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Does the Ratio of Na^+ and K^+ Affect the Hydrolytic and Pumping Activity of the Plasma Membrane H^+-Atpase from Maize (*Zea Mays* L.) Shoot?

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Abstract

Salinity limits plant growth and impairs agricultural productivity. In the first phase of salt stress, growth of plants is impaired predominantly by osmotic stress and only slightly by Na^+ effects. The aim of our work was to investigate whether Na^+ affects both hydrolytic and pumping activity of the plasma membrane H^+ ATPase. Sodium salinity leads to a shift of the Na^+/K^+ ratio resulting in a displacement of K^+ in the plant. It was shown that high cytoplasmatic Na^+ concentrations and low K^+ concentrations have an effect on the H^+ ATPase activity of the plasma membrane. Reduced H^+-ATPase activities caused a reduction of the acidification of the apoplast. This process limits the cell-wall extensibility and thus reduces growth after salt stress. For this reason, H^+-ATPase activity, which is important for cell elongation was measured under the influence of different Na^+/K^+ ratios. Plasma membrane was isolated from two days old maize shoots using the aqueous polymer two-phase technique. ATPase activity was determined by measuring the release of P_i. The H^+-pumping activity was revealed by absorbance quenching of acridine orange. Identical plant shoot material was used for ATPase extraction and effects of Na^+ and K^+ were tested *in vitro*. High concentration of 100 mM Na^+ decreased the hydrolytic H^+-ATPase activity to values of 80%, in comparison to high concentration of K^+ (100 mM) without Na^+. However, under comparable conditions the H^+ pumping activity was decreased to 30%.
Key Words: *Zea mays*, Na^+/K^+ ratio, Plasma membrane, H^+-ATPase, Leaf growth.

Özet

Na^+, K^+ oranı Mısır (*Zea mays* L.) Yapraklarında Hücre Zarı H^+-ATPase Hidrolitik ve Pompalama Aktivitesini Etkiler mi?

Tuzluluk bitki büyümesi ve tarımsal verimliliği sınırlar. Tuz stresinin ilk aşamasında, bitkilerin büyümesi ağırlıklı olarak ozmotik stresten ve az da olsa Na^+ iyonundan olumsuz yönde etkilenir. Çalışmamızın amacı, Na^+ un plazma zarı H^+-ATPaz'ının hem hidrolitik hem de pompalama aktivitesini etkileyip etkilemediğini araştırmaktır. Sodyum tuzluluğu bitki içerisinde Na^+/K^+ oranında değişime neden olur buda K^+ un azalmasına neden olur. Yüksek sitoplazmik Na^+ konsantrasyonları ve düşük K^+ konsantrasyonlarının plazma zarı H^+-ATPaz aktivitesi üzerine etkisi vardır. Azalan H^+-ATPaz aktivitesi apoplast asidifikasyonunda düşüşe neden olur. Bu süreç, hücre duvarı esnekliğini sınırlar ve tuz stresi sonrası büyüme azalır. Bu nedenle, hücre uzaması için önemli olan H^+-ATPaz aktivitesi, farklı Na^+/K^+ oranlarının etkisi altında ölçülmüştür. Plazma membranları iki fazlı sulu polimer tekniği kullanılarak iki günlük mısır sürgünlerinden izole edilmiştir. ATPaz aktivitesi serbest kalan P_i nin ölçülmesi ile tespit edilmiştir. H^+-pompalama aktivitesi akridin oranın absorbansı ile belirlenmiştir. İzole edilen hücre zarlarına *in vitro* koşullarında Na^+ ve K^+ verilmiştir. Sitoplazmik 100 mM yüksek Na^+ konsantrasyonu, 100 mM K^+ konsantrasyonu ile karşılaştırıldığında hidrolitik H^+-ATPaz etkinliği %80'e düşerken, H^+-pompalama aktivitesi %30'a düşmüştür.
Anahtar Kelimeler: *Zea mays*, Na^+/K^+ oranı, Hücre zarı, H^+-ATPaz, Yaprak gelişimi.

Introduction

Environmental stress caused by salinity is a serious factor limiting the productivity of agricultural crops, which are sensitive to the presence of high concentrations of salts in the soil. Although drainage and the supply of high–quality water can solve the problem, these measures are extremely costly and not applicable in extensive agriculture (Ruiz, 2001). Besides sustainable agricultural practices and soil amelioration, the improvement of salt resistance of crops has been proposed long ago (Epstein et al., 1980; Schubert, 1999). However, as salt resistance is a

multigenic character, only limited success in improving the salt resistance of crops has been achieved so far. Identification of physiological and biochemical processes in plants provide better understanding of salt resistance (Greenway and Munns, 1980; Cheeseman, 1988; Schubert et al., 2001; Schubert et al., 2009).

Maize (*Zea mays* L.) is considered to be a moderately salt-sensitive crop (Maas and Hoffman, 1977). The plant response to salinity depends upon developmental stage and plant parts (Maas et al., 1983). Salt stress causes a rapid and potentially lasting reduction in the rate of leaf growth (Munns, 1993). Munns (1993) has also proposed a biphasic model of growth response to salinity. The biphasic model of salt stress provided new physiological insight. According to this model, plant growth is impaired by osmotic stress in a first phase. This phase of growth reduction is probably regulated by inhibitory hormonal signals (e.g. ABA) from the roots (Rausch et al., 1996). In the second phase, Na^+ accumulates in older leaves, which show necrotic lesions and eventually die as a result of a fast increase of the Na^+ concentration (Fortmeier and Schubert, 1995).

We formerly reported (Sümer et al., 2004) that Na^+ also contributes to decreased maize growth in the first phase of salt stress. Leaf growth comprises cell elongation and cell division. Conditions for elongation of cells are especially the turgor and the cell wall extensibility (Boyer, 1987). However, under saline conditions turgor does not limit cell elongation (Arif and Tomos, 1993; Cramer et al., 1998). Under these conditions Cramer and Bowman (1992); Neumann (1993) showed that hardening of the cell wall represents the really limiting factor in elongation growth. Apoplast pH was suggested to play an important role in cell–wall loosening and growth (McQueen–Mason et al., 1993). In maize leaves, increased rates of growth are associated with increased acidification of the cell–wall space (Van Volkenburgh and Boyer, 1985; Jahn et al., 1996; Peters et al., 1998). Decreased cell–wall pH is generally thought to promote wall–loosening events necessary for growing cell enlargement (Rayle and Cleland, 1992; Cosgrove, 1997), and inhibition of cell–wall acidification may therefore reduce cell growth-rate. The plasma membrane H^+-ATPase is an electrogenic pump that directly couples ATP hydrolysis with the vectorial transport of H^+ into the apoplast (Briskin and Hanson, 1992). The plasma membrane H^+-ATPase has an important function for cell–wall acidification (Taiz, 1984; Kutschera, 1994). Reduced H^+ release by plasma membrane H^+-ATPase is a key factor for reduced leaf growth of some maize genotypes during the first phase of salt stress Pitann et al. (2009) but not for others (Hatzig et al., 2010).

The ionic similarities of Na^+ and K^+ enable Na^+ competition at transport sites for K^+ entry into the symplast and may result in K^+ deficiency (Maathuis and Amtmann, 1999). Plasma membrane ATPase activity is stimulated by K^+ because K^+ promotes the dephosphorylation of this enzyme (O'Neill and Spanswick, 1984; Gibrat et al., 1990). High cytoplasmatic Na^+ concentrations and relatively low K^+ concentrations can reduce plasma membrane H^+-ATPase activity. This could reduce apoplastic acidification and limit the cell–wall extensibility and thus growth according to the acid growth theory. For this reason, H^+-ATPase activity which is the most important enzyme for cell elongation was measured under the influence of varying Na^+/K^+ ratios. The aim of our work was to investigate whether Na^+ affects both hydrolytic and pumping activity of the plasma membrane H^+ ATPase.

Material and Methods

Plant cultivation and harvest

The experiment was carried out in a climate chamber. Light intensity was 400–500 µE $m^{-2}s^{-1}$ for 16 h, temperature was 25°C and 20°C for the dark period. The relative humidity was between 60–70%. Maize seedlings (*Zea mays* L. cv. Pioneer 3906) were imbibed in aerated 1 mM $CaSO_4$ for 1 d and germinated between two layers of filter paper at 28°C for 2 d in the dark. After four days, seedlings were transferred to 50 L plastic containers (360 plants per container) containing ¼ full strength nutrient solution. The full–strength nutrient solution had the following composition i.e.; mM, 2.5 Ca $(NO_3)_2$, 0.2 KH_2PO_4, 0.5 K_2SO_4, 0.6 $MgSO_4$, 5.0 $CaCl_2$; in µM, 1.0 H_3BO_4, 2.0 $MnSO_4$, 0.5 $ZnSO_4$, 0.3 $CuSO_4$, 0.005 $(NH_4)_6Mo_7O_{24}$, 200 Fe–EDTA. Since under natural condition NaCl is present in the soil, 1 mM NaCl was added into the nutrient solution on the next day. After the plants had been in ¼ concentrated nutrient solutions in 2 d, the harvest of the maize shoot began for plasma membrane isolation. At this time the plants had two leaves. The isolation was made at two-days-old *Zea mays* (cv. Pioneer 3906) shoots, because these contained a large part of elongation growth zone.

Plasma membrane isolation

Plasma membrane of maize shoots was isolated using two-phase partitioning according to Yan et al. (2002) with some modifications. Shoots were cut 1–2 cm above seeds and washed two times with chilled, deionised water. 50 g shoot fresh weight ground in 200 mL ice–cold homogenization buffer with a blender (adjusted to a grinding medium/tissue ratio of 4 mL g^{-1} fresh weight). The homogenization buffer contained 250 mM sucrose, 250 mM KI, 2 mM EGTA, 10% (v/v) glycerol, 0.5% (w/v) bovine serum albumin (BSA), 2 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM 2–mercaptoethanol, and 50 mM 1,3–bis(tris[hydroxymethyl]methylamino) propane (BTP), adjusted to pH 7.8 with 4–morpholineethanesulfonic acid (MES). The homogenate was filtered through two layers of miracloth (Calbiochem-Novabiochem, San Diego) and centrifuged in a swinging 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 5 mL 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 polyethylene glycol 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 phase stock was weighed and diluted to 6.1% (w/w, each polymer) with phase buffer (see above) 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 Para film and mixed by inversion (30 times). Phase separation was achieved at 4ºC by centrifugation at 720 *g* (Sorvall AH–629 rotor, 36 mL) for 23, 15 and 10 min, respectively. Contrary to Yan et al. (2002), after each separation the upper PEG phase was added to fresh dextran phase and after the last extraction the upper phase was diluted 1 : 5 with phase buffer (see above) and centrifuged again at 4ºC and 131.100 g for 40 min. The pellets were resuspended and diluted with resuspension buffer (250 mM sucrose, 3 mM KCl, 5 mM BTP/MES, and pH 7.8) and centrifuged again at 4ºC and 131,100 g for 40 min. The pellets were resuspended in resuspension buffer again, divided into aliquots and immediately stored in liquid nitrogen. Protein was quantified according to the method of Bradford (1976) using bovine serum albumin (Sigma) as a standard.

Enzyme assay

Hydrolytic ATPase activity was determined in 0.5 mL of 30 mM BTP/MES buffer (pH 6.5) 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 Na_2–ATP. The reaction was initiated by the addition of 1 to 3 µg of membrane protein, proceeded for 30 min at 30°C, and stopped with 1 mL of stopping reagent [5% (w/v) SDS (sodium dodecyl sulfate), 0.7% (w/v) $(NH_4)_2MoO_4$) and 2% (v/v) concentrated H_2SO_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 dihydrate, 2% [w/v] sodium meta–arsenite, and 2% [w/v] glacial acetic acid] was added to prevent the measurement of phosphate liberated by ATP hydrolysis under acidic conditions (Baginski et al., 1967). Color development was completed after 30 min and A_{820} was measured by means of a spectrophotometer (Carry 4 Bio, Company Varian). ATPase activity was calculated as phosphate liberated in excess of the boiled–membrane control.

Measurement of 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). The change of the quenching was continuously monitored with a spectrophotometer (Carry 4 Bio, Varian Australia Pty Ltd., Mulgrave, Victoria, Australia). The assay mixture contained 5 mM BTP/MES (pH 6.5), 100 mM KCl, 0.05% (w/v) Brij 58, 0.001% (w/v) valinomycin, 7.5 µM AO, and 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 20 min), the reaction was initiated by addition of 5 mM Mg–ATP (mixture of $MgSO_4$ and Tris–ATP, adjusted to pH 6.5 with BTP). The established pH gradient was completely collapsed by 10 µM gramicidin. The assay was conducted at 25°C. To study the influence of Na^+/K^+ ratios on the H^+-ATPase and pumping activity,

salt was supplied in all treatments under *in vitro* conditions. The salt concentrations of the reaction media under different variations are presented in Table 1.

Table 1. Various Na^+/K^+ ratios at 100 mM established in the assay using KNO_3, KCl, $NaNO_3$ and NaCl

Na^+/K^+ (mM)	**Salt concentration and form**
0/100	50 mM KCl + 50 mM KNO_3
25/75	25 mM NaCl + 25 mM KCl + 50 mM KNO_3
50/50	50 mM NaCl + 50 mM KNO_3
75/25	50 mM NaCl + 25 mM $NaNO_3$ + 25 mM KNO_3
100/0	50 mM NaCl + 50 mM $NaNO_3$

Statistical calculations are given as means of four replicates ± SE. Significant differences were calculated and given at *** P = 0.1%, or ** P = 1%, or * P = 5% level, respectively.

Results and Discussion

To determine the purity of the plasma membrane fraction isolated from maize shoots, the ATP hydrolytic activity was analyzed in the presence of nitrate, azide, molybdate and vanadate, which are inhibitors of tonoplast H^+-ATPase, mitochondrial H^+-ATPase, unspecific phosphatases and plasma membrane H^+-ATPase, respectively (Faraday and Spanswick, 1992; Yan et al., 2002). Nitrate and azide showed no inhibitory effects on the ATPase hydrolytic activity of the membrane fraction at both pH 6.5 and 8.0, indicating that the membrane isolated was almost free from tonoplast or mitochondrial membranes. Molybdate showed an inhibitory effect of 13.5% at pH 6.5, suggesting the presence of a small amount of unspecific phosphatases. Furthermore, ATP hydrolytic activity of the membrane fraction was inhibited by vanadate by 91.8% (Tab.1.). These data demonstrate that the plasma membrane fraction obtained from maize shoots was of high purity. Nevertheless, to exclude a contamination effect, the activity measurements were performed with an inhibitor complex solution (nitrate, azide, and molybdate) with and without vanadate. The difference is defined as hydrolytic activity of plasma membrane H^+-ATPase (Yan et al., 1998).

Table 2. Effect of various enzyme inhibitors on the H^+-ATPase activity of isolated plasma membrane vesicles isolated from maize shoots. The values represent means (± SE) of four isolations.

	pH 6.5		**pH 8.0**	
	H^+-ATPase activity	**(%)[a]**	**H^+-ATPase activity**	**(%)[a]**
Control	0.738 ± 0.045	100	0.177 ± 0.020	100
Nitrate (50mM)	0.764 ± 0.031	104.1	0.164 ± 0.017	92.4
Azide (1mM)	0.750 ± 0.032	102.0	0.181 ± 0.019	102.4
Molybdate (1mM)	0.638 ± 0.039	86.5		
Vanadate	0.060 ± 0.003	8.2		

[a] Relative enzyme activity

The plasma membrane specific hydrolytic H^+-ATPase activity decreased with increased NaCl concentration both with 100 mM K^+ and with 50 mM K^+ similarly (Fig.1.). Despite sufficient K^+ concentrations (50–100 mM K^+), the 100 mM NaCl treatment diminished the plasma membrane specific H^+-ATPase activity about 15% in relation to the control (without NaCl).

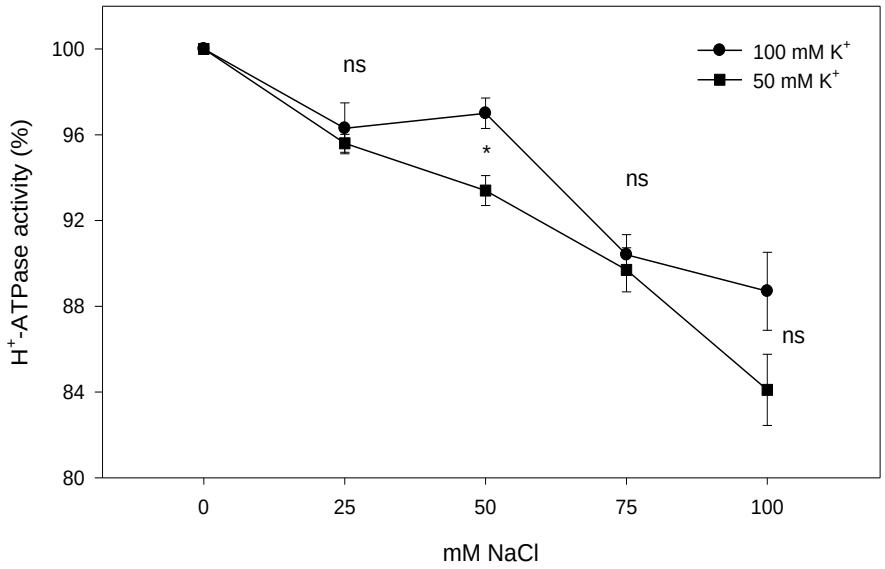

Figure 1. Effect of increasing NaCl in the reaction medium on the relative H^+-ATPase activity of plasma membrane vesicles from maize shoots, depending on K^+ concentration in reaction medium (100 mM K^+= 50 mM KNO_3 + 50 mM KCl; 50 mM K^+= 50 mM $KN0_3$). 100% correspond to 100 mM K^+, 0.676 ± 0.04 µmol P_i (mg Protein)$^{-1}$ min^{-1} and to 50 mM K^+, 0.641 ± 0.02 µmol P_i (mg Protein)$^{-1}$ min^{-1}. The values represent means (± SE) of four independent isolations.

It was important to keep the anion form and concentration (50 mM Cl^- and 50 mM NO_3^-) constant in all treatments (see material and methods). By increasing Na^+ concentration and decreasing K^+ concentration the H^+-ATPase activity both, in the biochemical optimal (6.5) and in the physiological relevant pH range (7.0) decreased in parallel (Fig. 2.). The H^+-ATPase activity decreased in relation to control (0 Na^+, 100 mM of K^+) significantly only by addition 50 mM Na^+ and 50 mM K^+ to the reaction medium (pH 6.5). Under the complete replacement of K^+ by 100 mM Na^+ the H^+-ATPase activity was reduced to about 30% with high significance (P = 0.1%)

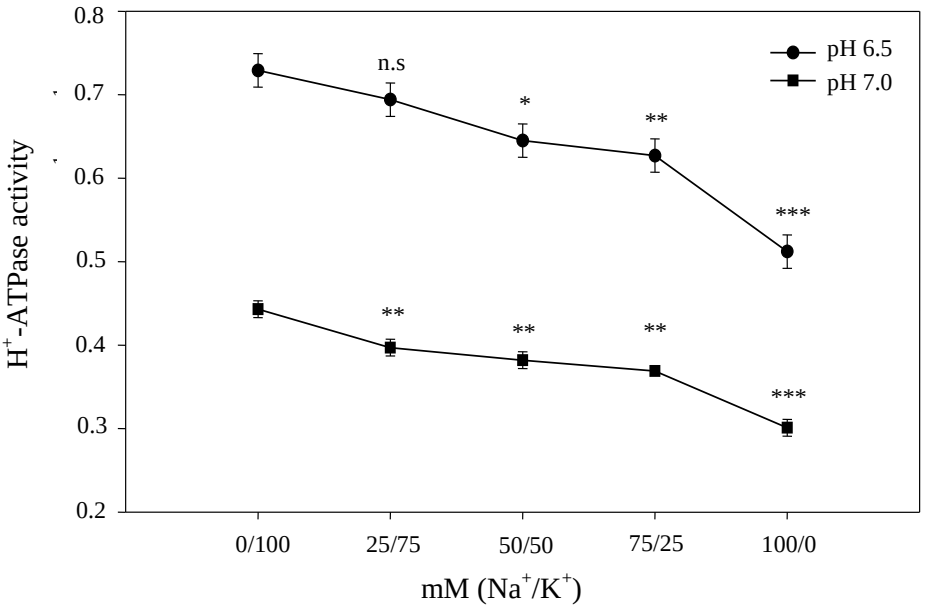

Figure 2. Effect of various Na^+/K^+ ratios in reaction medium at different pH values on the H^+-ATPase activity of isolated plasma membrane vesicles from maize shoots. The values represent means (± SE) of four independent isolations.

Besides hydrolytic activity, H^+-pumping activity of the plasma membrane was also determined. 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 min, which may reflect active H^+ influx into plasma

membrane vesicles. Maximum quenching was measured 50 min after initiation of the H^+ pumping. At this time net H^+ transport across the plasma membrane was 0 and H^+ influx due to active pumping and H^+ efflux because of leakage reached equilibrium. This parameter indicates the steepest pH gradient that can be created by H^+-pumping activity (Wakeel et. al., 2011).

For measurements of the pumping activity in the reaction medium were kept constant (200 mM Cl^-) as the H^+ transport of the H^+-ATPase strongly depends on the anions. Table 3 shows that with sufficient K^+ concentration in the reaction medium the loading of the vesicles with 100 mM of Na^+ had no significant influence on the H^+ pumping activity (initial rate) and on the maximal pH gradient (pH).

Table 3. Effect of cations on the H^+ transport of plasma membrane vesicles from maize shoots. The reaction medium contained 200 mM KCl and 100 mM NaCl + 100 mM KCl. Pumping activity was determined using the acridine orange (AO) technique. The values represent means (± SE) of four independent isolations.

	Initial rate of pumping	**pH gradient**
200 mM K^+	0.015 ± 0.002	0.046 ± 0.001
100 mM Na^+ + 100 mM K^+	0.013 ± 0.001 (ns)	0.052 ± 0.003 (ns)

ns = differences are not significant

As described under Material and methods, both the salt concentrations and the salt forms in the reaction medium were varied in the way that the anions (50 mM Cl^- and 50 mM NO_3^-) remained constant. Figure 3. shows that the pumping activity and the maximal pH gradient were strongly affected by increasing Na^+ and decreasing K^+ concentration.

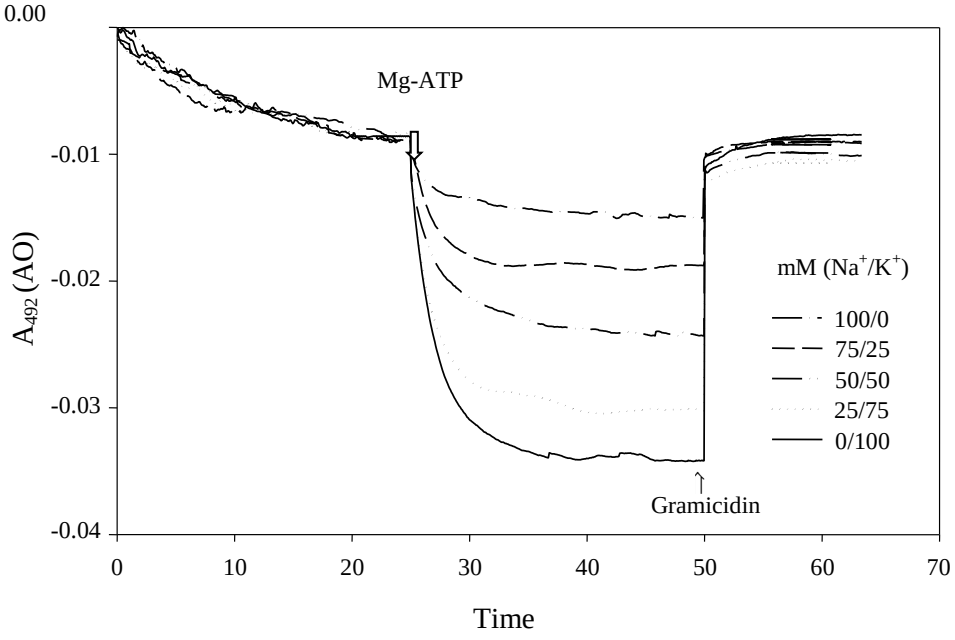

Figure 3. Effect of various Na^+/K^+ ratios in reaction medium on H^+ transport of plasma membrane vesicles from maize shoots. The pH gradient formation was monitored by absorbance quenching (A_{492}) of acridine orange (AO). At assay pH 6.5, intravesicular acidification was initiated by addition of 5 mM Mg-ATP and terminated by 10 µM gramicidin. Representative results from four independent measurements are shown.

The hydrolytic activity of the H^+-ATPase and the H^+ transport of the plasma membrane H^+-ATPase are shown in Figure 4. It is recognized that all measured parameters decreased significantly at 50 mM Na^+ and 50 mM K^+ in relation to the control (0 Na^+, 100 mM K^+). It is remarkable that at these concentrations (50/50) H^+ transport was more inhibited than the hydrolytic activity. With a complete replacement of K^+ by 100 mM Na^+ in the reaction medium the hydrolytic activity decreased in relation to control (100 mM K^+) by about 30%, while H^+ transport decreased about 80%.

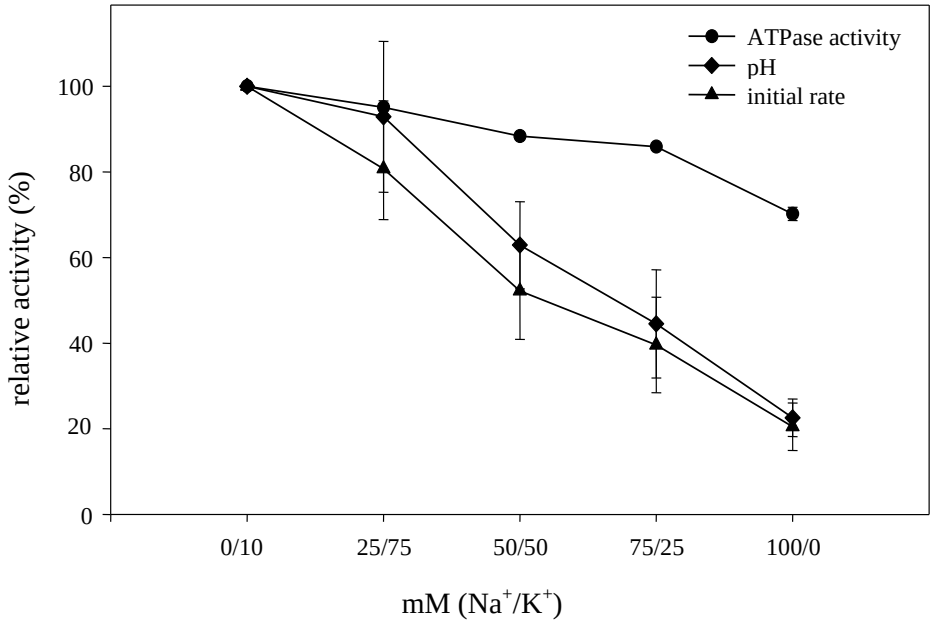

Figure 4. Effect of different Na^+/K^+ ratios in reaction medium on relative H^+-ATPase activity and relative H^+ transport of plasma membrane vesicles from maize shoots. 100% correspond to the activity without NaCl addition. The values represent means (± SE) of four independent isolations.

Conclusion

Increased cytoplasmatic Na^+/K^+ ratio results in decreased pumping activity of the plasma membrane H^+ ATPase and thus may decrease cell-wall acidification and reduce plant growth.

Information: This study was prepared from Ph.D. thesis.

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