GREENING BARLEY SEEDLINGS UNDER HIGH TEMPERATURE

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Summary. The influence of heating on the structure and functional activity of photosynthetic membranes in greening barley seedlings was studied. It was observed that plants respond differently under different heat treatments (40, 45 and 50^0C). It was also observed that elevated temperature (40^0C) enhances the stability of thylakoid membrane, reducing overall membrane fluidity. Interaction and mutual regulation of xanthophyll cycle activity and membrane fluidity was considered. The connection between structure dynamic and photosynthetic function of thylakoid membranes under heat shock was discussed.

Keywords: barley, chlorophyll *a* fluorescence, fluidity, heat stress, thylakoid membrane, xanthophylls.

Abbreviations: Ax —antheraxanthin, Chl – chlorophyll, DPH —1.6-diphenyl 1.3.5-hexatriene, F_m – maximum chlorophyll fluorescence yield, F_v – variable chlorophyll fluorescence, PQ—plastoquinone pool, PSII—photosystem II, Q_A, Q_B – primary and secondary quinone-type electron acceptors, Vx —violaxanthin, Yield — effective quantum yield of PSII photochemistry, Zx—zeaxanthin.

INTRODUCTION

Wild plants are exposed to various unfavorable factors of nature and human activity. Stress factors, such as high and low temperature, drought, high light, oxidative stress, upset many physiological processes and suppress the photosynthetic activity of plants (Lichtenthaler, 1998). The electron transport in chloroplasts is known to be the most

sensitive photosynthetic process (Carpentier, 1999; Bukhov, Mohanty, 1999). The complex of PSII is the most susceptible to high temperature, primarily due to the release of Mn^{2+}, Ca^{2+}, Cl^{-} from the oxygen-evolving complex and peripheral 18, 24, 33 kDa proteins. Such structural and functional impairments lead to a decrease of the oxygen evolving capacity and of the Q_A reoxidation, as well as the rate of electron transport from Q_B to the PQ pool. These effects could be associated with a modification of the thylakoid membrane fluidity (Bukhov, Mohanty, 1999; Rohacek, Bartak, 1999).

Thylakoid membrane is a relatively fluid system. It is essential for the photosynthetic processes involving lateral, rotational and transmembrane diffusion. However, this characteristic of the membrane is very vulnerable to high light intensity and increased temperature rate. Heat induced membrane damage is attributed to lipid hyperfluidity, which alters lipid–protein interactions and subsequently causes protein denaturation (Páli, Garab, 2003).

Membrane fluidity could be regulated by xanthophyll cycle activity (Eskling, Arvidsson, 1997). Havax and Gruszecki observed on potato leaves that zeaxanthin (Zx) accumulation in the absence of any inhibitory treatment, markedly affected several characteristics of the photosynthetic apparatus, such as the stability PSII to heat and the plastoquinon diffusion in the thylakoid lipid matrix (Havax, Gruszecki, 1993). Carotenoids of higher plants could also stabilize and photoprotect the lipid phase of the thylakoid membranes. The resulting interaction of the xanthophyll molecules and the membrane lipids leads to a decrease in the membrane fluidity, an increase in membrane thermostability and a lowered susceptibility to lipid peroxidation (Havaux, 1998).

Light-induced biogenesis of the plastid, maturation of the thylakoid membranes during greening, related structural reorganization, as well as the response of mature thylakoids to different stress factors, such as extreme temperatures or intense light, are active areas in the photosynthesis research. Thus, greening plant is a good model of plant development.

Studying the formation of the pigment-protein-lipid assemblies is a way to better understanding of the nature and the functional role of structural and functional changes taking place in photosynthetic membranes under stress (Páli, Garab, 2003). Data about the formation and the development of photosynthetic membranes in greening seedlings under high temperature are almost absent.

In the present work the thermoinduced changes in the structure of the thylakoid membrane, the function of linear electron flows through PS II and the xanthophyll cycle activity in greening barley leaves were studied. A connection between structural disturbances of thylakoid membranes and changes in the activity of electron-transport chain under heat shock was observed.

MATERIALS AND METHODS

Barley seedlings (*Hordeum vulgare* L.) were grown on tap water in the dark for 7 d at 22°C. After this time etiolated seedlings were transferred to continuous light (120 μmol $m^{-2}s^{-1}$) and allowed to green for 24 hours. Heat treatment (40, 45, 50^0C for 3 hours) was performed in air thermostat on previously greening leaves for 3 hours. After heating, seedlings were allowed to acclimate at 22°C (the same light condition was used).

Thylakoids were isolated according to Robinson and Yocum, 1980 and suspended in 40 mM HEPES (pH 7.6), 0.33 M sorbitol, 5 mM $MgCl_2$, 1 mM KH_2PO_4, 5 mM NaCl.

Photosynthetic pigments from barley leaves were extracted with 80% acetone according to the Lichtenthaler method (Lichtenthaler, 1987).

Xanthophyll pigments were separated and quantified by HPLC according to the method of Gilmore and Yamomoto (1991).

Lipid microviscosity was derived from the fluorescence polarization measurments using 1.6-diphenyl 1.3.5-hexatriene (DPH) fluorescence polarization probe (Shinitzky, Barenholz, 1978). Mesurments were carried out at room temperature on fluorescence spectrophotometer "Solar" (Belarus) at exiting wave-length 360±5 nm and emission wave-length 460±10 nm.

Chlorophyll *a* (Chl *a*) fluorescence was measured at room temperature with fluorometer PAM 201 (Walz, Germany). Before measurement, the samples were adapted to darkness within 15 minutes. Measured light (650 nm, 0.04 μmol quanta m^{-2} s^{-1}) modulated with low frequency (8 kG) exited the initial fluorescence level F_o. Light 1-s pulse (3500 μmol quanta m^{-2} s^{-1}) induced an enhancement of the fluorescence to the maximal level F_m. Effective quantum yield of PSII photochemistry (Yield) was calculated on the basis of slow Chl fluorescence kinetic, measured with actinic light (665 nm, 480 μmol quanta m^{-2} s^{-1}) and periodical light pulses (3500 μmol quanta m^{-2} s^{-1}) using the following expression: Yield=$(F_m'-F_t)/F_m$, $F_v/F_m=(F_m-F_o)/F_m$, $(F_i-F_o)/F_{v,}$ (Krause, Weis, 1991).

Statistical significance was assessed using the Student's *t*-test.

RESULTS

The influence of elevated temperatures on photosynthetic pigments accumulation during greening was studied. The heat treatment at 50^0C (3 hours) led to a 50% suppression in Chl(*a*+*b*) accumulation as compared to the control (Fig.1 A). It should be noted that during the subsequent 24 hours acclimation of the treated leaves, the Chl*(a*+*b*) level remained almost invariable, while in control seedlings Chl(*a*+*b*) content grew ten times. Such treatment induced an increase in the total carotenoids con-

Fig.1a

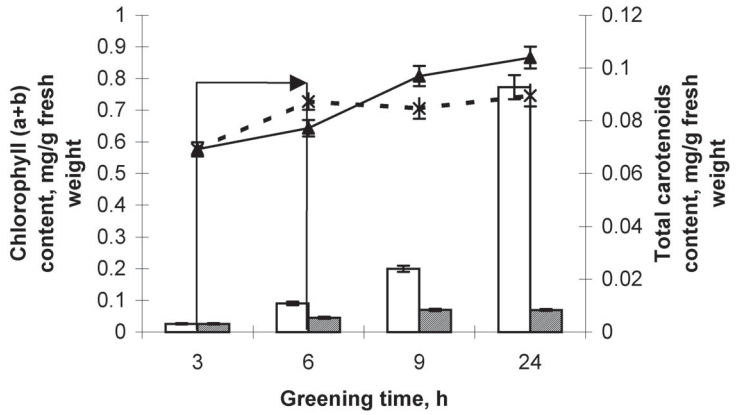
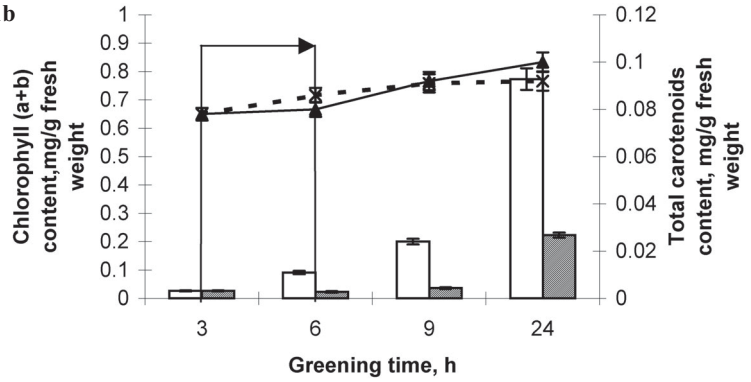


Fig.1b



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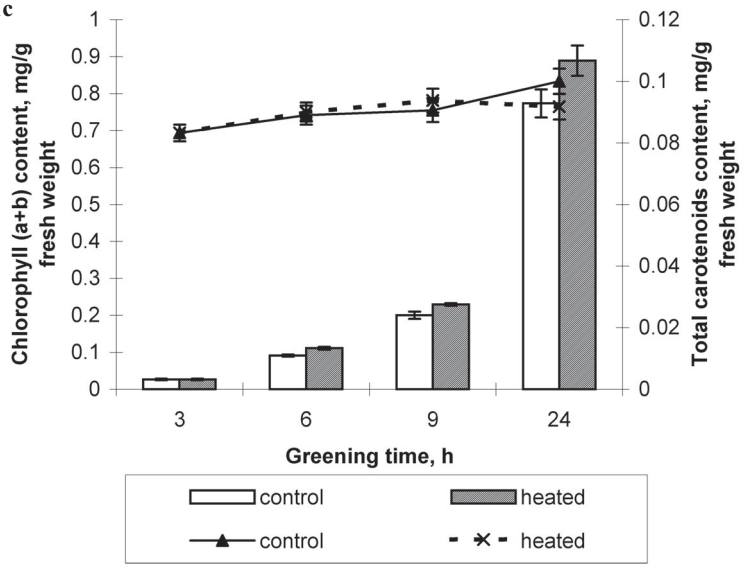

Fig.1 Changes in chlorophyll *(a+b)* content (columns) and total carotenoids (lines) under heating (3 hours) and consequent acclimation (A– 50^0C, B– 45^0C, C– 40^0C)

Table 1. Changes in total protein and chlorophyll (a+b) content under 40^0C heating (3 hours) and consequent acclimation at 22^0C

Greening time, hours	Total protein content in control leaves, mkg/g fresh weight	Total protein content in heated leaves, mkg/g fresh weight	Chlorophyll (a+b) content in control leaves, mg/g fresh weight	Chlorophyll (a+b) content in heated leaves, mg/g fresh weight
0	286±5	286±5	0	0
3	237±6	237±6	0.0262±0.0020	0.0262±0.0020
6	228±3	229±6	0.0909±0.0027	0.1111±0.0040
9	236±5	242±3	0.2002±0.0096	0.2293±0.0043
24	202±9	206±9	0.7733±0.0380	0.8891±0.0410

tent. After 3 hours acclimation the level of carotenoids in heated leaves was lower than in the control (Fig.1 A).

45^0C (3 hours) heating caused 75% reduction in Chl (*a*+*b*) content (Fig.1 B). Chl*(a*+*b*) accumulation was observed in treated leaves during a following adaptation of 24 hours greening. However, the control level has not been reached. The total carotenoids content in the leaves investigated increased during heating and remained at the same level in first acclimation time. During the 24 hours greening the total carotenoids content did not exceed the control (Fig.1 B).

Greening seedlings, undergone the heating at 40^0C, demonstrated 22% activation in Chl (*a*+*b*) accumulation as compared to the control (Fig.1 C, Table 1). During the consequent acclimation at 22^0C the difference between treated and control leaves was gradually reduced. The level of total carotenoids remained almost stable (Fig.1 C).

Such reaction of the greening seedlings to a heating of 40^0C seems uncommon and requires subsequent investigations to indicate the protective mechanism. Hence we decided to research the thermoinduced structural dynamic of thylakoid membrane. Total protein content was studied. There were no notable distinctions between heated and control leaves (Table 1).

It is well known that fluidity appears to be maintained and regulated by the photosynthetic organism to ensure optimal efficiency of the membrane-associated photosynthetic processes (Põli, Garab, 2003). Overall membrane viscosity was estimated using the steady-state fluorescence anisotropy of build-in probe (DPH). As Fig.2 indicates, DPH fluorescence anisotropy increased during the greening process. After heat treatment, DPH fluorescence anisotropy decreased. During the consequent acclimation at 22^0C, the difference between treated and control leaves reduced (Fig.2).

The effect of elevated temperature (40^0C) on the accumulation of xanthophyll-cycle pigments was investigated. The accumulation of xanthophyll-cycle pigments during chloroplast biogenesis at 22°C was examined by calculating the content of violaxanthin (Vx), antheraxanthin (Ax), and zeaxanthin (Zx) as a percentage of the

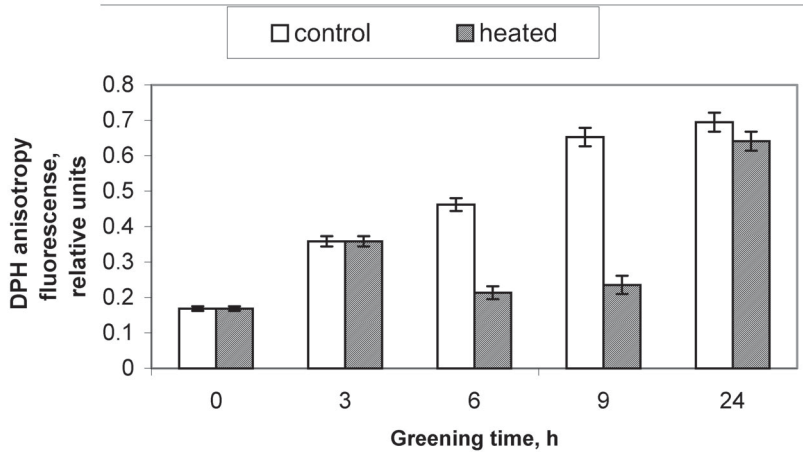

Fig.2 Changes in steady-state anisotropy fluorescence under 40^0C heating (3 hours) and consequent acclimation at 22^0C

total xanthophyll-cycle pool size (Vx+Ax+Zx) and plotted as a function of the greening time (Fig. 3). In the control, the relative content of violaxanthin increased as a function of the greening time (Fig.3 A). This occured concomitantly to the decrease in the proportion of antheraxanthin and zeaxanthin (Fig.3 A, B). After 24 h greening, zeaxanthin represented only about 1.5% of the total xanthophyll pool (Vx+Ax+Zx). Analyses of heated leaves at various greening stages indicated that the relative content of Vx increased and Ax decreased as compared to the control. After acclimation, Vx returned to the control level. Zx proportion reduced faster than in control, and

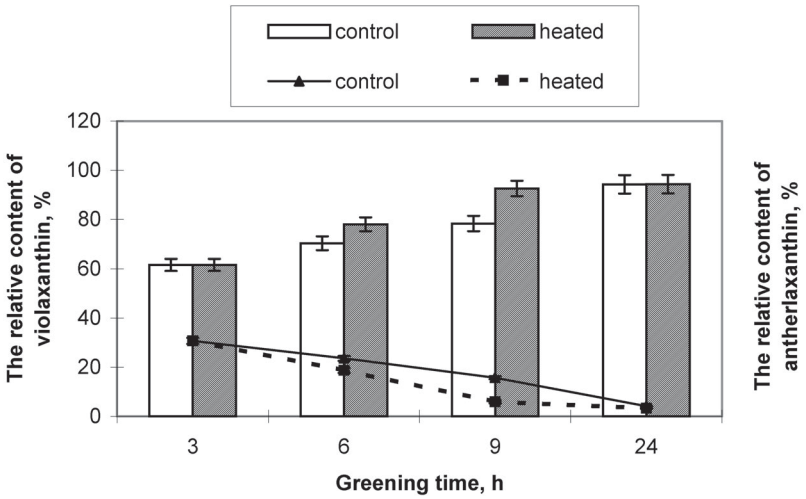

Fig.3: A: Changes in the relative content of violaxanthin (columns) and antheraxanthin (lines) under 40^0C heating (3 hours) and consequent acclimation at 22^0C

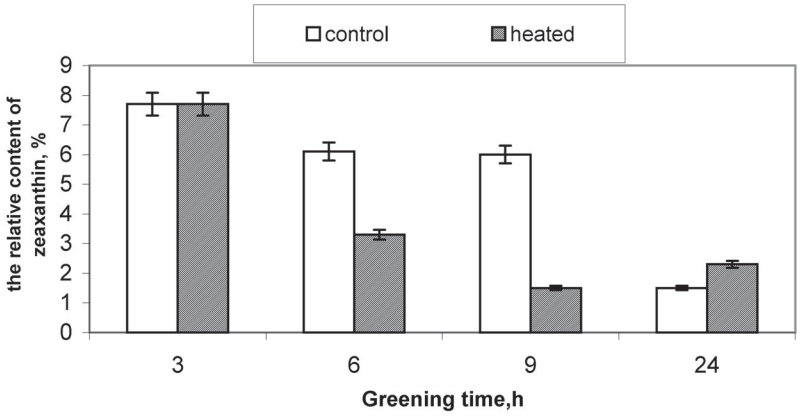

Fig.3: B: Changes in the relative content of zeaxanthin under 40^0C heating (3 hours) and consequent acclimation at 22^0C

after 24 h greening Zx represented about 2.3% of the total xanthophyll pool, which was 1.5 times higher than in the control leaves.

Functional characteristics of the PSII were investigated using the method of kinetics curves of the variable fluorescence of the chlorophyll *a.* The potential (Fv/Fm) and the effective (Yield) quantum yield of PSII photochemical reactions increased during the greening (Fig.4 A). Both Fv/Fm and Yield decreased immediately after heating and were restored in the 24 h acclimation. The ratio between non-active β-centers of PS II and active α-centers was estimated by means of $(F_i-F_o)/F_v$ parameter. As Fig. 4 B indicates, it grew twice after heating, but during the subsequent acclimation this parameter went down, which reflects the transformation of в- into б- center.

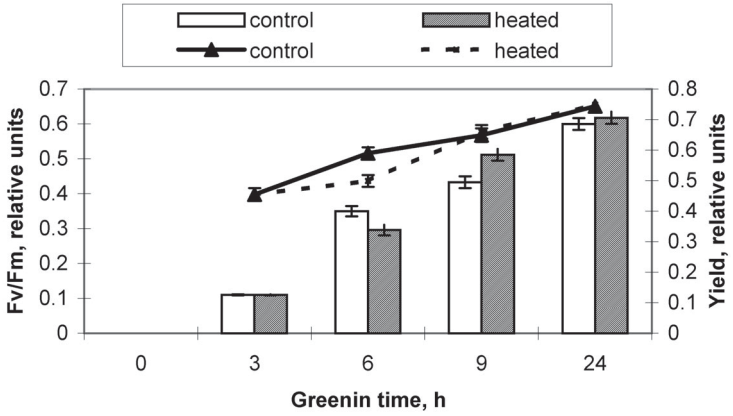

Fig.4 A: Changes in the potential (Fv/Fm) (columns) and effective (lines) (Yield) quantum yield of PSII photochemical reactions after heating (40^0C) and during subsequent acclimation at 22^0C

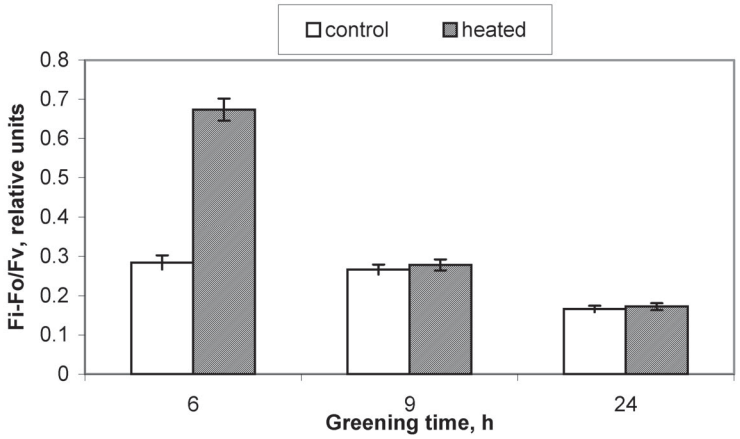

Fig. 4 B: Changes in the $(F_i-F_o)/F_v$ parameter after heating (40^0C) and during subsequent acclimation at 22^0C

DISCUSSION

Results obtained demonstrated different schemes of stress reactions proceeding in greening plants under heating at 40, 45, 50^0C. Data obtained correspondent to the general stress concept, according to which one has to differentiate among the plant's stress responses in four phases: response, restitution, end and regeneration phases (Fig.5) (Lichtenthaler, 1998). At the beginning of a stress, plants respond with a

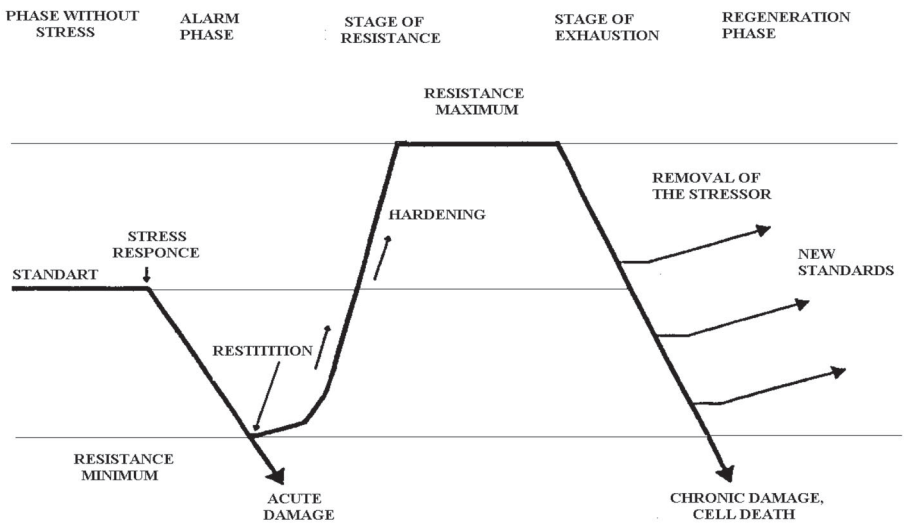

Fig.5 General concept of the phase sequences and responses induced in plants by stress exposure (Lichtenthaler, 1998)

decline of physiological functions and, as consequence, their vitality. At the restitution phase the adaptation and repair processes start and plant resistance increases. End phase or stage of exhaustion occurs when long-term stress takes place. Then the adaptation capacity of plant is overcharged. The last regeneration phase comes when stressor is removed and damages are not too severe. Partial or full regeneration of the physiological function occurs.

The resistance maximum was exceeded during the treatment at 50^0C. It led to a full plant exhaustion and death. 45^0C heating provoked an overcoming of the resistance stage. Therefore, plant stress took place. It induced a decline of the physiological functions and, probably, an activation of the protective mechanisms. At 40^0C the resistance maximum was not reached and regeneration phase occurred. After the removal of the stressor, plants regenerated and moved to new physiological standards.

40^0C is a temperature, which does not provoke any dramatic damage effects or plant death, but activates some physiological processes in plants. Therefore, this treatment was selected for the investigation of stress response and protective mechanisms.

The fluidity of the thylakoid membrane regulates the photosynthetic processes, assuring the essential mobility for membrane molecules (Quartacci, Pinzino, 2000). According to results obtained, membrane fluidity value was lowest at the phase of the prolamellar bodies (etiolated leaves). After 3 h greening, at the stage of planar thylakoid membranes formation, membrane fluidity value rose. The increase lasted until mature thylakoids appeared (24 h greening). The increase in membrane fluidity, observed during the biogenesis process, could be associated to the change in grana/stroma regions ratio. Chloroplast, containing mainly grana thylakoid fraction, characterized by anisotropy polarization value twice as much as that in stroma thylakoids fraction (Ford, Barber, 1983). According to Murphy et al. (1986) during chloroplasts biogenesis, grana thylakoids quantity is extended. This could be the reason for the rise of the PSII photochemical activity, as grana thylakoids is known to be enriched by the active α-center of PSII (Melis, 1991). In fact, the relative content of active α-type PS II was increased during greening process, as Fig. 4 B indicates.

Literature data confirm that in green leaves high temperature induces rise in the membrane fluidity and a lateral diffusion of the membrane lipids (Havaux, 1998). It is suggested that the membrane permability increases during heat treatment, which results in a decrease in the proton gradient formation across the thylakoid membrane and a suppression of the linear electron flow (Carpentier, 1999). The results of our investigations were unexpected. The membrane fluidity in heated leaves decreased as compared to the control. Such change in the membrane viscosity could be due to the variation of the grana and stroma thylakoids ratio. It was observed that the quantity of grana thylakoids in green leaves is enhanced when exposed to damaging temperature (Gounaris, Brain, 1984). Therefore, the increase in thylakoid membrane microviscosity under heating could be provoked by the increased ratio of grana and

stroma thylakoids. Photochemical activity of PS II was suppressed under heat treatment and then restored fast, exceeding the control level. It was supposed that the changes in the photochemical activity reflect the structure modification of PS II, namely the transformation of α- to β-type center under heating and return the process during the subsequent acclimation.

Current evidence suggest that when plants are exposed to potentially harmful environmental conditions, such as strong light and/or elevated temperatures, the violaxanthin and the products of its enzyme de-epoxidation, antheraxanthin and zeaxanthin, partition between the light-harvesting complexes and the lipid phase of the thylakoid membranes. The resulting interaction of the xanthophyll molecules and the membrane lipids brings about a decrease in membrane fluidity, an increase in membrane thermostability and a lowered susceptibility to lipid peroxidation (Havaux, 1998).

Our results suppose that heat treatment stabilizes membrane by means of xanthophyll cycle activity. Data obtained indicate that high temperature causes decreased membrane fluidity correlated with reduction of the zeaxanthin relative content. The last contradicts some literature data according to which the accumulation of zeaxanthin reduces the membrane fluidity and is a protective reaction to the stress effect (Montane, Tardy, 1998; Havaux, 1998). The reduction of the zeaxanthin relative content is assumed to be provoked by the inhibition of violaxanthindeepoxidase.

Data indicate that the interface between the major protein and the lipid components plays a key role in the process of greening. It improves the protection against elevated temperature and provides a suitable lipid environment that serves both the structural flexibility and the stability for lateral organization and the rearrangement of thylakoid membrane.

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