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Salt stress induces programmed cell death in *Thellungiella halophila* suspension-cultured cells

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ABSTRACT

Thellungiella halophila (*T. halophila*) suspension-cultured cells were used to gain knowledge of the pathway of programmed cell death (PCD) in halophytes under salt stress. Several apoptotic-like features occurred in *T. halophila* cells after exposure to 300 mM NaCl, including the retraction of the plasma membrane from the cell wall, nuclear condensation, DNA laddering and the release of cytochrome *c* accompanying the increase of caspase 3-like protease activity. This process resulted in ultrastructural changes of mitochondria and Golgi bodies, and autophagy was also induced by high salinity stress. DNA laddering and caspase 3-like activity were inhibited prior to the inhibition of cell death by a specific caspase 3 inhibitor, Ac-DEVD-CHO. The results indicate that 300 mM NaCl stress-induced PCD in *T. halophila* is similar to animal apoptosis, and this process occurs partly through a caspase 3-like dependent pathway.

Introduction

Programmed cell death (PCD) is an active process of autophagy induced by a change in cellular homeostasis that usually requires protein synthesis and signal transduction. In animals, the most common form of PCD has been termed apoptosis. The hallmarks of apoptosis include chromatin condensation, internucleosomal DNA cleavage, membrane blebbing, the formation of apoptotic bodies and their engulfment by phagocytosis (O'Brien et al., 1998; Fath et al., 1999). PCD in plants is responsible for removal of redundant, misplaced, or damaged cells, which contributes significantly to both development and maintenance of these multicellular organisms. PCD takes place during developmental processes as well as in response to various stimuli, including fungal toxins, biotic and abiotic stresses, and chemical agents (Korthout et al., 2000; Mlejnek and Procházka, 2002).

In the past decades, the signaling pathway of PCD has been gradually elucidated. Mitochondria play a key role in the regulation of PCD. Release of Cyt *c* from mitochondria is a pivotal event in the apoptosis of animal cells, as it drives the assembly of a high-molecular weight caspase-activating complex in the cytoplasm, which leads to the morphological changes of typical apoptosis (Yang et al., 1997). Recently, the involvement of mitochondria in plant PCD has been demonstrated in a number of systems (Carimi et al., 2003; Krause and Durner, 2004; Vacca et al., 2007; Chen et al., 2009), although the release of Cyt *c* could not induce PCD in other systems (Yu et al., 2002). In animal systems, specific caspases are activated in the execution phase of PCD (Hengartner, 2000). However, no caspase gene homologs have been found in plants to date (Sanmartín et al., 2005) and the nature of caspase-like proteases in plants is the subject of considerable debate. It is unknown whether caspase-like molecules are widely involved in plant PCD, especially in abiotic stress-induced cell death program.

Salt stress is one of the most serious problems in agriculture in arid and semi-arid areas (Katsuhara and Kawasaki, 1996). Possible mechanisms of salt-induced plant PCD have been elucidated previously. For example, the salt-induced PCD is mediated by ion disequilibrium in *Arabidopsis* (Huh et al., 2002) and in tobacco (Shabala et al., 2007; Shabala, 2009). Several candidates, including reactive oxygen species (ROS), antioxidant enzymes, mitochondria permeability transition and the secondary messenger Ca^{2+} were suggested to be involved in the signaling pathway of salt-induced plant PCD (Lin et al., 2005, 2006; Li et al., 2007; Chen et al., 2009). However, our current knowledge of signals involved in salt-induced PCD is incomplete and mostly limited to low salinity for the systems in which glycophytes are primarily used. Certain novel adaptive responses may be overlooked when using a glycophyte as the exclusive model, as halophytes may have evolved unique mechanisms or regulatory pathways that are not found in glycophytes, which would primarily present a stress response (Vera-Estrella et

Abbreviations: Cyt *c*, cytochrome *c*; DMSO, dimethyl sulfoxide; PCD, programmed cell death; ROS, reactive oxygen species.

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al., 2005). *Thellungiella halophila* (*T. halophila*), a close *Arabidopsis* relative, has attracted growing interest as a model for research into plant abiotic stress tolerance (Amtmann et al., 2005). In contrast to *Arabidopsis*, *Thellungiella* is a true halophyte and can survive for several months and produce viable seeds, even in the presence of 500 mM NaCl (Inan et al., 2004). Until recently, knowledge of how plant PCD occurs in halophytes under salt stress has remained relatively obscure. However, we are now able to employ a halophyte model in which the role of cell components can be investigated in detail.

In this work, we established a system consisting of salt-induced PCD in *T. halophila* suspension cells. We investigated some morphological and biochemical features occurring in the process of PCD. Whether the caspase 3-like protease was involved in salt-induced PCD was also investigated.

Materials and methods

Suspension cell culture and treatments

Seeds of *Thellungiella halophila* ecotype Shandong were obtained from the Arabidopsis Biological Resource Center (ABRC). For callus induction, leaves were surface sterilized by a 1 min immersion in 70% alcohol and 10 min in 4% sodium hypochlorite, washed with sterile water, then cut into 3–5 mm segments, and cultured on callus induction medium at a photoperiod of 16 h light (23 °C) and 8 h dark (18 °C). The callus induction medium contained MS (Murashige and Skoog, 1962) basal medium, 4.52 μM 2, 4-D and 2.22 μM 6-BA, 30 g/L sucrose and 8 g/L agar, pH 5.8. The calluses were sub-cultured monthly. After four sub-cultures, the calluses were transferred into liquid suspension culture medium containing MS basal medium supplemented with 2.26 μM 2, 4-D and 2.22 μM 6-BA and 30 g/L sucrose (pH 5.8). The suspension was sub-cultured every 8 days by transferring 10 mL of culture into 40 mL of fresh medium in 250 mL Erlenmeyer flask. Cells from cultures in the exponential phase of growth (4–5 day-old cultures) were utilized in the experiments. During the entire experimental period, the cells were maintained in a growth chamber at 23 °C in the dark on a gyratory shaker (120 rpm).

For treatments, the designated concentration of NaCl was added to 10^6 cells/mL determined with a hemocytometer, and cells were incubated under normal culture conditions. A specific caspase 3 inhibitor, Ac-DEVD-CHO (Sigma), was dissolved in dimethyl sulfoxide (DMSO). To achieve the maximum inhibitory effect, 100 μM Ac-DEVD-CHO [which was found to be effective in plants (Vacca et al., 2006)] for *in vitro* (see Figs. 1B, 4 and 5 in "Results" section) was added to the medium 1 h prior to salt stress treatment. The final concentration of DMSO in the culture medium was approximately 0.1%. Control cells were used with the addition of 0.1% DMSO.

Cell viability

Cell viability was measured using trypan blue staining as described in de Pinto et al. (1999). More than 600 cells were counted and three independent experiments were performed.

Transmission electron microscopy

For transmission electron microscopy, cells were collected by centrifugation (2000 g, 5 min) and fixed in 3% glutaraldehyde for 24 h at 4 °C. Afterwards, cells were fixed for 2 h at 4 °C with 1% osmium tetroxide in 0.1 M cacodylate buffer, dehydrated in a graded ethanol series and then embedded in araldite resin. Ultrathin sections were cut with an ultramicrotome, stained with uranyl acetate in ethanol for 30 min and observed at 100 kV in JEM-1230 TEM.

TUNEL assay

A TUNEL assay was performed with a commercially available TUNEL kit (G3250, Promega) according to the manufacturer's instructions. Treated and control cells were immobilized on the slides by L-polylysine, fixed 30 min with freshly buffered 4% paraformaldehyde and washed twice in PBS (pH 7.4). Cells were treated for 15 min with proteinase K (20 μg/mL, Sigma) before 3′-OH end labeling. Nuclei were stained with DAPI (Sigma) in PBS containing 0.1% (v/v) triton x-100 at 1 μg/mL for 10 min. Finally, cells were observed under a fluorescence microscope (Olympus AX 80, Japan). Cells with TUNEL-positive nuclei are considered as apoptotic, the total cell number was counted based on DAPI staining. More than 600 cells were counted and three independent experiments were performed.

DNA laddering analysis

Cells were harvested by centrifugation (2000 × *g*, 5 min) and the resulting pellets were then homogenized in liquid nitrogen. DNA was extracted by the cetyltrimethylammonium bromide (CTAB) method as described previously (Tada et al., 2001). Total DNA was dissolved in TE buffer (pH 8.0), and after incubation with DNase-free RNase A at 37 °C for 20 min, equal amounts of DNA samples (10 μg) were loaded on a 1.8% agarose gel and stained with ethidium bromide after electrophoresis.

Detection of Cyt c release

Mitochondria and cytosol extracts were prepared from the suspension cultures according to Balk et al. (1999). Protein concentration was determined by the Bradford method (1976). For Cyt *c*

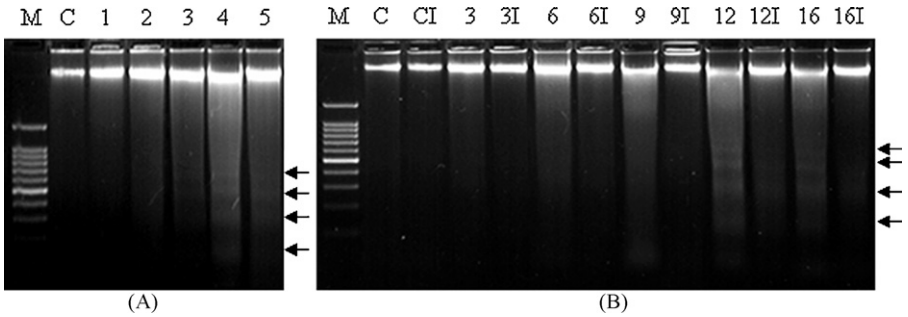

Fig. 1. DNA laddering in *T. halophila* suspension cells after salt treatment. Arrows indicate DNA ladders. (A) DNA laddering after 12 h treatment with different concentrations of NaCl. Lane M, 100 bp DNA marker; Lane C, control; Lane 1-5, cells stressed with 50, 100, 200, 300 and 400 mM NaCl, respectively. (B) DNA laddering after treatment with NaCl + Ac-DEVD-CHO. Lane M, 100 bp DNA marker; Lane C, control; Lane CI, cells treated with 100 μM Ac-DEVD-CHO for 1 h. Lanes 3, 6, 9, 12 and 16, cells stressed with 300 mM NaCl for 3, 6, 9, 12 and 16 h. Lanes 3I, 6I, 9I, 12I and 16 I, cells pretreated for 1 h with 100 μM Ac-DEVD-CHO before the incubation with 300 mM NaCl for 3, 6, 9, 12 and 16 h.

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detection, 20 μg of protein was separated by 15% (w/v) SDS-PAGE. Pre-stained molecular weight markers (Cat#SM0441, Fermentas) were used as standards. The separated proteins were then transferred onto a Hybond ECL nitrocellulose membrane (Amersham Pharmacia) and labeled with antibodies against Cyt *c* (7 H 8.2 C 12, Pharmingen, San Diego) used at a concentration of 1 mg/mL and incubated for 2.5 h. After three 5-min washes in TTBS, the membrane was incubated with peroxidase-conjugated goat-anti-mouse IgG in blocking solution for 2 h. The blot was further washed as indicated above, and the immunolabeled proteins were detected using Enhanced chemiluminescence (ECL) reagents (BeyoECL Plus, China). The blots were then re-probed with anti-actin antibody (Santa Cruz, CA), in order to verify equal protein loading.

Measurements of caspase 3-like activity

Cytosolic fractions from normal and salt-treated cells were used for the assay of caspase 3-like protease according to the manufacturer's instruction ('Caspase 3 Activity Assay Kit', Beyotime, China). Equal amounts of total protein extracts (15 μg) were incubated for 2 h at 37 °C with the synthetic tetrapeptide DEVD-p-nitroaniline (pNA), and the addition of the substrate resulted in a signal caused by the caspase 3-dependent cleavage of the chromophore pNA from the labeled substrate. Caspase 3-like activity was measured at 405 nm. Enzymatic activity was expressed as a percentage of activity present in control extracts. Each measurement was carried out with three independent experiments.

Statistical analysis

Three independent experiments were performed. All data are presented as the mean ± standard deviation (SD). Comparison between two groups was analyzed using paired-samples *t*-tests using SPSS 10.0. Results were considered statistically significant when $P < 0.05$ (*).

Results

Salt stress induces a cell death program in *T. halophila* suspension culture cells.

DNA laddering

Nuclear DNA fragmented after 12 h exposure to 50–400 mM NaCl. Clear DNA ladders were observed only for the 300 mM NaCl treatment (Fig. 1A). Then, we examined the time effects of the 300 mM NaCl treatment on the process of DNA fragmentation. As shown in Fig. 1B, DNA ladders appeared at 9 h, and in an obviously progressive manner (9–16 h). We also investigated the role of Ac-DEVD-CHO, a specific caspase 3 inhibitor, on salt stress-induced DNA fragmentation at times up to 16 h after salt treatment. When 100 μM Ac-DEVD-CHO was added to the medium 1 h before incubation with 300 mM NaCl, however, no obvious DNA ladder occurred.

Cell viability and TUNEL-positive nuclei

To evaluate the effect of salt stress on cell viability, we examined *T. halophila* suspension cells treated with different concentrations of NaCl for 12 h. As shown in Fig. 2, substantial cell death induced by NaCl was not apparent until treated with 300 mM NaCl. The percentages of cell death and TUNEL-positive nuclei were about 39% and 32% within 12 h after exposure to 300 mM NaCl, respectively. When a higher dose (400 mM) of NaCl was applied, the percentage of cell death kept increasing to about 51%, but the percentage of TUNEL-positive nuclei decreased quickly to about 14%. Together with DNA laddering data (Fig. 1A), 300 mM is determined to be the most effective NaCl concentration to induce PCD in this system. To further assess salt-induced cell death in *T. halophila*, cell death and TUNEL-positive nuclei were tested at different time points after exposure to 300 mM NaCl. The role of Ac-DEVD-CHO on cell death was also investigated. The results showed the presence of TUNEL-positive nuclei in control cells, salt-treated cells and Ac-DEVD-CHO pretreated cells (Fig. 3). It was noted that the caspase 3 inhibitor effectively blocked the presence of TUNEL-positive nuclei. The data (Fig. 4) showed that cell death at 6 and 12 h after salt treatment was about 2- and 4-fold that of control cells, respectively. The number of TUNEL-positive nuclei increased slightly in the beginning and increased distinctly within 12 h after treatment to about 8-fold that of untreated cells. The percentage of TUNEL-positive nuclei reduced significantly to about 21% ($P < 0.05$) in contrast to that of salt-stressed cells without Ac-DEVD-CHO (about 32%). A small but statistically significant ($P < 0.05$) prevention of cell death (71.31% of cell survival within 12 h after treatment) resulted from pretreatment with 100 μM Ac-DEVD-CHO in contrast with that of salt-alone-treated cells (61.34% of cell survival). Cell viability (cell survival) in the control cells during the experimental period was always more than 90%.

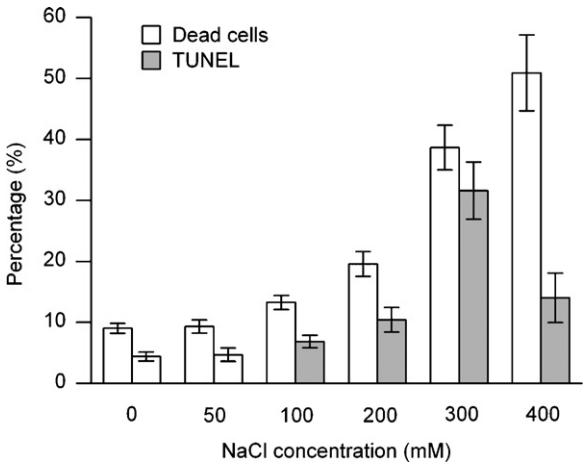

Fig. 2. Effect of salt stress on cell death and TUNEL-positive nuclei in *T. halophila* suspension cells. Cells were treated with 0, 50, 100, 200, 300 and 400 mM NaCl for 12 h.

Activation of caspase 3-like activity

To investigate whether caspase 3-like protease was involved in signaling to salt stress-induced PCD, *T. halophila* cells were treated with 300 mM NaCl in the absence or presence of pretreatment with 100 μM Ac-DEVD-CHO. As shown in Fig. 5, salt treatment induced an increase in caspase 3-like activity of up to 4.5-fold at 12 h compared to controls. However, in the cells pretreated with Ac-DEVD-CHO for 1 h before salt treatment, the caspase 3-like activity was significantly inhibited (at 3, 6 and 12 h, $P < 0.05$).

Release of Cyt c from mitochondria into cytosol

During apoptosis in animal cells, the release of Cyt *c* occurs before visible morphological changes. In this work, the content of Cyt *c* was detected in mitochondria fractions and cytosol fractions at times up to 6 h after treatments. Protein hybridization (Fig. 6) revealed that Cyt *c* (about 14 kDa) was detected mainly in the mitochondria of control cells, whereas it was detected only in the cytosol fraction of stressed cells. Release of Cyt *c* from mitochondria was detected as early as 1 h after salt treatment. There was a distinct reduction in the concentration of Cyt *c* in the mitochondria after 1 h of salt stress, whereas the band relative to the Cyt *c* released

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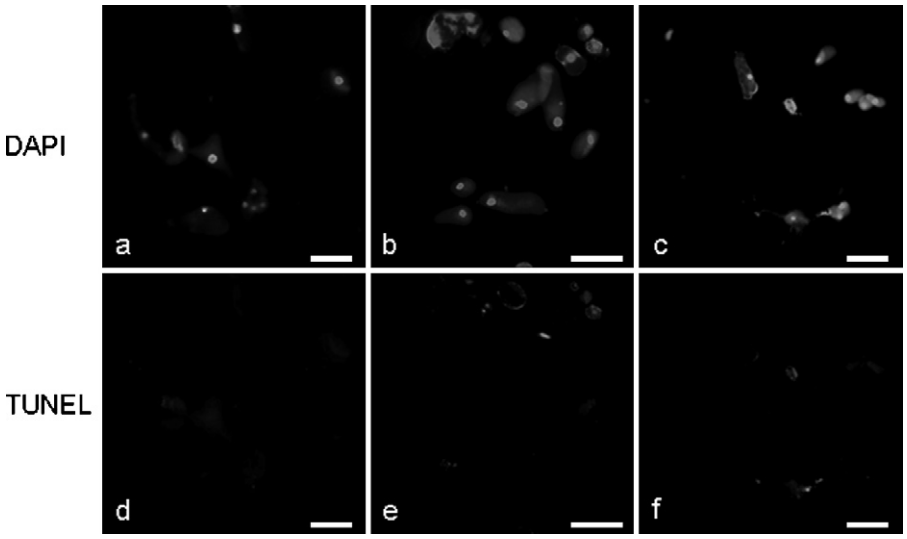

Fig. 3. Images of the TUNEL or DAPI labeled signals detected in *T. halophila* cells. (A and D), control cells; (B and E), cells treated with 300 mM NaCl for 12 h; (C and F), cells pretreated with 100 μM Ac-DEVD-CHO for 1 h before the incubation with 300 mM NaCl for 12 h. Bar = 60 μm.

into the cytosol was evident at this time. The release of Cyt *c* into cytosol increased markedly with time, reaching a maximum at 6 h, earlier than DNA laddering.

Stress causes ultrastructural changes in T. halophila suspension cells

Transmission electron microscopy confirmed the induction of apoptotic-like morphological changes in *T. halophila* cells. The ultrastructure of salt-stressed cells (Fig. 7C–H) was compared with that of normal cells (Fig. 7A and B). The change in the plasma membrane was noticeable. At the early stage of PCD, the plasma membrane began to detach from the cell wall and dense-material-containing vacuoles occupied up to 80–90% of the cell volume, and the cytoplasm appeared electron-opaque (Fig. 7C) while control cells showed normal ultrastructure with intact cell walls and cytoplasm (Fig. 7A). In stressed cells, the plasma membrane had retracted from the cell wall with abundant vesicles present near the plasma membrane (Fig. 7E). In normal cells, the Golgi body had a typical morphology of five or six closely stacked cisternae asymmetrically differentiated from the *cis* to *trans* faces, with small secretion vesicles budding from the *trans* end (Fig. 7B). In stressed cells, however, the *trans* face was often observed with several attached and adjacent translucent vesicles, giving the appearance of some enlarged vesicles that detached from the Golgi (Fig. 7D). DNA fragmentation starts in morphologically normal nuclei. We observed crescent-shaped nuclei (Fig. 7C) and fragmented nuclei (Fig. 7G). Cells at the early stage of stress showed swollen mitochon-

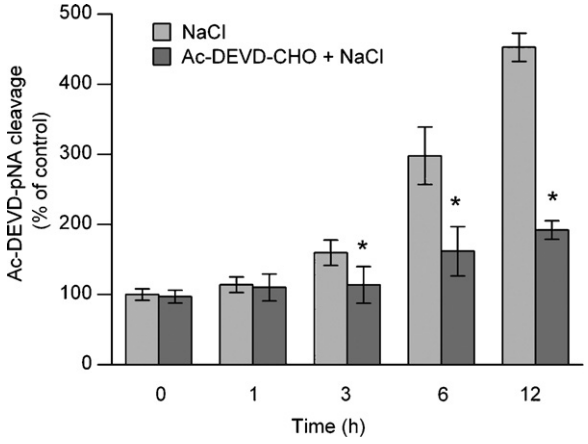

Fig. 5. Activation of caspase 3-like protease in 300 mM NaCl stressed *T. halophila* suspension cells. Caspase 3-like protease activity was expressed as the percentage of the activity of control cells (0 h), which was given a value of 100. The variability of caspase 3-like activity in the control cells during the 0–12 h period was always less than 5%. Data represent the mean (±SD) of three independent measurements. $^{*}P < 0.05$ compared with NaCl alone (paired-samples *t*-test).

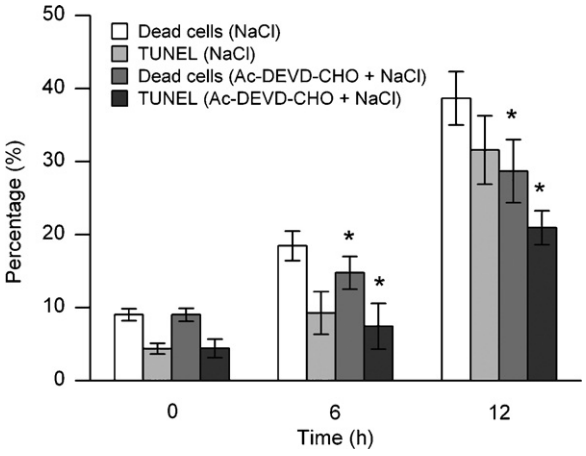

Fig. 4. Time-dependent cell death and TUNEL-positive nuclei in 300 mM NaCl stressed cells. The following cells were used: control cells, salt-stressed cells and cells pretreated for 1 h with 100 μM Ac-DEVD-CHO before the incubation with 300 mM NaCl. Cell viability in control cells and pretreated cells without salt treatment during the 0–12 h period was always more than 90%. Data represent the mean (±SD) of three independent measurements. $^{*}P < 0.05$ compared with NaCl alone (paired-samples *t*-test).

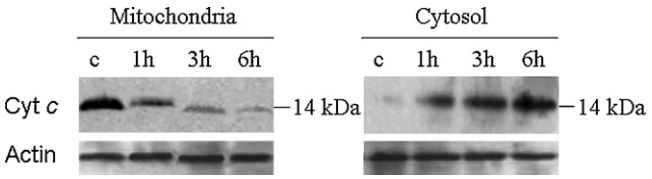

Fig. 6. Protein hybridization analysis of Cyt *c* release from mitochondria into cytosol. Lane c, control cells; Lane 1, 3 and 6 h, cells under 300 mM NaCl stress for 1, 3 and 6 h. Equal loading was verified by anti-actin immunoblotting.

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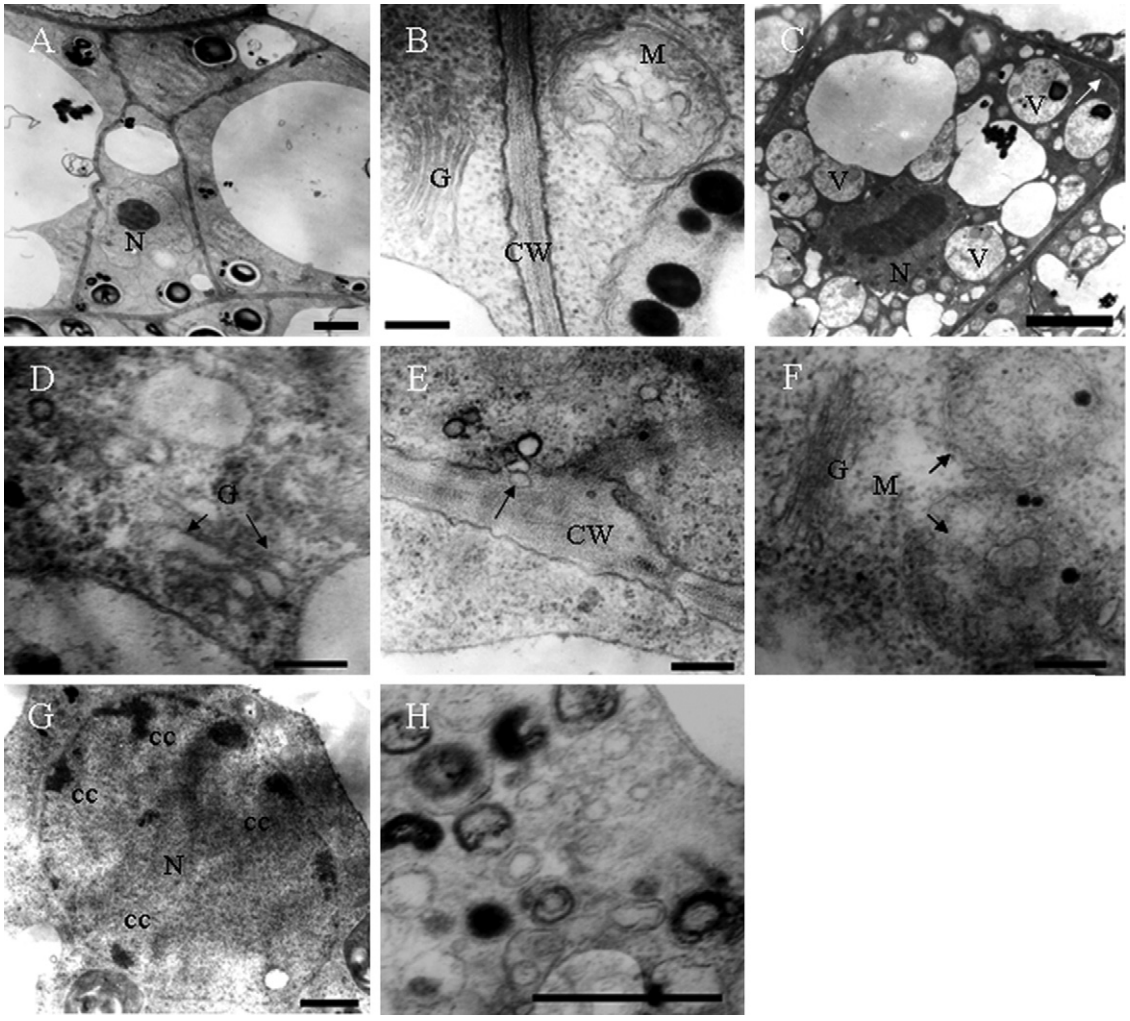

Fig. 7. Transmission electron micrographs (TEM) of untreated cells (A and B) and cells stressed with 300 mM NaCl for 6 h (C–F) and for 12 h (G and H). (A) Untreated cell with normal cell wall and nucleus. (B) Untreated cell showed ellipse-shaped normal mitochondria and a typical morphology of normal Golgi body. (C) Cytoplasm appeared electron-opaque and a limited degree of plasma membrane retracting from cell wall (arrow). Crescent-shaped nuclei was observed. (D) Golgi body under salt stress, arrows point to several attached and adjacent translucent vesicles that detached from the Golgi. (E) Shrinkage of the plasma membrane from the cell wall and presence of vesicles between plasma membrane and cell wall. Arrow points to vesicles present near the plasma membrane. (F) Stressed mitochondria with obscure cristae. Arrows point to the swelling mitochondria. (G) Chromatin condensation in stressed nucleus. (H) Crescent-shaped mitochondria. N, nucleus; G, Golgi body; M, mitochondria; CW, cell wall; PM, plasma membrane; V, vacuole; cc, chromatin condensation. Bar = 2 μm (A and C), 200 nm (B, D, E, and F), 1 μm (G), 500 nm (H).

dria with obscure cristae (Fig. 7F), while mitochondria in severely stressed cells were frequently crescent-shaped (Fig. 7H) in contrast with ellipse-shaped normal mitochondria with legible cristae (Fig. 7B). In this work, TEM photos also provided new evidence for the presence of autophagic vacuoles in severely salt-stressed plant cells. As shown in Fig. 8, impaired organelles and other cytoplasmic components were enclosed by vacuoles, creating double membrane autophagosomes, which indicates autophagy. Black arrows point to some membrane-bound body that was being engulfed into the vacuole at different stages of plant PCD (Fig. 8A, C and D). When cells were stressed for 12 h, the large vacuole which had engulfed impaired organelles and other inclusions was present (Fig. 8B), but autophagic bodies were not observed within normal cells (Fig. 7A). It is interesting that particulate matter and large vesicles between the plasma membrane and cell wall were also observed (Fig. 8B)

Discussion

The simplified system 'cell culture/abiotic or biotic stress' has been widely used to understand the basic mechanisms of plant biological responses in plants (McCabe et al., 1997; Zuppini et al., 2006). In plants, specific cell death with the morphologic features of the protoplast shrunk from the cell wall and the degradation of DNA, and the involvement of the activation of caspase-like molecules and the release of Cyt *c*, are considered apoptotic-like PCD (Reape and McCabe, 2008). In this work, we found that specific features of 300 mM NaCl stress-induced cell death are similar to animal apoptosis, including cytoplasmic shrinkage and chromatin condensation, DNA laddering, Cyt *c* release and activation of caspase 3-like protease activity.

A key process by which eukaryotic cells respond to and survive various stresses is vacuolar autophagy. Autophagy is a catabolic process by which eukaryotic cells degrade portions of their own cytoplasm. This process is conserved among yeast, animal, and plant cells, and has also been known in plants for a number of years to be involved in cellular architectural (re) modeling that occurs during differentiation and development (Bassham et al., 2006). Whether autophagy functions in cell survival or cell death depends on the level of autophagy induced during a given physiological condition (Pattingre et al., 2005). In previous descriptions of such structures in plants, Wang et al. (1996) reported apoptotic-like bodies during sloughing of root cap cells, and membrane-bounded bodies containing cellular organelles and other material were described by Gunawardena et al. (2001) in

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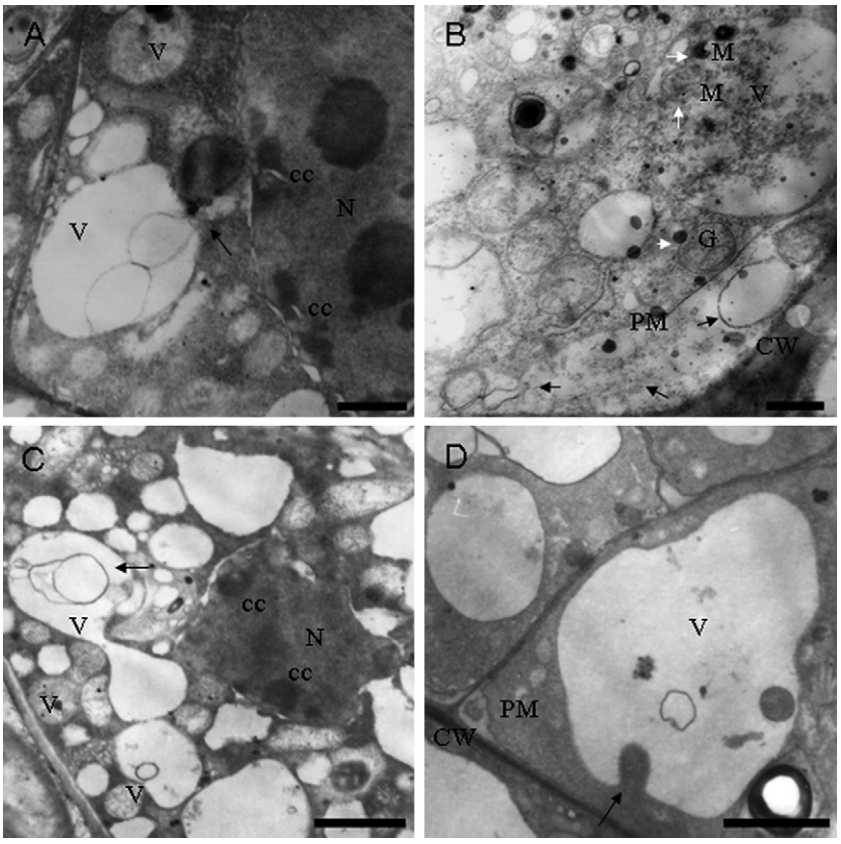

Fig. 8. Ultrastructural features of 300 mM NaCl stress-induced autophagy occurring at 6 h (A), 12 h (B), 16 h (C) and 24 h (D) in *T. halophila* suspension cells. Cytoplasm was being engulfed into the vacuole in (A), (C) and (D) and arrows points to the site of autophagy. White arrows in (B) pointed at vacuoles containing cellular organelles and other inclusion bodies. Particulate matter and irregularly shaped matter between the plasma membrane and the cell wall were observed (black arrows). V, vacuole; N, nucleus; CW, cell wall; PM, plasma membrane; cc, chromatin condensation; G, Golgi body; M, mitochondria. Bar = 1 μm (A and C), 500 nm (B) and 2 μm (D).

their study of aerenchyma formation in maize roots. Recently, Liu et al. (2005) demonstrated that autophagy plays a pivotal role in controlling HR-PCD, which provided a close association between HR-PCD and autophagy. A more recent study using autophagy-defective mutant plants demonstrated that autophagy is required for tolerance of drought and salt stress in plants (Liu et al., 2009). TEM photos provided evidence for a close link between salt stress-induced plant PCD and autophagy in our work. Here, we report that *T. halophila* suspension cells can degrade their cytoplasmic contents by autophagic bodies, which are induced by 300 mM NaCl stress, suggesting this autophagic vacuole is an integral part of salt stress-induced PCD since no autophagic vacuole was present in normal cells (Fig. 7A) or necrotic cells (data not shown). Thus, autophagic vacuoles may be involved in hydrolysis of impaired organelles and other materials to recycle cellular components such as nucleotides and amino acids. We also observed particulate matter and large vesicles between the plasma membrane and cell wall, and how this occurred remains to be elucidated.

Mitochondria play a key role in cellular metabolism and also in the regulation of PCD. One of the pivotal mitochondria events, the release of Cyt *c*, has been well established in animal cells (Orrenius, 2004). The release of Cyt *c* might be important in plant cell death, and our current knowledge of the release of Cyt *c* in plant PCD is accumulating, although the role of Cyt *c* on plant cell death is under debate (Yu et al., 2002). In this work, protein hybridization showed that Cyt *c* was released from mitochondria into the cytosol early during PCD and could be detected in cytosol as early as 1 h after salt treatment, which is earlier than DNA laddering. The Cyt *c* release observed in cells treated with severe salt stress is consistent with the hypothesis that the molecular mechanism of apoptosis execution in plants and animals is evolutionarily conserved (Stein and Hansen, 1999). Caspase-dependent as well as caspase-independent apoptosis has been described in animal cells, and the presence of caspase 3-like activities is one of the peculiar biochemical features of PCD (Matsumura et al., 2000). Functional homologues of animal caspases have not been identified to date in plant cells (Woltering et al., 2002). However, caspase 3-like protease has recently been shown to be activated in plants (reviewed in Bonneau et al., 2008). In support of a role of caspase 3-like activity in plant PCD, experiments in *T. halophila* showed that 100 μM Ac-DEVD-CHO could block the induction of caspase 3-like protease activity and of a DNA ladder prior to the inhibition of cell death. Synthetic tetrapeptide inhibitors, such as Ac-DEVD-CHO, are commonly used to block PCD in animal cells and, recently, have been used in plants for *in vitro*. Sun et al. (1999) found that DNA laddering induced by menadione in tobacco cells was completely inhibited by 100 μM Ac-DEVD-CHO. Ac-DEVD-CHO has been demonstrated to inhibit pathogenesis in plant cells (del Pozo and Lam, 1998; Richael et al., 2001) and to suppress cell death of pollen tubes in *Papaver* (Thomas and Franklin-Tong, 2004). More recently, Vacca et al. (2006) reported that Ac-DEVD-CHO largely prevented heat shock-induced PCD in tobacco cells. All of these findings imply that caspase-like activities may be part of the core mechanism of plant PCD (Bonneau et al., 2008), although caspase-like independent PCD exists in several plant systems (Fukuda, 1997; Krzymowska et al., 2007). In our work, when cells were pretreated with Ac-DEVD-CHO before salt treatment, a decrease in the amount of cells with TUNEL-positive nuclei was observed (Fig. 3). This observation, coupled with the changes of DNA laddering, cell viability and caspase 3-like activity (Figs. 1, 4 and 5 in "Results" section, respectively)

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provides evidence for the involvement of caspase 3-like pathway in salt stress-induced PCD in *T. halophila*. Our data indicate that the release of Cyt *c* from mitochondria is earlier than DNA laddering, and caspase 3-like protease is upstream of cell death in 300 mM NaCl treated cells. It is suggested that high salinity induced a cell death program in *T. halophila* cells that occurs with Cyt *c* release from mitochondria partly through a caspase 3-like dependent pathway.

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