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Amiloride Inhibition of Vacuolar Na^+/H^+ Antiporter Enhance Salt Stress in *Zea mays* L. Seedlings

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Abstract: The aim of this study to show the importance of salt intracellular compartmentation as a tolerance mechanism by inhibition of Na^+/H^+ antiporter system. In this research 4 day/old *Zea mays* L. seedlings (var. single cross 704) were exposed to 200 and 300 mM NaCl with and without 100 and 200 micromolar amiloride. After 48 h, the roots and shoots of seedlings were harvested separately. The changes of total Na^+absorption, the amount of malondialdehyde and the activity of antioxidant enzymes such as guaiacol peroxidase, ascorbate peroxidase and catalase were analysed. The results indicated that Na^+ absorption has been increased by salt stress but was not influenced by amiloride. Malondialdehyde content and the activity of antioxidant enzymes such as guaiacol peroxidase, ascorbate peroxidase and catalase were increased in salt stressed plants specially in plants treated with salt and amiloride. Therefore salt stress has caused osmotic and oxidative stress in plants and amiloride as inhibitor of vacuolar Na^+/H^+ antiporter has been increased salt stress. Therefore we concluded that Na^+ compartmentation in the cell is very important to reduce its damage in the cytosol and vacuolar Na^+/H^+ antiporter has an essential role in Na^+ homeostasis in the cell by exporting excess Na^+ to the vacuole.

Key words: Na^+/H^+ antiporter, amiloride, salt pretreatment, *Zea mays*

INTRODUCTION

Salt stress is one of the major constrains limiting crop productivity in the world (Quesada *et al*., 2000). There are many vast areas that are not suitable for agriculture due to their high salinity. Plants behavior against salinity are different. Glycophytes are sensitive and do not grow on salty soils; but halophytes are salt tolerant. Many salt tolerance mechanisms have been identified in these plants. Na^+ extrudation and enhanced producing of osmoregulators and antioxidant enzymes activity (Parida and Das, 2005). Recently it has been realized that these plants have also an active Na^+/H^+ antiporter system in their vacuolar and plasma membrane of the cells which function to efflux Na^+ from the cytoplasm to prevent Na^+ toxic cellular effects (Blumwald *et al*., 2000; Hasegawa *et al*., 2000). The Na^+/H^+ antiporter transports Na^+ ions into the vacuole using the H^+ gradient at the tonoplast which is maintained by (V-ATPase) H^+ /ATPase and (V-PPase) H^+/PPase. In most glycophytes the overall activity of the Na^+/H^+ antiporter system is extremely low, but is present (Gruwel *et al*., 2001). In recent years significant advances have been made toward understanding the structure and function of Na^+/H^+ exchangers as well as the mechanisms underlying the exchange reaction and its regulation (Qiu *et al*., 2003; Blumwald *et al*., 2000; Hasegawa *et al*., 2000). Amiloride has been known as an inhibitor of Na^+/H^+ antiporter activity in tonoplast. Other amiloride derivatives such as N(ethyl-N-isopropyl amiloride) (EIPA), 5 (N-methyl-N-isobutyl amiloride) (MIA) and 5 (N-N-dimethyl amiloride) also the show same inhibition effect. Amiloride blocks Na^+ influx into the vacuole against protons efflux from it, therefore the rate of acidification increases considerably and gives rise to a more acidic vacuolar pH. These findings suggest that amiloride-sensitive Na^+/H^+ antiporter constitutes a permanent drain of the ATPase-generated H^+ gradient a cross the tonoplast. Hence, inhibition of this process increases the steady state concentration of vacuolar protons (Viehweger *et al*., 2002; Qiu *et al*., 2004; Darley *et al*., 2000). Treatment the plants with amiloride under salt conditions causes Na^+ ions to accumulate into the cytosol and salt stress to enhance. Therefore in this research we tried to show the importance of the salt intracellular compartmentation as a tolerance mechanism by inhibition of this antiporter system.

MATERIALS AND METHODS

This experiment has been done in autumn of 2006 at the Physiological and Biochemical Laboratories, in the Department of Biology of Faculty of Science of Urmia University. The seeds of *Zea mays* L. (cv. single cross 704) were cleaned, selected by the size, washed with water

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and detergent and finally with distilled water and incubated in 25°C to germinate. After 4 days, the seedlings with the same size were selected again and nine seedlings (as a replicate) translated to the mediums containing 200 and 300 mM NaCl with or without 100 and 200 μM amiloride in an aerated and controlled condition (light density 14000 lux and day/night temperature 20/25°C and humidity 60%). To show the effect of amiloride and salt stress on the plants, the changes of Na^+ absorption, amount of malondialdehyde and the activity of antioxidant enzymes such as guaiacol peroxidase, ascorbate peroxidase and catalase were examined.

Measurement of Na^+ absorption: To do this experiment, 4 day old seedlings of *Zea mays* were exposed to 200 and 300 mM NaCl with and without 100 and 200 μM amilorid during the period of 48 h (at above mentioned condition). At the end of treatment the seedlings were harvested and the amount of Na^+ absorption in growth solution of each replicate was determined by flame photometer (model 405 made by Fater Electronic com).

Changes of lipid peroxidation: Lipid peroxidation was determined by measuring the amount of MDA produced by the thiobarbituric acid reaction as described by Heath and Packer (1968). The crude extract was mixed with the same volume of a 0.5% (w/v) thiobarbituric acid solution containing 20% (w/v) trichloracetic acid. The mixture was heated at 95°C for 30 min and then quickly cooled in an ice-bath. The mixture was centrifuged at 3000 g for 10 min and the absorbance of the supernatant was monitored at 532 and 600 nm. After subtracting the non-specific absorbance (600 nm), the MDA concentration was determined by its molar extinction coefficient (155 mM^{-1} cm^{-1}) and the results expressed as μmol MDA g^{-1} FW.

Assay of enzyme activity: Shoot and root fragments were excised from the seedlings of controls and treated plants with amiloride and NaCl. The 0.5 g FW. was homogenized at 4°C in 3 mL of extraction buffer (0.05 M Tris-HCL buffer (pH 7.5), 3 mM $MgCl_2$, 1 mM EDTA and 1.5% (w/v) PVP with mortar and pestle. The extraction buffer used for the APX assay contained 0.2 mM ascorbate. The extract was used for assay of antioxidant enzymes activities (Beaudoin *et al*., 2000).

GPX activity: GPX activity was determined according to Upad hyaya *et al*. (1985). The reaction mixture contained 2.5 mL of 50 mM phosphate buffer (pH 6.1), 1 mL of 1% hydrogen peroxide, 1 mL of 1% guaiacol and 20 μL enzyme extract. The reaction was started by addition of enzyme extract and the increase in absorbance recorded at 420 nm for 1 min.

APX activity: APX activity was determined according to the method of Chen and Asada (1989) with some modification. The 1 mL reaction mixture was composed of 50 mM phosphate buffer (pH 7.0) containing 0.1 mM EDTA, 0.5 mM ascorbate, 1.54 mM hydrogen peroxide and 50 μL enzyme extract. The oxidation of ascorbate was followed by the decrease in the absorbance at 240 nm.

CAT activity: CAT activity was assayed by measuring the rate of disappearance of hydrogen peroxide using the method of Maehly and Chance (1959). The reaction mixture contained 2.5 mL of 50 mM phosphate buffer (pH 7.4), 1 mL of 1% hydrogen peroxide and 50 μL enzyme extract. The decrease in hydrogen peroxide was followed as a decline in absorbance at 240 nm.

RESULTS AND DISCUSSION

Na^+ absorption: Na^+ absorption by the roots of *Zea mays* seedlings has been increased according to Na^+ concentration in the medium and has not been influenced by amiloride. Na^+ uptake has increased significantly at 300 mM NaCl, it was 2 times higher than 200 mM NaCl (Fig. 1). Differences between other treatments were not significant ($p \geq 0.05$). The results showed that the amount of Na^+ uptake depends on environmental salt concentration and its influx is often unavoidable, because

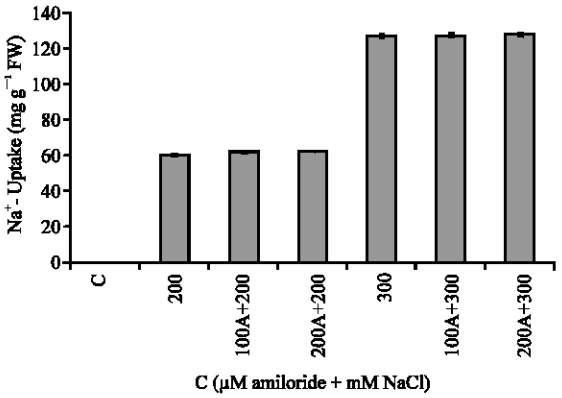

Fig. 1: The changes of Na^+ absorption of *Zea mays* seedlings roots exposed to different concentrations of NaCl (200 and 300 mM) with and with out amiloride (100 and 200 μM) during the period of 48 h. The values represent the mean of three replicates±SE

it enters into the cell through K^+ channels and accumulate in the cell, therefore the plant cells try to reduce its toxicity in the cell by extrudation it out of the cell or efflux it to the vacuole (Blumwald *et al*., 2000).

Na^+/H^+ vacuolar antiporter system is a mechanism that helps plants to efflux intracellular Na^+ to the vacuole and decreases its ionic oxidative effects. Amiloride has been realized that blocks this antiporter system and causes more Na^+ to accumulate into the cytosol but does not influence Na^+ influx. Therefore this experiment refers to the importance of this antiporter system in Na^+ compartmentation in the cell.

Lipid peroxidation: To show the effect of salt stress and amiloride on lipid peroxidation in shoots of *Zea mays* seedlings, MDA content was measured (Fig. 2). The amount of MDA of shoots as an indicator of lipid peroxidation has been increased by salt stress and amiloride has influenced its production. Its content in plants treated with 300 mM NaCl and 200 μM amiloride was the highest (it was 8.1 times higher than control and 2 times higher than plants treated only with 300 mM NaCl) (Fig. 2). Amiloride as an inhibitor of Na^+/H^+ antiporter causes Na^+ ions to accumulate into the cytosol and lipid peroxidation to enhance. Lipid peroxidation occur when enzymes do not control many oxidative chemical reactions and some of the highly reactive products attack and modify proteins, DNA and membrane lipids (Parida *et al*., 2005). MDA is cytotoxic and generally more stable than Reactive Oxygen Species (ROS) and can cause extensive damage to proteins and cellular constituents (Winger *et al*., 2005). Antioxidative enzymes protect plant cells from the potential oxidative damages (Kuk *et al*., 2003).

GPX activity: GPX activity in roots and shoots of *Zea mayz* seedlings was remarkably increased in plants exposed to 200 and 300 mM NaCl and 200 μM amiloride has enhanced it significantly. Its activity in roots and shoots of plants treated with 300 mM NaCl and 200 μM amiloride was 5.4 and 6.2 times higher than controls, respectively (Fig. 3).

APX activity: APX activity in roots and shoots of plants was increased by salt stress and amiloride. Its level in roots and shoots of plants treated with 300 mM NaCl and 200 μM amiloride was 3.5 and 4.7 times higher than controls, respectively (Fig. 4).

CAT activity: Catalase activity in roots and shoots of *Zea mayz* seedlings has been increased significantly by salt stress and has been influenced by amiloride. Its

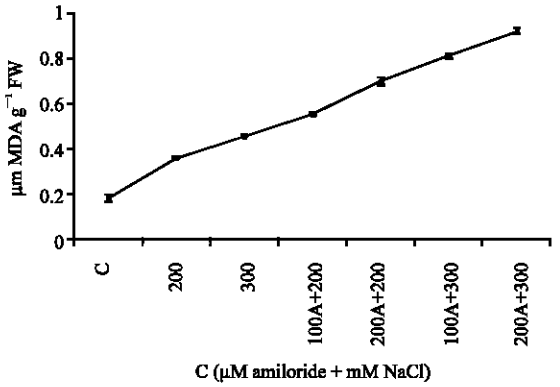

Fig. 2: The changes of total malondialdehyd of *Zea mays* seedlings shoots exposed to different concentrations of NaCl (200 and 300 mM) with and with out amiloride (100 and 200 μM) during the period of 48 h. The values represent the mean of three replicates±SE

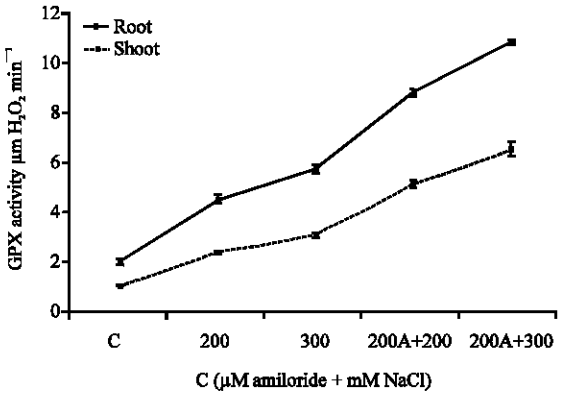

Fig. 3: The changes of guaiacol peroxidase activity of *Zea mays* seedlings shoots exposed to different concentrations of NaCl (200 and 300 mM) with and with out amiloride (200 μM) during the period of 48 h. The values represent the mean of three replicates±SE

activity in roots and shoots of plants exposed to 300 mM NaCl with 200 μM amiloride was the highest (3.3 and 5 times higher than controls, respectively) (Fig. 5). Due to the results of this experiment, activity of antioxidant enzymes in plants treated with NaCl and amiloride has been increased. It indicates that induced activity of antioxidative enzymes in salt stress condition protect the plants against oxidative damages, by scavenging O_2^-, H_2O_2, OH^- and other active oxygen species (Hong *et al*. 2000; Dagmar *et al*., 2001). Plants have evolved antioxidant systems to protect cellular membranes and organelles from damaging effects of Active Oxygen

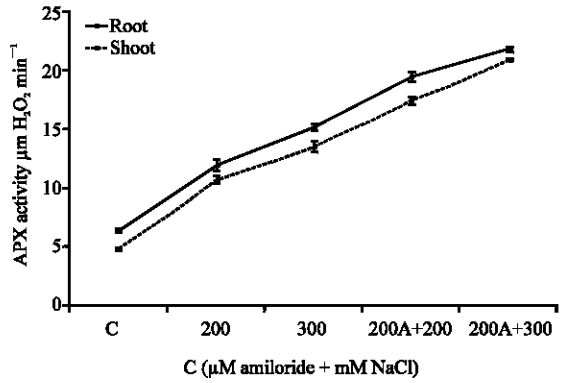

Fig. 4: The changes of ascorbate peroxidase activity of *Zea mays* seedlings shoots exposed to different concentrations of NaCl (200 and 300 mM) with and with out amiloride (200 µM) during the period of 48 h. The values represent the mean of three replicates±SE

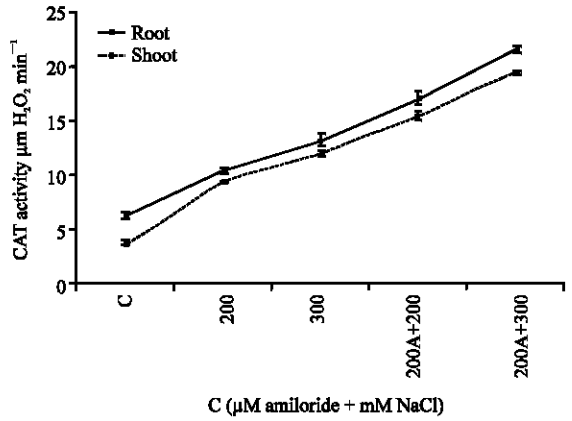

Fig. 5: The changes of catalase activity of *Zea mays* seedlings shoots exposed to different concentrations of NaCl (200 and 300 mM) with and with out amiloride (200 µM) during the period of 48 h. The values represent the mean of three replicates±SE

species (AOS). Antioxidant enzymes can react with active oxygen species and nautralize the activity of AOS (Kuk *et al.* 2003). Tolerant plants possess higher antioxidant enzymes activities in stress conditions (Harinasut *et al.*, 2003) that protect proteins, DNA molecules and membrane lipids from all kinds of active radicals damages (Neto *et al.*, 2005; Ashraf and Harris, 2004).

CONCLUSIONS

Under salt conditions plants suffer from both higher osmotic and oxidative ionic stresses and treatment the plants with amiloride enhances this effect. The results of this research indicated that amiloride increases the oxidative damage generated by Na^+ accumulation due to inhibition of vacuolar Na^+/H^+ antiporter. Therefore vacuolar Na^+/H^+ antiporter has an essential role in compartmentation of Na^+ and ionic homeostasis in the cell under saline conditions. Efflux of Na^+ in vacuoles is important for salt resistance. Therefore an over expression of a tonoplast Na^+/H^+ antiporter in plants can increase significantly salt tolerance in transgenic plants.

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