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The role of polyamines in the regulation of the plasma membrane and the tonoplast proton pumps under salt stress

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ABSTRACT

Polyamine content (PAs) often changes in response to abiotic stresses. It was shown that the accumulation of PAs decreased in roots treated for 24 h with 200 mM NaCl. The role of polyamines (putrescine – PUT, spermidine – SPD and spermine – SPM) in the modification of the plasma membrane(PM) H^+-ATPase (EC 3.6.3.6) and the vacuolar(V) H^+-ATPase (EC 3.6.3.14) activities in cucumber roots treated with NaCl was investigated. 24 h treatment of seedlings with 50 μM PUT, SPD or SPM lowered the activities of proton pumps in both membranes. The decreased H^+-ATPase activity in plasma membranes isolated from the PA-treated roots was positively correlated with a lower level of PM-H^+-ATPase *CsHA3* transcript. However, transcript levels of PM-H^+-ATPase *CsHA2* and V-ATPase subunit A and c in roots treated with 50 μM PAs were similar to those in the control. Additionally, treatment of plants with salt markedly increased the activity of the PM- and V-H^+-ATPases. However, exposure of plants to 20% PEG had no effect on these activities. These data suggest that, under salt stress conditions, the increase in H^+-ATPase activities is caused mainly by the ionic component of salt stress. It seems that the main role of the PAs in the 24 h salt-treated cucumber plants could be a result of their cationic character. The PA levels decreased when concentration of Na^+ increased, so action of PAs contributes to ionic equilibrium. Moreover, the decrease in the concentration of polyamines, which inhibit the PM-H^+-ATPase and the V-H^+-ATPase, at least under the studied conditions, seems to be beneficial. Thus, plants can increase salinity tolerance by modifying the biosynthesis of polyamines.

Introduction

Plants are susceptible to various stress factors under wild conditions. A high concentration of salt in the soil, i.e. salt stress, is one such stressor. Salt stress is a complex abiotic stress in which both ionic and osmotic components are involved (Alvarez et al., 2003). Polyamines (PAs) play a key role in plant responses to salinity. Putrescine (PUT), spermidine (SPD) and spermine (SPM) are the main PAs found in all living cells (Galston and Sawhney, 1990; Groppa and Benavides, 2008). PAs are polycationic cellular molecules at physiological pH (Alcázar et al., 2006; Kusano et al., 2008) and bind to phenolic acids or macromolecules, e.g. DNA, RNA, various proteins and phospholipids of cell membranes (Galston and Sawhney, 1990). Moreover, they interact with negatively charged parts of membranes. These interactions stabilize the membranes under stress conditions. A number of authors have reported the relationship of PA metabolism with plant responses to various environmental stresses. Changes in the accumulation of PAs during deficiency of potassium, magnesium or boron ions in the environment (Smith, 1984; Sarjala, 1996; Watson and Malmberg, 1996; Camacho-Cristóbal et al., 2002; Sarjala and Kaunisto, 2002; Camacho-Cristóbal et al., 2005), acidity (Young and Galston, 1983), stress caused by low or high temperature (Roy and Ghosh, 1996; Sarjala et al., 1997; Pillai and Akiyama, 2004), drought (Smith, 1985; Turner and Stewart, 1986; Yamaguchi et al., 2007), UV treatment (Smith et al., 2001; An et al., 2004; Zacchini and de Agazio, 2004) and the presence of heavy metals (Weinstein et al., 1986; Groppa et al., 2001, 2003; Zhao et al., 2008) have been observed. Research by Flores and Galston (1984), Feirer et al. (1998), Tiburcio et al. (1986a, b), Erdei et al. (1996) and Santa-Cruz et al. (1997a) demonstrated a rapid increase of PA levels under osmotic stress. There are no explicit data concerning the effect of salinity on biosynthesis of PAs. An increase in the concentration of PAs (PUT, SPD and SPM) was observed by Basu et al. (1988), Basu and Ghosh (1991), Friedman et al. (1989) and Kirshnamurthy and Bhagwat (1989) under salt stress conditions. Jiménez-Bremont et al. (2007) and Zapata et al. (2004) observed accumulation of SPD and SPM, but not PUT, under salt stress. However, a decrease in PA levels under salinity

Abbreviations: BSA, bovine serum albumin; BTP, bis-tris propane; FW, fresh weight; MES, 2-(N-morpholino)ethanesulfonic acid; PAs, polyamines; PM, plasma membrane; PUT, putrescine; SDS, dodecyl sulphate sodium salt; SPD, spermidine; SPM, spermine; T, tonoplast; TRIS, hydroxymethylaminomethane; V, vacuolar

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was reported by Prakasch et al. (1988), Friedman et al. (1989) and Santa-Cruz et al. (1997a, b). Ionic and osmotic components are two elements of salt stress. PAs play a key role as osmoprotectants during osmotic stress. Enzyme-conjugated polycations (osmoprotectants) stabilize and protect the membranes (Besford et al., 1993). Accumulation of Na^+ cations occurs during long periods of salt stress. PAs that occur as polycations in the cytoplasm are responsible for maintaining the cation–anion equilibrium. Lowering the concentration of PAs in the cytoplasm results in simultaneous removal of excess cations from the cytoplasm (Reggiani et al., 1993; Willadino et al., 1996; Santa-Cruz et al., 1997a, b).

In addition to appropriate osmotic pressure, the control of intracellular ionic homeostasis is a key factor that decides plant tolerance to salinity (Niu et al., 1993). Plants adapted to salinity maintain a low concentration of Na^+ in the cytosol by the active exclusion of sodium ions into the apoplast and vacuole catalyzed by specific Na^+/H^+ antiporters operating in the plasma membrane (PM) and tonoplast (T). The driving force for this secondary active transport is generated by the PM-H^+-ATPase and the vacuolar (V) H^+-ATPase (Palmgren, 1991; Ratajczak, 2000). Both enzymes are responsible for maintaining a suitable concentration of sodium ions in the cytosol. The flow of protons plays a significant role in ion homeostasis. Previous results have confirmed the stimulation of PM and T proton pumps during salt stress (Niu et al., 1993; Perez-Prat et al., 1994; Barkla et al., 1995; Binzel, 1995; Kłobus and Janicka-Russak, 2004; Janicka-Russak and Kłobus, 2007; Kabała and Kłobus, 2008). This salt-dependent activation is controlled at the transcriptional and posttranslational levels. Accumulation of mRNAs of both PM-H^+-ATPase (Niu et al., 1993; Perez-Prat et al., 1994; Janicka-Russak and Kłobus, 2007) and V-H^+-ATPase (Narasimhan et al., 1991) under NaCl is positively correlated with plant tolerance to salt stress.

In this present study, we attempt to explain the role of PAs (PUT, SPD and SPM) in modification of the activity of the PM-H^+-ATPase and the V-H^+-ATPase in cucumber roots subjected to salt stress.

Materials and methods

Cucumber seeds (*Cucumis sativus* L. var. Wisconsin), germinated 48 h in darkness at 25 °C, were transferred to a nutrient medium for 5 days and then for 1 day to a nutrient solution with the addition of 200 mmol/dm^3 NaCl or 50 μmol/dm^3 PUT, SPD, SPM or 20% PEG or with no addition (the control). The composition of the nutrient solution was described previously by Kłobus et al. (1988). It contained: 5 mmol/dm^3 $Ca(NO_3)_2 \times 4\ H_2O$; 5 mmol/dm^3 KNO_3; 1 mmol/dm^3 KH_2PO_4; 1 mmol/dm^3 $MgSO_4 \times 7\ H_2O$ and microelements: 75 μmol/dm^3 ferric citrate; 10 μmol/dm^3 $MnSO_4 \times 5\ H_2O$; 5 μmol/dm^3 H_3BO_4; 1 μmol/dm^3 $CuSO_4 \times 5\ H_2O$; 0.01 μmol/dm^3 $ZnSO_4 \times 7\ H_2O$; 0.05 μmol/dm^3 $Na_2MoO_4 \times 2\ H_2O$. The plants were grown hydroponically with a 16 h photoperiod (180 μmol m^{-2} s^{-1}) at 25 °C during the day and 22 °C during the night. The relative humidity in both the light and dark was 70%.

PM vesicles were isolated from cucumber root microsomes by phase partitioning according to the procedure of Larsson (1985) as modified by Kłobus (1995). A 16 g phase system containing 6.2% (w/w) Dextran T500, 6.2% (w/w) polyethyleneglycol 3350, 330 mmol/dm^3 sorbitol, 5 mmol/dm^3 KCl, and 5 mmol/dm^3 bis-tris propane (BTP)-2-(N-morpholino)ethanesulfonic acid (MES) (pH 7.5) was used. The PMs obtained by this procedure were composed mainly of right-side-out vesicles and were used in the determination of hydrolytic ATPase activity. Some vesicles were turned to the inside-out oriented form by the method of Johansson et al. (1995) and used for the measurements of ATP-dependent H^+ transport in PMs.

T vesicle isolation was performed as described previously (Kabała and Kłobus, 2001). The microsome fraction was overlaid on a discontinuous sucrose density gradient (20%, 28%, 32% and 42% w/w). The vesicles obtained after this isolation were used to determine both ATPase activity and ATP-dependent proton transport in V membranes.

The hydrolytic activities of both the vanadate-sensitive ATPase (PM-H^+-ATPase) and nitrate-sensitive ATPase (V-H^+-ATPase) were determined according to the procedure of Gallahger and Leonard (1982), as modified by Sze et al. (1992). The reaction mixture contained about 50 μg protein (PM or T), 33 mM hydroxymethylaminomethane (Tris)–MES (pH 7.5), 3 mM ATP, 2.5 mM $MgSO_4$, 50 mM KCl, 1 mM NaN_3, 0.1 mM Na_2MoO_4, $\pm$200 μM Na_3VO_4, $\pm$50 mM $NaNO_3$, and 0.02% Triton X-100. PM-H^+-ATPase activity was expressed as the difference between the activity measured in the absence and presence of Na_3VO_4. T-H^+-ATPase activity was expressed as the difference between the activity measured in the absence and presence of $NaNO_3$. The P_i released during the reaction was determined according to Ames (1966) with 0.2% (w/v) sodium dodecyl sulphate included to prevent precipitation (Dulley, 1975).

H^+ transport activity was measured spectrophotometrically as a drop in acridine orange absorbance at 495 nm (A_{495}). For the PMs, the assay medium contained: PM vesicles (about 50 μg protein), 25 mmol/dm^3 BTP-MES (pH 7.5), 330 mmol/dm^3 sorbitol, 50 mmol/dm^3 KCl, 0.1% bovine serum albumin (BSA), 10 μmol/dm^3 acridine orange and 0.05% Brij 58, according to Kłobus and Buczek (1995). The T vesicles (about 50 μg protein) were mixed with 20 mmol/dm^3 Tris–MES (pH 7.2), 250 mmol/dm^3 sucrose, 50 mmol/dm^3 KCl and 10 μmol/dm^3 acridine orange according to Kabała and Kłobus (2001). Proton transport in both membranes (PM and T) was initiated by the addition of 3 mmol/dm^3 Mg-ATP. For each combination, passive proton movement through the membrane was determined without ATP in the reaction medium.

Protein was measured according to method of Bradford (1976) in the presence of 0.02% Triton X-100 with BSA as the standard.

Sodium ion content was determined spectrophotometrically (atomic absorption spectrophotometer Perkin-Elmer 3300) in dry root tissues digested with concentrated HNO_3 in a microwave system.

Free PAs were measured according to Flores and Galston (1982). Root tissue (1 g) was homogenized in 10 ml 5% $HClO_4$ with a mortar and pestle. The homogenate was kept in an ice bath for 1 h and then centrifuged at 48,000*g* for 20 min. The supernatant was used to derivatize and quantify the soluble-free PAs. The derivatization of PAs was carried out with benzoyl chloride; 5 ml of supernatant was mixed with 10 ml of 2 N NaOH and 100 μl of benzoyl chloride. The mixture was vortexed and incubated for 20 min at room temperature. Next, 20 ml of saturated NaCl was added and benzoyl-PAs were extracted with 20 ml diethyl ether. The sample was centrifuged at 1500*g* for 5 min. Finally, 5 ml of the ether phase were evaporated and redissolved in 0.5 ml of methanol. PA standards were prepared similarly to plant samples. The PA contents were analyzed by HPLC. PAs were eluted with 64% methanol through a 4.6 × 250 mm reverse phase C-18 column at flow rate of 1 ml/min at room temperature. The absorbance was measured by an UV–vis detector (Pharmacia LKB) at 254 nm.

Total RNA was isolated from 50 mg of roots with Tri Reagent (Sigma). The concentration and the purity of isolated RNA were determined spectrophotometrically measuring the absorbance at 260 and 280 nm. To evaluate the expression of PM- and V-H^+-ATPase genes, as well as actin and 18S RNA (internal standards) genes, semi-quantitative RT-PCR analyses (Titan one tube RT-PCR system, Roche) with specific primers for each gene were performed.