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Effects of salinity on activities of H^+-ATPase, H^+-PPase and membrane lipid composition in plasma membrane and tonoplast vesicles isolated from soybean (*Glycine max* L.) seedlings

YU Bing-jun[1,2,*], LAM Hon-ming[2,*], SHAO Gui-hua[2,3], LIU You-ling[1]

(1. College of Life Sciences, Nanjing Agricultural University, Nanjing 210095, China. E-mail: bjyu@njau.edu.cn; 2. Department of Biology, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong, China; 3. Institute of Crop Breeding and Cultivation, Chinese Academy of Agricultural Sciences, Beijing 100081, China)

Abstract: The effects of NaCl stress on the H^+-ATPase, H^+-PPase activity and lipid composition of plasma membrane (PM) and tonoplast (TP) vesicles isolated from roots and leaves of two soybean cultivars (*Glycine max* L.) differing in salt tolerance (Wenfeng7, salt-tolerant; Union, salt-sensitive) were investigated. When Wenfeng7 was treated with 0.3% (W/V) NaCl for 3 d, the H^+-ATPase activities in PM and TP from roots and leaves exhibited a reduction and an enhancement, respectively. The H^+-PPase activity in TP from roots also increased. Similar effects were not observed in roots of Union. In addition, the increases of phospholipid content and ratios of phospholipid to galactolipid in PM and TP from roots and leaves of Wenfeng7 may also change membrane permeability and hence affect salt tolerance.

Keywords: salinity; *Glycine max* L.; plasma membrane; tonoplast; H^+-ATPase; H^+-PPase; membrane lipid composition

Introduction

Soil salinity is a major abiotic stress hampering plant growth and development hence reducing agricultural crop productivity (Apse, 1999; Zhu, 2002; Munns, 2002; Zhao, 2003). Salinity leads to decrease of photosynthesis, inhibition of growth, and accelerated senescence in plants (Liu, 1998; Herralde, 1998; Zhu, 2001; Lacerda, 2003). The mechanism of salt tolerance in glycophytes mainly depends on differential ion uptake in roots and compartmentation of ions in tissue, organ, cellular and sub-cellular levels. The net effect is to reduce salt accumulation in cytoplasm, and yet maintain osmotic and ionic homeostasis (Liu, 1998; Zhu, 2001; Greenway, 1980; Gong, 1999). Among all sub-cellular compartments, biomembranes are the major sensitive sites susceptible to salinity injury (Liu, 1998). Regulation of membrane-bound enzymes (e.g. H^+-ATPase and H^+-PPase) and membrane lipid composition will be helpful for plants to maintain membrane integrity and its function under salinity stress, and this may contribute to plant salt tolerance (Surjus, 1996). In higher plants, both the salt extrusion from the cell and the salt compartmentation into vacuoles are carried out by antiporters, which make use of proton pumps (P-type H^+-ATPase, V-type H^+-ATPase and V-type H^+-PPase) to provide the driving force for ion transport (Zhao, 2003).

Glycine max L., a middle salt-tolerant plant, is one of major crops in China (Liu, 1998; Shao, 1993). There are several reports on the changes in activities of H^+-ATPase in plasma membrane (PM), H^+-PPase in tonoplast (TP) and lipid composition in responses of plant to salinity (Gong, 1999; Lin, 1996; Ballesteros, 1998; Wu, 1998; Yu, 1999; Ma, 2002). But the study on *G. max*, especially the comparative research on soybean cultivars differing in salt tolerance has not been undertaken. The soybean cultivars Wenfeng7 and Union are salt-tolerant and salt-sensitive, respectively (Guo, 1998). In this paper, we performed comparative analysis using soybean cultivars Wenfeng7 and Union. The effects of salinity stress on the H^+-ATPase and H^+-PPase activities and lipid composition of PM and TP vesicles isolated from roots and leaves of this two soybean cultivars seedlings are examined, and their salt tolerance are also discussed.

1 Materials and methods

1.1 Plant materials

Soybean seeds (*G. max* L.) of two cultivars (salt-tolerant Wenfeng7 and salt-sensitive Union) were germinated as described previously (Yu, 2001). The nutrient solution (half strength Hoagland) was replaced every 2—3 d. When the first pair of true leaves of the seedlings was fully expanded, plants of the salt-treated group were transferred to nutrient solution supplemented with 0.3% (w/v) NaCl. After treatment for 3 d, partial roots and leaves of the control and salt-treated seedlings were cut for the preparation of PM and TP vesicles. The remaining seedlings of the salt-treated group were transferred to nutrient solution supplemented with 0.6% (w/v) NaCl for 3 d, and then sampled as before for PM and TP preparation.

1.2 Preparation of PM and TP vesicles

The procedures were performed according to the method

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described before(Yu, 1999; 1997). The excised roots and leaves were homogenized in a mortar with a pestle in the buffer contained 250 mmol/L D-mannitol, 25 mmol/L HEPES-Tris(pH 7.6), 5 mmol/L EGTA, 5 mmol/L EDTA, 10 mmol/L KF, 2 mmol/L PMSF, 1.5% PVP(w/v), 0.5% BSA(w/v), 15 μg/L BHT, 5 mmol/L $K_2S_2O_5$, 1 mmol/L DTT. The ratio of the medium to the tissue was 3 ml/g. The homogenate was filtered through cheesecloth and centrifuged at 500 × g for 10 min to remove unbroken cells and the cell wall fractions. The supernatant was recentrifuged at 12000 × g for 15 min, and the resulting supernatant was further centrifuged at 60000 × g for 30 min. The pellet was gently suspended in the suspension buffer consisting 250 mmol/L D-mannitol, 2.5 mmol/L HEPES-Tris (pH 7.6), 1 mmol/L EDTA, 1 mmol/L PMSF, 10 μg/L BHT, 1 mmol/L DTT, layered over a 22%/36%/45% (w/v) cushion and centrifuged at 70000 × g for 2 h. The interfaces of 36%—45% and 22%—36%, referred to as the PM and TP vesicles respectively, were collected. The partial PM and TP vesicles were used for assay of their activities of H^+-ATPase in PM, H^+-ATPase and H^+-PPase in TP. The rest of the membrane vesicles were centrifuged at 70000 × g for 30 min to remove the sucrose, then the membrane pellet were resuspended in the suspension buffer and stored frozen at −20℃ for membrane lipid analysis.

1.3 Assay of activities of H^+-ATPase in PM and TP

The activities of H^+-ATPase in PM, H^+-ATPase in TP were determined by quantitating the inorganic phosphate that was released from ATP as described previously(Yu, 1999). The reaction mixture contained 30 mmol/L HEPES-Tris(pH 6.5 for PM and 7.5 for TP, respectively), 6 mmol/L $MgSO_4$, 50 mmol/L KCl, 0.1 mmol/L ammonium molybdate, 1 mmol/L NaN_3, 50 mmol/L KNO_3 (for PM) and/or 0.1 mmol/L Na_3VO_4(for TP), and 3 mmol/L Na_2-ATP in a final volume of 0.5 ml. The reaction was started by addition of Na_2-ATP and terminated by addition 1 ml of 1% (w/v) ammonium molybdate and 1 mmol/L H_2SO_4 after incubation at 37℃ for 20 min. The liberated Pi was analyzed by the Fiske-Subbarow reagent, using KH_2PO_4 as the standard.

1.4 Assay of activities of H^+-PPase in TP

According to the method described previously (Yu, 1997), the reaction mixture contained 30 mmol/L HEPES-Tris(pH 7.8), 50 mmol/L KCl, 0.5 mmol/L $MgSO_4$, 0.1 mmol/L Na-molybdate, 0.3 mmol/L Na_4PPi-Tris(pH 7.8). After incubation at 37℃ for 30 min, the reaction was stopped and the liberated Pi was analyzed as the above.

1.5 Extraction and measurements of membrane lipids

Polar lipids were extracted from the isolated membrane according to Brown and Dupont (Brown, 1989). Phospholipid was quantified according to the method described by Hu *et al*.(Hu, 1993), and the liberated Pi was analyzed by the Fiske-Subbarow reagent, using KH_2PO_4 as the standard. Galactolipid was quantified by the method of Roughan and Batt(Roughan, 1968), using galactose as the standard.

1.6 Protein determination

Protein was determined by the method of Bradford (Bradford, 1976) with BSA as the standard.

1.7 Data analysis

Comparisons between means were carried out by one-way ANOVA(*F*-ratio test).

2 Results and discussion

2.1 Effects of salinity on activities of H^+-ATPase in PM and TP, H^+-PPase in TP isolated from roots and leaves of *G. max* seedlings

Under 0.3% NaCl stress for 3 d, the PM H^+-ATPase activities from roots and leaves of two soybean cultivars were all decreased compared with the control plants. However, the decrease in Wenfeng7 was much higher than that in Union. Under the same treatment, TP H^+-ATPase activities increased significantly in roots and leaves of Wenfeng7, while the changes in roots and leaves of Union were not consistent (Table 1). The main deleterious effects of salinity on plants included ion toxicity and osmotic stress, and the former is a dominant stress factor during prolonged salinity treatment (Munns, 2002). The most effective pathway of alleviating the ion toxicity under salt stress is to maintain ion homeostasis across the cell by controlling salt ions that intruding the plant cell(Zhu, 2001). Adjustment of the activities of membrane-bound enzymes(such as H^+-ATPase and H^+-PPase) may be helpful to enhance salt tolerance in plants under salinity stress. In the salt tolerant Wenfeng7, a decrease in PM H^+-ATPase activity and an increase in TP H^+-ATPase activity were observed in both roots and leaves under salt stress. An increase in TP H^+-PPase activity in its roots was also found (Table 1). On the other hand, the salt sensitive Union did not exhibit similar changes. These data suggested that the salt tolerance of Wenfeng7 may be partially related to the increase of ion pumping activity at TP although the ion pumping activity at PM was reduced under salt stress. By actively compartmenting ions into TP, the cell of Wenfeng7 seedlings under salt stress can maintain a low level of toxic ions in the cytoplasm and yet establish a negative osmotic potential gradient for water uptake.

2.2 Effects of salinity on contents of phospholipid and galactolipid in PM and TP isolated from roots and leaves of *G. max* seedlings

Under prolonged NaCl treatment (0.3% NaCl for 3 d and 0.6% NaCl for another 3 d), the contents of phospholipid in PM and TP from roots and leaves of salt tolerant Wenfeng7 were all increased, compared to the untreated plants. By contrast, the same measuring parameters in salt sensitive Union were mostly decreased, when compared to the untreated controls (Table 2). The contents of

galactolipid in PM and TP from leaves of both cultivars were all elevated, and the increase in Union were more prominent than that in Wenfeng7. On the other hand, the galactolipid contents in PM and TP from roots were mostly decreased, except in PM of Union (Table 3). In general, the ratios of phospholipid to galactolipid in PM and TP isolated from roots and leaves of salt-tolerant Wenfeng7 seedlings were higher than that in the control plants, while Union exhibited the opposite trend (Fig.1 and Fig.2).

Table 1 Changes in H^+-ATPase activity in PM and TP and H^+-PPase activity in TP isolated from roots and leaves of Wenfeng7 and Union seedlings under salt stress

Tissues	Plants	H^+-ATPase activity, μmol Pi.mg^{-1} protein.h^{-1}				H^+-PPase activity, μmol PPi dehydrolysed.mg^{-1} protein.h^{-1}	
		PM		TP		TP	
		Control	0.3% NaCl	Control	0.3% NaCl	Control	0.3% NaCl
Root	Wenfeng7	31.87 ± 0.88(100)	16.32 ± 0.78** (51.21)	10.63 ± 0.90(100)	16.42 ± 0.72* (154.47)	4.41 ± 0.50(100)	9.29 ± 0.54* (210.66)
	Union	27.36 ± 1.52(100)	24.85 ± 0.61(90.83)	7.93 ± 0.50(100)	7.23 ± 0.93(91.17)	4.86 ± 0.33(100)	3.31 ± 0.23* (68.11)
Leaf	Wenfeng7	15.22 ± 0.75(100)	9.64 ± 0.56* (63.34)	1.88 ± 0.25(100)	4.50 ± 0.02** (239.36)	13.08 ± 0.37(100)	12.33 ± 0.02(94.27)
	Union	15.84 ± 0.41(100)	14.00 ± 0.09* (88.38)	2.94 ± 0.02(100)	5.58 ± 0.02** (189.80)	14.12 ± 0.09(100)	13.73 ± 0.01(97.24)

Notes: Results are the mean ± S.D. of 3—4 replicates (percentage compared to control); ** and * indicate significant levels of $P < 0.01$ and $P < 0.05$ respectively, when compared NaCl treated samples to the corresponding controls

Table 2 Changes in phospholipid contents in PM and TP isolated from roots and leaves of Wenfeng7 and Union seedlings after prolonged NaCl treatment.

Tissues	Plants	Phospholipid contents, μmol.mg^{-1} protein			
		PM		TP	
		Control	0.3% NaCl	Control	0.3% NaCl
Root	Wenfeng7	0.229 ± 0.00(100)	0.308 ± 0.024* (134.50)	0.140 ± 0.017(100)	0.158 ± 0.005* (112.86)
	Union	0.401 ± 0.036(100)	0.353 ± 0.033* (88.03)	0.215 ± 0.040(100)	0.182 ± 0.013* (84.65)
Leaf	Wenfeng7	0.229 ± 0.018(100)	0.249 ± 0.019(108.73)	0.189 ± 0.044(100)	0.230 ± 0.00* (121.69)
	Union	0.237 ± 0.002(100)	0.253 ± 0.014(106.75)	0.224 ± 0.066(100)	0.158 ± 0.035** (70.54)

Notes: Results are the mean ± S.D. of 3—4 replicates (percentage compared to control); ** and * indicate significant levels of $P < 0.01$ and $P < 0.05$ respectively, when compared NaCl treated samples to the corresponding controls

Table 3 Changes in galactolipid contents in PM and TP isolated from roots and leaves of Wenfeng7 and Union seedlings after prolonged NaCl treatment

Tissues	Plants	Galactolipid contents (nmol.mg^{-1} protein)			
		PM		TP	
		Control	0.3% NaCl	Control	0.3% NaCl
Root	Wenfeng7	100.33 ± 2.12(100)	86.24 ± 2.00* (85.96)	85.70 ± 2.21(100)	42.59 ± 2.16** (49.70)
	Union	51.96 ± 1.02(100)	59.42 ± 0.98* (114.36)	122.54 ± 5.67(100)	72.47 ± 1.46* (59.14)
Leaf	Wenfeng7	86.59 ± 3.23(100)	89.57 ± 1.34(103.44)	34.79 ± 0.87(100)	49.34 ± 1.69* (141.82)
	Union	63.75 ± 1.14(100)	171.49 ± 4.56** (269.00)	41.43 ± 0.94(100)	68.21 ± 2.11** (164.64)

Notes: Results are the mean ± S.D. of 3—4 replicates (percentage compared to control); ** and * indicate significant levels of $P < 0.01$ and $P < 0.05$ respectively, when compared NaCl treated samples to the corresponding controls

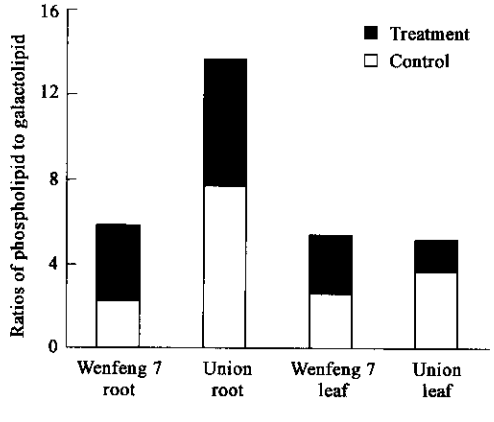

Fig. 1 Changes in ratios of phospholipid to galactolipid in PM isolated from roots and leaves of Wenfeng7 and Union seedlings after prolonged NaCl treatment

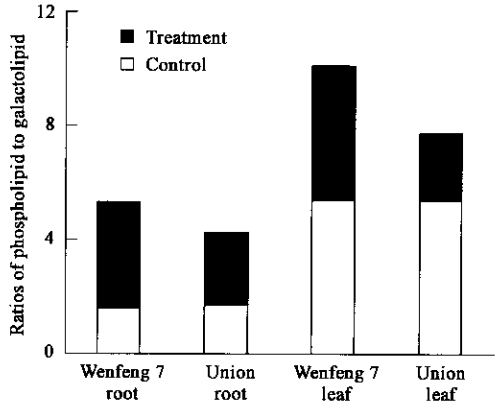

Fig. 2 Changes in ratios of phospholipid to galactolipid in TP isolated from roots and leaves of Wenfeng7 and Union seedlings after prolonged NaCl treatment

The changes in the contents of phospholipid and galactolipid in biomembranes, and the index of unsaturated fatty acids (IUFA) will affect the fluidity and permeability of membranes. It was suggested that phosphatidycholine (PC) and phosphatidylethanolamine (PE) (two major components of phospholipid in plants) were inversely related to chloride accumulation in grape roots (Kuiper, 1968a), and high galactolipid contents (such as monoglycosyldiacyl-glycerol, MGDG and diglycosyldiacylglycerol, DGDG) in grape roots increased the absorption and transportation of chloride

(Kuiper, 1968b). Stuiver(Stuiver, 1993) reported that the high unsaturated fatty acids in lipids facilitated Na^+ and Cl^- entering the cell across plasma membrane. The role of Ca^{2+} in alleviating salt stress upon barley seedlings was partly due to the increase of phospholipid contents and decrease of galactolipid contents in PM and TP vesicles isolated from its roots(Yu, 1998).

Previous study showed that one possible mechanism of salt tolerance in both cultivated soybeans(*G. max*) and wild soybeans(*G. soja*) is related to the lower absorption (in roots) and transportation(to aerial parts) of Na^+ and Cl^- in seedlings(Yu, 2001; An, 2002). Huang(Huang, 1996) reported a decrease in the phospholipid contents and increases in the ratio of saturated to unsaturated fatty acids and enthalpy of lipid phase transition in PM from leaves of soybean cv. Kaoshing seedling under salt stress. The salt-acclimatized Kaoshing seedlings exhibited higher H^+-ATPase activity in PM from roots compared to the control and non-acclimatized plants(Huang, 1998). Surjus *et al*.(Surjus, 1996) found that when soybean cv. Hodgson seedlings was treated with 25 mmol/L NaCl, the main lipid changes were observed in the root microsomal fraction where the phospholipid and sterol contents decreased by 50%, and the saturated fatty acids($C_{16:0}$ and $C_{18:0}$) and the IUFA in PM of roots increased by 13.3% and decreased by 26.0%, respectively. Hong and Pak(Hong, 1999) suggested that the contents of chiro-inositol-containing phospholipids were all decreased in apical, elongating and mature parts of hypocotyls and roots of soybean cv. Williams seedlings under salt stress.

One major difference between the salt tolerant Wenfeng7 and salt sensitive Union is related to the changes in lipid composition of PM and TP from roots and leaves of seedlings under salt stress. When Wenfeng7 seedlings were subjected to NaCl stress, the increase in phospholipid contents and ratios of phospholipid to galactolipid in PM and TP from its roots and leaves will help to reduce the membrane permeability, and hence affect the transportation of salt ions across the biomembranes.

3 Conclusions

Under salinity stress, the H^+-ATPase activities in PM from roots and leaves of two soybean cultivars decreased compared to the control plants. For the salt tolerant cultivar Wenfeng7, the activities of H^+-ATPase in TP from roots and leaves, and the activities of H^+-PPase in TP from roots were all increased, but the salt sensitive cultivar Union exhibited the opposite trend. By actively compartmenting salt ions into TP, the cell of Wenfeng7 seedlings under salt stress can maintain a lower level of toxic ions in the cytoplasm and yet establish a negative osmotic potential gradient for water uptake. The increases of phospholipid contents and ratios of phospholipid to galactolipid in PM and TP from roots and leaves of Wenfeng7 will lead to reduction of membrane permeability and salt ion transportation across the biomembranes. The above mentioned may be helpful for soybean cultivar Wengeng7 to tolerate salinity stress.

Abbreviations: BHT: butylated hydroxytoluene; BSA: bovine serum alumin; DTT: dithiothreitol; EDTA: ethylene diamine tetraacetic acid; EGTA: ethylene glycol bis(β-aminoethyl ether)-N, N'-tetraacetic acid; HEPES: N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid; PM: plasma membrane; PMSF: phenylmethylsulfonyl fluoride; PVP: polyvinylpyrrolidone; TP: tonoplast.

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