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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*

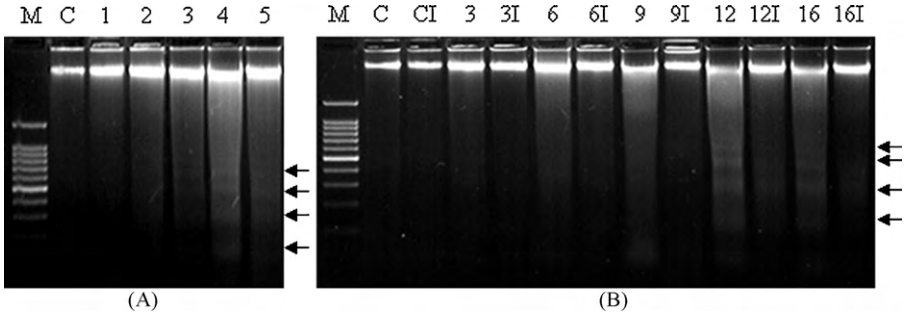

Fig. 1. DNA laddering in *T. halophila* suspension cells after salt treatment. Arrows indicate DNA ladders. (A) DNA laddering after 12 h treatment with different concentrations of NaCl. Lane M, 100 bp DNA marker; Lane C, control; Lane 1-5, cells stressed with 50, 100, 200, 300 and 400 mM NaCl, respectively. (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.