Involvement of poly(ADP-ribose) polymerase and activation of caspase-3-like protease in heat shock-induced apoptosis in tobacco suspension cells

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Abstract The cleavage of poly(ADP-ribose) polymerase (PARP) by caspase (casp)-3 is an essential link in the apoptotic pathway in animal cells. In plant cells, however, there is no authentic evidence for the similar role that PARP may play during apoptosis. Using a heat shock (HS)-induced apoptosis system of tobacco cells, we found that immediately after a 4 h heat treatment, PARP was cleaved to form an 89 kDa signature fragment, while DNA laddering appeared only after a 20 h recovery following the HS. An activation of casp-3-like protease was also observed. The results suggest that apoptosis in plants and animals may share common mechanisms. On the other hand, when cells were preincubated with 4 mM 3-aminobenzamide or 2–8 mM nicotinamide, the specific inhibitors of PARP, before HS treatment, apoptotic cell death was reduced significantly. Our results thus imply that PARP may also be involved in apoptosis in a different way from the casp-related events.

Key words: Poly(ADP-ribose) polymerase; Apoptosis; Caspase-3-like protease; 3-Aminobenzamide; Nicotinamide; Tobacco

1. Introduction

Apoptosis, a form of programmed cell death, is widely used to describe an active gene-dependent process in which the cell directs its own destruction [1]. Apoptosis has been observed in various animal cells and was found to play a fundamental role in normal development, maintenance of homeostasis, pathological processes and in response to various stresses [1]. In plants, apoptosis was also found to be involved in various developmental events, such as differentiation of xylem, programmed abortion of floral organ in unisexual plants and suspensor degeneration during embryonic development [2,3]. Furthermore, apoptosis was found to be correlated to the hypersensitive response of plants to pathogens as well [4,5].

Poly(ADP-ribose) polymerase (PARP), a very abundant chromatin protein in animal nuclei [6], occurs rather scarcely in plant cells [7,8]. This nuclear enzyme is activated by DNA breaks, catalyzing poly(ADP-ribosyl)ation of chromosomal proteins using intracellular NAD as substrate [9]. The physiological function of PARP concerns DNA repair, replication and genome integrity [6,9]. In apoptotic animal cells, PARP, known as a death substrate, is degraded to form an 89 kDa signature fragment. PARP cleavage by caspase (casp)-3 is a well-characterized event in the apoptotic pathway of animal cells [10,11]. On the other hand, shortly before PARP cleavage, the activation of PARP which leads to poly(ADP-ribosyl)ation of nuclear proteins was shown during the initial stage of apoptosis [12,13]. It is why the role that PARP may play in apoptosis has become an important aspect in apoptotic study.

It is commonly believed that the apoptotic events are conserved in various types of organisms. Although PARP has been reported to occur in some plant species and recently PARP genes were cloned from *Zea mays* and *Arabidopsis thaliana* [7,14], there is no convincing and direct evidence thus far for the involvement of PARP in programmed plant cell death.

In this study, we address the involvement of PARP in apoptosis by examining the characteristic cleavage of PARP and by using specific PARP inhibitors 3-aminobenzamide (3-AB) and nicotinamide (NIC). Our results suggested that like in apoptotic animal cells, both activation and specific cleavage of PARP are essential for the progression of apoptosis induced by heat shock (HS) in tobacco suspension cells.

2. Materials and methods

2.1. Cell culture

Tobacco (*Nicotiana tabacum*, cultivar BY-2) suspension cells were cultured in MS basic medium supplemented with 0.6 mg/l 2,4-dichlorophenoxy acetic acid at 27°C under rotation (130 rpm). Cells were subcultured every 5 days. Exponentially growing cells were used for experiments.

2.2. Treatment of tobacco suspension cells

Flasks containing tobacco cells were immersed in water baths at 44°C under rotation of 130 rpm for 4 h. After heat treatment, cells were returned to the normal culture conditions at 27°C for 20 h for recovery. 3-AB (Sigma) and NIC (Sigma), when used, were added to medium 30 min before HS treatment.

2.3. Test of cell death

After stained with 0.5% trypan blue in phosphate-buffered saline (PBS) for 1 min, cells were observed under a light microscope. The cell death percentage was calculated based on trypan blue exclusion.

2.4. Agarose gel electrophoresis of DNA

Tobacco DNA electrophoresis was conducted according to Chen et al. [15]. In brief, cells were frozen in liquid nitrogen, ground to a fine powder, then extraction buffer (200 mM Tris–HCl, pH 7.5, 25 mM

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Abbreviations: PARP, poly(ADP-ribose) polymerase; HS, heat shock; 3-AB, 3-aminobenzamide; NIC, nicotinamide; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling; casp, caspase

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EDTA, 250 mM NaCl, 0.5% sodium dodecyl sulfate (SDS)) was added. After phenol/chloroform extraction followed by ethanol precipitation, DNA was transferred to TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and RNase digestion was then conducted. DNA was loaded on a 1.6% agarose gel and run at 5 V/cm for 4 h, and was then visualized by ethidium bromide staining.

2.5. In situ detection of DNA specific cleavage by the terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling (TUNEL) method

The TUNEL procedure was performed according to the manufacturer's instructions (Boehringer Mannheim, Germany). 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 (0.1% Triton X-100 in 0.1% sodium citrate) 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. Finally, slides were rinsed with PBS and observed under a fluorescence microscope (Leica DMRB).

2.6. Test of percentage of apoptosis

Cells were stained with TUNEL reagent and 4′,6′-diamidino-2-phenyl-indole (DAPI), respectively. Cells with TUNEL positive nuclei were considered as apoptotic, the total cell number was counted based on DAPI staining, and the percentage of apoptotic cells was thus calculated.

2.7. Immunoblotting of PARP

Cell extracts were prepared as described by Sallmann et al. [16]. 10% SDS–PAGE was then conducted. The resolved polypeptides were transferred to a nitrocellulose membrane (Whatman) which was then incubated with primary anti-PARP (Boehringer Mannheim, Germany) and subsequently the secondary antibody (goat against rabbit) conjugated with alkaline phosphatase. The antibody-labeled bands were visualized by the color development in a solution containing nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate.

2.8. Assay of casp-3-like protease

Cytosolic fractions from normal and heat-treated cells were used for the assay of casp-3-like protease, which was carried out following the manufacturer's instruction (Calbiochem, USA). An appropriate volume of cytosol (10 μg total cytosol protein equivalents) was added to 100 μl assay buffer (50 mM HEPES pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol). After incubated at 37°C for 10 min, 100 μM Ac-DEVD-pNA was added. Absorbance at 405 nm was measured every 5 min. The activity was calculated according to the manufacturer's description.

3. Results

3.1. PARP cleavage in apoptotic tobacco cells as demonstrated by Western blotting

In animal cells, PARP cleavage by casp-3 is a central event in the apoptotic cascade and was found to precede DNA fragmentation. The cleavage of the 113 kDa molecules led to the formation of an 89 kDa signature fragment. In our Western blot analysis, as shown in Fig. 1B, a similar pattern of PARP cleavage was observed in apoptotic tobacco cells induced by HS. Strikingly, it was found that immediately after heat treatment without recovery, the typical PARP cleavage occurred, whereas at the same time point and even after 10 h recovery, there was not even a shred of sign of DNA fragmentation which became distinct only after 20 h recovery (Fig. 1A). Our study thus provided convincing evidence for the occurrence of endogenous PARP cleavage which is probably a constitutive apoptotic event of programmed cell death in tobacco cells, and an indispensable link of the apoptotic pathway shared by both animals and plants.

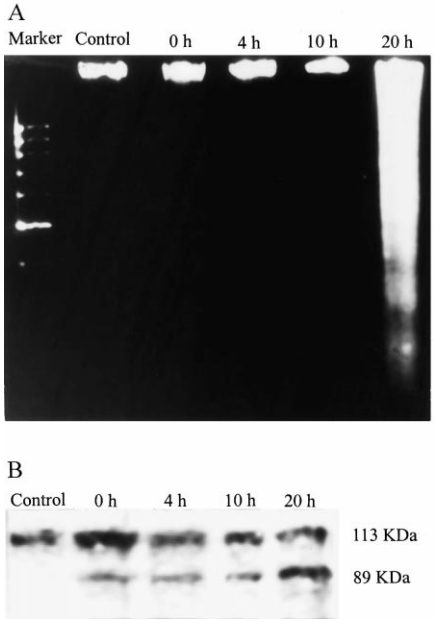

Fig. 1. Specific cleavage of PARP preceded DNA fragmentation during HS-induced apoptosis in tobacco cells. A: DNA fragmentation at different recovery times during HS-induced apoptosis. Control cells (lane 1) and cells with the indicated recovery time periods after HS treatment (lanes 2–5) were processed for DNA fragmentation analysis. B: Time course of HS-induced PARP cleavage as detected by immunoblotting. Lane 1, control. Lanes 2–5, 0, 4, 10 and 24 h recovery, respectively.

3.2. Casp-3-like activity in heat-treated tobacco cells

In our study, a synthetic tetrapeptide sequence Ac-DEVD-pNA based on the casp-3 cleavage site in the PARP molecule was used for the assay of casp-3-like protease activity in tobacco cells. This peptide is usually used in the substrate preference study and the K_{cat}/K_m is 218 000 [17], thus indicating a high specificity. As shown in Fig. 2, Casp-3-like activity in the cytosolic fraction of apoptotic tobacco cells induced by HS increased with the recovery time after heat treatment, and

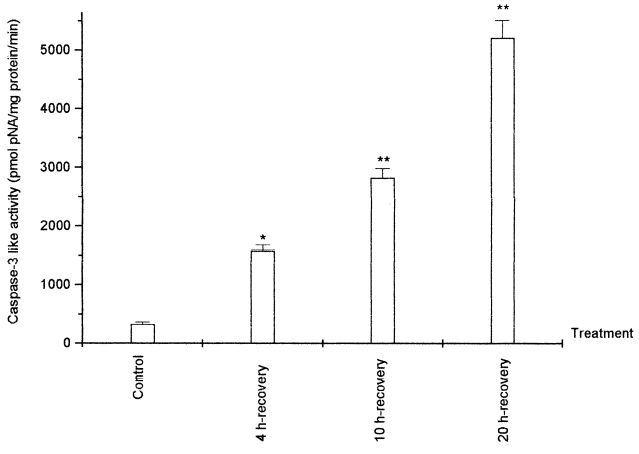

Fig. 2. Activation of casp-3-like proteases during HS-induced apoptosis in tobacco cells. Casp-3-like protease activity was assayed after 4, 10 and 20 h recovery post 4 h heat treatment using cytosol from control and heat-treated induced cells. Data are represented as mean ± S.E.M. Statistical analysis was done by a two-tailed Student's *t*-test. $*P < 0.01$ and $**P < 0.005$ vs. control. Values of $P < 0.05$ were considered significant.

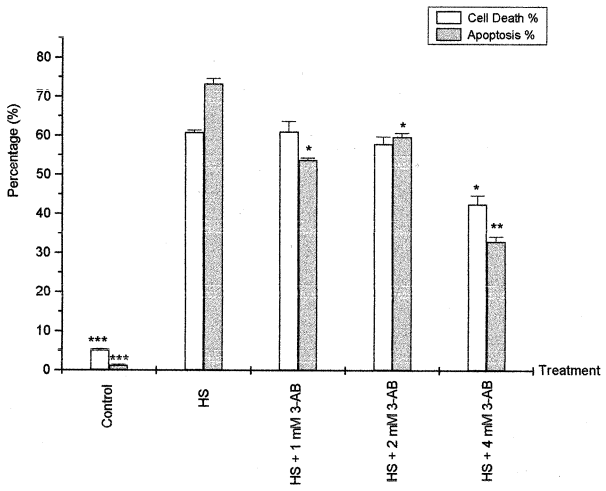

Fig. 3. Effect of 3-AB on cell death and apoptosis in HS-treated cells. Cells preincubated with 0 mM, 2 mM or 4 mM 3-AB were treated with HS (44°C, 4 h). After 20 h recovery, cell death was assayed by trypan blue staining, and apoptosis was determined by DAPI staining and the TUNEL assay. Data are represented as mean ± S.E.M. Statistical analysis was done by a two-tailed Student's *t*-test. $*P<0.05$, $**P<0.005$ and $***P<0.001$ vs. HS. Values of $P<0.05$ were considered significant.

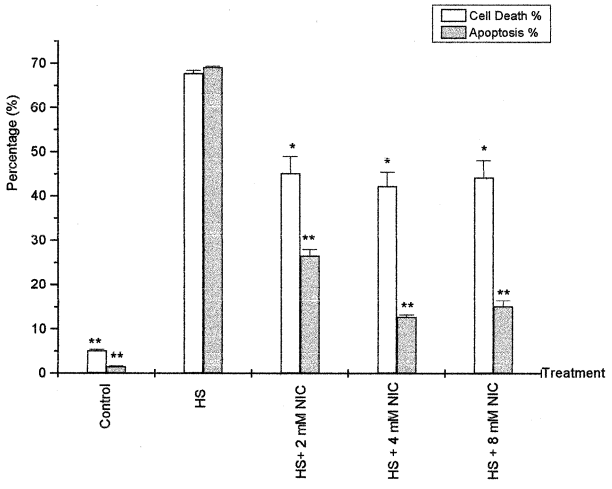

Fig. 4. Effect of NIC on cell death and apoptosis in HS-treated cells. Cells preincubated with 2 mM, 4 mM or 8 mM NIC were treated with HS (44°C, 4 h). After 20 h recovery, cell death was assayed by trypan blue staining, and apoptosis was determined by DAPI staining and the TUNEL assay. Data are represented as mean ± S.E.M. Statistical analysis was done by a two-tailed Student's *t*-test. $*P<0.05$ and $**P<0.001$ vs. HS. Values of $P<0.05$ were considered significant.

casp-3-like protease activity after 4, 10 and 20 h recovery was 3.69, 7.43 and 14.5 times higher than that in control cells, respectively. The results indicated a remarkable activation of casp-3-like protease during HS-induced apoptosis in tobacco cells.

3.3. *Effect of 3-AB and NIC pretreatment on cell death and apoptosis in HS-treated tobacco cells*

As illustrated in Fig. 3, in heat-treated cells (44°C, 4 h, 20 h recovery) the percentages of cell death and apoptosis were about 61 and 73, respectively. When 1 or 2 mM 3-AB was preincubated with cells for 30 min before HS, no change of the percentage of cell death was observed, while the percentage of apoptotic cells reduced to a certain extent. When 4 mM 3-AB was used to pretreat the cells, the percentage of both cell death and apoptosis decreased significantly (about 30% cell death and 55% apoptosis decrease was seen). The results suggested that activation of PARP is required for cell death and

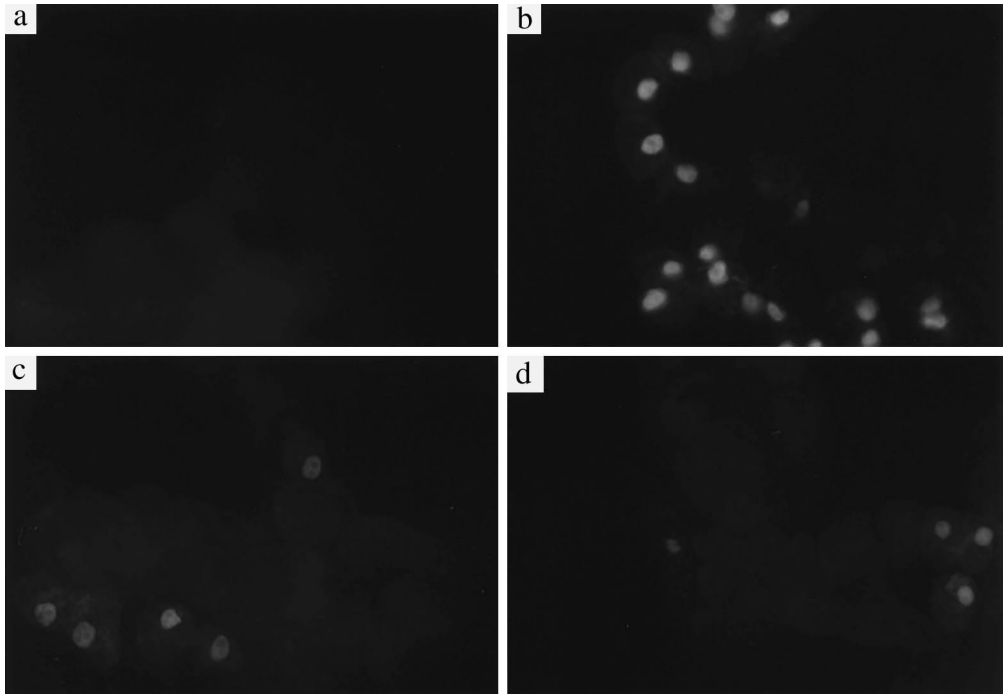

Fig. 5. Pretreatment with PARP inhibitors protected tobacco suspension cells from apoptosis as detected by the TUNEL method. (a) Control cells. (b) HS-treated cells. (c,d) Cells pretreated with 4 mM 3-AB or 4 mM NIC for 30 min respectively before HS treatment.

apoptosis. In the case of NIC, the effect of PARP inhibition was similar but the drop in cell death and apoptotic percentage was even sharper. When cells were pretreated with 4 mM NIC, the percentages of cell death and apoptosis were only about 42 and 13, respectively, whereas in heat-treated cells without NIC preincubation, the percentages were about 67 and 69 for cell death and apoptosis, respectively (Fig. 4). Again apoptosis was found to be more sensitive to PARP inhibition as compared with the non-apoptotic cell death.

3.4. The inhibition of DNA fragmentation by 3-AB and NIC as detected by the TUNEL assay

The TUNEL assays which enable in situ detection of nuclear DNA strand breaks via visualizing DNA 3′-OH nicks are widely used in examination of apoptosis in both animal and plant cells. As shown in Fig. 5a, in normal control cells, almost no 3′-OH nick end was labeled but only very weak autofluorescence in the cell wall area was seen following the TUNEL procedure. While in heat-treated cells, a great deal of nuclei was TUNEL positive, showing a fluorescence (Fig. 5b). In cells preincubated with 4 mM 3-AB or 4 mM NIC, however, the amount of TUNEL positive nuclei was significantly reduced, suggesting an obvious repression of apoptosis by these PARP inhibitors in HS-treated tobacco cells (Fig. 5c,d).

3.5. Inhibition of DNA laddering by PARP inhibitors

Another way to detect DNA fragmentation in apoptotic cells is agarose gel electrophoresis of nuclear DNA which shows distinct ladders of DNA fragments increasing in size by multiples of 180–200 bp and was thus named as DNA laddering. DNA laddering is commonly used as a characteristic biochemical hallmark of apoptosis. As shown in Fig. 6 (lane 2), DNA extracted from heat-treated cells formed typical ladders after electrophoresis, while no apoptotic DNA fragmentation was seen for DNA from the control. However, in the presence of 3-AB or NIC, no apoptotic DNA fragmentation was observed, and a faint successive smear of randomly degraded DNA was formed instead (Fig. 6, lanes 3–5). This is probably a reflection of the high ratio of necrosis to apoptosis in cells preincubated with PARP inhibitors particularly with NIC as shown in Figs. 3 and 4.

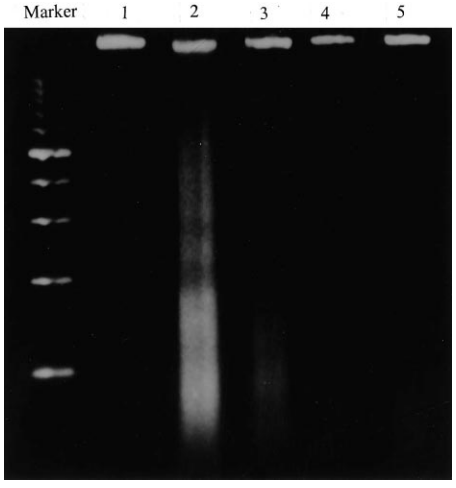

Fig. 6. Inhibition of DNA laddering in cells pretreated with 3-AB and NIC prior to HS treatment. Lane 2, DNA from control cells. Lane 3, DNA from HS-treated cells (44°C, 4 h, 20 h recovery). Lanes 4–6, DNA from cells pretreated with 4 mM 3-AB, 4 mM and 8 mM NIC respectively prior to HS (44°C, 4 h, 20 h recovery).

4. Discussion

It is widely accepted that apoptotic mechanisms in plants and animals may share common components leading to conserved cellular events. In animal cells, PARP was identified as an important substrate of casp-3, an apoptosis executor. The activation of casp-3 and correspondingly the proteolytic inactivation of PARP by casp-3 were considered as important hallmarks of apoptosis [18]. At the early stage of apoptosis, PARP was found to be cleaved to form an 89 kDa fragment. However, there was no direct and authentic evidence for the existence of a similar event during apoptosis in plant cells. Solomon et al. found that, in H_2O_2-treated soybean cells, novel cysteine proteases were activated, as evidenced by using a number of fluorogenic peptides, such as z-Gly-Gly-Arg-AMC, which were chosen according to known activities against papain, and by using the inhibitor 4-[2-aminoethyl]-benzesulfonylfluoride which inhibited both PCD and the novel proteases in addition to papain. They concluded that cysteine proteases were involved in PCD in plants [19]. In a previous study, we established a cell-free system of apoptosis which consists of the isolated nuclei from healthy tobacco cells and the cytosolic fraction prepared from apoptotic tobacco cells as induced by HS treatment. We found that in vitro apoptosis was selectively inhibited by iodoacetamide, a cysteine-alkylating agent, suggesting also the involvement of cysteine protease in apoptosis of plant cells [20]. Furthermore, the possible occurrence of casp-like protease during plant apoptosis was implicated by using specific synthetic tetrapeptide inhibitors and substrates. Pozo and Lam reported that in extracts prepared from tobacco plants undergoing TMV-induced hypersensitive reaction, a significant augmentation in Ac-YVAD-AMC (a specific substrate of casp-1) cleavage activity was observed. This enhancement was abolished when 0.5 mM of Ac-YVAD-CHO (inhibitor of casp-1) or Ac-DEVD-CHO (inhibitor of casp-3) was added to the reaction mixture [21]. In tobacco protoplasts, Ac-DEVD-CHO was found to inhibit DNA laddering which usually occurred during apoptosis induced by menadione [22]. Moreover, we also found that both casp-3-like protease and casp-6-like protease were activated during apoptosis in HS-induced tobacco protoplasts (unpublished data). The results imply that casp-like proteases may be involved in programmed plant cell death. Furthermore, when purified bovine PARP was added to the extracts of fungus-infected cowpea tissues exhibiting the hypersensitive reaction, the exogenous PARP was found to be proteolytically cleaved into fragments of 77, 52, 47 and 45 kDa, but not into the 89 kDa signature fragment shown in animal cells [23]. The cleavage of bovine PARP was completely inhibited by iodoacetamide and partially by Ac-DEVD-CHO, the specific casp-3 inhibitor. In this study, we reported a remarkable activation of casp-3-like protease, which may account for the specific cleavage of endogenous PARP in HS-induced apoptosis of tobacco cells. Taken together, our data provided new convincing evidence for the involvement of PARP and casp-3-like protease in apoptosis of plant cells.

On the other hand, the activation of PARP at the initial stage of apoptosis has been reported [12,13]. The activated PARP may lead to the poly(ADP-ribosyl)ation of important nuclear proteins which are required for the progression of apoptosis [24]. Also, PARP activation may affect the NAD

pool level, which may result in the exhausting of ATP and energy [25]. PARP activation was found to occur prior to the cleavage of PARP by casp-3. Inhibition of PARP by specific inhibitors may interfere with this event [12,26]. The inhibition of apoptotic tobacco cell death by 3-AB and NIC found in our study supported the above-mentioned requirement of PARP activation for the progression of apoptosis. Our results were in accord with the attenuation of H_2O_2-induced soybean cell death by 3-AB and NIC as reported by Amor et al. [26]. However, the role that PARP may play at the initial stage of apoptosis is not clearly defined, and further studies are thus required.

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