

# Cell cycle phase-specific death response of tobacco BY-2 cell line to cadmium treatment

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## ABSTRACT

**The character of programmed cell death (PCD) in plants differs in connection with the context, triggering factors and differentiation state of the target cells. To study the interconnections between cell cycle progression and cell death induction, we treated synchronized tobacco BY-2 cells with cadmium ions that represent a general abiotic stressor influencing both dividing and differentiated cells *in planta*. Cadmium induced massive cell death after application in all stages of the cell cycle; however, both the progression and the forms of the cell death differed pronouncedly. Apoptosis-like PCD induced by cadmium application in the S and G2 was characterized by pronounced internucleosomal DNA fragmentation. In contrast, application of cadmium in M and G1 phases was not accompanied by DNA cleavage, indicating suppression of autolysis and non-programmed character of the death. We interpret these results in the context of the situation *in planta*, where the induction of apoptosis-like PCD in the S and G2 phase might be connected with a need to preserve genetic integrity of dividing meristematic cells, whereas suppression of PCD response in differentiated cells (situated in G1/G0 phase) might help to avoid death of the whole plant, and thus enable initiation of the recovery and adaptation processes.**

*Key-words:* abiotic stressor; PCD suppression; programmed cell death (PCD).

## INTRODUCTION

Programmed cell death (PCD) fulfils several basic functions in plants and other multicellular organisms: it participates in tissue differentiation and morphogenesis; plays a key role in the defense against different pathogens and stressors; and safeguards genetic and physiological integrity of individual cells. Depending on the respective context of cell death, the triggering factors and the mechanisms substantially differ. Morphogenesis-related PCD is topologically well-predetermined and usually regulated by internal factors, in

particular phytohormones (e.g. Fath *et al.* 2000; Obara & Fukuda 2004). The hypersensitive response is induced by pathogen elicitors and mostly localized at the site of infection to prevent pathogen spreading through the plant (Dangl, Dietrich & Richberg 1996; Kadota *et al.* 2004). On the contrary, abiotic stress conditions affect usually multiple tissues and organs simultaneously. The programmed death of certain cells probably primarily serves to maintain physiological and genetic integrity and to ensure the survival of the whole plant. Elimination of genetically impaired cells by PCD can be expected to be essential mainly for meristems, especially their 'stem' cells that divide repeatedly. It contrasts to morphogenesis- and pathogen-related programmed death, where PCD impacts predominantly either differentiating or differentiated cells (mostly situated in G1/G0 phase of the cell cycle).

The response of plant cells to various triggers thus probably depends on both their differentiation state and their position in the cell cycle, similar to the situation known from animals. Although the connections between PCD and cell cycle were investigated in plant systems, the studies focused on inducers that were more relevant for differentiated cells (Herbert *et al.* 2001; Kadota *et al.* 2004). Whereas only fragmentary data are available for dividing plant cells, the induction of PCD in animal cells was described in detail in the context of a sophisticated system of cell cycle checkpoints, which predominantly serve to prevent cancer transformation and uncontrolled proliferation of the cells (Francis 2003).

It is technically very difficult to study the causal connections between cell cycle and PCD directly in meristematic plant tissues, because only a small population of cells divides, and because these divisions are asynchronous. Consequently, most of the available data have been obtained using suspension cultures as models, in particular the tobacco BY-2 cell line (Nagata, Nemoto & Hasezawa 1992). Progressive cell death could be induced in these cells in response to various forms of abiotic stresses such as oxidative stress (Reichheld *et al.* 1999; Houot *et al.* 2001; Jang *et al.* 2005; De Pinto *et al.* 2006), carbon starvation (Bolduc & Brisson 2002), high temperature (Jang *et al.* 2005) and cadmium treatment (Kuthanova *et al.* 2004). Similarly, a rapid decrease in cell viability was induced by pathogen elicitors (Kadota *et al.* 2004) or certain phytohormones (ethylene – Herbert *et al.* 2001;

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jasmonic acid – Swiatek *et al.* 2002). The cellular responses to these factors often strongly differed in a concentration-dependent manner, with clear manifestation of either programmed or necrotic cell death features (Houot *et al.* 2001; De Pinto *et al.* 2006; Reape, Molony & McCabe 2008). The relationship between cell death and cell cycle progression has been studied in two different experimental systems and in response to different inducers, such that the results are hardly comparable. Kadota *et al.* (2004) demonstrated that cryptogein (a bacterial elicitor) triggered the expression of defense-related genes and cell death only when applied in G1 and S phase, whereas the response was substantially suppressed when the elicitor was administered during G2 and M phase. The authors suggested that this result reflected a natural suppression of hypersensitive cell death in dividing cells, such as meristems, *in planta*. By contrast, cryptogein-induced cell death in BY-2 cells in G1 was consistent with the induction of hypersensitive cell death in mesophyll cells that are in G0 or G1 phase (Kadota & Kuchitsu 2006). In a different experimental set-up, Herbert *et al.* (2001) focused on immediate induction of PCD during cell cycle progression. Upon treatment with ethylene, the cells displayed peaks of mortality for application of the hormone (administered in two different time points) in the S phase and at the G2/M transition. The dead cells displayed apoptotic features such as DNA fragmentation as detected by the ApoTag test (Oncor, Gaithersburg, MD, USA) visualizing 3'OH termini (Herbert *et al.* 2001).

This rapid timing of the ethylene-induced PCD contrasts with the effects of cadmium ions on BY-2 cells, as Fojtova & Kovarik (2000) demonstrated that typical apoptotic DNA fragmentation induced by low concentrations (50  $\mu\text{M}$   $\text{CdSO}_4$ ) became detectable as late as several days after the treatment. Rapid death, induced by high concentrations (1 mM  $\text{CdSO}_4$ ), was comparable in speed with the ethylene response described by Herbert *et al.* (2001), but this death was without the typical symptoms of PCD (Fojtova & Kovarik 2000; Kuthanova *et al.* 2004; Kuthanova, Opatrny & Fischer 2008). This comparison suggests that the features of PCD induced by the 'senescence trigger' ethylene can be hardly generalized for other PCD inducers more relevant for dividing cells.

To get insight into more general forms of PCD, global abiotic stressors – such as cadmium – can be studied in the context of cell-cycle progression. Therefore, we followed the long-term response of synchronized, mitotic active BY-2 cells to cadmium that was administered at specific points of the cell cycle. We observed that incidence of internucleosomal DNA fragmentation, an indicative marker of apoptotic PCD, strongly depended on the phase of the cell cycle, in which cadmium was applied. The observed cross-talk between cell cycle and PCD is interpreted as a reflection of evolutionary ancient response of plant cells that is targeted to ensure genetic integrity in mitotic cells. Simultaneous suppression of PCD in differentiated cells might, on the other hand, support survival of the whole plant body.

## MATERIALS AND METHODS

### Plant material

The tobacco cell line BY-2 (*N. tabacum* L. cv. Bright Yellow 2) was maintained in modified Murashige & Skoog medium and subcultured every 7 d according to Nagata *et al.* (1992). The cells were cultivated at 25 °C in darkness in orbital shaker IKA 125B (IKA Labortechnik, Staufen, Germany) at 150 r.p.m. (orbital diameter 4 mm).

### Synchronization

The BY-2 cells (10 times diluted stationary, 7-day-old culture) were synchronized with aphidicolin (APC, final concentration 15  $\mu\text{M}$ ), a reversible inhibitor of DNA polymerase  $\alpha$ , or hydroxyurea (HU, final concentration 4 mM), a ribonucleotide reductase inhibitor. The method for synchronization (Nagata *et al.* 1992) was slightly modified. After 24 h of incubation of BY-2 cells in the medium containing APC/HU, the anti-replicative drug was removed by four successive washes with 3% sucrose.

### Mitotic index (MI) determination

Cell cycle progression after releasing cells from APC/HU was monitored by measuring MI (the sum of cells in mitosis as a percentage of all cells). To determine the MI and visualize the nuclear morphology, Hoechst 33258 substance (Invitrogene, Carlsbad, CA, USA) was used. Two  $\mu\text{L}$  of stock solution (10 mg  $\text{mL}^{-1}$  Hoechst 33258 in dimethylsulphoxide) was added to 1 mL of the suspension culture. At least 500 cells were counted in each of three replicate slides per sampling time.

Nuclear morphology was observed using an epifluorescence microscope (Olympus Provis AX70; Olympus, Olympus Opt. Co., Japan) with excitation filter of 340–380 nm and barrier filter of 400 nm. The images were collected and processed using image analysis LUCIA 4.51 G system (Laboratory Imaging, Prague, Czech Republic).

### 5-Bromo-2'-deoxy-uridine (BrdU) incorporation

DNA synthesis, indicating S-phase of cell cycle, was detected by *in situ* fluorescence using 5-bromo-2'-deoxy-uridine Labelling and Detection Kit II (Roche Diagnostic GmbH, Mannheim, Germany). The procedure was performed with modifications according to manufacturer's instructions and Sano *et al.* (1999). After incorporation of BrdU into cells (for 30 min at 37 °C), the cells were fixed in 3.7% (w/v) paraformaldehyde in PMEG buffer (50 mM PIPES, 2 mM  $\text{MgSO}_4$ , 5 mM EGTA and 2% glycerol, pH 6.8) overnight at 4 °C. After fixation, cells were washed three times for 10 min in phosphate-buffered saline (PBS; 0.15 M NaCl, 2.7 mM KCl, 1.2 mM  $\text{KH}_2\text{PO}_4$ , 6.5 mM  $\text{Na}_2\text{HPO}_4$ ), digested with 1% (w/v) Cellulase Onozuka R-10 (Serva, Heidelberg, Germany), 0.1% (w/v) Pectolyase Y-23 (Kyowa Chemical Products, Osaka, Japan) in PMEG buffer

containing 0.4 M mannitol for 5 min and rinsed again with 1% (w/v) Triton X-100 in PBS for 15 min. After treatment with a blocking solution (0.1 mM glycine and 0.5% (w/v) bovine serum albumin in PBS) for 10 min, cells were attached to poly-L-lysine-coated coverslips and incubated with mouse monoclonal anti-BrdU antibody, clone BMG 6H8 (Roche Diagnostic GmbH) for 1 h according to manufacturer's protocol. After washing three times in PBS, cells were incubated with a tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-mouse antibody (Sigma-Aldrich, St Louis, MO, USA; diluted in PBS 1:200) were applied for 1 h. Specimens were washed three times in PBS, and nuclear DNA was stained with Hoechst 33258. The BrdU incorporation rate (%) is presented as percentage of BrdU-positive cells versus total Hoechst-stained nuclei.

### Cadmium treatment

For cadmium treatment, the cells in the exponential (3 d after subculture) or stationary (7 d after subculture) phase of growth were inoculated into culture medium containing 50  $\mu\text{M}$  CdSO<sub>4</sub>. For the cytotoxic studies during cell cycle progression, CdSO<sub>4</sub> (to final concentration 50  $\mu\text{M}$ ) was applied into the cultivation medium at following hours after the removal of HU: 1, 5, 7 and 10.5 h (in the S phase, late G<sub>2</sub> phase, M phase and early G<sub>1</sub> phase, respectively).

### Determination of cell viability

Changes in cell viability were monitored by fluorescein diacetate staining (FDA-Molecular Probes Inc., Eugene, OR, USA). Forty microlitres of stock solution (0.2% w/v FDA in acetone) were added to 7 mL of culture medium; this staining solution was mixed 1:1 with BY-2 cells. At least 500 cells were counted immediately after addition of FDA in triplicate per sampling time.

### Determination of DNA fragmentation

DNA fragmentation was evaluated by DNA electrophoresis and terminal deoxynucleotidyl transferase (Tdt)-mediated deoxy-uridinetriphosphate (dUTP)-nick labelling (TUNEL) reaction. Total DNA was isolated by the cetyltrimethylammonium bromide method described by Fojtova & Kovarik (2000). The integrity of DNA was evaluated by electrophoresis in 1.8% agarose gel in the presence of ethidium bromide.

The TUNEL method was used to detect 3'OH termini in nuclear DNA. The procedure was performed according to the method described by Jones *et al.* (2001) using the TMR red (red fluorescence) *in situ* cell death detection kit (Roche Diagnostic GmbH).

## RESULTS

### The effect of cadmium on BY-2 cells in exponential and stationary phase

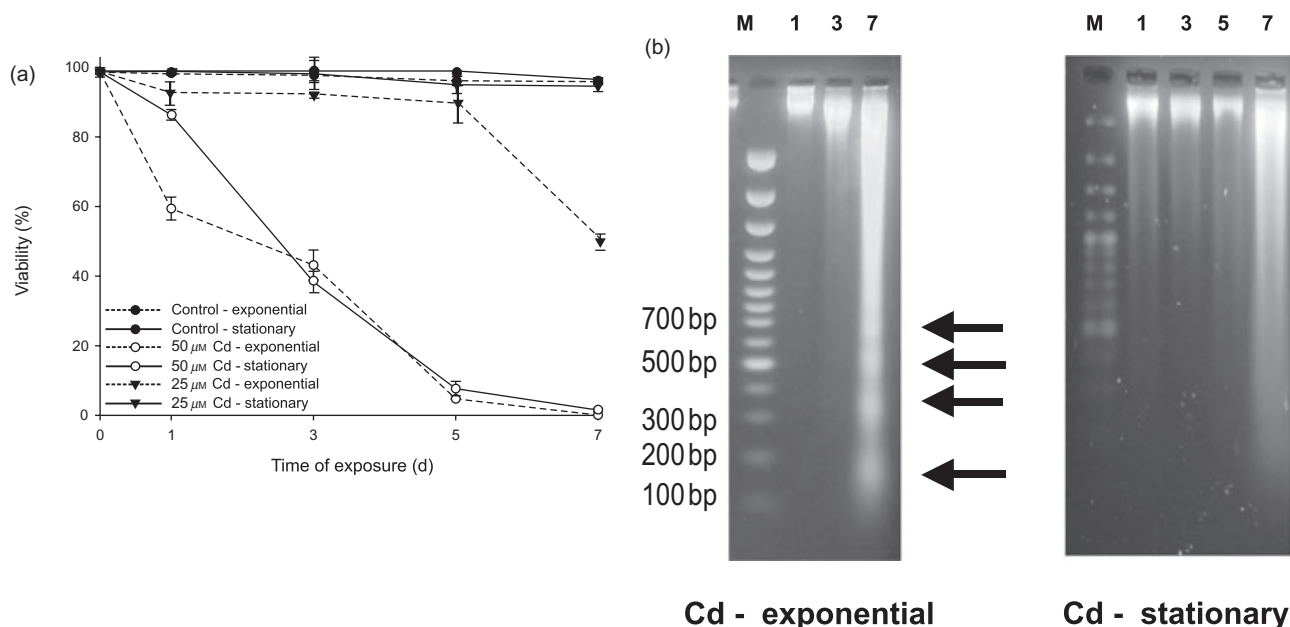
BY-2 cell suspension cultures in exponential and stationary phase were used as models for dividing (meristematic) and

somatic (differentiated) cells to study the differences in the induction of cell death by the general stressor, cadmium. Whereas 1 mM CdSO<sub>4</sub> induced quick cell death (within few hours) irrespective of the culture phase (data not shown), the treatment with 25  $\mu\text{M}$  CdSO<sub>4</sub> caused decline in viability (from the fifth day) only in the exponential cells. In case of 50  $\mu\text{M}$  CdSO<sub>4</sub>, the viability decreased gradually in both exponential and stationary cultures (Fig. 1a). Whereas the stationary cells died without typical features of PCD becoming manifest, a significant portion of the dividing (exponential phase) cells clearly exhibited different PCD symptoms after several days of the treatment, such as fragmented nuclei and fragmented DNA as detected by TUNEL reaction (data not shown) or DNA electrophoresis (Fig. 1b). As the symptoms characteristic for PCD were detectable only in the dividing cells and only in certain portion of these cells, we studied the induction of cell death using a synchronized BY-2 culture, in order to follow potential relations between the quality of the death response and cell cycle progression.

### Synchronization of tobacco BY-2 cells by HU and APC

The standard protocol used for synchronization of BY-2 cells combines APC, an inhibitor of DNA polymerase  $\alpha$ , with anti-microtubular agents such as propyzamide or oryzalin (Nagata *et al.* 1992). As APC is a relatively expensive compound and also exhibits undesirable PCD inductive effects (Matthew *et al.* 2007), we searched for milder antireplicative alternatives. We tested HU, a well-known inhibitor of ribonucleotide reductase (Adam & Lindsay 1967) and compared the result with the standard protocol using APC by following mitotic indices over the time (Fig. 2a). The BY-2 cells were arrested in the G<sub>1</sub>/S by 15  $\mu\text{M}$  APC or by 4 mM HU. For the APC treatment, the maximal MI (approximately 55%) was reached 7 h after the removal of the inhibitor (Fig. 2a). For HU, a maximum of about 35% was reached with a similar time course. In both cases practically all cells (90–95%) had undergone the cell division within the same period of 6 h, as concluded from the cell density measurements (data not shown) and the MI curves (Fig. 2a). In general, the cells tolerated the application of both compounds without any significant decrease of viability (95–98%). As the APC and HU synchronization were similarly effective, but the duration of individual cell mitoses was prolonged after APC treatment (see Discussion), we routinely used 4 mM HU for synchronization in subsequent experiments, which appeared to be less invasive synchronization drug. In addition to MI scoring, the sufficient level of synchronization with 4 mM HU was verified by BrdU labelling of S-phase nuclei (Sano *et al.* 1999; Fig. 2a).

Further experiments with lower HU concentration showed that 2 mM HU partially synchronized cell divisions even without washing out (MI peak of about 15% appeared after approximately 28 h), and 1 mM concentration of HU was not sufficient to block cell cycle progression at all (data



**Figure 1.** Effects of  $\text{CdSO}_4$  treatment on exponential and stationary BY-2 cells. (a) Viability of untreated cells and cells treated with 25 and 50  $\mu\text{M}$   $\text{CdSO}_4$  in exponential and stationary phase of growth. (b) Integrity of genomic DNA of cells treated with 50  $\mu\text{M}$   $\text{CdSO}_4$ —agarose electrophoresis. The numbers 1, 3, 5, 7 denote the days of exposure to 50  $\mu\text{M}$   $\text{CdSO}_4$ . The arrows indicate specific DNA degradation into oligonucleosomal fragments. M, molecular mass marker.

not shown). These findings indicate that 4 mM HU practically represents the minimal effective, and therefore the least invasive concentration.

### The effect of cadmium on synchronized BY-2 cells

#### Cell cycle progression

The effect of 50  $\mu\text{M}$   $\text{CdSO}_4$  on the cell cycle/mitosis progression was studied after addition of this compound into the cultivation medium at different time points after the release from the HU treatment; 1 h (S phase), 5 h (predominantly G2 phase), 7 h (M-phase) following HU removal (Fig. 2b–d). Application of cadmium in early G1 phase (at 10.5 h after HU removal) was not included in this assessment, because it could not influence mitosis progression.

The cadmium application in the S phase delayed the mitotic peak by 2 h compared with the synchronized cells cultivated in the absence of cadmium (Fig. 2b). In contrast, the cadmium treatment administered during G2 phase (Fig. 2c) did not shift the time of the mitotic peak, but increased the total MI integral and the MI peak to about 40%. The fastest response to cadmium was detected in the cells treated in M phase (Fig. 2d), where the treatment resulted in rapid decrease of the MI to 5%, reflecting quick disintegration of the mitotic structures.

Potential long-term effects of cadmium on cell division were followed through up to 30 h after the release from the HU-induced block of S phase. In contrast to untreated control cells, where a second peak of MI (up to about 10%) could be observed (about 12 h after the first peak), the

mitotic indices of all cadmium-treated samples never exceeded 2% (data not shown).

#### Viability

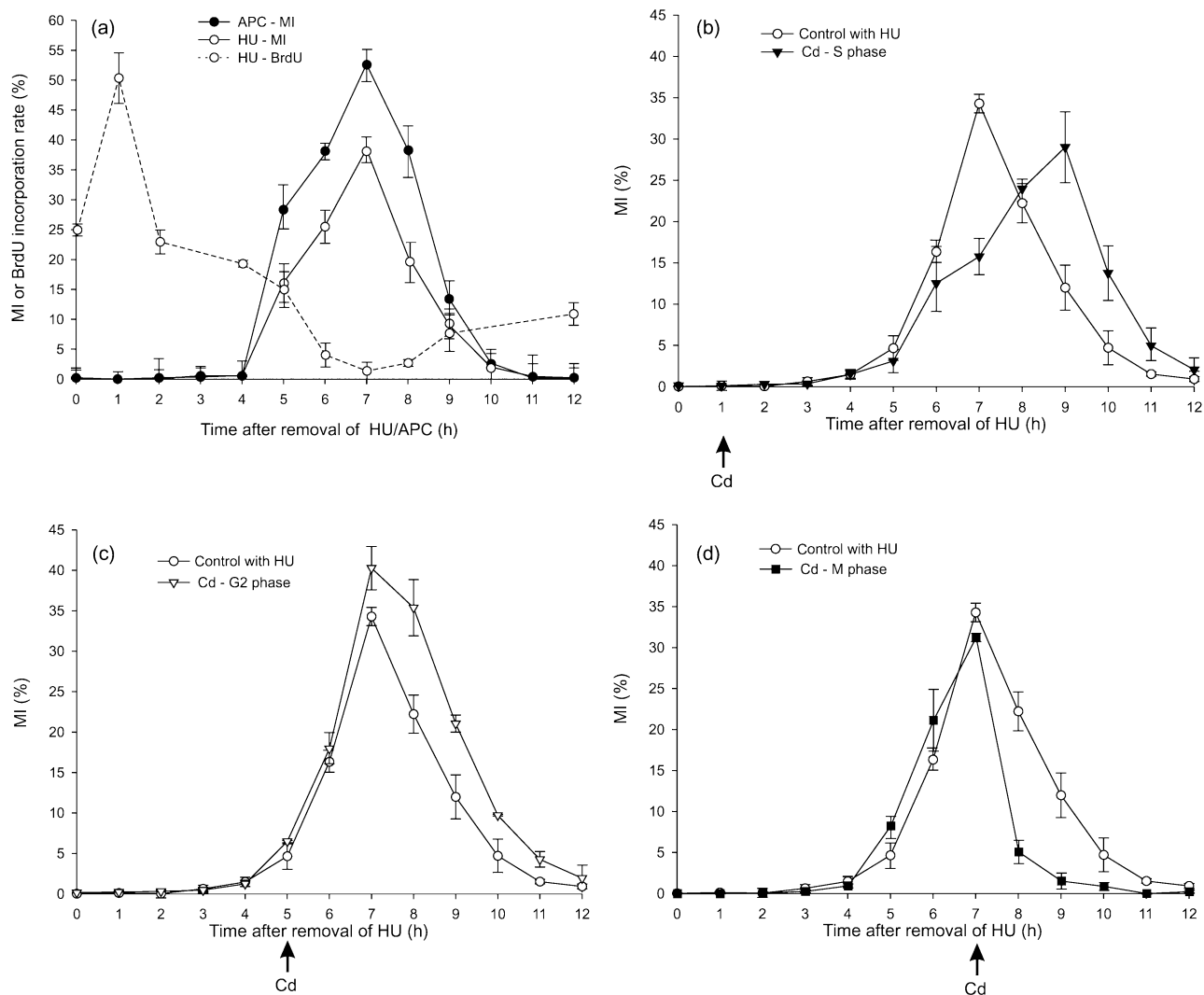
The viability of both control cell populations (either untreated or treated with HU) was high over the whole cultivation period (about 98% until day 7). Treatment with cadmium decreased cell viability as early as during the first day after the exposure (viability dropped to 20–60%). The cell cycle phase of cadmium application clearly influenced the speed of the viability decrease (Fig. 3). Application of cadmium in M phase induced the most dramatic loss of viability, down to 20%, whereas application during S or G1 phases had a significantly milder effect.

The subsequent long-term cultivation of the cells in the medium containing cadmium resulted in a progressive decrease of viability. All the cells died within 7 d. However, the morphological, biochemical and molecular characteristics of this death response differed specifically depending on the phase of cell cycle when cadmium had been applied.

#### DNA fragmentation

Fragmentation of genomic DNA is regarded to be one of the indicators of apoptosis-like PCD. To analyse the character of the cell death induced by cadmium that was applied in different phases of cell cycle, two different techniques were applied to detect DNA fragmentation; TUNEL reaction and electrophoretic detection of DNA laddering.

The TUNEL reaction was used to monitor DNA fragmentation *in situ* (Fig. 4). Both control samples (untreated



**Figure 2.** Synchronization of BY-2 cells and the effect of  $50 \mu\text{M}$   $\text{CdSO}_4$  on mitotic index (MI) progression. (a) Synchronization efficiency; MI and S phases [5-bromo-2'-deoxy-uridine (BrdU)] after  $4 \mu\text{M}$  hydroxyurea (HU) treatment and mitotic indices after treatment with  $15 \mu\text{M}$  aphidicolin (APC). (b–d) The affect of  $50 \mu\text{M}$   $\text{CdSO}_4$  on mitotic index progression compared with control cells (synchronized with HU);  $\text{CdSO}_4$  was applied at particular phases of cell cycle (S, G2 and M phases, respectively). MI values represent the mean of two (in case of synchronization with HU and APC) or three (cadmium treatments) independent experiments  $\pm$ SE.

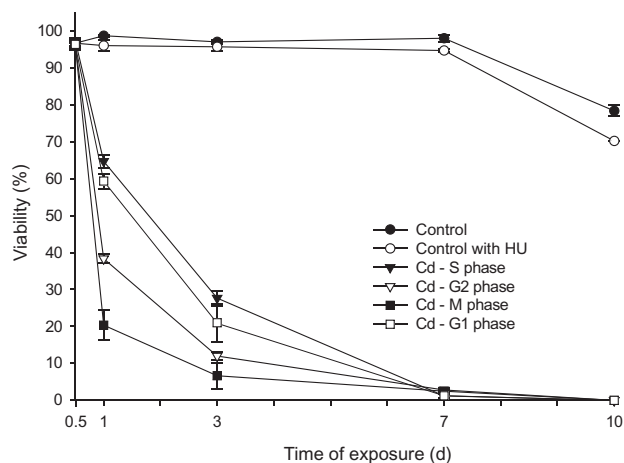
or synchronized by HU) showed maximally about 1% of TUNEL-positive nuclei during the whole cultivation period. In contrast, the cell population treated with  $50 \mu\text{M}$  cadmium in the S phase exhibited up to 50–60% of TUNEL-positive nuclei at the end of the cultivation interval (Fig. 4d). Cells treated with  $50 \mu\text{M}$  cadmium in G2 phase showed 10% of TUNEL-positive nuclei at day 7 with further increase to about 40% after 10 d of treatment. In contrast, the percentages of TUNEL-positive nuclei in variants treated with cadmium in M and G1 phase hardly exceed 10% at the end of the cultivation period.

Agarose gel electrophoresis was used to study the character of DNA fragmentation in the cell populations; the results supported the finding obtained by the TUNEL reaction: no DNA fragmentation was observed in untreated synchronized control cells (Fig. 5a). The specific DNA

degradation into oligonucleosomal fragments was observed in the cell culture with cadmium applied in the S phase (Fig. 5b) and G2 phase (Fig. 5c) after 7 and 10 d of exposure to cadmium. This internucleosomal DNA fragmentation was not detectable until the majority of cadmium-treated cells had died. In contrast, only a very small fraction of the DNA extracted from cells treated with cadmium in M and G1 phase (Fig. 5d,e) was fragmented.

### Nuclear morphology

Nuclear fragmentation, an additional symptom of PCD, was observed after cadmium treatment of non-synchronized culture in up to 40% of cells during 7 d of the treatment. Using a synchronized culture, fragmented nuclei were found not only after cadmium treatment in G2 and S phase



**Figure 3.** Viability of BY-2 cell treated with  $50 \mu\text{M}$   $\text{CdSO}_4$  at particular phases of cell cycle. Cell viability during 10 d cultivation interval after synchronization compared with untreated control and control treated with hydroxyurea (HU) in the absence of Cd. Each value represents the mean of three independent experiments  $\pm$ SE.

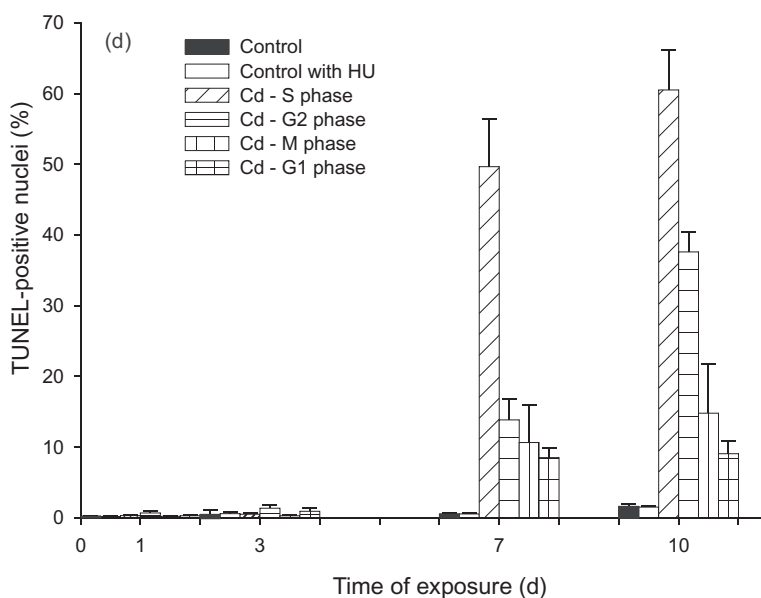
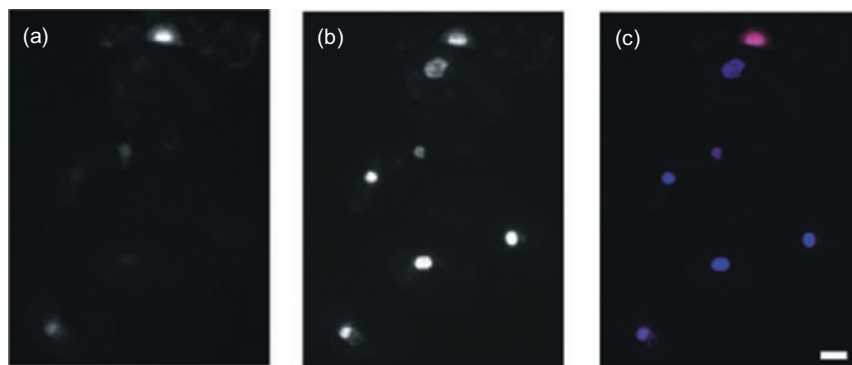
(up to 35% on day 7), but morphologically similar; either malformed or fragmented nuclei appeared frequently also in the cells that had been treated with cadmium in M phase (in up to 85% of the cells as early as 3 h after the cadmium application). In contrast to interphasic nuclei (either rounded or prolonged, with the latter often observed after synchronization with both APC and HU; Fig. 6a,b), nuclei in the cells treated with cadmium in M phase displayed granulated chromatin and various nuclear disorders/aberrations resembling destroyed late telophasic structures (Fig. 6c), which could be misclassified as fragmented interphasic nuclei observed in G2 and S variant (Fig. 6d).

## DISCUSSION

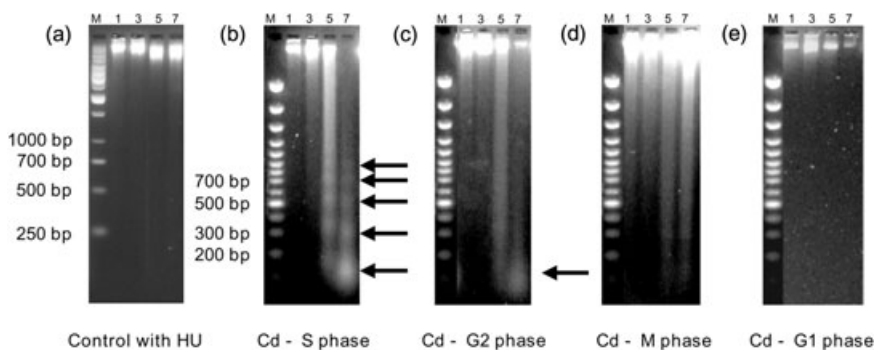
### Efficient and mild synchronization of tobacco BY-2 cells by HU

Before studying the connections between cell cycle phase and cadmium-induced cell death, we tested the effects of the anti-replicative drugs themselves, to minimize the potential interference with the effects of cadmium treatment. The invasiveness and side effects of APC and HU

## TUNEL- TMR red Hoechst 33258



**Figure 4.** DNA fragmentation in BY-2 cells treated with  $50 \mu\text{M}$   $\text{CdSO}_4$  as detected by TUNEL reaction. (a–c) *In situ* fluorescence in cells treated with cadmium in the S phase: (a) TUNEL-positive nuclei; (b) Hoechst 33258 stained nuclei; and (c) merged picture. (d) The frequency of TUNEL-positive nuclei during 10 d of cadmium treatment (after application at particular phases of cell cycle) compared with untreated controls and controls treated with hydroxyurea (HU) in the absence of cadmium. Each value represents the mean of three independent experiments  $\pm$ SE. Size bars =  $20 \mu\text{m}$ .



**Figure 5.** Time course of DNA cleavage in BY-2 cells treated with 50  $\mu\text{M}$   $\text{CdSO}_4$  at particular phases of cell cycle. (a) Control cell synchronized with hydroxyurea (HU). (b) Cells treated with 50  $\mu\text{M}$   $\text{CdSO}_4$  in the S phase. (c) Cells treated with 50  $\mu\text{M}$   $\text{CdSO}_4$  in the G2 phase. (d) Cells treated with 50  $\mu\text{M}$   $\text{CdSO}_4$  in the M phase. (e) Cells treated with 50  $\mu\text{M}$   $\text{CdSO}_4$  in the G1 phase. The numbers 1, 3, 7, 10 denote the days of culture/exposure to 50  $\mu\text{M}$   $\text{CdSO}_4$ . The arrows indicate the specific DNA degradation into oligonucleosomal fragments. M, molecular mass marker.

could be hardly envisaged, as these compounds completely differ in their mode of action: APC blocks DNA synthesis via inhibition of the activity of  $\alpha$ -polymerase (Bryant *et al.* 1992), whereas HU is an inhibitor of ribonucleotide reductase, one of the key enzymes of deoxyribonucleotide triphosphate (dNTP) synthesis (Adam & Lindsay 1967).

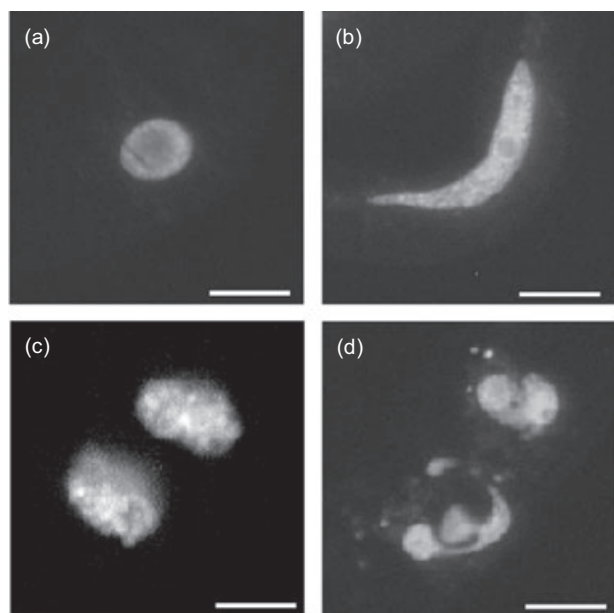
The synchronization efficiency of APC (Nagata *et al.* 1992) and HU (Doležel, Lucretti & Schubert 1994) was comparable; practically all cells in the population had divided during the mitotic phase lasting approximately 5 to 6 h (see Fig. 2a). However, the MI peak reached higher

values after APC treatment, which might be erroneously interpreted as better synchronization. As approximate duration of individual mitosis (2.5–3 h; Swiatek *et al.* 2002) is substantially shorter as compared with the mitotic phase period in the cell population (the width of the MI peak, i.e. 5 to 6 h), individual cells did not enter mitosis simultaneously, but within the interval of a few hours. Subsequently, the higher and broader MI peak reflects more frequent overlap of the final phases of the first mitoses in the population with the initial phases of the later ones. It indicates that the duration of individual cell mitoses was prolonged after APC treatment.

Prolonged duration of mitosis generally reflects troubles in cell cycle progression (Paulovich, Toczyski & Hartwell 1997), which corresponds to recently demonstrated increased induction of cell death after APC-mediated block of cell cycle progression in human cancer cells (Matthew *et al.* 2007). Moreover, the shape and the height of MI curve after synchronization with APC resembled the curve of HU-synchronized cells treated with cadmium in G2 phase (compare Fig. 2a and Fig. 2c), and, therefore, some effects of cadmium might have been masked or mimicked if APC had been used for synchronization. In conclusion, our data document higher suitability of 4 mM HU for reliable and fine synchronization of BY-2 cells with reduced invasiveness and side effects as compared with APC.

### Cadmium induced specific types of cell death depending on the cell-cycle phase of its application

The application of cadmium ions induced massive cell death in all experimental variants. However, both the progression and the quality of this death response pronouncedly differed depending on the cell-cycle position at the time of cadmium application. Although ‘apoptosis-like’ PCD, induced by cadmium application in the S and G2, was characterized by the presence of pronounced internucleosomal DNA fragmentation (and fragmented nuclei), the other two cell-death types, induced by cadmium application in M and



**Figure 6.** Changes in nuclear morphology of BY-2 cells treated with 50  $\mu\text{M}$   $\text{CdSO}_4$ . (a) Rounded nuclei of untreated control cells, (b) elongated nuclei of synchronized control cells, (c) fragmented late telophasic nuclei of cells that had been treated with cadmium in M phase for 3 h, (d) fragmented nuclei, 7 d after cadmium application in the S phase. Staining with Hoechst 33258, size bars = 10  $\mu\text{m}$ .

G1, were not accompanied by DNA fragmentation. As the absence of DNA cleavage indicates suppression of hydrolytic enzymes activity/release (see following discussion), we call the slow cell death – induced by cadmium application in G1 – as a ‘non-lytic G1 cell death’. In contrast, cadmium application in M phase resulted in a rapid ‘mitotic cell death’, characterized by fragmented late telophasic nuclei, which presumably reflected quick disruption of the mitotic apparatus.

The response of BY-2 cells to the bacterial elicitor cryptogein also depended on the cell cycle position at the time of its application. However, defense-related gene expression and cell death were induced only in the S and G1 phase, whereas this response was suppressed in G2 and M phase (Kadota *et al.* 2004). The differences between the cryptogein and cadmium effects are probably connected with the target sites of their action. Whereas the effect of cryptogein is highly specific, cadmium, as a general stressor, affects numerous metabolic processes independently of the cell-cycle phase. Cadmium was reported to cause oxidative stress (Sandalio *et al.* 2001; Olmos *et al.* 2003), to inhibit the activity of several antioxidative enzymes (Piqueras *et al.* 1999; Olmos *et al.* 2003) and to alter membranes function by inducing changes in their lipid composition (Skorzynska-Polit & Krupa 2006). Cadmium was also found to cause point mutations and chromosomal aberrations (Zhang & Xiao 1998), which could be connected with the ability of cadmium ions to displace zinc ions – for instance, in the zinc fingers proteins (Predki & Sarkar 1992) – involved in DNA repair (Hartwig & Schwerdtle 2002). A rapid cadmium-induced DNA damage has also been recently documented by the comet assay in various organs of potato plantlets (Gichner *et al.* 2006).

As cadmium affects DNA integrity and also induces rapid disintegration of mitotic structures, cell-death induction might be specific in the S and M phase of cell cycle. However, the difference between the G1 and G2 responses, i.e. apoptosis-like PCD induced in G2 phase and non-lytic G1 cell death, was unexpected and thought-provoking.

Our recent results indicate that even intact untreated BY-2 cells are predisposed with all the enzymatic machinery necessary for immediate triggering of internucleosomal DNA fragmentation. This fragmentation was clearly detectable within a day after rapid killing of the cells by mechanical disintegration, which precluded programmed activation of the enzymes (Kuthanova *et al.* 2008). In contrast, the DNA fragmentation observed during the apoptosis-like PCD lasted several days, indicating slow process of programmed vacuolar collapse (Groover & Jones 1999; Kuthanova *et al.* 2008). In this respect, the absence of DNA fragmentation (after application of cadmium in M and G1 phase) suggests special mechanism of cell dying, which suppresses release (or activity) of hydrolytic enzymes and prevents cell autolysis. As different plant PCD types are believed to share a singular event – the action of the vacuole, whose collapse releases sequestered hydrolases and causes cell autolysis (Jones 2001) – we assume that the mitotic and non-lytic G1 cell death were of non-programmed character.

Specific induction of PCD in response to cadmium application in the S and G2 phase might be connected with passing through the cell cycle checkpoints, canalizing further fate of the cells either to recover or to die via programmed suicide. Three basic DNA damage checkpoints, acting in different stages of cell cycle, are known in yeast, animal and plant cells: one at G1/S transition; a second in the S phase; and a third at the G2/M transition. Integrity of DNA, affected by cadmium (Zhang & Xiao 1998; Gichner *et al.* 2006), is a subject of control mainly before entering to S phase and to mitosis (Sobkowiak & Deckert 2004). With respect to the cell cycle checkpoints mentioned earlier, the G2/M transition appears to be the best candidate for the induction of specific apoptosis-like death of our BY-2 cells, as only cells treated with cadmium in the preceding cell cycle phases (i.e. in S and G2) exhibited apoptotic DNA fragmentation.

### Biological relevance

The character of our BY-2 cell death responses induced by cadmium in G1 and G2 phases (the non-lytic slow cell death and the apoptosis-like PCD, respectively) strongly contrast with the effect of cryptogein, whose cell-death inducing effect was very strong in G1, and suppressed in G2 (Kadota *et al.* 2004). Kadota & Kuchitsu (2006) suggested that the defense and PCD-inducing responses to cryptogein might be suppressed *in planta* in dividing cells. By contrast, differentiated cells (mesophyll cells of mature leaves) – situated in G0 or G1 phase – are expected to strongly respond to the elicitors by induction of the hypersensitive cell death, primarily serving to inhibit spreading of pathogens from the site of infection. Cadmium and, hypothetically, also other abiotic stressors impairing numerous cell processes usually affect cells unselectively in a whole plant. Therefore, the plant response to such factors must dramatically differ from the localized hypersensitive cell death response induced by the pathogen infection. If all affected cells responded ‘hypersensitively’ to the cadmium (or other abiotic stressors) by induction of massive PCD, the whole plant body would quickly die. In order to avoid the plant death and to enable initiation of the recovery and adaptive processes, induction of PCD might be suppressed in differentiated somatic cells. This explanation is suggested by stably high viability of stationary cells treated with lower (25  $\mu\text{M}$ ) concentration of  $\text{CdSO}_4$  (contrasting with decreasing viability of exponential cell treated with the same  $\text{CdSO}_4$  concentration; Fig. 1a) and the character of non-lytic cell death induced in G1 phase. The slowness of this (G1) death response together with the absence of DNA fragmentation indicates non-programmed character of this death, which might result from successive accumulation of variable cell damages.

On the other hand, in response to a stressor, which induces not only metabolic malfunctions, but also genotoxic damage, a plant needs to preserve the genetic integrity of meristematic cells. Therefore, genetically (or physiologically) impaired cells in meristems should be efficiently



removed, to allow subsequent proper development and reproduction of the plant. Impaired meristematic cells can be efficiently eliminated by PCD, as suggested from the apoptotic features of cell death induced by cadmium application in the S and G2 phase, which directly precede mitosis.

The PCD responses to ethylene (Herbert *et al.* 2001) or elicitors (Kadota & Kuchitsu 2006) represent sophisticated advanced mechanisms probably targeted predominantly to differentiated somatic cells, and are very likely the highly specialized products of a long and intricate evolutionary process connected with multicellularity. It must have evolved from much simpler and more global mechanisms. PCD has probably evolved in colony-forming single cell organisms such as bacteria, yeast or slime molds to effectively survive starvation and/or pathogen attack. In the context of starvation, nutrients released from a portion of 'altruistic' cells undergoing PCD were redistributed to ensure survival (alternatively accompanied by sporulation) of the remaining cells that would be the founder inoculum ensuring the survival of the colony (Severin & Hyman 2002). In combination with nutrient redistribution, it was necessary to safeguard the genetic integrity of the surviving cells (e.g. eliminate non-mating or aberrantly mating cells in yeast; Severin & Hyman 2002). Thus, elimination of genetically impaired cells was a central task to be solved already at the transition from protist 'immortality' towards multicellularity. In fact, the separation between the (immortal) germ line and the (differentiating, mortal) *soma* is a form of programmed 'genetic suicide' of the latter ones. This view has been a central element of developmental biology since this aspect was uncovered by Weismann (1892). The cell-cycle dependency of PCD induced by the general stressor cadmium can be interpreted as a manifestation of mechanisms that ensures genomic integrity in cycling cells that dates back to the primordial stages of multicellularity.

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