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H1 Histone in Developing and Aging Coleoptiles of Etiolated Wheat Seedlings

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Abstract—It has been established that the DNA and H1 histone contents in aged coleoptile of 8-day-old etiolated wheat seedling are about 40 and 30%, respectively, lower than those in young seedlings. H1 histone in wheat seedlings is represented as six electrophoretically different subfractions. The ratios of H1 histone subfractions in wheat coleoptile and initial leaf are similar. In contrast to some animal cells, apoptosis in wheat coleoptile is not accompanied by changes in the set and ratios of H1 histone subfractions. Aging of coleoptiles is associated with a progressive diminution of the H1 histone and DNA contents. H1 histone/DNA ratio in aged coleoptile is 1.5-2-fold higher than that in the young organs. Therefore, the content of H1 histone in chromatin of coleoptile decreases with age more slowly than DNA content.

Key words: aging, apoptosis, coleoptile, H1 histone, DNA fragmentation, ontogenesis, organoptosis, plant, wheat

Programmed cell death (PCD) is a universal event that accompanies ontogenesis; it is common for multicellular and some unicellular organisms [1]. Apoptosis is one of the types of PCD in the cells of mammals [2] and higher plants [3]. It is accompanied by significant changes in morphology of the nucleus and cytoplasmic structures, activation of specific nucleases and proteases, and internucleosomal fragmentation of nuclear DNA. Apoptosis has been observed in various plant tissues (corn endosperm, barley aleurone layer, differentiating xylem, root tip envelope, leaves on hypersensitive response due to pathogen attack, etc.) [3]. Apoptotic internucleosomal DNA fragmentation and expressed fragmentation of cytoplasm with transposition of some mitochondria as constituents of specific single-membrane vesicles formed into cellular vacuole were observed recently in aging wheat seedling coleoptiles [4, 5].

Despite the fact that apoptosis in plants and animals has many common characteristics [4], it has some specific features in plants. For example, two stages of chromatin condensation were observed, but chromatin bodies (ν-bodies) were not detected in plants [6, 7].

The functional role of H1 histone in the structural organization of chromatin and its changes in the process of genome functioning are rather well studied in animals [8]. But data on the participation of H1 histone in apoptosis in animals are still few and contradictory [9, 10]. In plants the state and functional role of H1 histone in apoptosis is practically unknown.

In this work, the contents of H1 histone and its subfractions as well as of DNA in developing and aging wheat coleoptiles were investigated. The coleoptile has drawn our special attention because it is a unique useful model of organoptosis (programmed organ death [11]) in plants.

MATERIALS AND METHODS

Growing and partial growth synchronization of seedlings of Mironovskaya 808 variety of winter wheat (*Triticum aestivum* L.) was performed as described earlier [4]. Etiolated seedlings (50-300 plants) of different age were cut off, and coleoptiles and initial leaves were isolated and used for analysis.

To isolate total histones, coleoptiles cut into small fragments with scissors were frozen in liquid nitrogen, ground in a porcelain mortar with pestle, and extracted twice with 0.2 M H_2SO_4 with treatment in an MSE (England) ultrasonic disintegrator (3 times for 30 sec each at 16-20 μ). Histones were precipitated from the total extract obtained with addition of trichloroacetic

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acid (TAA) up to 18%. The collected precipitates were washed with acetone and analyzed by PAGE.

H1 histone was isolated from the preparation of total histones obtained from wheat coleoptiles and initial leaves as well as from fraction of partially purified nuclei obtained from these plant organs.

H1 histone from fraction (precipitate) of total histones was obtained by 2-4-fold extraction with 0.74 M $HClO_4$ with ultrasonic treatment as was done for the isolation of total histones.

The procedure for isolation of partially purified nuclei is based on the combination of the method of fractionation of wheat tissue homogenates suggested by Kirnos et al. [5] and known methods of nuclei isolation from animal cells modified by us [12]. Coleoptiles or initial leaves of wheat seedlings were cut with scissors and ground in a porcelain mortar with pestle in the cold with three volumes (relative to the tissue mass) of solution containing 0.4 M sucrose, 20 mM TEA-buffer, pH 7.5, 30 mM KCl, 6 mM $MgCl_2$, 1 mM EDTA, and protease inhibitors such as 1 mM phenylmethylsulfonyl fluoride and 1 mM iodoacetate (buffer 1). The homogenate was filtered through 4-layers of gauze, the plant debris was ground again in two volumes of buffer 1, and the extract obtained was filtered. Combined extracts were centrifuged for 15 min at 600*g*. The precipitate obtained (taken as a fraction of partially purified nuclei) was washed with buffer 1 and used for 2-3-fold H1 histone extractions with 0.74 M $HClO_4$ after removal of aliquots needed for DNA determination. Combined supernatants obtained after removal of partially purified nuclei were used for isolation of H1 histone that could appear in cytoplasm on apoptosis [13]; the total protein was precipitated from supernatants by 18% TAA and extracted with 0.74 M $HClO_4$ or, *vice versa*, $HClO_4$ was added to supernatants to concentration equal to 0.74 M, and then H1 histone was precipitated with TAA added to 18% concentration.

To isolate H1 histone directly from coleoptiles or leaves the tissues were ground thoroughly in a porcelain mortar with pestle in liquid nitrogen, thawed in a small volume of buffer 1, and the homogenate was centrifuged for 15 min at 600*g*. The precipitate was extracted with a minimal volume of $HClO_4$ (1 ml) by adding concentrated $HClO_4$ up to the concentration needed. The suspension was treated with ultrasound in a similar way as was done on the isolation of total histones, and it was then clarified by 5 min centrifugation at 600*g*. These extractions were repeated from one to three times.

To monitor the completeness of the H1 histone extraction, additional extracts from plant homogenates or partially purified nuclei with buffer containing 125 mM Tris-HCl-buffer, pH 6.8, 15% glycerol, 2% β-mercaptoethanol, and 1% SDS were subjected to PAGE [14]. These extractions were carried out for 5 min at 100°C. Since H1 histone in the additional extract was not detected, we conclude that H1 histone in our experiments was extracted completely.

H1 histone was precipitated from extracts by addition of 3.5 volumes of acidified acetone (with 0.03 volume of concentrated HCl) in the cold and stored overnight. The precipitates obtained were washed with acetone and analyzed by electrophoresis.

Electrophoresis of histone preparations was performed in SDS polyacrylamide gels by Laemmli's procedure [14] in 5% concentrating and 15% separating gels. Gels were stained with Coomassie brilliant blue R-250 after electrophoresis.

Densitometry and analysis of electrophoregrams were done using the Scione computing program.

Histones were quantitatively determined on membrane filters (Synpor, Czech Republic) by a modification of the method of Schaffner and Weissmann [15]. Histones (3-5 μl) were applied onto the filters and the spots were fixed with 20% TAA, washed with 5% TAA on a filter funnel with a water aspirator pump, and stained for 10 min with 0.2% Amido black 10B solution in 7% acetic acid. Filters were washed with 7% acetic acid to background discoloration and then with water. Zones containing stained proteins were cut out and eluted with solution containing 50% ethanol, 25 mM NaOH, and 5 mM EDTA. The protein content in the spots was determined by measurements of the eluate absorbance at 630 nm against respective eluates from the control zones of the same filter and using a calibration curve.

DNA content in coleoptiles was measured spectrophotometrically [16] after its isolation by the procedure of Schmidt and Thannhauser [17]. The DNA in the fraction of partially purified nuclei was determined using the same scheme but without treatment of acid-insoluble material with organic solvents.

RESULTS

DNA content in coleoptiles of various age. It is known that during development of wheat seedlings the replicative DNA synthesis in coleoptiles stops after the coleoptile is broken through with the initial leaf (on the fourth day of seedling life) [4], and then the coleoptile ages quickly and dies. Programmed death of coleoptiles (organoptosis) is completed in about two weeks of the seedling life. We chose the following time points: 1) the fourth day of the seedling life, the period when the initial leaf breaks through the coleoptile, this period coinciding with cessation of DNA replicative synthesis [4] and seemingly being the beginning of coleoptile organoptosis; 2) the eighth day of the seedling life that is a period of marked internucleosomal DNA fragmentation in the coleoptile, DNA fragmentation being a marker of apoptosis [4]; this is one of the terminal stages of apoptosis in plants and animals [6] and it is already irreversible; 3)

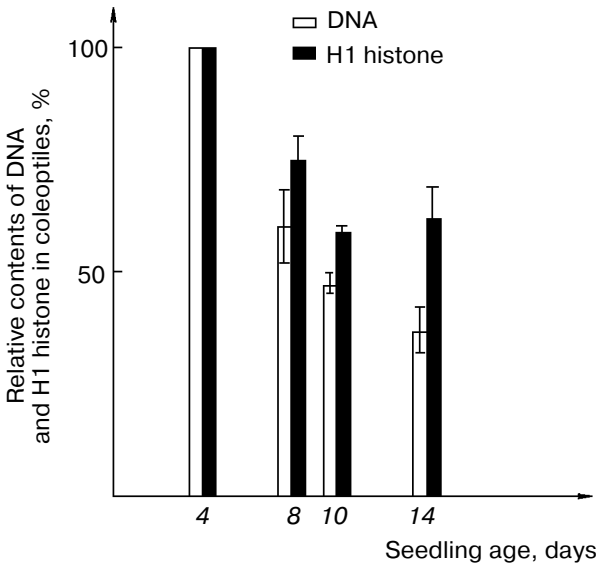

Fig. 1. Relative contents of H1 histone and DNA in coleoptiles of etiolated wheat seedlings of various age. The H1 histone and DNA contents in coleoptile of the 4-day-old seedlings are taken as 100%. The mean values of results obtained in three experiments are presented. Standard errors are shown.

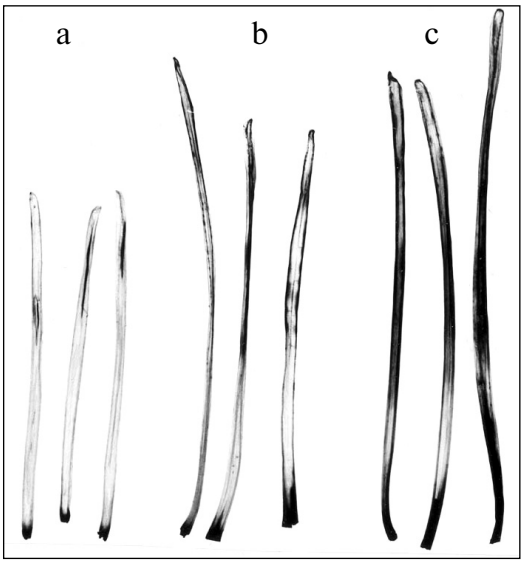

Fig. 2. Coleoptiles of etiolated wheat seedlings cut and stained with trypan blue in the various periods (days) after beginning of seed germination: a) 4; b) 8; c) 14.

10th and 14th days of the seedling life that correspond to the termination of coleoptile organoptosis.

Data on the relative DNA content in developing and then aging coleoptile are shown in Fig. 1. If the DNA content in coleoptile of the 4-day-old seedling is taken as 100%, it is seen that with age (in the 14-day-old seedling) it decreased by about 70%. This may be due to the age cessation of DNA synthesis accompanied by most probable rapid DNA decay in aging coleoptiles.

The coleoptiles (stained with trypan blue) of 4-, 8-, and 14-day-old seedlings are represented in Fig. 2. Trypan blue stains only the cells with distorted membranes and walls. Coleoptiles of the 4-day-old seedlings remain unstained except for the cutting areas. In 8-day-old seedlings, stained zones are seen both in apical and middle coleoptile parts. In the 14-day-old seedlings, the coleoptile is stained practically along the full length of the organ. It is not ruled out that cells in the inner layers of the coleoptile tissue are not available for the stain.

The staining data correspond to results on DNA content in various coleoptile parts (Fig. 3). The amount of DNA decreases with seedling age in all coleoptile parts (thirds) analyzed. Thus, in fact, apoptosis proceeds in the whole coleoptile.

We keep in the mind that coleoptile tissue may be quite heterogeneous in degree of the involvement of various cells in apoptosis; in each given period it may be represented by at least three cellular populations (native cells, apoptotic cells at the different stages of apoptosis, and dead cells still possessing the cell wall). The ratio between these cellular populations changes during seedling life: in 4-day-old seedling the coleoptile cells are represented mainly by living cells, some of which are already apoptotic, whereas in 14-day-old seedling most coleoptile cells contain decayed nuclear material and they are already empty. This is observed in electron microscopy investigations of coleoptiles in seedlings of various age [5, 18].

DNA content in both fractions, of partially purified nuclei from coleoptile cells (Fig. 4) and of whole coleoptiles (Fig. 1), decreases with seedling age; in 10- and 14-

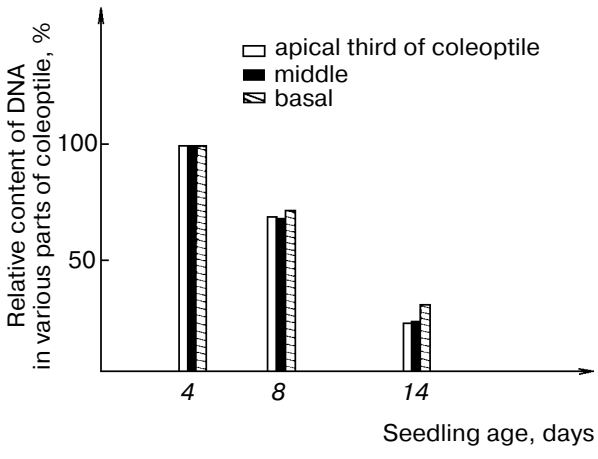

Fig. 3. Relative content of DNA in the apical, middle, and basal thirds of coleoptiles of etiolated wheat seedlings of various age.

day-old seedlings this decrease in nuclear fraction is less strong than that in whole coleoptiles.

Thus, in aging wheat coleoptile the further strong DNA degradation with liberation of acid-soluble products seems to take place along with or after internucleosomal DNA fragmentation detected earlier [4]. This may be associated with the death of some cells in coleoptiles even in the early period of young seedling life.

Characterization of H1 histones from coleoptiles of etiolated wheat seedlings. The electrophoregram of total histones isolated from coleoptiles of 3-day-old wheat seedlings by extraction with 0.2 M H_2SO_4 is presented in Fig. 5A. Electrophoregrams of histones from initial leaf of the 3-day-old wheat seedlings (Fig. 5B) and rat liver nuclei (Fig. 5C) are given for comparison. To determine the position of H1 histone, the total histone preparations from coleoptiles and initial leaves obtained by 0.2 M H_2SO_4 extraction were additionally extracted with 0.74 M $HClO_4$ and analyzed by electrophoresis (Fig. 5, D and E). Wheat H1 histone and core histones have lower electrophoretic mobilities than the respective histones from rat liver hepatocytes. H1 histone and core histones from wheat initial leaf and coleoptile are electrophoretically similar. This means that H1 histone in wheat plant does not have organ specificity, at least in electrophoretic mobility. H1 histone from wheat coleoptiles differs significantly from H1 histone of rat liver hepatocytes in the set and ratios of subfractions.

H1 histone from wheat coleoptiles is represented by at least six components (subfractions). They are denominated by letters from *a* to *f* in accordance with decrease in their molecular mass values. The H1 histone subfractions *b* and *f* in wheat plant are represented most strongly, whereas subfraction *e* is expressed to lesser degree. Only five subfractions in wheat H1 histone were observed earlier [19, 20]. A maximum of seven H1 subfractions was detected in human beings [9]. Six H1 histone subfractions in $HClO_4$-extracts from tobacco tissues were detected by electrophoresis at low pH values [21].

Because H_2SO_4 extraction does not provide complete isolation of H1 histone from plants [22], we have used for quantitative determinations a standard method of specific extraction of H1 histone with $HClO_4$ [23] with acid concentration increased up to 0.74 M in correspondence to [24]. As shown in our control experiments (see "Materials and Methods"), the method used provides for practically complete H1 histone extraction from wheat seedling tissues.

Content of H1 histone and its subfractions in developing and aging wheat coleoptiles. The amount of H1 histone in aging coleoptile in the 8-day-old wheat seedling is by about 30% less than that in young (4-day-old) seedlings; in the 10-day-old seedlings it corresponds only to about 60% of that in young plants (Fig. 1). Nevertheless, we did not observe electrophoretically the accumulation of relatively high-molecular mass products

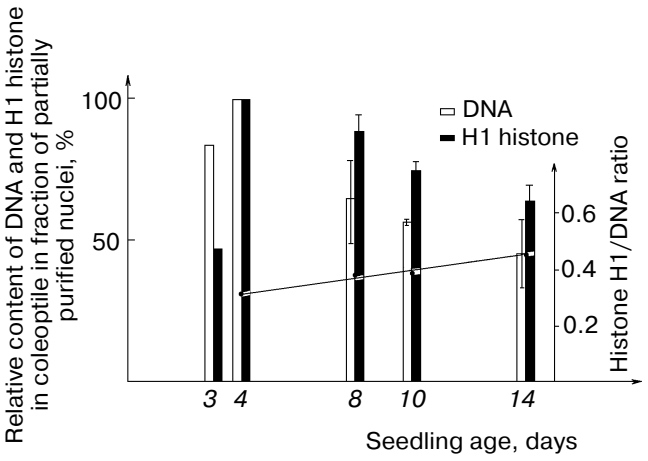

Fig. 4. Relative content of H1 histone and DNA in coleoptiles and histone H1/DNA ratio in fraction of partially purified nuclei from coleoptiles of etiolated wheat seedlings of various age. H1 and DNA contents in nuclear fraction of coleoptile of the 4-day-old seedlings were taken as 100%. The mean data of three experiments are given and the relative determination errors are shown.

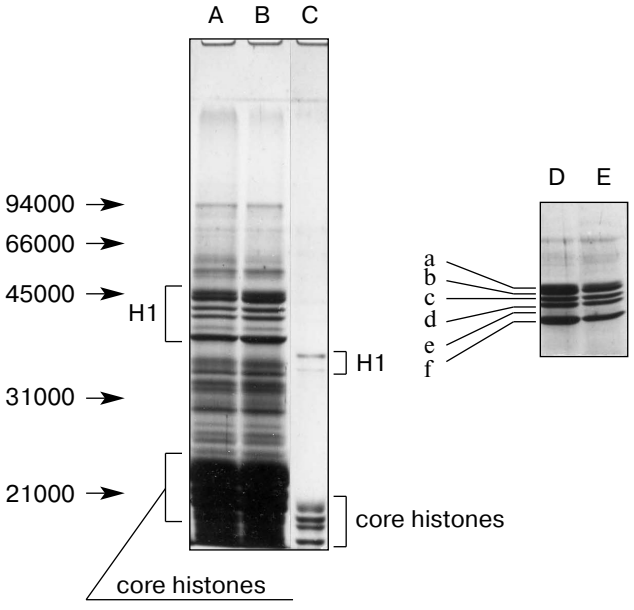

Fig. 5. Electrophoregrams of total histones from coleoptiles (A) and initial leaf (B) of etiolated 3-day-old wheat seedlings and nuclei of rat liver hepatocytes (C). Histones were extracted with 0.2 M H_2SO_4. The positions of reference proteins are shown by arrows. Electrophoregrams of H1 histones isolated from initial leaf (D) and coleoptile (E) of etiolated wheat seedlings. Histones were extracted with 0.74 M $HClO_4$.

of the H1 histone degradation with aging. This may be due to specific degradation of H1 histone in the coleoptile cells. The same character of age changes in H1 histone content in coleoptiles was observed also in the fraction of partially purified nuclei (Fig. 4). But in this particular case the age-dependent decrease in the H1 histone content was less expressed compared with that in whole coleoptiles. H1 histone was not detected in the "cytoplasm" fraction.

Data on the relative content of H1 histone subfractions (one of many experiments) in developing and aging coleoptile are shown in Fig. 6. Because subfractions *a* and *b* were not always well separated, we calculated the relative share of these components combined. The relative share of each H1 histone subtraction was unchanged (in the limits of the experimental error) during coleoptile development and aging for the period from 4 to 14 days of the seedling life (Fig. 7a). This was registered in three independent experiments. Similarly, the electrophoretic patterns of H1 histone subfractions from partially purified nuclei were not changed also during coleoptile development and aging (Fig. 7b).

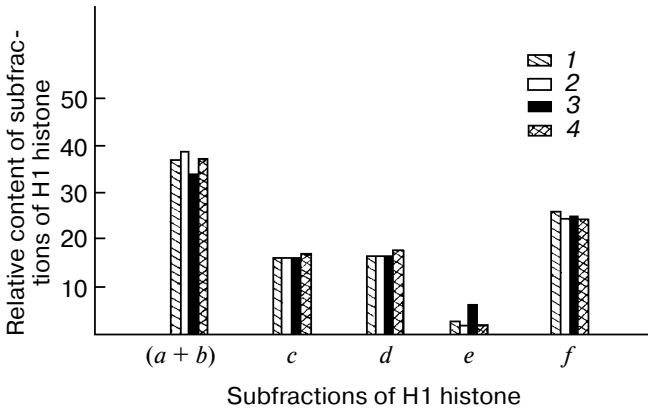

Fig. 6. Content of subfractions (*a* + *b*, *c*, *d*, *e*, and *f*) in the H1 histone preparation isolated from coleoptiles of etiolated 4- (*1*), 8- (*2*), 10- (*3*), and 14-day-old (*4*) wheat seedlings (% of the total H1 histone).

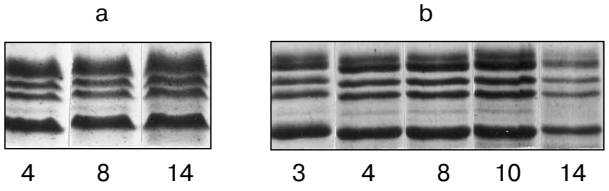

Fig. 7. Electrophoregrams of H1 histones isolated from whole coleoptile (a) and fraction of partially purified nuclei from coleoptile (b) of etiolated wheat seedlings of various age. Numerals indicate seedling age (days).

H1 histone/DNA ratios in coleoptiles of etiolated wheat seedlings of various age

Seedling age, days	4	8	14
H1 histone/ DNA ratio	0.30	0.41	0.52

Thus, development and aging of coleoptile is accompanied by decrease in the H1 histone content in this plant organ. This decrease is equal in all H1 histone subfractions.

H1 histone/DNA ratios in developing and aging coleoptiles. It is known that H1 histone takes part in chromatin compaction. On the other hand, chromatin condensation is one of the markers of apoptosis. Therefore, it was important to investigate the state of nucleoprotein during organoptosis of coleoptiles in etiolated seedlings by determination of the H1 histone/DNA ratio. This parameter increases from 0.30 to 0.52 in coleoptiles in the period from 4 to 14 days of seedling life (see table). The H1 histone/DNA ratio equal to 0.2 is specific for chromatin that contains one H1 histone molecule per nucleosome [8]. The observed almost twofold increase in this ratio should correspond to an increase in H1 histone amount of two molecules per nucleosome. The same character of changes in the H1 histone/DNA ratio was observed in the fraction of partially purified nuclei from coleoptiles, but this increase was less expressed (the value increased in a nuclear fraction and whole coleoptiles by 1.5- and 1.7-times, respectively).

Thus, decrease in the content of H1 histone in aging coleoptiles seems to proceed later compared with DNA degradation; this seems to result in increase in the relative H1 histone content in coleoptile cells.

DISCUSSION

Whole coleoptiles of etiolated wheat seedlings in the process of their development were used as a research object, which is a physiologically more adequate one than the tissue cultures used frequently. Because parenchyma is a predominant tissue in coleoptile, the results on H1 histone behavior and the conclusions made seem to be associated mainly with this particular tissue. The partial synchronization of the seedling growth performed and the synchronous DNA synthesis observed in coleoptiles during the few early cell cycles [4] suggest that most cells in coleoptiles enter apoptosis synchronously during

organoptosis. Therefore, the coleoptile of wheat seedlings is a very useful natural model for investigation of apoptosis in plants.

The lysine-rich H1 histone has been found in practically all eukaryotic organisms. Its structure and functional role is relatively well investigated in mammals. H1 histone has a central globular hydrophobic domain with a conservative amino acid sequence and highly charged nonstructural N- and C-ends. This H1 histone plays a key role in the supercoiling of linker DNA between nucleosomes in the chromatin fibril; it defines the distance between nucleosomes and takes part in formation of the highest chromatin structures [8]. Unfortunately, the data on the structure of plant H1 histones are still scanty and contradictory. Partial amino acid sequences of H1 histones have been established in corn, wheat, tomato, tobacco, *Arabidopsis*, and pea plants. Plant H1 histones are mainly similar to animal analogs, but they are much longer due to their long N- and C-ends [25]. This, in particular, may be responsible for lower electrophoretic mobility of H1 histone from wheat coleoptiles compared with the respective animal proteins.

Many H1 histone subfractions are observed in animals and plants; expression of their genes during growth and differentiation is regulated in a different way [26]. We have detected six H1 histone subfractions in wheat coleoptiles. This is one subfraction more than mentioned earlier [19] and similar to the H1 histone set in tobacco plant [21]. It is known that H1 histone subfractions are different in exchange velocity and their ability to compact chromatin [8]. The ratio of H1 histone subfractions is changeable. It can be different in the various cell types, depends on the physiological state of the organism, organs, and tissues [26, 27]. In particular, in plant cells it changes on various stresses [28]. Because in some animal cells the content of two H1 histone subfractions was changed on apoptotic DNA fragmentation, it was suggested that the individual H1 histone subfractions or their modification status may serve as a signal for the predominant splitting of DNA with endonuclease at specific chromatin sites [9, 29]. These experiments were performed with animal cell cultures and special methods for separation of H1 histone subfractions were developed. We have clearly separated H1 histone subfractions from wheat coleoptiles using classical electrophoresis. This made it possible to analyze the ratios of H1 histone subfractions in wheat coleoptiles during its organoptosis. The ratios of the H1 histone subfractions in coleoptile and initial leaf are similar and they do not change during the whole period of the seedling life observed.

On the contrary, we have found that amount of total H1 histone (per coleoptile) changed significantly during coleoptile development and aging. It is known that H1 histone is a substrate for caspase 3 [29], and the proteolytic enzymes are strongly activated on apoptosis in plants [30, 31]. The removal of H1 histone may be essential to the progress of apoptosis. The protease specific to homologous but not animal H1 histone with maximal activity was detected in coleoptiles of the 3-4-day-old seedlings before the initial leaf breaks through the coleoptile (probably just before the initiation of organoptosis) [32]. We suppose that due to this proteolysis of the H1 histone the share the chromatin is partially unwound and in such state it may be favorable for apoptotic internucleosomal DNA fragmentation. We have observed that the content of H1 histone in coleoptiles at the time of strong internucleosomal fragmentation of chromatin (in the 8-day-old seedling) decreased by almost 30%.

On the other hand, while the total H1 histone content in coleoptile decreases the ratio of H1 histone to high-molecular mass (acid-insoluble) DNA increases with age. The H1 histone/DNA ratio in 14-day-old seedlings is 1.5-2 times higher than that in 4-day-old seedlings. These results do not depend on either the method of H1 histone extraction or the material used (plant tissue or partially purified nuclei). In contrast to the data on the translocation of histones into the cytoplasm in apoptotic animal cells [13], we did not detect H1 histone in the "cytoplasm" fraction from coleoptiles of etiolated wheat seedlings in various periods of seedling development and coleoptile aging.

Similarly to that in whole coleoptiles, the H1 histone/DNA ratio increase with age was observed also in the fraction of isolated coleoptile nuclei. It seems that decay of H1 histone is to some extent retarded in surviving cells; this seems to be quite specific for the apoptosis in coleoptiles. H1 histone may take part in the known apoptotic chromatin condensation, but in some animal cells, this process may be mediated by some other proteins but not H1 histone [10]. It is known that *in vitro* H1 histone inhibits the loss of chromatin fragments from nuclei treated with nuclease [33]. This protein is able to form aggregates from oligonucleosomes with small nucleosome number [34]. Unlike the HMG proteins associated strongly with chromatin in apoptotic cells but being excreted from necrotic cells, the H1 histone stays always in the cell remnants, being associated with DNA [35]. H1 histone activates a specific DNase in apoptotic animal cells [36]; if it takes place in coleoptile cells, the aging increase in the H1 content relative to DNA along with an increase in chromatin condensation seems to speed up also the DNA degradation in it.

Thus, when DNA hydrolysis in coleoptiles is already active the H1 histone degradation may be to some extent retarded. In this particular case, the nucleosomes kept in the nucleus may possess higher H1 histone content. It is worth noting that there is no privilege or specificity for any H1 histone subfraction in the retardation of the elimination. This means that coleoptile organoptosis is accompanied by the proteolysis of each of six H1 histone subfractions, proceeding with a more or less similar rate.

Taking into account the results of the electron microscopy studies [5, 18], the increase in the H1 histone/DNA ratio in aging coleoptiles may have other causes as well. Many of the "empty" cells formed in the apoptotic tissue, are, in fact, not empty. These cells in old coleoptiles have quite dense periphery material and they contain the fragments of degraded nuclei enriched with H1 histone. The components of such thin cells may be present in the fraction of partially purified nuclei; it modifies the chromatin composition in surviving cells (the share of such cells in the 14-day-old seedlings does not exceed 30%). In this particular case, the apparent change in the H1 histone/DNA ratio will be observed but it is not a specific property of nuclear chromatin in the surviving living cells.

Anyway, our results show that the amount of H1 histone relative to DNA increases in the cells of aging wheat coleoptile dying of apoptosis. The functional significance of this event is still unknown. It is interesting that H1 histone has antimicrobial activity [37] and, therefore, it was suggested that this protein released from destroyed cells may, at least, protect an organism against extracellular infections inducing apoptosis.

It is known that even in the late stages of coleoptile development (8-day-old seedling) most DNA is still of relatively high molecular weight, only a small amount of DNA is located in the oligonucleosomal chromatin fragments [4]. The morphology of nuclei in surviving cells is not significantly changed either [18]. At the same time, the DNA content in coleoptile diminishes constantly (Fig. 1) and the number of empty cells increases (Fig. 2 and [5, 18]). This may mean that at each moment, the main pool of the living cells in coleoptiles is at the early stage of apoptosis or senescence, but a small share of the coleoptile cells is at the late apoptosis stage when specific apoptotic features are observed. Because after internucleosomal DNA degradation apoptosis may proceed very quickly, the cells with terminal apoptosis may represent a very small share in the general pool of coleoptile cells. This could in part explain why unlike synchro-nized animal apoptotic cell cultures [9, 29], we did not observe changes in the content of the individual H1 histone subfractions in developing and aging wheat coleoptiles.

As a whole, the results reported here show that apoptosis in wheat coleoptiles follows the more or less standard scenario observed in animal cells: the cells enter into apoptosis and die in turn. The retardation of the H1 histone decay compared with DNA degradation in aging coleoptiles is still mysterious, and it seems to be a specific feature of apoptosis in plants, which do not eliminate the remnants of the apoptotic cells completely due to the strong cell walls.

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