BIOLOGIA PLANTARUM 56 (2): 377-382, 2012

BRIEF COMMUNICATION

Na^+/H^+ antiport activity in plasma membrane and tonoplast vesicles isolated from NaCl-treated cucumber roots

K. KABAŁA and M. JANICKA-RUSSAK*

Department of Plant Physiology, Institute of Plant Biology, University of Wrocław
Kanonia 6/8, 50-328 Wrocław, Poland

Abstract

Sodium/proton antiporter activity in the plasma membrane and tonoplast of cucumber seedling roots treated with 200 mM NaCl for 24 h was determined. It was observed that plasma membrane and tonoplast antiporter activity was only present in membranes from salt-treated plants. In addition, the plasma membrane antiporter protein was present in membranes after induction with NaCl, whereas tonoplast antiporter protein was observed in control and at elevated level in NaCl-treated plants. Moreover, based on the affinity of studied antiporter proteins to sodium ions, it could be assumed that excess sodium ions are firstly translocated from the cytosol to the vacuole and then excluded to the apoplast through the plasma membrane.

Additional key words: antiporter proteins, salt stress, sodium compartmentalization.

Soil salinity is a major abiotic stress that limits plant growth and productivity. A high concentration of Na^+ is harmful to plants because of its interference with K^+ nutrition and, consequently, alteration of enzyme activities and cellular metabolism (Ward *et al*. 2003). In order to prevent accumulation of toxic amounts of Na^+ in the cytosol, active Na^+ efflux into the apoplast and its compartmentalization inside the vacuole occur. Since Na^+ pumps are absent in higher plant cells, Na^+/H^+ antiport in both the plasma membrane and the tonoplast is needed to translocate Na^+ against electrochemical gradient (Apse and Blumwald 2007). Na^+/H^+ antiport has been described in many different plants (Blumwald *et al.* 2000, Parks *et al.* 2002, Shi *et al.* 2002, Fukuda *et al.* 2004, Wu *et al.* 2007, Queiros *et al.* 2009). As Na^+ exclusion from the cytoplasm is dependent on the electrochemical gradient of membranes formed by the proton pumps, regulation of those enzymes plays an important role in salt tolerance. It has been documented that NaCl treatment strongly induced the activities of two membrane proton pumps: plasma membrane H^+-ATPase and vacuolar H^+-ATPase (Barkla *et al*. 1995, Kłobus and Janicka-Russak 2004). It is well known that salinity markedly elevates the content of endogenous abscisic acid (ABA) in plants (La Rosa *et al.* 1985, 1987, Janicka-Russak and Kłobus 2006). ABA is known as a stress hormone, which mediates responses to salt stress. Studies using ABA-deficient mutants indicate that salt stress signal transduction is controlled by both ABA-dependent and ABA-independent pathways (Agarwal and Jha 2010). Moreover, we showed earlier that ABA treatment of cucumber plants increased activity of plasma membrane and tonoplast H^+-ATPases (Janicka-Russak and Kłobus 2006).

Molecular analyses in *Arabidopsis* have led to the identification of a plasma membrane localized SOS1 (salt overly sensitive 1) and vacuolar NHX1 (Na^+/H^+ antiporters) upregulated at the genetic level in response to NaCl (Apse *et al.* 1999, Gaxiola *et al.* 1999, Shi *et al.* 2000, 2003). Sodium efflux through SOS1 is mediated by the SOS3-SOS2 complex (Qiu *et al.* 2002). In many plant tissues a salt-inducible shift in the cytoplasmic calcium content has been observed (Knight *et al.* 1997, Blumwald *et al.* 2000, Netting 2000, Xiong *et al.* 2002), suggesting its involvement in the signalling pathway under NaCl stress conditions. The Ca^{2+} signals are sensed by calcium-

Received 23 November 2010, *accepted* 22 June 2011.
Abbreviations: ABA - abscisic acid; BSA - bovine serum albumin; BTP - bis-tris propane; DAB - 3,3'-diaminobenzidine; DTT - dithiothreitol; EDTA - ethylenediaminetetraacetic acid; MES - 2-(N-morpholino)ethanesulfonic acid; PMSF - phenylmethylsulfonyl fluoride; SDS - sodium dodecyl sulfate; SOS - salt overly sensitive; Tris - tris(hydroxymethyl)aminomethane.
* Corresponding author; fax: (+48) 71 3754118, e-mail: russak@biol.uni.wroc.pl

binding proteins (SOS3). After binding with Ca^{2+}, SOS3 changes its conformation and interacts with SOS2, a Ser/Thr protein kinase, and activates its substrate phosphorylation (Bertorello and Zhu 2009). The activated SOS3-SOS2 complex then phosphorylates SOS1 and activates its antiporter activity (Chinnusamy *et al.* 2005). The SOS pathway is not limited to *Arabidopsis* but is widespread in higher plants, including both glycophytes and halophytes (Mahajan *et al.* 2008).

SOS2 kinase and SOS1 antiporter have emerged as important regulatory components that may affect other proteins involved in responses to salinity as well as to other stresses. SOS2 has been shown to interact with protein phosphatase ABI2 (Ohta *et al.* 2003) and with H_2O_2 signalling molecules: protein nucleoside diphosphate kinase and catalases (Verslues *et al.* 2007), connecting salt stress responses with ABA signalling and oxidative stress, respectively. It is well known that salt stress induces production of reactive oxygen species (ROS). ROS can act as signalling molecules, but at high concentrations they disrupt the cell metabolism. Many studies have indicated that activities of antioxidant enzymes are correlated with plant tolerance to salt stress (Lin and Pu 2010, Maia *et al.* 2010). It has also been confirmed that both SOS1 and SOS2 are involved in the connection between the ion homeostasis pathway and a pathway controlling ROS detoxification under salt stress (Zhu *et al.* 2007). Chung *et al.* (2008) postulated that SOS1 plays a role in regulating extracellular ROS generation at a very early signalling step of a signal transduction pathway common to several abiotic stresses.

In *Arabidopsis thaliana*, the NHX family of Na^+/H^+ antiporters comprises six members, AtNH1-6, sharing some basic structural similarities. Yokoi *et al.* (2002) clearly indicated that both AtNHX1 and AtNHX2 mediate vacuolar sequestration of Na^+, and together with AtNHX5 are salt tolerance determinants. Yamaguchi *et al.* (2005) have shown that cation selectivity of AtNHX1 is regulated by calmodulin in a Ca^{2+}- and pH-dependent manner. Binding of calmodulin to the antiporter decreases its Na^+/H^+ exchange activity. The importance of vacuolar Na^+ compartmentation in plant salt tolerance has been demonstrated in transgenic plants over-expressing the *NHX1* gene. It has led to the generation of glycophytes, including tomato, canola, rice, maize and wheat, able to survive and reproduce in the presence of high NaCl levels (Yamaguchi and Blumwald 2005).

Studies using *Arabidopsis* wild type and mutants *sos1*, *sos2* and *sos3* revealed that tonoplast NHXs are activated by SOS2, but are independent of SOS3 (Qiu *et al.* 2004). These results indicated coordinated regulation of the sodium antiporters in the vacuolar and plasma membranes. Qiu *et al.* (2004) suggested that reduced activity of one exchanger may be compensated by enhanced activity of another. Recent research has demonstrated that salt stress enhances a direct interaction between SOS2 and B subunits of vacuolar H^+-ATPase and stimulates proton transport activity, suggesting that regulation of V-ATPase activity is an additional key function of SOS2 in promotion of salt tolerance (Batelli *et al.* 2007).

Activity of Na^+/H^+ antiporters can be demonstrated *in vitro* in purified membrane vesicles. In this study, H^+-dependent Na^+ transport was monitored with the aim to present evidence for the induction of a specific Na^+/H^+ exchange and to show different roles for the transporters in the vacuolar and plasma membranes because of their different affinities to Na^+.

Cucumber (*Cucumis sativus* L. cv. Krak) seeds, germinated in darkness for 2 d at 25 °C, were grown in a medium composed of 5 mM KNO_3, 5 mM $Ca(NO_3)_2$, 1 mM KH_2PO_4, 1 mM $MgSO_4$, 75 μM ferric citrate, 10 μM $MnSO_4$, 5 μM H_3BO_4, 1 μM $CuSO_4$, 0.01 μM $ZnSO_4$, 0.05 μM Na_2MoO_4 (pH 6.5). After 6 d seedlings were transferred to the nutrient solution without (control) or with 200 mM NaCl for 24 h. The plants were grown hydroponically under a 16-h photoperiod (irradiance of 180 μmol m^{-2} s^{-1}), day/night temperature of 25/22 °C and relative humidity about 70 %.

Highly purified plasma membrane vesicles were isolated from cucumber roots according to Kłobus (1995). The system contained 6.2 % (m/m) *Dextran T500*, 6.2 % (m/m) polyethylene glycol 3350, 330 mM sorbitol, 5 mM KCl, 5 mM BTP-MES (pH 7.5). The plasma membranes, mainly composed of right-side-out vesicles, were turned to the inside-out oriented form by the method of Johansson *et al.* (1995). Tonoplast vesicle-enriched fractions were isolated from cucumber roots according to Kabała and Kłobus (2001). The microsomes were overlaid on a discontinuous sucrose density gradient consisting of 20, 28, 32 and 42 % (m/m) sucrose.

Proton transport (pH gradient generation) was measured spectrophotometrically as a drop in acridine orange absorbance at 495 nm (A_{495}). Plasma membrane vesicles (about 20 μg of protein) were incubated for 5 min at 25 °C with 25 mM BTP-MES (pH 7.5), 330 mM sorbitol, 50 mM KCl, 0.1 % BSA, 10 μM acridine orange and 0.05 % *Brij 58*, according to Kłobus and Buczek (1995). Tonoplast vesicles (about 20 μg protein) were incubated for 5 min at 25 °C with 20 mM TRIS-MES (pH 7.2), 250 mM sucrose, 50 mM KCl and 10 μM acridine orange, according to Kabała and Kłobus (2001). The reaction in both membranes was initiated by the addition of 3 mM Mg-ATP. For every combination, passive proton movement through the membrane was determined without ATP in the reaction medium. To stop the gradient formation, 1 unit of hexokinase and glucose per cm^3 of mixture was added. After 20 s of enzyme treatment, 20 mM Na-gluconate was introduced and the recovery of acridine orange absorbance was determined. Every variation in the reaction conditions is specified in the figures.

For Western blot analysis, plasma membrane or tonoplast proteins (15 μg) were incubated in buffer containing 2 % (m/v) SDS, 80 mM DTT, 40 % (m/v) glycerol, 5 mM PMSF, 10 mM Tris, 1 mM EDTA and 0.05 % (m/v) bromophenol blue for 30 min at room

temperature, and separated on 10 % SDS-polyacrylamide gel for 60 min (135 V, 20 mA) (Laemmli 1970). After electrophoresis, proteins were electrotransferred for 80 min (60 V, 220 mA) to nitrocellulose membrane using *SV10-EB10* blotting apparatus (*Sigma-Aldrich*, St. Louis, USA). The transfer buffer contained 25 mM Tris, 150 mM glycine and 10 % (v/v) methanol. To identify the plasma membrane Na^+/H^+ antiporter, the blots were incubated overnight (8 °C) with antibody against Na^+/H^+ exchanger (mouse monoclonal exchanger 1, *Abcam,* Cambridge, UK). The antiserum was diluted 750-fold. After repeated washing the nitrocellulose membrane was incubated at room temperature for 1 h with 1:4000 diluted secondary antibody (rabbit polyclonal to mouse IgG, conjugated to horseradish peroxidase, *Abcam*) and visualized by staining with 3,3'-diaminobenzidine (DAB). To identify the tonoplast Na^+/H^+ antiporter, the blots were incubated overnight (8 °C) with antibody (rabbit polyclonal to NHX, tonoplast exchanger, *Abcam*). The antiserum was diluted 1000-fold. After repeated washing the nitrocellulose membrane was incubated at room temperature for 1 h with 1:4000 diluted secondary antibody (goat polyclonal to rabbit IgG, conjugated to horseradish peroxidase, *Abcam*) and visualized by staining with DAB. Protein content was estimated by the method of Bradford (1976) in the presence of 0.02 % *Triton X-100* with bovine serum albumin as a standard.

It is well known that the Na^+/H^+ antiport can only operate under high transmembrane electrochemical proton gradient. In plasma membrane and tonoplast vesicles isolated from roots of cucumber seedlings, significant stimulation of plasma membrane and tonoplast ATP-dependant proton pump under salinity was demonstrated (Table 1). After 60 s, ATP-dependent proton transport was inhibited by the inclusion of hexokinase, which causes depletion of ATP from the medium in the presence of glucose. Subsequent addition of Na-gluconate into the samples containing plasma membrane or tonoplast vesicles isolated from salt-stressed plants dissipated the transmembrane pH gradient, observed as a rapid increase in acridine orange absorbance (Fig. 1*A*,*B*). When Na-gluconate was introduced into the assay medium with membrane vesicles obtained from the control plants, recovery of absorbance was not observed (Fig. 1*A*,*B*). This suggests the presence of antiporter activity in cell membranes of salt-treated cucumber roots. From published data it is known that Na^+/H^+ antiporter activity is observed in glycophytes only after salt stress induction as a part of the adaptive mechanism (Ballesteros *et al.* 1997, Fukuda *et al.* 1998). However, in halophytes, the antiporter is constitutively expressed and is present in both NaCl-treated plants and plants growing under NaCl-free conditions. Studies using amiloride were done to confirm if an increase of A_{495} absorbance, after introduction of sodium salt into the nutrient solution, might result from sodium/proton antiport. The amiloride has been shown to inhibit Na^+/H^+ exchange systems in plants and animals (Blumwald *et al.* 1987, Barkla *et al.* 1995, Darley *et al.* 2000, Venema *et al.* 2002). In our studies pre-incubation of membrane vesicles with 500 μM amiloride completely inhibited Na^+/H^+ antiport activity (Fig. 1*C*,*D*). These results confirmed the presence of Na^+/H^+ antiport protein in the plasma membrane and tonoplast isolated from cucumber roots treated with 200 mM NaCl for 24 h. Similar observations were made by Katz *et al.* (1986), Barkla *et al.* (1995) and Ballesteros *et al.* (1997).

To support the above observation, Western blot analysis with antibodies raised against plasma membrane and tonoplast Na^+/H^+ antiporter was carried out. These antibodies detected a protein in the plasma membrane fraction obtained from salt-treated cucumber roots (Fig. 2*A*). No noticeable cross-reactivity with the above-mentioned antibodies was observed in plasma membrane from the control plants. The similar result was obtained by Wu *et al*. (2007), who found that the plasma membrane antiporter was significantly up-regulated under salt stress. On the other hand, we have observed that in samples containing tonoplast protein, antibody raised against tonoplast antiporter detected proteins in membranes obtained from both control and salt-treated plants. However, the number of detected proteins in the tonoplast was significantly higher in vesicles obtained from NaCl-treated cucumber seedlings. Therefore, it seems quite probable that the tonoplast antiporter in cucumber roots is constitutively expressed, although its synthesis greatly increases under salinity. Xia *et al.* (2002) also observed that abundance of the tonoplast antiporter NHX1 from *Beta vulgaris* increased after salt treatment.

The activation of Na^+/H^+ exchange can be accounted for by either an increase in the number of antiporter molecules in membranes or modulation of the activity of pre-existing antiporter protein molecules. It is interesting

Table 1. Effects of 200 mM NaCl in the nutrient solution on the ATP hydrolysis [μmol(Pi) mg^{-1}(protein) min^{-1}] and steady state H^+ transport [ΔA_{495} mg^{-1}(protein) min^{-1}] of ATPases in the plasma membrane and tonoplast. Means ± SD, n = 6.

NaCl [mM]	Plasma membrane			Tonoplast		
	ATP hydrolysis	H^+ transport ATP-dependent	passive efflux	ATP hydrolysis	H^+ transport ATP-dependent	passive efflux
0	2.6 ± 0.23	0.216 ± 0.012	0.006 ± 0.002	0.8 ± 0.09	0.112 ± 0.006	0.000
200	7.8 ± 0.70	0.325 ± 0.017	0.009 ± 0.003	2.3 ± 0.23	0.257 ± 0.009	0.000

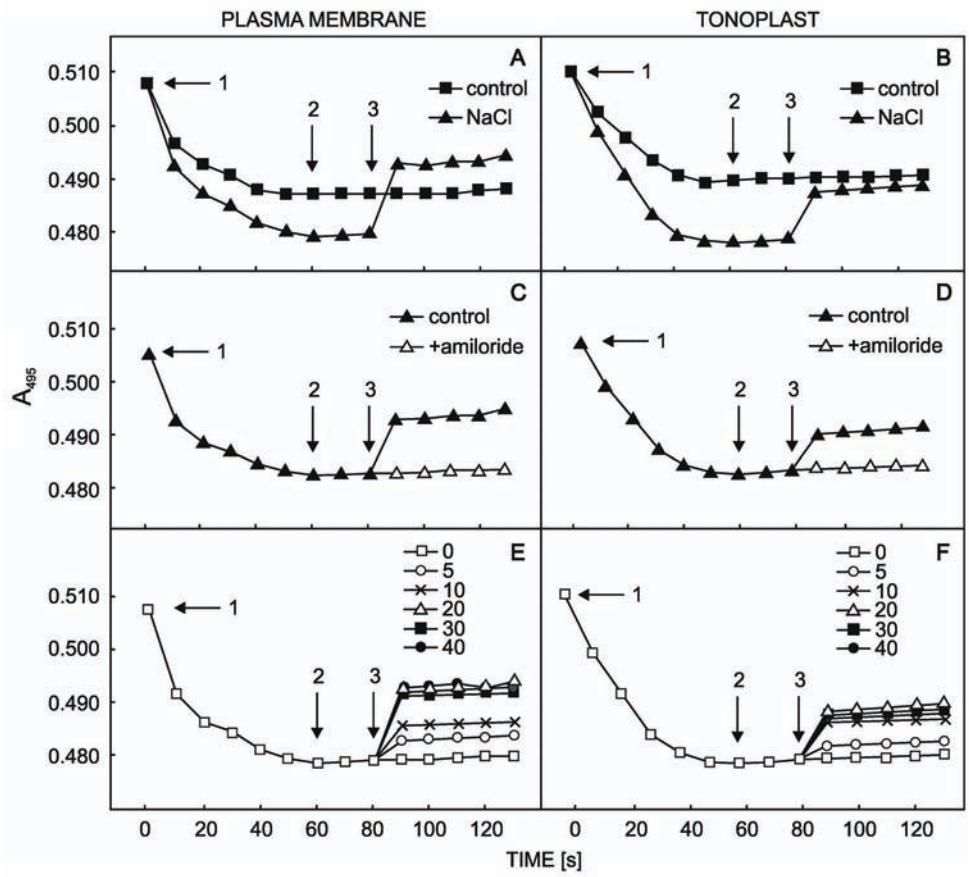

Fig. 1. Activity of Na^+/H^+ antiporter monitored as a drop of acridine orange absorbance. Effect of Na^+ on the dissipation rate of the H^+ gradient generated by the plasma membrane H^+-ATPase (*A*) or tonoplast H^+-ATPase (*B*) isolated from control plants and plants treated for 24 h with 200 mM NaCl. Activities measured in the presence and in the absence of 500 μM amiloride in plasma membrane (*C*) and tonoplast (*D*) vesicles isolated from 200 mM NaCl-treated cucumber roots. Effects of different concentration of Na-gluconate (5, 10, 10, 30, or 40 mM) on the dissipation rate of the H^+ gradient generated by the plasma membrane H^+-ATPase (*E*) or tonoplast H^+-ATPase (*F*) in vesicles isolated from roots treated for 24 h with 200 mM NaCl. *Arrows* indicate additions of 3 mM ATP and $MgSO_4$ (1), 1 U cm^{-3} hexokinase (2) and 20 mM Na-gluconate (3). Values presented are representative for the results obtained in three independent experiments with each experiment done in triplicate.

that in tonoplast vesicles Na^+/H^+ antiport activity was detected only in membrane from salt-treated plants, despite the fact that this protein was detected by Western blot analysis in membranes from both salt-treated and untreated cucumber roots (Fig. 2*B*). It seems probable that post-translational modification turns this activity on. It is known that under salt stress conditions the SOS pathway has a key role in regulating ion transport. Moreover, it has been documented that SOS2 targets the Na^+/H^+ exchanger in the plasma membrane (Shi *et al*. 2000, Qiu *et al*. 2002) and tonoplast (Qiu *et al*. 2004, Batelli *et al*. 2007).

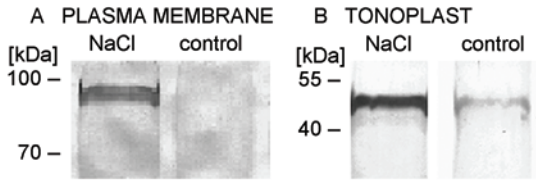

Fig. 2. Western blot of plasma membrane (*A*) and tonoplast (*B*) protein (obtained from control plants and plants treated with 200 mM NaCl) with antibodies raised against plasma membrane Na^+/H^+ exchanger (*A*), and tonoplast NHX, Na^+/H^+ exchanger (*B*). Location of molecular mass standard is indicated on the left. The figures presented are representative for the results obtained in three independent experiments.

In our experimental work, dependence of absorbance changes of acridine orange (Na^+ dependent H^+ efflux) on sodium concentration in the reaction mixture was observed (Fig. 1*E*,*F*). The increase in A_{495} was proportional to the concentration of Na-gluconate in the study. Dissipation of the transmembrane pH gradient through Na^+/H^+ antiporters displayed different saturation kinetics in the plasma membrane and tonoplast of cucumber roots (Fig. 1*E*,*F*). Kinetics studies showed that plasma membrane and tonoplast antiporters have K_m values for Na^+ of 10 and 5 mM, respectively. These findings suggest that tonoplast Na^+/H^+ antiporter transporting Na^+ to the vacuole has higher affinity to Na^+ than protein located in the plasma membrane. It may be assumed that under salt stress conditions in cucumber roots sodium ions are first removed to the vacuole and then to the apoplast.

In conclusion, in cucumber plants plasma membrane and tonoplast antiporter activity is present only in membranes of salt-treated plants. Moreover, sodium ions in excess are firstly transported from the cytosol to the vacuole and then to the apoplast.

References

Agarwal, P.K., Jha, B.: Transcription factors in plants and ABA dependent and independent abiotic stress signaling. - Biol. Plant. **54**: 201-212, 2010.

Apse, M.P., Aharon, G.S., Snedden, W.A., Blumwald, E.: Overexpression of a vacuolar Na^+/H^+ antiport confers salt tolerance in *Arabidopsis*. - Science **285**: 1256-1258, 1999.

Apse, M.P., Blumwald, E.: Na^+ transport in plants. - FEBS Lett. **581**: 2247-2254, 2007.

Ballesteros, E., Blumwald, E., Donaire, J.P., Belvera, A.: Na^+/H^+ antiport activity in tonoplast vesicles isolated from sunflower roots induced by NaCl stress. - Physiol. Plant. **99**: 328-334, 1997.

Barkla, B.J., Zingerelli, L., Blumwald, E., Smith, J.A.C.: Tonoplast Na^+/H^+ antiport activity and its energization by the vacuolar H^+-ATPase in the hallophytic plant *Mesembryanthemum crystallinum* L. - Plant Physiol. **109**: 549-556, 1995.

Batelli, G., Verslues, P.E., Agius, F., Qiu, Q., Fujii, H., Pan, S., Schumaker, K.S., Grillo, S., Zhu, J.-K.: SOS2 promotes salt tolerance in part by interacting with the vacuolar H^+-ATPase and upregulating its transport activity. - Mol. cell. Biol. **27**: 7781-7790, 2007.

Bertorello, A.M., Zhu, J.K.: SIK1/SOS2 networks: decoding sodium signals *via* calcium-responsive protein kinase pathways. - Eur. J. Physiol. **458**: 613-619, 2009.

Blumwald, E., Cragoe, E.J., Poole, R.J.: Inhibition of Na^+/H^+ antiport activity in sugar beet tonoplast by analogs of amiloride. - Plant Physiol. **85**: 30-33, 1987.

Blumwald, E., Aharon, G.S., Apse, M.P.: Sodium transport in plant cells. - Biochim. biophys. Acta **1465**: 140-151, 2000.

Bradford, M.M.: A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principles of protein dye binding. - Anal. Biochem. **72**: 248-254, 1976.

Chinnusamy, V., Jagendorf, A., Zhu, J.K.: Understanding and improving salt tolerance in plants. - Crop Sci. **45**: 437-448, 2005.

Chung, J.S., Zhu, J.K., Bressan, R.A., Hasegawa, P.M., Shi, H.: Reactive oxygen species mediate Na^+-induced *SOS1* mRNA stability in *Arabidopsis*. - Plant J. **53**: 554-565, 2008.

Darley, C.P., Wuytswinkel, O.C.M., Woude, K., Mager W.H.: *Arabidopsis thaliana* and *Saccharomyces cerevisiae NHX1*genes encode amiloride sensitive electroneutral Na^+/H^+ exchangers. - Biochem J. **351**: 241-249, 2000.

Fukuda, A., Yazaki, Y., Ishikawa, T., Koike, S., Tanake, Y.: Na^+/H^+ antiporter in tonoplast vesicles from rice roots. - Plant Cell **39**: 196-201, 1998.

Fukuda, A., Nakamura, A., Tagiri, A., Tanaka, H., Miyao, A., Hirochika, H., Tanaka, Y.: Function, intracellular lokalization and importance in salt tolerance of a vacuolar Na^+/H^+ antiporter from rice. - Plant Cell Physiol. **45**: 146-159, 2004.

Gaxiola, R.A., Rao, R., Sherman, A., Grisafi, P., Alper, S.L., Fink, G.R.: The *Arabidopsis thaliana* proton transporters, AtNhx1 and Avp1, can function in cation detoxification in yeast. - PNAS **96**: 1480-1485, 1999.

Janicka-Russak, M., Kłobus, G.: Modification of plasma membrane and vacuolar H^+-ATPase in response to NaCl and ABA. - J. Plant Physiol. **164**: 295-302, 2006.

Johansson, F., Olbe, M., Sommarin, M., Larsson, C.: Brij 58, a polyoxyethylene acyl ether, creates membrane vesicles of uniform sidedness. A new tool to obtain inside-out (cytoplasmic sideout) plasma membrane vesicles. - Plant J. **7**: 165-173, 1995.

Kabała, K., Kłobus, G.: Characterization of the tonoplast proton pumps in *Cucumis sativus* L. root cells. - Acta Physiol. Plant. **23**: 55-63, 2001.

Katz, A., Kaback, H.R., Avron, M.: Na^+/H^+ antiport in isolated plasma membrane vesicles from the halotolerant alga *Dunaliella salina*. - FEBS Lett. **202**: 141-144, 1986.

Kłobus, G.: The role of plasma membrane-bound activities in nitrate transport into sealed plasma membrane vesicles from *Cucumis sativus* L. roots. - In: Baluška, F., Čiamporová, M., Gašparíková, O., Barlow, P.W. (ed.): Structure and Function of Roots. Pp. 133-140. Kluwer Academic Publishers, Dordrecht 1995.

Kłobus, G., Buczek, J.: The role of plasma membrane oxidoreductase activity in proton transport. - J. Plant Physiol. **146**: 103-107, 1995.

Kłobus, G., Janicka-Russak, M.: Modulation by cytosolic components of proton pump activities in plasma membrane and tonoplast from *Cucumis sativus* roots during salt stress. - Physiol. Plant. **121**: 84-92, 2004

Knight, H., Trewavas, A.J., Knight, M.R.: Calcium signalling in *Arabidopsis thaliana* responding to drought and salinity. - Plant J. **12**: 1067-1078, 1997.

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. - Nature **227**: 680-685, 1970.

La Rosa, P.C., Handa, A.K., Hasegawa, P.M., Bressan, R.A.: Abscisic acid accelerates adaptation of cultured tobacco cells to salt. - Plant Physiol. **79**: 138-142, 1985.

La Rosa, P.C., Hasegawa, P.M., Rhodes, D., Clithero, J.M.: Abscisic acid stimulated osmotic adjustment and its involvement in adaptation of tobacco cells to NaCl. - Plant Physiol. **85**: 174-185, 1987.

Lin, K.H., Pu, S.F.: Tissue- and genotype-specific ascorbate peroxidase expression in sweet potato in response to salt stress. - Biol. Plant. **54**: 664-670, 2010.

Mahajan, S., Pandey, G.K., Tuteja, N.: Calcium- and salt-stress signaling in plants: shedding light on SOS pathway. - Arch. Biochem. Biophys. **471**: 146-158, 2008.

Maia, J.M., Costa de Macedo, C.E., Voigt, E.L., Freitas, J.B.S., Silveira, J.A.G.: Antioxidative enzymatic protection in leaves of two contrasting cowpea cultivars under salinity. - Biol. Plant. **54**: 159-163, 2010.

Netting, A.G.: pH, abscisic acid and the integration of metabolism in plants under stressed and non-stressed conditions: cellular responses to stress and their implication for plant water relations. - J. exp. Bot. **343**: 147-158, 2000.

Ohta, M., Guo, Y., Halfter, U., Zhu, J.K.: A novel domain in the protein kinase SOS2 mediates interaction with the protein phosphatase 2C ABI2. - PNAS **100**: 11771-11776, 2003.

Qiu, Q.S., Guo, Y., Dietrich, M., Schumaker, K.S., Zhu, J.K.: Regulation of SOS1, a plasma membrane Na^+/H^+ exchanger in *Arabidopsis thaliana*, by SOS2 and SOS3. - PNAS **99**: 8436-8441, 2002.

Qiu, Q.S., Guo, Y., Quintero, F.J., Pardo, J.M., Schumaker, K.S., Zhu, J.K.: Regulation of vacuolar Na^+/H^+ exchange in *Arabidopsis thaliana* by the Salt-Overly-Sensitive (SOS) pathway. - J. biol. Chem. **279**: 207-215, 2004.

Queirós, F., Fontes, N., Silva, P., Almeida, D., Maeshima, M., Gerós, H., Fidalgo, F.: Activity of tonoplast proton pumps and Na^+/H^+ exchange in potato cell cultures is modulated by salt. - J. exp. Bot. **60**: 1363-1374, 2009.

Parks, G.E., Dietrich M.A., Schumaker K.S.: Increased vacuolar Na^+/H^+ exchange activity in *Salicornia bigelovii* Torr. in response to NaCl. - J. exp. Bot. **53**: 1055-1065, 2002.

Shi, H., Ishitani, M., Kim, C., Zhu, J.K.: The *Arabidopsis thaliana* salt tolerance gene *SOS1* encodes a putative Na^+/H^+ antiporter. - PNAS **97**: 6896-6901, 2000.

Shi, H., Quintero, F.J., Pardo, J.M., Zhu, J.K.: The putative plasma membrane Na^+/H^+ antiporter SOS1 controls long-distance Na^+ transport in plants. - Plant Cell **14**: 465-477, 2002.

Shi, H., Lee, B., Wu, S.J., Zhu, J.K.: Overexpression of a plasma membrane Na^+/H^+ antiporter gene improves salt tolerance in *Arabidopsis thaliana*. - Natur. Biotechnol. **21**: 81-85, 2003.

Venema, K., Quintero, F.J., Pardo, J.M., Donaire, J.P.: The *Arabidopsis* Na^+/H^+ exchanger AtNHX1 catalyzes low affinity Na^+ and K^+ transport in reconstituted liposomes. - J. biol. Chem. **277**: 2413-2418, 2002.

Verslues, P.E., Batelli, G., Grillo, S., Agius, F., Kim. Y.S., Zhu, J., Agarwal, M., Katiyar-Agarwal, S., Zhu, J.K.: Interaction of SOS2 with nucleoside diphosphate kinase 2 and catalases reveals a point of connection between salt stress and H_20_2 signaling in *Arabidopsis thaliana*. - Mol. cell. Biol. **27**: 7771-7780, 2007.

Ward, J.M., Hirschi, K.D., Sze, H.: Plants pass the salt. - Trends Plant Sci. **8**: 200-201, 2003.

Wu, Y., Ding, N., Zhao, X., Zhao, M., Chang, Z., Liu, J., Zhang, L.: Molecular characterization of *PeSOS1*: the putative Na^+/H^+ antiporter of *Populus euphratica.* - Plant mol. Biol. **65**: 1-11, 2007.

Xia, T., Apse M.P., Aharon, G.S., Blumwald, E.: Identification and characterization of NaCl-inducible vacuolar Na^+/H^+ antiporter in *Beta vulgaris*. - Physiol. Plant. **116**: 206-212, 2002.

Xiong, L., Schumaker, K.S., Zhu, J.K.: Cell signaling during cold, drought, and salt stress. - Plant Cell **14**: 165-183, 2002.

Yamaguchi, T., Aharon, G.S., Sottosanto, J.B., Blumwald, E.: Vacuolar Na^+/H^+ antiporter cation selectivity is regulated by calmodulin from within the vacuole in a Ca^+- and pH-dependent manner. - PNAS **102**: 16107-16112, 2005.

Yamaguchi, T., Blumwald, E.: Developing salt-tolerant crop plants: challenges and opportunities. - Trends Plant Sci. **10**: 615-620, 2005.

Yokoi, S., Quintero, F.J., Cubero, B., Ruiz, M.T., Bressan, R.A., Hasegawa, P.M., Pardo, J.M.: Differential expression and function of *Arabidopsis thaliana* NHX Na^+/H^+ antiporters in the salt stress response. - Plant J. **30**: 529-539, 2002.

Zhu, J., Fu, X., Koo, Y.D., Zhu, J.K., Jenney, F.E., Jr., Adams, M.W.W., Zhu, Y., Shi, H., Yun, D.-J., Hasegawa, P.M., Bressan, R.A.: An enhancer mutant of *Arabidopsis salt overly sensitive 3* mediates both ion homeostasis and the oxidative stress response. - Mol. cell. Biol. **27**: 5214-5224,. 2007.