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

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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) (Domínguez *et al.* 2004), which is under genetic control (Kuo *et al.* 1996; Wang *et al.* 1996; Bissenbaev *et al.* 2004; Domínguez *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 internucleosomal 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).

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Preparation of nuclear and cytoplasmic extracts

Nuclear and cytoplasmic extracts were prepared as described previously (Domínguez *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 (–20°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 visualised 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 $CaCl_2$, 1 mM $MgCl_2$ 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 $CaCl_2$, 1 mM $MgCl_2$ 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 $KMnO_4$. 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 $KMnO_4$ 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. The reaction was stopped with 20 mM EDTA 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 GA_3 on the DNA fragmentation of aleurone layer cells showed that treatment by 1 μM GA_3 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 GA_3 (Bissenbaev *et al.* 2004). It seems likely that wheat aleurone cell death is initiated after 48 h of GA_3 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 Ca^{2+} and Mg^{+2} is not required, whereas for form A both divalent metal cations are necessary, i.e., form A is a Ca^{2+}/Mg^{+2}-dependent DNase. In contrast, the GA_3-activated DNase form C was observed only in the presence of Ca^{2+} cations, i.e., Dnase form C is Ca^{2+}-dependent (Bissenbaev *et al.* 2004). Interestingly, the addition of 0.01-1 μM ABA to the incubation medium entirely eliminated the GA_3-stimulated fragmentation of DNA to oligonucleosomal fragments (**Fig. 1B**).

In order to further analyse the involvement of nuclear endonucleases in GA_3-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 Ca^{2+} and 5 mM Mg^{2+} 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 Ca^{2+} and 5 mM Mg^{2+} ions (**Fig. 2A**, lanes 3-6). This data suggests that wheat aleurone nuclei contain an endogenous Ca^{2+}/Mg^{2+}-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 Ca^{+2}/Mg^{+2} for activation and is inhibited by Zn^{+2}. In contrast, DNase II activity is independent of added Ca^{+2}/Mg^{+2} and has a pH optimum of 5. DNase I and II produce different 5′ and 3′ terminal motifs. DNAase I produces DNA oligomers with 5′ phosphates and 3′ hydroxyl, while DNase 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 Ca^{2+} or Mg^{2+} alone or in combinations of both cations. In the presence of 1 mM $CaCl_2$ DNA fragmen-

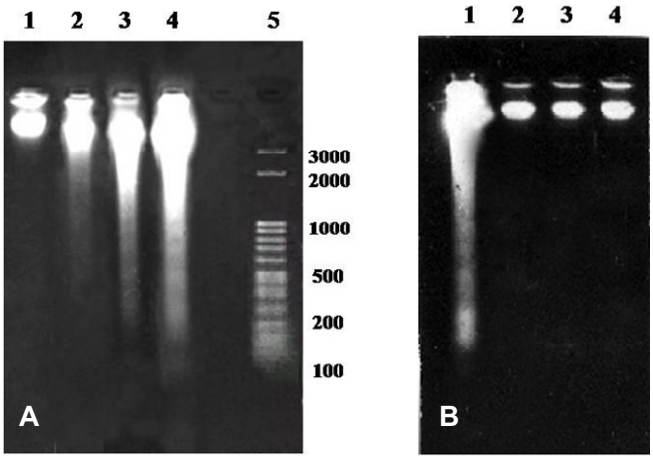

Fig. 1 Effect of GA on the cellular fragmentation of DNA in wheat aleurone cells. (**A**) Time dependence of cellular DNA fragmentation after GA-treatment. 1 - 24 h; 2 - 48 h; 3 - 72 h; 4 - 96 h; 5 - Marker (bp); (**B**) Dose effect of ABA on GA-induced cellular DNA fragmentation; aleurone layers were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

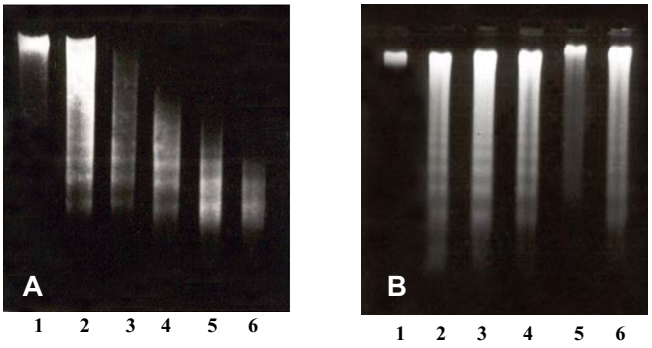

Fig. 2 Analysis of DNA fragments from wheat aleurone nuclei by agarose gel electrophoresis. (**A**) Time course of cellular DNA fragmentation by the isolated nuclei extracts in the presence of 1 mM $CaCl_2$ and 5 mM $MgCl_2$. 1 - freshly isolated nuclei, 2 - 15 min; 3 - 30 min; 4 - 45 min; 5 - 60 min; 6 - 75 min. (**B**) Inhibition of Ca^{+2}/Mg^{+2}-dependent fragmentation of cellular DNA by EGTA and EDTA. 1 - Ca^{+2}- and Mg^{+2}-free medium; 2 - 1 mM Ca^{+2}; 3 - 1 mM Ca^{+2} + 5 mM Mg^{+2}; 4 - 5 mM Mg^{+2}; 5 - 1 mM Ca^{+2} + 5 mM Mg^{+2} + 25 mM EGTA; 6 - 1 mM Ca^{+2} + 5 mM Mg^{+2} + 50 mM EDTA.

tation increased (**Fig. 2B**, lane 2). The combined addition of 1 mM $CaCl_2$ and 5 mM $MgCl_2$ to the incubation medium significantly increased DNA fragmentation compared with the control (Ca^{2+}-free medium) and compared with the effect of Ca^{2+} alone. Incubation of isolated nuclei in the presence 50 mM EDTA decreased the level of fragmented DNA. In contrast, the addition of 25 mM EGTA to the incubation medium almost completely prevented nuclear DNA fragmentation (**Fig. 2B**, lane 5). These results strongly suggest the presence of two types of endogenous DNases that are Ca^{+2}/Mg^{+2} and Ca^{+2}-dependent and are localized in the nucleus of aleurone cells undergoing PCD.

To characterize the DNase activity localized in the nuclei of aleurone cells, nuclear and cytoplasmic extracts were prepared from cells untreated and treated with GA_3 for 72 h. The in-gel activity assay revealed three distinct protein bands with nuclease activity (A, B and C forms) in the cell-free extracts from GA_3-treated wheat aleurone cells (**Fig. 3**, lane 1). The in-gel analysis of nuclease activity of cytoplasmic extracts from aleurone cells treated with GA_3 for 72 h revealed the presence of the B form of DNase. 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 μM

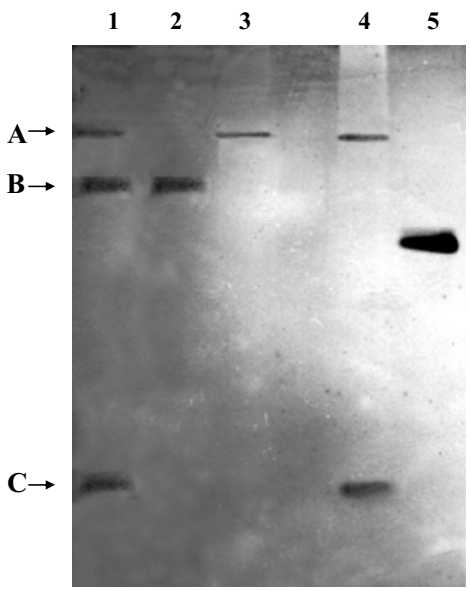

Fig. 3 Effects of 1 μM GA on the activation of deoxyribonucleases in wheat aleurone cells. Aleurone cells were incubated for 72 h after treatment and prior to gel electrophoresis analysis. 1 - Whole cell-free extracts from GA-treated wheat aleurone cells; 2 - Cytoplasmic extracts from GA-treated wheat aleurone cells; 3 - Nuclear extracts prepared from aleurone cells untreated with GA_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.

GA_3 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_3 in the incubation medium. On this basis the results demonstrated the nuclear location 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_3, clearly demonstrating that GA_3 regulates its expression by a functional signal transduction pathway in response to the hormone. It seems likely that a GA_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_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_3-treated cells significantly accelerated the process of

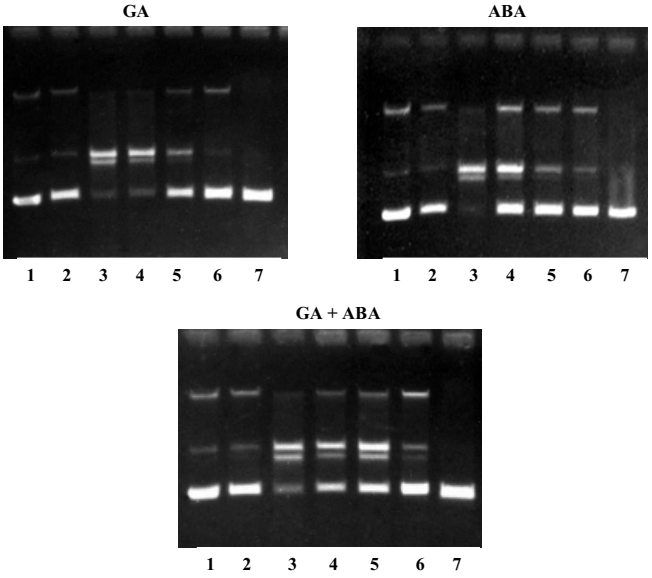

Fig. 4 Effect of divalent cations on the activity of DNA glycosylases in wheat aleurone cells. Aleurone cells were incubated for 72 h after treatment prior to gel electrophoresis analysis. Dosage of GA - 1 μM; Dosage of ABA - 5 μM. 1 - Control; 2 - 0.027 μg of Nfo; 3 - 0.012 μg of Fpg; 4 - 1 mM Ca^{2+}; 5 - 5 mM Mg^{2+}; 6 - 5 mM Mg^{2+} + 10 mM EDTA; 7 - 1 mM Ca^{2+} + 10 mM EGTA.

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 GA_3-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 $KMnO_4$. **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 $KMnO_4$, 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), formamido-pyrimidines from duplex DNA generating apurinic/apyrimidinic (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 H_2O_2 or $KMnO_4$ (Ishchenko *et al.* 2002). This result suggests that oxidatively damaged DNA was obtained by the $KMnO_4$-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 GA_3 for 72 h results in considerable enhancement of the Ca^{2+}-dependent form of a DNA glycosylase, and does not affect the Mg^{2+}-dependent forms (**Fig. 4**, lanes 4-5). The addition of only 5 μM ABA, the native antagonist of GA_3, to incubation media significantly inhibited GA_3-induced Ca^{2+}-dependent DNA glycosylases. As shown in **Fig. 4**, the presence of the divalent cation chelator EDTA strongly inhibits the GA_3-induced DNA glycosylase. The addition of the specific calcium ion chelator, 10 mM EGTA, entirely blocked GA_3-induced activity of the DNA glycosylases (**Fig. 4**, lane 7).

CONCLUSIONS

The evidence from results obtained during the present study suggests that the GA_3-stimulation of PCD in wheat seed aleurone cells may involve activation of DNA glycosylases. However, under the experimental conditions described, GA_3 activated only Ca^{2+}-dependent DNA glycosylases. These results may indicate a key role for DNA excision repair enzymes in hormone-regulated PCD of wheat aleurone cells. GA_3-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, occured at later stages of apoptosis. It is expected that prior to PCD, the GA_3-mediated inhibition of antioxidative defense enzymes increase intracellular concentrations of ROS (Bissenbaev *et al.* 2007). The down-regulation of ROS defense enzyme activities in GA_3-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′-OH and 5′-P extremities, which in turn serve as substrates for endo- and exonucleases. As we described above, GA_3 in our model system induces the activation of endogenously 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 GA_3-dependent nuclear DNase is the primary reason for oligonucleosomal fragmentation of DNA in PCD of cells in the aleurone tissue of germinating wheat seed.

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