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BRIEF COMMUNICATION

Differences in protein patterns in suspension cultures of *Taxus cuspidata* induced by cerium

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Abstract

The changes in soluble proteins induced by Ce^{4+} were analyzed in suspension cultures of *Taxus cuspidata* using two-dimensional polyacrylamide gel electrophoresis. The ultrastructure of cells obviously changed at day 4 after addition of Ce^{4+}. Large amount of nuclear DNA fragments of about 200 bp were observed. Thirteen protein spots were different between the cultures grown with and without Ce^{4+} at day 4 as well as at day 6 after addition of Ce^{4+}.

Additional key words: Ce^{4+}, programmed cell death, protein, two-dimensional gel electrophoresis.

It is well known that programmed cell death is an active form of cell death in plants and animals during development or as a cellular response to stress (Greenberg 1996, Pennel and Lamb 1997). Many evidences showed that the reactive oxygen species (ROS) play important roles in programmed cell death (Desikan *et al.* 1996, Breusegem *et al.* 2001) by induction of programmed cell death or destruction of the cells as an executive factor (Jabs 1999, Vandenabeele *et al.* 2000). Lots of specific proteins are synthesized during programmed cell death in plant cells (Pennel and Lamb 1997, Tarchevsky 2001). Recently, we found that Ce^{4+}, an ion with a strong oxidative ability, could induce the programmed cell death in suspension cultures of *Taxus cuspidata* (Yuan *et al.* 2001). However, the mechanism of the alteration of gene expression associated with the Ce^{4+}-induced programmed cell death of *Taxus cuspidata* is unclear. In this work, two-dimensional electrophoresis was used to analyze the variations of proteins in suspension cultures of *Taxus cuspidata* induced by Ce^{4+} so as to have a better understanding of the apoptotic process.

Cell line of *Taxus cuspidata* from the Institute of Botany, Chinese Academy of Sciences, was sub-cultured in liquid BS medium (Gamborg *et al.* 1968) containing 30 g dm^{-3} sucrose, 5 μM naphthylacetic acid (NAA) and 0.01 μM 6-benzyladenine (BA). The pH of the medium was adjusted to 5.8 using 0.1 M NaOH and the medium was autoclaved at 121 °C for 20 min. Suspension cultures were grown at 25 °C in dark at agitation 105 rpm for 5 generations. Samples (5 cm^3) from the 5th generation were transferred into 250 cm^3 flasks containing 100 cm^3 B5 medium, then Ce^{4+} [$Ce(NH_4)_2(NO_3)_6$] (1 mM) was added at day 7. The control culture contained ammonium sulfate (1 mM) and sodium nitrate (1 mM).

Suspension cultures (1 cm^3) were centrifuged at 1 100 *g* for 10 min, the sediment was collected, dissolved in 10 mM phosphate buffer of pH 7.4 and washed three times with the same buffer with continuous bubbling to prevent the cells from possible aggregation. The cells were re-suspended in 0.2 cm^3 10 mM phosphate buffer (pH 7.4) and stained with propidium-iodine (50 μg cm^{-3}) and *Hoechst 33342* (50 μg cm^{-3}) for 15 min. Cell

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Abbreviations: CHAPS - 3-(3-chloamidopropyl) dimethylammonio-1-propanesulfonate; CTAB - cetyltrimethyl ammonium bromide; DTT - dithiothreitol; 2-D - two-dimensional; EDTA - ethylenediaminetetraacetic acid; IEF - isoelectric focusing; PAGE - polyacrylamide gel electrophoresis; PCD - programmed cell death; SDS - sodium dodecyl sulphate.

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suspensions (0.05 cm^3) were dropped on a slide and viewed under a fluorescent microscope (*Nikon E800*, Tokyo, Japan).

Total nuclear DNA was extracted following the method of Katsuhara and Kawasaki (1996) with a slight modification. Fresh cells (2 g) were ground into powder in liquid N_2. Powdered cells (200 mg) were dissolved in 0.6 cm^3 lysis buffer (pH 8.0) consisting of cetyltrimethyl ammonium bromide (CTAB) (2 % m/v), Tris-HCl (10 mM), EDTA (20 mM), NaCl (1.4 mM), and β-mercaptoethanol (2 % v/v) in a sterilized Eppendorff tube. The mixture (0.6 cm^3) of phenol, chloroform and isoamylol (24:24:1, v/v/v) was added to denature the proteins. Then the mixture was centrifuged at 9 000 *g* for 10 min, the supernatant was collected and precipitated by addition of 0.36 cm^3 isopropanol. The precipitate was re-dissolved in 0.5 cm^3 TE buffer (10 mM Tris-HCl, 20 mM EDTA), then RNAase (1 μg cm^{-3}) was added to digest RNAs at 37 °C for 30 min. Afterwards, the mixture was cooled down to 4 °C and isopropanol (0.3 cm^3) was added to precipitate DNA. The DNA precipitate was collected by centrifugation and dissolved in 0.5 cm^3 TE buffer (10 mM Tris-Cl, 1 mM EDTA) of pH 8.0. DNA samples (0.01 cm^3) were placed on an agarose gel (2.0 % m/v) stained with ethidium bromide (0.5 μg cm^{-3}) for electrophoresis under 5.0 V cm^{-1} and observed under UV radiation.

For extraction of proteins cell suspensions (100 cm^3) were washed 3 times with 50 cm^3 10 mM cold phosphate buffer (pH 7.4) then grounded in liquid N_2. Cell powders (0.2 g) were dissolved in 0.5 cm^3 lysis buffer consisting of urea (8 M), 3-(3-chloamidopropyl) dimethylammonio-1-propanesulfonate (CHAPS) (4 %, m/v) and Tris-base (40 mM) in a sterilized Eppendorf tube at 4 °C for 1 h. The mixture was centrifuged at 4 °C and 15 500 *g* for 20 min, the supernatant was mixed with three volumes of ice-cold acetone at -20 °C for 2 h. The mixture was centrifuged at 4 °C and 9 000 *g* for 10 min and the sediment was freeze-dried. The dried cytosolic proteins were dissolved in a buffer (300 cm^3) containing urea (8.0 M), CHAPS (2.0 %, m/v), dithiothreitol (DTT) (30 mM), *Pharmalytes* (2.0 %, v/v) and small amount of bromphenol blue. Protein content was determined according to the Bradford (1976) method.

Two-dimensional electrophoresis of proteins was performed on the *Hoefer DALT Vertical System* (*Amersham Pharmacia Biotech*, Piscataway, USA). The isoelectric focusing (IEF) gels (pH 3 - 10, 18 cm long) were re-hydrated with the proteins (200 - 300 μg) dissolved in 0.3 cm^3 rehydration buffer and focused in a *IPGphor* (*Amersham Pharmacia Biotech*, Piscataway, USA) apparatus. The IPG strips were incubated in a SDS equilibration buffer [50 mM Tris-HCl of pH 8.8, 6 M urea, 30 % (v/v) glycerol, 2 % (m/v) SDS, 1 % (m/v) dithiothreitol (DTT), trace of bromophenol blue] for

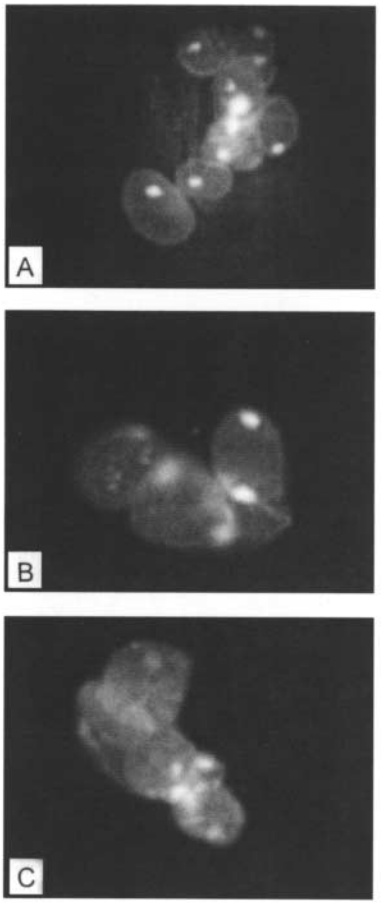

Fig. 1. Morphological changes of nuclei of suspension culture of *Taxus cuspidata*. *A* - control culture; *B* - Ce^{4+}-treated culture at day 4; *C* - Ce^{4+}-treated culture at day 6.

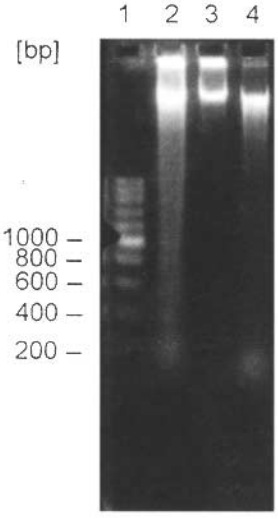

Fig. 2. Agar gel electrophoresis of nuclear DNA fragments from suspension cultures of *Taxus cuspidata*. *Lane 1* - DNA markers of 200 bp interval; *lane 2* - total DNA from the Ce^{4+}-treated culture at day 4; *lane 3* - DNA from control culture; *lane 4* - total DNA from the Ce^{4+}-treated culture at day 6.

20 min. After being equilibrated, the proteins were separated on 12.5 % SDS-polyacrylamide gels (250 × 200 × 1 mm) at 30 mA and silver-stained by *Protein-Silver Staining Kit* (*Amersham Pharmacia Biotech*). The 2-D PAGE image was analyzed on a computer using the *Image Master 2-D* software (*Amersham Pharmacia Biotech*).

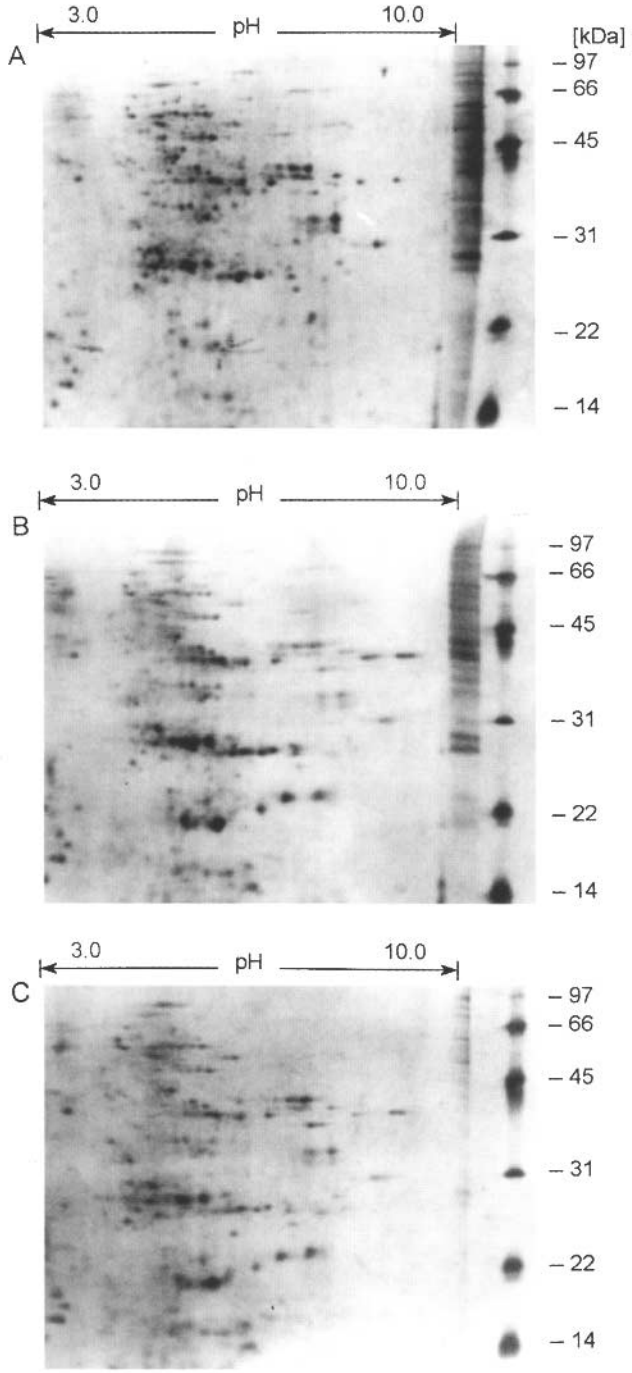

Fig. 3. Two-dimensional gel electrophoresis of the expressed proteins from suspension culture of *Taxus cuspidata*. *A* - from control culture; *B* - from the Ce^{4+}-treated culture at day 4; *C* - from the Ce^{4+}-treated culture at day 6.

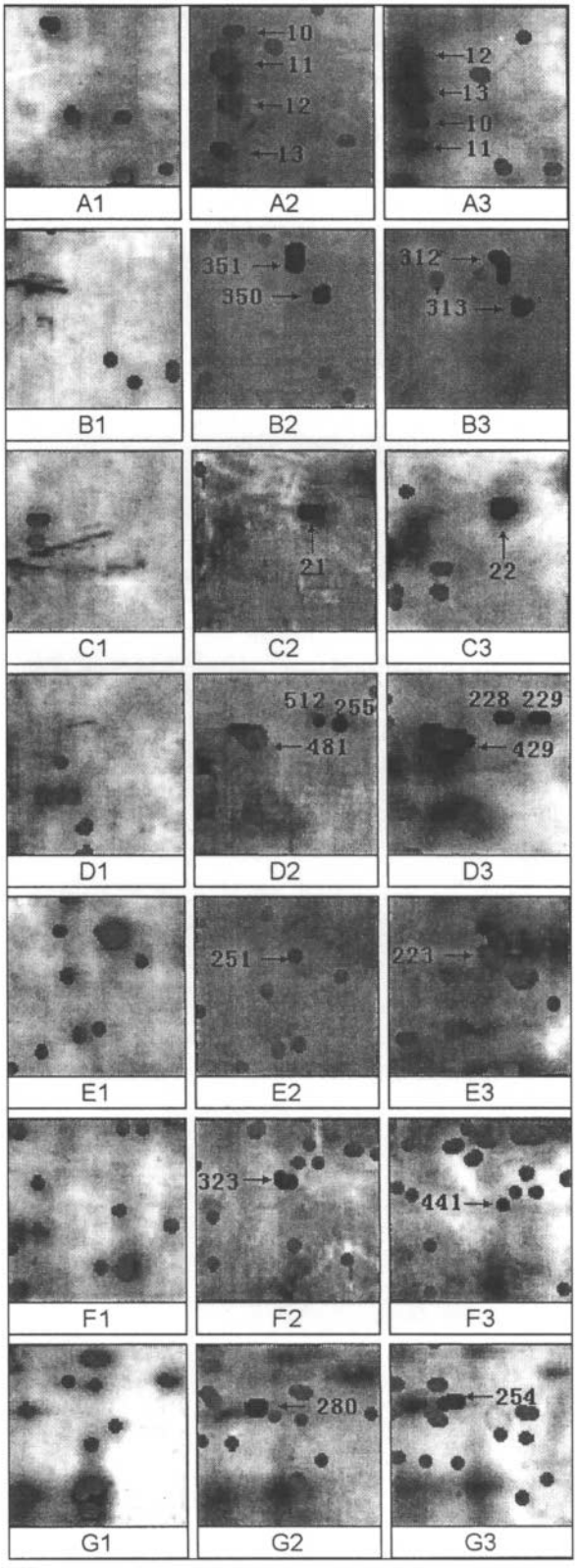

Fig. 4. Contrast of the protein spots different in the Ce^{4+}-treated and control cultures. A1 - G1 - from the control culture at day 4; A2 - G2 - from the Ce^{4+}-treated cells at day 4; A3 - G3 - from the Ce^{4+}-treated cells at day 6. The pictures were created based on different parts of Fig. 3 by using the *ImageMaster* software.

Propidium-iodine (PI) and *Hochest 33342* were purchased from *Sigma* (St. Louis, USA). Isoelectric focusing (IEF) gel and *Pharmalytes* were from *Amersham Pharmacia* (Piscataway, USA). All other chemicals used were of analytical grade and obtained commercially.

At days 4 and 6 after addition of Ce^{4+} obvious nuclear fragments were observed (Fig. 1). In contrast, no nuclear fragments were visible in the control culture. Also, DNA ladders, a characteristics of programmed cell death, occurred in the cells at 4 and 6 d after addition of Ce^{4+} (Fig. 2). The two-dimensional electrophoresis showed approximately 600 protein spots (Fig. 3). However, some differences existed between the proteins from the culture systems with and without Ce^{4+}. Thirteen of protein spots that were observed existed only in the Ce^{4+}-treated plants (Fig. 3). The pictures of specific protein spots created by using the *ImageMaster* software are shown in Fig. 4 and their volume, pI and molecular mass are listed in Table 1. The production of the "new" proteins might be ascribed to the programmed cell death induced by Ce^{4+}. The distributions of proteins from the Ce^{4+}-treated cultures at days 4 and 6 were almost the same, indicating tkat the protein expression was relatively stable within a certain period.

Programmed cell death has been widely studied in plant cells induced by some additives (Navarre and Wolpert 1999, Pedroso *et al.* 2000). Metal ions have been shown to be a kind of effective elicitors to induce cell death (Ikegawa *et al.* 1998, Pan *et al.* 2001). Ce^{4+} was reported to hydrolyze double-strand DNA and induce programmed cell death in suspension cultures of *Taxus cuspidata* (Shen *et al.* 2001, Yuan *et al.* 2001). In addition, the programmed cell death might also be induced as a result of the damage of DNA. It has been reported that the oxidative stress imposed on cells can activate highly sensitive reactions and induce programmed cell death (Levine *et al.* 1994, Cunnigaipur *et al.* 2001). Thus, the programmed cell death induced by Ce^{4+} might be attributed to its strong oxidative ability.

The isoelectric points and molecular masses of the proteins observed on gels after 2 D-electrophoresis varied mainly between (pH 4.0 - 8.0) and (40 - 80 kDa), respectively (Fig. 3). According to the distribution of proteins, they can be grossly divided into three groups: the proteins with approximately the same expression between the Ce^{4+}-treated and control culture, possibly can be ascribed to the basic metabolisms or to the "housekeeping genes" the proteins whose amounts decreased after addition of Ce^{4+}, are likely relevant to cell growth and division, and the proteins unavailable in control culture but occurred in the Ce^{4+}-treated systems, are probably produced as a result of the programmed cell death.

Table 1. Characteristics of protein spots observed in the Ce^{4+}-treated cultures. The data were calculated using the *ImageMaster* software.

Spot No.	Ce^{4+} at day 4 volume	pI	Mr [kDa]	Spot No.	Ce^{4+} at day 6 volume	pI	Mr [kDa]
10	10.272	3.3	31.2	12	19.199	3.2	29.9
11	23.530	3.3	29.1	13	28.753	3.2	27.5
12	14.071	3.3	26.4	10	16.215	3.2	25.6
13	13.739	3.3	23.4	11	13.798	3.2	23.9
21	15.815	6.6	21.4	22	26.331	6.6	21.6
251	7.159	5.4	17.5	223	8.698	5.3	18.0
255	7.656	6.8	10.8	229	11.681	6.9	11.6
280	10.466	7.7	50.7	254	13.460	7.7	50.5
323	7.761	5.7	29.5	441	5.943	5.8	28.1
350	13.775	4.4	17.0	313	17.303	4.3	17.1
351	18.960	4.3	19.4	312	22.564	4.2	19.6
481	30.530	6.4	10.0	429	49.322	6.4	10.4
512	5.758	6.7	11.1	228	12.379	6.7	11.7

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