Decline in leaf growth under salt stress is due to an inhibition of H^+-pumping activity and increase in apoplastic pH of maize leaves

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Abstract

In this study, salt-induced changes in the growth rate of maize (*Zea mays* L.) were investigated during the first phase of salt stress. Leaf growth was reduced in the presence of 100 mM NaCl, and effects were more pronounced for the salt-sensitive cv. Pioneer 3906 in comparison to the hybrid SR03. While hydrolytic activity of plasma membrane remained unaffected, H^+-pumping activity was reduced by 47% in Pioneer 3906, but was unchanged in SR03. Changes in apoplastic pH were detected by ratiometric fluorescence microscopy using the fluorescent dye fluorescein isothiocyanate-dextran (50 mM). Pioneer 3906 responded with an increase of 0.2 pH units in contrast to SR03 for which no apoplastic alkalization was found. With respect to the hypothesis that the apoplastic pH is influenced by salinity, it is suggested that salt resistance is partly achieved due to efficient H^+-ATPase proton pumping, which results in cell-wall acidification and loosening.

Key words: cell-wall acidification / H^+-ATPase / ratio imaging / salinity / *Zea mays*

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1 Introduction

Soil salinity is one of the major environmental constraints limiting agricultural production worldwide. Already up to 20% of the world's arable land and up to 50% of all irrigated land are adversely affected by salinity mainly due to nonadapted irrigation practices (*FAO*, 2008). In most saline environments, NaCl is the predominant salt species, that causes growth reduction in nonresistant plants. The decline in shoot growth on salt-affected soils is due to an inhibition of cell division and cell elongation caused by osmotic effects, ion toxicity, and mineral disturbances in plants (*Tester* and *Davenport*, 2003). However, the various deleterious effects of salinity on growth are still not completely understood yet (*Munns* and *Tester*, 2008).

A concept for understanding of salt-induced growth repression has been put forward by *Munns* (1993) and can be characterized by the biphasic model of growth response to salinity. In the first phase, growth is reduced by osmotic stress, while the second phase is mainly characterized by ion toxicity. While there are only small genetic variations in growth due to osmotic effects, sensitive and resistant genotypes can be distinguished within the second phase, with resistant genotypes maintaining a higher growth rate under saline conditions (*Munns*, 1993; *Sümer* et al., 2004). To avoid a disorder of ion homeostasis under saline conditions, plant cells have to maintain low cytoplasmic Na^+ concentrations and concurrent high K^+ concentrations. Salt exclusion at the plasma membrane of root cells (*Greenway* and *Munns*, 1980; *Schubert* and *Läuchli*, 1990), sequestration in xylem parenchyma cells (*Fortmeier* and *Schubert*, 1995) and in leaf-cell vacuoles and cell walls (*Blumwald*, 2000), ion retranslocation from the leaves, and ion leaching from the leaf apoplast by precipitation (*Tester* and *Davenport*, 2003) are mechanisms to reduce the salt burden of plants under saline conditions. In glycophytes, sensitivity is associated with an inability to prevent or tolerate elevated NaCl concentrations in the shoots. Salt resistance may be achieved by salt sequestration in vacuoles. At the same time, high NaCl concentrations in vacuoles require the accumulation of compatible osmolytes in the cytosol (*Yang* and *Lu*, 2005). When the vacuolar storage capacity is surpassed, rising cytoplasmic concentrations of NaCl may result in enzyme inhibition or apoplastic NaCl accumulation may result in turgor loss. The latter had originally been proposed by *Oertli* (1968), but seems to be of subsidiary significance because turgor pressure remains unchanged under saline conditions (*De Costa* et al., 2007).

According to the acid-growth theory, the acidification of the leaf apoplast is the major requirement to increase cell-wall extensibility, which controls extension growth (*Rayle* and *Cleland*, 1970; *Hager* et al., 1991). This theory is supported by the finding that auxin mediates the acidification of the apoplast below a pH of 5.5 (*Van Volkenburgh* and *Cleland*, 1980), thus stimulating cell elongation in *Avena* coleoptiles (*Hager*, 2003), maize, and pea (*Jacobs* and *Ray*, 1976). A lowered apoplastic pH is presumed to activate wall-loosening enzymes such as expansins, thus enhancing cell growth (*Cosgrove*, 2000).

Several environmental conditions affect plant growth (*Munns* et al., 2000) by altering apoplastic acidification. For example, growth inhibition by water stress is accompanied by an

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increase in apoplastic pH and a decrease in acidification rate (*Van Volkenburgh* and *Boyer*, 1985). Apoplastic alkalization can be understood as a general response to various stresses such as drought or salinity (*Felle* and *Hanstein*, 2002). It is assumed that leaf-growth reduction in the first phase of salt stress is caused by a reduced plasma-membrane H^+-ATPase pumping activity (*Zörb* et al., 2005b). Therefore, H^+ extrusion into the apoplast is reduced. Furthermore, modifications of the enzyme and the expression of minor isoforms occur under the influence of salt stress as shown for maize (*Zörb* et al., 2005a).

The objective of this work was to investigate the inhibition of leaf growth in the first phase of salt stress associated with alterations in plasma-membrane H^+-ATPase activity. In addition to the effect of salt stress on the H^+-extrusion capability of two maize genotypes differing in salt resistance, *in vivo* changes of apoplastic pH of intact leaves were investigated. The results provide insights into the possible relation between proton extrusion and plant growth under salinity and may contribute to a deeper understanding of maize salt resistance.

2 Material and methods

2.1 Plant cultivation

Maize (*Zea mays* L. cv. Pioneer 3906 and a newly developed hybrid SR03; *Schubert* and *Zörb*, 2005) seeds were soaked in aerated 1 mM $CaSO_4$ solution for 1 d and germinated at 25°C in the dark between two layers of filter paper moistened with 0.5 mM $CaSO_4$. After 4 d, seedlings were transferred to containers with 4.5 L of a 0.25 concentrated nutrient solution. After 2 and 4 d of cultivation, the concentration of the nutrient solution was increased to 0.5 and full-strength, respectively. The full-strength nutrient solution had the following composition: 2.0 mM $Ca(NO_3)_2$, 0.2 mM KH_2PO_4, 1.0 mM K_2SO_4, 0.5 mM $MgSO_4$, 2.0 mM $CaCl_2$, 1mM NaCl, 1.0 μM H_3BO_4, 2.0 μM $MnSO_4$, 0.5 μM $ZnSO_4$, 0.3 μM $CuSO_4$, 0.01 μM $(NH_4)_6Mo_7O_{24}$, 200 μM Fe-EDTA.

The NaCl treatment was started when the full nutrient concentration was applied. NaCl was added in 25 mM increments daily until a final concentration of 100 mM NaCl was reached. Plants were grown in a growth chamber 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 70%. To investigate the effect of salinity on shoot growth, plant cultivation was extended to 23 d.

2.2 Plant harvest

To determine shoot growth during salt treatment, length of all leaf blades and shoot height were measured daily with a ruler. Plants were harvested 8 d after application of 100 mM NaCl by separating the shoot from the root, and fresh weights were determined. Plant material for cation analysis was homogenized by grinding the tissue in liquid nitrogen with mortar and pestle. Ground material was stored at –80°C. The concentrations of Na^+, K^+, Ca^{2+}, and Mg^{2+} were determined after dry-ashing (480°C) by means of atomic-absorption spectrophotometry (SpectrAA 220 FS, Varian, Mulgrave, Victoria, Australia). For plasma-membrane isolation, plants were harvested 8 d after application of 100 mM NaCl by cutting the three youngest leaves, which developed under salinity treatment, and separating the first 10 cm of the basal leaf blade. Plant material for membrane isolation was cooled to 4°C and processed immediately. For pH measurements, the youngest, fully developed leaf was excised 8 d after reaching the maximum salinity level at 100 mM NaCl. Plant material was treated immediately.

2.3 Plasma-membrane isolation

Plasma membrane was isolated with two-phase partitioning according to *Yan* et al. (1998). After removing the midribs, leaves were cut and washed three times with chilled, deionized water and ground in ice-cold homogenization buffer in a blender (Waring Blender). The homogenization buffer contained 250 mM sucrose, 250 mM KI, 2 mM EGTA, 10% (v/v) glycerol, 0.5% (w/v) bovine serum albumin, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 mM 2-mercaptoethanol, 1% (w/v) polyvinylpyrrolidone, and 50 mM 1,3-bis (tris[hydroxymethyl]-methylamino) propane (BTP), adjusted to pH 7.8 with MES. The homogenate, adjusted to a grinding medium–to–tissue ratio of 4 mL (g fresh weight)$^{-1}$, was filtered through two layers of Miracloth (Calbiochem-Novabiochem, San Diego) and centrifuged in a swing-out bucket rotor at 11,500 *g* (AH 629 rotor, 36 mL, Sorvall Products, Newtown, CT) for 10 min at 0°C. The supernatants were centrifuged at 87,000 *g* for 35 min.

The microsomal pellets were resuspended in phase buffer (250 mM sucrose, 3 mM KCl, and 5 mM KH_2PO_4, pH 7.8). The microsomal membrane preparation was fractionated by two-phase partitioning in aqueous dextran T-500 (Sigma) and PEG-3350 (Sigma) according to the method of *Larsson* (1985). Stock solutions of polymers were prepared with concentrations of 20% and 40% (w/w) for dextran and polyethylene glycol, respectively. The concentration of the dextran stock solution was determined by means of optical rotation. The phase stock was weighed and diluted to 6.1% (w/w, each polymer) with phase buffer to a final weight of 32 g. Polymers in "start tubes" were, however, diluted to 26 g. Six grams of microsomal resuspension (in phase buffer) were added to the upper phase of each start tube. The tubes were sealed with Parafilm and mixed by inversion (30 times). Phase separation was achieved by centrifugation at 4°C and 720 *g* (Sorvall AH-629 rotor, 36 mL) for 23 min followed by two washing steps in identical phases. Centrifugation times for the second through fourth separation were 15, 10, and 5 min, respectively. The upper phases obtained after four separations were diluted with phase buffer (see above) and centrifuged at 151,200 *g* for 40 min. The pellets were washed with resuspension buffer (250 mM sucrose, 3 mM KCl, 5 mM BTP/MES, pH 7.8) and pelleted again. The pellets were resuspended in resuspension buffer, divided into aliquots, and immediately stored in liquid nitrogen. Protein was quantified according to the method of Bradford (*Larsson*, 1985) using bovine serum albumin (Sigma) as a standard.

2.4 Enzyme assays

Hydrolytic ATPase activity was determined in 0.5 mL of 30 mM BTP/MES buffer containing 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 (Sigma), and 5 mM disodium-ATP. The reaction was initiated by the addition of 3 μg of membrane protein at 30°C and stopped after 30 min with 1 mL of stopping reagent [2% (v/v) concentrated H_2SO_4, 5% (w/v) SDS, and 0.7% (w/v) $(NH_4)_2MoO_4$)] followed immediately by 50 μL of 10% (w/v) ascorbic acid. After 10 min, 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] were added to prevent the measurement of phosphate liberated by ATP hydrolysis under acidic conditions. Color development was completed after 30 min and *A*820 was measured by means of a spectrophotometer (Carry 4 Bio, Varian Australia Pty Ltd., Mulgrave, Victoria, Australia). ATPase activity was calculated as phosphate liberated in excess of a boiled-membrane control.

2.5 pH gradient

The formation of a pH gradient across the plasma membrane of inside-out vesicles was measured as the quenching of *A*492 by acridine orange (AO). Quenching was continuously monitored 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, 1 mM sucrose, (adjusted to pH 6.5 with BTP), 0.05% (w/v) Brij 58, and 50 μg of membrane protein in a final volume of 1.5 mL. Brij 58 was used to create inside-out vesicles (*Baginski* et al., 1967). After equilibration of the membrane vesicles with the reaction medium (about 20 min), the reaction was initiated by the addition of Mg-ATP (mixture of $MgSO_4$ and disodium-ATP, adjusted to pH 6.5 with BTP) to give a final concentration of 5 mM. The reaction temperature was 25°C.

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

Plasma-membrane proteins were separated by SDS PAGE using the system of *Laemmli* (1970). Membrane vesicles (7 μg membrane protein) were solubilized in SDS-loading buffer containing 0.25 mM Tris-HCl, pH 7.4; 20% (w/v) SDS; 20% (v/v) glycerol; 0.2 m dithiothreitol, 0.002% (w/v) bromocresol blue, and 10 mM PMSF. The standard markers for protein molecular mass were purchased from Sigma. After 30 min shaking at room temperature (22°C), samples were loaded on a discontinuous SDS-polyacrylamide gel [6% (w/v) acrylamide stacking gel and 10% (w/v) acrylamide separating gel].

For the western-blot analysis, after separation by SDS PAGE, samples were transferred to PVDF membrane filters (0.2 μm, Pall Specialty Materials, Port Washington, NY) using a semidry blotting system with a buffer containing 250 mM Tris (pH 8.3, adjusted with NaOH), 0.92 M glycine, and 10% (v/v) methanol for 1.5 h at room temperature and at a current intensity of 0.8 mA cm^{-2}. For the identification and quantification of plasma-membrane H^+-ATPase, the blot with plasma-membrane proteins was incubated with a polyclonal antibody (kindly supplied by 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*). The antiserum was diluted 1 : 3000 in TBS-T buffer [1 mM Tris-HCl (pH adjusted to 8.0 with NaOH), 15 mM NaCl, and 0.1% (v/v) Tween 20], and incubation was carried out for 1 h at room temperature followed by an incubation at 4°C overnight. After rinsing in TBS-T, the PVDF membrane filters were incubated at room temperature for 2 h with a 1 : 30,000 (v/v) diluted secondary antibody (alkaline phosphatase-conjugated anti-rabbit IgG, Sigma). After rinsing in TBS-T, the filters were incubated for 5 min in a buffer containing 100 mM Tris-HCl (pH 9.5, adjusted with NaOH), 100 mM NaCl, and 5 mM $MgCl_2$ and stained subsequently. For quantification of plasma-membrane H^+-ATPase, the blots were scanned (Scanner PowerLook 1120, Umax) and the H^+-ATPase immunoreactive bands were quantified densitometrically with software (TINA, Raytest Isotopenmessgeräte, Straubenhardt, Germany).

2.7 Leaf preparation and ratio imaging

The excised leaves were washed with deionized water, and leaf apoplast was infiltrated with 50 μM fluorescein isothiocyanate (FITC)-dextran (MW = 10,000, Sigma-Aldrich, Munich, Germany) by means of the vacuum-infiltration technique (*Mühling* and *Läuchli*, 2000). Subsequently, leaves were washed again with deionized water to remove adhering dye and cut into segments of approximately 4 cm^2. Leaf segments were placed upside down between an object plate and coverslip and used to measure fluorescence-emission intensity.

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 was 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 for pH by using the 20× objective (Leica pH 1; 20× / 0.40). Ratios were converted to pH values by *in vivo* calibration for pH (*Mühling* et al., 1995). The fluorescence of the whole image, resulting from the intercellular spaces (apoplast) of the intact leaf epidermis, was detected (see Fig. 4) and used for the ratio imaging.

2.8 Statistical treatment

Values are given as means ± standard error of at least three replicates. Significant differences were calculated using Student's t-test.

3 Results

3.1 Effect of salinity on leaf development and shoot growth

Salinity primarily affected shoot growth and leaf development. Salt treatment (100 mM NaCl, which was continued for 8 d after the application of the maximum-salinity treatment) significantly decreased shoot fresh weight of both maize genotypes (Tab. 1). The genotypes differed significantly with SR03 producing the maximum and Pioneer 3906 the minimum shoot fresh weight under saline conditions. Visual damages typical for ion toxicity of leaves were not observed. Instead, the leaves of the salt-treated plants appeared dark-green, characteristic for the first phase of salt stress (not shown).

Table 1: Shoot fresh weights per plant and relative shoot fresh weights compared to control plants of two maize genotypes as affected by salinity: Pioneer 3906 with 1 mM NaCl treatment (control), Pioneer 3906 with 100 mM NaCl treatment (+NaCl), SR03 with 1 mM NaCl treatment (control), SR03 with 100 mM NaCl treatment (+NaCl). The values represent means ± SE of four independent experiments. Significant differences ($p \leq 5\%$) between genotypes and treatments are indicated by different letters.

Salt treatment	Shoot fresh weight / g plant^{-1}	Relative shoot fresh weight / %
Pioneer 3906 (control)	67.79 ± 1.84 a	
Pioneer 3906 (+NaCl)	26.66 ± 2.78 c	39.33
SR 03 (control)	55.24 ± 3.58 b	
SR 03 (+NaCl)	30.42 ± 1.18 c	55.07

A significant effect of salt stress was only apparent from leaf 5 onwards for both Pioneer 3906 (Fig. 1A) and SR03 (Fig. 1B). Maximal leaf length attained by Pioneer 3906 was reduced by 10%, 30%, and 50% for the 5th to 7th leaves, respectively, whereas growth reduction was less pronounced for SR03 with 5%, 20%, and 35%, respectively. We chose to use leaf 5 to 7 and leaf 7 from 23 d–old plants as our experimental system for plasma-membrane isolation and pH determination, respectively, because salt-stress effects on leaf development and growth were more distinct in the younger leaves.

3.2 Effect of salinity on Na^+ and K^+ concentration

While Pioneer 3906 showed a slightly increased K^+ concentration during salt treatment (Tab. 2), K^+ was significantly reduced by 100 mM NaCl in SR03. On the other hand, a significant increase in Na^+ concentration of salt-treated plants was obvious in comparison to the corresponding control plants. As for salt-treated plants, a six-fold lower Na^+ concentration was revealed in SR03 than in Pioneer 3906 (Tab. 2). Nevertheless, Na^+ was within the range of nontoxic concentrations in both genotypes.

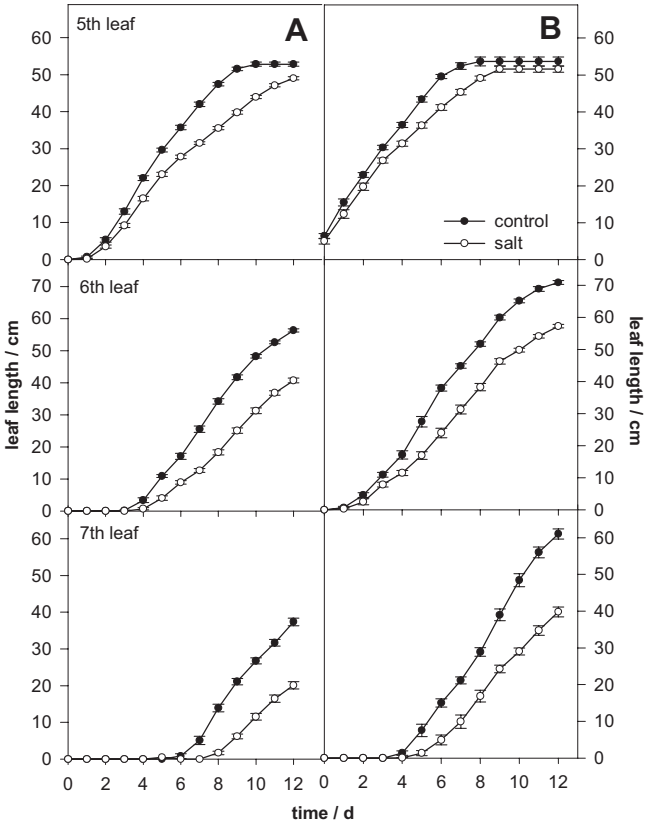

Figure 1: Effect of salinity on leaf length of two maize genotypes (A) Pioneer 3906 and (B) SR03. Plants were grown at 1 mM NaCl (control, ●) or 100 mM (salt, ○). Data represent results of nondestructive leaf-length measurements of the 5th to 7th leaf level on a daily basis starting with the first application of NaCl (day 1). The values represent means ± SE of four independent experiments.

Table 2: Effect of 8 d treatment with 100 mM NaCl on K^+ and Na^+ concentrations of leaves of *Zea mays* cv. Pioneer 3906 and hybrid SR03. The values represent means ± SE of three independent experiments. Significant differences ($p \leq 5\%$) between treatments are indicated by different letters.

Salt treatment	Shoot	
	K^+	Na^+
	/ mg (g DM)$^{-1}$	
Pioneer 3906		
control	31.82 ± 0.86 a	0.08 ± 0.03 a
+NaCl	43.50 ± 1.31 c	18.01 ± 1.16 c
SR03		
control	33.17 ± 4.11 a	0.04 ± 0.00 a
+NaCl	21.08 ± 1.39 b	2.89 ± 0.16 b

The concentrations of Ca^{2+} and Mg^{2+} remained unaffected by salt treatment (data not shown).

3.3 Isolation of plasma membrane from maize leaves

For the investigation of plasma-membrane H^+-ATPase, plasma membrane was isolated from the three youngest, fully

developed leaves of two maize genotypes grown under saline and nonsaline conditions. To determine the purity of the plasma-membrane fraction and to eliminate an underestimation due to contamination by tonoplast H^+-ATPase and mitochondrial H^+-ATPase, the azide- and nitrate-sensitive ATPase activities at pH 8.0 were analyzed. In addition, a possible contamination of molybdate-sensitive unspecific phosphatases at pH 6.5 was excluded. The inhibitor-sensitive ATPase hydrolytic activity of each membrane fraction was calculated by subtracting the ATPase hydrolytic activity in the presence of the inhibitor from the activity of the control at each assay pH. The results showed similar plasma-membrane purity as reported by *Zörb* et al. (2005b; data not shown).

3.4 Plasma-membrane H^+-ATPase hydrolytic and pumping activities

To investigate the effect of salinity on plasma-membrane H^+-ATPase activity of maize leaves, the difference in ATP hydrolytic activities of the membrane with and without the presence of vanadate (0.1 mM) was measured and this specific difference defined as hydrolytic activity of plasma-membrane H^+-ATPase. The data indicated that ATP hydrolysis by the plasma-membrane H^+-ATPase in maize leaves was not affected by salinity, irrespectively of the salt resistance of both genotypes (Tab. 3).

Plasmalemma H^+-pumping activity was monitored as absorbance decrease (ΔA_{492}) of acridine orange (AO). After initiation of H^+ pumping by addition of Mg-ATP, there was rapid quenching, which eventually reached a constant level. Absorbance was completely restored after addition of 5 μM gramicidin (Fig. 2). Compared with salt treatment of Pioneer 3906 (Fig. 2A), absorbance quenching of AO caused by plasma-membrane vesicles from control plants was more rapid at the beginning and reached a higher level after 140 min. For SR03, no significant differences in absorbance quenching of AO between vesicles from stressed and non-stressed leaves were obvious (Fig. 2B). Two parameters, initial rate and maximum quenching (pH gradient), were used to characterize the plasma-membrane H^+ pumping. The initial rate of H^+ pumping was determined according to the quenching rate within the 1st minute, which reflects active H^+ influx into plasma-membrane vesicles (*Yan* et al., 1998). Maximum quenching was measured 100 min after initiation of the H^+ pump. At this time, net H^+ transport across the plasma membrane was no longer apparent and H^+ influx due to active pumping and passive H^+ efflux reached equilibrium. This parameter indicates the steepest pH gradient that can be created by H^+-pumping activity. At assay pH 6.5, the initial rate of H^+ pumping by plasma-membrane ATPase from salt-treated Pioneer 3906 was reduced by 47% in comparison with control (Tab. 3). On the other hand, such a change in pumping activity was not apparent for vesicles from SR03 when grown under saline conditions (Tab. 3).

For inside-out vesicles, the establishment of a pH gradient is determined by both active H^+ influx (pumping) and passive H^+ efflux. After 140 min, the equilibrated pH gradient was completely collapsed by 5 μM gramicidin (Fig. 2). According to the initial rate and, therefore, the formation of the pH gradient across the vesicle membrane, the salt-treated Pioneer 3906 showed a 30% reduced capability to build up a pH gradient compared to control (Tab. 3). In contrast, SR03 was capable to maintain a similar pH gradient independently of salinity. To determine passive H^+ efflux from plasma-membrane vesicles, we measured the degradation of the pH gradient after specifically inhibiting H^+ pumping by addition of 500 μM vanadate. Because the degradation rate of the pH gradient depends on the gradient itself, a comparison between treatments should be made at the same pH gradient. Therefore, we stopped H^+ pumping when the pH gradients of plasma-membrane vesicles were identical. After addition of vanadate, the established pH gradient was degraded quickly and then reached a relative constant level. This resting pH

Table 3: Hydrolytic activity of plasma-membrane ATPase and H^+ transport in vesicles isolated from two maize genotypes grown under control and salt-stress conditions.
Membranes were isolated from leaves of 3-week-old maize: Pioneer 3906 with 1 mM NaCl treatment (control), Pioneer 3906 with 100 mM NaCl treatment (+NaCl), SR03 with 1 mM NaCl treatment (control), SR03 with 100 mM NaCl treatment (+NaCl). The assay was conducted at 25°C, pH 6.5, using 50 μg of membrane protein. The values represent means ± SE of three independent experiments. Significant differences ($p \leq 5\%$) between treatments are indicated by different letters.

Salt treatment	Hydrolytic activity	Active H^+ transport		Passive H^+ transport	
		initial rate	pH gradient	initial rate	$t_{1/2}$
	/ μmol P_i mg^{-1} min^{-1}	/ ΔA_{492} min^{-1}	/ ΔA_{492}	/ ΔA_{492} min^{-1}	/ min
Pioneer 3906 (control)	0.31 ± 0.12 a	0.017 ± 0.001 a	0.07 ± 0.00 a	0.006 ± 0.002 a	2.38 ± 0.20 a
Pioneer 3906 (+NaCl)	0.35 ± 0.10 a	0.009 ± 0.003 b	0.05 ± 0.00 b	0.007 ± 0.002 a	2.43 ± 0.20 a
SR03 (control)	0.41 ± 0.20 a	0.016 ± 0.001 a	0.05 ± 0.01 a	0.006 ± 0.002 a	1.95 ± 0.30 a
SR03 (+NaCl)	0.54 ± 0.10 a	0.016 ± 0.001 a	0.05 ± 0.01 a	0.008 ± 0.001 a	1.95 ± 0.12 a

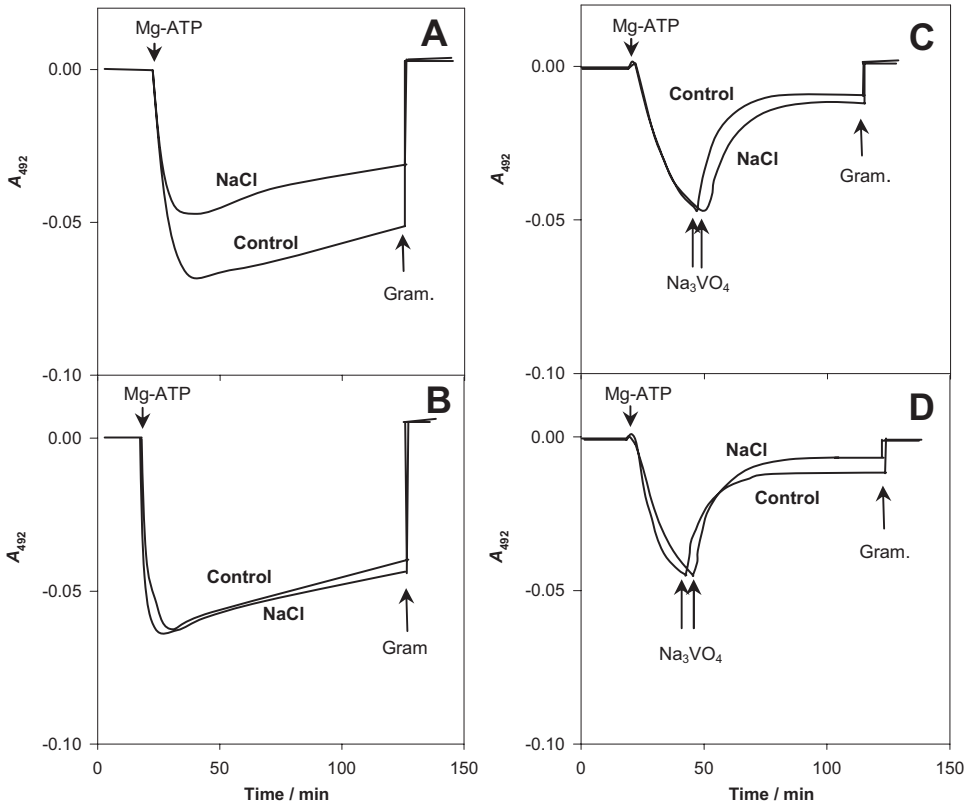

Figure 2: Comparison of active H^+ transport (A, B) driven by plasma-membrane H^+-ATPase and passive H^+ transport (C, D) across the plasma membrane of inside-out vesicles. Membrane vesicles were isolated from the maize genotypes Pioneer 3906 (A, C) and SR03 (B, D). For the comparison of active H^+ transport, the pH-gradient formation across vesicle membranes was monitored as $\Delta A492$ of AO. At assay pH 6.5, intravesicular acidification was initiated by addition of 5 mM Mg-ATP. For 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 μM gramicidin (Gram.).

gradient was completely collapsed by gramicidin (Fig. 2). Two parameters were used to characterize the pH-gradient degradation. The degradation rate within the 1st minute after addition of vanadate was measured as the initial rate to describe how rapidly the pH-gradient degradation starts. The time in which half of the established pH gradient was degraded ($t_{1/2}$), was determined to characterize the time course of degradation. Compared with control, both the initial degradation rate for plasma-membrane vesicles and $t_{1/2}$ were unaffected by salt treatment in Pioneer 3906 and SR03 (Tab. 4). Both parameters indicate an identical passive H^+ transport in vesicles derived from control and salt-treated plants.

3.5 Effect of salt stress on apoplastic acidification in growing leaf tissues

To assess the physiological relevance of salinity-induced changes of plasma-membrane H^+-ATPase pumping activity, the apoplastic pH in growing tissue of maize leaves was measured using the pH-dependent fluorescent dye FITC-dextran. Fluorescence microscopy confirmed that the dye accumulated selectively in the cell walls of maize leaves. Figure 3 shows the fluorescence-intensity signals after ratiometric conversion (490 nm : 440 nm) from the leaves of Pioneer 3906 (Fig. 3A) and SR03 (Fig. 3B) under nonsaline and saline conditions. Tissue differences became obvious between the epidermal cells and stomatal cavity, with higher fluorescent intensities and pH in the latter (Fig. 3), due to the infiltration process. The mean fluorescent ratio measured in leaf segments of both maize cultivars was 0.5 ± 0.01 (n = 12 plants, three segments per plant) under nonsaline conditions. Salt treatment resulted in a significantly lower fluorescent ratio (0.4 ± 0.01) for Pioneer 3906, whereas no changes occurred for SR03. Likewise, the color obtained for the apoplastic pH differed for the two treatments of Pioneer 3906 (Fig. 3A), with that of nonsaline conditions being yellowish-green and thus indicating a pH of 5.5 ± 0.01, and that of saline conditions being bluish-green, indicative of a pH of 5.7 ± 0.02 (Tab. 4A). In contrast, no effect on the apoplastic pH was found for SR03 (Tab. 4B).

Table 4: Effect of salinity on apoplastic pH in the growing zone of the youngest, expanding leaf of Pioneer 3906 and SR03 after 8 d of salt treatment (100 mM NaCl). pH values were measured by means of fluorescent microscopy after infiltration with FITC-dextran (50 μM). The values represent means ± SE of four independent experiments after ratiometric fluorescence microscopy. Significant differences ($p \leq 5\%$) between treatments are indicated by different letters.

Salt treatment	Apoplastic pH	
	Pioneer 3906	SR03
Control	5.5 ± 0.01 a	5.8 ± 0.04 a
100 mM NaCl	5.7 ± 0.02 b	5.8 ± 0.03 a

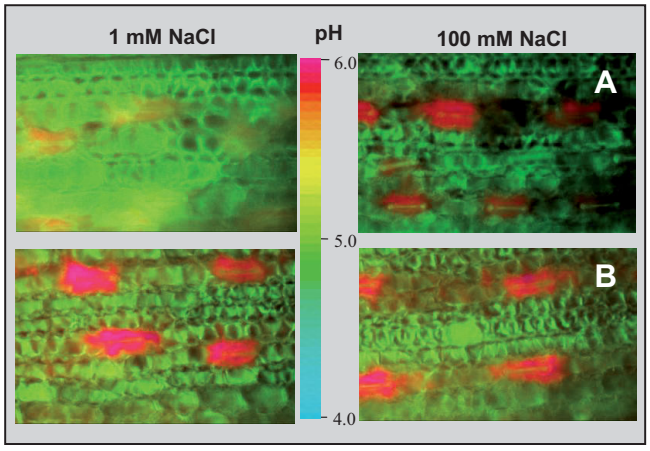

Figure 3: Fluorescent ratio images of mesophyll cells and stomata of intact leaves of *Zea mays* L. cv. Pioneer 3906 (A) and hybrid SR03 (B). The image presents the ratio (490 mm : 440 mm) after excitation at 490 nm and 440 nm. Apoplastic pH was calculated by *in vivo* calibration according to *Mühling* et al. (1995). The images were taken with a 20× objective (Leica pH 1; 20× / 0.40). The dye (FITC-dextran, 50 μM) was infiltrated into the leaf apoplast using the vacuum-infiltration technique.

4 Discussion

The acid-growth theory states that H^+ secretion into the apoplast is an essential premise for cell-wall loosening and plant growth (*Hager*, 2003). Cell-wall acidification is realized by plasma-membrane H^+-ATPase and activates wall-loosening expansins, xyloglucan hydrolases, and xyloglucan endotransglycosylases (*Cosgrove*, 2000). Findings of *Stahlberg* and *Van Volkenburgh* (1999) as well as of *Fan* and *Neumann* (2004) indicate that increased cell-wall acidification is associated with increased extension growth. Under saline conditions, reduced H^+ pumping by plasma-membrane H^+-ATPase may be a key factor for reduced leaf growth of maize during the first phase of salt stress according to the model suggested by *Munns* (1993). To elucidate the role of plasma-membrane H^+-ATPase and apoplastic pH in the growth reduction of maize leaves during the first phase of salt stress, the well-characterized maize cultivar Pioneer 3906 (*Schubert* and *Läuchli*, 1990; *Fortmeier* and *Schubert*, 1995) and the maize hybrid SR03, which is able not only to effectively exclude Na^+ but also to withstand osmotic stress (*Schubert* and *Zörb*, 2005), were used in a comparative approach.

4.1 Shoot growth of maize is influenced by salt resistance in the first phase of salt stress

Short-term NaCl treatment guaranteed that the effects measured can be mainly ascribed to osmotic stress in the first phase of salt stress (*Munns*, 1993; *Sümer* et al., 2004) without reaching toxic Na^+ concentrations (Tab. 2; *Fortmeier* and *Schubert*, 1995). Shoot growth of both maize cultivars was significantly reduced after the application of 100 mM NaCl for 8 d (Tab. 1). However, a comparison of both genotypes revealed that growth reduction was not uniform but more pronounced for Pioneer 3906 than for SR03 (Tab. 1; Fig. 1). These growth differences are in agreement with findings of *De Costa* et al. (2007), who showed that short-term salinization (100 mM NaCl) resulted in a significant shoot reduction of salt-sensitive maize, whereas more resistant genotypes were less affected.

4.2 Adaptation of plasma-membrane H^+-ATPase under salinity

Several authors reported that plasma-membrane H^+-ATPase is involved in adaptation to salinity in moderately resistant glycophytes (*Kerkeb* et al., 2001a). To examine this involvement, plasmalemma vesicles were isolated from maize leaves by using a method previously reported (*Zörb* et al., 2005b).

In the present study, plasma-membrane-H^+-ATPase-mediated ATP hydrolysis was not affected by NaCl treatment of both maize genotypes Pioneer 3906 and SR03 (Tab. 3). This result is in agreement with findings of authors who reported that hydrolytic activity was either not modulated or increased (*Ayala* et al., 1996). However, compared with SR03 under salinity, H^+ pumping of the plasma-membrane H^+-ATPase was strongly decreased in Pioneer 3906 (Fig. 2A), which may be concluded from a reduced initial rate of H^+ pumping and the lower maximum pH gradient established (Tab. 3), which is in agreement with findings of *Zörb* et al. (2005b). This higher pH gradient was not explained in terms of a reduced H^+ permeability of this membrane under salinity (Fig. 2; Tab. 4). *Sibole* et al. (2005) described a positive relation between salt resistance and H^+-ATPase enzyme activity, which was confirmed by the result that H^+-pumping activity remained unaffected by high-salt treatment of the more resistant SR03 (Tab. 3).

The possible mechanisms involved in the adaptation of plasma-membrane H^+-ATPase to saline conditions in case of SR03 may include (1) an increase in enzyme concentration by transcriptional or translational regulations (*Niu* et al., 1993), (2) a modulation of the turnover rate (*Hager* et al., 1991), (3) a differential expression of isoforms of this enzyme (*Zörb* et al., 2005b), or (4) the modification of the lipid environment and plasma-membrane permeability (*Kerkeb* et al., 2001b).

4.3 Salinity-induced changes of plasma-membrane H^+-ATPase affect apoplastic pH and growth

Even though shoot growth is inhibited by salt stress, turgor maintenance is complete, especially in leaf tissue (*De Costa* et al., 2007). For many tissues, it has been demonstrated that increased rates of growth are often due to acidification of the apoplastic space (*Rayle* and *Cleland*, 1992; *Peters* et al., 1998). This process relies on the H^+ extrusion by the plasma-membrane H^+-ATPase into the leaf apoplast, which then induces wall loosening required for elongation (*Hager*, 2003). Growing leaves of Pioneer 3906 showed stronger apoplastic acidification under nonsaline conditions compared to salt-treated plants (Fig. 4). In particular, the increase of osmolarity typical for salt-stress conditions resulted in apoplastic alkali-

zation of up to 0.5 pH units in leaves of barley (*Felle* et al., 2005) and bean (*Felle* and *Hanstein*, 2002). It is noteworthy that those experiments were conducted with nonadapted plants, which cannot thoroughly be assigned to conditions representative for this study. Since salt-adapted plants adjust to saline condition by, *e.g.*, long-term modulation H^+-ATPase activity (*Hartung* and *Radin*, 1989), ionic effects seem to be more likely for nonadapted plants. This change of apoplastic pH is exactly the pH change that induced elongation growth in maize coleoptiles following IAA treatment (*Peters* and *Felle*, 1991). Thus an inversion of the acid-growth argument would permit the conclusion that in growing tissues a pH increase of about half a unit is sufficient to inhibit growth, as in fact was observed for Pioneer 3906. Therefore, the reduction in shoot growth of Pioneer 3906 (Tab. 1) by high apoplastic pH observed in this investigation may have been caused by the shift in the H^+-pumping activity of the plasma-membrane H^+-ATPase (Tab. 3). In contrast, *Neves-Piestun* and *Bernstein* (2001) failed to show salt-induced alterations of the apoplastic pH in maize leaves, which was ascribed to the unchanged H^+-pumping activity of the plasma-membrane H^+-ATPase. On the other hand, apoplastic pH remained unaffected by salinity in the salt-resistant hybrid SR03 (Tab. 4) as did H^+-pumping activity (Tab. 3). Consequently, growth performance of SR03 was superior compared to Pioneer 3906 under salinity (Fig. 1).

As recently demonstrated for intact tomato plants, *Wilkinson* and *Davies* (2008) induced immediate stomatal closure and growth reduction by the application of buffers adjusted to "stressful" pH between 6.4 to 7.0. This is in line with other studies, which indicate that lowering the apoplastic pH correlates well with an instant growth activation of oat (*Schopfer*, 1989) and maize (*Peters* et al., 1998). Likewise, drought-induced growth reduction was consistent with apoplastic alkalization (*Fan* and *Neumann*, 2004). In accordance, it can be concluded from this study that a salt stress–induced pH increase (Tab. 4) was related to the reduced leaf growth of Pioneer 3906 (Tab. 1). In contrast, no relation between apoplastic pH and growth reduction occurred in SR03.

However, as the apoplastic pH values under nonsaline and saline conditions were comparable for SR03, it is suggested that a direct effect of apoplastic pH on cell expansion cannot account for the observed growth reduction in the more salt-resistant cultivar. In fact, other growth-controlling factors such as ABA or expansins, as well as xyloglucan hydrolases and xyloglucan endotransglycosylases (*Cosgrove*, 2000) may be adversely affected by salinity. As already shown by *De Costa* et al. (2007), genetic differences in leaf growth of maize were related to increasing leaf ABA concentration, implying that ABA may be involved in growth regulation of maize under salinity. Besides reports of the growth-inhibiting role of ABA under salt and drought stress (*Cramer* et al., 1998), there is considerable evidence in the literature showing that ABA could be involved in conferring salt resistance (*Hasegawa* et al., 2000; *Mäkelä* et al., 2003; *Fricke* et al., 2004). Even though higher shoot ABA concentrations are mostly linked to a more alkaline apoplastic pH, this may not be in contradiction to the pH values measured in this study. It rather seems that in resistant genotypes, the maintenance of a low apoplastic pH may well be possible despite high ABA concentrations.

5 Conclusion

We have shown that salinity reduces apoplastic acidification in leaves of salt-sensitive maize due to the reduction of H^+-pumping activity of the plasma-membrane ATPase. The increase of apoplastic pH may cause reduced activation of wall-loosening enzymes, thus causing shoot-growth reduction. There is evidence that other factors than reduced cell-wall acidification may be responsible for growth inhibition of resistant maize genotypes in the first phase of salt stress.

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