BIOLOGIA PLANTARUM 51 (4): 759-763, 2007

BRIEF COMMUNICATION

Plasma membrane ultrastructure in embryogenic cultures of orchardgrass during NaCl stress

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Abstract

Embryogenic cultures of *Dactylis glomerata* L. were subjected to NaCl stress by culturing for 4 passages in Schenk and Hildebrandt (SH) medium containing 30 μM dicamba and 200 mM NaCl. Ultrastructural studies indicated invaginations and disruptions of the plasma membrane. Membrane-bound vesicles were observed in the cytoplasm of NaCl treated cells and their occurrence were increased with the culture age.

Additional key words: *Dactylis glomerata* L., *in vitro* cells, membrane damage, plasmolysis, salt stress, vesiculation.

Salinity is one of the major factors limiting plant development and crop productivity. Damage to plants exposed to salinity has been ascribed to ion toxicity, water deficit, nutrient imbalance and oxidative stress (Zhu 2001, Panda and Upadhyay 2004, Molassiotis *et al.* 2006). There have been a fair number of ultrastructural investigations on the effects of salinity on whole plants (Mitsuya *et al.* 2000, Yamane *et al.* 2003, Parida *et al.* 2003). However, a relatively small number of studies have been carried out with *in vitro* grown cells/tissues (*e.g*. Diaz de Leon 1982, Nyman *et al.* 1987, Dutta Gupta *et al.* 1992, Molassiotis *et al.* 2006). In particular, embryogenic cells with dense cytoplasm and meristematic activity represent an ideal system to assess the physiological effects of salt and /or water stress at the cellular level as compared with the heterogeneous whole plants (Lerner 1985, Niknam *et al.* 2004). Ultrastructural changes in cells that accompany adaptation to NaCl or NaCl tolerance may aid in understanding the mechanism(s) of tolerance. Plant cell and tissue culture have also proved to be useful in selecting cell lines and regenerating plants tolerant to NaCl in order to assist breeding programs for salinity resistant crops (Cherian and Reddy 2003, Elavumoottil *et al.* 2003).

Cell membranes are the primary target of plant stresses (Hasegawa *et al.* 2000). Studies on membrane behavior during stress are considered important as membranes represent selective barriers separating protoplasm from the environment. Furthermore, compartmentation of metabolites within cells is controlled by membranes (Palta 1990). Whole plant cells often react upon a salt stress by increased electrolyte leakage (Leopold and Willing 1984) and by increased vesiculation of the plasma membrane (Bonghanmi *et al.* 2003).

Cellular mechanisms for NaCl tolerance must involve regulation of ion transport across the plasma membrane in concert with the vesicular transport to reduce cytoplasmic sodium. The existence of a vesicle mediated system for solute transport from the vacuole to the plasma membrane was observed first in the secretory salt glands of mangrove species (Shimony *et al.* 1973) and reviewed by Echeverria (2000). It is believed that as the salt accumulates in the cells, it becomes compartmentalized within the vacuoles and is secreted when vesicles fuses and becomes incorporated with the plasma membrane (Blumwald and Gelli 1997, Battey *et al.* 1999, Echeverria 2000). The scarce *in vitro* studies on NaCl induced ultrastructural changes indicate alterations of cell walls (Dutta Gupta *et al.* 1992), accumulation of lipids along the cell wall (Dutta Gupta *et al.* 1992), mitochondria with poorly developed cristae and a higher degree of vacuolation (Diaz de Leon 1982, Nyman *et al.* 1987, Dutta Gupta *et al.* 1992).

The objective of this work was to study the behavior of plasma membrane at the ultrastructural level during NaCl stress in embryogenic cultures of orchardgrass (*Dactylis glomerata* L.).

Received 22 August 2005, *accepted* 15 July 2006.
Abbreviations: SH - Schenk and Hildebrandt; PM - plasma membrane; CW - cell wall; Vs - vesicle; RER - rough endoplasmic reticulum.
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Young leaf segments of *Dactylis glomerata* L. genotype 'Embryogen-P', which produces somatic embryos in high number (Conger and Hanning 1991) were used to initiate *in vitro* cultures. Orchardgrass with its high embryogenic potential from leaf segments represents a model species among the gramineous plants for *in vitro* stress studies. Cultures were initiated and maintained on SH medium (Schenk and Hildebrandt 1972) containing 30 μM 3,6-dichloro-2-methoxybenzoic acid (dicamba) and 0.8 % agar (hereafter referred to as SH-30). Embryogenic calli were exposed to 0 and 200 mM NaCl. A concentration of 200 mM was chosen as minimal lethal concentration of NaCl in this species (Dutta Gupta *et al.* 1992, 1995, Dutta Gupta 1999). At this concentration, cultures were allowed to grow for a total period of 56 d. The cultures were incubated in the dark at 25 °C and transferred to fresh medium at 14-d intervals.

For ultrastructural studies, samples of 50 globular embryos from the embryogenic cultures were collected from untreated or NaCl-treated cultures for fixation at 14, 28 and 56 d of culture. They were fixed in 2 % glutaraldehyde solution buffered with 0.1 M sodium cacodylate, pH 7.2, for 6 h at room temperature. Vacuum (1013 - 1520 kPa) was applied for 1 h to facilitate the penetration of the fixative. After fixation, samples were washed three times with buffer and post fixed in 1 % (m/v) osmium tetroxide for 2 h at 4 °C. Dehydration was carried through an acetone graded series and propylene oxide. Tissue samples were embedded in Spurr's resin (Spurr 1969) in flat molds. Silver-grey ultrathin (50 - 80 nm) sections were cut with a diamond knife using a *LKB* ultrathin microtome (*GE Healthcare Bio-Sciences*, New Jersey, USA) and stained in uranyl acetate and post stained in lead citrate. Observations were made by using a *Jeol 100 CX* (Tokyo, Japan) transmission electron microscope. Photographs were taken from upper median part of globular embryos at three random sites in three different sections and representative pictures are presented.

Ultrastructural observations were made from the upper median part of the globular embryos obtained from both control and treated cultures. This allowed comparisons which were not affected by the differential development of embryogenic cultures. It has been suggested that similar types and developmental stages of cells must be compared in such studies (Ciamporova and Mistrik 1993). Further, our previous work on ultrastructure revealed that glutaraldehyde in buffered regime maintained the sub-cellular structures as close as possible to their native form (Dutta Gupta *et al.* 1992). Similar kind of tissue processing in the present work ruled out the possibility of artifact generation in sub-cellular structure.

The plasma membrane of control cells was smooth or occasionally had minor undulations (Fig. 1*A*). No space was seen between the cell wall and plasma membrane. In contrast, NaCl induced considerable abnormalities, leading to the damage of the plasma membrane (Fig. 1*B*,*D*,*E*,*F*). The intensity of damage varied from cell to cell. However, no major changes in patterns were observed at various culture intervals. In most of the cells invaginations of plasma membrane were common. Periplasmic space appeared between the cell wall and plasma membrane (Fig. 1*E*). Absence of periplasmic space in the control cells indicate that osmotic damage was not triggered during tissue processing for transmission electron microscopy. However, differences in water relations of stressed cultures to control samples may affect tissue processing. Plasma membrane invaginations were observed in NaCl treated plants of *Oryza sativa* (Pareek *et al.* 1997), *Artiplex nummularia* (Niu *et al.* 1996) and cultured cells of tobacco (Niu *et al.* 1996). As suggested by Niu *et al.* (1996) plasma membrane invagination may function in membrane preservation during the plasmolysis/deplasmolysis cycle which results from cellular osmotic stress.

Invaginations were also observed in the tonoplasts of some of the cells. The tonoplasts of these vacuoles were connected to the cytoplasm at a narrow isthmus (Fig. 1*B*). Whorls of membranous materials within the invagination also show dark-light-dark layering. Membranous vesicles were found near (Fig. 1*C*) or continuous with the plasma membrane (Fig. 1*D*). Fig. 1*D* also depicts electron dense fibrilar tufts which were not parallel to the rest of the microfibrils in the cell wall indicating alterations of cell wall structures during NaCl stress. Stretching of the plasma membrane with electron dense material in the paramural region was clearly seen (Fig. 1*E*). Extensive damage to the plasma membrane could be seen in some cells (Fig. 1*F*). These observations point to the expansion induced lysis of plasma membrane during altered osmotic behavior. It has been suggested that the plasma membrane is subjected to mechanical stresses resulting from surface area changes during osmotic contraction/ expansion (Steponkus *et al.* 1981, Wolfe and Steponkus 1981, Gordon-Kamm and Steponkus 1984, Steponkus 1985). The lowered elasticity of membranes ultimately leads to membrane damage at a critical level (Steponkus 1985). The present ultrastructural observations on NaCl-induced membrane damage commensurate with our previous work on callus water relations using pressure-volume analysis (Auge *et al.* 1989, Dutta Gupta *et al.* 1995). Increased values of average and maximum elastic modulus of membranes obtained during salinization of orchardgrass were indicative of a change in membrane area achieved by elastic contraction/expansion (Dutta Gupta *et al.* 1995). Thus, NaCl induced membrane damage may be due to the process referred to as expansion induced lysis, similar to that of freeze-thaw cycle (Dowgert and Steponkus 1984, Gnanapragasam and Vasil 1992).

It also appears that the susceptibility of membranes to damage was not solely due to toxic effects of salt. Symptoms of plasmolysis as characterized by the appearance of space between the cell wall and plasma membrane suggest that NaCl induced ultrastructural effects may possibly represent those associated with osmotic stress. The simultaneous occurrence of

plasmolysis and membrane lysis in the present study contradicts the findings of Pareek *et al.* (1997). In their study, plasmolysis and increased cytoplasmic vesiculation were seen only in response to NaCl stress, while discontinuity and damage in the plasma membrane were observed in response to high temperature stress.

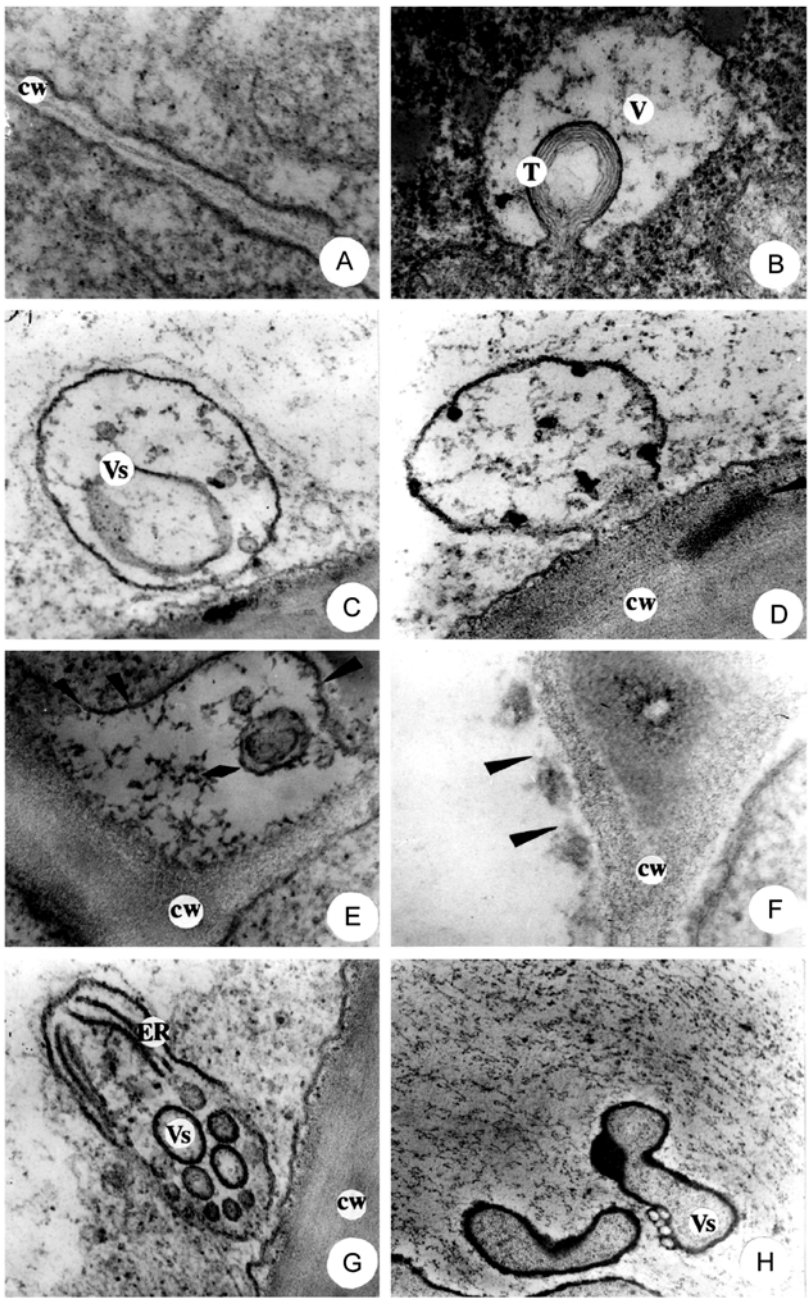

Fig. 1. *A* - Control cell showing plasma membrane without any undulation. 56-d-old embryogenic cultures maintained on SH-30 without NaCl (×17000). *B* - NaCl treated cell after 28 d of culture showing invagination of the tonoplast (T) in the vacuole (V). The tonoplast of this vacuole also show multi-membranous structures (× 20000). *C* - Portion of a cell cultured on NaCl for 28 d showing membrane-bound vesicle (Vs) near the plasma membrane (× 30000). *D* - Cell at day 28 on 200 mM NaCl showing extensive invagination of plasma membrane. Also note tufts of microfibrils (*arrow*) in the cell wall (CW) (× 45000). *E* - Stretching of plasma membrane (*arrow*) of a cell cultured on NaCl at 56 d from the cell wall (CW) with electron dense materials in the paramural region (*double arrow*) (× 30000). *F* - Cell with a disrupted plasma membrane (*arrows*) at day 56 on 200 mM NaCl (× 45000). *G* - Portion of a cell cultured on 200 mM for 56 d showing group of vesicles (Vs) surrounded by endoplasmic reticulum (ER) near the plasma membrane (× 30000). *H* - Vesicles (Vs) from NaCl treated cultures at 56 d showing electron dense osmiophilic deposits (× 10000).

Increased vesiculation was observed in 28- and 56-d-old NaCl treated cultures. Vesicles observed in the cytoplasm may have their origin from the invaginations of plasma membrane and/or dilation of the endoplasmic reticulum cisternae. Invaginations of the plasma membrane and increased values of elastic modulus during salinization (Dutta Gupta *et al.* 1995) suggest endocytotic origin of vesicles. However, group of small vesicles surrounded by the endoplasmic reticulum with prominent ribosomes along the membrane were also observed (Fig. 1*G*). Sometimes the extrusions contain a core of electron dense material (Fig. 1*H*) which is most likely composed of lipids deleted from the plasma membrane. These findings indicate an exocytotic mechanism. Further studies are warranted for conclusive evidence in support of the existence of endocytotic and exocytotic vesicles.

In conclusion, the present study provides EM evidence for salt-induced damage to the plasma membrane of plant cell cultures and increased vesiculation in the cytoplasm during NaCl stress.

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