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N. M. TOPCHII[1], V. D. SAKALO[2], O. M. TISHCHENKO[2], V. M. KURCHII[2]

[1]Taras Shevchenko National University of Kyiv, Ukraine, Kyiv 01033, 64 Volodimirska St.,
e-mail: topchiy_nataliya@univ.kiev.ua
[2]Institute of Plant Physiology and Genetics of the National Academy of Sciences of Ukraine,
Ukraine, Kyiv 03022, 31/17 Vasylkivska St.

Cytokinin influence on the nDNA and enzyme activity of sucrose metabolizing pathway during senescence of sugar beet (*Beta vulgaris L.*) leaves

Wpływ cytokinin na jądrowe DNA i aktywność enzymów w procesach metabolicznych w starzejących się liściach buraka cukrowego (*Beta vulraris* L.)

SUMMARY

The effect of phytohormones 6-benzyladenine (6-BA) and zeatin on the metabolic processes in the sugar beet leaves senescence has been investigated. We have found that leaf senescence is accompanied by destruction of nucleus and degradation of nuclear DNA; decrease in the level of soluble proteins and chlorophyll; inactivation of sucrose synthesis by sucrose phosphate synthase (SPS); increase in the hexokinase activity. The treatment of plants with 6-BA) and zeatin regulates these processes in the senescence leaves of sugar beet. Cytokinins (6-BA and zeatin) increased the chlorophyll content in the senescence (45–60-day-old) sugar beet leaves and the level of soluble proteins during all ontogenesis stages.

6-BA activated SPS during all ontogenesis stages, but zeatin only in the senescence (45–60-day-old) sugar beet leaves. 6-BA and zeatin inhibited hexokinase, its activity was substantially increased during leaves senescence. Also cytokinins retarded the destruction of nuclei and degradation of nuclear DNA in the senescence (45–60-day-old) leaves.

STRESZCZENIE

W pracy badano wpływ fitohormonów 6-benzyloadeniny i zeatyny na procesy metaboliczne w starzejących się liściach buraka cukrowego. Wykazaliśmy, że starzeniu liści towarzyszy destruk-

cja jądra i degradacja jądrowego DNA; obniżenie poziomu rozpuszczalnych białek i chlorofilu; inaktywacja syntezy sacharozy przez syntazę fosforanu sacharozy (SPS); wzrost aktywności heksokinazy. Traktowanie rośli 6BA i zeatyną reguluje te procesy w starzejących się liściach buraka cukrowego. Cytokininy (6BA i zeatyna) podwyższały zawartość chlorofilu w starzejących się (45–60 dniowych) liściach buraka cukrowego i poziom rozpuszczalnych białek podczas wszystkich etapów ontogenezy. 6BA aktywował SPS podczas wszystkich etapów ontogenezy, a zeatyna tylko w starzejących się (45–60 dniowych) liściach. 6BA i zeatyna hamowały heksokinazę; jej aktywność znacznie wzrastała podczas starzenia się liści. Cytokininy opóźniały też destrukcję jąder i degradację jądrowego DNA w starzejących się (45–60 dniowych) liściach.

K e y w o r d s: *Beta vulgaris* L., DNA, hexokinase, cytokinins, sucrose phosphate synthase

INTRODUCTION

Senescence is an important physiological process occurring during different stages of the plant life cycle. In most cases it is accompanied by leaves tissue dehydration, increase in the activity of nucleases and proteinases, destruction of photosynthetic pigments, RNA, and lipids [2, 7]. Deep biochemical and structural changes which may occur at natural senescence, are bound to a metabolism of sucrose, which is being not only the major integrate component of cells, but also is the regulatory molecule, supervising an expression of many genes. Changes of concentration of sugar, their accumulation in leaves, in connection with loss of donor function, can lead to decrease in an expression of photosynthesis genes and stimulation of senescence [3, 13].

The process of sucrose synthesis, carried out by sucrose phosphate synthase (SPS, EC 2.4.1.14) is bound to function the photosynthetic apparatus. At any modification of photosynthesis, as shown in leaves of barley, first of all changes the speed of sucrose synthesis and its outflow in an ear [1].

The first enzyme including hexose into metabolism and, thus, determining the endogenous level of sugars in photosynthetic tissues, is hexokinase (EC 2.7.1.1). It is known that a role of hexokinase, as regulatory enzyme, consists in a regulation of photosynthesis, growth and senescence [3]. Hexokinase by phosphorylation of glucose not only forms the substrates for glycolysis but also takes part in the aging regulation [18].

The study of activity of enzymes that catalyse sucrose synthesis and metabolism during senescence leaves of sugar beet (*Beta vulgaris L.,*) is important in respect to the synthesis regulation and the transport of sucrose, the regulation of the ripening.

The senescence may be induced or modified by different factors and controlled by phytohormones [18], for example kinetins regulate metabolism and control senescence processes. During senescence the balance of phytohormones is constantly changed partially decreasing in kinetins, auxins and gibberellins. It is known that kinetins inhibit senescence and retard the chlorophyll content [9], nevertheless the data on the changes in the activity of SPS during senescence and possibility of their hormonal regulation are poor presented in literature.

In this paper, we have studied the effects of 6-BA and zeatin on nucleus statements, the nuclear DNA fragmentation, sucrose synthesis and its metabolism in the senescence leaves of sugar beet.

MATERIAL AND METHODS

Sugar beet plants cv. Uladovskaya odnosemennaya – 35 (UO-35) were grown under natural conditions in the pots filled with 15 kg of dark-grey soil fertilized with the nutrient mixture elaborated at the Institute of the Sugar Beet (Ukraine). This nutrient medium included (substance

g x kg^{-1} of the soil in each pot): $Ca(NO_3)_2$ (1.22), KNO_3 (0.2), $NaNO_3$ (0.33), KCl (0.11), K_2HPO_4 (0.32), H_3BO_3 (0.003), $Fe_2(SO_4)_3 \cdot 9H_2O$ (0.08), $MnSO_4 \cdot H_2O$ (0.02), $MgSO_4 \cdot 7H_2O$ (0.54), $CaCO_3$ (0.4).

Twice during the growing period at the stage of leaves of 5-7-day-old and 25 days of old leaves were sprayed with tested solutions: $4x10^{-5}$ M 6-BA and $1x10^{-6}$ M zeatin, "Serva" (Germany). Control plants were treated with water. Leaves 5-, 25-, 45-, 60-day-old were taken for studies. SPS was isolated in accordance to the method of Huber [5].

Leaf samples (500 mg) were ground in 4 ml 50 mM Mops-NaOH, pH 7.5, supplemented with 15 mM $MgCl_2$, 6 mM DTT, 1 mM EDTA, and 0.1% (w/v) Triton X-100. The homogenate was squeezed through a nylon cloth and centrifuged at 20000 g for 15 min. The supernatant was placed in teflon tubes filled with Sephadex that was equilibrated with the homogenization buffer devoid of Triton X-100. These tubes were placed into the centrifuge tubes and, after loading the protein extract, centrifuged for 5 min at 5000 g. The obtained extract (4–5 ml) was used immediately for protein determination [11] and enzyme activity assays.

The incubation mixture at substratum saturated level: 50 µmol Mops-NaOH, pH 7.5, 10 µmol UDPG, 40 µmol glucose 6-phosphate, 10 µmol fructose 6-phosphate, 15 µmol $MgCl_2$, 2.5 µmol DTT and 45 µl of the enzyme preparation. The total volume of incubation mixture was 70 µl; time of the incubation was 30 min at 35°C and stopped by the addition of 70 µl 30% KOH. Unreacted fructose was destroyed by boiling with the alkali for 10 min. SPS activity was determined by the resorcine method [15] from the amount of sucrose produced. Enzyme activity was expressed as µmol of sucrose formed per 1 mg of protein.

Hexokinase was isolated and its activity was evaluated in accordance to [10]. The tissue of leaves (1 g) were ground in 15 ml of 0.1 M Tris-HCl buffer, pH 7.5, containing 0.3 M KCl, 5 mM $MgCl_2$, 1 mM EDTA and 10 mM DTT. After centrifugation at 20000 g during 20 min, supernatant was fractionated with ammonium sulfate at saturation from 30 to 70%. Precipitate obtained was dissolved in a small volume of isolation medium diluted 5-fold and dialyzed against the same buffer.

Incubation mixture (2.4 ml) contained (mM) Tris-HCl, pH 8.0 – 50; $MgCl_2$ – 5, ATP – 5, $NADP^+$ – 1, glucose – 5, glucose-6-phosphate dehydrogenase – 1 unit and 500-600 µg enzyme preparation. Glucose was included after pre-incubation. The reaction was run at 37°C. Enzyme activity was measured using a Specord M40 spectrophotometer (Carl Zeiss, Germany) at 340 nm in 1-cm cuvette by the rate of NADPH formation. Enzyme activity was calculated using the coefficient of molar extinction, which is $6.2x10^3$ for NADPH. Glucose-6-phosphate dehydrogenase was purchased from Ferak (Germany), other reagents, from Serva (Germany).

The content of chlorophyll was determined by unmaceration method after extraction of leaf discs (fixed mass and area) by dimethylsulfoxide at 65° C during 4 h [4]. Optical density was measured at 645, 652 and 663 nm using Specord M-40 spectrophotometer (Carl Zeiss, Germany).

Nuclei were isolated from cells of sugar beet leaf tissue (10–20 g) according to [6]. The integrity of nuclei was controlled with light microscope NU-2 (ZEIS JENA, Germany) at different stages of the growth cycle and their quantity was estimated in Goriaev box after coloration in 2%-acetoorcein solution. This method allows to receive unbroken intact nuclei; they had no fragments of cytoplasmic structures.

The purified DNA was digested for 1 h at 37°C with RNAase A (Serva) (1µg RNAase A : 1 µg DNA). Clear DNA fragmentation was separated in 1.7 % agarose gel [17]. The agarose gel was stained with 0.5 µg ml^{-1} ethidium bromide and DNA visualized under UV illumination.

RESULTS

Senescence is the final stage of plant vegetative and reproductive development. This stage is especially characterized by partial destruction of nuclei. In the

senescent leaves (60-day-old), their contents are about 65% of maximum quantity revealed in 5-day-old leaves. At the same time under the influence of cytokinins destructive processes in nuclei of senescent leaves of sugar beet were retarded: 6-BA constrained destruction of nuclei on 77.3%, zeatin – on 72.4% (Fig.1).

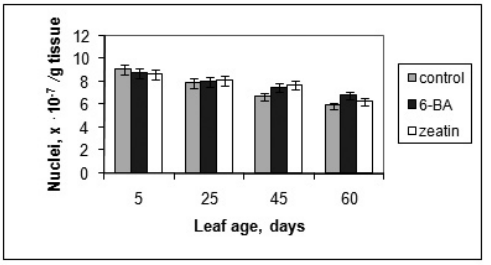

Figure 1. Effect of phytohormones on the quantity of nuclei in the sugar beet leaves during ontogeny

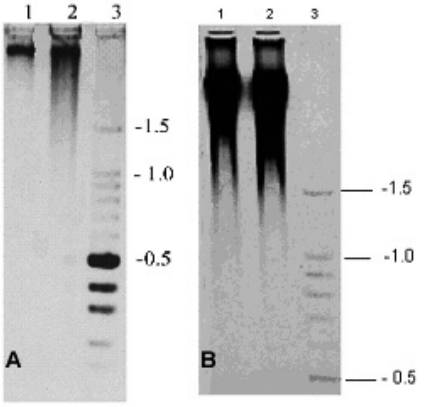

Figure 2. Electrophoregram of nuclear DNA in 1.7%-agarose gel during sugar beet ontogeny. A: 1 – nuclear DNA of leaves (5-day-old), 2 – nuclear DNA of leaves (25-day-old), 3 – marker of molecular weight – 100 bp DNA Ladder ("Promega", USA) along with pUC19 DNA/ MspI Marker, 23 ("Fermentas", Lithuania); B : 1 – nuclear DNA of leaves (45-day-old), 2 – nuclear DNA of leaves (60-day-old), 3 – marker of molecular weight – 100bp DNA Ladder ("Promega", USA).

During sugar beet leaf senescence there occurs gradual degradation of nuclear DNA, however, oligonucleosomal DNA fragmentation does not detect right up to full loss of chlorophyll (Fig. 2, A, B). It is noted that distribution of the spectrum of fragments during senescence of sugar beet leaves does not correspond to a pattern of DNA fragmentation that is revealed during destruction of cells by to a necrosis.

The treatment of 60-day-old sugar beet leaves with 6-BA and zeatin retarded processes of nuclear DNA fragmentation (Fig. 3).

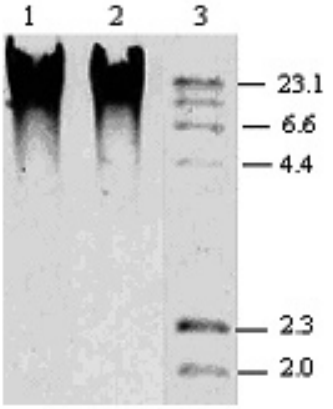

Figure 3. Electrophoregram of nuclear DNA in 0.8 %-agarose gel from the sugar beet 60-day--old leaves treated with zeatin (1) and 6-BA (2), marker of molecular weight – DNA of λ-phage after hydrolysis by HindIII (3).

The maximum level of soluble proteins in leaves of a sugar beet was found in 25-day-old leaves. Senescence of leaves was accompanied by decrease in the protein content and in the leaves of 60-day-old leaves its content consisted of a half of maximum. Application of growth regulators led to increase in the protein content: 6-BA to 17–28% and zeatin to 24–39% (Table 1).

Table 1. The influence of 6-BA and zeatin on the content of soluble proteins and chlorophyll *a* in the sugar beet leaves

Leaf age, days				
Variants	5	25	45	60
Soluble proteins (mg/g FW tissue)				
Control	42.4±0.6	59.5±0.5	37.6±0.4	30.6±0.2
6-BA	49.4±0.2	67.6±0.8	48.0±2.0	39.0±0.4
Zeatin	58.8±0.8	73.6±1.2	40.6±0.8	42.0±0.8
Chlorophyll *a*, mg/dm^2				
Control	4.13±0.1	4.63±0.05	1.55±0.03	1.20±0.02
6-BA	3.21±0.3	4.47±0.1	2.10±0.05	1.40±0.07
Zeatin	3.15±0.4	4.58±0.06	2.20±0.04	2.09±0.03

The content of chlorophyll was also changed in the senescence leaves. In the 60-day-old leaves the chlorophyll *a* made up about 26% of one maximal level that was found in the 25-day-old leaves. Cytokine preparations did decrease chlorophyll destruction: the chlorophyll content under the influence of 6-BA reached in a senescent yellow leaf 31.0 % from maximal, under the influence of zeatin – 41% (Table 1).

The state of photosynthetic system is closely related with functional activity of enzyme of sucrose biosynthesis – SPS. The activity of SPS is not constant. The maximal specific activity was revealed in the 25-day-old leaves. In a senescent leaf (60-day-old) specific activity of SPS constituted 26% of maximum (Fig. 4). In the leaves (45- and 60-day-old) treated by 6-BA the activity of SPS was 2–3-fold higher in comparison to non-treated. At the same time in the experiments with zeatin in young leaves (5-, 25-day-old) no changes in the SPS activity were observed. Thus, exogenous cytokinins 6-BA, zeatin had the essential influence on SPS activity, sustaining on the higher level a specific activity of enzyme in mature and senescent leaves.

In young (5-day-old) leaves exogenous 6-BA and zeatin increased hexokinase activity to 288 and 176% correspondingly. Nevertheless, the total level of activity was not great. 6-BA activation of hexokinase in young leaves was accompanied also by rising of activity of SPS, which is impossible to tell about zeatin. In the process of ripening and senescence of leaves the activity of a hexokinase essentially increased and in 60-day-old leaves its level in 15.7 times exceeds one of 5-day-old leaves. 6-BA and zeatin inhibited the hexokinase activity to 40% in

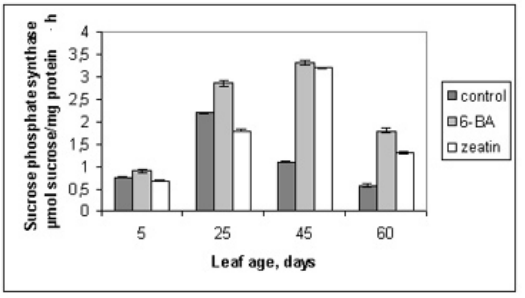

Figure 4. Effect of 6-BA and zeatin on the specific activity of sucrose phosphate synthase in the sugar beet leaves during ontogeny

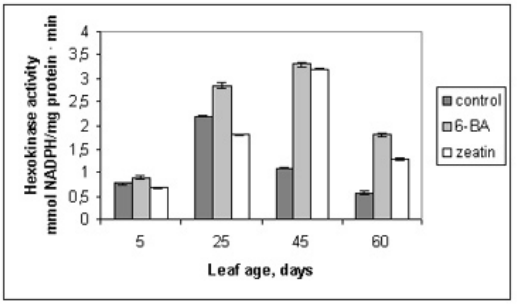

Figure 5. Effect of 6-BA and zeatin on the specific activity of hexokinase in sugar beet leaves during ontogeny

senescence leaves. Thus, nature cytokine hormones reduce a degree of hexokinase activity in senescence leaves (Fig. 5).

DISCUSSION

During leaf senescence the quantity of nuclei decreased and their content in the senescent leaves was lower 1.5-fold in comparison with young leaves at the beginning of the plant vegetation.

Leaves senescence, when it occurs in ordinary conditions, is accompanied by destruction and decreasing in chlorophyll content, chromatin modification, nuclear destruction, oligonucleosomal nuclear DNA fragmentation, which are apoptosis markers [14, 16, 20]. Also senescence is the programmed process and it can be modulated by different factors including phytohormones [14, 18].

It is known that cytokinins phytohormones can delay the leaves senescence, but is not clear whether they take part in the degradation nuclei and nuclear DNA. Hence, we studied the influence of 6-BA and zeatin on the changes in the quantity of nuclei and molecules of nuclear DNA. In the 60-day-old leaves the decreasing in the quantity of nuclei was 1.5-fold lower in comparison with green leaves but oligonucleosomal fragmentation was not detected (see Fig. 1). In the experiments with programmed cell death in the first wheat leaf DNA fragmentation was revealed only in apical parts of leaf blade but not in the basal ones [18]. Nuclear DNA degradation can be caused first of all by chromatin breaking. This process requires the presence of more than 1,000 molecules of water and cations per nucleosomes under physiological conditions [12].

We put forward a hypothesis that cytokinins can take part in the stabilization of DNA molecules via controlling the expression of endonucleases genes in the senescent leaves.

Based on our data we can suppose that during programmed cell death of sugar beet leaves there occurs fragmentation in the nucleosome spacers of more than 180 bp (Fig. 2–3). There are some sites of DNA hydrolysis changed within linker. It is possible to note, for example, that in yellow leaves *Bauhinia purpurea* low molecular weight DNA fragments began to occur, but legible oligonucleosomal fragmentation is revealed only during the analysis of this biopolymer in yellow--brown leaves [23].

Similar changes in the polymerous DNA molecules reflect their physiological functions that are connected with gradual transport of organic and mineral substances to young growing leaves and generating reproductive organs. Nevertheless, partial DNA fragmentation in the senescence organs of plants can be reliable criteria of programmed cell death (see Fig. 2 and 3).

In the senescence leaves, where are visible changes in polymerity of nuclear DNA molecules, under the influence of 6-BA and zeatin the fragmentation of this biopolymer was not great (Fig. 3). These data prove that cytokinins take part in maintenance of integrity and stabilization of DNA molecules. It is possible that they control an expression of endonucleases genes and their accumulation in the nuclei of senescent leaves.

It is shown that cytokines and auxins inhibit processes of senescence and increase leaf greening [9], whereas, the data about carbohydrate metabolism are extremely severe. It is found that during tobacco leaves senescence phytohormones taking part in the metabolism regulation, interact with sugars, regulate transport of assimilates from leaves [19].

As our data suggest (Fig. 4), the functional activity of SPS in the matured and senescence leaves has been increased 2–3-fold under the influence of 6-BA and zeatin in comparison to the control plants (45-, 60-day-old) of sugar beet leaves. At the same time, in the young leaves (5-day-old) little activation of this enzyme by 6-BA and its absence in the leaves treated by zeatin can be explained by the fact that SPS subordinated the to complex system of endogenous regulation, is closely connected with photosynthesis. The content of chlorophyll in young leaves was also not changed (see Table 1). During natural senescence in the 45-, 60-day-old leaves exogenous cytokinins retarded the chlorophyll destruction. Such regularity was also found in the rice leaves under water deficit [22]. Nevertheless, in our experiments during natural senescence of sugar beet leaves exogenous 6-BA and zeatin activated SPS, while in the work of Yang et al. [22] cytokinins reduced the activity of this enzyme and the level of remobilization of carbon reserves. It is possible that during senescence of leaves under water deficit the essential role in the regulation of sucrose activity depended on the concentration of cytokinins in the cells.

The content of proteins was decreased in the senescence leaves. Under the action of exogenous 6-BA and zeatin the level of proteins was little increased. The protein synthesis and enzymatic activity are closely interrelated. There is a need to note that the maximal activity of SPS, the content of chlorophyll and proteins were the highest in young 25-day-old leaves, which created conditions of higher degree of cellular metabolism.

The opposite situation was visible in the study of hexokinase activity. Increase in the hexokinase activity is connected with senescence, because the products of hexokinase activity – phosphorylated sugars decrease genes expression, that are responsible for synthesis of chlorophyll-bonded proteins [3]. Decreasing in gene expression of hexokinase leads to retardation of senescence processes [21].

In young (5-day-old) leaves the activity of hexokinase was not higher, which can be explained by active growth processes. At the same time increase in its activity is connected with growth inhibition. Increase in the hexokinase activity by phytohormones during this period can suggest their participation in the supply

by phosphorylated sugar of intensive metabolic processes. During the senescence, when the hexokinase activity is increased, 6-BA and zeatin inhibited this process (see Fig. 5).

In conclusion, the obtained data suggest that cytokinins 6-BA and zeatin increased the levels of soluble proteins during all ontogenesis stages of leaves and thus preserved chlorophyll destruction in the old leaves. 6-BA activated SPS during all ontogenesis, zeatin only in 45-, 60-day-old sugar beet leaves. Cytokinins inhibited the hexokinase activity. Exogenous 6-BA and zeatin retarded the breakdown of nuclei and degradation of nuclear DNA.

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