# Cleavage of lamin-like proteins in in vivo and in vitro apoptosis of tobacco protoplasts induced by heat shock

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**Abstract Apoptosis in heat shock-treated tobacco protoplasts was evidenced by DNA fragmentation, flow cytometric analysis and activation of caspase 3-like protease. Furthermore, an in vitro apoptosis system was established which reproduced the apoptotic events. Western blotting analysis using an antibody against lamin A and C showed that in both in vivo and in vitro systems lamin-like proteins were cleaved into a 35-kDa fragment, and that lamin-like protein degradation precedes DNA fragmentation. Moreover, we found a 22.8-fold increase in caspase 6-like activity in cytosol of heat-treated protoplasts as compared with the control.** 

*Key words:* Lamin-like protein; Heat shock; Tobacco protoplast; Apoptosis; Cell-free system

## 1. Introduction

Apoptosis, as a genetically programmed process in which a cell directs its own destruction, was first described by Kerr et al. in 1972 [1]. It plays a fundamental role in the normal course of development, maintenance of homeostasis, and in response to pathogens. The main signal pathway of apoptosis has been illustrated in animal cells. Recently it has been confirmed that apoptosis also exists in plant cells, and that the process shares many common morphological and biochemical characteristics with that in animal cells [2,3]. This implies that apoptosis in plant cells may be based on similar molecular mechanisms. However, there are only few reports regarding the mechanism of apoptosis in plant cell.

In animal cells the proteolytic cleavage of lamins leading to the collapse of the nucleus is an important event in the apoptotic program. In plant cells there are also lamin-like proteins occurring in nuclei [4,5]. However, little is known about the fate of these proteins during apoptosis of plant cells. Recently we reported that lamin C-like protein was degraded during apoptosis in menadione-treated tobacco cells [6]. Yet more detailed evidence is needed to verify if the cleavage of lamin-like proteins is a common step of the apoptotic pathway. In this study we chose heat shock (HS) as an efficient inducer of apoptosis and established both intact protoplast and a cell-free system to study the apoptotic changes of lamin-like proteins. Our results provide evidence that the cleavage of lamin-like proteins is a constitutive apoptotic event occurring in the nuclei of plant cells, and the cleavage of lamin-like proteins was probably via the proteolytic activity of caspase 6-like protease.

## 2. Materials and methods

### 2.1. Plant material and protoplast preparation

BY-2 tobacco (*Nicotiana tabacum*) cell suspension was cultured in MS medium supplemented with 2,4-D (0.6 mg/ml) on a 4-day subculture cycle at 26°C. Protoplasts were isolated according to the method described previously [7]. Protoplasts were stationary cultured at 25°C.

### 2.2. HS treatment

For heat treatment, tubes containing protoplasts were immersed in water baths at 44 or 48°C for a certain time period; each group had three replicates. After heat treatment protoplasts were returned to a 25°C incubator and cultured for the desired time period for recovery. Protoplasts cultured at 25°C without heat treatment were used as controls.

### 2.3. Agarose gel electrophoresis of DNA

DNA extraction was conducted following the method of Ryerson and Heath [8]. DNA was loaded on a 1.4% agarose gel and run at 5 V/cm for 3 h. DNA fragments were visualized using ethidium bromide staining.

### 2.4. Fluorescence microscopy

Protoplasts and nuclei were stained with 5 μg/ml 4,6-diamidino-2-phenylindole diacetate (DAPI). Nuclear morphological changes were observed using a fluorescence microscope (Nikon Fluopho). The nuclei showing chromatin condensation and nuclear collapse were calculated as apoptotic protoplasts and nuclei.

### 2.5. Flow cytometric analysis

Nucleus preparation and flow cytometry measurements were carried out according to the methods described by Yanpaisan et al. [9]. $10^5$ nuclei were treated with 1000 units of RNase at 37°C for 30 min and stained with 20 μg/ml propidium iodide. The samples were tested using a Coulter Elite Flow Cytometer. Data were treated using the Coulter Elite workstation 4.0 software (Coulter Corp.).

### 2.6. In vitro apoptotic system

Tobacco protoplasts (normal and heat-treated) were homogenized in lysis buffer (40 mM HEPES–KOH, pH 7.5, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.1 M sucrose), then ground tenderly for 10 min. The homogenate was centrifuged at $100\,000 \times g$ for 30 min. The resulting supernatant was collected as the cytosolic fraction and used for the in vitro apoptotic system. All steps of cytosol extraction were carried out on ice.

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*Abbreviations:* DAPI, 4,6-diamidino-2-phenylindole; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; HS, heat shock; PCD, programmed cell death

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Nuclei ($2.5\times10^7$) isolated from normal protoplasts were incubated with the cytosol ($9.0\times10^7$ cell equivalents) prepared from the normal protoplasts and heat-treated protoplasts at 28°C for 4 h. Then the hallmarks of apoptosis were examined.

*2.7. In situ detection of DNA fragmentation by the TUNEL procedure*

Tobacco protoplasts were immobilized by polylysine on the slides. The TUNEL assay (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) was carried out following the manufacturer's description (Boehringer Mannheim).

*2.8. Extraction of lamin-like proteins*

Extraction of lamin-like proteins was carried out according to the methods of McNutly and Saunders [4].

*2.9. Western blotting*

Western blotting analysis was carried out according to the methods of McNutly and Saunders [4].

*2.10. Detection of the activity of caspase-like proteases*

Cytosol prepared from normal and heat-treated protoplasts was used for the caspase-like protease activity assay. The assay was conducted following the manufacturer's description (Calbiochem, USA).

*2.11. Statistical analysis*

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

## 3. Results

*3.1. Induction of apoptosis by HS in tobacco protoplasts*

Based on our previous study of apoptosis induction in tobacco suspension cells by HS [10], a milder heat treatment was applied to trigger programmed cell death (PCD) in tobacco protoplasts. When heated at 44°C for 2 h and incubated at 25°C for 24 h for recovery, about 45% apoptotic protoplasts were observed whereas in control protoplasts the apoptotic death was very low (Fig. 1). The apoptotic death of protoplasts was evidenced by the detection of stereotypic hallmarks of apoptosis including DNA laddering (Fig. 2) and by flow cytometric analysis (Table 1). Strikingly, the percentages of apoptosis measured by DAPI staining and flow cytometry were very close to each other (Fig. 1 and Table 1) and were parallel to the extent of apoptotic DNA fragmentation as well (Fig. 2).

Furthermore, the activation of caspase 3-like protease was also demonstrated under the inductive conditions using a synthetic tetrapeptide Ac-DEVD-pNA, a specific substrate of caspase 3 and of caspase 7 to a certain extent. The activity of caspase 3-like protease in HS-treated protoplasts was $216.9\pm13.8$ pmol/min, while that in controls was only $12.3\pm1.4$ pmol/min, showing a very significant activation or induction of this protease.

Since the different HS treatments triggered PCD to similar degrees, the treatment at 44°C for 2 h was used for the experiments in the later study of the in vitro apoptosis and the cleavage of lamin-like proteins.

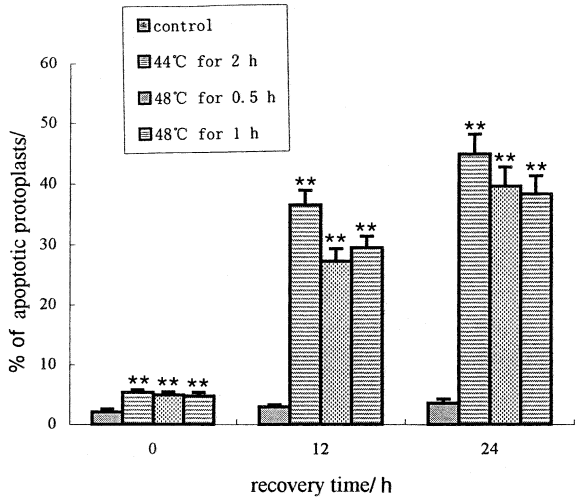


Fig. 1. Percentage of apoptosis in heat-treated tobacco protoplasts. At different recovery times protoplasts were stained with 5 μg/ml DAPI. Protoplasts that contained nuclei showing chromatin condensation and nuclear collapse were counted as apoptotic protoplasts. Values are the means of three separate experiments. Error bars indicate standard deviations. The level of significance was $^{**}P<0.01$ (Fisher's PLS).

*3.2. Apoptosis in cell-free system*

There is little information regarding the utilization of cell-free systems in apoptosis studies of plant cells. To study the cleavage of lamin-like proteins which are located in nuclei, we established a cell-free system which consists of the isolated nuclei ($2.5\times10^7$) from healthy tobacco cells and the cytosolic fraction ($9.0\times10^7$ cell equivalents) prepared from the apoptotic protoplasts induced by HS treatment (44°C for 2 h with 24 h recovery). After 2 or 4 h incubation at 28°C, the percentages of apoptotic nuclei in the cell-free system were measured by DAPI staining and flow cytometry. Interestingly, the results were almost the same as for in vivo apoptosis (Table 2).

In addition, DNA extracted from nuclei incubated with apoptotic cytosol showed distinct laddering characteristic of all apoptotic cells, whereas that from the nuclei incubated with non-apoptotic cytosol showed no fragmentation as shown in Fig. 3. Moreover, the TUNEL assay showed a bright yellow-green fluorescence in the nuclei incubated with the apoptotic cytosol extracted from heat-treated protoplasts (44°C, 2 h; 24 h recovery), while nothing can be seen from the nuclei incubated with the normal cytosol (not shown). The results thus suggest that the cell-free apoptotic system established in this study reproduced the characteristics of an apoptotic program.

*3.3. Cleavage of lamin-like proteins during in vivo and in vitro apoptosis in tobacco protoplasts induced by HS*

In animal cells lamin-like proteins cleaved during apoptosis leading to the collapse of the nucleus. The cleavage of lamin-like proteins is one of the most important links in the apo-

Table 1
Apoptotic percentages of protoplast nuclei detected by cytometric analysis

| Treated group | Recovery time (h) | Apoptosis (%) | G0/G1 (%) | S (%) | G2/M (%) |
|---|---|---|---|---|---|
| Control | | $4.2\pm0.3$ | $70.3\pm6.2$ | $8.1\pm0.5$ | $17.4\pm1.2$ |
| 44°C for 2 h | 24 | $49.2\pm3.2$** | $26.8\pm1.8$ | $14.9\pm1.3$ | $9.1\pm0.4$ |
| 48°C for 0.5 h | 24 | $44.6\pm3.0$** | $22.5\pm2.3$ | $21.7\pm1.7$ | $11.2\pm0.5$ |
| 48°C for 1 h | 24 | $42.7\pm4.1$** | $21.4\pm1.8$ | $25.6\pm2.0$ | $10.3\pm1.1$ |

Values are the means of three separate experiments ± S.D. The level of significance was $^{**}P<0.01$ (Fisher's PLS).


Fig. 2. Detection of DNA fragmentation in heat-treated tobacco protoplasts. The recovery time was 16 h. Lane 1, DNA molecular markers (λ/*Hin*dIII+*Eco*RI); lane 2, protoplasts heated at 44°C for 2 h; lanes 3 and 4, protoplasts heated at 48°C for 0.5 h and 1 h respectively; lane 5, control protoplasts.

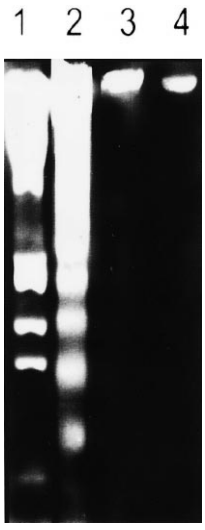


Fig. 3. DNA fragmentation in cell-free system. The nuclei were incubated with cytosol at 28°C for 4 h. Cytosol$^0$ and cytosol* represent cytosol prepared from control and heat-treated (44°C for 2 h) protoplasts respectively. Lane 1, DNA molecular markers (λ/*Hin*dIII+*Eco*RI); lane 2, cytosol*+nuclei; lane 3, cytosol*+nuclei+5 mM iodoacetamide; lane 4, DNA from cytosol$^0$+nuclei.

ptotic pathway and precedes DNA fragmentation. In this study, lamin-like proteins were cleaved into 35-kDa fragments in the in vivo and in vitro heat-treated apoptotic system of tobacco protoplasts, as illustrated in Fig. 4A. The degradation of the 65-kDa lamin-like protein in apoptotic protoplasts took place under all three HS conditions (44°C for 2 h, 48°C for 0.5 and 1 h). Moreover, the 65-kDa lamin-like protein extracted from nuclei that were incubated for 4 h with cytosol prepared from HS protoplasts was also cleaved leading to the formation of a 35-kDa fragment. The cleavage of the 65-kDa lamin-like protein appeared as early as 8 h after heat treatment, while at the same time point no sign of DNA fragmentation was seen. DNA fragmentation became apparent at 16 h after heat treatment (Figs. 4B and 5). The results are similar to previous results obtained with thymocyte [11]. These data convincingly indicated that cleavage of the 65-kDa lamin-like protein is an indispensable link in the apoptotic pathway in tobacco cells.

### *3.4. Caspase 6-like activity in HS-treated tobacco protoplasts*

In animal cells, caspase 6 catalyzes the cleavage of lamin A. A synthetic tetrapeptide with the sequence Ac-VEID-pNA based on the caspase 6 cleavage site in lamin A is usually used as a substrate of caspase 6. The $K_{cat}/K_m$ for this peptide substrate is 168 000, indicating a high specificity. Using Ac-VEID-pNA as a substrate to conduct a similar assay in the tobacco protoplast system, we found that the activity of caspase 6-like protease in the cytosolic fraction of HS-induced apoptotic protoplasts was 209.6 pmol/min, while that in normal cytosol was only 9.2 pmol/min. It shows a 22.8-fold increase. The results thus suggested an activation or induction of caspase 6-like protease during apoptosis induced by HS treatment. Moreover when 100 nM Ac-VEID-CHO, a specific inhibitor of caspase 6, was added to the assay system, it completely abolished the augmentation of caspase 6-like protease activity shown in apoptotic cytosol.

Our study thus demonstrated that cytosol prepared from protoplasts committed to apoptosis contains a caspase 6-like protease activity that resembles the caspase 6 in animal cells. The occurrence of the protease in tobacco cells accounts for the cleavage of lamin-like proteins observed in our study. And in plant cells this caspase 6-like protease may also lie in the middle of an apoptotic cascade serving as an apoptosis executor similar to its counterpart in animal cells, the well characterized caspase 6.

Table 2
The percentages of apoptotic nuclei in cell-free system of tobacco protoplasts as measured by DAPI staining and flow cytometry

| Treatment | DAPI staining (%) | Flow cytometry (%) |
|---|---|---|
| Cytosol$^0$+nuclei | 4.1 ± 1.0 | 6.1 ± 0.3 |
| Cytosol*+nuclei | 37.4 ± 4.0** | 42.3 ± 3.2** |

The nuclei were incubated with cytosolic fractions prepared from normal and heat-treated (44°C for 2 h, 24 h recovery) protoplasts at 28°C for 4 h. Values are the means of three separate experiments ± S.D. The level of significance was $^{**}P < 0.01$ (Fisher's PLS).

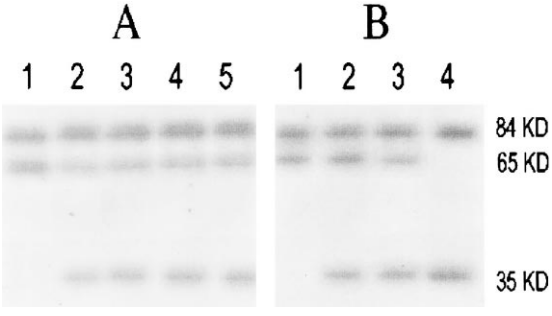


Fig. 4. Cleavage of lamin-like protein in assayed by Western blotting. A: Lamin-like protein degradation in in vivo and in vitro apoptosis systems. The recovery time was 16 h. Lane 1, control protoplasts; lanes 2–4, lamin-like proteins from protoplasts treated at 44°C for 2 h, 48°C for 0.5 and 1 h respectively; lane 5, lamin-like proteins from nuclei incubated with cytosol prepared from heat-treated (44°C for 4 h) protoplasts at 28°C for 4 h. B: Time course of lamin-like protein cleavage. The recovery times were 0, 8, 16 and 24 h for lanes 1, 2, 3 and 4 respectively.


Fig. 5. Time course of DNA fragmentation in heat-treated protoplasts (44°C for 2 h). Lane 1, DNA molecular markers (λ/*Hin*dIII+*Eco*RI); lanes 2–5, DNA extracted at 0, 8, 16 and 24 h after the onset of recovery respectively.

## 4. Discussion

It is commonly accepted that the basic features of apoptosis are conserved in animal and plant cells. Many links of the apoptotic pathway in animal cells have been very well characterized, particularly the action of caspases, a group of specific cysteine proteases. However, little information is available about this aspect of the apoptotic mechanism in plant cells. In one of our previous studies we reported that apoptosis induced by HS in tobacco cells was inhibited by iodoacetamide, a cysteine alkylating agent, but not by TLCK, pepstatin and leupeptin, suggesting an involvement of cysteine protease in apoptosis of plant cells [12]. Moreover, we also found that AC-DEVD-CHO, a specific inhibitor of caspase 3 in animal cells, completely abolished DNA fragmentation in apoptotic tobacco protoplasts induced by menadione [6] implying that a caspase 3-like cysteine protease may play a role in apoptosis of plant cells. Cysteine proteases have been found to be involved in PCD of soybean cells induced by oxidative stress and by pathogen infection in soybean plants [13]. The activation of cysteine proteases was found to be associated with hypersensitive resistance (HR), a form of PCD. And some of the cysteine proteases are similar to caspases in having specificity for Asp residues as evidenced by the inhibitor study [14].

Caspase 3 is considered to be one of the apoptosis executors in the middle of the apoptotic cascade and is activated by the apical triggering proteases such as caspase 8/10, caspase 3 activates the downstream DNA fragmentation factor which initiates the fragmentation of DNA [15]. In this study we directly detect the activity of a caspase 3-like protease using a synthetic tetrapeptide substrate Ac-DEVD-pNA, which is a mimic of the sequence in poly(ADP-ribose) polymerase where caspase 3 specifically cleaves. It is very clear that there must be a cysteine protease which recognizes the peptide sequence and cleaves it at the specific aspartic acid site functionally resembling caspase 3 in animal cells. The activation of this caspase 3-like protease in the cytosolic fraction of tobacco protoplasts found in this study is in accord with the proteolytic cleavage of the death substrate by caspase 3 in animal cells, which is widely accepted to be a hallmark of apoptosis [16]. Furthermore, since most of the parameters used for apoptosis detection so far are based on DNA fragmentation (such as DNA laddering, TUNEL assay, comet assay, DAPI staining and flow cytometry), it would be appropriate to add the activation of caspase 3-like protease to the list.

In this paper we studied the cleavage of lamin-like proteins during in vivo and in vitro apoptosis in plant cells. Lamina-like structure and lamin-like proteins have been reported to exist in plant cells suggesting a similarity between animal and plant cells in the lamin-related structure and function. In this study, a 65-kDa lamin-like protein was found to be cleaved forming a 35-kDa fragment during in vivo and in vitro apoptosis induced by HS. The same phenomena were found in our previous study on the menadione-induced apoptosis in tobacco protoplast [6]. Whether the 35-kDa cleaved product can be considered to be the signature apoptotic fragment and a hallmark of plant apoptosis is not clear at the present time and more studies on different plants and different apoptosis systems are required.

The assay of cysteine protease activity using Ac-VEID-pNA as substrate revealed the occurrence and activation of a unique cysteine protease of plant origin in apoptotic tobacco cells. Recognition of this tetrapeptide sequence and specific cleavage after aspartic acid suggest that this protease is a caspase 6-like protease.

The data shown in this paper provide evidence for the occurrence of the caspase 6- and caspase 3-like proteases and their activation during apoptosis in plant cells. Further studies on the identification and characterization of these endogenous proteases and their protein targets are necessary for elucidating the apoptotic pathway in plant cells.

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