Journal of Experimental Botany, Vol. 59, No. 8, pp. 2233–2240, 2008
doi:10.1093/jxb/ern090 Advance Access publication 23 April, 2008
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Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Is internucleosomal DNA fragmentation an indicator of programmed death in plant cells?

Andrea Kuthanova, Zdenek Opatrny and Lukas Fischer*

Charles University in Prague, Faculty of Science, Department of Plant Physiology, Vinicna 5, CZ 128 44 Prague 2, Czech Republic

Received 8 January 2008; Revised 3 March 2008; Accepted 4 March 2008

Abstract

Specific DNA fragmentation into oligonucleosomal units occurs during programmed cell death (PCD) in both animal and plant cells, usually being regarded as an indicator of its apoptotic character. This internucleosomal DNA fragmentation is demonstrated in tobacco suspension and leaf cells, which were killed immediately by freezing in liquid nitrogen, and homogenization or treatment with Triton X-100. Although these cells could not activate and realize the respective enzymatic processes in a programmed manner, the character of DNA fragmentation was similar to that in the cells undergoing typical gradual PCD induced by 50 μM $CdSO_4$. This internucleosomal DNA fragmentation was connected with the action of cysteine proteases and the loss of membrane, in particular tonoplast, integrity. The mechanisms of DNase activation in the rapidly killed cells, hypothetical biological relevance, and implications for the classification of cell death are discussed.

Key words: Non-PCD internucleosomal DNA fragmentation, programmed cell death, protease inhibitor, tobacco BY-2 cell line, vacuolar integrity.

Introduction

The process of programmed cell death (PCD) is based on the actively controlled degradation of intracellular components and facilitates removal of unwanted, incorrect, or damaged cells from multicellular organisms. PCD fulfils several essential functions in plant life: terminal differentiation of cells and sculpting tissues in plant development, responses to abiotic and biotic stress (e.g. hypersensitive response), and senescence connected with the reutilization of nutrients. Although individual processes differ in the triggering factors, it was suggested that all share a singular event: the action of the vacuole. Vacuole collapse, releasing sequestered hydrolases, is regulated by the cell itself and probably represents the 'point of no return'—the moment of cell death (Jones, 2001).

Not only the triggers, but also the executive phase and typical hallmarks of PCD, differ under different occasions. The classification of PCD has proved to be difficult; specialized types of PCD; autophagy, paraptosis or mitotic catastrophe differ from the apoptosis first identified both morphologically and in their mechanisms (Bröker *et al*., 2005). In animal apoptosis, specific proteases (mainly cytosolic cysteine/aspartate proteases caspases) activate executive enzymes involved in the digestion of cells from the inside (reviewed in Kumar, 2007). One of the first biochemical symptoms/indicators of animal apoptosis to be identified was the specific internucleosomal fragmentation of chromosomal DNA (Wyllie *et al*., 1980), although it may not be detectable, even in cells which undergo morphologically typical apoptosis (Oberhammer *et al*., 1993).

Cleavage of genomic DNA during apoptotic PCD is realized in two subsequent steps; an early cleavage into high molecular weight fragments (in sizes consistent with chromatin loop domains) and, later, an intense fragmentation, usually forming oligonucleosomal fragments (Brotner *et al*., 1995), which can be detected by DNA electrophoresis (in the whole tissue or cell population). *In situ* excessive DNA fragmentation can be visualized in individual cells by TUNEL reaction (TdT-mediated dUTP nick-end labelling; Gavrieli *et al*., 1992), however, without the possibility to distinguish between internucleosomal and random cleavage.

* To whom correspondence should be addressed. E-mail: lukasf@natur.cuni.cz
Abbreviations: PCD, programmed cell death; TUNEL, TdT-mediated dUTP nick-end labelling.

Several different DNA endonucleases have proved to be responsible for producing both large and small DNA fragments. The majority of those already identified mainly in animals, for example, DNase I (Oliveri *et al*., 2001), DNase II (Barry and Eastman, 1993), or CAD/CPAN/DFF40 (Enari *et al*., 1998) are activated by caspases. However, there are also some DNases, which belong to the caspase-independent biochemical pathway; for example, L-DNase II (Torriglia *et al*., 1998) or endonuclease G (Li *et al*., 2001), which are associated with intracellular acidification. Moreover, activation of some plant DNases involved in internucleosomal fragmentation during PCD can also be mediated by serine proteases (Ye and Varner, 1996). Serine proteases were also involved in internucleosomal fragmentation observed during necrotic (non-programmed) death of animal cells (Dong *et al*., 1997).

In this study, we present to our knowledge, the first description of non-PCD internucleosomal fragmentation of DNA in plant cells exposed to different treatments causing immediate cell death; freezing in liquid nitrogen, homogenization, and treatment with a detergent Triton X-100. Biochemical and cytological analysis of the rapidly killed cells was performed and compared with the cells treated with 50 μM and 1 mM $CdSO_4$ causing either programmed or non-programmed cell death (Fojtová and Kovařík, 2000; Kuthanová *et al*., 2004; Yakimova *et al*., 2006). Based on the results, a mechanism of the non-programmed DNase activation is proposed and our results are discussed with respect to their possible biological relevance and practical consequences for cell death classification.

Materials and methods

Plant material

The tobacco BY-2 cell line (*Nicotiana tabacum* L. cv. Bright Yellow 2) was maintained in the modified MS medium (Murashige and Skoog, 1962) and subcultured every seventh day according to Nagata *et al*. (1992). Three-day-old cell cultures (in the exponential phase of growth) were used in all experiments. Tobacco plants (cv. Samsun) were grown on solidified MS medium at 25 °C under a 16/8 h light/dark photoperiod and an irradiance of 120 W m^{-2} (leaves of 8-week-old plants were used in the experiments).

Induction of cell death

Freezing in liquid nitrogen: BY-2 cells (either untreated or just after the addition of $CdSO_4$ to a final concentration of 1 mM) or detached tobacco leaves (from 3-week-old plants) were killed by immediate freezing in liquid nitrogen for 3 min, followed by quick thawing at 60 °C (for 5 min).

Mechanical killing: BY-2 cells, filtered via Nalgene filter equipment (Nalgene Nunc International, Rochester, NY, USA) with a nylon mesh (20 μm) to reduce the culture volume by approximately 20 times, were killed mechanically (in 2 ml Eppendorf tubes) by homogenization with two steel balls in the mixer mill Retsch MM301 (Retsch Gmbh, Haan, Germany, 5 min, 25 oscillations s^{-1}, at 25 °C).

Following the treatments mentioned above, the cultures (of dead cells) were incubated at 25 °C for 1–7 d in darkness on a shaker. Frozen and thawed tobacco leaves were kept in a sterile tube with a small amount of MS medium to provide high humidity.

$CdSO_4$ and Triton X-100 treatment: $CdSO_4$ (at a final concentration of 50 μM and 1 mM) or Triton X-100 (at a final concentration of 5%) supplemented the cultivation medium. The cultures were further cultivated under standard cultivation conditions for 1–7 d. The viability of cells was determined using fluorescein diacetate staining according to (Wildholm, 1972).

Protease inhibitors

Protease inhibitors supplemented BY-2 cultures just before killing in liquid nitrogen, and DNA fragmentation was assessed 1 d afterwards. Inhibitors used were, inhibitor of cysteine proteases E-64c (in 1% methanol) at final concentrations of 10 μM and 50 μM (Moriyasu and Ohsumi, 1996); serine (cysteine) protease inhibitor PMSF (phenylmethanesulphonyl fluoride, in dimethyl sulphoxide) at final concentrations of 1 mM and 10 mM, and serine protease inhibitor aprotinin (in water) at final concentrations of 0.10 μM and 0.50 μM.

Evaluation of DNA integrity

Genomic DNA was isolated from 250 mg fresh weight of filtered BY-2 cells or tobacco leaves as described by Fojtová and Kovařík (2000).

The presence of oligonucleosomal fragments was evaluated by electrophoresis (at 0.1–0.2 V cm^{-1}) in 1.8% agarose gel in the presence of ethidium bromide.

TUNEL reaction, terminal deoxynucleotidyl transferase (Tdt)-mediated deoxyuridinetriphosphate (dUTP)-nick labelling, was used to detect 3′-OH termini in fragmented nuclear DNA. The procedure was performed according to Jones *et al*. (2001) using the TMR-red (red fluorescence) *in situ* cell death detection kit (Roche Diagnostic GmbH, Mannheim, Germany).

Cell integrity assessment

The integrity of dying BY-2 cells was evaluated under a confocal microscope TCS SP2 AOBS (TCS NT, Leica, Heidelberg, Germany) equipped with Nomarski optics (DIC). The membrane system was visualized either using transgenic line BY-GV7, stably expressing GFP-AtVam3p fusion protein localized in the vacuolar membranes (Kutsuna and Hasezawa, 2002) or by an FM4-64 probe (Molecular Probes) used for the endomembrane system. FM4-64 (dissolved in DMSO) was added to the BY-2 culture to a final concentration of 32 μM just before the treatment with $CdSO_4$ or 24 h before freezing. The vacuolar integrity was, in all cases, evaluated 24 h after FM4-64 addition, when it should preferentially stain vacuoles according to Kutsuna and Hasezawa (2002). The cells were observed under a confocal microscope TCS SP2 AOBS equipped with ArKr laser and a filter set for TRITC and filter set for FITC. Objective lenses Plan Apo (magnification 63×, numerical aperture 1.2) were used for all observations.

Results

DNA fragmentation

Internucleosomal fragmentation of genomic DNA was studied in tobacco cells subjected to different conditions inducing either rapid or slow cell death. Integrity of the

total DNA at different times after the treatment was analysed by agarose gel electrophoresis and, in selected cases, by TUNEL reaction.

Cell death induction and electrophoretic detection of internucleosomal fragmentation: DNA isolated from untreated tobacco BY-2 cells was never fragmented into oligonucleosomal units and formed a single, high molecular weight band (Fig. 1A) even after prolonged cultivation for 2 weeks (data not shown). To induce slow, programmed cell death of the cells, the culture was treated with 50 μM $CdSO_4$, causing a gradual decrease in cell viability between the second and the fifth days of the treatment (Fojtová and Kovařík, 2000; Kuthanová *et al*., 2004). Specific oligonucleosomal fragments were clearly detected after 7 d; i.e. approximately from the time when

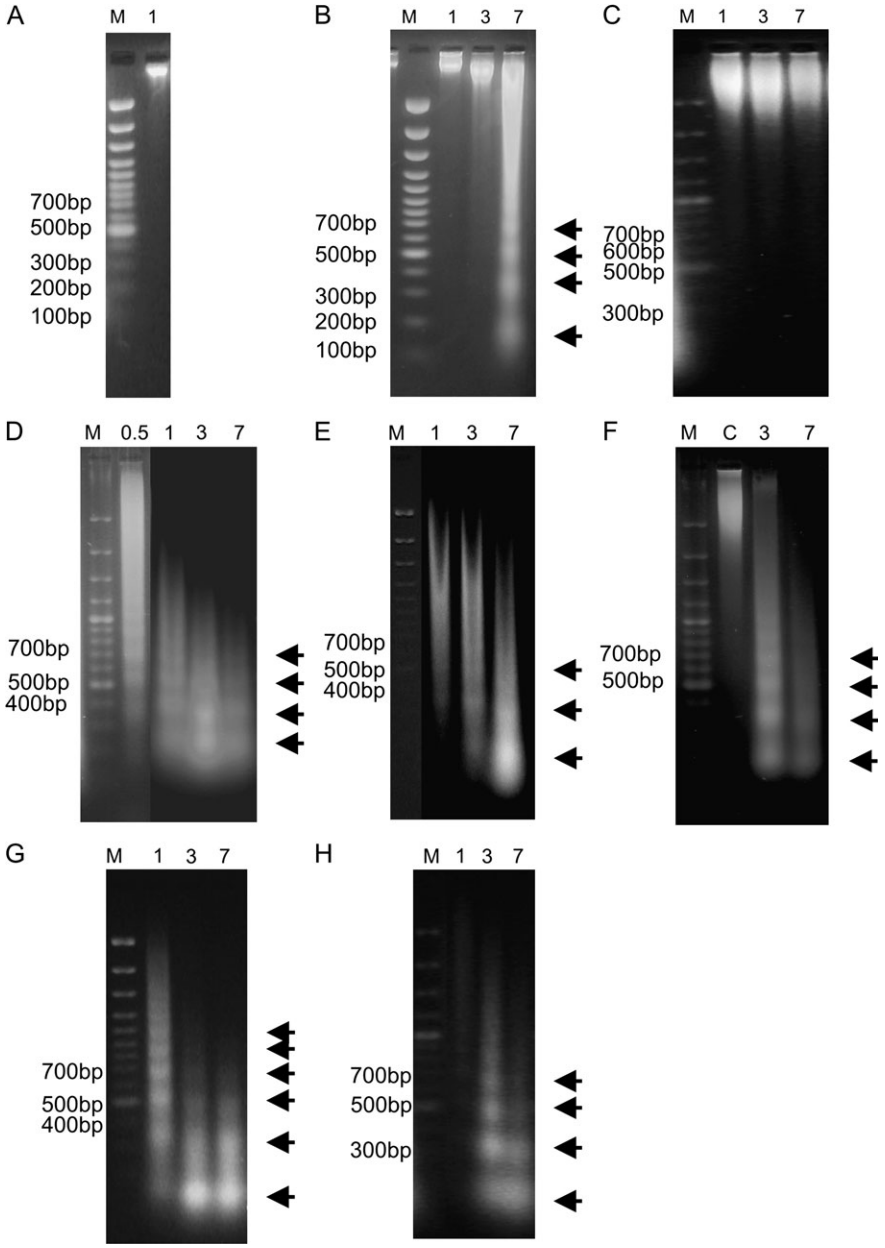

Fig. 1. Internucleosomal fragmentation of tobacco genomic DNA during 7 d after different treatments. (A) Untreated control BY-2 cells in the exponential phase of growth, (B) BY- 2 cells treated with 50 μM $CdSO_4$, (C) BY-2 cells treated with 1 mM $CdSO_4$, (D) BY-2 cells killed by freezing in liquid nitrogen and further cultivated after thawing at 25 °C, (E) BY-2 cells killed by liquid nitrogen and further cultivated at 25 °C in the presence of 1 mM $CdSO_4$, (F) tobacco leaves frozen in liquid nitrogen and cultivated at 25 °C, (G) BY-2 cells killed mechanically by homogenization in a mixer mill and further cultivated at 25 °C, (H) BY-2 cells treated with 5% Triton X-100. DNA isolated from the cells (representative samples from 2–5 independent experiments) was separated in 1.8% agarose gel in the presence of ethidium bromide. Arrows indicate positions of oligonucleosomal fragments. Line markers: M, molecular mass marker; C, untreated control leaves; 0.5, 1, 3, 7, d of culture (exposure in case of $CdSO_4$ and Triton X-100 treatments).

practically all cells in the culture died as determined by fluorescein diacetate staining (Fig. 1B). BY-2 cells treated with 1 mM $CdSO_4$ died quickly within several hours. DNA isolated from these cells during subsequent cultivation for 7 d remained without or with almost undetectable fragmentation (Fig. 1C). Freezing of BY-2 cells in liquid nitrogen, followed by quick thawing resulted in immediate cell death. DNA isolated from these cells (subsequently cultivated at 25 °C) was fragmented as early as the first day after thawing. The progressive fragmentation continued, yielding shorter oligonucleosomal fragments, during prolonged cultivation (Fig. 1D). When $CdSO_4$ (at 1 mM final concentration) was added to the culture just before freezing, the progression of DNA fragmentation was partially slowed as compared to the culture frozen without $CdSO_4$ (Fig. 1E). Fragmentation of DNA isolated from tobacco leaves frozen in liquid nitrogen, thawed, and further cultivated at 25 °C was clearly detectable from the third day of cultivation after the thawing, although particular oligonucleosomal fragments were less evident (Fig. 1F). Mechanical disintegration of BY-2 cells by homogenization in a mixer mill or the addition of Triton X-100 to a final concentration of 5% also resulted in pronounced internucleosomal fragmentation of DNA isolated from the cultures during subsequent cultivation at 25 °C for 1–7 d (Fig. 1G, H, respectively).

TUNEL reaction: TUNEL reaction was used in parallel with electrophoretic analysis for *in situ* visualization of fragmented DNA in selected treatments. In rapidly killed cells (by freezing in liquid nitrogen) the fluorescent signal was observed in almost 50% nuclei (referred as TUNEL positive nuclei) by 3 d after freezing (almost 80% of nuclei were malformed after the treatment; Fig. 2). In the culture treated with 50 μM $CdSO_4$ the frequency of TUNEL-positive nuclei reached about 40% after 7 d (Fig. 2D). Practically no TUNEL-positive nuclei were detected in untreated control cells and in the cells treated with 1 mM $CdSO_4$ (Fig. 2D; Kuthanová *et al.*, 2004).

Morphology of the cells

To assess the impact of structural changes for the induction of DNA fragmentation, the cellular, and in particular vacuolar, integrity were analysed after staining with the membrane probe FM4-64 and using Nomarski differential contrast. To confirm the specificity of FM4-64 staining, the cellular integrity was evaluated in the BY-GV7 transgenic cell line with GFP targeted into the tonoplast (Kutsuna and Hasezawa, 2002). Untreated exponential BY-GV7 culture (as well as the untransformed BY-2 line) was characterized by long files of small isodiametric cells with apparent cytoplasmatic strands and several middle-sized vacuoles (Fig. 3A–D). Treatment with 50 μM $CdSO_4$ stopped cell division and resulted in typical elongation during the first 3 d of the treatment (Fig. 3E–H). In the cells, few large and numerous small spherical vacuoles/vesicles were visible

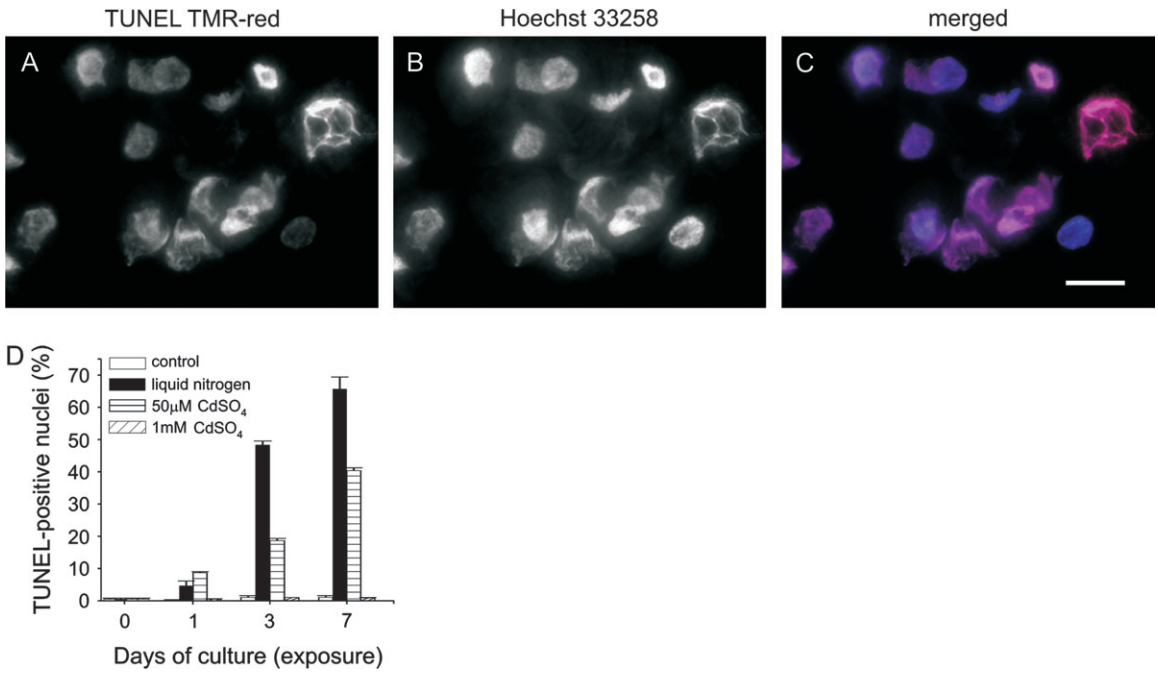

Fig. 2. Detection of DNA fragmentation *in situ* by TUNEL reaction. (A–C) BY-2 cells killed by liquid nitrogen and cultivated after thawing at 25 °C for 3 d, (A) TUNELpositive nuclei in red, (B) nuclei stained by Hoechst 33258 in blue, (C) merged picture; (D) frequency of TUNEL-positive nuclei during 7 d cultivation of untreated BY-2 cells, cells killed by freezing in liquid nitrogen, and cells treated with 50 μM and 1 mM $CdSO_4$. Scale bars=20 μm. Histograms represent the mean +SE of three independent experiments.

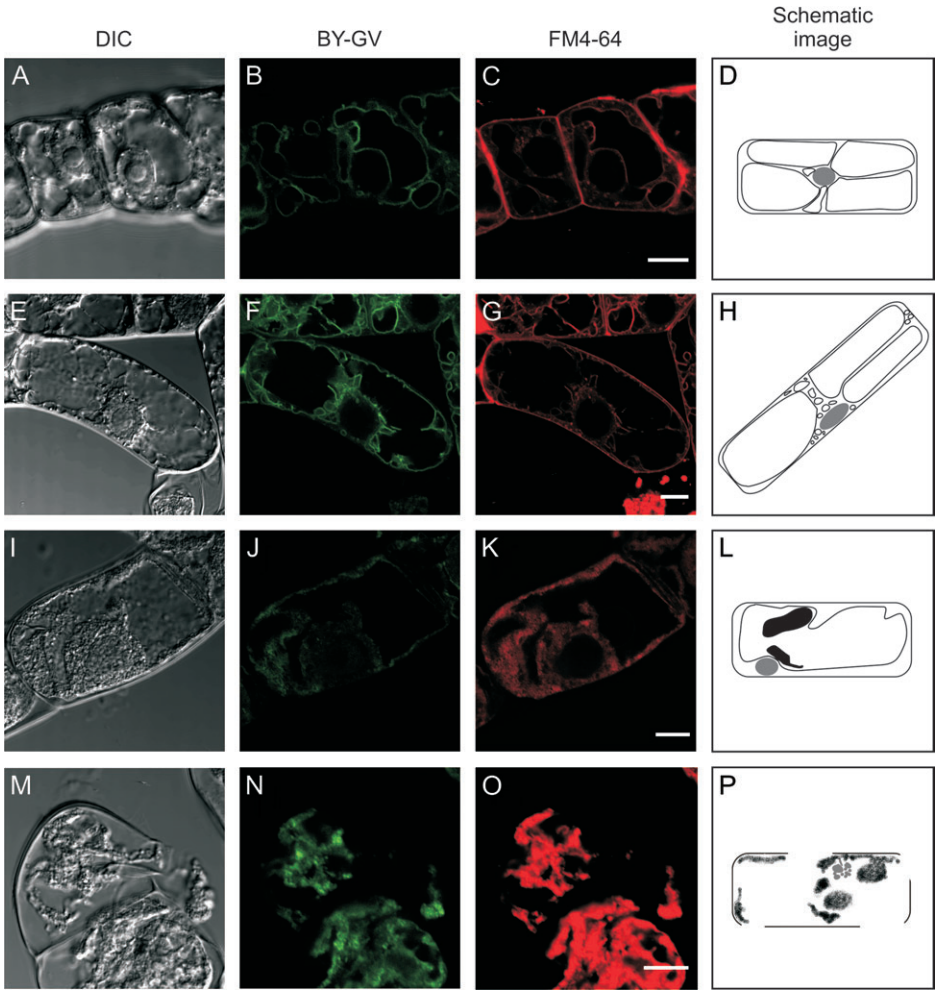

Fig. 3. Morphology of BY-GV7 cells on 1 d after different treatments. (A–D) Untreated cells, (E–H) elongated cells in the presence of 50 μM $CdSO_4$ with small rounded vacuoles, (I–L) plasmolysed dead cells in the presence of 1 mM $CdSO_4$ with preserved vacuolar membranes, (M–P) disintegrated cells with diffusional signal of tonoplast after freezing in liquid nitrogen. (A, E, I, M) Nomarski differential contrast, (B, F, J, N) tonoplast targeted GFP signal, (C, G, K, O) staining with vacuolar membrane probe FM4-64, (D, H, L, P) schematic figure of membrane system. Scale bars=10 μm.

in the cytoplasm (Fig. 3F–H). Treatment of BY-GV7 (BY-2) cells with 1 mM $CdSO_4$ resulted in cell death within a few hours, characterized by protoplast shrinkage. The internal architecture of the cells was not visually disturbed; vacuoles, cell walls, and plasma membranes looked to be integral, even though the cells underwent plasmolysis (Fig. 3I–L). Cells rapidly killed by either freezing in liquid nitrogen (Fig. 3M–P) or direct mechanical disintegration (data not shown) lost their integrity immediately. Disruption of vacuoles and cell walls, and swelling of membranes was observed just after the treatment. The FM4-64 signal, a membrane-staining probe, was dispersed as well as the GFP signal in the BY-GV7 transgenic cells (Fig. 3N, O). Similar disintegration of membranes was observed after several hours of Triton X-100 treatment (data not shown).

Protease inhibitors

Proteases play an important role in PCD and are usually responsible for DNase activation. In order to examine the involvement of different proteases in the internucleosomal fragmentation in rapidly killed cells, specific protease inhibitors were tested. While cysteine protease inhibitor E-64c strongly suppressed DNA fragmentation in a concentration-dependent manner (Fig. 4A), aprotinin, predominantly inhibiting serine proteases, and PMSF, inhibiting serine and, partially, at higher concentrations, cysteine proteases as well (Renier *et al*., 2004), had only minor effects on DNA fragmentation in BY-2 cells killed by freezing in liquid nitrogen (Fig. 4B, C, respectively). The solvents (methanol and dimethyl sulphoxide) used for inhibitor stock solutions did not influence internucleosomal fragmentation at all (data not shown).

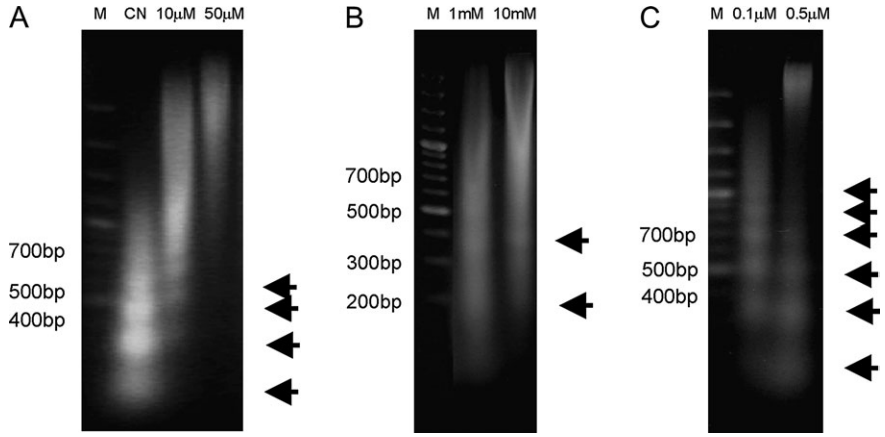

Fig. 4. Effect of protease inhibitors on DNA fragmentation in BY-2 cells frozen in liquid nitrogen and incubated for 1 d in the presence of (A) cysteine protease inhibitor E-64c (10 μM, 50 μM), (B) serine (partially also cysteine) protease inhibitor PMSF (1 mM, 10 mM), and (C) serine protease inhibitor aprotinin (0.10 μM, 0.5 μM). DNA (representative samples from 2–4 independent experiments) was separated in 1.8% agarose gel in the presence of ethidium bromide. Arrows indicate the positions of oligonucleosomal fragments Line markers: M, molecular mass marker; CN, control cells (frozen and thawed in liquid nitrogen) with no inhibitor added.

Discussion

DNA fragmentation into oligonucleosomal units was originally described during apoptosis in animal cells (Wyllie *et al*., 1980). In plant cells, progressive internucleosomal fragmentation was documented during programmed cell death (PCD) in abiotically stressed tobacco BY-2 cell line cultivated at low temperature (4 °C; Koukalová *et al*., 1997) or treated with 50 μM $CdSO_4$ (Fojtová and Kovařík, 2000). Although electrophoretic detection of internucleosomal fragmentation is usually considered as a typical symptom and indicator of certain PCD types, this fragmentation in plant cells which demonstrably did not undergo PCD (Fig. 1D, F, G, H) was clearly documented. Besides, using the TUNEL reaction (Gavrieli *et al*., 1992) the presence of fragmented DNA was confirmed *in situ* in the majority of nuclei (Fig. 2). The internucleosomal fragmentation was observed after three different treatments causing rapid cell death, but it did not accompany moderately quick death (within several hours) of cells treated with 1 mM $CdSO_4$ (Fojtová and Kovařík, 2000; Fig. 1C). Since the presence of 1 mM $CdSO_4$ itself did not substantially inhibit DNA fragmentation in cells killed by freezing (Fig. 1E), morphological differences between the cells in individual treatments were sought. All three treatments inducing rapid internucleosomal fragmentation were characterized by a loss of integrity of the membrane system, in particular the tonoplast, and this was clearly visible under the confocal microscope using both the membrane staining probe FM4-64 and tonoplast targeted GFP in the BY-GV7 (Kutsuna and Hasezawa, 2002) transgenic cells (Fig. 3M–P). By contrast, in cells treated with 1 mM $CdSO_4$, killed within several hours, the tonoplast integrity seemed to be preserved (Fig. 3I–L) corresponding with no DNA fragmentation in these cells. Intensive changes in the vacuolar system (splitting of vacuoles) accompanied stepwise induction of PCD in the cells treated with 50 μM cadmium (Fig. 3E–H) and preceded the loss of the cell integrity and internucleosomal fragmentation both observed in the late phases of cell death.

The morphological analysis thus indicated that internucleosomal fragmentation was connected with the loss of vacuole integrity, in agreement with the general assumption that vacuole collapse, releasing sequestered hydrolases, is a common mechanism of the majority of plant PCD types (Jones, 2001). Regulated vacuolar collapse accompanied by the release of hydrolytic enzymes was repeatedly documented during plant PCD; xylem differentiation (Groover and Jones, 1999; Kuriyama, 1999), death of cells in the aleurone layer (Bethke *et al*., 1999), somatic embryogenesis (Filonova *et al*., 2000), and the hypersensitive reaction (Hatsugai *et al*., 2004). Our results indicate, that the executive phase of PCD, normally induced by regulated (programmed) vacuole collapse, can be mimicked by its artificial violent disintegration.

After artificial vacuole disintegration in our experiments, either active DNases or activating proteases might have been released from the impaired vacuoles. In the case of xylem differentiation, ZEN 1 DNase is transported into vacuoles directly in an active form just prior to vacuolar collapse, and played a major role in the subsequent non-specific nuclear DNA degradation (Ito and Fukuda, 2001). By contrast, in typical animal apoptosis, inactive DNase proenzymes, for instance specific CAD nucleases (Enari *et al*., 1998), DNase I (Oliveri *et al*., 2001), and DNase II (Barry and Eastman, 1993), are activated by cysteine proteases caspases. Alternative caspase-independent pathways, which involve serine proteases, can also mediate the activation of DNases, as in the case of L-DNase II in animal cells (Torriglia *et al*., 1999). In plants the mechanism of DNase activation is

less well understood. Using specific protease inhibitors Kusaka *et al*. (2004) reported the involvement of cysteine proteases during toxin-induced PCD in plant cells. Caspases were not identified in plants, but plant-specific cysteine proteases called vacuolar-processing enzymes (VPEs) are supposed to substitute their function, as was demonstrated during the PCD triggered by tobacco mosaic virus (Hatsugai *et al*., 2004; Kuroyanagi *et al*., 2005). Since cysteine protease inhibitor E-64c effectively blocked DNA fragmentation accompanied by vacuolar disintegration (Figs 3, 4), VPEs present in the vacuole might be good candidates for the DNase activation in our experiments. However, DNA fragmentation mediated by VPEs was not internucleosomal during virus-induced PCD (Hatsugai *et al*., 2004), suggesting that different DNases might participate in DNA cleavage in our experiments. Although the precise effectors of DNA cleavage and DNase activation remain unknow, the results indicate that the same enzymatic apparatus might be involved in realization of the internucleosomal fragmentation during both slow programmed cell death and rapid accidental death in our tobacco cells. It contrasts with the situation in animals, where typical apoptotic internucleosomal fragmentation is mediated by cysteine proteases caspases, while in necrotically (non-programmed) dying cells, DNases were activated by serine proteases (Dong *et al*., 1997).

Plant cells (at least those used in our study) appeared to be predisposed for quick autolysis, for example, there was an enzymatic apparatus ready to mediate quick specific DNA cleavage triggered by either rapid accidental vacuole disintegration or programmed vacuolar collapse. What could the reason be for this predisposition? Since mechanical damage of plant tissues is relatively common and such injuries might serve as an entry for many pathogens, quick autolysis of damaged cells could represent another component of the plant defensive mechanism complementary to hypersensitive cell death. Although the biological relevance remains unclear, our results document that internucleosomal fragmentation in rapidly killed tobacco cells is hardly distinguishable from that specific for PCD. Therefore, DNA fragmentation into oligonucleosomal units should not be considered as an indicator of PCD without parallel evaluation of morphological changes of intracellular components (in particular, the progression of the loss of vacuolar integrity) especially in quickly dying cells. Moreover, our findings should be taken into account in the interpretation of any results based on the detection of PCD-related protease or DNase activities in cell-free extracts.

Acknowledgements

We are grateful to Professor Seiichiro Hasezawa (The University of Tokyo) for providing us with the BY-GV7 tobacco cell line. The work was supported by grants from the Ministry of Education, Youth and Sports of the Czech Republic (MSM 0021620858, LC06034, and LC06004).

References

Barry MA, Eastman A. 1993. Identification of deoxyribonuclease II as an endonuclease involved in apoptosis. *Archives of Biochemistry and Biophysics* **300,** 440–450.

Bethke PC, Lonsdale JE, Fath A, Jones RL. 1999. Hormonally regulated programmed cell death in barley aleurone cells. *The Plant Cell* **6,** 1033–1046.

Bröker LE, Kruyt FAE, Giaccone G. 2005. Cell death independent of caspases: a review. *Clinical Cancer Research* **11,** 3155–3162.

Brotner CD, Oldenburg NBE, Cidlowski JA. 1995. The role of DNA fragmentation in apoptosis. *Trends in Cell Biology* **5,** 21–26.

Dong Z, Saikumar P, Weinberg JM, Venkatachalam MA. 1997. Internucleosomal DNA cleavage triggered by plasma membrane damage during necrotic cell death. Involvement of serine but not cysteine proteases. *The American Journal of Pathology* **151,** 1205–1213.

Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S. 1998. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* **391,** 43–50.

Filonova LH, Bozhkov PV, Brukhin VB, Daniel G, Zhivotovsky B, von Arnold S. 2000. Two waves of programmed cell death occur during formation and development of somatic embryos in the gymnosperm, Norway spruce. *Journal of Cell Science* **113,** 4399–4411.

Fojtová M, Kovařík A. 2000. Genotoxic effect of cadmium is associated with apoptotic changes in tobacco cells. *Plant, Cell and Environment* **23,** 531–537.

Gavrieli Y, Sherman Y, Ben-Sasson SA. 1992. Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. *Journal of Cell Biology* **119,** 493–450.

Groover A, Jones AM. 1999. Tracheary element differentiation uses a novel mechanism coordinating programmed cell death and secondary cell wall synthesis. *Plant Physiology* **119,** 375–384.

Hatsugai N, Kuroyanagi M, Yamada K, Meshi T, Tsuda S, Kondo M, Nishimura M, Hara-Nishimura I. 2004. A plant vacuolar protease, VPE, mediates virus-induced hypersensitive cell death. *Science* **305,** 855–858.

Ito J, Fukuda H. 2002. ZEN1 nuclease is a key enzyme in the degradation of nuclear DNA during programmed cell death of tracheary elements. *The Plant Cell* **14,** 3201–3211.

Jones AM. 2001. Programmed cell death in development and defense. *Plant Physiology* **125,** 94–97.

Jones AM, Coimbra S, Fath A, Sottomayor M, Thomas H. 2001. Programmed cell death assays for plants. *Methods in Cell Biology* **66,** 437–451.

Koukalová B, Kovařík A, Fajkus J, Široký J. 1997. Chromatin fragmentation associated with apoptotic changes in tobacco cells exposed to cold stress. *FEBS Letters* **414,** 289–92.

Kumar S. 2007. Caspase function in programmed cell death. *Cell Death and Differentiation* **14,** 32–43.

Kuriyama H. 1999. Loss of tonoplast integrity programmed in tracheary element differentiation. *Plant Physiology* **121,** 763–774.

Kuroyanagi M, Yamada K, Hatsugai N, Kondo M, Nishimura M, Hara-Nishimura I. 2005. Vacuolar processing enzyme is essential for mycotoxin-induced cell death in *Arabidopsis thaliana*. *Journal of Biological Chemistry* **280,** 32914–32920.

Kusaka K, Tada Y, Shigemi T, Sakamoto M, Nakayashiki H, Tosa Y, Mayama S. 2004. Coordinate involvement of cysteine protease and nuclease in the executive phase plant apoptosis. *FEBS Letters* **578,** 363–367.

Kuthanová A, Gemperlová L, Zelenková S, Eder J, Macháčková I, Opatrný Z, Cvikrová M. 2004. Cytological changes and alterations in polyamine contents induced by cadmium in tobacco BY-2 cells. *Plant Physiology and Biochemistry* **42,** 149–156.

Kutsuna N, Hasezawa S. 2002. Dynamic organization of vacuolar and microtubule structures during cell cycle progression in synchronized tobacco BY-2 cells. *Plant and Cell Physiology* **43,** 965–973.

Li LY, Luo X, Wang X. 2001. Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature* **412,** 95–99.

Moriyasu Y, Ohsumi Y. 1996. Autophagy in tobacco suspension-cultured cells in response to sucrose starvation. *Plant Physiology* **111,** 1233–1241.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15,** 473–97.

Nagata T, Nemoto Y, Hasezawa S. 1992. Tobacco BY-2 cell line as the 'HeLa' cell in the cell biology of higher plants. *International Review of Cytology* **132,** 1–30.

Oberhammer F, Wilson JW, Dive C, Morris ID, Hickman JA, Wakeling AE, Walker PR, Sikorska M. 1993. Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. *The EMBO Journal* **12,** 3679–3684.

Oliveri M, Daga A, Cantoni C, Lunardi C, Millo R, Puccetti A. 2001. DNase I mediates internucleosomal DNA degradation in human cells undergoing drug-induced apoptosis. *European Journal of Immunology* **31,** 743–751.

Renier AL, van der Hoorn Leeuwenburgh MA, Bogyo M, Joosten MHAJ, Peck SC. 2004. Activity profiling of papain-like cysteine proteases in plants. *Plant Physiology* **135,** 1170–1178.

Torriglia A, Negri C, Chaudun E, Prosperi E, Courtois Y, Counis MF, Scovassi AI. 1999. Differential involvement of DNases in HeLa cell apoptosis induced by etoposide and long term-culture. *Cell Death and Differentiation* **6,** 234–244.

Torriglia A, Perani P, Brossas JY, Chaudun E, Treton J, Courtois Y, Counis MF. 1998. L-DNase II, a molecule that links proteases and endonucleases in apoptosis, derives from the ubiquitous serpin leukocyte elastase inhibitor. *Molecular and Cellular Biology* **18,** 3612–3619.

Wildholm JM. 1972. The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. *Stain Technology* **47,** 189–194.

Wyllie AH, Kerr JF, Currie AR. 1980. Cell death: the significance of apoptosis. *International Review of Cytology* **68,** 251–306.

Yakimova ET, Kapchina-Toteva VM, Laarhoven LJ, Harren FM, Woltering EJ. 2006. Involvement of ethylene and lipid signalling in cadmium-induced programmed cell death in tomato suspension cells. *Plant Physiology and Biochemistry* **44,** 581–589.

Ye ZH, Varner JE. 1996. Induction of cysteine and serine proteases during xylogenesis in *Zinnia elegans*. *Plant Molecular Biology* **30,** 1233–1246.