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Research Article

Hyperthermia-induced apoptosis and the inhibition of DNA laddering by zinc supplementation and withdrawal of calcium and magnesium in suspension culture of tobacco cells

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Abstract. In the present paper we report examination of stereotypic hallmarks of apoptosis in heat-treated tobacco cells. Hyperthermia (44 °C, 4 h) caused apoptosis in 53.6% of cells when assayed 24 h after heat treatment. The induction of apoptosis by heat treatment was confirmed by flow cytometric assay. Cytological observations revealed condensation of the cytoplasm and nucleus, as well as nuclear collapse. DNA ladders were observed in DNA extracted from heat-treated cells, whereas DNA from control cells remained undegraded. The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay revealed that 51.8% of the heat-treated cells (44 °C, 4 h) show positive reaction after a 24-h recovery. When cells were cultured in a medium supplemented with 0.4–5.0 mM $ZnSO_4$, internucleosomal DNA fragmentation induced by heat shock was completely negated. Strikingly, when cells were cultured in Ca^{2+} and/or Mg^{2+} free medium for 44 h followed by heat treatment, DNA laddering was not observed. The results suggest hyperthermia-induced apoptosis and a correlation between the regulation of endonucleases and heat shock signal in apoptotic tobacco cells.

Key words. Tobacco cell; heat shock; apoptosis; DNA laddering; TUNEL; zinc.

Apoptosis, a form of programmed cell death (PCD), is a genetically programmed process in which the cell directs its own destruction. Apoptosis was first described by Kerr et al. in 1972 and has been observed in various animal cell types [1]. More recently it was found that apoptosis also exists in plants [2–5].

Apoptosis plays a fundamental role in the normal course of development, maintenance of homeostasis, pathological process and response to environmental stress. In plants, apoptosis was found to occur in different processes such as suspensor degeneration, xylem differentiation, aerenchyma formation, sloughing of root cap cells and floral organ abortion in unisexual plants. There are also some reports suggesting that hypersensitive reactions correlate with apoptosis [6].

Apoptosis can be triggered by a variety of intrinsic and extrinsic signals, such as radiation, free radicals, heat shock and so on. Hyperthermia was found to induce apoptosis in many animal cell types [7, 8], but the mechanism underlying it is not well defined as yet [9, 10]. Heat shock response is highly conserved. It has been described in a wide range of organisms from

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Escherichia coli to humans. The response of cells to hyperthermia is well characterized, and it was found that different organisms share similar mechanisms in heat shock response [11]. In plants, only a few reports mention the effect of heat shock on cell killing and DNA cleavage [2, 4]. The knowledge of heat shock response accumulated in previous studies will aid in better understanding the mechanism of heat-induced apoptosis in plants. It is therefore worthwhile establishing an experimental model for revealing the apoptotic pathway in heat-treated plant cells. Furthermore, it has been reported that Ca^{2+}/Mg^{2+}-dependent nuclease plays an important role in apoptosis. The study of the regulation of this enzyme by metal ions will shed crucial light on our understanding of the apoptotic mechanism in plant cells. Here we reported heat shock-induced apoptosis in a suspension culture of tobacco cells. This is evidenced by the appearance of stereotypic morphological and biochemical hallmarks of apoptosis, including cytoplasmic and nuclear condensation, DNA degradation and nuclear collapse. In addition, we provide evidence that zinc supplementation and withdrawal of calcium and/or magnesium restrain DNA laddering induced by heat treatment.

Materials and methods

Cell culture. A tobacco (*Nicotiana tabacum*, cultivar BY-2) cell suspension culture was initiated from cotyledon explants. Cells were cultured in MS medium supplemented with 2,4-D (0.6 mg/ml) at 27 °C under rotation (120 rpm). Cells were subcultured every 5 days. Exponentially growing cells were used for experiments.

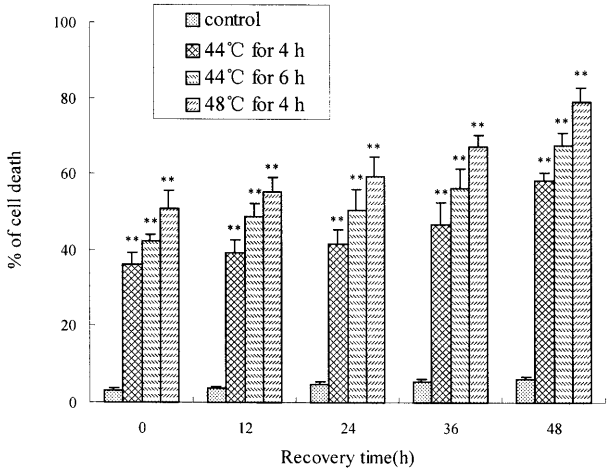

Figure 1. Percentage of cell death of heat-treated tobacco cells. Values are the means of three separate experiments. Error bars indicate standard deviations. The level of significance was (**) $P < 0.01$ (Fisher's protected least-significant-difference, PLS).

Heat treatment. For heat treatment, flasks containing cultured cells were immersed in water baths at 44 °C and 48 °C under rotation (120 rpm); each group had three replicates. After heat treatment cells were returned to a 27 °C incubator and cultured for the desired time period for recovery. Cells cultured at 27 °C without heat treatment were used as controls.

Test of cell death. Cells were transferred from flask to a slide and stained with 0.5% Trypan Blue. After 1 min staining, cells were observed under a microscope. Dead cells were stained blue, whereas living cells remained unstained. The percentage of cell death was obtained by calculating the ratio of dead cells to total cells.

Agarose gel electrophoresis of DNA. Tobacco DNA was extracted according to the method of Mittler and Lam [12]. Identical amounts of DNA were loaded on a 1.4% agarose gel and run at 5 volts (V)/cm for 3 h. DNA fragments were visualized using ethidium bromide staining.

In situ detection of DNA cleavage by the TUNEL procedure. Cells were brought to a slide and immobilized by polylysine. The TUNEL assay was performed according to the manufacturer's (Boehringer Mannheim) instructions. The slides were observed under a fluorescence microscope (Nikon UFX-II), and the cells showing a positive reaction were scored.

Preparing single particles for flow cytometry. Single particles were prepared in the form of nuclei. Two grams of cells (control cells or heat-treated cells after 24-h recovery) pelleted by centrifugation were suspended in 2 ml of nuclei isolation buffer (10 mM 2-[N-morpholino]ethanesulfonic acid (MES), 0.2 M sucrose, 2.5 mM EDTA, 2.5 mM DTT, 10 mM NaCl, 10 mM KCl, 0.02% Triton X-100, pH 7) and ground gently at 4 °C for 15 min. The homogenate was then filtered through a 25-μm nylon mesh to remove the cell debris. The filtrate fractions were pooled and centrifuged at 115*g* to pellet the nuclei. The prepared nuclei were washed once with nuclei isolation buffer (without Triton X-100).

Flow cytometry measurements were carried out according to the methods described by Yanpaisan [13]. Nuclei (10^5) were treated with 1000 units of RNase at 37 °C for 30 min and stained with 20 μg/ml of propidium iodide. The samples were tested using a Coulter Elite Flow Cytometer. Data were treated using the MULTICYCLE software program.

Percentage of apoptosis. Cells were stained with 5 μg/ml 4′,6′-diamidino-2-phenyl-indole (DAPI) and observed under a fluorescence microscope (Nikon UFX-II). The ratio of cells showing nuclear condensation and collapse to total cells was calculated as the percentage of cells undergoing apoptosis.

Statistical analysis. Data were analyzed using a one-way analysis of variance (ANOVA). The protected least-significant-difference test was performed using Fisher's test to analyze differences. Statistical probability was considered significant with $P < 0.05$ or $P < 0.01$.

Results

Effect of heat shock on cell death. Tobacco cells were heated at 44 °C or 48 °C, then further incubated at 27 °C for recovery. Cell death was determined by Trypan Blue staining at different recovery time points (fig. 1). When cell death was determined 24 h after heat treatment, the percentage of cell death was 41.7 in cells heated at 44 °C for 4 h, whereas that in cells heated at 48 °C for 4 h was 59.5. After a 48-h recovery incubation, the percentage of cell death reached 58.2 in cells treated at 44 °C for 4 h; that in cells heated at 48 °C for 4 h was 79.1. In normal control cells, however, the percentage of cell death was very low, as shown in figure 1. Statistical analysis showed that the difference between the percentage of cell death in heat-treated cells and that in control cells was significant ($P < 0.01$). Our data suggest that heat treatment at 44 °C for 4 h provided a modest stress for inducing apoptosis in tobacco cells, and this treatment was used for most of the experiments in this study.

Detection of DNA laddering in heat-treated tobacco cells. DNA laddering is commonly accepted as an important biochemical hallmark of apoptosis. Thus it is critical to test whether heat treatment induces DNA laddering in tobacco cells. As illustrated in figure 2, agarose gel electrophoresis analysis of DNA extracted from heated cells showed distinct ladders of DNA fragments increasing in size by multiples of 180 bp, whereas no DNA fragmentation is shown in DNA from the control cells. The results suggest activation of endonuclease by mild heat treatment.

In situ detection of DNA cleavage by the TUNEL procedure in heat-treated tobacco cells. The TUNEL procedure now is widely used in studies of apoptosis. This method enables in situ detection of nuclear DNA strand breaks by visualizing DNA 3′-OH nicks. As illustrated in figure 3, lane 2, 51.8% of the hyperthermia-treated cells (44 °C, 4 h; 24-h recovery) were TUNEL-positive, showing a bright yellow-green fluorescence in the nuclei (value is the mean of three separate experiments), whereas in control cells only very weak autofluorescence in the cell wall area was observed following the TUNEL procedure (fig. 3, lane 1).

The revelation of accumulated DNA 3′-OH nicks using the TUNEL procedure combined with the display of DNA laddering in cells treated with an appropriate level of hyperthermia provided convincing evidence that heat shock triggers apoptosis involving DNA fragmentation in cultured tobacco cells.

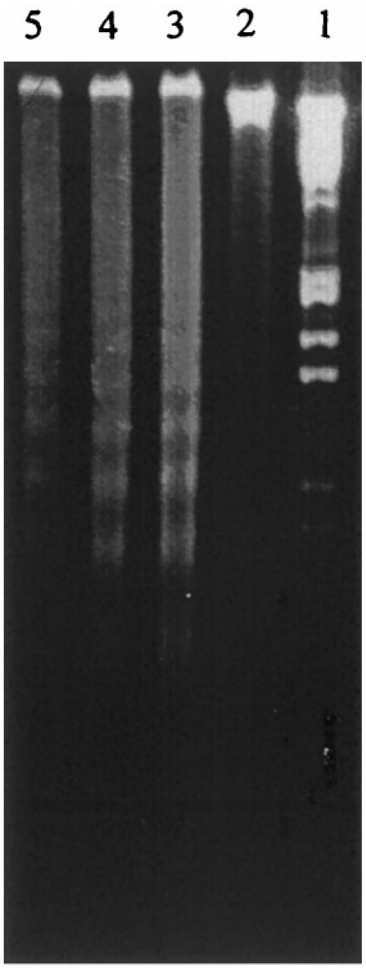

Figure 2. Detection of DNA fragmentation in heat-treated tobacco cells. DNA was extracted after 12-h recovery: Lane 1, DNA molecular markers (λ/Hind/III + Ecol I); lane 2, control cells; lane 3, cells heated at 44 °C; for 4 h; lane 4, cells heated at 44 °C for 6 h; lane 5, cells heated at 48 °C for 4 h.

Morphological changes during heat-induced cell death and the percentage of apoptosis. The general appearance of heat-treated cells was observed using a light microscope. It is striking that in cultured tobacco cells heat shock always causes plasmolysis (fig. 4), which mirrors condensation and shrinkage of the cytoplasm, an essential character of programmed cell death in both animals and plants. DAPI staining revealed nuclear changes during apoptosis induced by heat shock, including nuclear condensation (fig. 4b and c) and collapse (fig. 4d). Since the culture of tobacco cells used in this study was not synchronized, it is not surprising that response of these cells to heat treatment was different. In our experiments, a series of phases of nuclear changes emerged at the same time from intact nuclei to the appearance of

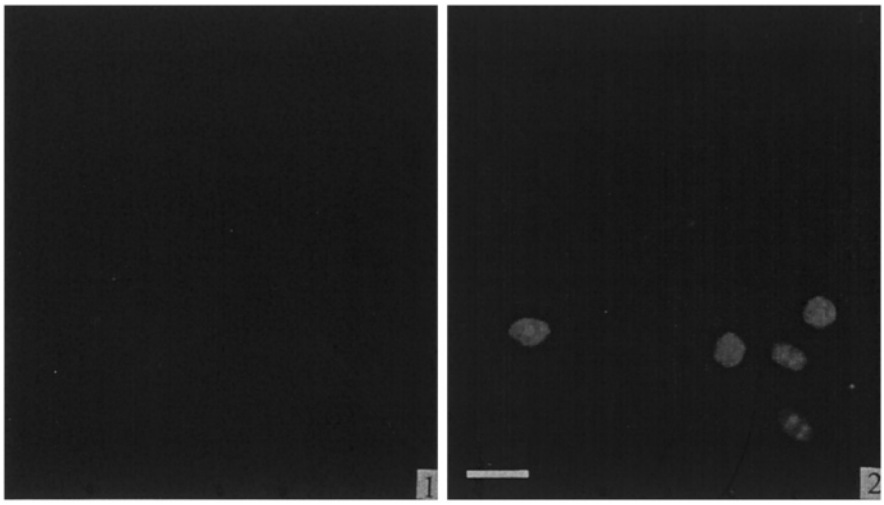

Figure 3. In situ detection of DNA cleavage in heat-treated tobacco cells. Lane 1, control cells; lane 2, cells heated at 44 °C for 4 h and recovered for 24 h. Bar, 40 μm.

distinct nuclear bodies, which invites a comparison with the apoptotic bodies in animal cells undergoing apoptosis.

The percentage of apoptotic cells was determined by DAPI staining at different time points of recovery (fig. 5). The cells showing nuclear condensation and collapse were counted as apoptotic cells. After a 24-h recovery, the percentage of apoptotic cells was 53.6 in cells heated at 44 °C for 4 h, whereas that in cells heated at 48 °C for 4 h was 44.5. In control cells, on the other hand, the percentage of apoptosis was very low, which can be seen from figure 5. Statistical analysis showed that the difference between the percentage of apoptosis in heat-treated cells and that in control cells was significant ($P < 0.01$).

Quantitative analysis of heat-induced apoptosis by flow cytometry. Heat-induced apoptotic cells and normal cells were quantitatively analyzed using flow cytometry. When the DNA content of the nucleus is less then that at G0/G1 phase, the cell is considered to be apoptotic. As shown in table 1, cells cultured under normal conditions did not undergo apoptosis, whereas the percentage of apoptosis in cells heated at 48 °C for 4 h was 54.4, and that in cells heated at 44 °C for 4 and 6 h was 66.5 and 62.3, respectively. Statistical analysis showed that the difference between the percentage of apoptosis in heat-treated cells and that in control cells was significant ($P < 0.01$). The results further supported the conclusion drawn from the detection of a series of stereotypic hallmarks of apoptosis, including DNA laddering, DAPI staining and TUNEL assay, that heat shock can induce apoptosis in suspension culture of tobacco cells.

Effect of zinc supplementation on DNA fragmentation in heat-treated tobacco cells. Zinc is among the important regulators of apoptosis. It was found that zinc could prevent apoptosis in peripheral blood lymphocytes and bovine aortic endothelial cells [14, 15]. It is worth testing whether Zn^{2+} demonstrates antiapoptotic effects in plant cells treated with heat shock. Thus, tobacco cells were cultured in MS medium supplemented with 0.1, 0.4, 2.0 and 5.0 mM $ZnSO_4$ and treated with heat (44 °C, 4 h), then recovered at 27 °C for 24 h. DNA was extracted from cells cultured in regular and Zn^{2+}-supplemented medium and analyzed by agarose gel electrophoresis. As illustrated in figure 6, Zn^{2+} supplementation at concentrations of 0.4, 2 and 5 mM showed distinct preventive effects on DNA laddering, whereas 0.1 mM $ZnSO_4$ has a weaker inhibiting effect.

Effect of withdrawal of calcium and magnesium on DNA laddering in heat-treated tobacco cells. Ca^{2+}- and Mg^{2+}-dependent endonuclease was found to be responsible for DNA fragmentation at the linker sites between nucleosomes during apoptosis of thymocytes [16]. To verify whether it also plays a role in DNA fragmentation during apoptosis in plants, tobacco cells were transferred to a medium in which Ca^{2+} and/or Mg^{2+} were withdrawn and cultured for 44 h at 27 °C. Cells were then treated with heat shock (44 °C, 4 h) and recovered for 24 h at 27 °C. DNA extracted from these cells was analyzed by agarose gel electrophoresis. It was found that withdrawal of calcium and/or magnesium prevented DNA laddering induced by heat shock. The results implied that Ca^{2+}- and Mg^{2+}-dependent endonuclease plays a fundamental role in apoptosis of plant cells.

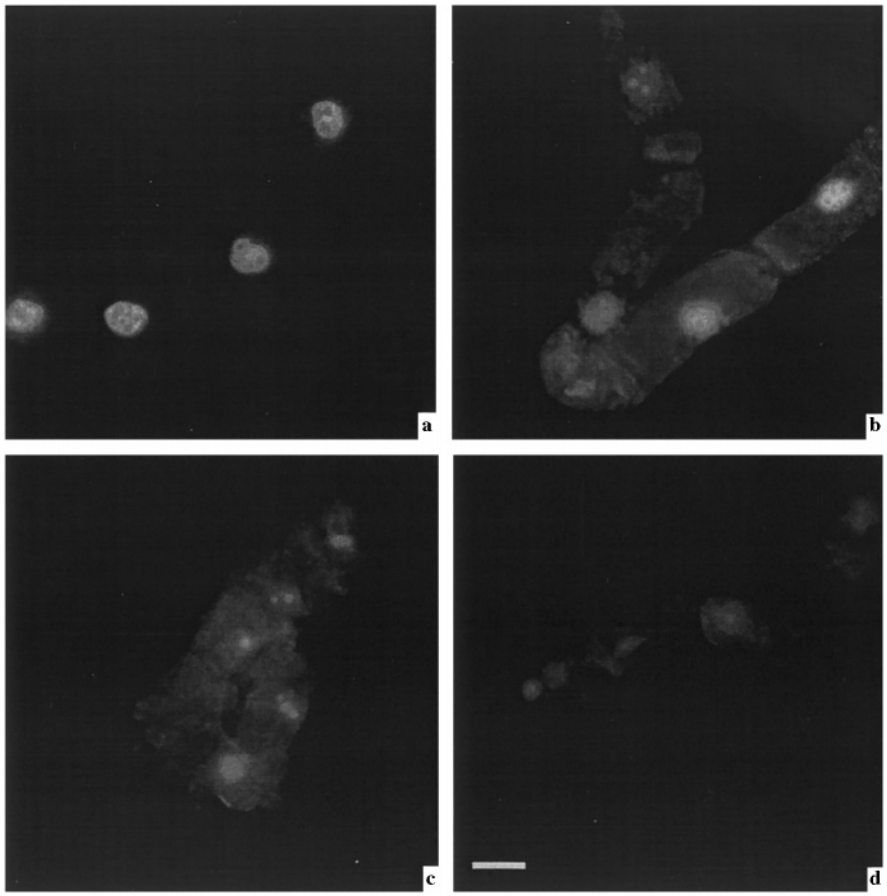

Figure 4. Morphological changes of cells after heat shock. (*a*) normal cells; (*b*) nuclear condensation; (*c*) serious nuclear condensation; (*d*) nuclear collapse. Plasmolysis can be seen in *a*, *b* and *c*. Bar, 40 μm.

The preventive effect of both zinc supplementation and withdrawal of calcium and/or magnesium on DNA fragmentation indicated that Ca^{2+}, Mg^{2+} and Zn^{2+} are important regulators of programmed cell death in plants.

Discussion

Heat shock was reported to be an extrinsic signal that triggers apoptosis in many cell types of animals. Recently heat shock (42 °C, 4 h) was found to trigger 55% cell death and to induce DNA laddering in tomato protoplasts [4]. In cultured carrot cells, it was found that heat treatment (55 °C, 10 min) caused 90% cell death, which showed cell condensation and shrinkage. In these heat-treated carrot cells DNA cleavage was revealed by the TUNEL method, but evidence for DNA laddering was faint [2]. In this study, strong evidence for the heat shock induced apoptosis in cultured tobacco cells was provided. A modest heat treatment (44 °C, 4 h) resulted in condensation of cytoplasm, DNA fragmentation and nuclear disintegration and collapse. All these cellular changes are stereotypic hallmarks of apoptosis in both animal and plant cells which are commonly used for diagnosing apoptosis. It was found that mild hyperthermia is able to induce apoptosis, and that this may be prevented by synthesis of heat-induced proteins in animal system [17]. In our study we found that mild heat shock can induce apoptosis in plant cells. This, we believe, may exist virtually under natural conditions, especially for plants in subtropical and tropical areas. It is promising that apoptosis induced by hyperthermia in plant cells may also be prevented by overexpression of heat-induced proteins. This will be of importance in the development of thermotolerance in crop plants. The heat-induced apoptosis system established in this study may be used for this purpose as well as for studying the molecular mechanisms of apoptosis in plants.

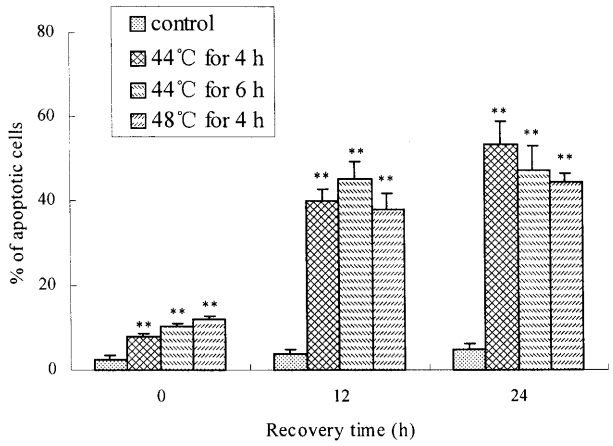

Figure 5. Percentage of apoptosis in heat-treated tobacco cells. Values are the means of three separate experiments. Error bars indicate standard deviations. The level of significance was (**) $P < 0.01$ (Fisher's PLS).

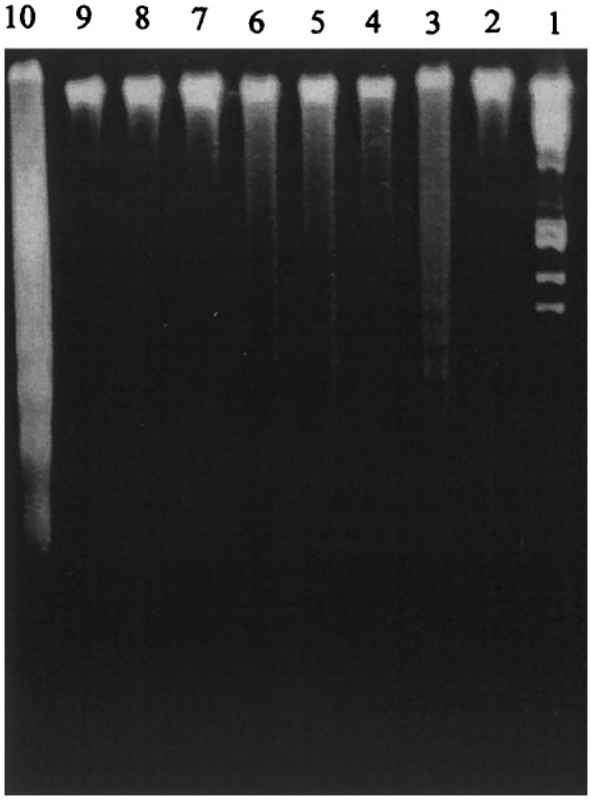

Figure 6. Inhibition of DNA laddering by zinc supplementation and withdrawal of calcium and/or magnesium in heat-treated tobacco cells. Lane 1, DNA molecular markers (λ/Hind III + Ecol I); lane 2, DNA from cells cultured in normal MS medium at 27 °C; lanes 3–6, DNA from cells exposed to hyperthermia (44 °C, 4 h) 1 h after being transferred to MS medium supplemented with 0.1, 0.4, 2 and 5 mM $ZnSO_4$, respectively; lanes 7–9, DNA from cells cultured in Ca^{2+}- and/or Mg^{2+}-free MS medium for 44 h then exposed to hyperthermia (44 °C, 4 h): lane 7, Ca^{2+}-free medium; lane 8, Mg^{2+}-free medium; lane 9, Ca^{2+}- and Mg-free medium; lane 10, DNA from cells cultured in MS medium and exposed to heat shock (44 °C, 4 h). DNA was extracted from heat-treated cells after a 24-h recovery.

At the present time the mechanism by which heat shock induces apoptosis is not well determined. Two lines of thinking are proposed to elucidate the pathway through which heat shock triggers apoptotic death in mammalian cells. Many reports provide evidence that heat shock-induced accumulation of nascent unfolded polypeptides and denatured proteins result in cell death [18]. On the other hand, heat shock proteins (HSPs) induced by heat stress and some chemical agents were found to act as apoptosis inhibitors in many systems. Mosser et al. (1997) found that heat-induced apoptosis and poly (ADP-ribose) polymerase (PARP) cleavage were inhibited in HSP-70 expression cell clones (the parental cell line was the human acute lymphoblastic leukemia T-cell line PEER) [19]. Moreover, HSP-70 was found to inhibit caspase-3 processing. In cells where HSP expression was not constitutionally elevated, the relevance of HSP function in apoptosis has not been established [20, 21]. Controversial reports regarding the concomitant expression of HSP and development of apoptosis even made things more complicated. Elucidation of the mechanism by which hyperthermia triggers apoptosis awaits further study.

Table 1. Quantitative analysis of heat-induced apoptosis in tobacco cells by flow cytometric assay.

Treated group	Recovery time (h)	Apoptosis (%)	G0/G1 (%)	S (%)	G2/M (%)
Control		3.1 ± 0.4	70.3 ± 4.7	9.1 ± 0.7	17.5 ± 3.8
44 °C for 4 h	24	66.5 ± 5.3**	19.3 ± 4.1	11.4 ± 1.9	2.8 ± 0.2
44 °C for 6 h	24	62.3 ± 3.2**	16.8 ± 2.6	15.3 ± 1.5	5.6 ± 0.9
48 °C for 4 h	24	54.4 ± 7.5**	20.9 ± 6.7	14.5 ± 3.6	10.2 ± 1.3

Values are the means of three separate experiments $\pm$ SD (10,000 nuclei were counted for each sample). The level of significance in apoptosis (%) was ** $P < 0.01$ (Fisher's PLS).

Pandey et al. (1997) found that DNA fragmentation in nuclei isolated from 5123tc rat hepatoma cells required the presence of calcium and magnesium ions. The existence of a novel calcium/magnesium-dependent 97-kDa endonuclease was responsible for DNA cleavage, and this enzyme was also inhibited by millimolar concentrations of zinc [22]. Moreover, Zn^{2+} supplementation was found to prevent apoptosis in other animal cells [15, 16]. This effect has been attributed to inhibition of a Ca^{2+}- and Mg^{2+}-dependent endonuclease [23]. The inhibition of heat shock-induced DNA laddering by zinc supplementation and withdrawal of Ca^{2+} and/or Mg^{2+} found in our study is probably based on the same mechanism. In addition, we studied the effect of phenylmethysulfonyl fluoride (PMSF), a cysteine and serine protease inhibitor, on heat shock-induced cell death and DNA laddering. Neither inhibition nor stimulation by PMSF was found in terms of heat-induced DNA laddering (data not shown). The result is consistent with the findings of McCabe et al. in carrot cells. These results led to a postulation that in tobacco cells the terminal step of the apoptotic pathway, that is DNA fragmentation, is one of the sites with which heat shock signals connect possibly via activation of endonuclease. Recently zinc was found to be a potent inhibitor of caspase-3 in Molt 4 leukemia cells [24]. Since in our study zinc supplementation was found to prevent apoptosis induced by heat shock in tobacco cells, it is worthwhile studying the zinc effect on PARP-cleaving proteases in heat shock-induced apoptotic plant cells.

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