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Salt-Stress Induces Lipid Degradation and Lipid Phase Transition in Plasma Membrane of Soybean Plants[1]

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ABSTRACT : Soybean seedlings were grown under NaCl-stress and non-stress conditions. To induce salt stress, the water potential of Hoagland solution, which was used to culture the soybean plants, was adjusted to -1.6 MPa daily with saturated NaCl solution. Leaf plasma membranes were isolated and assayed for lipid content, phospholipid class composition, and the fatty acid composition of total phospholipids of plasma membranes. Lipid phase transition temperature and lipoxygenase activity were also measured. The plasma membrane contained a higher concentration of phospholipid than that of glycolipid. The phospholipid(PL) contained higher contents of saturated fatty acids than that of unsaturated fatty acids. Under salt-stress, the ratio of saturated/unsaturated fatty acids became higher, and the enthalpy of lipid phase transition in leaf plasma membranes was raised . A large increase in the lipoxygenase activity in salt-stressed plasma membranes may have contributed to the decline in PL. These results suggest that the plasma membrane of leaves from soybean plants grown under the salt-stress became solidified, and this solidification of the plasma membrane might be the limiting factor that restricts plant growth under salt-stress environments.

KEY WORDS : **Salt-stress, Phospholipid, Fatty acid, Lipid-phase Transition, Lipoxygenase.**

INTRODUCTION

Plasma membrane plays an important role in cellular activities such as ion transport, proton-pumping ATPase, signal transduction, and metabolic functions (Taiz and Zeiger, 1991). It has been reported that senescence (Bilsen and Hoekstra, 1993; Yao *et al.*, 1991) and environmental stresses including low temperature (Hetherington *et al.*, 1988; Lynch and Steponkus, 1987; Kenrick and Bishop, 1986), water deficit (Norberg and Liljenberg, 1991; Bell and Mullet, 1991; Navari-Izzo *et al.*, 1989),and salinization (Hurkman *et al.*, 1989; LaRosa *et al.*, 1985; Greenway and Munns, 1980) adversely affect the biochemical structure and physiological functions of the plasma membrane in plants. The specific effects of salinization on the plasma membrane are still uncertain. It has been shown that salinization results in biochemical and physiological changes in the plasma membrane. These changes are summarized as follow: (1) Displacement of Ca^{++} ion by Na^{+} ion that increases the permeability (Cramer *et al.*, 1985), (2) Activation of the proton-pumping ATPase that consequently decreases turgor pressure (Niu *et al.*, 1993), (3) Induction of biosynthesis of a specific protein, 60 kD (Fisher *et al.*, 1994), or 150 kD protein (Sadka *et al.*, 1991) that

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plays an important role in ionic homeostasis or osmotic adjustment (Sadka *et al.*, 1989), (4) Biosynthesis of a protein which is similar to a mammalian glutathione peroxidase (Holland *et al.*, 1993) and is associated with lipid peroxidation (Huang and Chen,1995), (5) Biosynthesis of abscisic acid that is associated with adaptation of plants to salt-stress (LaRosa *et al.*, 1985). In addition, changes in lipid compositions may play an important role in regulating the activities and functions of the plasma membrane. In this study, it was attempted to determine whether alternations of lipid components and structure of plasma membrane are important in limiting the ability of plants to grow under salt-stress conditions.

MATERIALS AND METHODS

Plant culture

Soybean (*Glycine max.* (L.) Merr. cv Kaoshing #10) seeds were germinated for three days in vermiculite. Seedlings were transplanted to pots containing a full strength of Hoagland solution. After 7 days, one tenth of full strength of Hoagland solution was used for the cultures, and for one group of seedling, the water potential of the solution was adjusted to -1.6 MPa with saturated NaCl solution. The water potential of the nutrient solution was checked and adjusted daily to the desired value by using a thermocouple psychrometer described by Huang *et al.* (1975). The growth conditions were: Day/night temperature, 30/25 ℃; photoperiod, 8 hrs; light intensity, 392 μmol m^{-2} s^{-1}. The leaves in middle part of plant were harvested for assays or measurements four days after imposing the salt-stress treatment.

Preparation of plasma membrane

Isolation procedures followed the methods of Kjellbom and Larsson (1984) with little modification. Leaf samples were homogenized in buffer containing 0.25 M sucrose, 24 mM Tris-HCl pH 7.5, 3 mM EDTA, and 25 mM DTT. Homogenates were filtered through four layers of cheesecloth and centrifuged at 1000 g for 10 min. The supernatant was then centrifuged at 12,000 g for 15 min to remove organelles and nuclei. The resultant supernatant was finally centrifuged at 80,000 g for one hour in a Hitachi SCP 7OH centrifuge to obtain microsomal fractions. The microsomes were then partitioned using a two-phase system following the procedures of Widell (1987). Purity of the plasma membranes was checked by assaying the activity of a marker enzyme, vanadate-sensitive H^+-ATPase.

Determination of membrane protein

Plasma membrane protein was extracted and quantified by the method of Kjellbom and Larsson(1984). Bovine Serum Albumin protein was used as a standard.

Extraction and assay of lipoxygenase

Lipoxygenase was extracted from the plasma membrane and purified according to the methods of Bowsher *et al.* (1992) with little modification. The pellet of plasma membrane was suspended in 30 ml of buffer containing 20 mM Tris-HCl (pH 7.2), 10% glycerol, and 2% Triton X-100 at 4 ℃ with constant stirring for two hours. This suspension was then dialyzed against buffer containing 20 mM Tris-HCl (pH 7.2), 10% glycerol, and 0.5% Triton

X-100 for 24 hours. The dialyzed fraction was centrifuged at 9250 g for 10 min. The crude enzyme in the supernatant fraction was then partially purified by precipitation using ammonium sulfate from 25% to 60 % saturation at 4 ℃. The precipitate was removed by centrifugation, then dissolved in a minimum volume of extraction buffer and dialyzed overnight against the same buffer at 4 ℃. This dialyzed fraction was used to assay enzyme activity. Activity of lipoxygenase was measured spectrophotometrically as the increase in A_{235} nm caused by the formation of a conjugated diene from di- or trienoic fatty acids structure. The enzyme extracts were incubated with 100 μM linoleic acid (or linolenic acid) in 100 mM sodium acetate buffer, pH 7.0 at 25 ℃ (modified from Geerts *et al.*, 1994). Protein concentration was determined as described by Bradford (1976).

Extraction and separation of lipids

Membrane lipids were extracted according to the methods of Bligh and Dyer (1959), and separated by TLC according to the method of Miquel and Dubacq (1992) with little modification. Phospholipids and glycolipids were separated using the solvent system chloroform : acetone : methanol : acetic acid : water (50 : 20 : 10 : 10 : 5, v/v). Neutral lipids were chromatographed using the solvent system petroleum ether : diethylether : acetic acid (70 : 30 : 0.4, v/v). The separated lipids were located under UV light after the TLC plate was sprayed with 0.001% primulin. The individual lipid classes were verified by the method of Parkin and Kuo (1989). The identified lipid individual were scraped from the plate and quantified by assays of lipid-phosphorous and lipid-galactose fractions.

Extraction and separation of fatty acids

Fatty acids from the phospholipid fraction were transmethylated and analyzed by GLC (Shimadzu) on a silica capillary column of PEG 20 M. The quantitation of fatty acid methyl esters as performed with flame ionization detector (FID). The injector and FID temperatures were 270 and 300 ℃, respectively, and column temperature was programmed from 190 to 230 ℃ at 5 min^{-1}. Heptadecanoic acid was used as internal standard (Parkin and Kuo, 1989; Miquel and Dubacq, 1992).

Determination of phase transition of plasma membrane

The lipid phase transition temperature of the plasma membrane was determined calorimetrically according to Vertucci (1992) with slight modification. The extracted lipid was sealed in aluminum DSC pans, and then the pans were loaded into a Perkin-Elmer DSC-4. The calorimeter was calibrated for temperature between -90 and -160 ℃ with methylene chloride and Indium standards. Samples were initially cooled to -150 ℃ at a rate 10 ℃ per min. Thermogram were then recorded during heating at a rate 10 ℃ per min from -150 to 40 ℃. The onset temperatures of the transition were determined, and the enthalpy of lipid transition was calculated.

RESULTS

Total lipid content of plasma membranes from soybean seedlings grown under the salt-stress was reduced about 64% compared with that in control seedlings (Table 1). Two major groups of lipids, polar and neutral, were detected in plasma membranes. The polar fraction

consisted of phospholipids and glycolipids. Phospholipid was the major fraction and neutral lipid was least abundant. All lipid fractions were reduced when plants were grown under salt-stress condition. Phospholipid, glycolipid , and neutral lipid in plasma membranes of salt-stressed plants were reduced about 29%, 20% and 4% , respectively, compared with those in control plants (Table 1).

Table 1. Lipid composition of the plasma membranes isolated from leaf tissue of soybean seedlings.

Lipids	Treatment	
	Control (%)	% deceased by salt-stress
Polar lipids:		
Phospholipids	53	29
Glycolipids	35	20
Neutral lipids	12	4

Decrease (%)* =1— [Phospholipid or Glycolipids or Neutral lipids]$^{\text{salt-stress}}$ / [Phospholipids or Glycolipids or Neutral lipids]$^{\text{control}}$

Four classes of phospholipid components, phosphatidyl ethanolamine (PE), phosphatidyl choline(PC), phosphatidyl serine (PS), and phosphatidyl inositol (PI), were quantified. Among these components, PE and PC were the major-, PS and PI were the minor-phospholipid class. Although these phospholipids were all reduced by the salt-stress treatment, the ratios of PE/PC, PE/PS, and PE/PI were increased from 2.14, 5.04, and 6.65 in control plants to 2.70, 6.52, and 11.32 in salt-stressed plants, respectively. Thus, the ratio of PE to total phospholipid was increased in plants grown under salt-stress conditions (Table 2).

Table 2. Phospholipid composition of plasma membranes in leaf tissue of soybean seedlings.

Treatment	Phospholipids (μg/100 g fresh weight)			
	PE	PC	PS	PI
Control (-0.8 MPa)	55.49 ± 5.3	25.51 ± 3.4	11.02 ± 0.9	8.35 ± 1.7
Salt-stress (-1.6 MPa)	34.07 ± 3.3	12.62 ± 1.7	5.22 ± 0.8	3.01 ± 0.2

Each value shown in tables is the mean of triplicates.
PE: Phosphatidyl ethanolamine
PC: Phosphatidyl choline
PS: Phosphatidyl serine
PI: Phosphatidyl inositol

Palmitic acid was the most abundant, and palmitoleic acid the least abundant fatty acid found in the fatty-acyl moieties of phospholipids. Between the two saturated fatty acid detected, the concentration of palmitic acid was much higher than that of stearic acid. Among the four kinds of unsaturated fatty acids detected, the concentration of oleic acid was the greatest compared with the three other unsaturated fatty acids, palmitoleic acid, linoleic acid, and linolenic acid (Table 3).

Table 3. Fatty acids in acyl chains of phospholipid extracted from plasma membranes of soybean leaf cells.

	Fatty acid mol (%)					
	16:0	18:0	16:1	18:1	18:2	18:3
Control (-0.8 MPa)	51.20±8.1	12.39±1.8	5.99±1.5	13.01±2.2	8.34±0.9	7.67±1.8
Salt-stress (-1.6 MPa)	58.67±8.2	21.00±2.6	4.76±4.5	7.78±0.84	4.04±0.5	1.23±0.4

16:0 = Palmitic acid
16:1 = Palmitoleic acid
18:0 = Stearic acid
18:1 = Oleic acid
18:2 = Linoleic acid
18:3 = Linolenic acid

Overall, the contents of saturated fatty acids were increased, and those of unsaturated-fatty acids decreased respectively by the salt treatment. The ratios of 16:1/16:0, (18:1 + 18:2 + 18:3)/18:0, and total unsaturated /saturated fatty acid in phospholipid were decreased by salt-stress(Table 4). This result correlated with of lipoxygenase activity in plasma membranes. As shown in Table 4, this enzyme activity was increased about 16-fold in salt-stressed plants. The enthalpy (heat content) in total plasma membrane lipids was also raised from about 57 J/g to 69 J/g by the salt-stress treatment (Table 5). The data in Table 5 indicate that salt-stress damaged the nature of the plasma membrane from a fluid mosaic to a more gel-solid state.

Table 4. Lipoxygenase activity and ratio of unsaturated/saturated fatty acid in plasma membrane of soybean leaf cells.

Treatment	Enzyme specific activity (μ mol/min mg protein)	16:1/16:0	Unsat. 18C/18:0	Total unsat./sat.
Control (-0.8 MPa)	0.09	0.12	2.32	0.55
Salt-stress (-1.6 MPa)	1.48	0.08	0.62	0.22

Unsat. 18 C = 18:1 + 18:2 + 18:3
Total unsat. = 16:1 + 18:1 + 18:2 + 18:3
Total sat. = 16:0 + 18:0

Table 5. Enthalpy change of lipids extracted from the plasma membrane of soybean leaf cells.

Treatment	Total lipid content (μg / 100 g fresh weight)	Enthalpy (J/g)
Control (-0.8 MPa)	289.5	56.7
Salt-stress (-1.6 MPa)	126.6	68.9

DISCUSSION

Salt-stress treatment (-1.6 MPa) caused soybean plants to wilt. However, they recovered to their original growth rate when placed in normal nutrient solution at a water potential of

-0.8 MPa. Phospholipid was the major component of lipids in the plasma membrane, and its concentration was reduced much more than other the two kinds of lipid components by the salt-stress treatment (Table 1). Therefore, phospholipid was the key constituent of the plasma membrane assayed and studied in this investigation. Regarding the fatty acid composition of plasma membrane phospholipids, salt-stress treatment decreased the proportion of unsaturated- and increased the proportion of saturated-fatty acids (Table 3). The decline in the proportion of unsaturated fatty acids in phospholipid may be due to enhancement of lipoxygenation of polyunsaturated fatty acids by the salt-treatment. This possibility is supported by the data in Table 4 which showed that salt-stress greatly increased the activity of lipoxygenase. This could result in an increase in the ratio of saturated/ unsaturated fatty acids in acyl side chains of phospholipids. Corresponding with the decrease in unsaturated fatty acids in phospholipids, the enthalpy of the lipid phase transition indicates that plasma membranes became more rigid and solidified after salt-stress treatment. Existence of domains of liquid in the solid-gel state could cause the plasma membrane to lose its osmo-regulatory function. It has been reported that salt-stress at water potential -1.6 MPa enhanced ion, K^+ (^{86}Rb), leakage across the plasma membrane of soybean plants (Huang and Chen 1995). Therefore, it may be concluded that salt-stress results in the solidification of the plasma membrane and a lose in differential permeability.

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鹽逆境使大豆幼苗細胞膜中脂質分解及脂質相的改變

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摘　　要

大豆生長於鹽逆境情況下，其葉細胞膜中磷脂濃度比醣脂及中性脂的濃度高。而磷脂中所含的飽和脂肪酸濃度增加，而不飽和脂肪酸的濃度反而相對減低。不飽和脂肪酸濃度的減低與 Lipoxygenase 活性的增加有關。由於鹽逆境情況下，此酵素分解不飽和脂肪酸活性的增加，及飽和脂肪酸濃度的提高，使飽和脂肪酸的濃度與不飽和脂肪酸的濃度的比例增加。另外細胞膜的脂質 Enthalpy 也有增加的現象。這些結果顯示出，大豆生長於鹽逆境情況下其脂質相有改變的現象，也就是細胞膜有固體化的現象。而此細胞膜的固體化作用，導致細胞失去其對水分或養分滲透壓的調控作用，這才是致使植物不能生長於鹽逆境土壤中的主要因素。

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