Gibberellin-induced Nuclear Ca\(^{2+}\)-dependent Deoxyribonucleases and DNA Glycosylases Regulate DNA Degradation of Wheat Aleurone Cells Undergoing Programmed Cell Death

Amangeldy K. Bissenbaev\(^{1} \ast \) • Murat K. Saparbayev\(^{2} \)

\(^{1}\) Department of Molecular Biology and Genetics, Faculty of Biology, Kazakh National University named al-Farabi, 480078, Almaty, al-Farabi 71, Kazakhstan
\(^{2}\) CNRS UMR8126 Groupe «Réparation de l’ADN», Université Paris-Sud, Institut de Cancérologie Gustave Roussy, F-94805 Villejuif Cedex, France

Corresponding author: \ast Amangeldy.Bissenbaev@kaznu.kz

ABSTRACT

The nature of DNA fragmentation was studied in isolated nuclei of wheat aleurone layer cells. Endogenous Ca\(^{2+}\)- and Mg\(^{2+}\)-dependent deoxyribonucleases (DNase) were present in the nuclei. Analysis of DNase activity in the nuclear and cytoplasmic fractions revealed the nuclear location of A and C forms of DNases. Activity of the B-form enzymes was found exclusively in the cytoplasm. Experimental evidence demonstrated that activation of B and A nucleases did not depend on the presence of gibberellic acid (GA\(_3\)) in the incubation medium, while activation of C-form DNase was dependent on the presence of GA\(_3\). The stimulatory effect of GA\(_3\) on the activation of Ca\(^{2+}\)-dependent DNA-N-glycosylases, the key DNA repair enzyme, was demonstrated. The evidence suggests that concurrent activation of DNA repair enzymes and GA\(_3\)-dependent nuclear DNases is likely to be the underlying cause of oligonucleosomal fragmentation of DNA during programmed cell death in wheat aleurone layer cells.

Keywords: abscisic acid, gibberellic acid, AP-endonucleases, nuclease, nucleus

Abbreviations: AP, apurinic/apyrimidinic; DNase, deoxyribonuclease; Fpg, formamidopyrimidine-DNA glycosylase; Nfo, Endonuclease IV; PCD, programmed cell death; ROS, reactive oxygen species

INTRODUCTION

The cereal aleurone is one of the most intensively studied tissues in plant biology because of its importance in malting and brewing. Chemists and biologists have carried out detailed investigations of cereal grain functions for nearly 200 years (Bethke et al. 1997).

The aleurone layer of a cereal grain is a highly specialized tissue that synthesizes and secretes hydrolases (including α-amylase), which in turn mobilizes endosperm reserves during germination (Fincher 1989). Gibberellic acid (GA\(_3\)) is a plant hormone that serves to regulate the synthesis and secretion of hydrolases (Fincher 1989; Bissenbaev et al. 1992), thus producing simple carbohydrates to support the initial germination and growth of the seedling. GA\(_3\) not only promotes the metabolic activation of dormant aleurone cells but also accelerates their programmed cell death (PCD) (Dominguez et al. 2004), which is under genetic control (Kuo et al. 1996; Wang et al. 1996; Bissenbaev et al. 2004; Dominguez et al. 2004).

The key features of PCD include early cell membrane blebbing, generalized chromatin condensation, compaction of cytoplasmic organelles and internucleosomal DNA cleavage (Krishnamurthy 2000). DNA fragmentation in particular has been used as a marker of apoptosis and is a critical feature of PCD.

Various nucleases have been identified in plant cells undergoing PCD. Two prominent and several minor nuclease activities were detected in dying pericarp and nucellar cells of maize, with deoxyribonuclease (DNase) activity also detected in dying endosperm cells (Young and Gallie 2000). It remains to be determined whether one or more of the observed nuclease activities is responsible for the inter-nucleosomal degradation of endosperm DNA.

Cellular metabolism based on aerobic respiration generates reactive oxygen species (ROS), which are central to the regulation of PCD (Gechev et al. 2006). Indeed, an increased level of ROS triggers cell death in both plant and animal tissues suggesting the almost universal nature of the role of free radicals. ROS also induces the oxidative damage of DNA, resulting in strand breaks, and base and sugar modifications (Ischenko and Saparbaev 2002). Oxidative DNA damage induces a robust DNA repair response, characterized by excision of modified bases and/or nucleotides. Cleavage of double-stranded DNA also activates DNA repair enzymes, including DNA-N-glycosylases and AP-endonucleases (Roldán-Arjona and Ariza 2009).

The goal of the present work was to investigate the function of nuclear DNases and DNA-glycosylases in ontogenetically PCD of aleurone layer cells in wheat seeds.

MATERIALS AND METHODS

Plant material: Preparation of wheat aleurone layers

Aleurone layers were isolated from grains of wheat (Triticum aestivum, variety Kazakhstanskaya 4) collected from the 2003 harvest. Aleurone layers were prepared from de-embryonated seeds as described previously (Bissenbaev et al. 2007) and incubated in media containing 10 mM CaCl\(_2\) and 1 µM GA\(_3\) (Sigma-Aldrich, St. Louis, USA) and/or 5 µM ABA (Sigma-Aldrich) and incubated up to 96 h at room temperature in the dark with constant agitation (125 rpm).
Preparation of nuclear and cytoplasmic extracts

Nuclear and cytoplasmic extracts were prepared as previously described (Dominguez et al. 2004) with some modifications. The freshly isolated aleurone cells (500-1000 cells) were ground under liquid nitrogen using a mortar and pestle and then resuspended in 15-20 ml of buffer A containing 15 mM Tris-HCl, pH 7.4, 60 mM KCl, 15 mM NaCl, 0.34 M sucrose, 0.15 mM spermine (Sigma-Aldrich), 0.5 mM spermidine (Sigma-Aldrich), 0.2 mM phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich), 0.25% (v/v) Triton X-100 (Sigma-Aldrich) and 15 mM β-mercaptoethanol (Sigma-Aldrich) at +4°C.

The homogenate was filtered through a nylon mesh (Millipore®) with a pore size of 60 μm. Cellular fractionation was performed by carefully placing the clarified homogenate in an equal volume of 30% (v/v) Percoll prepared in buffer A and centrifuged at 7000 rpm for 15 min. The upper phase was retained as the cytoplasmic fraction, the Percoll phase was discarded, and the nuclei-enriched pellet was harvested and washed in buffer A before resuspending in 100 μl of extraction buffer containing 15 mM Tris-HCl, pH 7.4, 40 mM KCl and 20% (v/v) glycerol. After extraction on ice for 30 min and centrifugation at 13,000 rpm for 20 min at 4°C, the supernatant containing the nuclei was recovered. The protein concentrations in extracts were measured by the Bradford assay (Bradford 1976).

DNA extraction and electrophoresis

Isolated nuclei were lysed in 0.7 ml of ice cold buffer containing 5 mM Tris-HCl, pH 8.0, 20 mM EDTA, 0.5% (w/v) Triton X-100, 2% (w/v) sodium dodecyl sulphate (SDS), 1.4 M NaCl, 0.2% (w/v) β-mercaptoethanol and incubated for 30 min at 60°C. The DNA was extracted with equal volumes of chloroform and centrifuged for 10 min at 10,000 rpm. The aqueous phase was transferred to a new tube and 2/3 volumes of ice-cold isopropanol (%0°C) was added and centrifuged for 10 min at 5000 rpm. The supernatant was discarded, and the pellet containing dried DNA was resuspended in 500 μl of TE buffer (10 mM Tris-HCl, pH 7.5 and 0.1 mM EDTA). The resulting suspension was extracted with equal volumes of phenol: chloroform (1:1) mixture and after 15 min centrifuged at 5000 rpm for 10 min. Sodium acetate (pH 7.0, 1/10 vol) was added to the isolated aqueous phase followed by the addition of 2.5 volumes of ice-cold ethanol. DNA was then allowed to precipitate at –20°C. After centrifugation the pellet containing DNA was dried and resuspended in 50 μl of TE buffer. DNA samples were analyzed by 1.8% agarose gel electrophoresis at 70V for 2 h. Agarose gels were stained with 10 μg/ml (w/v) ethidium bromide and DNA was visualized by ultraviolet fluorescence.

Nuclease activity gels

In-gel nuclease activity assay was performed according to the method described previously (Fath et al. 1999) with some modifications. Cytoplasmic and nuclear extracts (120 μg of protein) obtained as described above were subjected to 12.5% SDS-PAGE gels containing 0.5 mg ml–1 DNA extracted from leaves of wheat grains germinated for 4 days and 0.5% (w/v) bovine albumin at 4°C and 30 mA plate. After electrophoresis gels were washed twice in a buffer containing 10 mM Tris-HCl (pH 6.0), 25% isopropanol, 1 mM CaCl2, 1 mM MgCl2 and 1 mM DTT then washed for 30 min with the same buffer but without isopropanol. Rinsed gels were incubated in the buffer containing 10 mM Tris-HCl pH 6.0, 1 mM CaCl2, 1 mM MgCl2 for 24 h at 37°C. To detect DNase activity the gels were stained with 10 μg/ml (w/v) ethidium bromide for 15 min. DNase activities were visualized under ultraviolet light as clear DNA-depleted non-fluorescent bands in a fluorescent background.

DNA modifications and DNA incision assay

Oxidatively damaged DNA was produced by treatment of the supercoiled plasmid DNA with KMnO4. To generate DNA containing oxidized bases, 2 mg/ml of pBluescript II plasmid DNA in 10 mM degassed phosphate buffer, pH 7.5, was treated with 40 mM KMnO4 at 0°C for 5 min as described by Ishchenko et al. (2002). The DNA samples were desalted on a Sephadex G50 column equilibrated in 10 mM Tris-HCl, pH 7.5 and 1 mM EDTA. The standard assay mixture for damage-specific incision activity (20 μl final volume) contained 0.8 mg of chemically modified pBluescript II plasmid in 20 mM HEPES-KOH (pH 7.5), 50 mM KCl, 1 mM EDTA, limiting amounts of aleurone nuclear extract (0.02 μg of protein). Incubations were carried out at 37°C for 5 min and terminated by addition of 20 μl 0.2 M phenylmethylsulfonylfluoride (PMSF, Sigma-Aldrich) and products were separated by electrophoresis on a 0.8% agarose gel in the presence of ethidium bromide (0.5 mg/ml).

Statistical analyses

All experiments described here were repeated at least three times. Similar results and identical trends were obtained each time. The data reported here are from a single experiment.

RESULTS AND DISCUSSION

A time course study of the action of GA3 on the DNA fragmentation of aleurone layer cells showed that treatment by 1 μM GA3 strongly enhanced DNA fragmentation but only after incubation of aleurone cells for 72 h. When incubation time was increase up to 96 h further DNA fragmentation occurred. Under these conditions different sized fragments were observed to form characteristic internucleosomal band ladders during agarose gel electrophoresis (Fig. 1A).

The data supports our previous proposal that apoptotic characters of wheat aleurone PCD are initiated by GA3 (Bissenbaev et al. 2004). It seems likely that wheat aleurone cell death is initiated after 48 h of GA3 treatment and reaches a maximum level 96 h after treatment. This process is accompanied by internucleosomal DNA fragmentation that is characteristic of apoptosis (Gilchrist 1998).

In a previous report we demonstrated the presence of three types of DNase, denoted as A, B and C forms, in the aleurone layer of wheat seeds. For DNase form B, the presence of Ca2+ and Mg2+ is not required, whereas for form A both divalent metal cations are necessary, i.e., form A is a Ca2+/Mg2+-dependent DNase. In contrast, the GA3-activated DNase form C was observed only in the presence of Ca2+ cations, i.e., Dnase form C is Ca2+-dependent (Bissenbaev et al. 2004). Interestingly, the addition of 0.01-1 μM ABA to the incubation medium entirely eliminated the GA3-stimulated fragmentation of DNA to oligonucleosomal fragments (Fig. 1B).

In order to further analyse the involvement of nuclear endonucleases in GA3-induced PCD of wheat aleurone cells, we studied DNA fragmentation in isolated nuclei. Fig. 2A (lane 1) shows that DNA remains intact in freshly isolated nuclei. Incubation of nuclei with 1 mM Ca2+ and 5 mM Mg2+ ions for 15 min significantly increased the DNA fragmentation. Under these conditions different sized DNA fragments formed characteristic internucleosomal ladders when electrophoresed on agarose (Fig. 2A, lane 2). Most DNA was fragmented in 75 min following incubation of isolated nuclei in the presence of 1 mM Ca2+ and 5 mM Mg2+ ions (Fig. 2A, lanes 3-6). This data suggests that wheat aleurone nuclei contain an endogenous Ca2+/Mg2+-dependent DNase responsible for DNA fragmentation.

As mentioned above, nucleases associated with PCD in animal cells differ in their cation requirements. DNase I, which has been cloned and sequenced from rat, human and mouse genomes (Hess 1996), requires Ca2+/Mg2+ for activation. Other animal cells differ in their cation requirements. DNase I, which has been cloned and sequenced from rat, human and mouse genomes (Hess 1996), requires Ca2+/Mg2+ for activation. In order to further analyze the involvement of nuclear endonucleases in GA3-induced PCD of wheat aleurone cells, we studied DNA fragmentation in isolated nuclei. DNAase I and II produce different 5’ and 3’ terminal motifs. DNAase I produces DNA oligomers with 5’ hydroxyl, while DNAase II produces 5’ hydroxyl and 3’ phosphates (Wyllie 1980; Lewin 2004).

To study the effects of divalent cations on DNA fragmentation, the aleurone cell nuclei were incubated for 30 min either with Ca2+ or Mg2+ alone or in combinations of both cations. In the presence of 1 mM CaCl2 DNA fragment-
Effect of Ca\textsuperscript{2+} alone. Incubation of isolated nuclei in the presence of Ca\textsuperscript{2+}/Mg\textsuperscript{2+} and Ca\textsuperscript{2+}-dependent and are localized in the nucleus of aleurone cells undergoing PCD.

The presence of two types of endogenous DNases that cleave DNA. In contrast, the addition of 25 mM EGTA to the incubation medium caused an increase in DNA glycosylase and an AP endonuclease (Gros et al. 2002). Interestingly, in the nuclear extracts of untreated aleurone cells only one protein band corresponding to the A form of the nuclease was present (Fig. 3, lane 3). Finally, the addition of 1 mM Ca\textsuperscript{2+} to the incubation medium was accompanied with a change in the spectrum of nucleases in nuclear extracts since an additional DNA-negative electrophoretic band corresponding to the C form nuclease was found in addition to the A form (Fig. 3, lane 4). Therefore, activation of B and A nucleases did not depend on the presence of GA\textsubscript{3} in the incubation medium. On this basis the results demonstrated the nuclear localization of A and C forms, whereas the activity of B-nuclease was detected exclusively in the cytoplasm. Activation of the C form of nuclease in nuclear extracts occurs only in the presence of GA\textsubscript{3}, clearly demonstrating that GA\textsubscript{3} regulates its expression by a functional signal transduction pathway in response to the hormone. It seems likely that a GA\textsubscript{3}-dependent increase of nuclear C-nuclease activity might be a principal cause of internucleosomal fragmentations of DNA during PCD in wheat aleurone layers.

As was noted above, cellular energetic oxidative metabolism generates ROS such as superoxide, hydrogen peroxide, hydroxyl radical, nitrogen monoxide, and unsaturated fatty acid radicals. Evidence continues to accumulate to support the paradigm that free oxygen radical-mediated damage to hereditary material (DNA) is the underlying cause of apoptosis. It has been shown that one of the primary factors which leads to DNA damage and mutations is oxidative stress (Beckman and Ames 1997). The levels of oxygen-free radicals are increased during oxidative stress and this in turn induces various levels of DNA damage such as 8-oxyguanine, formamidopyrimidines, thymine glycols. It is thought that during evolution, cells developed several DNA repair pathways to remove the products of DNA damage and restore the integrity of genetic material. One of the key and most universal DNA repair pathways is base excision repair (BER), which is initiated by the joint action of a DNA glycosylase and an AP endonuclease (Gros et al. 2002).

Previously we have shown that the increase in intracellular concentration of ROS, resulting from the GA\textsubscript{3}-mediated inhibition of enzymes involved in the antioxidative defense system, serves to trigger PCD (Bissenbaev et al. 2007). The decrease in activities of metabolic enzymes in GA\textsubscript{3}-treated cells significantly accelerated the process of

Fig. 1 Effect of GA on the cellular fragmentation of DNA in wheat aleurone cells. (A) Time dependence of cellular DNA fragmentation after GA-treatment. 1 - 24 h; 2 - 48 h; 3 - 72 h; 4 - 96 h; 5 - Marker (bp); (B) Dose effect of ABA on GA-induced cellular DNA fragmentation; aleurone layers were incubated for 96 h after treatment prior to gel electrophoresis. 1 - GA (1 \mu M); 2 - GA (1 \mu M) + ABA (0.01 \mu M); 3 - GA (1 \mu M) + ABA (0.1 \mu M); 4 - GA (1 \mu M) + ABA (1 \mu M).

Fig. 2 Analysis of DNA fragments from wheat aleurone nuclei by agarose gel electrophoresis. (A) Time course of cellular DNA fragmentation by the isolated nuclei extracts in the presence of 1 mM CaCl\textsubscript{2} and 5 mM MgCl\textsubscript{2}. 1 - freshly isolated nuclei; 2 - 15 min; 3 - 30 min; 4 - 45 min; 5 - 60 min; 6 - 75 min. (B) Inhibition of Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-dependent fragmentation of cellular DNA by EGTA and EDTA. 1 - Ca\textsuperscript{2+}- and Mg\textsuperscript{2+}-free medium; 2 - 1 mM Ca\textsuperscript{2+}; 3 - 1 mM Ca\textsuperscript{2+} + 5 mM Mg\textsuperscript{2+}; 4 - 5 mM Mg\textsuperscript{2+}; 5 - 1 mM Ca\textsuperscript{2+} + 5 mM Mg\textsuperscript{2+} + 25 mM EDTA; 6 - 1 mM Ca\textsuperscript{2+} + 5 mM Mg\textsuperscript{2+} + 50 mM EDTA.

Fig. 3 Effects of 1 \mu M GA on the activation of deoxyribonucleases in wheat aleurone cells. Aleurone cells were incubated for 72 h after treatment and prior to gel electrophoresis analysis. 1 - Whole cell-free extracts from GA-treated wheat aleurone cells; 2 - Cytoplasmic extracts from GA-treated wheat aleurone cells; 3 - Nuclear extracts prepared from aleurone cells untreated with GA\textsubscript{3}; 4 - Nuclear extracts prepared from GA-treated wheat aleurone cells; 5 - commercial deoxyribonuclease. A, B and C arrows indicate the protein bands with DNase activity.
ROS production which in turn leads to PCD of wheat aleurone cells. In contrast, the aleurone layers incubated in the presence of ABA do not undergo PCD and maintain high levels of ascorbate peroxidase and superoxide dismutase activities as compared to the cells incubated in GA₃-containing media (Bissenbaev et al. 2007).

Bifunctional DNA glycosylases/AP lyases produce single-strand breaks at the site of a damaged base in double-stranded DNA. This type of break converts supercoiled covalently closed circular DNA to open circular or linear forms of DNA, which can be separated by agarose gel electrophoresis. We used this approach to elucidate the DNA glycosylase activity towards plasmid DNA (pBluescript II) treated with KMnO₄. Fig. 4 shows typical agarose gel electrophoresis to measure the incision activity of the proteins in nuclear extracts on plasmid DNA substrates. E. coli formamidopyrimidine-DNA glycosylase (Fpg) efficiently incises supercoiled plasmid DNA treated by KMnO₄, although no incision was detected when using E. coli AP endonuclease (Nfo) (Fig. 4, lanes 3 and 2). Fpg acts both as a DNA glycosylase and an AP-lyase. The DNA glycosylase activity of Fpg excises oxidized purines including 7,8-dihydro-8-oxoguanine (8-oxoG), formamidopyrimidines from duplex DNA generating apurinic/pyrimidinic (AP) sites which are then converted to single-strand break points by the AP lyase function of Fpg. In contrast, Nfo is more efficient towards DMS/alkali-treated plasmid DNA, and to much lesser extent on DNA treated with H₂O₂ or KMnO₄ (Ishchenko et al. 2002). This result suggests that oxidatively damaged DNA was obtained by the KMnO₄-treatment of plasmid DNA. Thus, oxidized plasmid DNA is a good substrate for measuring activities of oxidative damage-specific DNA glycosylases.

The results have shown that the incubation of isolated aleurone layers in the presence of 1 μM GA₃ for 72 h results in considerable enhancement of the Ca²⁺-dependent form of a DNA glycosylase, and does not affect the Mg²⁺-dependent forms (Fig. 4, lanes 4-5). The addition of only 5 μM ABA, the native antagonist of GA₃ to incubation media significantly inhibited GA₃-induced Ca²⁺-dependent DNA glycosylases. As shown in Fig. 4, the presence of the divalent cation chelator EDTA strongly inhibits the GA₃-induced DNA glycosylase. The addition of the specific calcium ion chelator, 10 mM EGTA, entirely blocked GA₃-induced activity of the DNA glycosylases (Fig. 4, lane 7).

CONCLUSIONS

The evidence from results obtained during the present study suggests that the GA₃-stimulation of PCD in wheat seed aleurone cells may involve activation of DNA glycosylases. However, under the experimental conditions described, GA₃ activated only Ca²⁺-dependent DNA glycosylases. These results may indicate a key role for DNA repair enzymes in hormone-regulated PCD of wheat aleurone cells. GA₃-induced PCD of aleurone cells by the fragmentation of genomic DNA to oligonucleosomal fragments. Degradation of chromatin to oligonucleosomal fragments, which is a characteristic apoptotic cell death, occurred at later stages of apoptosis. It is expected that prior to PCD, the GA₃-mediated inhibition of antioxidant defense enzymes increase intracellular concentrations of ROS (Bissenbaev et al. 2007). The down-regulation of ROS defense enzyme activities in GA₃-treated cells significantly increased ROS levels in the cell and correspondingly led to the accumulation of oxidative DNA base damage in the genome. Products of oxidative DNA base damage are the main substrates for DNA glycosylases and/or AP endonucleases in the BER pathway for DNA repair (Saparbaev et al. 2002). These DNA repair enzymes pro-duce single-strand breaks at the 3'- and 5'-P extremities, which in turn serve as substrates for endo- and exonucleases. As we described above, GA₃ in our model system induces the activation of endogeneously localized nuclear endonucleases. It is tempting to speculate that concurrent activation of DNA repair enzymes which lead to the accumulation of single-strand DNA breaks and GA₃-dependent nuclear DNase is the primary reason for oligonucleosomal fragmentation of DNA in PCD of cells in the aleurone tissue of germinating wheat seed.

ACKNOWLEDGEMENTS

The editorial assistance of Prof. Colin R. Wellings (The University of Sydney, Australia) is gratefully acknowledged.

REFERENCES

Beckman KB, Ames BN (1997) Oxidative decay of DNA. The Journal of Biological Chemistry 272, 19633-19636
Gibberellin-induced DNA degradation of wheat aleurone cells. Bissenbaev and Saparbayev


Saparbaev M, Sidorkina OM, Jurado J, Privezentzev CV, Greenberg MM, Laval J (2002) Repair of oxidized purines and damaged pyrimidines by E. coli Fpg protein: different roles of proline 2 and lysine 57 residues. Environmental and Molecular Mutagenesis 39, 10-17


Young TE, Gallie DR (2000) Programmed cell death during endosperm development. Plant Molecular Biology 44 (3), 283-301