of a newly developed maize hybrid SR 03 does not change and therefore accounts for its resistance during the first phase of salt stress. It has been proposed in some studies that the events that limit leaf growth following osmotic stress are controlled by root-sourced signals. Inhibition of leaf growth by salt stress is a complex mechanism and may involve multiple signals. So it was assumed that the salt resistance of SR 03 may involve a signal distinct from that of salt-sensitive ones or differs in pathway of a common signal. The hormonal signal abscisic acid (ABA) accumulates in plant tissues under salt stress and the maize genotypes differing in salt resistance have shown variations in ABA synthesis. In addition there are also some indications that the stress hormone jasmonic acid (JA) also takes part in osmotic stress signaling.

The aims of the current study were to test the hypotheses that I) salt stress stimulates the synthesis of JA in the root and shoot tissues of maize genotypes II) leaf growth and acidification of the leaf apoplast in maize genotypes during the first phase of salt stress are controlled by long-distance signaling of JA and/or ABA; III) differential acidification of the leaf apoplast in maize genotypes in response to salt stress is caused by differential expression of plasmalemma H^+-ATPase isoforms; IV) variation of maize genotypes in salt resistance during the first phase of salt stress depends on the type of hormone signal and/or the sensitivity to these signals.

The results obtained in this study support the following conclusions:

1. Increased concentrations of JA in root tissues of only salt-sensitive Acoss 8023 showed that JA signaling is different in maize genotypes of variable sensitivity during the first phase of salt stress. JA improved Na^+ exclusion at the root surface.

2. The expansion of young leaves was significantly reduced during the first phase of salt stress and the reduction was more pronounced in salt-sensitive Pioneer 3906 as compared to the salt-resistant SR 03. A similar pattern of leaf growth inhibition by exogenous ABA in both maize genotypes supports the hypothesis that leaf growth during the first phase of salt stress is controlled by ABA. In comparison to salt-sensitive Pioneer 3906, leaf expansion of salt-resistant SR 03 was less sensitive to ABA-induced inhibition and is responsible for the resistance of SR 03 during the first phase of salt stress.

3. The decrease in cell-wall acidification due to lower plasmalemma H^+-ATPase proton pumping during the first phase of salt stress is a major cause of reduction in leaf expansion of salt-sensitive Pioneer 3906. The results reported in this study provide evidence that the partial inhibition of plasmalemma H^+-ATPase proton pumping in leaves of Pioneer 3906 under salt stress involves ABA signaling, which down-regulates the transcription of *MHA3*. The data support the conclusion that *MHA3* is an efficient isoform of H^+-ATPase with higher H^+/ATP coupling ratio. Unchanged levels of enzyme concentration in the membranes of the ABA and salt-treated plants show that the ABA-induced down regulation of *MHA3* is compensated with the up-regulation of an unknown inefficient isoform.

4. In contrast, H^+-ATPase proton pumping and cell-wall acidification remained unaffected in leaves of salt-resistant SR 03 during the first phase of salt stress. A differential response of *MHA3* transcription to ABA reduced the sensitivity of SR 03 to ABA-induced inhibition of leaf growth and therefore contributed to the salt resistance of SR 03.

6 Zusammenfassung

Salzstress zählt zu den Umweltfaktoren, welche die landwirtschaftliche Produktion weltweit limitieren. In der ersten Phase des Salzstresses nimmt die Pflanzenwurzel osmotische Probleme im Boden wahr, in dessen Folge es zu einer Einschränkung des Wachstums hauptsächlich der jungen, noch nicht vollständig ausgewachsenen Blätter kommt. So zeigte der salzempfindliche Maisgenotyp Pioneer 3906 ein vermindertes Blattwachstum, welches auf eine reduzierte Pumpaktivität der Plasmalemma-H^+-ATPase und einer damit einhergehenden verminderten Ansäuerung des Blattapoplasten zurückzuführen ist. Ausgehend von der Säurewachstumstheorie ist diese ausbleibende Ansäuerung der Zellwand verantwortlich für eine verminderte Zellwandextensibilität und Zellstreckung. Im Gegensatz dazu konnte der neu entwickelte Maishybrid SR 03 die Zellwandansäuerung während der ersten Phase des Salzstresses aufrechterhalten, was sich in einem verbesserten Blattwachstum zeigte. Dieses scheint daher eine Möglichkeit zur Ausbildung von Salzresistenz zu sein.

In einigen Studien wurde bereits postuliert, dass wurzelbürtige Signale ein wichtiger Vermittler zwischen der Wahrnehmung des osmotischen Stresses und der dadurch vermittelten Hemmung des Blattwachstums darstellen. Für Salzstress ist bekannt, dass die Wachstumshemmung des Sprosses ein komplexer Mechanismus ist, in welchen viele verschiedene Signale involviert sind. Es wird vermutet, dass die bei SR 03 zu beobachtende Salzresistenz einen Signalweg beinhaltet, welcher sich von dem des salzempfindlichen Pioneer 3906 unterscheidet oder aber auf Veränderungen innerhalb der Signalweiterleitung zurückzuführen ist.

Unter abiotischen Stressbedingungen wie Trockenheit oder Salinität spielt in diesem Zusammenhang besonders das Phytohormon Abscisinsäure (ABA) eine wesentliche Rolle. Unter Salzstress beispielsweise unterscheiden sich verschiedene Maisgenotypen in Abhängigkeit von ihrem Resistenzeigenschaften in der Stärke der ABA-Synthese und letztlich der ABA-Akkumulation im Pflanzengewebe. Neben der Abscisinsäure gibt es aber auch Hinweise, dass auch das Stresshormon Jasmonsäure (JA) eine wichtige Rolle in der Signalweiterleitung bei osmotischem Stress spielt.

Vor diesem Hintergrund war das Ziel dieser Arbeit, für verschieden resistente Maisgenotypen zu überprüfen, ob in der ersten Phase von Salzstress:

1. die Synthese von JA in Wurzel- und Sprossgewebe stimuliert wird,
2. das Blattwachstum und die apoplastische Ansäuerung über Langstreckensignale mittels JA und/oder ABA vermittelt werden,
3. Unterschiede in der Ansäuerung des Blattapoplasten auf eine veränderte Expression unterschiedlicher Isoformen der Plasmalemma-H^+-ATPase zurückzuführen sind,
4. Unterschiede in der Salzresistenz von der Art des Signals und der Sensitivität gegenüber diesem Signal abhängen.

Abschließend lassen sich die aus dieser Arbeit erhaltenen Ergebnisse wie folgt zusammenfassen:

1. Eine erhöhte Konzentration an JA im Wurzelgewebe konnte nur für den salzempfindlichen Maisgenotypen Across 8023 festgestellt werden. Hieraus kann abgeleitet werden, dass der JA-Signalweg unterschiedlich in den verschieden resistenten Maisgenotypen in der ersten Phase von Salzstress abläuft. Durch Jasmonsäure kam es zwar zu einer verbesserten Natriumexklusion an der Wurzeloberfläche.

2. Das Wachstum der jungen Blätter aller Maisgenotypen war signifikant vermindert in der ersten Phase des Salzstresses. Dabei war das Sprosswachstum des salzempfindlichen Pioneer 3906 deutlich stärker beeinträchtigt als das des resistenteren SR 03. Durch die exogene Behandlung der beiden Maisgenotypen mit ABA konnte eine ähnliche Wachstumshemmung induziert werden. Dieses unterstützt die Hypothese, dass das Blattwachstum in der ersten Phase des Salzstresses durch ABA kontrolliert wird. Im Gegensatz zu Pioneer 3906 reagierte SR 03 auch hier deutlich weniger empfindlich mit einer Wachstumshemmung auf die ABA-Behandlung, so dass auf eine Beteiligung von ABA an der Ausbildung von Salzresistenz geschlossen werden kann.

3. Die Abnahme der Zellwandansäuerung bedingt durch eine verminderte Pumpaktivität der Plasmalemma-H^+-ATPase ist einer der Hauptursachen für das gehemmte

Blattwachstum bei Pioneer 3906 in der ersten Phase des Salzstresses. Die gezeigten Ergebnisse lassen darauf schließen, dass eine teilweise Hemmung der Pumpaktivität der Plasmalemma-H^+-ATPase in den Blättern von Pioneer 3906 auf eine Beteiligung von ABA zurückzuführen ist. So führte die Applikation von ABA auf Transkriptionsebene zu einer Runterregulation der Isoform *MHA3*. Die vorliegenden Ergebnisse erlauben den Schluss, dass innerhalb der Familie der Plasmalemma H^+-ATPasen bei Mais *MHA3* eine effiziente Isoform darstellt, welche sich durch eine erhöhte H^+/ATP-Kopplungseffizienz auszeichnet. Die unveränderte Enzymkonzentration in der Plasmamembran der mit ABA und Salz behandelten Pflanzen zeigt, dass die ABA-induzierte Runterregulation von *MHA3* in Pioneer 3906 durch die Hochregulation einer unbekannten, ineffizienten Isoform kompensiert werden muss.

4. Im salzresistenteren SR 03 blieben in der ersten Phase des Salzstresses die Pumpaktivität der Plasmalemma-H^+-ATPase sowie die Ansäuerung des Blattapoplasten unbeeinflusst. Durch die Behandlung mit ABA kam es auf Transkriptionsebene zu einer Hochregulation von *MHA3*. Hierdurch wurde bei SR 03 die Sensitivität gegenüber der ABA-induzierbaren Wachstumshemmung vermindert, welches zu einer verbesserten Salzresistenz beitrug.

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Gießen, June 7, 2011 **Ahmad Naeem Shahzad**

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