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5-Azacytidine decreases fragmentation of nuclear DNA and pigment formation in first leaf cells of barley seedlings

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Abstract

Realization of programmed cell death in senescence represents an activation/inactivation of the respective gene. Enzymatic methylation of nuclear DNA with the creation of 5methylcytosine is one of the mechanisms, which can regulate gene activity in animal and plant cells. 5azacytidine (5azaC) acts as an inhibitor of DNA methylation, and induces expression of a range of some genes including genes responsible for senescence. Fragmentation of nuclear DNA is one of the hallmarks of programmed cell death in apoptosis pathway in plant cells. The influence of 5azaC (100 μkg/ml) on nuclear DNS amount and its fragmentation in the first leaf cells of barley was studied. It was shown that in the first leaf cells of barley seedlings there is an apoptosis pathway of programmed cell death. It was also observed that nuclear DNA fragmentation under the 5azaC influence is strongly inhibited, and the DNA amount in the first leaf increases.

Synthesis and destruction of chlorophyll also play a significant role in programmed cell death in plants. It was shown that under the 5azaC influence, the absorption spectrum of a pigment does not change in leaves and coleoptiles in the light, whereas in the dark condition, these pigments are not created under the 5azaC influence.

Keywords: Apoptosis; DNA fragmentation; 5-azacytidine; Pigments; Barley seedlings

1. Introduction

Plants have various forms of programmed cell death: one of them is realized as an obligatory development and senescence programme, the other is a hypersensitive reaction. Apoptosis was found in mezofile cells of senescence leaves of *Philodendron hastatum, Epipermnum aureum, Bauhimia purpured, Delonix regia, Butea monosperma*. Young leaves of these plants do not demonstrate apoptosis (Vanyushin, 2001). Although there are few reports concerning the mechanisms of programmed cell death in plant cells, recent studies have shown several morphological and biochemical similarities between apoptosis and programmed plant cell death (Danon et al., 2000). These data suggest that programmed cell death in plants and animals may be based on a general cell death process. The one of this cell process is a repression/derepression of the respective gene by methylation of nuclear DNA. Enzymatic methylation of nuclear DNA with the creation of 5methylcytosine in CG and CNG sequences has been correlated with gene silencing associated with various phenomena such as genomic imprinting, transposon and X-chromosome inactivation, differentiation, and cancer in animal cells (Bird, 2002). In plant cells, 5mC amounts up to 30% of the cytosine residues in nuclear DNA. Methylation of nuclear DNA is also a very important phenomenon in plant cells. In plants, DNA methylation is species-, tissue-, and organelle specific. It changes with age and is regulated by phytohormons (Vanyushin, 2005). Cytosine methylation of genomic DNA in plants has been extensively studied with regard to gene silencing of transgenes and transposon elements (Martienssen and Colot, 2001). Reduction in DNA methylation led to heritable alterations in the flowering time, fertility, and in leaf and floral morphology (Jeddeloh et al., 1999).

5azacytidine (5azaC) acts as an inhibitor of replicate methylation in animal cells. The main inhibitory role of 5azacytidine in DNA methylation appears to arise from the ability of

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5azaC to differentiate among the systems, perhaps by activating previously dormant genes and triggering their expression (Bird, 2002). In plants, 5azaC also induces demethylation of genomic DNA and expression of a range of genes. In wheat seeds for example, 5azaC induces the expression of protein storage genes (Vanyushin, 2001). There are very many investigations about the influence 5azaC-induced DNS demethylation on apoptosis in tumor cells. An apoptotic protease-activating factor, which interacts with cytochrome C and caspase 9 in the ''apoptosome'' to mediate apoptosis, can be reactivated by 5azaC (Klein, 2004). But about 5azaC-induced DNS demethylation on apoptosis in plant cells there, is practically no information.

However, certain features observed in the plant cell-free system suggest a different nature of apoptosis pathway in plants and animals (Balk et al., 2003). It is connected with different environmental constraints of plant life-style. Thus synthesis and destruction of chlorophyll play a significant role in programmed cell death in plants. In this pathway, activation of many enzymes and specific factors are involved. Such environmental factor as light regulates the synthesis of many genes that are necessary for chlorophyll synthesis. Thus, the expression of the FLP gene, which product as a regulator of chlorophyll synthesis is controlled by light (Falciatore et al., 2005). The pool size of two Mg^{2+} containing porphyrins and protophyrins, that are chlorophyll precursors, increased from dark to light (Kropat et al., 2000). But light can lead to the expression of nuclear genes through their methylation. For example, light-induced demethylation of a distant regulatory site correlated with increased levels of PEPSase mRNA levels in *Zea mays* (Langdale et al., 1991). Such demethylation factors as 5azaC can involve and regulate the process of chlorophyll synthesis. But there is very little information about it. In this study we investigated the 5azaC influence on two important phenomena involved in plant cell senescence: the nuclear DNA apoptotic fragmentation, and pigment formation in cells of barley seedlings.

2. Materials and methods

2.1. Plants materials

Barley seedlings (*Hordeun vulgare* L. cv Abava) were placed on the water filter paper in a plastic cuvette and soaked in H_2O. After germination for 36 h in the darkness at 25 °C, seedlings were placed in another analogical plastic cuvette with an aquatic solution (control) or in the 5azaC solution (100 μg/ml ''Sigma'').

The solution was changed each 12 h, since 5azaC is unstable in aqueous solution. Then at a certain time after germination, similarly developed etiolated seedlings were washed, and their first leaves and coleoptiles were separated on the ice. The age of seedlings was determined from the moment of soaking of seeds in water.

2.2. DNA quantification

First leaves in different ages were fixed in the 96% ethanol and homogenized. Mixture was centrifuged 3 times and homogenized with 96% ethanol, acetone, and ether to white powder for remove the lipid components. 0.005M NaOH, 2M $HClO_4$ were added to this powder and this material was incubated to 60 °C for 3 h, for destruction of nucleic acids to nucleotide components. Then this hydrolyzate was centrifuged 2000 *g* for 10 min 2 ml 3% diphenylamine was added for 1 ml from supernatant and this mixture was incubated to 30 °C for 16 h for obtained the complex of desoxyribose with diphenylamine. Absorption of the blue solution was measured by spectrophotometer at 600 nm and 700 nm. DNS amount was measured with calibration line from standard thymus DNS (''Sigma'') in this protocol.

2.3. Nuclear DNA extraction

The plant material was frozen with liquid nitrogen, and was carefully homogenated. The standard lysing solution (50 mM Tris–HCl buffer (pH 8), 25 mM EDTA, 1% (w/v) sodium dodecyl sulphate) and inhibitors of proteases (0.5M $Na_2S_2O_5$, 2β-mercaptoethonol, 0.12% diethylpyrocarbonate) was added to the obtained powder, and the solutions were mixed and incubated for 1 h at 45 °C. NaCl was added to the solution to get the concentration of 1 M. The mixture was deproteinised by shaking with chloroform: isoamyl alcohol (10:1). It was centrifuged 5000 *g* for 10 min at the room temperature. The top phase was replaced in other tubes. DNA was pelleted by adding 3 volumes of 96% ethanol to the top aqueous phase and centrifuged at the room temperature for 5 min. The pellet was washed with 70% ethanol, and centrifuged again for 2 min. The resuspended DNA was placed in sterile distilled water with ribonuclease A (50 mkg/ml ''Sigma'') at 40 °C for 30 min and centrifuged 10 000 *g* for 5 min. Extracted DNA was conserved at −20 °C.

2.4. DNA electrophoresis

Extracted DNA was analyzed by means of electrophoresis for 2 h in 1% (w/v) agaroses gel, voltage 5 V/sm^{-1} in TAE buffer (pH 8) with the 0.1 μkg/ml ethidium bromide for visualization of DNA.

2.5. Spectrophotometry of pigments

In order to extract the pigments in mg of $MgCO_3$ and 1 ml of 80% acetone were added to the 200 *g* of the plant material and mixed. This mixture was centrifuged 3 times at 3000 *g* for 10 min, and the supernatant was obtained. Acetic extracts of the pigments obtained in this way were analyzed by spectrophotometer, using a RYE Unikam SP8400 UV/VIS. The absorption spectrum was recorded from 350 nm to 700 nm.

3. Results

3.1. Influence of 5azacytidine on the growth of some organs of etiolated barley seedlings

As a model for investigation of chemistry-induced hypomethylation of nuclear DNA on intact plant, barley seedlings were used. A barley seedling, as well as a wheat one, is a unique model for investigating the molecular process of cell ageing in whole organs. Coleoptiles and the first leaves which were grown in standard conditions, represent the population of synchronically dividing cells. In growing leaves, the endoreplication processes of genome do not occur. Synthesis of nuclear DNA is replicate and discrete in the first leaf cells (Kirnos et al., 1999).

Influence of 5azacytidine (100 μkg/ml) on the growth dynamics of the first leaf, coleoptiles, and root was investigated (data are not shown). The data were represent for 6-day-old seedlings (144 h) (Fig. 1). In this case, the treatment with 5azaC significantly inhibits the growth of maximally long roots (up to 60%), of the first leaf (up to 30%), and of the coleoptiles (to 15%). As it is shown, inhibition of growth in roots is the strongest. Analogical data were also obtained