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SPONTANEOUS AND INDUCED APOPTOSIS IN SALT STRESSED EMBRYOGENIC SUSPENSION CULTURES OF *DACTYLIS GLOMERATA* L.

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ABSTRACT. In multicellular organisms cell death accompanies many biological processes involving cell proliferation and development. There is clear evidence that cell death during plant development and interactions with the environment involves PCD. Apoptosis in plants and plant cells in culture has received much less attention than its animal counterpart. In present study we investigate DNA fragmentation, which is the hallmark of apoptosis, at different physiological and stress-related conditions in embryogenic suspension cultures of *Dactylis glomerata L.* We used heat shock (42^{o}C for 6 h) and NaCl in different concentrations (5, 10 or 15 g.L^{-1}). Here we demonstrate that suspension cultures from *D.glomerata L.* show spontaneous apoptosis without heat and NaCl treatment, but 5 g.L^{-1} NaCl block the spontaneous apoptosis. We find induced apoptosis when treating with NaCl in a concentration 10 g.L^{-1} without heat stress as well as in heat shocked cultures treated with 5 or 10 g.L^{-1} NaCl.

KEY WORDS. spontaneous and induced apoptosis, Dactylis glomerata, salt stress, suspension cultures

INTRODUCTION

In multicellular organisms cell death accompanies many biological processes involving cell proliferation and development. Programmed cell death (PCD) is an active process, genetically controlled where, following various signal pathways, cells condense and shrink, their DNA is cut at specific sites, but the membranes maintain their continuity (Lodish et al. 2004). In plants, selective cell death is necessary for growth and survival and can occur on a local or large scale (Pennell et al., 1997). There is clear evidence that cell death during plant development and interactions with the environment involves PCD. Apoptosis was shown to occur in cell cultures under

oxidative stress and cell cultures after abiotic stress. High salt concentration causes two types of stress on the plant cell, an ionic stress and an osmotic stress. The ionic stress is mainly caused by high Na^+ and Cl^- concentrations, which leads to an influx of ions into the plant cell and an altered ion homeostasis. The osmotic stress is caused by lowering of the soil water potential leading to a reduced water uptake and eventually to cellular dehydratation. Increased concentration of charged elements in the cytosol may change the hydration sphere of macromolecules and thus affects their conformation or charge interactions. The most notable response observed in plants exposed to water deficit is the high accumulation of novel proteins. In addition, the osmotic stress leads to increased production of reactive oxygen species (ROS), which are involved in the induction of PCD (Sarafian et al., 1994).

The aim of the present study is to investigate DNA fragmentation, which is the hallmark of apoptosis, at different physiological and stress-related conditions in embryogenic suspension cultures of *Dactylis glomerata L.*

MATERIAL AND METHODS

Cell culture. For callus initiation *Dactylis glomerata* L. genotype Embryogen-P was used (Conger et al. 1991). *In vitro* suspension cultures were initiated from embryoids using one month callus according to Conger et al. 1989 on liquid SH30 medium containing 0 (SH30), 5 (SH3005), 10 (SH3010) or 15 (SH3015) $g.L^{-1}$ NaCl, respectively. All cultures were subcultured at 2-week intervals and maintained in a dark place at 25^{o}C on a rotary shaker (105 rpm).

DNA extraction and analysis. Cells and embryos from 2-week-old suspension cultures were ground in a mortar with liquid nitrogen. Isolation buffer (100 mMtris-HCl pH 8, 500 mM NaCl, 1% PVP 25000, 50 mM EDTA) was added to broken cells. After incubation with 10% SDS (1/5 v/v) at 65^{o}C for 10 min the proteins were precipitated with cold 5M CH_3COOK for 20 min at 0 ^{0}C. Aqueous phase was removed after centrifugation, mixed with cold isopropanol (2/3 volumes) to precipitate nucleic acids and centrifuged at 12000 g65 ^{0}C for 15 min. The pellet was washed with 76% ethanol, 10 mM ammonium acetate, air-dried and resuspended in TE (10 mM Tris-HCl pH 8, 1 mM EDTA). The DNA was resolved in 1.8% (w/v) agarose gel and stained with ethidium bromide.

RESULTS AND DISCUSSION

I. Embryogenic response

We examined the influence of NaCl on the induction of somatic embryogenesis of suspension cultures from *Dactylis glomerata* L. Our observations showed that the presence of 5 $g.L^{-1}$ had a positive effect on embryogenic response but the formation of somatic embryoids was inhibited when it was applied at concentration 10 $g.L^{-1}$. When NaCl was applied at concentration 15 $g.L^{-1}$ the somatic embryogenesis was completely arrested and organogenesis was induced.

II. Spontaneous apoptosis

Fig.1 shows the electrophoregrams from DNA isolated from suspension cultures SH30, SH3005; SH3010 and SH3015 without heat shock - lanes 1- 4.

The observed PCD in unstressed (SH30) control cultures (lane 1) is a normal stage of the process of somatic embryogenesis (during the degradation of polyembryogenic masses – PEMs and the elimination of embryo-suspensors). These cell suicide events ensure normal progression of somatic embryogenesis, i.e. transition from PEMs to somatic embryos and correct embryonic pattern formation, respectively (Filonova et al).

The presence of PCD in SH3010 (lane 3) could be explained with inhibitory effect of NaCl on embryogenic response. This salt concentration induces cell growth in an unorganized way. It is known that the embryoids are more resistant to apoptosis in comparison with unorganized non-embryogenic cells (LoSchiavo et al. 1999).

The lack of PCD in SH3005 suspensions (lane 2) is probably result at a later stage in development of somatic embryoids. Our preliminary studies showed that NaCl at 5 $g.L^{-1}$ accelerates the formation of somatic embryoids and shortens the period of embryogenic response (Odjakova et al, 1992).The lack of PCD in SH3015 (lane 4) could be a result of the blocked activity from the effectors in PCD signal cascade during organogenesis.

III. Induced apoptosis

Fig.1 – lanes 5-8 and fig.2 show electrophoregrams of DNA isolated from heat shocked suspension cultures SH30, SH3005, SH3010 and SH3015.

We demonstrate that some cultures may not respond to the inducing signals according their physiological state. If cells are set up for embryogenesis (SH30 - Fig.2 – lane 1, 5) they become insensitive to the heat shock apoptosis-inducing treatment. It is possible that heat stress does not induce PCD in somatic embryoids at an earlier stage of development (globular stage). However, when they reach late embryo stages they reacquire sensitivity (SH3005 - Fig.2 – lane 2, 6) (LoSchiavo et al. 1999).

The higher degree of PCD in SH3010 in comparison with SH3005 is due to the inhibitory effect of NaCl on embryogenic response (Fig.2 - lane 3, 7). The lack of PCD in SH3015 (lane 4, 8) could be a result of the blocked activity of the effectors in PCD signal cascade during organogenesis. Most distinct DNA - laddering in heat-treated cultures for 6 hours and following cultivation for 15 hours at room temperature is due to the fact that PCD is an active process in which it is needed enough time for the expression at the PCD pathway (Fig.1 - lanes 6, 7 and Fig.2 - lanes 6, 7) (LoSchiavo et al., 1999; Desikan et al.,1998).

CONCLUSIONS

1. Suspension cultures from *D.glomerata* show spontaneous apoptosis without heat and NaCl treatment.

2. 5 $g.L^{-1}$ NaCl block the spontaneous apoptosis in suspension cultures from *D. glomerata**3.*** Suspension cultures from *D. glomerata* show induced apoptosis when

they are treated with NaCl in a concentration 10 $g.L^{-1}$ without heat stress as well as heat shocked cultures treated with 5 or 10 $g.L^{-1}$ NaCl.

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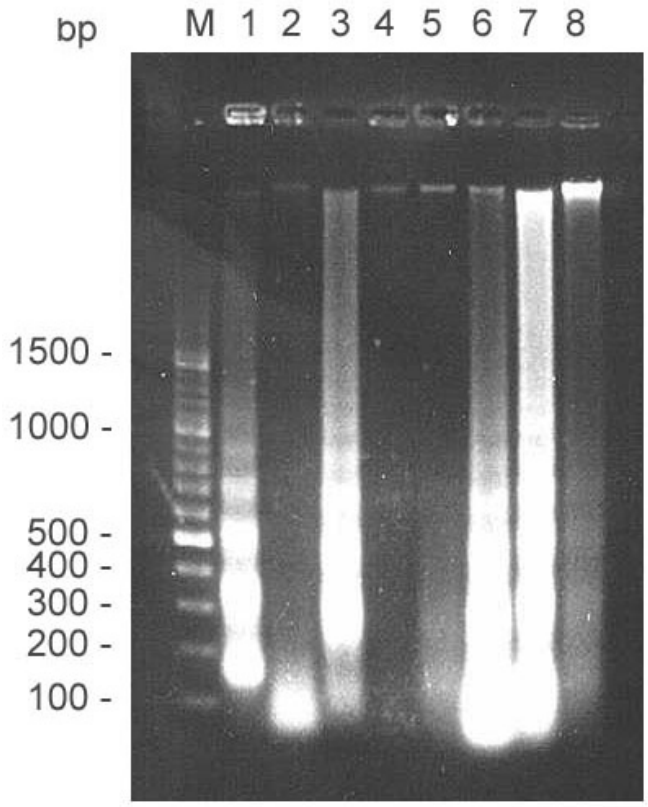

Fig. 1. DNA electrophoresis showing DNA - laddering. Lanes 1-4 refer to electrophoresis of DNA extracted from cells maintained at 25 ^{0}C; lanes 5-8 are treated at 42 ^{0}C for 6 h (+15 h at 25 ^{0}C, expression time).
SH30 – lanes 1, 5; SH3005 – lanes 2,6; SH3010 – lanes 3, 7; SH3015 – lanes 4, 8

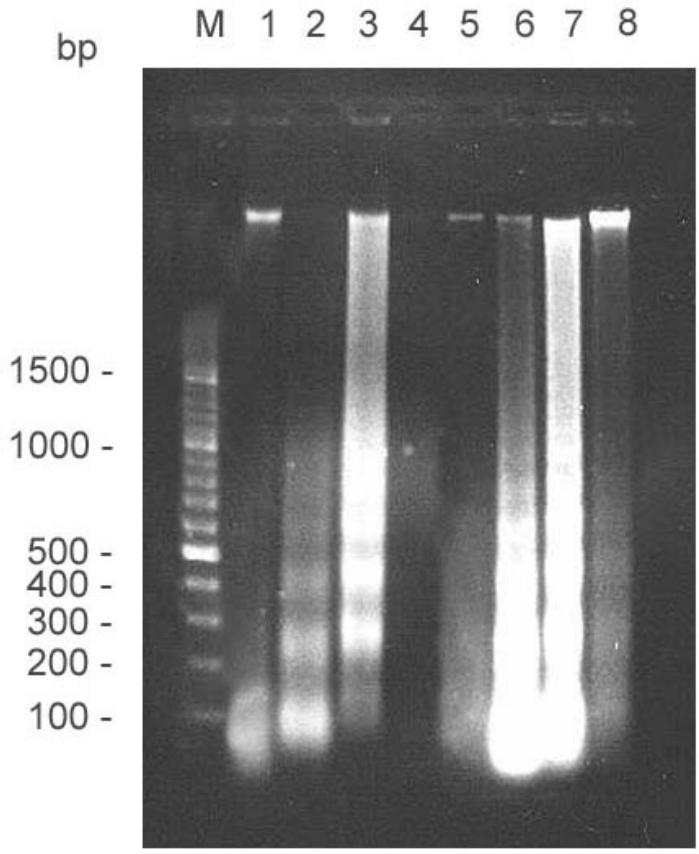

Fig. 2. DNA electrophoresis showing DNA degradation. Lanes 1-4 refer to electrophoresis of DNA extracted from cells treated at 42 ^{0}C for 6 h; lanes 5-8 are heat-treated (+15 h at 25 ^{0}C, expression time)
SH30 – lanes 1, 5; SH3005 – lanes 2,6; SH3010 – lanes 3, 7; SH3015 – lanes 4, 8