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# Effect of Ethylene Producer Ethrel and Antioxidant Ionol (BHT) on the Proteolytic Apparatus in Coleoptiles of Wheat Seedlings during Apoptosis

**L. I. Fedoreyeva, N. I. Aleksandrushkina, Ya. E. Dunaevsky, M. A. Belozersky, and B. F. Vanyushin***

*Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, Moscow 119992, Russia; fax: (095) 939-3181; E-mail: vanyush@belozersky.msu.ru*

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**Abstract**—It was established that total proteolytic activity in etiolated wheat seedlings changes in ontogenesis in cycles: peaks of proteolytic activity correspond to the 3rd, 5th, and 8th days of seedling growth, respectively. The maximum of proteolytic activity preceded the maximum of nuclease activity, which may be due to activation of nucleases by proteolytic enzymes. According to inhibitory analysis the cysteine and serine proteases play the main role in apoptosis in wheat coleoptiles. Growing of seedlings in the presence of ethrel stimulated apoptosis in the coleoptile, and it increased (almost 6-fold) the proteolytic activity in its cells. On the other hand, the antioxidant ionol (BHT) suppressed the induction of proteases, particularly at the second stage of coleoptile development, and it slowed down the increase in the nuclease activity after 6th day of the seedling life. It is suggested that phytohormones and antioxidants participate in regulation of apoptosis in the ageing coleoptile, directly or indirectly effecting the proteolytic apparatus in the coleoptile cells.

*Key words*: antioxidant, apoptosis, BHT, ethrel, ethylene, ontogenesis, plants, proteases, wheat

Apoptosis is a genetically determined programmed cell death that is an obligatory constituent part of plant development [1]. Apoptotic cell elimination accompanies plant development and it becomes apparent in different plant organs and tissues already at the early stages of ontogenesis. In particular, it occurs in coleoptile and initial leaf in developing wheat seedlings [1-4].

Three stages can be distinguished in the process of apoptosis: 1) an inductive one that depends on the nature of the death-inducing signal; 2) effector one when the mechanism of the cell suicide is activated, and 3) degradative one when irreversible biochemical and morphological changes take place in the cell [5]. In plant cell, like in animals, apoptosis is accompanied by pronounced condensation and margination of chromatin with subsequent nuclear decay [1, 6], internucleosomal fragmentation of nuclear DNA [2-4, 7], and the reorganization of the cytoplasm [8].

Cereal seedlings are a very useful model for investigation of apoptosis in plants [1-4]. First, the growth and development of these seedlings can be easily synchronized [9]; in particular, this is very important for the study of dynamics of many biochemical processes. Second, the individual organs in these plants are subjected to programmed death (organoptosis) [10] due to massed apoptosis. For example, the coleoptile in cereals functions only for a relatively short period and then it dies rapidly during seedling development.

Despite the fact that apoptosis is programmed in plant ontogenesis it may be modulated or induced by various environmental factors or agents including different infections [6, 7] and abiotic stressors (hypoxia, oxygen stress, and others [7, 11]. Apoptosis is effectively modulated by anti- and prooxidants [6, 11-13] and it can be under phytohormonal control [14-16]. In particular, ethylene (ethrel) stimulates [17] but BHT blocks apoptosis [13] in coleoptile of wheat seedling. Unfortunately, data on the influence of different phytohormones on apoptosis are very rare and fragmentary, and the molecular mecha-

*Abbreviations*: BHT) butylated hydroxytoluene, or 2,6-di-*tert*-butyl-4-phenol (ionol); DTT) dithiothreitol; NA) nuclease activity; PA) protease activity; PCMB) *p*-chloromercuribenzoate; PMSF) phenylmethylsulfonyl fluoride; ethrel) 2-chloroethylphosphonic acid.

* To whom correspondence should be addressed.

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nisms of apoptosis regulation in plants are still practically unknown.

Proteolytic enzymes are the main executors of apoptosis in animal cells, but their role in plant apoptosis still needs to be clarified. These enzymes seem to take part in each of three stages of apoptosis starting from the moment of the appearance of the apoptosis induction signal up to strong destruction of structural proteins and enzymes that maintain the cell homeostasis. Caspases draw main attention when cellular proteases are studied in animal apoptotic cells: these enzymes are shown to play a crucial role in apoptosis [18-20]. Unfortunately, there is no direct evidence that real caspases are present in plants; nevertheless, it was mentioned that in plants some caspase-like proteolytic enzymes were found [21, 22]. Information on the participation of other classes of proteases in plant apoptosis is very scarce.

In this particular work we have studied changes in proteolytic activities in wheat coleoptiles with strong apoptosis. The influence of exogenous antioxidant BHT (ionol) and plant growth regulator ethrel (2-chloroethylphosphonic acid) on these activities was also investigated.

## MATERIALS AND METHODS

Seeds of Mironovskaya 808 variety of winter wheat (*Triticum aestivum* L.) were germinated in darkness for 24 h in thermostat at 26°C on wet filter paper in a plastic cuvette; sprouted seeds were transferred into another cuvette, covered with a lid, and grown for 24 h in darkness at 26°C. Then, for the experimental plants, water as the medium was changed for BHT (Sigma, USA; 50 mg/liter, $2.3 \cdot 10^{-4}$ M) or ethrel ($10^{-2}$ M) solutions, and the growth of the seedlings was continued in darkness at 26°C for a few days. To prepare BHT water solution, solid BHT was dissolved in ethanol and added to boiling water to the concentration used, then the weakly opalescent liquid was cooled down to 26°C. An equivalent volume of ethanol was added to the water used for growing the control wheat seedlings. The ethrel solution was prepared by dilution of original 45.5% 2-chloroethylphosphonic acid and it was neutralized to pH 6.5 with addition of 0.5 M NaOH. The reagent solutions and water in dishes with seedlings were changed rapidly (for 1-2 min period) once a day for freshly prepared and plants were continued to be grown in darkness in a thermostat.

Seedlings were grown on water or in the presence of BHT in one thermostat, but in the presence of ethrel in another one. Seedlings of definite age (seedling age was estimated in days starting from the beginning of seed soaking) were thoroughly washed with water; coleoptiles were separated and used for isolation and analysis of DNA and determination of activities of hydrolytic enzymes. Each experiment (plant growing in the presence of each compound tested at specific concentration) was done at least three times and was accompanied by an independent control (plant growing in water under the same conditions).

To isolate DNA, the coleoptiles of etiolated wheat seedlings were thoroughly ground in a mortar with pestle in liquid nitrogen, a lysing solution (50 mM Tris-HCl, pH 7.5, 25 mM EDTA, 1% SDS) was added to the fine powder obtained, and the mixture was then incubated for 30 min at room temperature. Afterward NaCl was added to concentration 1 M and the mixture was deproteinized by careful shaking with chloroform–isoamyl alcohol mixture (10 : 1 v/v). After centrifugation for 10 min at 5000*g*, DNA was precipitated from the aqueous phase with the addition of three volumes of 96% ethanol and dissolved then in 50 mM Tris-HCl-buffer, pH 7.5, containing 25 mM EDTA. The DNA samples thus obtained were treated with DNase-free ribonuclease A (50 μg/ml) for 20 min at 37°C, and the DNA was precipitated again by addition of three volumes of 96% ethanol.

Similar aliquots of isolated and purified DNA preparations were electrophoresed for 2 h in 1.2% agarose gel at 2-3 V/cm in 0.09 M Tris-borate buffer, pH 8.3, containing 0.5 μg/ml ethidium bromide.

To isolate the protein extracts, wheat coleoptiles (200 mg) were homogenized at 4°C in 50 mM Tris-HCl buffer, pH 7.5, containing 0.8 M sucrose and 0.35 M NaCl. Cell debris separated by centrifugation was discarded, and the supernatant (extract) was dialyzed against 50 mM Tris-HCl buffer, pH 7.5.

Proteolytic activity was measured spectrophotometrically at 420 nm by the method described by Erlanger et al. [23] using 5 mM synthetic substrates for trypsin-like proteases (Bz-Arg-pNa) and aminopeptidases (Leu-pNa, Arg-pNa, Phe-pNa) as well as by the trinitrophenylation method [24] using 1% protein substrate (casein at pH 7.0). In the first case, the amount of enzyme that was able to increase absorption by 0.01 in a sample hydrolyzed under conditions described (1 h, 37°C, 0.1 M phosphate buffer, pH 8.0) was taken as one unit of the enzymatic activity. In the second case, one enzymatic activity unit corresponded to the enzyme amount that liberated 1 nmol amino groups under similar conditions (glycine was used as a standard).

Specific inhibitors of the enzyme active centers—PMSF ($2 \cdot 10^{-4}$ M), PCMB ($10^{-4}$ M), EDTA ($5 \cdot 10^{-3}$ M), and pepstatin ($10^{-5}$ M)—were used for protease classifications. The enzymatic preparations were preincubated for 1 h at 20°C in 0.1 M phosphate buffer, pH 8.0, then the substrate solution was added and residual activity was determined. The activity measured in reaction mixture containing 5% methanol was used as a control if inhibitors (PMSF, pepstatin) were dissolved in methanol.

Nuclease activity was measured spectrophotometrically at 260 nm using calf thymus DNA as a substrate ($A_{260}$ = 1.0 in 0.5 M acetate buffer, pH 5.0). DNA was

hydrolyzed at 37°C for 1 h. The enzyme amount that induced an increase in solution absorption by 0.01 under standard incubation conditions was taken as one enzymatic activity unit.

The protein concentration in solutions was measured by Bradford's method [25] or spectrophotometrically by absorption at 280 nm.

The diluted protein solutions were concentrated in Amicon cells (The Netherlands) using YM-10 filters.

## RESULTS AND DISCUSSION

To study the behavior of proteases in apoptotic plant cells, we used wheat coleoptiles that have been shown to be a very useful model for investigation of apoptosis in plants [2]. The combined comparative cytological and biochemical analysis of cells with detection of specific apoptotic features is the most reliable identification of apoptosis [1].

It was shown that development of etiolated wheat seedlings is accompanied by internucleosomal DNA fragmentation, which is a clear-cut marker of apoptosis. The first showings of apoptotic DNA fragmentation in coleoptile were observed in 6-day-old etiolated wheat seedlings but in 8-day-old seedlings it was expressed very strongly (Fig. 1a). Peculiar morphological changes specific for apoptosis in the cells of aging wheat coleoptile were observed earlier [1]: chromatin condensation and margination in the nucleus, cytoplasmic condensation and reorganization, and formation of specific vacuolar vesicles containing cytoplasm and active subcellular organelles [8, 13]. Thus, aging of organs in developing etiolated wheat seedlings involves apoptosis; this is in agreement with modern conceptions of the mechanisms of aging in plants [1].

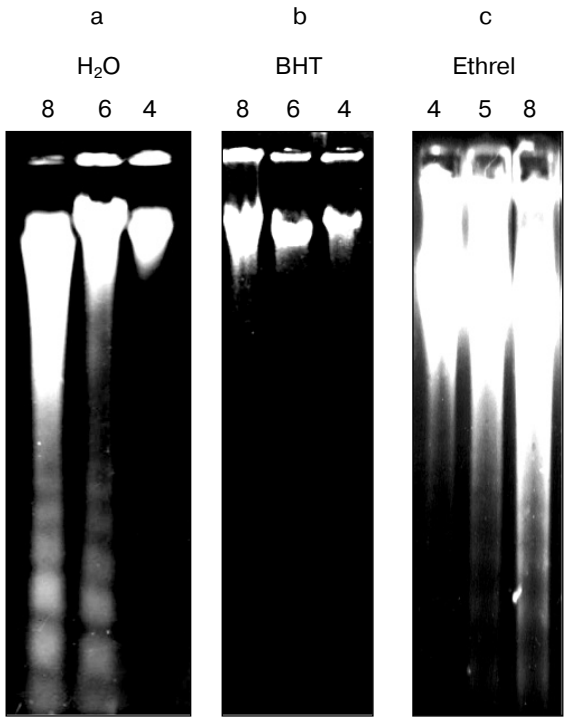


**Fig. 1.** Electrophoregrams of DNA isolated from coleoptiles of etiolated wheat seedlings. a) DNA from control seedlings; b) DNA from seedlings grown in the presence of BHT ($2.3 \cdot 10^{-4}$ M); c) DNA from seedlings grown in the presence of ethrel ($10^{-2}$ M). Numerals indicate the seedling age (days after beginning seed imbibition).

Unlike in control seedlings, apoptotic DNA fragmentation was not observed in coleoptiles of plants grown under similar conditions for the period investigated but in the presence of BHT in the medium (50 mg/liter) (Fig. 1b). It seems that BHT, known as a "geroprotector", increases the lifespan of the coleoptile. On the other hand, the "aging hormone" ethylene regulating expression of respective genes (for example, *dad*, ACA-synthetase gene, and others) controls the processes of aging and apoptosis also [16]. Ethylene formed in plants from the ethylene producer ethrel induces the beginning of internucleosomal DNA fragmentation much earlier (in 5-day-old seedlings) than it occurs in control seedlings grown without this plant growth regulator (Fig. 1c).

These changes in nuclear DNA in the cells of aging coleoptile preceded the changes in the total proteolytic activities and in the protease set. The dynamics of the total proteolytic activity (PA) measured by casein hydrolysis in the process of coleoptile development is represented in Fig. 2. Clearly expressed age-dependent cyclic changes in PA have been observed. Three peaks of PA can be distinguished: peak number one was observed on the third day of the seedling life; then PA decreases and it increases again starting from the fifth day of the seedling life; the third peak of PA begins from the eighth day of seedling life. The peaks of PA observed coincide with respective stages of the coleoptile development—growth and elongation (from 2nd to 4th day of the seedling life), beginning of aging and the start of the apoptosis program (from 5th to 8th day of seedling life), and terminal stage (after 8 days of seedling life) when mass programmed cell death occurs in coleoptiles.

Similarly to PA, the changes in total nuclease activity (NA) in coleoptile are also cyclic: a small increase in NA was observed in the period from the 3rd to 5th day of the seedling life, it drops on the 6th day, and then it strongly increases again (Fig. 3). It can be assumed that an increase in NA up to the fifth day of the seedling life is due to synthesis or activation of early apoptotic endonucleases hydrolyzing DNA into large fragments that appear on the electrophoregram as a wide diffuse DNA band. Subsequent strong increase in NA seems to be due to the action of nucleases responsible for internucleosomal

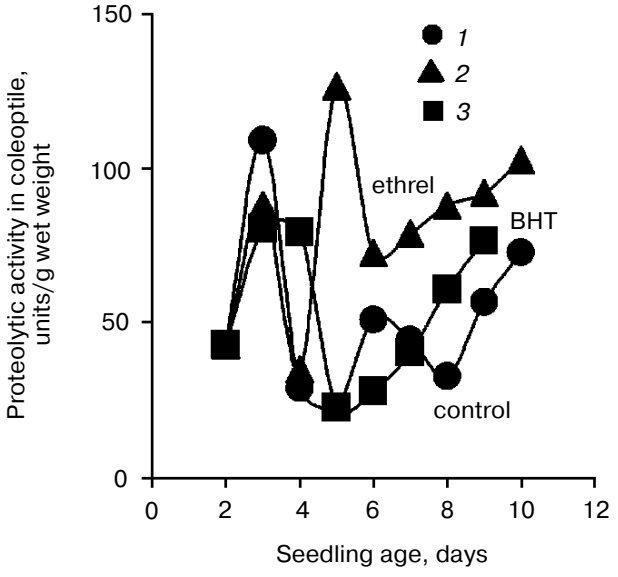


**Fig. 2.** Dynamics of changes in the proteolytic activity in coleoptiles of etiolated wheat seedlings grown on water (*1*), ethrel (*2*), or BHT (*3*) solutions. The data on the enzymatic activities in developing wheat coleoptiles represented are means of three independent experiments, and they are the result of at least three sample determinations in each experiment. Maximal standard deviation values (σ) were not higher than 10% of the means presented. The character of curves was similar to the represented ones when the specific activity values (units per mg protein) were calculated.

DNA fragmentation and more profound DNA degradation.

In animals the cysteine aspartate-specific proteases known as caspases functioning as the activated enzyme cascade play an important role in the triggering of programmed cell death [20]. It can be supposed that a similar mechanism of apoptosis induction is present in plants also.

The dynamics of PA with a significant share of cysteine protease activity in coleoptile is shown in Fig. 4. This PA was measured in the presence of dithiothreitol (DTT) using casein as the substrate. The character of changes in this PA (measured in the presence of DTT) in the process of the coleoptile development is similar to that of total PA determined under the standard conditions. But peaks observed in the first case were about 2- to 3-fold larger. This testifies to the possible participation of cysteine proteases in apoptosis in wheat coleoptiles. By inhibitory analysis it was shown that the basic level of PA and its increase in the apoptotic cells of wheat coleoptile are not associated with the action of aspartyl proteases, but they are due to a small extent to the action of metalloproteases, whose activities decreased by 65-70% on the 8th day of the seedling life (Table 1).

Cysteine and serine proteases are the main participants of the apoptotic process in wheat coleoptile; up to the 5th day of seedling life, the activity level of cysteine proteases is higher than that of serine proteases. As the coleoptile develops the activity of serine proteases increases by about 70%, but the activity of cysteine proteases decreases by 14%. Therefore, on the 8th day of the seedling life the activity level of serine proteases is even slightly higher than that of cysteine proteases. It should be mentioned that we have measured only the total changes in the activities of various classes of proteases. In fact, these changes to some extent may be due to degradation

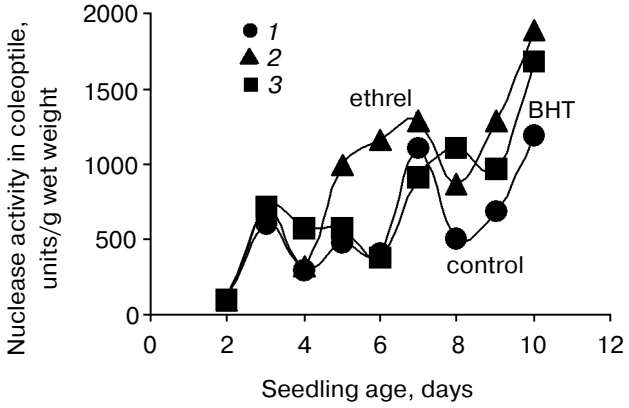


**Fig. 3.** Dynamics of changes in the nuclease activity in coleoptiles of etiolated wheat seedlings grown on water (*1*), ethrel (*2*), or BHT (*3*) solutions. Maximal standard deviation values (σ) were not higher than 10% of the means presented.

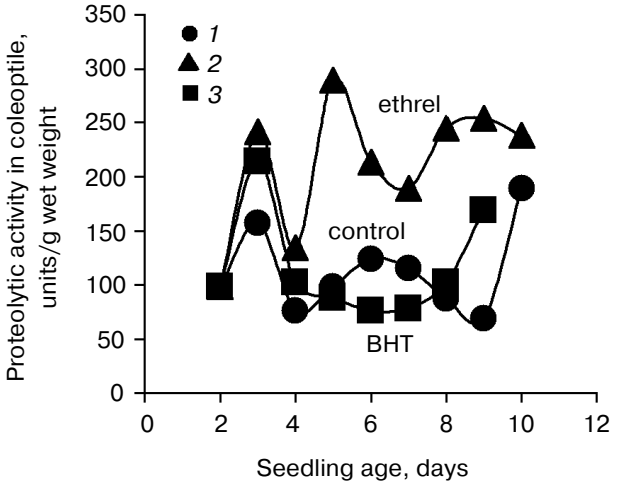


**Fig. 4.** Dynamics of changes in the proteolytic activity (measured in the presence of DTT) in coleoptiles of etiolated wheat seedlings grown on water (*1*), ethrel (*2*), or BHT (*3*) solutions. Maximal standard deviation values (σ) were not higher than 10% of means presented.

**Table 1.** Inhibition of proteolytic activity (%) in coleoptiles of etiolated wheat seedlings grown in the presence of BHT ($2.3 \cdot 10^{-4}$ M) or ethrel ($10^{-2}$ M)

| Seedling age, days after beginning seed imbibition | Medium | PMSF, $2 \cdot 10^{-4}$ M | PCMB, $10^{-4}$ M | EDTA, $5 \cdot 10^{-3}$ M |
|---|---|---|---|---|
| 3 | control | 30 | 50 | 20 |
| | BHT | 35 | 43 | 22 |
| | ethrel | 26 | 49 | 25 |
| 5 | control | 42 | 44 | 14 |
| | BHT | 39 | 46 | 15 |
| | ethrel | 33 | 45 | 22 |
| 8 | Контроль | 51 | 42 | 6 |
| | BHT | 48 | 44 | 8 |
| | ethrel | 46 | 45 | 9 |

**Table 2.** Total proteolytic activity (PA) in coleoptiles of etiolated wheat seedlings (PA in coleoptiles of similar seedlings grown in the presence of $10^{-2}$ M ethrel is indicated in parentheses)

| Seedling age, days after beginning seed imbibition | Activity (units) measured relatively to: | | | |
|---|---|---|---|---|
| | Bz-Arg-pNa | Leu-pNa | Arg-pNa | Phe-pNa |
| 3 | 16 (17.6) | 11.4 (11) | 11.2 (12) | 22.4 (21.6) |
| 4 | 17.6 (12) | 8.6 (11.2) | 11.3 (12) | 16.4 (22) |
| 6 | 13.9 (9.9) | 8.0 (7.6) | 9.5 (6.7) | 15.7 (14.6) |
| 8 | 15.5 (12.8) | 9.2 (7.5) | 9.6 (7.2) | 18.1 (14.7) |

Note: Maximal deviations from the mean values presented are not more than 3%.

of existing proteases as well as appearance of new proteases that seem to play a significant role in apoptosis. Aminopeptidase activity due to the action of metalloproteases with various specificity relative to the N-terminal amino acid residue decreased slightly (by 14-19%) at the early stages of seedling life (Table 2).

Hormonal signals are the leading elements of regulation of cellular differentiation and realization of total development program including programmed cell death (PCD) in plants. In this connection we have studied the changes in the activity of hydrolytic enzymes in the coleoptile cells of wheat seedlings grown in the presence of ethrel that is an ethylene producer. As can be seen (Fig. 2), ethrel strongly (up to 6-fold) increases PA in coleoptiles of wheat seedlings grown for more than four days in the presence of this plant growth regulator. Under these conditions the second peak of PA appeared earlier, it was clearly shifted from the 6th to the 5th day of the seedling life. This may be associated with more active coleoptile development and earlier appearance of initial marks of apoptosis such as internucleosomal nuclear DNA fragmentation (Fig. 1c) in wheat seedlings due to the ethylene formed from ethrel.

The main proteolytic activity in coleoptiles of seedlings grown with or without ethrel is due to serine proteases; the level of the cysteine protease activity was not changed during seedling development (Table 1), in general, but the third activity peak clearly expressed was observed on the 8th day of the seedling life (Fig. 4) if the enzymatic activity was measured in the presence of DTT. A significant increase in the level of serine protease activity (by 77%) observed in coleoptiles of seedlings grown in the presence of ethrel is mainly due to decrease in the level of metalloprotease activities. Activity of aminopeptidases and trypsin-like proteases that seem to be not involved in apoptosis in coleoptile was more strongly diminished in plants grown in the presence of ethrel (Table 2).

Judging from the data on the internucleosomal DNA fragmentation (Fig. 1), it can be concluded that ethrel as a producer of ethylene really accelerates the appearance of apoptosis and it seems to strongly intensify the apopto-

sis in wheat coleoptile. It is suggested that the ethrel-dependent apoptosis observed seems to be due mainly to PA induction and modulation. It cannot be ruled out that proteases induced by ethylene formed from ethrel can be the crucial triggering elements of apoptosis in plant cells.

Reactive oxygen species (ROS) trigger apoptosis in animal and plant cells [1]. A hypersensitive response to pathogen infection in plants is associated with accumulation of hydrogen peroxide that stimulates apoptosis [1]. It is known that in the presence of the antioxidant BHT (ionol) the formation of superoxide-anion is diminished [26] and apoptotic DNA fragmentation in etiolated wheat coleoptile is prohibited [13]; in other words, the diminution of the ROS content results in distortion of mechanisms regulating apoptosis.

The first maximum of PA in coleoptiles of etiolated wheat seedlings grown in the presence of BHT was shifted from the 3rd to the 4th day of seedling life compared with the control. It is most interesting that unlike control the second PA peak measured both in the presence and without DTT is practically absent in plants grown with BHT. These results coincide with known data showing that BHT inhibits apoptotic DNA fragmentation. Therefore, inhibition of the PA induction by antioxidant at the second stage of the coleoptile life (Figs. 2 and 4) may be one of the elements of the inhibitory antioxidant action on the apoptotic process in the aging wheat coleoptile.

Thus, antioxidants (BHT) and phytohormones (ethylene) directly or indirectly acting on the cell proteolytic apparatus can take part in the regulation of apoptosis in plants.

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## REFERENCES

1. Vanyushin, B. F. (2001) *Usp. Biol. Khim.*, **41**, 3-38.
2. Kirnos, M. D., Aleksandrushkina, N. I., and Vanyushin, B. F. (1997) *Biochemistry (Moscow)*, **62**, 864-869.
3. Kirnos, M. D., Aleksandrushkina, N. I., Shorning, B. Yu., Bubenshchikova, S. N., and Vanyushin, B. F. (1997) *Biochemistry (Moscow)*, **62**, 1348-1357.
4. Kirnos, M. D., Aleksandrushkina, N. I., Shorning, B. Yu., Kudryashova, I. B., and Vanyushin, B. F. (1999) *Fiziol. Rast.*, **46**, 48-57.
5. Roberts, L. R., Adjei, P. N., and Gores, G. J. (1999) *Cell Biochem. Biophys.*, **30**, 71-88.
6. Greenberg, J. T. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 12094-12097.
7. Ryerson, D. E., and Heath, M. C. (1996) *Plant Cell*, **8**, 393-402.
8. Bakeeva, L. E., Kirnos, M. D., Aleksandrushkina, N. I., Kazimirchyuk, S. B., Shorning, B. Yu., Zamyatnina, V. A., Yaguzhinsky, L. S., and Vanyushin, B. F. (1999) *FEBS Lett.*, **457**, 122-125.
9. Kirnos, M. D., Volkova, S. A., Ganicheva, N. I., Kudryashova, I. B., and Vanyushin, B. F. (1983) *Biokhimiya*, **48**, 1587-1595.
10. Skulachev, V. P. (2001) *Exp. Gerontol.*, **36**, 995-1024.
11. Jabs, T. (1999) *Biochem. Pharmacol.*, **57**, 231-245.
12. Shorning, B. Yu., Poleshchuk, S. V., Gorbatenko, I. Yu., and Vanyushin, B. F. (1999*) Izv.Ros. Akad. Nauk*, *Ser. Biol.*, No. 1, 30-38.
13. Bakeeva, L. E., Zamyatnina, V. A., Shorning, B. Yu., Aleksandrushkina, N. I., and Vanyushin, B. F. (2001) *Biochemistry (Moscow)*, **66**, 850-859.
14. He, C. J., Morgan, P. W., and Drew, M. C. (1996) *Plant Physiol.*, **112**, 463-472.
15. Groover, A., and Jones, A. M. (1999) *Plant Physiol.*, **119**, 375-384.
16. Jones, A. M. (2001) *Plant Physiol.*, **125**, 94-97.
17. Vanyushin, B. F., Shorning, B. Yu., Seredina, A. V., and Aleksandrushkina, N. I. (2002) *Fisiol. Rast. (Russ. J. Plant Physiol.)*, **49**, 558-564.
18. Rosen, A., and Casciola-Rosen, L. (1997) *J. Cell. Biochem.*, **64**, 50-54.
19. Kutsy, M. P., Kuznetsova, E. A., and Gaziev, A. I. (1999) *Biochemistry (Moscow)*, **64**, 115-126.
20. Hengartner, M. O. (2000) *Nature*, **407**, 770-776.
21. De Jong, A. J., Hoeberichts, F. A., Yakimova, E. T., Maximova, E., and Woltering, E. J. (2000) *Planta*, **211**, 656-662.
22. Korthout, H. A., Berecki, G., Bruin, W., van Duijn, B., and Wang, M. (2000) *FEBS Lett.*, **475**, 139-144.
23. Erlanger, B. F., Kokowsky, N., and Cohen, W. (1961) *Arch. Biochem. Biophys.*, **95**, 271-278.
24. Habeeb, T. S. (1966) *Analyt. Biochem.*, **14**, 328-336.
25. Bradford, M. M. (1976) *Analyt. Biochem.*, **72**, 248-254.
26. Shorning, B. Yu., Smirnova, E. G., Yaguzhinsky, L. S., and Vanyushin, B. F. (2000) *Biochemistry (Moscow)*, **65**, 1357-1361.