Cytochrome *c* release and caspase activation during menadione-induced apoptosis in plants

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Abstract We report here the detection of the release of cytochrome *c* from mitochondria into the cytosol during menadione-induced apoptosis in tobacco protoplasts. Western blot analysis indicated that the caspase specific inhibitors AC-DEVD-CHO (Ac-Asp-Glu-Val-Asp-aldehyde) and AC-YVAD-CHO (*N*-acetyl-Try-Val-Ala-aspartinal) inhibited the degradation of a caspase 3 specific substrate PARP (poly(ADP-ribose) polymerase), and they had no effect on the release of cytochrome *c*. Further study showed that menadione could not induce apoptosis of mouse liver nuclei in tobacco cytosol extract containing no mitochondria. However, when cytochrome *c* or mitochondria was added into the cytosol extract, apoptosis of mouse liver nuclei and the degradation of PARP could both be detected. The results provide strong evidence that menadione can induce apoptosis in tobacco protoplasts via the release of cytochrome *c* from mitochondria into the cytosol.

Key words: Apoptosis; Menadione; Cytochrome *c*; Poly(ADP-ribose) polymerase; Mitochondrion; Tobacco cell

1. Introduction

Apoptosis, a genetically controlled programmed cell death, has its special morphological and biochemical characteristics [1,2]. Recent studies show that cytochrome *c* plays an important role during apoptosis in animal cells. Cytochrome *c* combines with caspase 9 after its release from mitochondria into the cytosol, and thus activates the downstream caspase 3 to initiate apoptosis. In fact, bcl-2 inhibits apoptosis by inhibiting the release of cytochrome *c* [3–6].

The study on apoptosis in plant cells is still in an early stage. Although previous studies provided evidence that apoptosis may occur during development and differentiation, such as the tracheary element formation and disease resistance, little was known about its mechanism [7]. It is therefore an interesting issue to investigate whether apoptosis in plants and animals share common pathways.

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Abbreviations: AC-DEVD-CHO, Ac-Asp-Glu-Val-Asp-aldehyde; AC-YVAD-CHO, *N*-acetyl-Try-Val-Ala-aspartinal; PARP, poly-(ADP-ribose) polymerase; ICE, interleukin-converting enzyme; DAPI, 4,6-diamidino-2-phenylindole diacetate; MES, 4-[*N*-morpholino]ethanesulfonic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; EDTA, ethylenediaminetetracetic acid; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MS, Murashige-Skoog; 2,4-D, 2,4-dichlorophenoxyacetic acid; PMSF, phenylmethylsulfonyl

Our previous study showed that cytochrome *c* is an efficient inducer of apoptosis in plants. It is able to induce apoptosis of carrot protoplasts, and also induces apoptosis in mouse liver nuclei in carrot cytosol extract [8,9]. Based on these studies, we further investigated the inductive mechanism of cytochrome *c*. Because menadione can induce apoptosis in tobacco protoplasts, we studied the role of cytochrome *c* by using menadione as an inducer. Menadione-induced apoptosis in tobacco protoplasts resembles that in animal cells, and is inhibited by the caspase specific inhibitor AC-DEVD-CHO. Besides, bcl-2 inhibits menadione-induced apoptosis in animal cells [10–12].

In this paper, we report the release of cytochrome *c* from mitochondria into the cytosol in a time-depending manner as well as the degradation of PARP, a specific substrate of caspase 3, from 116 kDa into 84 kDa fragments during menadione-induced apoptosis in tobacco protoplasts. It is the first study to show the change of PARP in plant apoptosis since its first description [13,14]. Caspase belongs to a large ICE (interleukin-converting enzyme) protease family, and 11 members have been found in this family. The characteristic of caspases is the occurrence of cysteine in the active center, and this enables them to specifically cleave near the Asp site of their substrates. But caspases have different specificity for different substrates. In particular, caspase 3 is the most important member of the caspase family [3,5,15–17]. PARP is the specific substrate of caspase 3, and the degradation of PARP is an important event during apoptosis. PARP is also a nuclear protein, it is very important for DNA repairing and the maintenance of chromatin structure [16]. Experiments showed that AC-DEVD-CHO and AC-YVAD-CHO could inhibit the degradation of PARP, but could not inhibit the release of cytochrome *c*. Moreover, one study showed that menadione can not induce apoptosis of mouse liver nuclei in tobacco cytosol without the presence of mitochondria, while its function could subsequently be recovered by adding cytochrome *c* or mitochondria back into the cytosol. The above results indicate that menadione can induce apoptosis in tobacco protoplasts through the release of cytochrome *c* from mitochondria into the cytosol. Cytochrome *c* thus activates one or a few caspase-like protein(s)(CLP) in cytosol [5]. Our study provides important findings in the study of the mechanism of apoptosis in plants.

2. Materials and methods

2.1. Cell culture

A tobacco (*Nicotiana tobaccum*, cultivar BY-2) cell suspension culture was initiated from cotyledon explants and maintained in MS (Murashige-Skoog) medium supplemented with 1 mg/l 2,4-D (2,4-di-

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chlorophenoxyacetic acid) at 25°C. Subculture was done at weekly intervals.

2.2. Preparation of tobacco protoplasts and cytosol extract

Five g suspension culture tobacco cells (gross weight) cultured 5–7 days were added into enzymatic buffer (2% (w/v) cellulase, 0.5% macerozyme, 5 mmol/l MES, 6.8 mmol/l $CaCl_2$, 11 mmol/l KH_2PO_4, 0.6 mol/l mannitol, 0.4% polyvinylpyrrolidone (PVP), pH 5.8), and the cells were maintained in a rotary incubator at a speed of 50–100 rpm for 2 h at 28°C in darkness. The protoplasts were collected by centrifugation at $120\times g$ for 5 min. The sediment was washed twice with washing buffer (0.2 mol/l sorbitol, 0.2 mol/l mannitol, 0.05 mol/l MES, pH 5.8–6.0), then re-suspended in 0.6 M sucrose buffer. The floating protoplasts were centrifuged at $120\times g$, then washed twice with a washing buffer, and suspended in buffer A (20 mmol/l HEPES-KOH, 10 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 1 mmol/l EDTA, 1 mmol/l EGTA, 1 mmol/l DTT, 200 mmol/l sucrose). The protoplasts were homogenized appropriately; aprotinin and leupeptin were added into the buffer A at a concentration of 6 mg/ml and 8 mg/ml respectively. After centrifugation at $100\,000\times g$ (Beckman TLS-55) for 2 h at 4°C, the soluble cytosol was collected, and the solution was called mitochondria-free cytosol extract (MF cytosol extract).

2.3. Preparation of mouse liver nuclei

The preparation and purification of interphase mouse liver nuclei were according to the description of Blobel [18]. The nuclei were suspended in nuclear reserve buffer (10 mmol/l PIPES, 80 mmol/l KCl, 20 mmol/l NaCl, 250 mmol/l sucrose, 5 mmol/l EGTA, 0.5 mmol/l spermidine, 0.2 mmol/l spermine, 50% glycerol), centrifuged at $124\,000\times g$ (Beckman TLS-55) and counted before storing in −80°C.

2.4. Preparation of tobacco protoplast mitochondria

Mitochondria were isolated as described by Menassa et al. [19]. The purified mitochondria were diluted and stained with Janus Green, counted under the microscopy and stored at −80°C.

2.5. Induction of apoptosis in tobacco protoplasts

(A) The tobacco protoplasts were re-suspended to achieve a concentration of 1×10^5 cells/ml, then menadione was added to a final concentration of 100 μmol/l. The tobacco protoplasts were incubated at 22°C, and samples were taken at 0 h, 1 h, 2 h, 4 h, 6 h, and 8 h.

(B) Tobacco protoplasts were re-suspended to achieve a concentration of 1×10^5 cells/ml, then menadione was added to a final concentration of 100 μmol/l, in addition, 100 μmol/l AC-DEVD-CHO and 10 μmol/l AC-YVAD-CHO were added. The protoplasts were incubated at 22°C, and samples were taken for analysis at 1 h, 2 h, 4 h, 6 h, and 8 h.

2.6. Induction of apoptosis in mouse liver nuclei in tobacco cytosol

(A) 1×10^5 cells/ml mouse liver nuclei were added into the tobacco cytosol extract, and menadione was added to a final concentration of 100 μmol/l.

(B) The procedure is similar to (A), except that an addition of 1×10^6 cells/ml of mitochondria was applied to cytosol extract.

(C) Cytochrome *c* (Sigma) was added at a final concentration of 2 μmol/l to the tobacco cytosol extract, and 1×10^5 cells/ml of mouse liver nuclei were added. Samples were taken after incubation for 4 h in the induction system. To detect the inhibition by AC-DEVD-CHO, 100 μmol/l AC-DEVD-CHO was added into the three induction systems respectively, and samples were taken after incubation for 4 h.

2.7. Detection of the release of cytochrome c during menadione-induced apoptosis in tobacco protoplasts

Tobacco protoplasts were taken after induction for indicated time as previously stated, while mitochondria and cytosol extract were prepared. The proteins were dissolved in standard SDS sample buffer, and SDS-PAGE using 12% gel was then performed. The resolved polypeptides were transferred to a nitrocellulose membrane (Whatman), were first incubated for 1 h in a blocking buffer (3% BSA in TBS), then incubated with the primary antibody (monoclonal antibodies against cytochrome *c*) for 3 h at 37°C, the membrane was washed thoroughly with TBS, and then incubated with 1:1000 diluted Biotin-conjugated goat anti-mouse IgG (Pharmingen) for 2 h at 37°C. The nitrocellulose membrane was further incubated with 1:1000 diluted alkaline phosphatase-conjugated avidin (Pharmingen) for 15 min at room temperature. The antibody labeled bands were visualized by the color development in a solution containing 5-bromo-4-chloro-3-indolyl-phosphate-toluidine salt and *p*-nitrotetrazolium chloride.

2.8. Detection of the degradation of PARP in tobacco protoplasts

Tobacco protoplasts were re-suspended after incubation for indicated time with washing buffer (0.2 mol/l mannitol, 0.2 mol/l sorbitol, 5 mmol/l MES, and pH 5.8–6.0) and centrifuged for 5 min at $120\times g$. The cells were washed once in TBS and the sediment was kept. The sediment was re-suspended in a nuclei isolation buffer (10 mmol/l MES, 0.2 mol/l sucrose, 2.5 mmol/l EDTA, 25 mmol/l DTT, 10 mmol/l NaCl, 10 mmol/l KCl, 0.1 mmol/l spermine, pH 5.2) containing 0.02% (v/v) Triton X-100. The mixture was then homogenized manually using a mortar for 15 min at 4°C. The suspension was filtered first through a layer of cheese cloth and then through a polycarbonate filter (Sigma) of 15 μm pore size. Finally the nuclei were collected by centrifugation at $400\times g$ for 10 min and re-suspended in nuclear isolation buffer without Triton X-100. The 'number' of nuclei was determined by counting with a hemocytometer.

2.9. DNA analysis

DNA was extracted from tobacco protoplasts following the method of Ryerson et al. [9]. Identical amounts of DNA samples were run on a 1.5% (w/v) agarose gel at 50 V for 1 h. Oligonucleosomal fragments of DNA were visualized under ultraviolet illumination after staining with 0.63 mg/l ethidium bromide.

3. Results

3.1. The degradation of genomic DNA and the formation of 'DNA ladder' during menadione-induced apoptosis in tobacco protoplasts

At 0 h, genomic DNA degradation was not observed (Fig. 1, lane 1). The degradation of DNA began after 2 h incubation (Fig. 1, lane 2), and a 'DNA ladder' started to appear at 4 h, and it's ladder pattern became very clear at 6 h (Fig. 1, lanes 3, 4). The 'DNA ladder' could still be seen after 8 h incubation (Fig. 1, lane 5). However, when AC-DEVD-CHO was added, the formation of 'DNA ladder' was inhibited after 4 h incubation (Fig. 1, lane 6).

3.2. The release of cytochrome c from mitochondria into cytosol

Western blot analysis showed that cytochrome *c* was

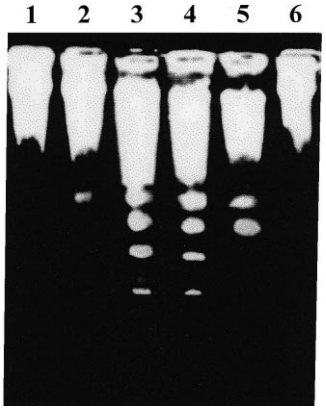

Fig. 1. Agarose gel analysis of tobacco protoplasts for DNA fragmentation. Lane 1, protoplasts after induction for 0 h with menadione. Lane 2, protoplasts after induction for 2 h with menadione. Lane 3, protoplasts after induction for 4 h with menadione. Lane 4, protoplasts after induction for 6 h with menadione. Lane 5, protoplasts after induction for 8 h with menadione. Lane 6, 100 μmol/l AC-DEVD-CHO was added and incubated for 4 h.

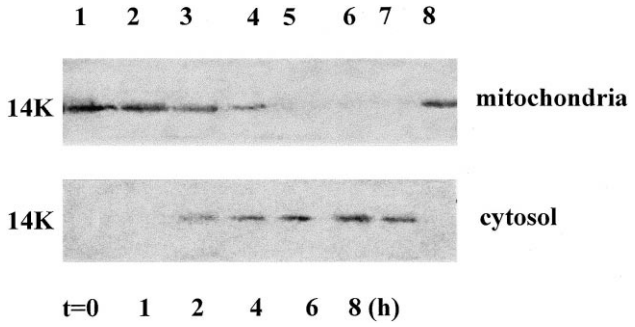

Fig. 2. Western blot analysis showed the changes of cytochrome *c* concentration in mitochondria and cytosol during menadione-induced apoptosis in tobacco protoplasts. Lane 1, 0 h. Lane 2, 1 h. Lane 3, 2 h. Lane 4, 4 h. Lane 5, 6 h. Lane 6, 8 h. Lane 7, with 100 μmol/l AC-DEVD-CHO, 4 h. Lane 8, control, 4 h, without menadione.

present in mitochondria at 0 h and 1 h (Fig. 2, lanes 1, 2). The concentration of cytochrome *c* in mitochondria did not change significantly while those existed in cytosol increased dramatically after incubation for 2 h (Fig. 2, lane 3). At 4 h, the concentration of cytochrome *c* in the mitochondria and the cytosol reached its minimum and maximum levels respectively, and these levels were maintained until 6 h and 8 h respectively (Fig. 2, lanes 4, 5, 6). The results also showed that the release of cytochrome *c* could not be inhibited in the presence of 100 μmol/l AC-DEVD-CHO (Fig. 2, lane 7).

3.3. The degradation of PARP in tobacco protoplasts

Western blot analysis showed that PARP was not degraded at 0 h, 1 h, and 2 h (Fig. 3A, lanes 1, 2, 3), while at 4 h, a 116 kDa band of PARP was degraded into 84 kDa fragments (Fig. 3A, lane 4). After 6 h and 8 h, PARP was degraded almost completely (Fig. 3A, lanes 5, 6). The degradation of PARP was inhibited by AC-DEVD-CHO and AC-YVAD-CHO (Fig. 3A, lanes 7, 8). The degradation of PARP was

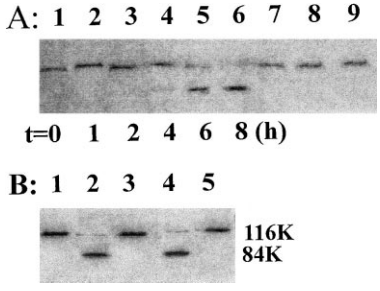

Fig. 3. Western blot analysis showed the specific degradation of PARP. A: Lane 1, 0 h, with menadione in tobacco protoplasts. Lane 2, 1 h, with menadione in tobacco protoplasts. Lane 3, 2 h, with menadione in tobacco protoplasts. Lane 4, 4 h, with menadione in tobacco protoplasts. Lane 5, 6 h, with menadione in tobacco protoplasts. Lane 6, 8 h, with menadione in tobacco protoplasts. Lane 7, 4 h, with menadione and 100 μmol/l AC-DEVD-CHO in tobacco protoplasts. Lane 8, 4 h, with menadione and 10 μmol/l AC-YVAD-CHO in tobacco protoplasts. Lane 9, the PARP of tobacco nuclei was not degraded after induction for 4 h in nuclei reserve buffer. B: Lane 1, 4 h, with menadione in tobacco cytosol extract without menadione. Lane 2, 4 h, with menadione in tobacco cytosol extract with mitochondria. Lane 3, 100 μmol/l AC-DEVD-CHO with menadione and mitochondria in tobacco cytosol extract. Lane 4, 4 h, with 2 μmol/l cytochrome *c* in tobacco cytosol extract. Lane 5, 4 h, with 2 μmol/l cytochrome *c* and 100 μmol/l AC-DEVD-CHO in tobacco cytosol extract.

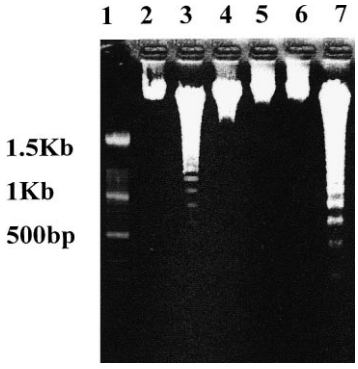

Fig. 4. Detection of the fragmentation of mouse liver nuclear DNA in tobacco cytosol extract. Lane 1, DNA marker with 100 bp intervals. Lane 2, control group, normal mouse liver nuclei. Lane 3, 4 h, with menadione and mitochondria in tobacco cytosol extract. Lane 4, 4 h, with menadione, mitochondria and 100 μmol/l AC-DEVD-CHO in tobacco cytosol extract. Lane 5, 4 h, with menadione in tobacco cytosol extract. Lane 6, 4 h, with cytochrome *c* and 100 μmol/l AC-DEVD-CHO in tobacco cytosol extract. Lane 7, 4 h, with cytochrome *c* in tobacco cytosol extract.

not found when the tobacco nuclei were incubated in nuclei reserve buffer (Fig. 3A, lane 9).

3.4. Menadione can not induce apoptosis of mouse liver nuclei in cytosol extract without mitochondria

After menadione was added into the MF cytosol at a final concentration of 100 μmol/l, 1×10^5 cells/ml mouse liver nuclei were mixed with the drug-treated cytosol. The DNA of the mouse liver nuclei was not degraded after an incubation of 4 h at 22°C (Fig. 4, lane 5), DAPI staining showed that nuclear chromatin was distributed evenly (Fig. 5 (1)), Western blot analysis showed that PARP was not degraded (Fig. 3B, lane 1).

3.5. Menadione could induce apoptosis of mouse liver nuclei in cytosol extract with mitochondria

After menadione was added into the MF cytosol at a final concentration of 100 μmol/l, 1×10^5 cells/ml mouse liver nuclei were mixed with the drug-treated cytosol. The genomic DNA of the mouse liver nuclei was degraded and showed a clear 'DNA ladder' (Fig. 4, lanes 3, 4). DAPI staining showed the condensation and margination of nuclear chromatin (Fig. 5(2)). We detected in addition the degradation of PARP in the mouse liver nuclei (Fig. 3B, lane 2). The specific degradation of PARP was inhibited by AC-DEVD-CHO (Fig. 3B, lane 3).

3.6. Cytochrome c-induced apoptosis in mouse liver nuclei and the degradation of PARP in mouse liver nuclei

Addition of cytochrome *c* to the MF cytosol at a final

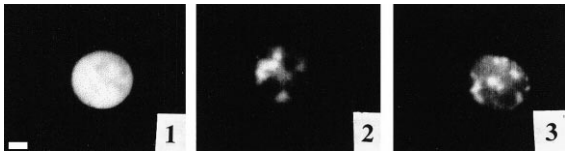

Fig. 5. Morphological changes of mouse liver nuclei in tobacco cytosol extract (bar = 10 μm). (1) 4 h, with menadione in tobacco cytosol extract without mitochondria. (2) 4 h, with menadione and mitochondria in tobacco cytosol extract with mitochondria. (3) 4 h, with cytochrome *c* in tobacco cytosol extract.

concentration of 2 μmol/l can induce typical hallmarks of apoptosis in mouse liver nuclei, including the condensation and margination of the chromatin and the formation of 'DNA ladder' (Fig. 4, lanes 6, 7). Western blot showed degradation of PARP in mouse liver nuclei, and this degradation was inhibited by AC-DEVD-CHO (Fig. 3B, lanes 4, 5).

4. Discussion

Apoptosis has been observed in plant cells during the process of tissue differentiation, the formation of tracheary elements and disease resistance [7]. Wang et al. (1996) found that AAL could induce apoptosis in plant protoplasts, and the formation of 'DNA ladder' was detected for the first time [20]. In comparison to the naturally occurring apoptotic process (in vivo), chemically induced apoptosis is highly synchronized and thus a highly yield of apoptotic body can be obtained. This enables us to study the mechanism of apoptosis in plants more easily. However, because enough yields of apoptotic cells could not be obtained, 'DNA ladder' could only be observed by isotope labeling during AAL-induced apoptosis in tobacco protoplasts. A large quality of apoptotic cells could be obtained using menadione as an inducer, and the menadione-induced apoptosis was inhibited by AC-DEVD-CHO and PMSF (phenylmethylsulfonyl), which indicated the possible participation of caspase in menadione-induced apoptosis in plants [10]. Therefore, we took a further step to study the function of cytochrome *c* in menadione-induced apoptosis in plants.

Although previous study showed that PARP also exists in plants, little is known about its changes during apoptosis in plants [13,14]. Our study indicated that PARP was specifically cleaved during menadione-induced apoptosis in plants. This not only suggests that PARP is involved in apoptosis in plants, but also provides evidence for the existence of caspase 3-like protease(s) (CLP) in plants (we use CLP for the convenience of description in this article). Moreover, the degradation of lamin, a caspase substrate, was also observed in menadione-induced apoptosis in plants [10]. So, the finding of the existence of caspase is in agreement with our previous study.

Further study showed that the activation of CLP is closely related with cytochrome *c*. As an important component in animal cells, cytochrome *c* is essential for the activation of caspases [3,5,6]. Many apoptotic inducers exert their function as a result of the release of cytochrome *c* from mitochondria into the cytosol [5]. We detected that CLP caused no PARP cleavage activity before the release of cytochrome *c* from mitochondria into the cytosol. A certain time was needed from the start of cytochrome *c* release to the degradation of PARP, which indicated that a series of reactions was involved. For the same reason, we still could not give a general conclusion that CLP is the only factor influencing the activity of PARP. Our results showed that PARP degraded in a step by step manner as cytochrome *c* was releasing gradually into the cytosol, and AC-DEVD-CHO and AC-DEVD-CHO could inhibit the degradation of PARP. AC-DEVD-CHO is a four polypeptide which contains the specific cleavage sites of caspase 3 and caspase 7. However, AC-DEVD-CHO does not inhibit the release of cytochrome *c*. This result also provides evidence that there are several CLP functions between the release of cytochrome *c* and the degradation of PARP.

Other evidence comes from the study of the apoptosis of mouse liver nuclei induced in tobacco cytosol extract. Our previous study showed that cytochrome *c* can induce apoptosis of mouse liver nuclei in carrot cytosol extract [9]. However, when menadione was added into the tobacco cytosol extract with no mitochondria, we found that menadione did not induce apoptosis of mouse liver nuclei. However, in the presence of mitochondria, menadione did induce not only the apoptosis of mouse liver nuclei, but also the degradation of PARP in mouse liver nuclei. The action of menadione and mitochondria can be replaced by exogenously adding the cytochrome *c*. These results showed that CLP exists in tobacco cytosol, and its activation depends on the inductive function of menadione through the release of cytochrome *c* from mitochondria into the cytosol. Additional work is required to test if this is a common pathway in different species of plants.

Although apoptosis has been described in several different species of animal cells, regulators of cell death have not been identified in the plant system. Recently, it is reported that Ced-4 protein from *Caenorhabditis elegans* and its human homology Apaf-1 share conserved structural and functional domains with plant resistance gene products [21–23]. These homologies suggest that a cell death mechanism may be conserved in plants and animals, and the regulators show little species specificity. Our present study shows that cytochrome *c* release and caspase activation are also important events during menadione-induced apoptosis in plants like that in animals [4,5,24]. The absence of the species specificity indicates that a common pathway may be shared by apoptosis in animals and plants.

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