

APPLICATION OF THE COMET ASSAY IN STUDIES OF PROGRAMMED CELL DEATH (PCD) IN PLANTS

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ABSTRACT

Programmed cell death (PCD) in plants is an intensively investigated process. One of the main characteristics of PCD in both animal and plant organisms is the non-random, internucleosomal fragmentation of nuclear DNA, usually analysed using total DNA gel electrophoresis or TUNEL method. In this paper we present application of the "comet assay" (Single Cell Gel Electrophoresis) for detection of nDNA degradation in studies of PCD during plant life cycle. We analyzed three types of tissue: anther tapetum, endosperm and mesophyll which were prepared in different ways to obtain a suspension of viable cells (without cell walls). The comet assay gives a possibility of examination of the nDNA degradation in individual cell. This method is significant for studies of the plant tissue differentiation and senescence especially in the cases when it is not possible to isolate large number of cells at the same developmental stage.

KEY WORDS: comet assay, programmed cell death (PCD) in plants, tapetum, endosperm, mesophyll, protoplasts.

INTRODUCTION

Programmed cell death (PCD) is a process of cellular self-disintegration which is caused by activation of an intrinsic suicide programme, genetically determined and highly conserved among multicellular eukariota (Goldstain 1998). Much less is known about the molecular mechanisms of PCD in plants but a variety of data strongly support the view that the processes may function similarly as apoptosis in animal cells.

It is well established that some plant cells or tissues are destined for death as the part of the normal development of the plant body. Programmed death can also be induced by certain environmental events, also in *in vitro* culture. Developmental processes in both vegetative and generative phases of the plant life are correlated with or are followed by death of some cells or tissues. There are some common examples of cells which die during the plant body development starting from the embryo stage, e.g. suspensor cells, xylem vessels (during vascular system differentiation in all plant organs, including the embryo), root cap cells, endosperm, anther tapetum, non-functional megasporangia, synergids, antipodals, ovule nucellus and pollen tubes after fertilization. The above processes were considered recently as examples of PCD in plants (Bell 1996; Charzyńska 1996; Greenberg 1996; Havel and Durzan 1996; Jones and Dangel 1996; Beers 1997; Pennell and Lamb 1997; Buckner et al. 1998; Richberg et al. 1998). The

in vitro culture of tracheary elements is a model system for this type of PCD (Mittler and Lam 1995; Fukuda 1996, 1997; Fukuda et al. 1998).

PCD in plants has been thought to occur also during senescence of leaves (see reviews by Hadfield and Bennett 1997, Nooden et al. 1997, and Inada et al. 1999), floral organs as well as during the response of the plant to pathogen infection (reviews: Mittler and Lam 1996; Ryerson and Heath 1996; Wang et al. 1996; Gilchrist 1997; Greenberg 1997; Blumwald et al. 1998; Del Pozo and Lam 1998).

Although many molecular and structural events involved in PCD were identified (e.g. Panawas and Rubinstein 1998; Gao and Showalter 1999; Susin et al. 1999) the internucleosomal fragmentation of the nuclear DNA (nDNA) is widely accepted as an universal hallmark of PCD both in animal (Bortner et al. 1995) and plant organisms. The nDNA fragmentation is correlated with condensation of the chromatin and occurs by activation of a specific endonuclease that cleaves the DNA in the linker regions between nucleosomes into oligonucleosomal fragments. The inter-nucleosomal cleavage has been also one of the main biochemical criteria used to distinguish PCD from necrosis (Bortner et al. 1995).

The methods most widely used to visualise the phenomenon of the nDNA cleavage are: 1) electrophoresis of the extracted nDNA on agarose gel to obtain the DNA "ladder" (Eastman 1995), 2) TUNEL (the terminal deoxynucleotidyl transferase-

mediated dUTP nick end in situ labelling method) – a histochemical method which detects the DNA breaks (nicks) in fixed tissue sections, based on the specific binding of dUTP by terminal deoxynucleotidyl transferase-(TdT) to 3'-OH ends of DNA. dUTP is labeled with a fluorochrome or with another marker which can be detected in situ by a histochemical reaction (Gavrieli et al. 1992).

Some aspects of PCD in higher plants and its hormonal regulation were recently presented using the above two methods during carpel senescence (Orzáez and Granell 1997), endosperm degradation (Young et al. 1997), leaf senescence (Yen and Yang 1998) and anther development (Wang et al. 1999). In all these studies the genomic DNA was extracted from samples of the tissue-heterogeneous organs, rather than from one type of cells. Isolation of such a large number of cells of one type (10^6 - 10^7 as a minimum) for the standard gel electrophoresis of DNA (without preparation-induced nDNA damage) is practically not possible.

The possibility of examination of the nDNA damage at the individual cell level is provided by the technique of single cell gel electrophoresis (called comet assay) which was introduced by Östling and Johanson (1984) for measuring the radiation damage in nDNA and for the estimation of the DNA repair capacity in human lymphocytes in culture. This fast and sensitive method for quantitative analysis of the nDNA damage in individual cells is at present widely used in different types of animal cells (Olive et al. 1993; Muller et al. 1994, 1996; reviewed by Jałoszyński et al. 1996). The procedure involves: embedding of living cells in agarose, lysis at either neutral or alkaline conditions and exposition to a weak electric field. The relaxed and broken, negatively charged, DNA fragments migrate from the nucleus towards the anode. Staining with a DNA-binding dye gives images reminding comets that are visible in light microscope. The "heads" of the comets are visible in places of the original localisation of the nuclei on the slide and they correspond to the amount of nDNA which still remains in the region of the nuclear matrix. The "tails" of the comet visualise the migrating relaxed DNA and the fragments that are liberated from the "head". The form of the comet depends on the degree and quality of the DNA damage – the degree of relaxation, the size of migrating fragments and conditions of electrophoresis.

The neutral conditions of lysis and electrophoresis, originally used by Östling and Johanson (1984), enabled to observe the effect of only double-strand breaks of DNA. Modifications introduced by Singh and collaborators (1988), together with the application of alkaline conditions during lysis and electrophoresis, gave the possibility to detect both single and double-strand breaks of nDNA in the studied cells. Fairbairn et al. (1995) presented a comprehensive review concerning the technical details of both versions of the "comet assay".

Our study (Charzyńska and Leśniewska 1998; Sikora 1998; Simeonova et al. 1998; Leśniewska 1999) have pioneered the application of the technique of the single cell gel electrophoresis for the analysis of the plant material. In this work we described adaptation of the "comet assay" for the analysis of nDNA fragmentation in viable plant cells from diverse plant tissues i.e. anther tapetum, endosperm and mesophyll.

MATERIAL AND METHODS

Plant material

Plants of *Ornithogalum virens* L. (Liliaceae), *Nicotiana tabacum* L. ecotype Petit Havana SR I (Solanaceae) and *Haem-*

anthus albiflos L. (Amaryllidaceae) were grown in the greenhouse under natural photoperiod conditions or in the growth chamber (16 h light/8 h dark and temperature cycle of 24°C day/22°C night; under illumination of continuous fluorescent light of 45 W/m²) at the Department of Plant Anatomy and Cytology of the Warsaw University. Three types of tissue were analysed: tapetum (*O. virens* and *N. tabacum*), endosperm (*H. albiflos*) and mesophyll (*O. virens* and *N. tabacum*).

COMET ASSAY

Both versions of the "comet assay" (the neutral and alkaline one) were applied by us to analyse the tapetum cells, whereas the alkaline version of the assay was used for the mesophyll protoplasts and endosperm. The neutral version (pH 8.3) was performed according to the procedure used by Grądzka and Szumiel (1996), which is based on the descriptions by Olive et al. (1991) and Müller et al. (1994). The alkaline version (pH>13) of the method was realized as described previously by Singh et al. (1988) and slightly modified by Kruszewski et al. (1996). The electrophoresis was performed in a horizontal gel electrophoresis unit.

1. Tissue preparation

The tissues were prepared in various ways, suitable for the types of analysed tissues.

a) **Tapetum.** Tapetum cells (the "natural protoplasts") were isolated from viable anthers. The stage of their differentiation was estimated in the light microscope: one anther of a given flower was gently squashed in PBS on a microscopic slide. Remaining five anthers from the same flower were placed in E-coup filled with 75 µl of PBS (stored on ice), then squashed with glass stick and pipetted 2-3 times in order to break cell aggregates into the cell suspension.

b) **Endosperm.** Nuclear and cellular endosperm of *Haemanthus albiflos* was isolated from ovules of different stages of development three weeks after completion of the florescence of the plants. Nuclear endosperm before cellularisation was taken from ovules 5-6 mm long, cellular endosperm in successive stage of development/senescence – from ovules containing 3-10 mm long embryos.

c) **Mesophyll.** To test whether the natural leaf senescence is correlated with DNA fragmentation in mesophyll cells we chose two plant species, *Ornithogalum virens* and *Nicotiana tabacum*, which represent different types of the leaf developmental pattern. The leaf blade of *O. virens* develops in a tip-to-base longitudinal gradient typical for monocotyledonous plants: the most basal part of the blade is meristematic, the middle one contains differentiated mesophyll cells, the distal older part – senescing tissue. The leaf blades were divided into basal and apical fragments and isolation of protoplasts from mesophyll cells of each part was performed. In *N. tabacum*, a dicotyledonous plant, mesophyll protoplasts were isolated from leaves of different developmental stages: from green leaves of young plants and from yellowing leaves of older plants. Leaf samples were taken from plants growing during the last eight hours in darkness (for elimination of starch mesophyll). Isolation of protoplasts was performed as described by Parys et al. (1998).

2. Embedding of the cells in agarose

The cell material before the lysis and electrophoresis must be placed on the agarose coated microscope slides and embedded in 1% low gelling-point agarose. Clean microscopic slides were coated with 0.5% agarose (Low ECC Agarose I-A, Sigma) in distilled water by using a pipette (100 µl per

slide) or by immersing the slides in this agarose solution. Agarose coated slides were air-dried at room temperature.

In the neutral version of the comet assay 1% low gelling-point agarose (type VII, Sigma) was prepared in distilled water; for the alkaline version we used a buffer consisting of 10 mM Tris and 2 mM EDTA (pH 8); both at 37°C. Embedding of the cell material in low-gelling agarose differs in some details, depending on the type of tissue analysed and on electrophoresis conditions.

a) Tapetum. 30 ml of the cell suspension was mixed with 90 µl of 1% low gelling-point agarose at 37°C (in a bath water). The mixture (100 µl) was pipetted on the agarose coated microscope slides under cover glasses and left to solidify on an aluminium plate on ice (for 1 min).

b) Endosperm. Agarose pre-coated microscope slides were covered with 1% low gelling-point agarose (500 µl pipetted carefully onto the slide; temperature 37°C) and nuclear endosperm was squeezed from ovules directly onto the agarose before its solidification. The older ovules were cut and cellular endosperm was removed into an E-coup and lightly crushed with a glass stick. 7 µl of the material was pipetted immediately on microscope slides (into low gelling agarose). In both of the above variants the preparations (not covered by microscopic cover slides) were left to solidify on ice.

c) Mesophyll. The suspension of mesophyll protoplasts was pipetted and embedded in 1% low gelling-point agarose covering the microscope slides, in the same way as described for the endosperm cells. The slides were left to solidify on ice.

3. Lysis and electrophoresis procedures

After solidification of agarose the cover glasses were carefully removed (see 2a) and the preparations (microscopic slides with cells embedded in the solidified agarose) were immersed in the lysing solution. Further manipulations have to be performed under red light (or at darkness) to protect DNA molecules sensitive to light after digestion of the chromatin proteins.

– *Neutral version of the "comet assay"* (lysis pH 8.3, electrophoresis pH 8.3)

Lysis. Preparations were immersed in the lysing solution (30 mM EDTA, 0.5% sodium sarcosylate, pH 8.3) for 10 min and then for 45 min in the same solution with the addition of 2.5 M NaCl (at the room temperature).

Electrophoresis. After lysis the preparations were immersed two times for 15 min in the electrophoretic buffer (TBE: 90 mM Tris/boric acid, 10 mM EDTA; pH 8.3) at 10°C. Electrophoresis was performed in the same buffer at 2.5 V/cm, for 5

min, at 10°C. Preparations were then washed in distilled water for 30 min.

– *Alkaline version of the "comet assay"* (lysis pH 10, electrophoresis pH 13)

Lysis. Preparations were immersed in the lysing solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris base, pH 10; 1% Triton X-100 was freshly added) for 1 h, at 4°C.

Electrophoresis. After lysis the preparations were immersed for 40 min in the electrophoretic buffer (300 mM NaOH, 1 mM Na₂EDTA, pH 13) at 4°C. Electrophoresis was conducted in the same buffer at 25 V and 500 mA for 30 min at 4°C. After electrophoresis the preparations were washed 3 times for 5 min to remove alkali and detergents in the neutralising buffer (0.4 M Tris, pH 7.4), and then in distilled water for 5 min.

4. DNA visualization

Staining. After washing (in both versions used) the material was stained immediately with DAPI (1 µg/ml H₂O; 100 µl), covered by the cover glass and placed in a humidity chamber in the refrigerator (at 4°C) for 12-18 hours or preparations were air-dried (at 37°C) and stored in black boxes in the refrigerator (at 4°C) for up to 2 weeks. Before staining they have to be rehydrate in distilled water for 10 min.

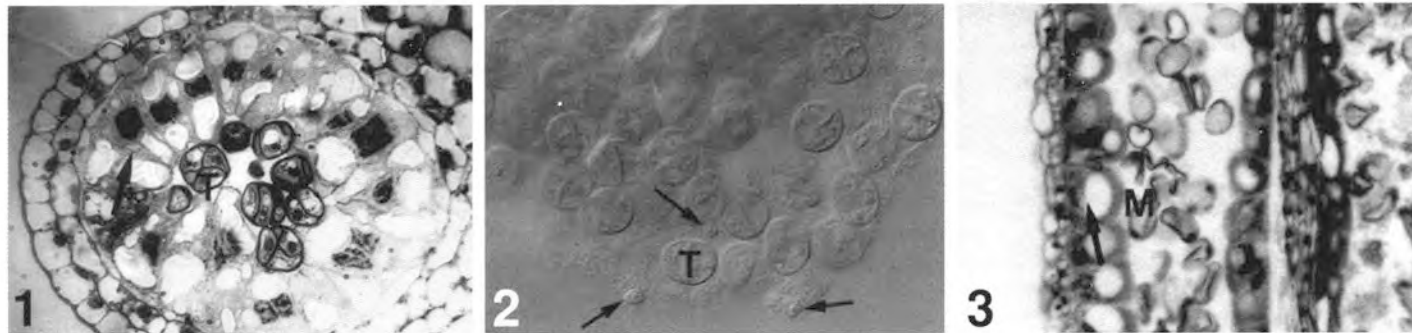
Microscopic observations and computer analysis were performed in: a) Labophot-2 epifluorescence microscope (NIKON, Japan), with the use of EFD-3 × 20 objective and 365/10 nm excitation filter for DAPI and 400 nm for the light emitted by the dye. The comet images were analysed with computer program (Kinetic Imaging, U.K., Comet User Guide, version 3, Nov 1994) and photographs were made directly from monitor, b) with NIKON fluorescence microscope using Emil computer program.

RESULTS

Results of the single cell gel electrophoresis (comet assay) on analysed tissues

Anther tapetum

The secretory tapetum in *Ornithogalum virens* and *Nicotiana tabacum* forms one layer of cells, which differentiate and undergo disintegration of the structure in a synchronous way during anther maturation. The life of tapetum cells is limited to a few days preceding cellular disintegration to a pollen coat. The developmental stages of tapetum cells were deter-



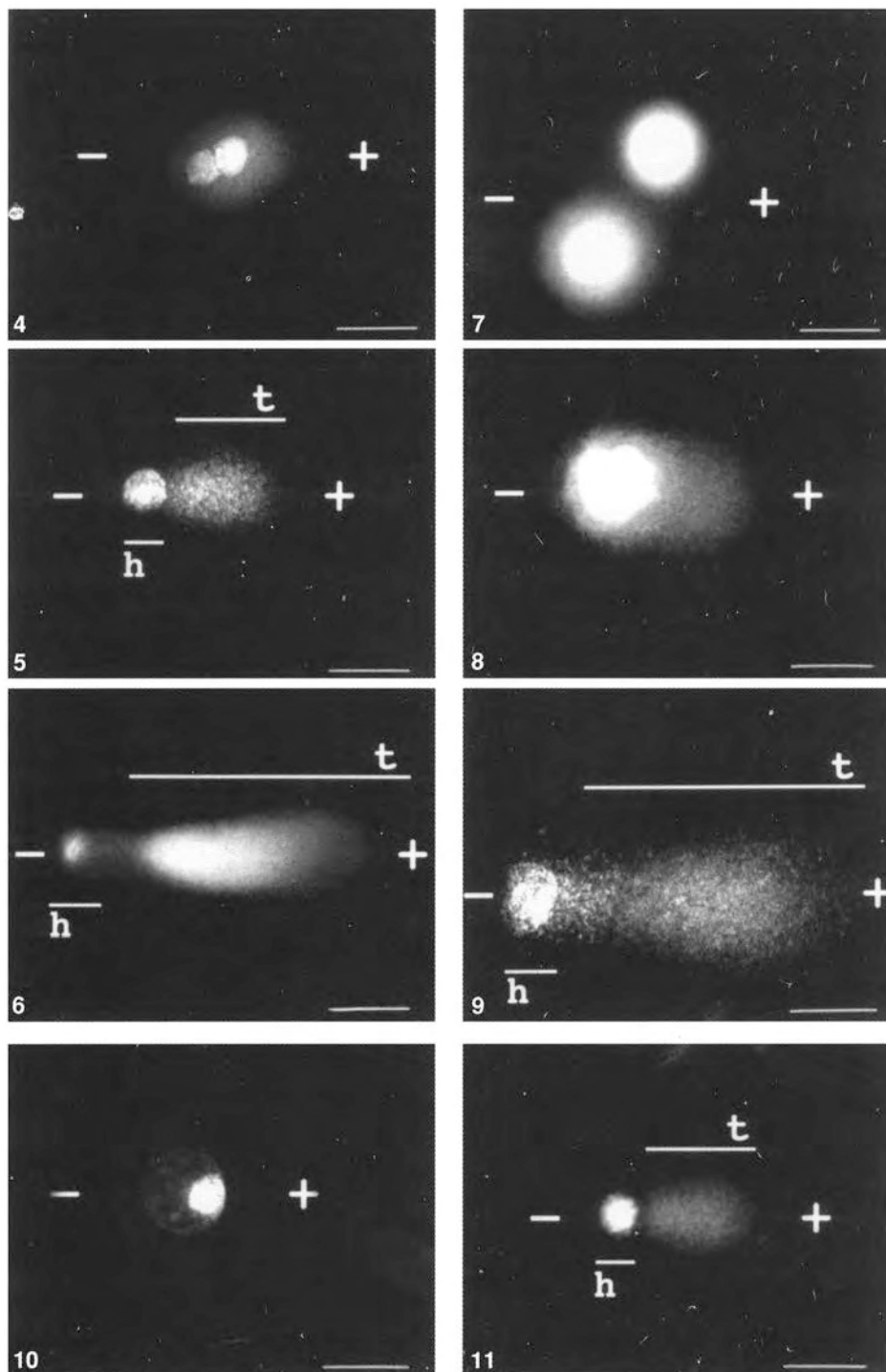
Figs 1-3. Anther of *Ornithogalum virens*.

Fig. 1. Semi-thin cross section of anther at the tetrad stage, stained with toluidine blue.

T – tetrad, arrow – tapetum cells, 230×.

Fig. 2. The interference-microscope image of squashed anther at the late tetrad stage. Arrows indicate tapetum protoplasts, T – tetrad, 230×.

Fig. 3. Longitudinal paraffin section of anther at the microspore stage, stained with Erlich hematoxylin, arrow – tapetum protoplast, M – microspore, 125×.



Figs 4-11. Some examples of the results of the comet assay (single cell gel electrophoresis) on the analyzed tissues.

Figs 4-6. Anther tapetum of *Ornithogalum virens*; Figs 7-9. Endosperm of *Haemanthus albiflos*; Figs 10 and 11. Mesophyll of *O. virens*. Bars = 1 μ m.

Figures present fluorescent images of studied cells nuclei after neutral version of electrophoresis (Fig. 6) and after alkaline version (the others). nDNA was staining by DAPI. "+" – anode, "-" – cathode; h – "comet head", t – "comet tail".

Figs. 4-6. Anther tapetum of *O. virens*.

Fig. 4. Binuclear tapetum cell at the meiocyte/young tetrad stage. There is no nDNA fragmentation.

Fig. 5. Tapetum at the microspore stage. The comet image indicates that nDNA degradation is already advanced.

Fig. 6. Tapetum at the young bicellular pollen stage. Strong fluorescence and long comet "tail" indicates that much of nDNA is degraded.

Figs 7-9. Endosperm of *Haemanthus albiflos*.

Fig. 7. Nuclear endosperm squeezed from 5 mm long ovule. There is no nDNA fragmentation.

Fig. 8. Endosperm short after cellularization. nDNA fragmentation has just started.

Fig. 9. Endosperm cell taken from ovule containing 3mm long embryo. Strong fluorescence and long comet "tail" indicate, that much of nDNA is already fragmented.

Figs 10 and 11. Mesophyll of *O. virens*.

Fig. 10. Mesophyll protoplast taken from basal part of the leaf blade. There is no nDNA fragmentation.

Fig. 11. Mesophyll protoplast taken from senescing apical part of the leaf blade. nDNA fragmentation is visible, but strong fluorescence of comet "head" shows, that much of DNA is still associated with nuclear matrix.

mined both from their structure and from the stage of development of meiocytes /microspores/ pollen grains (Fig. 1). Tapetum cells undergo poliploidization during the first meiotic prophase and stay binuclear. After cell wall dissolution which starts during the late tetrad stage the tapetal protoplasts retain their position in the anther loculus periphery during microspore and pollen development (Leśniewska 1999), Fig. 3. The natural protoplasts of tapetum, highly synchronised in differentiation, is a suitable material for isolation and preparation for the "comet assay" without the use of enzymes (e.g. Fig. 2).

We have analysed five stages of tapetum development which were correlated to development of the male germ line cells. There was no comet formation during electrophoresis of tapetum cells of *O. virens* and *N. tabacum* isolated from the anthers at meiocyte stage (Fig. 4). The damage of nDNA in tapetum cells (expressed by the "comets" formation during electrophoresis) appeared first at the old tetrad stage and increased during the long-lasting microspore stage (Fig. 5) and during the young bicellular pollen stage (Fig. 6), which is the stage of tapetal cell disintegration. Application of both versions of the "comet assay" gave similar results. Some differences are connected with conditions of the electrophoresis (see "Introduction")

Endosperm

We have not detected nDNA fragmentation in *Haemanthus albiflos* nuclear endosperm isolated from 5-6 mm long ovules (Fig. 7). After cellularization of endosperm we have found nuclei with different degree of DNA cleavage (see comets in Figs 8 and 9). This was a result of a gradual differentiation and death of the endosperm cells. The stage of differentiation of these cells is related to their location with respect to the developing embryo.

Mesophyll

There was no formation of "comets" during the electrophoresis of mesophyll protoplasts isolated from green leaves of *Nicotiana tabacum* (photo not included) and from basal parts of *Ornithogalum virens* leaves (Fig. 10). The "comets" were formed from nuclei of senescing mesophyll cells of yellow leaves of *Nicotiana tabacum* (photo not included) as well as from yellowing apical parts of leaves of *Ornithogalum virens* (Fig. 11). The images of comet "heads" and "tails" differ depending on the progress of nuclear DNA degradation.

DISCUSSION

The results presented above indicate that the "comet assay" (the single cell gel electrophoresis) offers a possibility to examine nDNA fragmentation in individual cells in suspension of a small population of cells carefully isolated from plant organs or in the plant protoplasts isolated by a standard technique from the examined plant tissue. PCD related nDNA fragmentation was recently documented both by TUNEL and standard gel electrophoresis during leaf senescence. "Ladders" of DNA were detected only in senescent yellow/brown leaves without any laddering in green, not senescent leaves (Yen and Yang 1998). These results confirmed indirectly that our version of the "comet assay" is a useful method for determination of the DNA damage typical for PCD. As mentioned earlier the procedure of single cell gel electrophoresis involves five important steps: isolation of viable cells; embedding of the cells in agarose; lysis either in neutral or alkaline conditions (to remove some of the cytoplasmic and nuclear proteins); exposition of the remaining nucleoids to a weak elec-

tric field (horizontal gel electrophoresis); visualisation of DNA. The requirement for the application of the method in plant material is the acquisition of a suspension of viable cells without cell walls. Successful application of the "comet assay" in the studies of nDNA damage of plant protoplasts (for the first time used by us for examination of the protoplasts isolated from the leaf mesophyll tissue) might be a tool for analysis of nDNA condition in plant protoplasts stimulated to somatic embryogenesis. The "comet assay", as being an easy, very sensitive and rapid technique, can also be used instead of the TUNEL method which is more time consuming and is connected with deep chemical changes in the cell material resulting from procedures of tissue fixation and sectioning.

The "comet assay" depending of its version (neutral or alkaline) enables to detect the initial single or double-strand breaks of nDNA which start in PCD nuclei before the internucleosomal cleavage and to compare them with those from undamaged cells. Recently two other types of DNA fragmentation occurring during the animal cell apoptosis were distinguished: fragmentation into larger not internucleosomal fragments (50-300 kbp lengths) and a single-strand cleavage (Walker et al. 1993; Bortner et al. 1995). The comet assay could be useful for the detection of PCD where the characteristic internucleosomal nDNA cleavage pattern is not present.

Our preliminary results indicate that in the anther tapetum and mesophyll cells the appearance of "comets" always coincides with changes in chromatin structure (Charzyńska and Leśniewska 1998; Simeonova et al. 1998; Leśniewska 1999). This leads to the conclusion that nDNA fragmentation and chromatin reorganization are symptoms of the same process of DNA disintegration. Application of the comet assay, which is a rapid and sensitive method of detection of DNA damage at the single cell level, should lead to a better understanding of the temporal relationships between changes in the chromatin structure, nDNA fragmentation and destruction of the nuclear function during plant PCD.

The portion of this study is also a part of PhD dissertation by J.L. at the Warsaw University.

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LITERATURE CITED

- BEERS E.P. 1997. Programmed cell death during plant growth and development. *Cell Death Differ.* 4: 649-661.
 BELL P.R. 1996. Megaspore abortion: a consequence of selective apoptosis? *Int. J. Plant Sci.* 157: 1-7.
 BLUMWALD E., AHARON G.S., LAM B.C-H. 1998. Early signal transduction pathways in plant-pathogen interactions. *Trends Plant Sci.* 3: 342-346.
 BORTNER C.D., NICKLAS B.E., OLDENBURG N.B.E., CIDLOWSKI J.A. 1995. The role of DNA fragmentation in apoptosis. *Trends Cell Biol.* vol. 5: 21-26.
 BUCKNER B., JANICK-BUCKNER D., GRAY J., JOHAL G.S. 1998. Cell-death mechanisms in maize. *Trends Plant Sci.* 3: 218-223.

- CHARZYŃSKA M. 1996. Programmed cell death in the ontogenesis of Angiosperms. *Folia Histochem. Cytobiol.* 34, suppl. 2: 57.
- CHARZYŃSKA M., LEŚNIEWSKA J. 1998. Structural and molecular aspects of programmed cell death of the anther tapetum. XV International Congress on Sexual Plant Reproduction. Wageningen, p. 18.
- DEL POZO O., LAM E. 1998. Caspases and programmed cell death in the hypersensitive response of plants to pathogens. *Curr. Biol.* 8: 1129-1132.
- EASTMAN A. 1995. Detection of internucleosomal DNA fragmentation. *Methods Cell Biol.* 46: 43-46.
- FAIRBAIRN D.W., OLIVE P.L., ONEILL K.L. 1995. The comet assay: a comprehensive review. *Mutation Res.* 339: 37-59.
- FUKUDA H. 1996. Xylogenesis: initiation, progression and cell death. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 47: 299-325.
- FUKUDA H. 1997. Programmed cell death during vascular system formation. *Cell Death Differ.* 4: 684-688.
- FUKUDA H., WATANABE Y., KURIYAMA H., AOYAGI S., SUGIYAMA M., YAMAMOTO R., DEMURA T., MINAMI A. 1998. Programming of cell death during xylogenesis. *J. Plant. Res.* 111: 253-256.
- GAO M., SHOWALTER A.M. 1999. Yariv reagent treatment induces programmed cell death in *Arabidopsis* cell cultures and implicates arabinogalactan protein involvement. *Plant J.* 19: 321-331.
- GAVRIELI Y., SHERMAN Y., BEN-SASSON S.A. 1992. Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* 119: 493-501.
- GILCHRIST D.G. 1997. Mycotoxins reveal connections between plants and animals in apoptosis and ceramide signaling. *Cell Death Differ.* 4: 689-698.
- GOLDSTAIN P. 1998. Cell death in us and others. *Science* 281: 1283.
- GRADZKA I., SZUMIEL I. 1996. Discrepancy between the initial DNA damage and cell survival after camptothecin treatment in two murine lymphoma L5178Y sublines. *Cell Biochem. Funct.* 14: 163-171.
- GREENBERG J.T. 1996. Programmed cell death: a way of life for plants. *Proc. Natl. Acad. Sci.* 93: 12094-12097.
- GREENBERG J.T. 1997. Programmed cell death in plant-pathogen interactions. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48: 525-545.
- HADFIELD A., BENNETT A.B. 1997. Programmed senescence of plant organs. *Cell Death Differen.* 4: 662-670.
- HAVEL L., DURZAN D.J. 1996. Apoptosis in plants. *Bot. Acta* 109: 268-277.
- INADA N., SAKAI A., KUROIWA H., KUROIWA T. 1999. Senescence program in rice *Oryza sativa* L. leaves: analysis of the blade of the second leaf at the tissue and cellular levels. *Protoplasma* 207: 222-232.
- JAŁOŚZYŃSKI P., KUJAWSKI M., SZYFTER K. 1996. The single cell gel electrophoresis – a useful technique for DNA damage studies (in Polish). *Post. Biol. Kom.* 23: 339-354.
- JONES A.M., DANGL J.L. 1996. Longjam at the Styx: programmed cell death in plants. *Trends Plant Sci.* 1 (4): 114-119.
- KRUSZEWSKI M., WOJEWÓDZKA M., IWANEŃKO T. 1996. Test kometkowy. 1. Teoria i praktyka (in Polish). Reports of Institute of Nuclear Chemistry and Technology (Seria B nr 1/96), Warszawa.
- LEŚNIEWSKA J. 1999. Differentiation and programmed death of secretory tapetum of *Ornithogalum virens* L., PhD dissertation at the Warsaw University. (in Polish).
- MITTLER R., LAM E. 1995. In situ detection of nDNA fragmentation during the differentiation of tracheary elements in higher plants. *Plant Physiol.* 108: 489-493.
- MITTLER R., LAM E. 1996. Sacrifice in the face of foes: pathogen-induced programmed cell death in plants. *Trends Microbiol.* 4: 10-15.
- MÜLLER W.U., BAUCH T., STREFFER C., NIEDEREICHHOLZ F., BÖCKER W. 1994. Comet assay studies of radiation-induced DNA damage and repair in various tumour cell lines. *Int. J. Radiat. Biol.* 65: 315-319.
- MÜLLER W.U., BAUCH T., WÓJCIK A., BOCKER W., STREFFER C. 1996. Comet assay studies indicate that caffeine – mediated increase in radiation risk of embryos is due to inhibition of DNA repair. *Mutagenesis* 11: 57-60.
- NOODEN L.D., GUIAMET J.J., JOHN I. 1997. Senescence mechanisms. *Physiol. Plant.* 101: 746-753.
- OLIVE P.L., WLODEK D., BANATH J.P. 1991. DNA double-strand breaks measured in individual cells subjected to gel electrophoresis. *Cancer Res.* 51: 4671-4676.
- OLIVE P.L., FRAZER G., BANATH J.P. 1993. Radiation-induced apoptosis measured in TK6 human B lymphoblast cells using the comet assay. *Radiation Res.* 136: 130-136.
- ORZÁEZ D., GRANELL A. 1997. DNA fragmentation is regulated by ethylene during carpel senescence in *Pisum sativum*. *Plant J.* 11: 137-144.
- ÖSTLING O., JOHANSON K.J. 1984. Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. *Bioch. Bioph. Res. Comm.* 123 (1): 291-298.
- PANAVAS T., RUBINSTEIN B. 1998. Oxidative events during programmed cell death of daylily (*Hemerocallis* hybrid) petals. *Plant Sci.* 133: 125-138.
- PARYS E., ROMANOWSKA E., SIEDLECKA M., POSKUTA J.W. 1998. The effect of lead on photosynthesis and respiration in detached leaves and in mesophyll protoplasts of *Pisum sativum*. *Acta Physiol. Plant.* 20(3): 313-322.
- PENNELL R.I., LAMB C. 1997. Programmed cell death in plants. *Plant Cell* 9: 1157-1168.
- RICHBERG M.H., AVIV D.H., DANGL J.L. 1998. Dead cells do tell tales. *Curr. Opin. Plant Biol.* 1: 480-485.
- RYERSON D.E., HEATH M.C. 1996. Cleavage of nuclear DNA into oligonucleosomal fragments during cell death induced by fungal infection or by abiotic treatments. *Plant Cell* 8: 393-402.
- SIKORA A. 1998. Changes of nDNA structure during differentiation and programmed death of plant cells (in Polish). MS Thesis, Dept. of Plant Anatomy and Cytology, Warsaw University.
- SIMEONOVA E., SIKORA A., MOSTOWSKA A., CHARZYŃSKA M. 1998. Progressive degradation of chloroplast structure during mesophyll senescence is accompanied by fragmentation of nuclear DNA. (in Polish). Proceeding of the 51 Congress of the Polish Botanical Society.
- SINGH N.P., MC COY M.T., TICE R.R., SCHNEIDER E.L. 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.* 175: 184-191.
- SUSIN S.A. et al. 1999. Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* 397: 441-446.
- WALKER P.R., KOKILEVA L., LE BLANC J., SIKORSKA M. 1993. Detection of initial stages of DNA fragmentation in apoptosis. *Bio-Techniques* 15: 1032-1040.
- WANG H., LI J., BOSTOCK R.M., GILCHRIST D.G. 1996. Apoptosis: a functional paradigm for programmed plant cell death induced by a host-selective phytotoxin and invoked during development. *Plant Cell* 8: 375-391.
- WANG M., HOEKSTRA S., VAN BERGEN S., LAMERS G.E.M., OPPERDIJK B.J., VAN DER HEIJDEN M.W., DE PRIESTER W., SCHILPEROORT R.A. 1999. Apoptosis in developing anthers and the role of ABA in this process during androgenesis in *Hordeum vulgare* L. *Plant Molec. Biol.* 39: 489-501.
- YEN CH.-H., YANG CH.-H. 1998. Evidence for programmed cell death during leaf senescence in plants. *Plant Cell Physiol.* 39(9): 922-927.
- YOUNG T.E., GALLIE D.R., DEMASON D.A. 1997. Ethylene-mediated programmed cell death during maize endosperm development of wild-type and shrunken 2 genotypes. *Plant Physiol.* 115: 737-751.

ZASTOSOWANIE METODY KOMETKOWEJ DO BADAŃ PROGRAMOWANEJ ŚMIERCI KOMÓRKOWEJ U ROŚLIN

STRESZCZENIE

W pracy przedstawiamy zastosowanie metody elektroforezy na żelu DNA pojedynczych komórek tzw. metody "kometkowej" (ang. "comet assay"), stosowanej na materiale zwierzęcym, do analizy PCD u roślin. Wybraliśmy do analizy trzy typy tkanek: tapetum pylnikowe, bielmo i mezofil, które w naturalnym procesie różnicowania lub starzenia organów ulegają programowanej śmierci. Analizowane tkanki były preparowane w odmienny sposób, zależny od ich organizacji komórkowej w celu otrzymania zawiesiny żywych, pozbawionych ścian komórek. Podaliśmy szczegółowo każdy z wariantów zastosowanej przez nas procedury oraz piśmiennictwo dotyczące metody. Metoda "kometkowa" jest szczególnie przydatna do badań zmian DNA, jeśli programowanej śmierci w rozwoju rośliny ulegają pojedyncze komórki lub niewielka ich liczba.

SŁOWA KLUCZOWE: metoda kometkowa, fragmentacja DNA, programowana śmierć komórkowa (PCD), tapetum, bielmo, mezofil, protoplasty.