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Research Paper

DNA fragmentation and change of nucleus in salt-treated cells of wheat root

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Abstract: Programmed cell death (PCD) or apoptosis is a key process in plant growth and development which may be induced by various abiotic and biotic stress factors including salt stress. Wheat (*Triticum aestivum* L.) seedling was treated with different concentrations of NaCl for certain times. Then we use histo-chemical and cyto-chemical methods order to study cell death morphology in root wheat. In our experiments using TUNEL reaction, Hoechst staining and DNA electrophoresis, features of PCD such as condensed chromatin, a condensed and marginal nucleus, fragmented DNA were observed. The results indicated that 500 mmol/L NaCl treatment induced apoptosis in root cells wheat. Root cells death may provide an adaptive advantage to plant to salt stress. it might be involved in plant tolerance against salt stress.

Keywords: Programmed cell death; Wheat (*Triticum aestivum* L.); Salt stress

Introduction

Programmed cell death (PCD) has been defined as a sequence of (potentially interruptible) events that lead to the controlled and organized destruction of the cell (Lockshin 2004).

In plants has also been identified in a number of developmental processes, including tracheary element formation (Obara 2001) degeneration of the suspensor (Giuliani, Consonni et al. 2002), cell death in wheat aleurone layers (Dom´ınguez, Moreno et al. 2004), and petal senescence(Xu, Chen et al. 2004) anther development (Wang 1999) . In addition, cell death also occurs in plants in response to stressful environments such as drought and salt (Katsuhara and Kawasaki 1996; Munne 2004). Which may help plants survive adverse stresses by eliminating cells, tissues or organs that render a plant more vulnerable to its environment (Huh, Damsz et al. 2002).

Typical hallmarks of PCD both in plants and animals include the fragmentation of the DNA into discrete fragments of about 180 bp (DNA laddering), condensation and shrinkage of the cytoplasm (Pennell 1997; van Doorn and Woltering 2005) cytochrome c leakage out of mitochondria, altered nuclear morphology and activation of proteases (Lam 1999; Danon, Delorme et al. 2000). Most of these morphological and ultra structural changes can be traced to the actions of the caspases, a family of intracellular cysteine proteases that become activated during the cell suicide response (Li 1997; Villa 1997).

Salt stress is known to have severe effects on plant growth and development (Greenway 1980; Bohnert 1995) . As plants are sessile organisms they must cope with changing environmental conditions by adapting to stress situations via various molecular and physiological processes among PCD (Huh, Matsumoto et al. 2002).

Plant cells, in contrast, display several unique features as compared with their animal counterparts, including a lack of 'true' caspases, the presence of a rigid cell wall and more importantly the lack of an active phagocytosis system. Other unique feature of plant cells include totipotency, chloroplasts, non-motility and numerous and some- times large vacuoles harbouring high levels of degradative enzymes. There is already ample evidence that several plant processes involve a form of PCD (e.g. senescence, xylem formation), but whether these genetically programmed events are apoptotic-like is not entirely clear (Lim et al., 2007).

One of the universal hallmarks of PCD is the nonrandom, internucleosomal fragmentation of nuclear DNA (nDNA), occurring as a result of a specific endonuclease activation (Brotner, Oldenburg et al. 1995) . This specific cleavage in the internucleosomal linker regions between nucleosomal cores resulting in the production of oligonucleosomal DNA fragments is one of the main criteria distinguishing necrosis from PCD (Brotner, Oldenburg et al. 1995).

The nDNA electrophoresis on agarose gel (DNA ladder) (Eastman 1995) and TUNEL assay (terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling) (Gavrieli, Sherman et al. 1992) are methods commonly used to detect the DNA cleavage characteristic for PCD. Both of these methods require sections or samples extracted from heterogeneous tissue or plant organ.

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In this study, we found the relations between PCD specific nDNA fragmentation, changes in chromatin condensation, degradation nucleus in root wheat cells.

Martial mad Methods

Sterilized wheat (*Triticum aestivum* L.) seeds were soaked in double-distilled H_2O for 1 day and transferred into a container filled with the Hoagland's nutrient solution (Hoagland 1950). Seedlings were treated with different concentrations of NaCl in the nutrient solution for certain times. The plants were allowed to grow under a constant condition of 16 h light/8 h dark at 28 C in the growth chamber.

The nuclear DNA was extracted by cetyltrimethyl ammonium bromide method (CTAB). DNA electrophoresis was carried out in a 2% agarose gel, stained with ethidium bromide (EB) an captured under UV light.

Segments of root tissue (5 mm) were fixed in an FAA mixture overnight and dehydrated through a graded ethanol series (50, 70, 80, 95%, 1 h each step), then to 100% ethanol for 1 h with two changes of the solution. The dehydrated tissues were then taken rough a graded xylene series (25%, 50%, and 100% for 1 h at each step).The tissues were embedded in 50% paraffin wax mixed with 50% xylene overnight at 59 8C. The specimens were finally placed in 100% paraffin wax at 59 8C for 1 day .and sectioned longitudinally on a microtome at thickness of 10µm.

Root sections were deparaffinized, rehydrated and stained with Hoechst 33258 for 5 min and examined under fluorescence microscope. Stained nuclei showed blue fluorescence when excited with UV beam.

After deparaffinization, samples were stained with haematoxylin and eosin (Harris 1994) and photographs were taken from samples and dimension cell were measured by software motic. NPRs were calculated using the equation:

NPR = nucleus area/ (total area of the cell – nucleus area)

Then, the average and standard deviation of the NPR in each region was computed and the results analysed by one-tailed ANOVA in order to statistically compare nucleus condensation in time 4,8 and 12 hours after 500 mmol/L NaCl concentrations.

Nuclear DNA fragmentation was identified by TUNEL terminal deoxynucleotide transferase-mediated dUTP nick-end labeling) staining, which detects free 3OH hydroxyl groups of degraded nuclear DNA(Gavrieli, Sherman et al. 1992).

Sections (5 µm) of samples embedded in paraffin were mounted on glass slides with poly-L-lysine and treated with the in situ TUNEL Kit according to the manufacturer's instructions to visualise 3'OH-labelled ends in the nuclei.'. After rinsing for 3 × 5 min with phosphate buffered saline (PBS) (pH7.4), the slides were counterstained with 3% methyl green and visualized with a light microscope.

Results

DNA fragmentation

The analysis of DNA fragmentation was carried by electrophoresis on 2% agarose gel (Fig 1). At concentrations 100,200,300 and 400 mmol/L NaCl no degradation of DNA was observed. DNA laddering occurred at concentrations 500 mmol/L NaCl which after 4h treatment was observed and became more significant after 8 and 12 h treatment. A ladder of about180 to 200 bp fragmented DNA was observed which shows enzyme-derived oligonocleosomal DNA fragmentation. We found that salt stress induced progressive DNA laddering in a dose- and time-dependent manner.

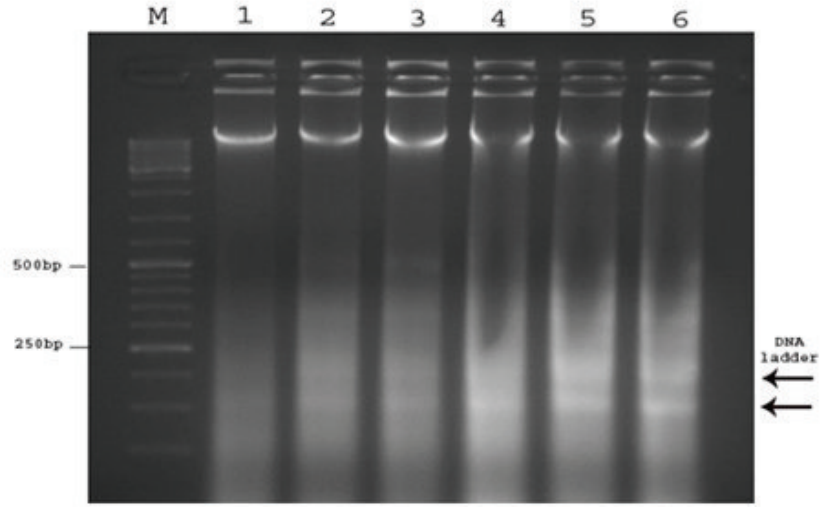

Figure 1. DNA laddering after 4 h treatment with different concentrations of NaCl. M, marker; 1, control; 2-6 stressed with 100-500 mmol/L NaCl, respectively. The arrow indicates the position of 180-200 bp fragments of fragmented DNA that generates a 'ladder' pattern in a gel.

Detection chromatin condensation and morphological changes

We evaluated the cell morphological changes by staining of the nuclei with the fluorescence HO . Untreated samples show a HO fluorescence homogenously distributed in the cell nuclei without chromatin condensation. The typical morphological features of apoptosis including chromatin condensation, margination and nuclear DNA fragmentation were observed in nuclei the root meristem cells treated samples. Also after 12h treatment degradation nuclei was observed (Fig 2).

NPR change

The decrease of NPR is mainly due to nucleus condensation was obvious at concentrations 500 mmol/L NaCl which nucleus condensation were significantly reduced at 4h after treatment ($P < 0.05$) (Fig 3).

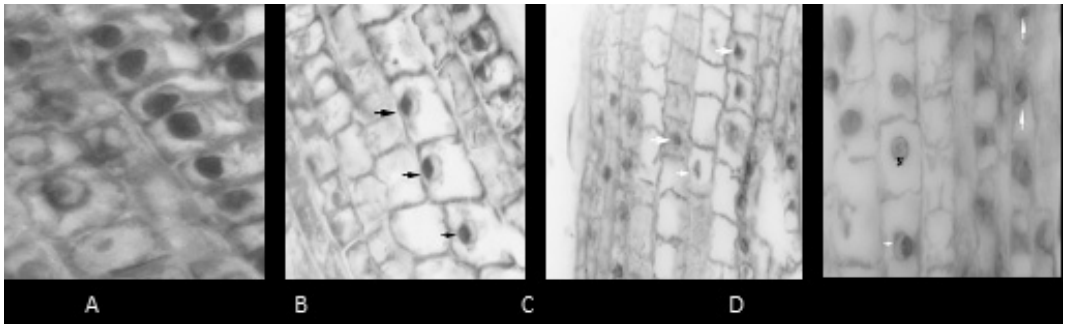

Figure 2. Hoechst -stained longitudinal sections wheat root tip cellsafter 500 mmol/L NaCl treatments. (A) control primary (40 x) (B) 4h, (C) 8h and (D) 12hours after treatment. arrows show Condensed nuclei and Cells undergoing programmed cell death (N), nucleus with lobed nucleus.

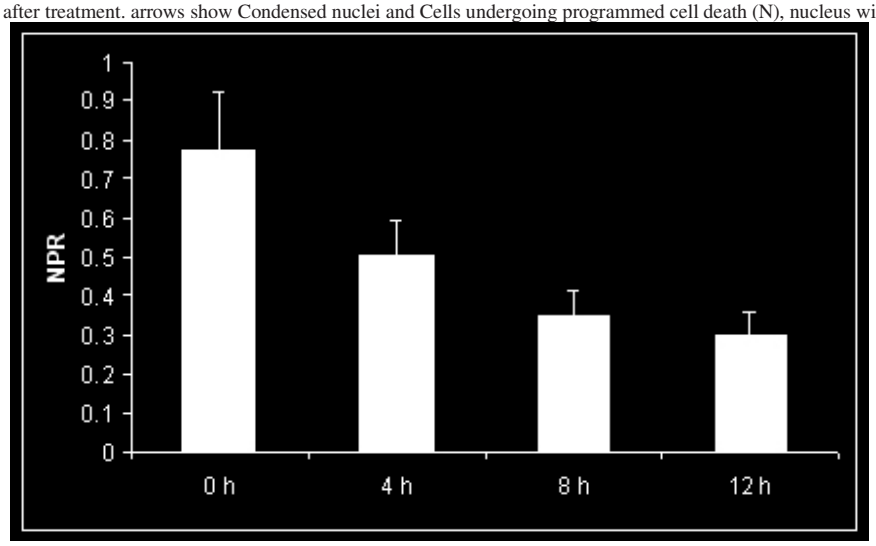

Figure 3. nucleo-plasmic ratio in root. NPR of cells in 500 mmol/L NaCl treatments.

TUNEL analysis

Another specific feature of PCD is the cleavage of DNA at internucleosomal sites by DNA endonucleases. In order to detect DNA fragmentation in situ, fragmented DNA was end labeled applying the TUNEL method. The results of TUNEL assay for tissue sections from the root tips of untreated and treated seedlings were shown in figure 3.As shown in figure no nuclues from control cells were found to be TUNEL positive. In contrast Under the NaCl stress and 4h after treatment nuclear DNA cleavage detected as TUNEL positive staining (brown sign al under light) was observed in cells root. Similar results were obtained with the root tips of seedlings subjected to 8 an 12 hours after treatment.

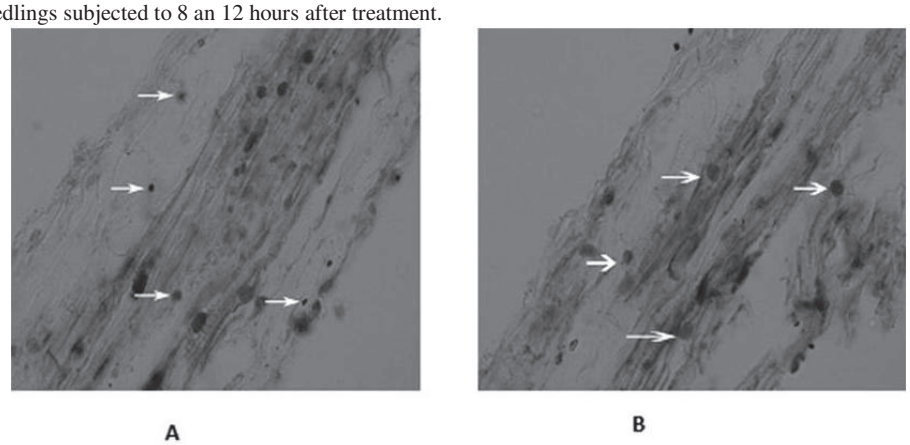

Figure 4. TUNEL staining in cells of longitudinal sections from the root tissues of untreat and salt treated. A) An untreated tissue section (TUNEL negative) b) a tissue section from root tips treated for 4 h. Note the presence of TUNEL-positive nuclei indicated by brown color and TUNEL negative nuclei indicated by green color.

Conclusion

Programmed cell death (PCD), the highly regulated dismantling of cells, is essential for plant growth and survival (Williams and Dickman 2008). Recent evidence indicates PCD plays key roles in adaptation plants to environmental conditions (Shabala

2009). Whereas PCD is well-studied in animals, our knowledge of the genetic mechanisms that regulate and execute plant cell death is limited. Recent biochemical and molecular genetic studies have revealed parts of the complex plant cell death network and broadened our understanding of the machinery controlling cell death in plants. In this study we use histo-chemical and cyto-chemical methods order to study cell death morphology in root wheat. The specific features of PCD such as condensed chromatin, a condensed and marginal nucleus, fragmented DNA in respone to salt stress were observed.

The biochemical hallmark of apoptosis is the fragmentation of the genomic DNA, an irreversible event that commits the cell to die(Palavan-Unsal 2005; van Doorn and Woltering 2005; Williams and Dickman 2008). In many systems, this DNA fragmentation has been shown to result from activation of an endogenous Ca2+ and Mg2+-dependent nuclear endonuclease (Krishnamurthy, Krishnaraj et al. 2000). This enzyme selectively cleaves DNA at sites located between nucleosomal units (linker DNA) generating mono- and oligonucleosomal DNA fragments. These DNA fragments reveal, upon agarose gel electrophoresis, a distinctive ladder pattern consisting of multiples of an approximately 180 bp subunit. it is demonstrated that salt strees in highly concentration and a few time lead to DNA laddering in cells root. DNA laddering has been used to diagnose PCD in animals and plants. Our finding accordance with Katsuhara which first time have reported induced cell death by salt stress in in barley roots (Katsuhara and Kawasaki 1996). Also reported occurrence DNA laddering during salt stress long-time and short time in rice root tips (Huh, Damsz et al. 2002; Li, Jiang et al. 2007; Liu, Fu et al. 2007). DNA laddering, one of the hallmarks of pcd, was visible as soon as 1 h after the onest of NaCl strees (Affenzeller 2009).while previus reports suggested that at least 4 h of salinity treatment was needed (Li, Jiang et al. 2007). affenzeller et al., 2009 also found that the observed DNA laddering occurred in NaCl but not in sorbitol-stressed cells (Affenzeller 2009). This indicates that the ionic rather then the osmotic component of salt stress led to activation of the endonuclease resulting in PCD.

Morphological features of apoptosis may be detected by various cytochemical and microscopic methods (Palavan-Unsal 2005). use of intercalatory agents such as Hoechst 3325 enables the detection of thecondensation and marginalization of chromatin in thenucleus by measuring the level of fluorescence, which is reduced during apoptosis. On the other hand, the bisbenzimidazole dye, Hoechst 33342, penetrates the plasma membrane and stains DNA in cells; without permeabilization. In contrast to normal cells, the nuclei of apoptotic cells have highly condensed chromatin that is uniformly stained by Hoechst 33342 (ORMEROD 1993). This can take the form of crescents around the periphery of the nucleus, or the entire nucleus can appear to be one or a group of featureless, bright spherical beads. These morphological changes in the nuclei of apoptotic cells may be visualized by fluorescence microscopy.after 4hours treatment with 500 ,stained samples showed nucleus condonsed and shrinkage which in times 8 , 12 after treatment condensed increased. Also marginal nucleus was significantly observed. Gladish et al in Pea (Pisum sativum) primary roots under wet or flooded conditions find condensed nuclei and abnormal cytoplasms (Glladish, JipingI et al. 2006). This is also observed in root meristem cells of rice under treatment PEG and NaCl (Liu, Fu et al. 2007). The nuclear degration was observed by katsuhara and Kawasaki when roots were exposed to more than 300 mM NaCl for 24h (Katsuhara and Kawasaki 1996) .katsuhara 1997 investigate the salt stress-induced cell death in barley roots (Katsuhara 1997).changes in nuclei of meristematic root cell of soybean in response to sever salinity.

Extensive DNA degradation is a characteristic event which occurs in the late stages of apoptosis (Williams and Dickman 2008). The DNA fragments can be cytochemically determined by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) of the 3'OH group (Palavan-Unsal 2005) .TUNEL reaction is more specific and sensitive method for apoptosis. We observed under salt stress root tip cells showing TUNEL-positive nuclei, Because TUNEL may not unequivocally discriminate between internucleosomal DNA fragmentation associated with apoptosis and random DNA cleavageassociated with necrosis, apoptotic DNA fragmentation have to be confirmed by other methods (Katsuhara and Kawasaki 1996; McCabe, Levine et al. 1997; Loo and Rillema 1998). Therefore, DNA laddering patterns were visualized on agarose gels. Wu et al (1996) show NaCl induces nuclear DNA fragmentation in primary root cells of wild-type (gl1) and mutant (sos1) seedlings based on TUNEL cytochemical analysis of cells in the root meristematic and elongation/differentiation zones. Based on the above analysis, it is obvious that the death of the meristematic cells of wheat root tips under salt stress is apoptosis.

We suggested occurred apoptosis maybe provide an adaptive advantage to salt stress It depend on time and concentration NaCl. It maybe helps to survive plant.

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