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ELECTRON AND PROTON TRANSPORT IN CHLOROPLASTS OF PEA PLANTS AFTER NIGHT EXPOSURES TO CHILLING TEMPERATURES

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Plant growth and development slow down when the temperature drops below a critical level (10—12 °C). One of the main mechanisms of plant adaptation to lower temperatures is an increase in the fluidity of the lipid phase of the membranes, which makes it possible to maintain the necessary membrane processes for survival, in particular, the activity of photosynthetic electron transport. In the temperate climate zone, plants often tolerate nighttime temperature drops to frost. Pea (*Pisum sativum* L.) refers to cold-resistant species that can withstand a prolonged decrease in temperature. In this work, pea plants were grown at a constant temperature of 20—22 °C for 11 days, then for 6 days at night, the plants were placed in chambers at a temperature of 6 °C. The temperature dependence of the photochemical activity in chloroplasts isolated from control and chilled leaves was studied. It was shown that the maximum value of the rate of uncoupled electron transport from water to potassium ferricyanide in control chloroplasts was observed at a temperature of 22 °C, i.e. at the temperature of plant growth. In the chloroplasts of plants subjected to night cooling, the temperature dependence of the uncoupled electron transport was shifted to lower temperatures and the maximum reaction rate was recorded at a temperature of about 12 °C. When measuring the value of light-induced proton uptake (ΔH^+) and the rate of O_2 uptake in the reaction of electron transfer from H_2O to methyl viologen in coupled chloroplasts, a sharp decrease in these parameters was observed with a change in the temperature of the reaction medium from 14 to 10 °C in the control, and from 10 to 6 °C in the experimental plants. The sharp decrease in the rate of photochemical reactions with decreasing temperature may be due to a phase transition of membrane lipids and a slowing down of diffuse processes. These results allow us to consider the ΔH^+ as an indicator of the fluidity of the lipid phase of chloroplast membranes in comparative studies. The transmembrane proton gradient (ΔpH), which was estimated by light-dependent quenching of the fluorescent label of 9-aminoacridine, exceeded the control level in chloroplasts of plants subjected to night cooling, which may be due to an improvement in the insulating function of the membranes. The data obtained indicate that when pea plants adapt to lower night temperatures, the state of the photosynthetic chloroplast membranes changes, which ensures the preservation of their functional activity.

Key words: *Pisum sativum* L., photosynthesis, chloroplasts, chilling, electron transport, low temperature adaptation, plastoquinone, proton exchange, transmembrane proton gradient.

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Low temperature is one of the most important ecological factors that may restrict plant growth during each stage of life from germination to maturity [1, 2]. Chilling includes the swelling of chloroplasts, deformation of thylakoid membranes, and decrease in number or size of starch granules [3—5]. The extent of temperature acclimation of photosynthetic processes differs among species. Some species are able to acclimate, whereas others are not [6, 7]. When adapted to chilling, cool-resistant plants develop essential tolerance to survival under cold stress through various levels of biochemical and cellular biological changes [5, 8]. The mechanisms underlying the acclimation of photosynthesis to low temperatures vary with the plant species, and a high photosynthetic acclimation potential to thermal conditions is associated with a highly plastic response of electron transport to temperature [7]. Plants grown at chilling temperature (0—15 °C) show maximum rates of photosynthesis at lower temperatures than do plants grown under warm temperature [8—10]. The mechanism for supporting of photosynthetic activity during cooling is maintaining membranes in a fluid state that allow them to resist low temperatures [1, 2, 5, 6].

The primary effects of low temperature on plant metabolism include the impact of cold on the fluidity of membrane lipids, which is likely to affect lipid-associated enzymes of plant membranes, membrane proteins themselves, cytoskeletal proteins, and soluble enzymes [11]. When the temperature drops below a critical level (12 °C), the physical state of the lipid membranes changes, a physical phase transition from a liquid crystal structure to a solid gel structure occurs and, therefore, the diffusion of mobile carriers in thylakoid membranes is significantly slowed down [12, 13]. The lateral movement of plastoquinone, the two-electron and two proton carrier providing electron transfer between the second and first photosystems and participating in proton transport and the formation of the transmembrane gradient of protons, is most vulnerable to the action of cold [14].

It is known that after short-term exposure to low temperature cold-resistance of plants increases [15]. This occurs, in particular, due to a change in the fatty acid composition of lipids and a decrease in the critical freezing temperature. In nature, in temperate regions, plants are often exposed to daily periodic temperature changes up to night frost.

The mechanisms of cold-tolerant plants resistance to these destructive changes are not fully understood. Still, the ability to adapt membrane reactions and modify metabolism at low temperature are among the processes which require further study.

In this work, we studied the effect of lowered night temperatures on the photosynthetic processes in pea plants (*Pisum sativum* L.) which is a cold-resistant plant. Its seeds begin to germinate at a temperature of +1... +2 °C, and seedlings can tolerate frosts of —4...—6 °C. Due to its high resistance to low positive temperatures, it is cultivated to the very northern border of agriculture (68° N), although when the temperature drops to 8—14 °C, some inhibition of growth begins, and productivity decreases by 10—30 % [16].

The aim of the work was to investigate the effect of regular drops of the night temperature on temperature dependence of electron and proton transport in the photosynthetic membranes isolated from the pea plants.

Materials and methods

The object of the study was pea (*Pisum sativum* L.) Damir 2 variety, obtained from the Institute of Agriculture of National Academy of Agrarian Sciences of Ukraine. Plants were grown on Pryanishnikov medium for 19 days (counting from the time of seeds soaking) at a temperature of 21 °C and a photoperiod of 12 hours. The illumination source was LB40 fluorescent lamps (Poltava, Ukraine) with a photosynthetic photon flux density in the PAR area (PPFD) of 200 μmol/(m^2 · s). PPFD at the plant level was determined using a USSQS spherical micro-quantum sensor and a LICOR (LI250) measuring device, USA.

Starting from 11th day of growing, the experimental group of plants was cooled daily over 6 days for 12 h at 6 °C during the night period.

Chloroplasts (class B, [17]) were isolated from the leaves of 17-day old seedlings according to the procedure published previously [18]. Freshly cut pea leaves of the middle tier were wrapped in moist filter paper and kept at 4—6 °C in the refrigerator for 2 hours. All procedure for the isolation of chloroplasts was carried out at a temperature of 0—4 °C. The cooled leaves were disrupted using a blender (BRAUN 250W, Germany) for 20 s in a medium of the following composition: 0.4 M sorbitol, 0.01 M NaCl, 0.01 M Tris-HCl, (pH 7.8) and 5 mM sodium isoascorbate. The homogenate was filtered through 2 layers of nylon fabric and centrifuged at 800 g for 3 min to precipitate large cell fragments. The supernatant was centrifuged at 3500 g for 10 min to obtain a chloroplast fraction. Chloroplast sediment was resuspended in the storage medium (0.4 M sorbitol, 2.5 mM $MgCl_2$, 10 mM NaCl, 10 mM Tris-HCl, pH 7.8) and used to determine the rate of photochemical reactions. Chlorophyll concentration in the chloroplast suspension was estimated according Arnon method [19].

The uncoupled electron transport from H_2O to $K_3Fe(CN)_6$ was determined as previously described [20] by light-induced pH-changes in the reaction media contained 200 mM sorbitol, 2.5 mM $MgCl_2$, 10 mM NaCl, 10 mM KCl, 0.5 mM of HEPES (pH 7.0), 1 mM tricine-NaOH, 5 mM NH_4Cl, 0.5 μM gramicidine, 1 mM $K_3Fe(CN)_6$, and chloroplasts (0.01 mg Chl/ml) (initial pH was 7.7).

The value of the light-induced oxygen evolution or its absorption in the Mehler reaction was investigated by the amperometric method using a Clark closed platinum electrode mainly as previously described [21]. The composition of the reaction medium was the same as when measuring isolated electron transport except that concentration of HEPES was 20 mM (pH 7.0) and 0.1 mM methyl viologen (MV) was used instead $K_3Fe(CN)_6$.

Light-induced proton uptake (ΔH^+, μmol H^+/mg Chl) [22] was registered in a glass thermostated cell using a glass electrode in the reaction medium contained 200 mM sorbitol, 2.5 mM $MgCl_2$, 10 mM NaCl, 10 mM KCl, 0.5 mM tricine-NaOH, 1 mM of MES and 0.5 mM of HEPES, 0.1 mM MV, and chloroplasts (0.05 mg Chl/ml). The photochemical activity of chloroplasts was induced by a white light of a KGM-250 halogen lamp (PPFD 500 μmol/(m^2 · s)). The amount of protons absorbed in the reaction was calculated from the magnitude of the light-induced changes in pH (δpH) and the buffer capacity of the reaction medium. The buffer capacity was determined by titrating the suspension with small (10 μmol)

amounts of 10 mM sodium hydroxide. The composition of the reaction medium was the same as when determining the electron transport rate.

The transmembrane proton gradient (ΔpH) was estimated by measuring the ΔpH-dependent fluorescence quenching of lipophilic label 9-aminoacridine (9-AA) at room temperature using an XE-PAM fluorometer (Walz, Germany) [23]. The composition of the reaction medium was the same as in the determination of ΔH^+, except that the concentration of chloroplasts corresponded to 10 μg Chl/ml, and the concentration of tricin-KOH (pH 8.0) was 20 mM. The ΔpH value was calculated according to [24] under the assumption that the internal volume of thylakoids is unchanged at 10 μl/mg Chl. The concentration of 9-AA in the reaction medium was 1 μM, the intensity of the exciting light — 1000 μmol/($m^2 \cdot s$).

ΔpH was estimated by 9-aminoacridine fluorescence quenching as described by Mills [24] except that the 9-aminoacridine concentration was 5 μM. The intensity of the measuring ray was 0.4—0.8 μmol/($m^2 \cdot s$), and the ΔpH was driven by a quartz halogen lamp filtered through a Corning 2-62 glass filter. ΔpH was calculated from the fractional quenching, q, according to $\Delta pH = \log [q/(1 - q)] + \log (V/v)$, where V is total reaction volume, and v is the internal volume of the thylakoids (assumed to be 10 μl/mg Chl).

Experiments were performed in 3—5 biological (n) and analytical replicates. Experimental data are presented as the arithmetic mean value (M) with a standard error $m = \frac{\sigma}{\sqrt{n}}$. Evaluation of the significance of differences between the samples was performed using two-sample t-test with different dispersions. Calculations were performed using Microsoft Excel 2010 program.

Results and discussion

The growth of pea plants, exposed to low night temperatures for six days, was suppressed compared with the control. The length of the seedlings and the leaf area markedly decreased (Table).

The effect of the night chilling on the photosynthetic processes has been investigated by determining the rate of the light-induced electron transport in thylakoid membranes isolated from subjected to low temperatures and control pea leaves. The electron transfer rate in chloroplast membranes is controlled by the level of the transmembrane proton gradient (the so-called photosynthetic control). In non-phosphorylating chloroplasts, the electron transfer rate is low and increases significantly under photophosphorylation, when the proton efflux through the proton channel of ATP-synthase is coupled with ATP synthesis accelerates and ΔpH decreases [25]. The same effect is achieved by using uncouplers — protonophores or ionophores which form channels in membranes, and facilitate the outflow

Growth indices of 17-day old pea seedlings in control and after six day night chilling

Variant	Plant height, cm	Leaf area of one plant, cm^2
Control plants	18±3	52±8
Plants subjected to low night chilling during 6 days	11±3	27±6

of protons from the inner space of thylakoids bypassing ATP-synthase [26], thereby eliminating the photosynthetic control. The uncoupled electron transfer in this case is not limited by proton transfer via ATP-synthase that allows one to estimate the electron transfer potential. In this work, we determined the rate of electron transfer from water to potassium ferricyanide in the presence of uncoupling concentrations of NH_4Cl and gramicidin.

Fig. 1 shows the temperature dependence of the uncoupled photosynthetic electron transport rate in thylakoids isolated from pea seedlings subjected to night cooling (2) in comparison with the control (1). The rate of the light-induced $H_2O \rightarrow K_3Fe(CN)_6$ reaction was determined after 5 min dark incubation in the reaction media under excitation at all indicated temperature values. The temperature dependences of the electron transport rate were bell-shaped with maximum at 20—24 °C for the control and 12—13 °C for thylakoids isolated from the night-chilled seedlings. It should be noted that uncoupled electron transport in thylakoids isolated from chilled seedlings only slightly exceeded the control in the temperature range 6—18 °C. At the same time, at temperatures of 20—30 °C, the electron transfer rate in uncoupled thylakoids isolated from chilled plants was significantly lower than in the control. This data suggest that regular drop of the growth temperature in the night is enough to induce the thylakoid membrane transformation in pea leaves.

Fig. 2 shows the rate of oxygen uptake in the reaction of photosynthetic electron transfer from water to methyl viologen (MV) in chloroplast suspension without the uncouplers. It can be seen that the rate of the $H_2O \rightarrow MV$ reaction at temperatures of 6—18 °C in chloroplasts isolated from chilled leaves was significantly higher than in the control.

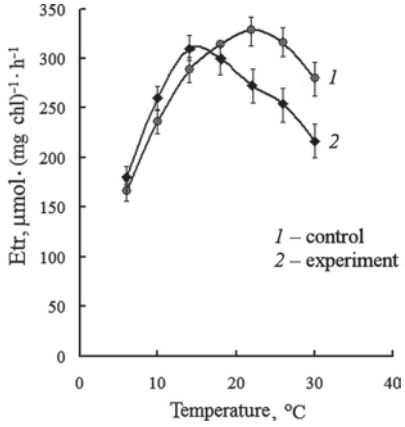

Fig. 1. The dependence of uncoupled electron transport on temperature of the reaction media: *1* — control, chloroplasts were isolated from control plants grown at 20—22 °C; *2* — experiment, chloroplasts were isolated from experimental plants grown at day/night air temperature of 20—22/6 °C. The rate of the light-induced $H_2O \rightarrow K_3Fe(CN)_6$ reaction was determined after 5 min dark incubation of chloroplasts in the reaction medium under excitation at all indicated temperature values. The reaction media contained 200 mM sorbitol, 2.5 mM $MgCl_2$, 10 mM NaCl, 10 mM KCl, 0.5 mM of HEPES (pH 7.0), 1 mM tricine-NaOH, 5 mM NH_4Cl, 0.5 μM gramicidine, 1 mM $K_3Fe(CN)_6$, and chloroplasts (0.01 mg Chl/ml) (initial pH was 7.7). The photochemical activity of chloroplasts was induced by a white light of a KGM-250 halogen lamp (PPFD 500 μmol/($m^2 \cdot s$)

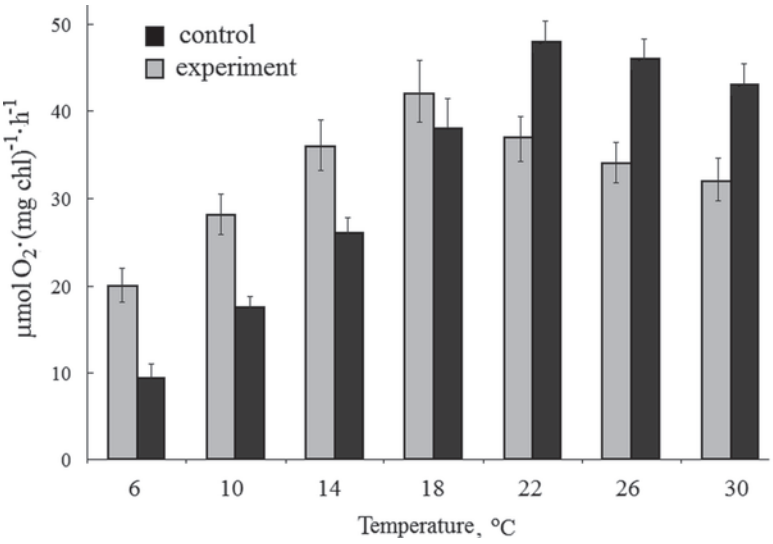

Fig. 2. The dependence of light-induced O_2 uptake in chloroplasts suspension on temperature of the reaction media in control and experiment. The reaction media contained 200 mM sorbitol, 2.5 mM $MgCl_2$, 10 mM NaCl, 10 mM KCl, 20 mM of HEPES (pH 7.0), 1 mM tricine-NaOH, and chloroplasts (0.01 mg Chl/ml) (initial pH was 7.7), (pH 7.0) and 0.1 mM methyl viologen (MV)

In general, as expected, electron transport in coupled membranes was considerably lower than in uncoupled chloroplasts over the entire temperature range tested. At temperatures above 18 °C, both in coupled and uncoupled chloroplasts, the electron transport rate in control samples exceeded the rate in chloroplasts isolated from chilled plants. In contrast to the uncoupled samples, the electron transfer rate at temperatures of 6—18 °C was significantly higher in chloroplasts isolated from chilled leaves than in the control (see Fig. 1 and 2). Since uncouplers remove photosynthetic control, i.e. the dependence of the electron transfer on ΔpH, the difference in the data in Fig. 1 and Fig. 2 may be related to a change in proton exchange in chloroplasts of chilling plants.

To determine the effect of low temperatures during plant growth on proton exchange in chloroplasts, we compared the light-dependent proton uptake (ΔH^+) and the transmembrane proton gradient (ΔpH) in chloroplast membranes isolated from leaves of control and chilled plants. Uptake of hydrogen ions in the stroma is associated with protonation of plastoquinone molecules ($Q + 2e^- + 2H^+ \rightarrow QH_2$) that are reduced on the acceptor region of PS2 and in the Q_i center of the cytochrome b_6f complex. This results in a decrease in pH of the intrathylakoid space (pH_{in}) and in an increase in pH of the stroma (pH_{out}) with formation of ΔpH [25].

Experimentally, the ΔH^+ value is determined by the light-dependent alkalization of a weakly buffered chloroplast suspension [22] (Fig. 3). After the light is turned on, the pH of the reaction medium rises to a certain stationary level, at which the absorption of protons by chloroplasts is compensated by their outward leak. After turning off the light, the pH of the external environment decreases to the initial one and is accompanied by the release of protons to the outside during deenergization of the membranes. The value of ΔH^+ depends on the integrity of the organelles.

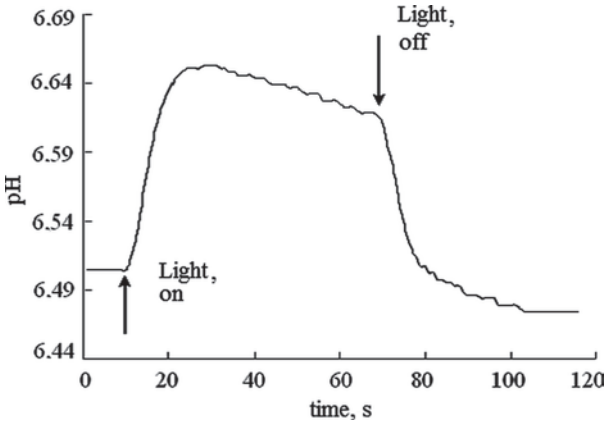

Fig. 3. pH changes in chloroplast suspension induced by illumination (PPFD 500 μmol/($m^2 \cdot s$))

Uncouplers (gramicidin, penetrating amines, etc.), as well as inhibitors of electron transport, suppress the light-dependent protons uptake. The maximal values of ΔH^+ (usually about 0.50 μmol H^+/mg Chl in the presence of phenazine methasulfate or about 0.25 μmol H^+/mg Chl in the presence of methyl viologen) are recorded at an external pH of 6.5—6.7. At pH values optimal for photophosphorylation (7.8—8.2), the value of ΔH^+ is not more than 0.1—0.15 μmol H^+/mg Chl.

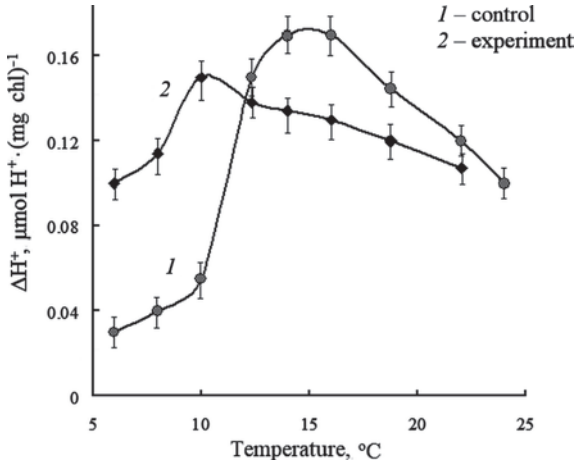

Fig. 4. The temperature dependence of the light-dependent proton uptake (ΔH^+) by chloroplasts isolated from pea plants grown at different temperature conditions: *1* — control; *2* — experiment. The reaction media contained 200 mM sorbitol, 2.5 mM $MgCl_2$, 10 mM NaCl, 10 mM KCl, 0.5 mM tricine-NaOH, 1 mM of MES and 0.5 mM of HEPES, 0.1 mM MV, and chloroplasts (0.05 mg Chl/ml)

The protons entering the thylakoid are bound by membrane buffer groups; the concentration of which (the intrathylakoid buffer capacity) determines the amount of light-induced proton uptake (ΔH^+) [27, 28]. The dependence of the ΔH^+ value on temperature in control thylakoids (curve *1*) and thylakoid isolated from chilled plants (curve *2*) is shown in Fig. 4. The maximal ΔH^+ value was registered at 14—17 °C in control thylakoids and at 10 °C in thylakoids of the night chilled plants. Generally, in thylakoids isolated from chilled leaves, the temperature dependence of the ΔH^+ value is shifted towards lower temperatures. The ΔH^+ value drops sharply in control chloroplasts when temperature of the reaction medium decreases from 14 °C to 10 °C and more smoothly reduced in chloroplasts from chilled plants while temperature decreasing from 10 to 6 °C. We can assume that a sharp drop of the ΔH^+ in control membranes near the critical temperature occurs due to a disorder of the transmembrane proton transfer by plastoquinone, lateral diffusion of which is greatly hampered at temperatures below 12 °C [14]. In the membranes isolated from chilled plants, ΔH^+ value is much higher at temperatures <12 °C than in control (see Fig. 4). This is probably due to an increase in the fluidity of thylakoid membranes in chilled leaves, which is indispensable for the lateral movement of plastohydroquinone (QH_2) and supporting the proton transfer. For normal operation, as we known, the membrane must be in a liquid crystal state. Therefore, in living systems, with a prolonged decline in the ambient temperature, an accumulation of unsaturated fatty acids is observed, which ensures a decrease in the phase transition temperature [13, 29].

Unlike control samples, the ΔH^+ in chloroplasts isolated from chilled leaves decreased with temperature dropping below 10 °C more smoothly then in control. This may be due to transformation of fatty acid composition of the thylakoid membranes in plants under night chilling. Since biological membranes usually consists of a large number of different lipids, they may not have a pronounced phase transition and the phase transition temperature shifts to lower values with an increase in the degree of unsaturation of the side chains of fatty acids.

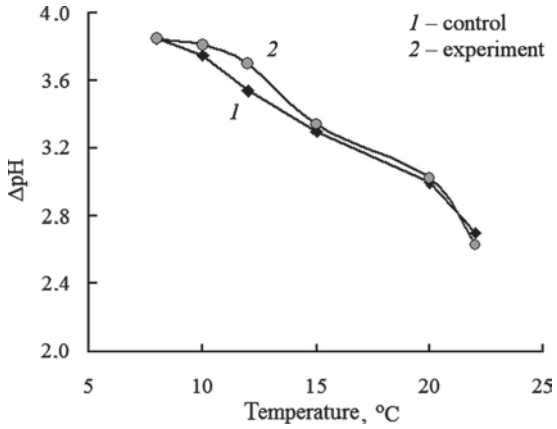

Fig. 5. The temperature dependence of transmembrane ΔpH in thylakoids isolated from pea plants grown at 20—22 °C (*1*) and day/night air temperature of 20—22/6 °C (*2*)

Thus the change of dependence of photochemical reactions on temperature may be an indicator of lipid phase fluidity of chloroplast membranes during comparative studies.

Light-induced proton uptake contributes to the formation of transmembrane ΔpH in thylakoids. ΔpH value depends on insulating properties of membranes which could change under chilling of plants. In this work, we determined the ΔpH value by the light-dependent quenching of the fluorescence of the lipophilic label 9-aminoacridine. ΔpH values were calculated from the light-induced 9-aminoacridine fluorescence quenching (PPFD 1000 μmol/($m^2 \cdot s$)).

The results presented in Fig. 5 show that ΔpH in chloroplasts isolated from control and chilled plants did not practically differ in the entire range of studied temperatures, except that at temperatures below 12 °C, ΔpH in chloroplasts of plants subjected to night cooling exceeded the control level. The results indicate an improvement in the insulating function of membranes in the chloroplasts of chilled plants.

Thus results obtained revealed that when pea plants adapt to lower night temperatures, the state of the photosynthetic chloroplast membranes changes, which ensures the preservation of their functional activity.

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ТРАНСПОРТ ЕЛЕКТРОНІВ І ПРОТОНІВ У ХЛОРОПЛАСТАХ РОСЛИН ГОРОХУ, ЯКІ ВИРОЩУВАЛИ ЗА УМОВ НІЧНОГО ЗНИЖЕННЯ ТЕМПЕРАТУРИ

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За температури нижчої від критичного рівня (10—12 °С) ріст і розвиток рослин гальмується. Одним з основних механізмів адаптації рослин до зниження температури є збільшення за цих умов плинності ліпідної фази мембран, що дає змогу підтримувати необхідну для виживання швидкість мембранних процесів, зокрема активність фотосинтетичного транспорту електронів. У зоні помірного клімату на рослини часто впливає нічне зниження температури аж до приморозків. Горох (*Pisum sativum* L.) належить до холодостійких видів, здатних витримувати тривале зниження температури. Ми вирощували рослини гороху за постійної температури 20—22 °С протягом 11 днів, після чого протягом 6 днів частину рослин у нічний час вміщували в камери з температурою 6 °С. Вивчали температурну залежність фотохімічної активності хлоропластів, ізольованих із листків контрольних і охолоджених рослин. Показано, що максимальне значення швидкості роз'єднаного транспорту електронів від води до гексаціаноферату(III) калію у контрольних хлоропластах спостерігалося за температури 22 °С, тобто за температури вирощування рослин, тоді як після нічного охолодження рослин крива температурної залежності зміщувалася в бік низьких температур, а максимальна швидкість реакції реєструвалася за температури близько 12 °С. У нероз'єднаних хлоропластах величина світлоіндукованого поглинання протонів (ΔH^+) і швидкість поглинання кисню в реакції перенесення електронів від H_2O до метилвіологену різко знижувалися при зміні температури реакційного середовища від 14 до 10 °С в контролі і більш плавно за температур від 10 до 6 °С в експериментальних рослин. Різке падіння швидкості фотохімічних реакцій при зниженні температури може бути зумовлене фазовим переходом мембранних ліпідів і уповільненням дифузійних процесів. Ці результати дають змогу розглядати показник ΔH^+ як індикатор плинності ліпідної фази мембран хлоропластів у порівняльних дослідженнях. При зниженні температури величина трансмембранного градієнта протонів (ΔpH), яку оцінювали за світлозалежним гасінням флуоресцентної мітки 9-аміноакридину, перевищувала контроль у хлоропластах охолоджених рослин, що може бути пов'язано з поліпшенням ізолюючої функції мембран. Отримані дані свідчать, що за адаптації рослин гороху до зниження нічних температур стан фотосинтетичних мембран хлоропластів змінюється, що забезпечує збереження їхньої функціональної активності.

Ключові слова: Pisum sativum L., фотосинтез, хлоропласти, охолодження, транспорт електронів, низькотемпературна адаптація, пластохінон, протонний обмін, трансмембранний протонний градієнт.