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Research Paper

Programmed Cell Death Induced by Salt Stress in Wheat Cell Suspension

Ali Rezaei, Mohammad Reza Amirjani and Majid Mahdiyeh
Department of Biology, Faculty of Sciences, Arak University, Arak 38156-8-8349, Iran, Fax: +98 861 4173406,
Email: m-amirjani@araku.ac.ir

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Abstract: Programmed cell death (PCD) plays an essential role in many aspects of plant development and growth. Also it a key element in response to stresses. In the present study, we investigated role salt stress in induced programmed cell death or apoptosis wheat (*Triticum aestivum* L.) suspension cell culture. Wheat suspension cells were exposed to 500 Mm NaCl and cell death was determined using several apoptosis assay. We observed morphological and biochemical apoptosis features such as nuclear condensation, DNA laddering, transferase mediated dUTP nick end labeling. Positive TUNEL and reduction cell viability results were obtained treatment wheat suspension cell. The results indicate that 500 mM NaCl can induced pcd in suspension cell.

Keywords: Programmed cell death; Salt stress; Wheat; DNA fragmenta

Introduction

Programmed cell death (PCD) is a highly regulated cellular suicide process for growth and survival in eukaryotes. it is a genetically determined process that occurs at all stages of the life cycle in plants, including growth, development, and death of the whole plant (van Doorn and Woltering 2005).

In plants, PCD occurs during development, such as during xylogenesis, embryogenesis, parenchyma formation, several plant reproductive processes, seed development and leaf senescence (Pennell 1997; Gray 2004)

In addition, PCD is well documented in relation to manifestation of hypersensitive response (HR) caused by the interaction between the host plant and an incompatible pathogen (Heath 2000)

Furthermore, PCD plays an important role during cell death in response to various abiotic stresses, including heat shock, toxic chemicals, ozone exposure, UV radiation and hypoxia (Danon and Gallois 1998;; Overmyer 2005; van Doorn and Woltering 2005)

Recent studies showed that morphological and biochemical hallmarks of PCD, like cytoplasmic shrinkage, chromatin condensation, cytochrome C leakage out of mitochondria, altered nuclear morphology, protease activation and DNA fragmentation are similar to those found in animal apoptosis (Danon A 2000; Krishnamurthy et al. 2000)

Salinity is a major threat to the sustainability of wheat production in irrigated and rainfed environments around the world (Ghassemi et al. 1995). As plants are sessile organisms they must cope with changing environmental conditions by adapting to stress situations via various molecular and physiological processes. 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).

This work was aimed on investigating the way of cell death observed in cultures of wheat suspension cells under salt stress and study characterizes feature pcd by cytochimical methods**.**

Martial mad Methods

Surface sterilized wheat (*Triticum aestivum* L.) seeds cultured on callus induction medium. The callus induction medium contained MS medium. After sub-cultures, the calluses were transferred into liquid suspension culture medium. The cells were maintained in a growth chamber at 23◦ C in the dark on a gyratory shaker (120 rpm). Cells in exponential growth phase were exposed to 500 Mm NaCl for various times under culture conditions. Control and treated cells were collected in the time interval of 4, 8 and 12 h.

Total genomic DNA was isolated from control as well as treated cells using CTAB method. The frozen suspension cells (100–500 mg) were ground into fine powder with liquid nitrogen and transferred into an extraction buffer. After thawing the mixture was incubated for 40 min at 65◦C and treated with extraction mixture (chloroform: phenol 9:1, w/w) and DNA was precipitated by adding 2-propanol (1:1, v/v). After centrifugation (500g, 25◦C, 5 min), washing (70% ethanol, w/w) and dissolving in TE buffer (1 mM EDTA, 10 mM Tris, pH 8,0) the solution was incubated with RNAase (at the final concentration 40μgml−1) for10 min at 65◦C.

To analyze nuclear morphological changes, cells were stained with the fluorescent dyes Hoechst 33342 (HO). Cells were incubated in darkness with 5 μg/ml HO (Sigma, USA) at room temperature for 10 minutes and observed by fluorescence microscopy using an excitation light of 350 nm.

After stained with 0.5% trypan blue in phosphate-bufer saline(PBS) for 1 min, cells were observed under a light microscope. 400 cells were counted and three independent experiments were performed

The TUNEL procedure was performed according to the manufacturer's instructions (In situ Apoptosis Detection Kit. Takara, Japan). In brief, cells were immobilized on the slides by polylysine, fixed with 4% paraformaldehyde (in PBS, pH 7.4) for 30 min

at room temperature. Slides were rinsed with PBS (pH 7.4), and then incubated in permeabilization solution for 2 min on ice. After rinsed twice with PBS (pH 7.4), 50 µl TUNEL reaction mixture was added and incubated for 1 h at 37◦C. Apply Anti-FITC HRP Conjugate at37º for 30 min. and wash with PBS 3 times. After coloring with DAB at room temperature for 10 min, terminate the reaction by washing with distilled water Stain the cells with 3 % methyl green. Mount and detect with a light microscope.

Results

DNA laddering

Cleavage of genomic DNA during apoptotic PCD is realized in two subsequent steps; an early cleavage into high molecular weight fragments (in sizes consistent with chromatin loop domains) and, later, an intense fragmentation, usually forming oligonucleosomal fragments (Brotner et al., 1995), which can be detected by DNA electrophoresis. Therefore, DNA laddering patterns were visualized on agarose gels. Control cells did not show DNA fragmentation while The DNA isolated from treated cells revealed DNA ladders consisting of multiples of 180 bp. DNA laddering occurs after 4 h treatment, and became more significant after 12 h treatment (Fig. 1).

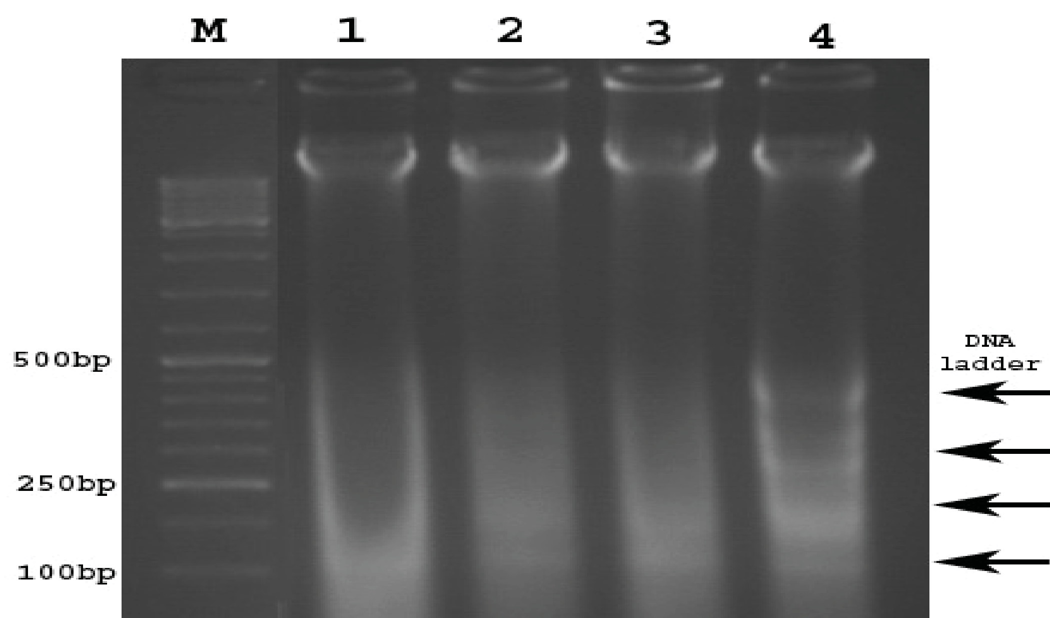

Figure 1. DNA laddering in wheat suspension cells. wheat (*Triticum aestivum* L.) suspension cells were treated with 500 mmol/L NaCl for 12 hours. Lane M, marker; lane 1, control, lan 2-4 cells stressed with 500 mmol/L for 4 h, 8 h and 12 h, respectively.

Nucleus Morphological changes during salt stress

One of the characteristics of apoptosis is the occurrence of morphological changes to the nucleus. In order to determine whether nuclear changes Hoechst staining were used and the morphology of nuclei of control and treated cells was compared. Result showed that control cells had a normal nuclear morphology while in treated cells the nuclei were smaller and showed chromatin condensation and DNA fragmentation (Fig 2). Also were observed early morphological features of PCD such as early cytoplasm shrinkage and nuclear condensation in 4 and 8 hours after treatment. In 12 after treatment cells showed fragmented nuclei in which the formed bodies connected by DNA-containing threads, with the remaining nucleus. These results show that salt-induced cell death in wheat cell suspension is accompanied by morphological changes to the nuclei that are characteristic of animal apoptosis. These changes included chromatin condensation, nucleolar architecture, resembling those observed during apoptosis induction in animal as well as human cells, described.

TUNEL-positive nuclei

In situ excessive DNA fragmentation can be visualized in cells by TUNEL reaction (TdT-mediated dUTP nick-end labelling. The TUNEL-positive cells were found in cell cultures treated with 500Mm NaCl and TUNEL signals, detected in salt stressed cells , provided further evidence for the onset of PCD. Treated cells indicated nucleus with brown in color (TUNEL-positive while the nucleus of control cells remain green in color (TUNEL-negative). As expected no evidence of DNA fragmentation was observed in control cells and the number of nuclei with evidence of DNA fragmentation increased in relation to the duration of salt stress (Fig 3).

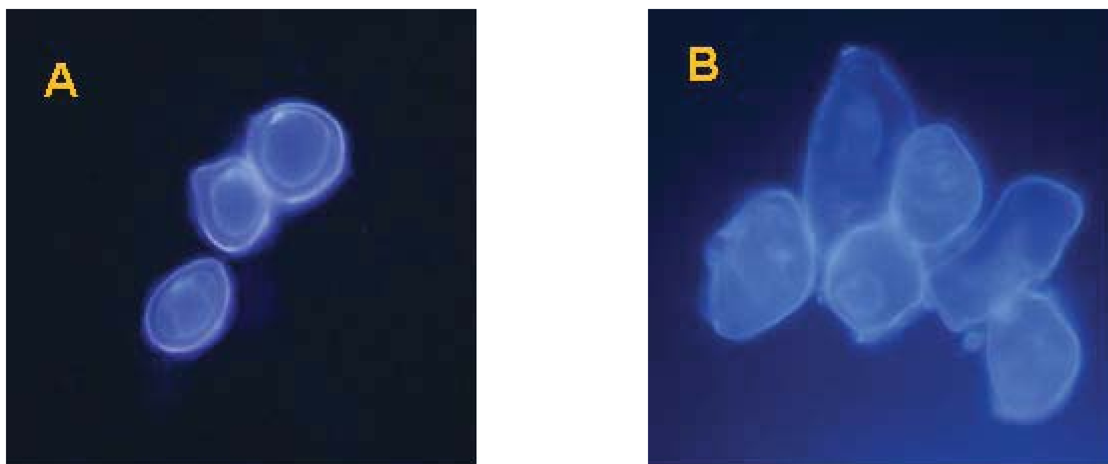

Figure 2. Morphological changes in wheat suspension cells. A: non-treated control cells. B:cells treated with 500 mmol/L NaCl for 4 hours. Cells from both treatments and control were stained with HO342.

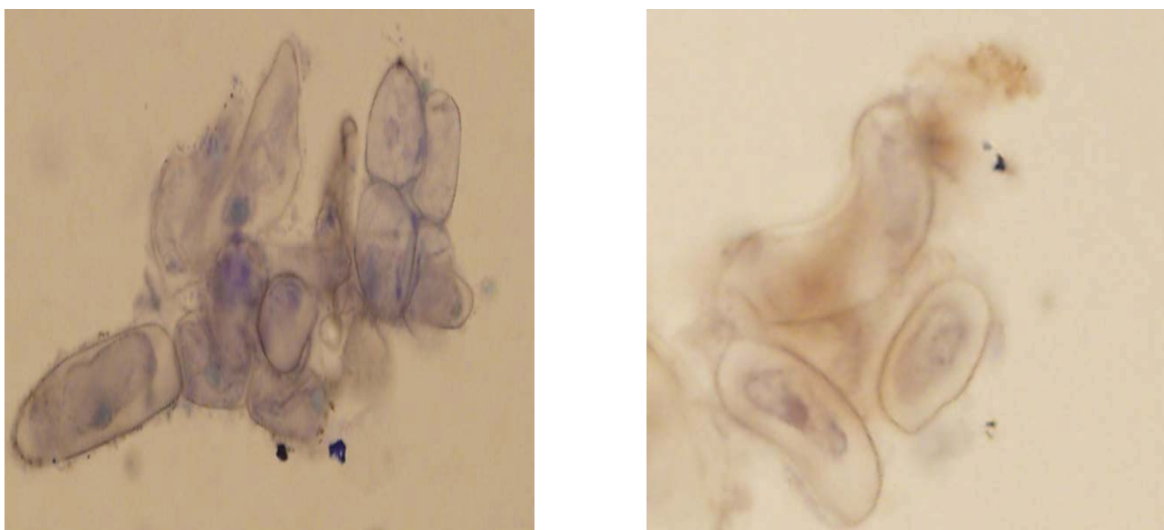

Figure 3. TUNEL assay of wheat suspension cells. A: non-treated control cells. B:cells treated with 500 mmol/L NaCl for 4 hours. Note the presence of TUNEL-positive nuclei indicated by brown color and TUNEL negative nuclei indicated by green color.

Effect of salt stress on viability wheat suspension cell

As it is shown in Fig. 1, the exposure of wheat cell suspension to 500Mm NaCl for 0–12 h resulted cell viability reduction compared with control cells. The maximum of the viability cell number was recorded in control cells. Compared with control cells, treated cells showed strong reduction in viability. An increase in the number of dead cells was first detected after 4 h of treatment cell. Showed that cell death at 4 and 8 h after salt treatment was about 1.5- and 2-fold that of control cells, cell viability decreased with time of treatment: within12 h after the treatment 88% cells are dead (Fig 4).

Conclusion and Discussion

Cultured cells have frequently been used to study PCD successfully (McCabe and Pennell 1996) .
Cell death triggered by biotic and abiotic stresses in plants can be classified as PCD or necrosis. There are several indications that plants employ a form of PCD similar to apoptosis, the most common form of PCD in animals that is defined by a distinct set of morphological and biochemical features such cell shrinkage, chromatin condensation (Steller 1995; McCabe and Pennell 1996; van Doorn and Woltering 2005). The presence of genomic DNA fragmentation, a hallmark of apoptosis, has been reported in various plant systems (Orzaez and Granell 1997; Wang 1999; De Jong et al. 2000).

Recently, several studies revealed that upon exposure to NaCl , some species of plant suspension cultures undergo PCD. reported that 300 mM NaCl induced a rapid decrease in cell viability and ultrastructural changes and DNA integrity, cytoplasmic shrinkage and chromatin condensation, DNA laddering in Thellungiella halophila suspension culture (Wang et al. 2010) . Also they observed 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) . In 2006 Lin et al. were evaluated effect salt stress on pcd (Lin et al. 2006). Result of them work were showed that treatment of tobacco protoplasts with NaCl could effectively induce PCD caused a reduction in the mitochondrial membrane potential and increased

ROS accumulation. We investigated DNA fragmentation by two methods using the TUNEL reaction and gel electrophoresis. After 4 h treatment cells showed DNA fragmentation the 180-bp DNA ladder increases in intensity with time. This observation indicates that plasma membrane alteration is an early indicator of cells undergoing DNA fragmentation since it occurs prior to the detection of DNA strand breaks by the TUNEL reaction. Cleavage of genomic DNA into small fragments is a hallmark of PCD in plant. DNA fragmentation due to endonuclease activity is very categorically established in apoptotic animal cells. Almost all the reports of the presence of endonucleases in plant systems are either indirect or indefinite with reference to their role in fragmenting DNA .in other study chromatin condensation and DNA strand breaks, which are considered the hallmarks of PCD, were observed in BY-2 suspension cultures treated with 150 µM $CdCl_2$. also induction of a 180-bp DNA ladder has been described in several plant species(Williams and Dickman 2008)

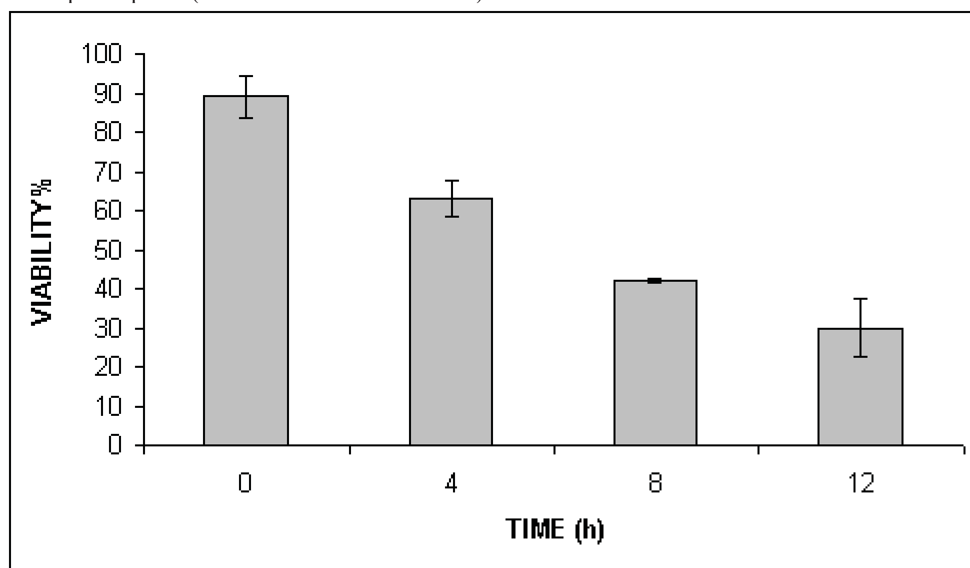

Figure 4. Effects of 500 mmol/L NaCl on viability of wheat suspension cells. Cell viability was assayed tripan blue staining at 0, 4, 8 and 12 h after treatment.

Morphologically, PCD, known as apoptosis, is generally characterized by a subset of changes such as chromatin and cytoplasm condensation (Vaux and Korsmeyer 1999) . Little is known about apoptosis in plants including the morphological changes(Danon et al. 2000) . Although some accumulating evidence suggests that some features of plant apoptosis such as nuclear disintegration and chromatin condensation triggered endogenously or environmentally are similar to those in animals (Vaux and Korsmeyer 1999; Danon et al. 2000). all major hallmarks of apoptosis following the treatment of wheat suspension cells were observed. Nuclei reported characteristic marks of chromatin condensation and degradation nucleus. Similar results were obtained experimentally in tobacco suspension cultures treated with 150 µM $CdCl_2$. PCD induced by EIX or staurosporine in *N. tabacum* cell suspension also cytoplasm shrinkage and nuclear condensation were observed. De Jong et al (2000) the main morphological features of apoptosis find in suspension cells Tomato (*Lycopersicon esculentum* Mill.) treatment with inducers of apoptosis in mammalian cells (De Jong et al. 2000). Those results showed that proteolysis plays a crucial role in apoptosis in plants.

Our results suggest that the cell death observed under salt stress wheat suspension cells characteristics of PCD. In our work for induce pcd we use concentration 500 Mm NaCl. Treatment of wheat suspension cells with concentration 500 Mm NaCl during 12 hours induces changes in nuclear morphology and internucleosomal DNA cleavage. We have showed which It morphological and functional similarities between plant and animal PCD.

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