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Research article

Leaf ion homeostasis and plasma membrane H^+-ATPase activity in *Vicia faba* change after extra calcium and potassium supply under salinity

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ABSTRACT

Salt stress in plants impacts apoplastic ion activities and cytosolic ionic homeostasis. The ameliorating effects exerted by calcium or potassium on compartmentation of ions in leaves under salinity are not fully understood. To clarify how calcium or potassium supply could ameliorate ion homeostasis and ATPase activities under salinity, 5 mM $CaSO_4$ or 10 mM K_2SO_4 were added with, or without, 100 mM NaCl for 7 d and 21 d to *Vicia faba* grown in hydroponics. The apoplastic pH was detected with Oregon Green dextran dye in intact second-uppermost leaves by microscopy-based ratio imaging. The cytosolic Ca^{2+}, Na^+, K^+ activities and pH were detected in protoplasts loaded with the acetoxy methyl-esters of Fura-2, SBFI, PBFI and BCECF, respectively, using epi-fluorescence microscopy. Furthermore, total Ca^{2+}, Na^+, K^+ concentrations and growth parameters were investigated. The ATPase hydrolyzing activity increased with time, but decreased after long salinity treatment. The activity largely increased in calcium-treated plants, but was depressed in potassium-treated plants after 7 d. The calcium supply increased *V*max, and the ATPase activity increased with salinity in a non-competitive way for 7 d and 21 d. The potassium supply instead decreased activity competitively with Na^+, after 21 d of salinity, with different effects on *K*m and *V*max. The confirmed higher ATPase activity was related with apoplast acidification, cytosol alkalinization and low cytosolic [Na^+], and thus, might be an explanation why extra calcium improved shoot and leaf growth.

1. Introduction

One strategy to reduce the adverse effects of salinity is to add nutrients such as calcium or potassium in order to ameliorate the nutritional deficiencies that usually occur under salinity (Shabala, 2002; Shabala et al., 2006; Sun et al., 2010). An adequate level of calcium and potassium in growing leaf cells can be beneficial for improving plant growth under salinity (Shabala et al., 2006; Munns and Tester, 2008). In that context, it can be suggested that salinity may disturb the essential Ca^{2+} functions without disturbing the overall tissue concentrations of Ca^{2+}, as the cytosolic Ca^{2+} activity, $[Ca^{2+}]_{cyt}$, is in nM range, whereas the overall tissue concentrations are in mM range (Felle, 1988; Plieth, 2001). Moreover, Na^+ competes with K^+ for intracellular influx, as these cations can be transported by the same proteins (reviewed by Demidchik and Maathuis, 2007; Flowers et al., 2010; and recently by Shabala, 2013).

Abbreviation: BCECF,AM, acetoxy methyl ester of 2', 7'-bis-(carboxy ethyl)-5-, (and 6)-carboxy fluorescein; $[Ca^{2+}]_{cyt}$, cytosolic free Ca^{2+} concentration; Fura-2,AM, acetoxy methyl ester of calcium binding benzofuran; $[K^+]_{cyt}$, cytosolic free K^+ concentration; *K*m, apparent enzyme-substrate affinity constant; $[Na^+]_{cyt}$, cytosolic free Na^{2+} concentration; PBFI,AM, acetoxy methyl ester of potassium-binding benzofuran; SBFI,AM, acetoxy methyl ester of sodium-binding benzofuran; pH_{apo}, apoplastic pH; pH_{cyt}, cytoplasmic pH; PM, plasma membrane; ST, short term of growth (7 d); LT, long term of growth (21 d); *V*max, reaction rate at saturating concentration of substrate.

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Calcium is established as a ubiquitous intracellular second messenger in plants, especially in response to abiotic and biotic stresses (Sanders et al., 2002; Reddy et al., 2011). The $[Ca^{2+}]_{cyt}$ homeostasis plays important roles in many aspects of plant growth and development. This role is especially important under salinity, according to the proper function of calcium in stress signaling pathways, which are usually closely linked to the cytosolic pH (pH_{cyt}) homeostasis (Kader and Lindberg, 2010). Furthermore, the acidification of the apoplast is an essential premise for plant growth (Hager, 2003; Pitann et al., 2009a), and can be regulated by Ca^{2+} through the Donnan interaction between Ca^{2+} and H^+ in the cell wall, in addition to effluxes of H^+ from the cells by the PM H^+-ATPase pump (Shabala, 2002). This pump activity is likely regulated by $[Ca^{2+}]_{cyt}$ homeostasis under stress (Fuglsang et al., 2007).

The intracellular $[Na^+]_{cyt}/[K^+]_{cyt}$ homeostasis is important for cell metabolism and is considered as a key component of salt resistance in plants under salinity (Maathuis and Amtmann, 1999; Chen et al., 2007; Munns and Tester, 2008; Sun et al., 2010). Due to the essential role of K^+ in the plant as a co-factor for many cytosolic enzymes, Na^+ toxicity results from its competition with K^+ at binding sites, and can inactivate the enzymes active sites and essential cellular functions (Maathuis and Amtmann, 1999). Highly salt-resistant species are able to maintain low $[Na^+]_{cyt}/[K^+]_{cyt}$ and $[Na^+]_{cyt}/[Ca^{2+}]_{cyt}$ ratios (Munns and Tester, 2008; Sun et al., 2010). Moreover, high concentrations of Na^+ may also replace Ca^{2+} from the membranes, leading to a decrease in the K^+/Na^+ selectivity (Cramer, 2002; Munns and Tester, 2008).

In order to maintain low $[Na^+]_{cyt}/[Ca^{2+}]_{cyt}$ and $[Na^+]_{cyt}/[K^+]_{cyt}$ ratios under saline conditions, an accumulation of excessive amounts of Na^+ in the cytosol should be prevented along with retention of physiological concentrations of $[Ca^{2+}]_{cyt}$ and $[K^+]_{cyt}$ (Maathuis and Amtmann, 1999; Munns and Tester, 2008; Sun et al., 2010). However, our understanding of how this is achieved by the plant is still limited. According to the plant's efficiency, or ability, to re-distribute various ions between apoplast, cytosol and vacuole, and especially by excluding Na^+ from the cytosol to prevent its toxic effect, the plant resistance may increase (Munns and Tester, 2008). There are different suggested mechanisms describing how the plant can do this: i) prevent excessive $[Na^+]_{cyt}$ accumulation by reducing Na^+ influx into the cytosol, or excluding Na^+ into the apoplast and/or into the vacuole (Blumwald and Poole, 1987; Kader and Lindberg, 2005; Demidchik and Maathuis, 2007; Shabala, 2013), ii) increase the ability of plants to retain Ca^{2+} and increase the resting $[Ca^{2+}]_{cyt}$ (Sun et al., 2010; Morgan et al., 2013) or iii) exhibit a greater capacity to retain $[K^+]_{cyt}$ (Maathuis and Amtmann, 1999; Shabala, 2002; Shabala et al., 2006; Chen et al., 2007; Sun et al., 2010). All those ion fluxes are mediated by antiporters, symporters or channels (i.e., Na^+/H^+ antiporters: excluding Na^+ from the cytoplasm under salinity stress), and are driven by the electrochemical H^+ gradient at the plasma membrane and tonoplast (Chen et al., 2007; Shabala, 2013). The ability to control $[Na^+]_{cyt}/[Ca^{2+}]_{cyt}$ and $[Na^+]_{cyt}/[K^+]_{cyt}$ homeostasis in salinity resistant plants is usually associated with a higher PM H^+-ATPase activity and proposed to be involved in salt resistance (Sun et al., 2010; Chen et al., 2012).

To our knowledge only few studies have considered the changes of free apoplastic ion activities in growing leaves under salinity, and how these changes are correlated with the cytosolic ion activities and PM H^+-ATPase activity. In order to better understand the plant's reactions under salinity, and how these reactions can affect plant growth, we then studied the compartmentation of ions under salt stress. In this context, we investigated the changes in pH_{apo}, pH_{cyt}, $[Ca^{2+}]_{cyt}$, $[Na^+]_{cyt}$ and $[K^+]_{cyt}$ homeostasis, PM H^+-ATPase activity, as well as the overall concentrations of Ca^{2+}, Na^+ and K^+ under salinity in leaves of field bean. These parameters were studied, with and without extra calcium or potassium supply to a control and to a saline nutrient solution with a sufficient amount of nutrients. The aim was to clarify how these additional supplies affect PM H^+-ATPase in keeping the desired ion homeostasis under salinity conditions and its relations to apoplastic, free cytosolic and overall ion concentrations.

2. Methods

2.1. Cultivation

Seeds of field bean (*Vicia faba* L. cv. Fuego) were soaked in an aerated $CaSO_4$ solution (0.5 mM) for 1 day at 25 °C and subsequently placed into quartz sand moistened with $CaSO_4$ (1 mM). After seed germination (9 days) seedlings were transferred to 25-L plastic containers (35 plants per container which decreased to 15 plants per container after taking the 1st sample) and were placed in a climate chamber at day/night temperature 20/15 °C for 14/10 h, light intensity 350 μmol s^{-1} m^{-2}, and 50–60% humidity. The aerated nutrient solution had the following composition: 2.0 mM $Ca(NO_3)_2$, 1 mM NaCl, 0.1 mM KH_2PO_4, 1.0 mM K_2SO_4, 0.2 mM KCl, 0.5 mM $MgSO_4$, 60 μM Fe-EDTA, 10 μM H_3BO_4, 2.0 μM $MnSO_4$, 0.5 μM $ZnSO_4$, 0.2 μM $CuSO_4$, 0.05 μM $(NH_4)_6Mo_7O_{24}$, but was diluted 1:4 from start of cultivation in order to avoid osmotic and salt shocks of the seedlings. The concentration was daily increased to reach a final concentration after 4 days of cultivation. Thereafter, extra 100 mM NaCl were added to the nutrient solutions with or without extra 5 mM $CaSO_4$ or extra 10 mM K_2SO_4 as a third portion on consecutive days until reaching the final desired concentrations of 1 or 101 mM NaCl with or without $CaSO_4$ (2 and 7 mM Ca^{2+} final concentrations) or K_2SO_4 (2.3 and 12.3 mM K^+ final concentrations) after 3 days in the nutrient solution. Thereafter, the plants were cultivated for 3 weeks. The nutrient solution was renewed twice a week to avoid nutrient depletion. After 7 d and 21 d from reaching the final concentrations the apoplastic measurement were performed on the second uppermost intact leaves and plant samples were taken for different analyses.

2.2. Determinations of pH_{apo}

The pH_{apo} was determined using fluorescence microscopy-based ratio imaging technique according to Geilfus and Mühling (2011). In intact plants from different treatments, each leaflet of the intact second uppermost leaf was infiltrated by using a syringe with 25 μM of Oregon Green 488 dye, conjugated to 10 kDa dextran (Invitrogen GmbH, Darmstadt, Germany), for pH_{apo} determination. Briefly, the indicator was inserted directly into the apoplast using a syringe (without a needle). By means of gentle pressure, the dye was loaded into the leaf entering the apoplast of the living plants through the stomata. The dextran containing dyes are large molecules, which are non-permeable to the cell membranes and do not enter the symplast, and can therefore be used for pH detection in the apoplast according to Geilfus and Mühling (2011, 2013).

Images were captured on the intact leaves after 2 h from infiltrating the Oregon Green 488 dye according to Geilfus and Mühling (2011), to allow the dye to diffuse through the leaf apoplast and to get rid of any excess water originated from the dye solution through the stomata. The images were captured as single images using a Leica inverted microscope (DMI6000B; Leica Microsystems, Wetzlar, Germany) connected to a DFC-camera (DFC 360FX; Leica Microsystems, Wetzlar, Germany) via a 20-fold magnification, 0.4 numerical aperture, dry objective (HCX PL FLUOTAR L, Leica Microsystems, Wetzlar, Germany). An HXP lamp (HXP Short Arc Lamp; Osram, München, Germany) was used for illumination at excitation wavelengths of 440/20 nm and 495/10 nm. The exposure

time was 25 mS for both channels. Fluorescence was detected at both excitation channels by using a 535/25 nm-emission band-pass filter (BP 535/25; ET535/25M; Leica Microsystems, Wetzlar, Germany) and a dichromatic mirror (LP518; dichotic T518DCXR BS, Leica Microsystems, Wetzlar, Germany).

Ratio images were calculated as a terms of pixel-by-pixel basis as F_{495}/F_{440} using LAS AF software (version 2.3.5, Leica Microsystems, Wetzlar, Germany). The background noise values were subtracted at each channel. The quantitative measurements were calculated from each image as the ratio of the mean intensity for the whole image as a region of interest.

For pH_{apo} calibration, fluorescence ratio images for pH_{apo} were displayed with spectral pseudo-color display hue from purple (no signal), over blue (lowest pH signal; pH 3.9), to pink (highest pH signal; pH 6.3) (Supplementary Fig. S1) set by the limits of an in vivo calibration which was obtained by infiltrating the intact leaves with Oregon Green 488 dextran buffered with 100 mM MES (2-[N-morpholino] ethanesulfonic acid) to a pH ranging from 3 to 7 (steps of 0.5 pH units) as shown in Geilfus and Mühling (2011).

2.3. Determinations of pH_{cyt}, $[Ca^{2+}]_{cyt}$, $[K^+]_{cyt}$ and $[Na^+]_{cyt}$

After 7 d and 21 d under salinity growth conditions, the pH_{cyt}, $[Ca^{2+}]_{cyt}$, and $[Na^+]_{cyt}$ were determined according to the methods cited by Morgan et al. (2013). In brief, the protoplasts were isolated from the second uppermost leaf by an enzymatic method. The isolated protoplast suspensions were divided into 4 portions, and each portion was loaded in darkness with one of the different dyes in the form of acetoxy methyl-ester (AM) (All used dyes were purchased from Invitrogen GmbH, Darmstadt, Germany). For pH_{cyt} determination, the tetra (acetoxy methyl) ester of bis-carboxyethyl-carboxyfluorescein (BCECF-AM) was loaded at 4 °C for 50 min (Kader et al., 2007). Moreover, for $[Ca^{2+}]_{cyt}$ determination the calcium binding benzofuran (Fura 2-AM) was loaded at 22 ± 1 °C for 3 h (Kader et al., 2007), and for $[Na^+]_{cyt}$ determination, the sodium-binding benzofuran isophthalate (SBFI-AM) was loaded at 22 ± 1 °C for 4 h (Kader and Lindberg, 2005). For $[K^+]_{cyt}$ determination the potassium-binding benzofuran isophthalate (PBFI-AM) was loaded at 22 ± 1 °C for 3 h according to Lindberg (1995).

Before measurements, samples were kept in darkness at room temperature for 30 min to allow the protoplasts to recover after centrifugation. An epi-fluorescence microscope (Axiovert 10; Zeiss, Oberkochem, Germany), supplied with an electromagnetic filter-exchanger, xenon lamp (XBO 75), photometer, microprocessor (MSP 201) and a personal computer was used to determine fluorescence intensity of the protoplasts after dye excitation at 340/380 nm for the Fura 2, PBFI and SBFI dyes and at 485/436 nm for the BCECF dye. Emission wavelengths were 510–550 nm for Fura 2 and BCECF, and 500–530 nm for PBFI and SBFI. All measurements were performed with a Planneofluar ×40/0.75 objective (Zeiss) for phase contrast. The ratio measurements were performed only with protoplasts of similar size and properly loaded only in the cytosol. Adjustment for signals and noise was made automatically. The effect of different dye concentration can be eliminated by means of ratio imaging. Micro-slides were covered with 0.2% poly-l-lysine (MW 150,000–300,000, Sigma) in order to attach protoplasts to their surfaces. For measurements, only protoplasts of similar size, with a dense cytoplasm were selected. The cell viability was always checked before, and after, the fluorescence measurements by measuring the presence of fluorescence inside the cells, because the dye hydrolysis is a good viability indicator, and also by checking the protoplasmic streaming and any visible change in size and shape of the protoplasts.

For pH_{cyt} *in situ* calibration, the BCECF-AM fluorescence ratio (485/436 nm) corresponding to different pH-values was provided on single protoplasts according to the methods cited by Kader et al. (2007). Measurements of BCECF-fluorescence standards were performed with protoplasts in suspension solutions at pH-values 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0. The standard measurements were undertaken 5–10 min after addition of 5 μM nigericin (Sigma) to equilibrate intracellular and extracellular concentrations of H^+.

An *in situ* calibration of the Fura-2-AM fluorescence ratio (340/380 nm) was provided on single protoplasts according to the methods cited by Sebastiani et al. (1999) and Kader et al. (2007). In brief, the Ca^{2+} concentrations were calculated according to the equation:

$$\left[Ca^{2+}\right]_{cyt} = K_d[(R - R_{min})/(R_{max} - R)]S_{f2}/S_{b2}nM$$

where:

K_d = dissociation value of the complex [Ca^{2+}-Fura 2].
R_{min} = fluorescence ratio values under Ca^{2+}-free conditions.
R_{max} = fluorescence ratio values under saturating Ca^{2+}conditions.
S_{f2}/S_{b2} = ratio of fluorescence values for Ca^{2+}-bound to Ca^{2+}-free indicator measured after excitation at 380 nm.

The mean values of *R*min, *R*max and S_{f2}/S_{b2} were determined from the *in situ* calibration experiments as cited by Sebastiani et al. (1999). In brief, a droplet of protoplast suspension (50 μl) was added to a micro-slide covered with poly L-lysine. After 5 min the fluorescence measurement at a single protoplast was started and the following solutions were added directly to the protoplast suspension: (a), 1 μl of 200 mM ethylene glycol-bis(beta-aminoethyl ether) *N*,*N*,*N*,*N*-tetra acetic acid (EGTA, Sigma), 1.5 M Tris (hydroxymethyl) amino methane (Tris) pH 8.7; (b) 2 μl of 80 μM Ca^{2+} ionophore A 23187 (Sigma); (c) 1 μl of 500 mM $CaCl_2$ when the minimum value of the ratio 340/380 nm was reached; (d) 2 μl of 125 mM $MnCl_2$ when the maximum value of ratio 340/380 was reached.

For $[Na^+]_{cyt}$ *in situ* calibration, the SBFI-AM fluorescence ratio (340/380 nm) corresponding to different Na^+ concentrations was obtained on single protoplasts according to the methods cited by Kader and Lindberg (2005). Measurements of SBFI-fluorescence standards were performed with protoplasts in separate suspension solutions with concentrations of 0, 25, 50, 75 and 100 mM NaCl. KCl was added to the suspension solutions to give a final concentration of 100 mM [Na^++K^+] to approximate physiological ionic strengths. The standard measurements were undertaken 5–10 min after addition of 10 μM gramicidin (Sigma) to equilibrate intracellular and extracellular concentrations of Na^+. As salt stress also induces cytosolic acidification, 5 μM nigericin was added to avoid a pH effect.

For $[K^+]_{cyt}$ *in situ* calibration, the PBFI-AM fluorescence ratio (340/380 nm), corresponding to different K^+ concentrations, was provided on single protoplasts as described by Lindberg (1995). Measurements of PBFI-fluorescence standards were performed with protoplasts in separate suspension solutions with concentrations of 0, 50, 100, 150 and 200 mM KCl. NaCl was added to the solutions to give a final concentration of 200 mM [Na^++K^+] to approximate physiological ionic strengths. The standard measurements were undertaken 5–10 min after addition of 10 μM gramicidin to equilibrate intracellular and extracellular concentrations of K^+. Nigericin was added at a final concentration of 5 μM to avoid pH effect.

2.4. Determinations of PM H^+-ATPase activity and kinetics

The plasma membranes (PMs) were isolated from the second uppermost leaves according to Larsson et al. (1994) with some

modification. Approximately 25 g of fresh leaves from the different treatments were collected after 7 d and 21 d and kept in −80 °C until use. Frozen leaves were directly homogenized in 50 ml ice-cold homogenization buffer with a *Broun*-hand-mixer. The homogenization buffer contained 250 mM sucrose, 5 mM EDTA, 5 mM dithiothreitol (DTT), 5 mM ascorbate, 0.2% (w/v) bovine serum albumin (BSA), 0.6% (w/v) polyvinylpyrrolidone (PVPP), 0.2% (w/v) casein (boiled for 10 min before supply), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 50 mM Mops-KOH, pH 7.5. The homogenate was filtered through 2 layers of Miracloth and centrifuged at 10,000 × g for 30 min at 4 °C. The supernatant was further centrifuged at 48,000 × g for 75 min. Thereafter, the obtained microsomal pellet was re-suspended in a 2 mL phosphate buffer, containing 250 mM sucrose, 5 mM KCl and 5 mM KH_2PO_4 (pH 7.8).

The microsomal fraction was then partitioned by a 3-steps procedure using a two-phase system (portions of exact 16 g final weight) containing 6.0% (w/w) dextran T-500 (Pharmacia Fine Chemicals), 6.0% (w/w) polyethylene glycol PEG 3350 (Sigma), 250 mM sucrose, 0.1 mM EDTA, 1 mM DTT, 5 mM KCl and 5 mM KH_2PO_4 (pH 7.8). Two grams of the re-suspended microsomal fraction was added to the upper phase of the two-phase system. After heavily shaking of the system, phase separation was achieved by centrifugation at 4 °C and 1500 × g for 4 min. The upper phase was then added to a new lower phase containing the PEG. This step was repeated two more times to get the pure plasma membranes in the upper phase. The finally obtained upper phase was diluted 10 folds with a medium containing 250 mM sucrose, 5 mM EDTA, 1 mM DTT and 10 mM Mops-Tris, pH 7. The pellet (plasma membrane proteins) was collected at 48,000 × g for 120 min and re-suspended in 1 ml of the same medium. The PM were immediately frozen and kept at −80 °C until use. Protein quantification was performed according to Bradford (1976). All steps were carried out at 4 °C.

After thawing the PM suspension at room temperature, the ATPase assays were performed by the method of Lindberg and Wingstrand (1985) with some modifications. To detect the hydrolytic activity in the presence of Mg-ATP, the assays were run in triplicate for 20 min at 35 °C in a 200 µl medium containing 30 µl PM suspension (80–140 µg protein per ml, pH 7), 20 µl 0.02% Brij 58, $MgCl_2$, 1, 1.5, 2.5 or 3.5 mM final concentrations, and 0.55, 1.1, 2.2 or 3.3 mM ATP (ATP-diTris, Sigma) final concentrations, as well as double-distilled water (to adjust the volume to 200 µl). To detect the activity in the presence of ATP and enzyme only, (background), $MgCl_2$ was replaced by double-distilled water. To detect the activity in the presence of K^+ + MgATP, 50 mM KCl final concentration was added. The reaction was started by addition of ATP buffer and stopped by addition of 20 µl of ice-cold 33% tri-chloro acetic acid (TCA). Then the tubes were directly transferred to ice-bath. The ATPase activity was determined as the release of inorganic phosphate (Pi) according to Lindberg and Wingstrand (1985). The *K*m and *V*max values were calculated by use of Hanes plots.

2.5. Determination of plant growth and overall ion concentrations

Plant samples were taken after 7 d and 21 d of salinity treatment (1 and 100 mM NaCl) and/or Ca^{2+} (0 and 5 mM $CaSO_4$) and/or K_2SO_4 (0 and 10 mM) treatments. Fresh and dry weights of the second uppermost leaves were measured. Two hundred mg of leaf dry weights were ashed overnight in an oven at 520 °C. The ash was dissolved in 2 mL 4 N HNO_3 with gentle heating. This suspension was adjusted to a volume of 10 mL with distilled water and filtered through a paper filter (Schleicher and Schuell, white, Germany). Thereafter, the Na^+, K^+ and Ca^{2+} concentrations were analyzed using atomic absorption spectrophotometer (SpectrAA-100, Varian, Springvale, Australia).

2.6. Statistics

The apoplastic experiments were performed 4 times (biological replicates). In each experiment and from each treatment, 3 plants ware used to detect the pH_{apo} using fluorescent microscopy-based ratio imaging technique with capturing more than 5 different image fields from each leaflet (more than 15 image replicates from each treatment). Moreover, the cytosolic experiments were performed 3 times (biological replicates), with more than 20 different single protoplasts measured in each experiment and from each treatment. Furthermore, the PM H^+-ATPase activity and kinetic experiments were performed 3 times (biological replicates). For growth character measurements 15 plants were used and for atomic absorption measurements 3 replicates were used. All collected data were statistically analyzed using factorial randomized complete block design and the means were compared using the least significant difference test (L.S.D.) at 5% level of probability to indicate treatment differences (Snedecor and Cochran, 1980).

3. Results

3.1. Influence of calcium and potassium supply on pH_{apo} in leaves

The pH_{apo} was detected in the intact second-uppermost leaves after 7 d (ST) and 21 d (LT), under control or saline conditions, with or without extra calcium or potassium supply during the cultivation.

A pH_{apo} alkalinization occurred in leaves in response to salinity stress (Fig. 1 and Supplementary Fig. S1). Calcium supply under saline condition decreased the alkalinization of the leaf apoplast at both ST and LT, but extra potassium supply did not change the pH_{apo} (Fig. 1). Under control condition the pH_{apo} was the same, irrespective of treatments.

3.2. Influence of calcium and potassium supply on cytosolic ionic homeostasis and pH in leaf protoplasts

The $[Ca^{2+}]_{cyt}$, pH_{cyt}, $[Na^+]_{cyt}$ and $[K^+]_{cyt}$ were detected in living protoplasts isolated from the second-uppermost leaves after ST and LT, under control or saline conditions, with or without extra calcium or potassium supply during the cultivation.

The results show that calcium or potassium supply during the cultivation affected the $[Ca^{2+}]_{cyt}$ in different ways. Calcium supply

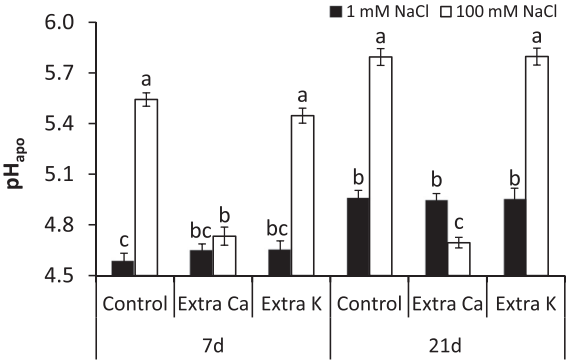

Fig. 1. The pH_{apo} in the intact second uppermost leaf of field bean plants cultivated at 1 or 100 mM NaCl for 7 d and 21 d, with and without extra-calcium (5 mM) or -potassium (10 mM) supply to the nutrient solution. Significant differences between the treatments within the same date were shown at $p < 0.05$ by LSD-test and the error bars indicate the mean standard error ($n \geq 60$).

increased the resting $[Ca^{2+}]_{cyt}$ in leaf protoplasts regardless of salinity (Fig. 2A). However, by potassium supply, the resting $[Ca^{2+}]_{cyt}$ only decreased after LT under control condition, while the other potassium treatments did not affect the resting $[Ca^{2+}]_{cyt}$. Whenever the resting $[Ca^{2+}]_{cyt}$ increased under salinity stress, an increase in pH_{cyt} alkalinization was simultaneously recorded. After LT, the pH_{cyt} alkalinization increased at all treatments (Fig. 2B).

As expected salinity treatments increased the $[Na^+]_{cyt}$ both after ST and LT compared with the corresponding controls. Calcium supply abolished this increase after both ST and LT, but potassium supply abolished it only after LT (Fig. 2C). Moreover, after ST, the $[K^+]_{cyt}$ decreased under salinity stress at different treatments (Fig. 2D). Interestingly, within the salinity treatments, calcium supply increased $[K^+]_{cyt}$ after ST, while decreased it after LT.

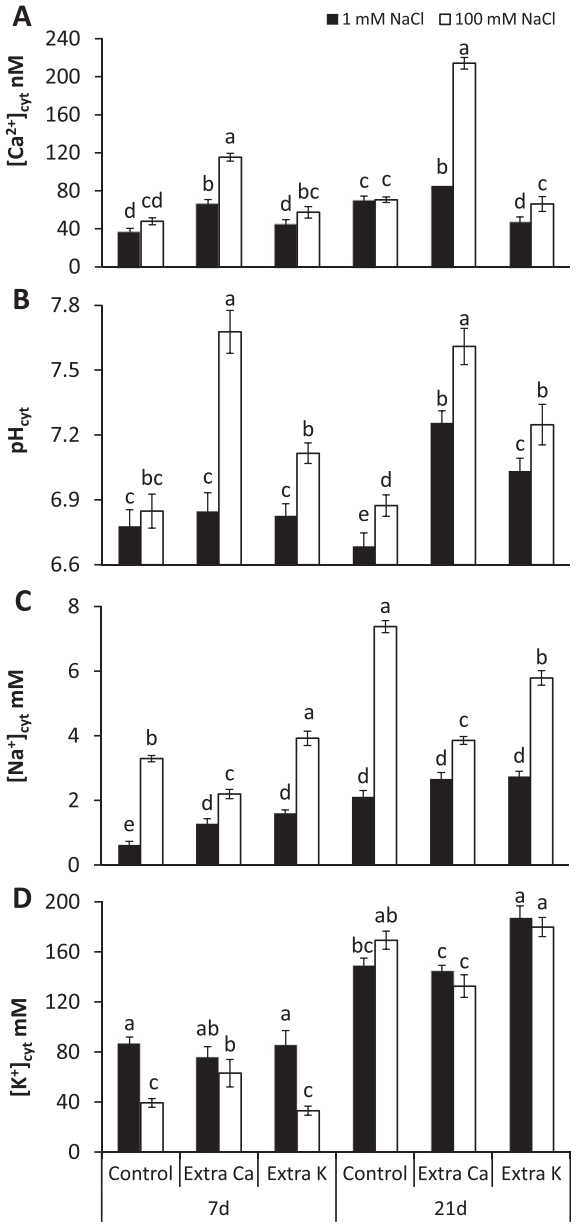

Fig. 2. The $[Ca^{2+}]_{cyt}$ (A), pH_{cyt} (B), $[Na^+]_{cyt}$ (C) and $[K^+]_{cyt}$ (D) in the protoplasts isolated from the second uppermost leaf of field bean plants cultivated at 1 or 100 mM NaCl for 7 d and 21 d, with and without extra-calcium (5 mM) or -potassium (10 mM) supply to the nutrient solution. Significant differences between the treatments within the same date were shown at $p < 0.05$ by LSD-test and the error bars indicate the mean standard error ($n \geq 60$).

Table 1
The $[Na^+]_{cyt}/[Ca^{2+}]_{cyt}$ ratios and the $[Na^+]_{cyt}/[K^+]_{cyt}$ ratios in protoplasts isolated from the second uppermost leaves of field bean plants cultivated at 1 or 100 mM NaCl for 7 d and 21 d, with and without extra-calcium (5 mM) or -potassium (10 mM) supply to the nutrient solution.

Treatments	$[10^{-3}\ Na^+]_{cyt}/[Ca^{2+}]_{cyt}$		$[Na^+]_{cyt}/[K^+]_{cyt}$	
	7 d	21 d	7 d	21 d
1 mM NaCl (Control)	17	31	0.1	0.1
100 mM NaCl (Saline condition)	69	104	0.8	0.4
Calcium supply	20	32	0.2	0.2
Calcium supply + 100 mM NaCl	19	18	0.3	0.3
Potassium supply	36	59	0.2	0.1
Potassium supply + 100 mM NaCl	68	88	1.2	0.3

Contrariwise, without salinity, potassium supply increased $[K^+]_{cyt}$ only after LT.

Upon salinity stress, with or without extra potassium, the $[Na^+]_{cyt}/[Ca^{2+}]_{cyt}$ ratios increased about 35% after ST (Table 1). However, when calcium was supplied, the same ratios decreased to approximately equivalent levels under both control and saline conditions and almost to the same level as for the control plants. Also after LT salinity stress, the $[Na^+]_{cyt}/[Ca^{2+}]_{cyt}$ ratios decreased with calcium or potassium supplies, but less with potassium (Table 1). Upon salinity stress, after ST the $[Na^+]_{cyt}/[K^+]_{cyt}$ ratio decreased with calcium supply but increased with potassium supply. After LT the $[Na^+]_{cyt}/[K^+]_{cyt}$ ratio was approximately the same in all salinity treated plants regardless of calcium or potassium supply.

Instead of measuring the proton pumping activity after different growth conditions, we calculated the pH difference between the apoplast and cytosol, which gives similar information. Under non-saline condition, the cytosolic-apoplastic pH difference was stable with or without calcium or potassium supply after ST and LT (Table 2). Upon salinity stress this pH difference decreased after ST and LT. On the other hand, by calcium supply under saline condition, the cytosolic-apoplastic pH difference increased after ST and LT to reach even a higher value than in the control plants, while with potassium supply this difference increased only after LT to reach the same value in the control plants.

3.3. *Influence of calcium and potassium supply on PM H^+-ATPase activity of leaves and kinetic parameters*

Under non-saline condition, the hydrolytic activity of the H^+-ATPase, in the presence of Mg^{2+} (1.0, 1.5, 2.5 or 3.5 mM $MgCl_2$) given *in vitro*, increased after LT compared with ST (Fig. 3A). The calcium supply increased the H^+-ATPase activity after ST and LT

Table 2
The cytosolic apoplastic pH differences in the second uppermost leaves of field bean plants cultivated at 1 or 100 mM NaCl for 7 d and 21 d, with and without extra-calcium (5 mM) or -potassium (10 mM) supply to the nutrient solution. The values represent means ± standard error ($n \geq 60$). Letters indicate the significant differences between the treatments within the same date at $p < 0.05$ by LSD-test.

Treatments	$(pH_{cyt} - pH_{apo})$	
	7 d	21 d
1 mM NaCl (Control)	2.1 ± 0.03 b	1.7 ± 0.01 b
100 mM NaCl (Saline condition)	1.4 ± 0.04 c	1.3 ± 0.01 c
Calcium supply	2.1 ± 0.05 b	2.3 ± 0.01 ab
Calcium supply + 100 mM NaCl	2.8 ± 0.04 a	2.9 ± 0.05 a
Potassium supply	2.1 ± 0.01 b	2.0 ± 0.01 b
Potassium supply + 100 mM NaCl	1.7 ± 0.04 c	1.7 ± 0.02 b

under both saline and non-saline conditions. In contrast, potassium supply under salinity increased the activity only after LT. Moreover, for control plants, the hydrolytic activity of the H^+-ATPase in presence of both Mg^{2+} and K^+ *in vitro* was much higher than in the presence of only Mg^{2+}.

After salinity the activity in presence of both Mg^{2+} and K^+ *in vitro* mainly increased after calcium supply in vivo, but decreased after potassium supply in vivo (Fig. 3B).

The kinetic parameters *K*m and *V*max were estimated by Hanes plots (MgATP v^{-1} versus MgATP) (Fig. 4), showing -apparent *K*m as an intersection with the X-axis. These plots show straight lines and Michaelis Menten kinetics for control, calcium and potassium treatments after ST (Fig. 4A) or LT (Fig. 4B–D). The calcium supply increased *V*max in both ST and LT plants (Table 3), as the slope of the line (1/Vmax) decreased (Fig. 4A). On the other hand, the extra potassium supply in vivo decreased *V*max both at ST and LT compared with control (Fig. 4A,B and Table 3). After extra Ca supply at ST and LT the lines intersect close to each other on the x-axis (-*K*m), with no significant difference, irrespective of salinity (Fig. 4A,D and Table 3). When LT plants were treated with potassium supply in vivo, Hanes plots for control and saline conditions showed parallel straight lines with different intersection on the x-axis (Fig. 4C). Thus, extra calcium did not influence *K*m, while potassium decreased it. The *K*m was higher for the combined treatment (potassium supply under saline condition) compared with potassium supply under control condition, which could reflect a competitive inhibition between K^+ and Na^+. On the contrary, the straight lines for calcium supply under control and saline conditions after LT intersect mainly on the x-axis (Fig. 4D) and show a non-competitive interaction with no difference in *K*m values (Table 3).

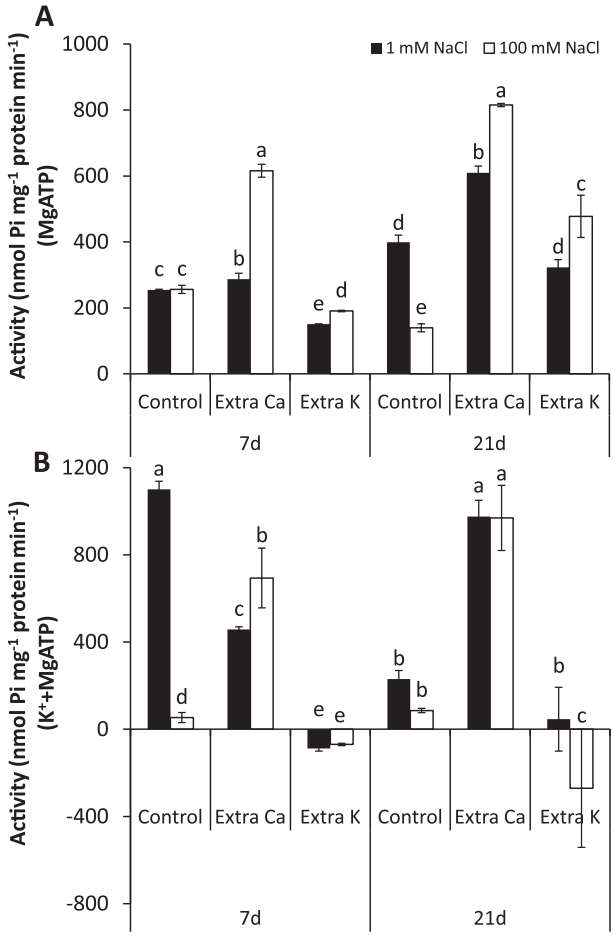

Fig. 3. Comparison of the PM H^+-ATPase hydrolytic activity in the presence of 3 mM MgATP (A), or 3 mM MgATP + 50 mM KCl (B) of plasma membranes vesicles derived from the second uppermost leaf of field bean plants cultivated at 1 or 100 mM NaCl for 7 d and 21 d, with and without extra-calcium (5 mM) or -potassium (10 mM) supply to the nutrient solution. Significant differences between the treatments within the same date were shown at $p < 0.05$ by LSD-test and the error bars indicate the mean standard error ($n = 9$).

3.4. Influence of calcium and potassium supply on total concentrations of Ca^{2+}, Na^+ and K^+ in the second uppermost leaves

The overall Ca^{2+} concentration of leaves didn't change significantly after salinity stress during ST and LT or when potassium was supplied, while calcium supply increased Ca^{2+} concentration (Fig. 5A). Here by, overall Ca^{2+} concentration was higher when calcium was supplied under control than under saline conditions. As expected the total Na^+ concentration increased under salinity stress regardless of different external treatments (Fig. 5B). However, both calcium and potassium supply under saline condition decreased this accumulation of Na^+ in the treated plant leaves. After ST under salinity the calcium exhibited a superior effect in decreasing the Na^+ accumulation compared with potassium. Salinity mainly decreased the overall K^+ concentration (Fig. 5C). The potassium supply at ST and LT increased the overall K^+ concentration under both control and saline conditions. At non-saline condition, the overall K^+ concentration increased with calcium supply only after ST, but did not change after LT.

The overall Na^+/Ca^{2+} ratios and Na^+/K^+ ratios after both ST and LT showed the same trend (Table 4). The ratios decreased with calcium or potassium supply when compared with plants grown under the same salinity level. Thus, only a reported increase in the overall Na^+/Ca^{2+} ratio was obtained when the potassium was supplied for LT under saline condition (For shoot ion concentrations and ion ratios after ST and LT, see Supplementary Fig. S2 and Supplementary Table S1).

3.5. Influence of calcium and potassium supply on fresh and dry weights

The fresh and dry weights of shoots and second-uppermost leaves were reduced in plants grown under salinity stress (Fig. 6). Moreover, after both ST and LT under saline condition, the shoot fresh and dry weights increased by calcium or potassium supply with no differences in-between treatments. On the other hand, the second-uppermost leaves fresh and dry weights increased only by calcium supply and not by potassium supply after both ST and LT under saline condition.

4. Discussion

4.1. Changes in apoplastic pH under saline conditions

According to the acid-growth-theory (Hager, 2003), a major requirement for increasing cell wall extensibility and leaf growth is to establish a low pH_{apo}. Under salinity the maintenance of a low apoplastic pH is drastically affected, leading to a pH_{apo} alkalinization (Hager, 2003). Such alkalinization was obtained in the leaves of maize plants growing for several days under NaCl stress (Pitann et al., 2009a, 2009b), and also in intact *V. faba* leaves within a few min after addition of NaCl to the roots (Geilfus and Mühling, 2011, 2013). Upon adding NaCl, also the chloride ions are transported and could cause a membrane hyperpolarization (Geilfus and Mühling, 2013). Additionally, the apoplastic pH can be alkalized as a

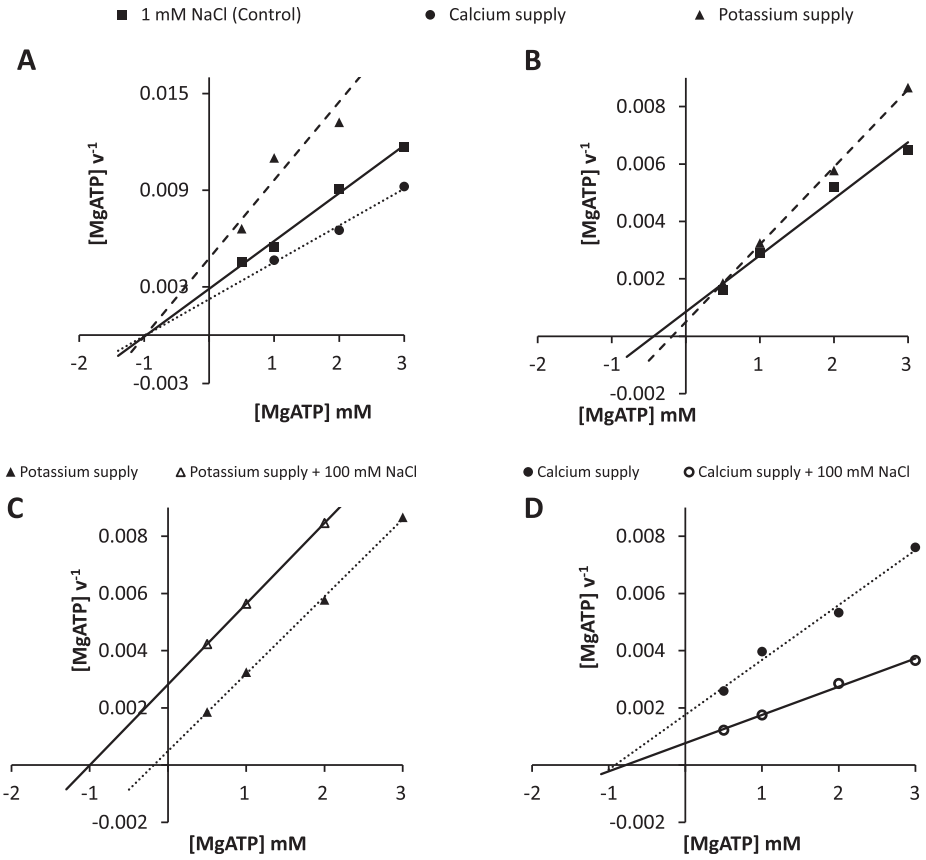

Fig. 4. Hanes plots showing the MgATPase activities in PM of bean leaves after cultivation for 7 d (A) or 21 d (B–D) at different conditions. A and B, Activity in control plants, after extra-potassium supply and after extra-calcium supply; C and D, Activity after extra-potassium supply at non-saline conditions, extra potassium supply at saline conditions, extra-calcium supply at non-saline conditions, and extra-calcium supply at saline conditions. Assays were run at 35 °C for 20 min. Activities are expressed in nmol Pi, mg protein^{-1}, min^{-1} ($n = 9$).

consequence of H^+/Cl^- symport (Geilfus and Mühling, 2011, 2013), but this has not been investigated here.

In the present investigation, results from salinity treatment of the plants for 7 d (ST; short-term of growth) or 21 d (LT; long-term of growth) corroborate the above mentioned findings. Thus, the second uppermost leaf of plants grown under saline condition exhibited a leaf apoplastic alkalinization. Only calcium supply, and not potassium supply, resulted in a more pronounced leaf apoplastic acidification (Fig. 1 and Supplementary Fig. S1). Following the acid-growth-theory, this finding could explain why calcium and not potassium supply under saline condition increased the leaves fresh and dry weights after both ST and LT (Fig. 6C and D).

Table 3
Kinetic characteristics of the PM H^+-ATPase vesicles derived from the second uppermost leaf of field bean plants cultivated at 1 or 100 mM NaCl for 7 d and 21 d, with and without extra-calcium (5 mM) or -potassium (10 mM) supply to the nutrient solution. The values represent means ± standard error ($n = 6$). Small letters indicate the significant differences between the treatments within the same date at $p < 0.05$ by LSD-test.

Treatments	*K*m (mM ATP)		*V*max (nmol Pi mg protein^{-1} min^{-1})	
	7 d	21 d	7 d	21 d
1 mM NaCl (Control)	0.90 ± 0.067 b	0.53 ± 0.075 b	333 ± 15.0 b	450 ± 50.0 c
100 mM NaCl (Saline condition)	0.61 ± 0.045 c	0.14 ± 0.052 c	267 ± 3.6 c	109 ± 1.0 e
Calcium supply	0.93 ± 0.026 b	0.91 ± 0.094 a	456 ± 20.7 a	590 ± 34.7 b
Calcium supply + 100 mM NaCl	1.04 ± 0.012 a	0.91 ± 0.091 a	355 ± 61.3 b	955 ± 45.5 a
Potassium supply	0.95 ± 0.031 b	0.19 ± 0.007 c	204 ± 10.0 d	385 ± 14.8 d
Potassium supply + 100 mM NaCl	0.66 ± 0.015 c	0.96 ± 0.042 a	222 ± 4.9 cd	387 ± 29.8 d

4.2. Calcium supply effects on cytosolic ion homeostasis in leaves

The acidification of the leaf pH_{apo} by calcium supply under salinity stress was combined with alkalinization of pH_{cyt}, increase of resting $[Ca^{2+}]_{cyt}$, decrease of $[Na^+]_{cyt}$, and increase of $[K^+]_{cyt}$ (Figs. 1 and 2). These changes are likely due to the higher PM H^+-ATPase activity obtained at extra calcium supply (Fig. 3A), in addition to a high overall Ca^{2+} concentration in the leaves after both ST and LT, which was found at the same growth conditions (Fig. 5A). The increases of the leaves resting $[Ca^{2+}]_{cyt}$ and overall Ca^{2+} under salinity (Figs. 2A and 5A) by extra calcium supply can inhibit the dominant transporters for Na^+ influx into cytosol (Tester and Davenport, 2003; Shabala et al., 2006; Demidchik and Maathuis, 2007; Sun et al., 2010) and reduce the K^+ leakage through the cell membrane (Cramer, 2002; Mühling et al., 1998). This is confirmed here by $[Na^+]_{cyt}$ decrease and $[K^+]_{cyt}$ increase (Fig. 2C and D) leading to $[Na^+]_{cyt}/[Ca^{2+}]_{cyt}$ and $[Na^+]_{cyt}/[K^+]_{cyt}$ homeostasis by calcium supply under salinity (Table 1). These results suggest that the calcium supply helps to stabilize the plasma membrane (Cramer, 2002), and stimulate the H^+-ATPase activity (Fig. 3A). This assumption is in accordance with previous findings

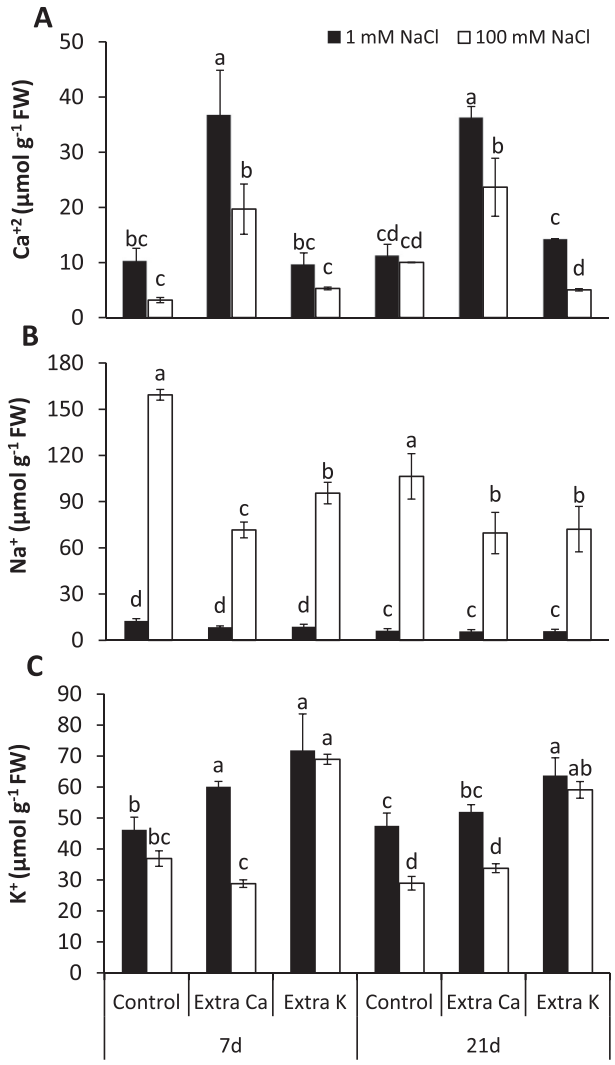

Fig. 5. Overall concentrations of Ca^{2+} (A), Na^{+} (B) and K^{+} (C) in the second uppermost leaf of field bean plants cultivated at 1 or 100 mM NaCl for 7 d and 21 d, with and without extra-calcium (5 mM) or -potassium (10 mM) supply to the nutrient solution. Significant differences between the treatments within the same date were shown at $p < 0.05$ by LSD-test and the error bars indicate the mean standard error ($n = 6$).

(Maathuis and Amtmann, 1999; Kader and Lindberg, 2005; Chen et al., 2007; Pitann et al., 2009b) indicating that a higher transporter activity at the plasma membrane is associated with higher H^{+}-ATPase activity. The higher H^{+}-ATPase activity, obtained by extra calcium supply, provides a higher electrochemical H^{+} gradient. This is confirmed by the larger difference between apoplastic and cytosolic pH after salinity and extra calcium (Table 2). An increased level of $[Ca^{2+}]_{cyt}$ activates the SOS pathway leading to increased Na^{+}/H^{+} antiport activity at the PM and tonoplast (Zhu, 2002).

Table 4
The Na^{+}/Ca^{2+} and Na^{+}/K^{+} ratios in the second uppermost leaf of field bean plants cultivated at 1 or 100 mM NaCl for 7 d and 21 d, with and without extra-calcium (5 mM) or -potassium (10 mM) supply to the nutrient solution.

	Na^{+}/Ca^{2+}		Na^{+}/K^{+}	
Treatments	7 d	21 d	7 d	21 d
1 mM NaCl (Control)	1.2	0.6	0.3	0.3
100 mM NaCl (Saline condition)	50.1	10.4	4.2	3.9
Calcium supply	0.2	0.2	0.4	0.1
Calcium supply + 100 mM NaCl	3.6	2.4	2.5	2.1
Potassium supply	0.9	0.1	0.2	0.1
Potassium supply + 100 mM NaCl	18.1	14.0	1.4	1.2

4.3. Effect of potassium supply on cytosolic ion homeostasis in leaves

Although extra potassium did not affect the pH_{apo} under condition of salt stress, the cytosol was alkalized in LT (Figs. 1 and 2B). A cytosolic alkalinization is considered as an indicator for improved plant-salinity tolerance to some extent (Kader and Lindberg, 2010), while a high pH_{apo} depresses the leaves growth (Hager, 2003). This can explain why only the whole shoots and not the leaves could maintain growth by potassium supply after ST and LT and saline condition (Fig. 6). Thus, the leaf growth may be more related to the pH_{apo} changes, than to pH_{cyt}. Furthermore, the increases in PM H^{+}-ATPase activity only after LT and saline condition were connected with $[K^{+}]_{cyt}$ increase and $[Na^{+}]_{cyt}$ decrease (Figs. 2 and 3). This observation suggests that the $[Na^{+}]_{cyt}/[K^{+}]_{cyt}$ homeostasis is more affected by PM H^{+}-ATPase activity and/or pH_{cyt} changes than by the pH_{apo} changes (Figs. 1A, 2B and 3A and Table 1). It is well known that maintenance of $[K^{+}]_{cyt}$ under saline condition is one of the key factors in determining the ability to tolerate salinity (Maathuis and Amtmann, 1999). Efflux of K^{+} under salinity mainly depends on lack of membrane integrity (Cramer, 2002; Mühling et al., 1998). The finding indicates that the $[Na^{+}]_{cyt}/[K^{+}]_{cyt}$ ratio is more important for salt resistance than the absolute $[Na^{+}]_{cyt}$ activity, as also suggested by other authors (Chen et al., 2007; Sun et al., 2010).

4.4. Effect of calcium and potassium supply on PM H^{+}-ATPase activity

Calcium showed a superior effect to potassium on increasing the PM H^{+}-ATPase activity, as the extra calcium supply increased the PM H^{+}-ATPase activity under saline and non-saline condition after both ST and LT, but potassium increased it only after LT and decreased the activity after ST (Fig. 3A). This could explain why calcium supply improved the cytosolic ion homeostasis after both ST and LT under salinity stress. When the H^{+}-ATPase activity was high, the apoplast acidified, and the cytosol further alkalized, which forms a larger difference between cytosolic and apoplastic pH. This is expected at a high proton–pump activity, when hydrolyzing activity is coupled to the proton pumping (Figs, 1A, 2B, 3A and Table 2).

In the presence of K^{+} *in vitro*, calcium supplied in vivo increased the PM H^{+}-ATPase activity, but, interestingly, this activity was largely reduced when potassium was supplied in vivo (Fig. 3B). At high external K^{+} and a high pH_{apo}, obtained at potassium supply under saline condition, the pH difference between apoplast and cytosol decreased (Table 2). This may reflect a decrease of the plasma membrane electro-chemical gradient generated by the PM H^{+}-ATPase. A decrease in pump activity should decrease Na^{+} exclusion by the PM Na^{+}/H^{+} antiporter and decrease K^{+} uptake (Haruta and Sussman, 2012). Therefore, under saline condition the potassium supply caused an increase of $[Na^{+}]_{cyt}$ and no effect the $[K^{+}]_{cyt}$ at ST (Fig. 2C and D), when the pH difference between apoplast and cytosol was low. On the other hand, $[Na^{+}]_{cyt}$ decreased when the H^{+}ATPase activity and pH difference between compartments increased after LT (Figs. 2C and 3A and Table 2). Under saline condition only the calcium supply caused a higher $[K^{+}]_{cyt}$ and lower $[Na^{+}]_{cyt}$ (Fig. 2C and D). The maintained K^{+} concentration at calcium supply may depend on less leakage of K^{+} (Cramer, 2002; Mühling et al., 1998). Therefore, it is likely that the

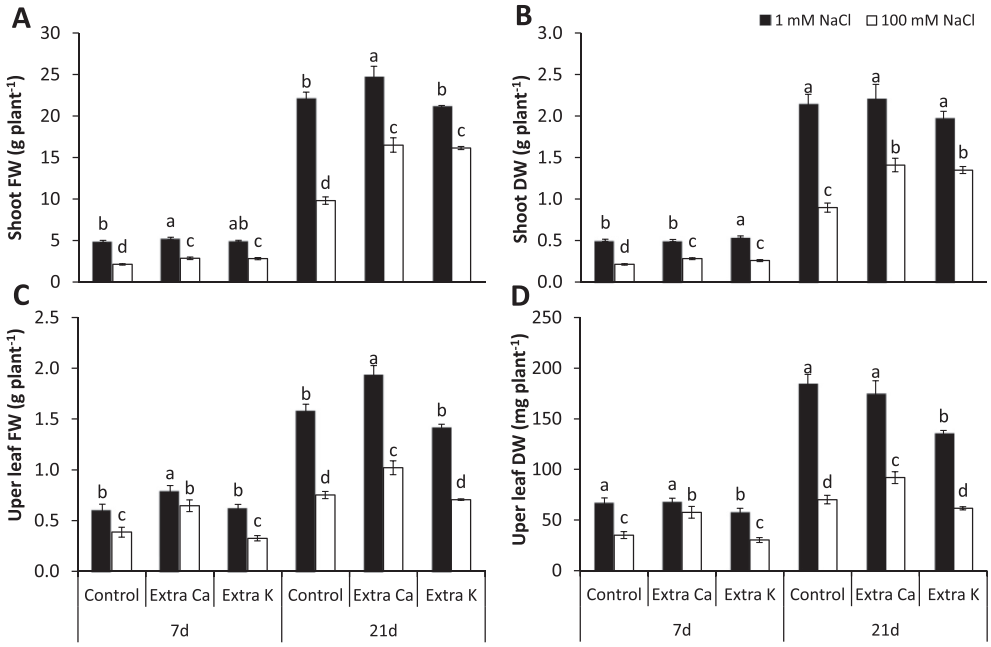

Fig. 6. Fresh weights (g plant^{-1}; A,C) and dry weights (g plant^{-1}; B and mg plant^{-1}; D) of shoots (A,C) and second uppermost leaf (B,D) respectively, of field bean plants cultivated at 1 or 100 mM NaCl for 7 d and 21 d, with and without extra-calcium (5 mM) or -potassium (10 mM) supply to the nutrient solution. Significant differences between the treatments within the same date were shown at $p < 0.05$ by LSD-test and the error bars indicate the mean standard error ($n = 30$).

proton pump mainly maintain the Na^+ exclusion and not the K^+ uptake.

4.5. *Effect of calcium and potassium supply on PM H^+-ATPase kinetics*

The kinetic parameters *K*m and *V*max were influenced in different way by calcium and potassium supply (Table 3). The obtained higher *K*m by potassium supply under salinity after LT reflects a lower affinity between enzyme and the substrate MgATP. Contrariwise, by extra calcium supply Km was not changed, but the enzyme activity and *V*max increased (Fig. 3A and Table 3). The increases in the activity may depend on that more enzymes units are expressed when calcium was supplied under salinity. In that context, it is well known that the cellular Ca^{2+} level has an important role in regulating gene expression and improving the transcriptional level of proteins under stress (Reddy et al., 2011). Also, the PM-proton pump has cation-binding pockets at the phosphorylation and nucleotide binding domains, which can bind both potassium and calcium and thereby modulate the activity (Ekberg et al., 2010). However, the interactions between Ca^{2+}-binding proteins and H^+-pumps in the PM require further investigation (Sun et al., 2010).

When the plants were treated with potassium in vivo, parallel lines were obtained reflecting a competitive inhibition of the H^+-ATPase by salinity after LT (Fig. 4C). On the other hand, when pretreated with calcium supply, there was a non-competitive interaction between calcium at saline and non-saline condition after LT, with no change in *K*m (Fig. 4D). The competition between K^+ and Na^+ may depend on that protons are used for both Na^+/H^+ antiport and K^+/H^+ symport. Such competition does not exist for the H^+-ATPase activity at calcium supply under saline condition, since Ca^{2+} uses other transporter proteins for efflux from the cytosol (Sanders et al., 2002).

In summary, the high PM H^+-ATPase activity will increase the rate of proton pumping, ultimately acidifying the pH_{apo}, alkalizing the pH_{cyt} and reducing the $[Na^+]_{cyt.}$ in addition to increasing the resting $[Ca^{2+}]_{cyt}$ and increasing the $[K^+]_{cyt}$ (model in Fig. 7). All these alterations introduced when calcium is supplied under saline conditions can improve what we can identify as an "apoplastic-cytosolic-plasma membrane" reciprocal system. This system is

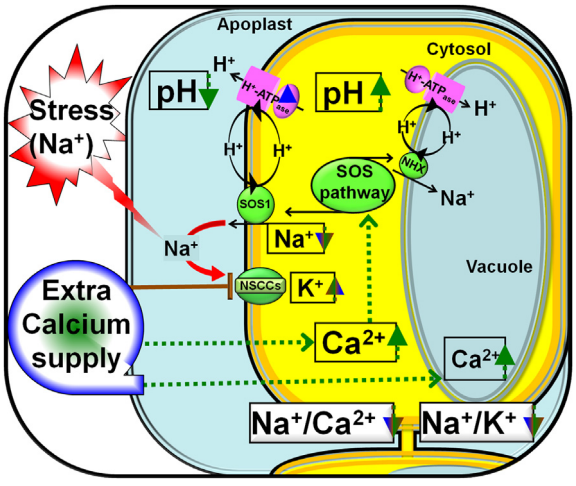

Fig. 7. A simplified model of ion transport in a *Vicia faba* second uppermost leaf at extra-calcium supply under saline conditions. The calcium supply increases the $[Ca^{2+}]_{cyt}$ in ST and LT under saline condition and leads to an activation of the PM H^+-ATPase, and thereby, an acidification of pH_{apo} and alkalinization of pH_{cyt}. Sodium can be taken up into plant cells by different types of NSCCs, non-selective cation channels, and also by the HKT, high affinity potassium transporter, family (Demidchik and Maathuis, 2007; Munns, and Tester, 2008; Shabala, 2013). The NSCCs are also mediating Ca^{2+} influx, and at high Ca^{2+}, it is likely that the influx of Na^+ is inhibited (Kader and Lindberg, 2005). The depolarization of PM by Na^+, may be inhibited by elevated H^+-ATPase activity (Blumwald and Poole, 1987), which prevents efflux of K^+ through NSCCs (Chen et al., 2007; Demidchik and Maathuis, 2007; Sun et al., 2010) and causes increased ion homeostasis. High calcium can prevent the toxic impact of Na^+ on PM permeability, which can decrease K^+ leakage from the cytosol (Mühling et al., 1998; Cramer, 2002). An increase in $[Ca^{2+}]_{cyt}$ activates the SOS system, which transports Na^+ out of the cytosol by SOS1 (antiporter at the PM) and NHX (antiporter at the tonoplast) (Tester and Davenport, 2003; Shabala, 2013) (increasing▲; decreasing▼).

likely to work simultaneously to improve the re-distribution of the ions between the cytosol and the apoplast and derive ion homeostasis. Thus, calcium supply under saline condition improves leaf growth (Fig. 6C and D).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2014.06.010.

Contributions

S.H. Morgan performed all the experiments and together with S. Lindberg and P.J. Maity the plasma membrane preparations and ATPase analyses.

S.H. Morgan wrote the text together with S. Lindberg, C-M. Geilfus and K. M. Mühling.

References

Blumwald, E., Poole, R., 1987. Salt-tolerance in suspension cultures of sugar beet. I. Induction of Na^+/H^+-antiport activity at the tonoplast by growth in salt. Plant Physiol. 83, 884–887.

Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dyebinding. Anal. Biochem. 72, 248–254.

Chen, J., Liu, Z., Shao, H., Qin, S., Zheng, Q., Liu, L., Mao, Y., 2012. Changes in H^+-ATPase activity of plasma membrane enriched vesicles isolated from physic nut roots and leaves of energy and medicinal plant, *Jatropha curcas* L., as an adaptation mechanism to salinity. J. Med. Plants Res. 6 (16), 3129–3142.

Chen, Z., Pottosin, I.I., Cuin, T.A., Fuglsang, A.T., Tester, M., Jha, D., Zepeda-Jazo, I., Zhou, M., Palmgren, M.G., Newman, I.A., Shabala, S., 2007. Root plasma membrane transporters controlling K^+/Na^+ homeostasis in salt stressed barley. Plant Physiol. 145, 1714–1725.

Cramer, G.R., 2002. Sodium calcium interactions under salinity stress. In: Lächli, A., Lüttge, U. (Eds.), Salinity: Environment-plants-molecules. Kluwer, Dordrecht, pp. 205–527.

Demidchik, V., Maathuis, F.J.M., 2007. Physiological roles of nonselective cation channels in plants: from salt stress to signalling and development. New. Phytol. 175, 387–404.

Ekberg, K., Pedersen, B.P., Sørensen, D.M., Nielsen, A.K., Veierskov, B., Nissen, P., Palmgren, M.G., Buch-Pedersen, M.J., 2010. Structural identification of cation binding pockets in the plasma membrane proton pump. Proc. Natl. Acad. Sci. U S A 107, 21400–21405.

Felle, H., 1988. Auxin causes oscillations of cytosolic free calcium and pH in *Zea mays* coleoptiles. Planta 174, 495–499.

Flowers, T.J., Galal, H.K., Bromham, L., 2010. Evolution of halophytes: multiple origins of salt tolerance in land plants. Funct. Plant Biol. 37, 604–612.

Fuglsang, A.T., Guo, Y., Cuin, T.A., Qiu, Q., Song, C., Kristiansen, K.A., Bych, K., Schulz, A., Shabala, S., Schumaker, K.S., 2007. *Arabidopsis* protein kinase PKS5 inhibits the plasma membrane H^+-ATPase by preventing interaction with 14-3-3 protein. Plant Cell 19, 1617–1634.

Geilfus, C.M., Mühling, K.H., 2011. Real-time imaging of leaf apoplastic pH dynamics in response to NaCl stress. Front. Plant Sci. 2, 13.

Geilfus, C.M., Mühling, K.H., 2013. Ratiometric monitoring of transient apoplastic alkalinizations in the leaf apoplast of living *Vicia faba* L. plants: chloride primes and PM-H^+-ATPase shapes NaCl-induced systemic alkalinizations. New. Phytol. 197, 1117–1129.

Hager, A., 2003. Role of the plasma membrane H+-ATPase in auxin-induced elongation growth: historical and new aspects. J. Plant Res. 116, 483–505.

Haruta, M., Sussman, M.R., 2012. The effect of a genetically reduced plasma membrane proton motive force on vegetative growth of *Arabidopsis*. Plant Physiol. 158, 1158–1171.

Kader, Md.A., Lindberg, S., 2005. Uptake of sodium in protoplasts of salt-sensitive and salt-tolerant cultivars of rice, *Oryza sativa* L. determined by the fluorescent dye SBFI. J. Exp. Bot. 56, 3149–3158.

Kader, Md.A., Lindberg, S., 2010. pH signaling in plants under salinity stress. Plant Signal. Behav. 3, 1–7.

Kader, Md.A., Lindberg, S., Seidel, T., Golldack, D., Yemelyanov, V., 2007. Sodium sensing induces different changes in free cytosolic calcium concentration and pH in salt-tolerant and -sensitive rice (*Oryza sativa*) cultivars. Physiol. Plant 130, 99–111.

Larsson, C., Sommarin, M., Widell, S., 1994. Isolation of highlypurified plant plasmamembrane and theseparation of inside-out and right-side out vesicles. Methods Enzymol. 228, 451–469.

Lindberg, S., 1995. *In situ* determination of intracellular concentrations of K^+ in barley (*Hordeum vulgare* L. cv. Kara), using the fluorescent probe PBFI-AM. Planta 195, 525–529.

Lindberg, S., Wingstrand, G., 1985. Mechanism for Cd^{2+} inhibition of ($K^+ + Mg^{2+}$) ATPase activity and $K^+(^{86}Rb^+)$ uptake in roots of sugar beet (*Beta vulgaris*). Physiol. Plant 63, 181–185.

Maathuis, F.J.M., Amtmann, A., 1999. K^+ nutrition and Na^+ toxicity: the basis of cellular K^+/Na^+ ratios. Ann. Bot. 84, 123–133.

Morgan, S.H., Lindberg, S., Mühling, K.H., 2013. Calcium supply effects on wheat cultivars differing in salt resistance with special reference to leaf cytosol ion homeostasis. Physiol. Plant 149, 321–328.

Munns, R., Tester, M., 2008. Mechanisms of salinity tolerance. Annu. Rev. Plant Biol. 59, 651–681.

Mühling, K.H., Wimmer, M., Goldbach, H.E., 1998. Apoplastic and membrane-associated Ca^{2+} in leaves and roots as affected by boron deficiency. Physiol. Plant 102, 179–184.

Pitann, B., Kranz, T., Mühling, K.H., 2009a. The apoplastic pH and its significance in adaptation to salinity in maize (*Zea mays* L.): comparison of fluorescence microscopy and pH-sensitive microelectrodes. Plant Sci. 176, 497–504.

Pitann, B., Schubert, S., Mühling, K.H., 2009b. Decline in leaf growth under salt stress is due to an inhibition of H^+-pumping activity and increase in apoplastic pH of maize leaves. J. Plant Nutr. Soil Sci. 172, 535–543.

Plieth, C., 2001. Plant calcium signalling and monitoring: pros and cons and recent, experimental approaches. Protoplasma 218, 1–23.

Reddy, A.S., Ali, G.S., Celesnik, H., Day, I.S., 2011. Coping with stresses. Roles of calcium- and calcium/calmodulin-regulated gene expression. Plant Cell 23, 2010–2032.

Sanders, D., Pelloux, J., Brownlee, C., Harper, J.F., 2002. Calcium at the crossroads of signaling. Plant Cell 14, S401–S417.

Sebastiani, L., Lindberg, S., Vitagliano, C., 1999. Cytoplasmic free calcium dynamics in single tomato (*Lycopersicon esculentum* L) protoplasts subjected to chilling temperatures. Physiol. Plant 105, 239–245.

Shabala, S., 2002. Ionic and osmotic components of salt stress specifically modulate net ion fluxes from bean leaf mesophyll. Plant Cell Environ. 23, 825–837.

Shabala, S., 2013. Learning from halophytes: physiological basis and strategies to improve abiotic stress tolerance in crops. Ann. Bot. 112, 1209–1221. http://dx.doi.org/10.1093/aob/mct205.

Shabala, S., Demidchik, V., Shabala, L., Cuin, T.A., Smith, S.J., Miller, A.J., Davies, J.M., Newman, I.A., 2006. Extracellular Ca^{2+} ameliorates NaCl-induced K^+ loss from *Arabidopsis* root and leaf cells by controlling plasma membrane K^+-permeable channels. Plant Physiol. 141, 1653–1665.

Snedecor, G.W., Cochran, W.G., 1980. Statistical Methods, seventh ed. Iowa Stat University Press, Ames, Iowa, USA.

Sun, J., Wang, M.J., Ding, M.Q., Deng, S.R., Liu, M.Q., Lu, C.F., Zhou, X.Y., Shen, X., Zheng, X.J., Zhang, Z.K., Song, J., Hu, Z.M., Xu, Y., Chen, S.L., 2010. H_2O_2 and cytosolic Ca^{2+} signals triggered by the PM H^+-coupled system mediate K^+/Na^+ homeostasis in NaCl-stressed *Populus euphratica* cells. Plant Cell. Environ. 33, 943–958.

Tester, M., Davenport, R., 2003. Na^+ tolerance and Na^+ transport in higher plants. Ann. Bot. 91, 503–527.

Zhu, J.K., 2002. Salt and drought stress signal transduction in plants. Annu. Rev. Plant Biol. 53, 247–273.