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Temperature sensitivity of photosystem II in isolated thylakoid membranes from fluridone-treated pea leaves

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Abstract: High temperature-induced changes in the photosystem II (PSII) activity of thylakoid membranes isolated from pea (*Pisum sativum* L. 'Ran') plants treated with low (10^{-8} M) and high (10^{-7} M) concentrations of fluridone were investigated. Pulse amplitude modulated (PAM) chlorophyll fluorescence and photosynthetic oxygen evolution (measured by polarographic oxygen rate electrode) were used to estimate the effect of high temperature on the functional activity of PSII. Higher temperature treatments lead to stronger inhibition of the flash oxygen evolution than the primary photochemistry of PSII (F_v/F_m). The inhibitory effect of higher temperature on the PSIIα centers is stronger than on the PSIIβ centers. The heat-induced damage in the PSIIα centers as well as in the donor side of all PSII centers (PSIIα and PSIIβ) is accentuated by fluridone.

Key words: Fluridone, PAM chlorophyll fluorescence, photosynthetic oxygen evolution, PSIIα centers, PSIIβ centers, carotenoids

1. Introduction

The photosynthetic apparatus in the thylakoid membranes of green plants is one of the main targets of heat stress in plants (Misra et al., 2001a, 2001b; Ducruet et al., 2007). The extent of damage to the photosynthetic apparatus caused by high temperature varies depending on plant species and ecotypes, growth and development of the plant, the growth environment, and dose and duration of temperature treatment (Misra and Misra, 1987; Havaux and Tardy, 1994; Misra et al., 1997, 2001a, 2001b; Sung et al., 2003). High temperature, otherwise denoted as heat stress, induces thylakoid membrane disorganization, resulting in the loss of photochemical functions (Pastenes and Horton, 1996; Misra et al., 1997, 2001a, 2001b). Photosystem II (PSII) is more susceptible to heat stress than photosystem I (PSI) (Berry and Björkman, 1980), altering both the donor (Enami et al., 1994) and the acceptor side of PSII (Cao and Govindjee, 1990). Elevated temperature leads to monomerization of the light-harvesting complex of the PSII (LHCII) (Mohanty et al., 2002). This process starts at 55 °C for isolated LHCII trimers (Garab et al., 2002), while in leaves (in vivo) it starts at lower temperatures between 45 and 47 °C (Takeuchi and Thornber, 1994).

Temperature is one of the most important environmental factors for photosynthetic organisms, having a significant impact on their temperature sensitivity (Haldimann and Feller, 2005). It also influences the lipid and carotenoid composition of the thylakoid membranes (Haldimann, 1996; Kłodawska et al., 2012). Carotenoids are integral components of the photosynthetic apparatus and play a crucial role in the photoprotection function (Havaux, 1998). These pigments are involved in the stabilization of the LHCII trimers and in the assembly of LHCII monomers (Formaggio et al., 2001; Standfuss et al., 2005). β-Carotene is a component of the reaction center of PSII, which stabilizes the structure and triggers the degradation of the D1 protein (Trebst and Depka, 1997). Violaxanthin also plays an important structural role in LHCII macroorganization (Horton et al., 1996) and the stabilization of the lipid phase of the thylakoid membranes (Havaux, 1998; Gruszecki and Strzałka, 2005). One approach to influencing the in vivo synthesis of the carotenoids is the use of bleaching herbicides (Hirschberg and Chamovitz, 1994). The target site for these herbicides is the enzyme phytoene desaturase, which catalyzes the desaturation of phytoene into phytofluene in the carotenoid biosynthesis pathway (Bartels and Watson, 1978). One of the representatives of this group of herbicides is fluridone. The treatment of etioplasts with bleaching herbicide (amitrole and norflurazon) leads to changes in lipid composition, fatty acid unsaturation, and a decrease of the lipid to protein ratio (Di Baccio et al., 2002). The

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essential role of β-carotene for the assembly of D1 protein into functional PSII was shown using norflurazon and fluridone (Trebst and Depka, 1997). The authors proposed that the bleaching of β-carotene in the reaction center of PSII destabilizes the structure and triggers the degradation of the D1 protein.

Our previous study revealed that fluridone treatment of pea plants causes a decrease in both carotenoid (Car) and total chlorophyll (Chl) content in a concentration-dependent manner (Dankov et al., 2009). It has been shown that changes in the pigment composition alter the functions of both photosystems, especially PSII. It is known that PSII in higher plants is not homogeneous. One widely studied aspect of PSII heterogeneity is antenna heterogeneity. Based on differences in antenna size, 2 types of PSII centers (PSIIα and PSIIβ) are distinguished (Apostolova and Misra, 2014). PSIIα centers display a lower chlorophyll a:b ratio and larger antenna size, and they are located in the appressed region of grana stacks forming a cluster of 3–4 PSIIα centers. However, PSIIβ centers have a smaller chlorophyll antenna size and form isolated units in the unappressed stroma-exposed region of thylakoid membranes. The interconversion of these 2 types of PSII centers takes place under different environmental and developmental conditions (Apostolova and Misra, 2014). Our previous investigation showed that fluridone can also alter PSII antenna complex organization (Dankov et al., 2009). The fluridone-induced changes in the photosynthetic apparatus influence plant development, leaf anatomy, and plastid ultrastructure (Popova and Riddle, 1996).

Taking into consideration the role of the composition and structural organization of the photosynthetic apparatus in its sensitivity to abiotic stress (Apostolova and Misra, 2014), in this study we assess the temperature sensitivity of PSII as a result of changes in the structural organization of the photosynthetic apparatus (in particular, the decrease in carotenoid content after fluridone treatment; Dankov et al., 2009). Isolated thylakoid membranes from fluridone-treated plants are used as model carotenoid-depleted thylakoid membranes for temperature sensitivity studies. Flash oxygen evolution, oxygen evolution under continuous illumination, and photochemistry of PSII measured by pulse amplitude modulated (PAM) fluorimetry were studied to estimate the inhibitory effect of higher temperature on the donor and acceptor side of PSII, as well as the effect on the PSIIα and PSIIβ centers in the photosynthetic apparatus of the plants treated with fluridone.

2. Materials and methods

2.1. Plant materials and preparation of thylakoid membranes

Plants from *Pisum sativum* L. 'Ran' were grown hydroponically in Hoagland solution under controlled conditions with a 16/8-h light/dark photoperiod in the presence of 10^{-8} M (LF) and 10^{-7} M (HF) fluridone (1-methyl-3-phenyl-5-(3-trifluoromethylphenyl)-4-(1H)-pyridone) (Sigma-Aldrich, USA). Fluridone was prepared as a 3 mM stock solution in 95% (v/v) ethanol and then diluted to the respective concentration in the growth medium, such that the ethanol concentration in the medium was less than 1% (v/v). The growth medium was replaced every 7 days.

Thylakoid membranes were isolated from 14-day-old pea plants as described by Harrison and Melis (1992). The leaves were ground in 50 mM tricine (pH 7.8), 10 mM NaCl, 5 mM $MgCl_2$, and 400 mM sucrose. The homogenate was passed through a filter and the filtrate was centrifuged at 5000 × *g* for 5 min. Thylakoid membranes were prepared by lysis of the chloroplasts in hypotonic buffer containing 50 mM tricine (pH 7.8), 10 mM NaCl, and 5 mM $MgCl_2$. These were harvested by centrifugation at 7000 × *g* for 5 min. The thylakoid membranes were suspended in 40 mM HEPES (pH 7.6), 10 mM NaCl, 5 mM $MgCl_2$, and 400 mM sucrose.

Chlorophyll and carotenoid content was estimated by the method of Lichtenthaler (1987). The pigments were extracted with 80% acetone. The absorption of the centrifuged extracts was measured on a spectrophotometer (Specord 210 Plus, Analytik-Jena AG, Germany). The following equations were used to determine the pigment concentrations:

chlorophyll a (μg/mL) = $12.25 \times A_{663.2} - 2.79 \times A_{646.8}$,

chlorophyll b (μg/mL) = $21.50 \times A_{646.8} - 5.10 \times A_{663.2}$,

total chlorophyll = chlorophyll a + chlorophyll b,

total carotenoids (μg/mL) = $(1000 \times A_{470} - 1.63 \times$ chl a $- 104.96 \times$ chl b$)/198$,

where A is absorbance.

2.2. Temperature treatment

The thylakoid membranes were suspended in a medium containing 40 mM HEPES (pH 7.6), 10 mM NaCl, 5 mM $MgCl_2$, and 400 mM sucrose. The chlorophyll concentration of the suspension taken for photosynthetic measurements was 150 μg/mL. The thylakoid membrane suspension was incubated in a water bath for 5 min at different temperatures (from 20 °C to 50 °C) and subsequently cooled to 20 °C in the dark for photosynthetic measurements.

2.3. Pulse amplitude modulated chlorophyll fluorescence

The above samples (thylakoid membrane suspension and 150 μg Chl mL^{-1}) were dark-adapted for 15 min with Chl fluorescence measurement with a PAM fluorometer (H. Walz, Germany). The F_0 level was measured with an

instrument frequency of 1.6 kHz and a measuring beam set at 0.12 μmol m^{-2} s^{-1} PFD. For the evaluation of maximal fluorescence level (F_m) in dark-adapted state (DAS) and F_m' in light-adapted state (LAS), saturating flashes of 3000 μmol m^{-2} s^{-1} PFD with a duration of 0.8 s were provided by a KL 1500 Schott lamp (Schott Glaswerke, Germany). The time interval between 2 consecutive flashes was 20 s. Actinic light illumination (250 μmol m^{-2} s^{-1} PFD) was provided by a second KL 1500 Schott lamp for the induction of photosynthesis. The following parameters were calculated: maximum quantum yield of primary photochemistry of PSII, $F_v/F_m = (F_m - F_0)/F_m$ (Kitajima and Butler, 1975); maximum ratio of quantum yields of photochemical and concurrent nonphotochemical processes in PSII, F_v/F_0; and effective quantum yield of PSII photochemistry, F_v'/F_m' (Roháček, 2002).

2.4. Oxygen evolution measurements

Oxygen flash yields and oxygen evolution under continuous illumination of thylakoid membrane suspension (150 μg Chl mL^{-1}) were measured using a home-built (Zeinalov, 2002) polarographic oxygen rate electrode as described by Apostolova et al. (2006). Thylakoid membranes (150 μg Chl mL^{-1}), which were in the suspension medium without any artificial electron acceptor and containing 100 μL of sample volume in a 2-mm suspension layer, were preilluminated with 25 flashes and then dark-adapted for 5 min before oxygen measurements. Oxygen flash yields were induced by saturating (4 J) and short ($t_{1/2}$ = 10 μs) periodic flash sequences with 650 ms of dark spacing between the flashes. Initial S_0 and S_1 state distribution, misses (α), and double hits (β) were determined by the fitting of the theoretically calculated yields according to the model of Kok et al. (1970), with the experimentally obtained oxygen flash yields using the least square deviation procedure. The initial oxygen burst was recorded after irradiation with continuous white light (450 μmol photons m^{-2} s^{-1}). The curves of the oxygen evolution under continuous illumination after oxygen burst exhibited biphasic exponential decay. The deconvolution of the oxygen burst decay was performed by fitting the function with 2 exponential components: $A_1e^{-(tk1)} = A_2e^{-(tk2)}$ where A_1, A_2 are amplitudes and k_1, k_2 are rate constants of the fast and slow components, respectively. In other words, the A_1/A_2 ratio corresponds to the proportion of the functionally active PSIIα centers in grana to PSIIβ centers in stroma lamellae (Ivanova et al., 2008; Zeinalov, 2010).

2.5. Statistical analysis

The results are mean values of from 3 to 5 independent experiments. The statistical differences between the means were determined using a 2-tailed paired Student's t-test. Values of $P < 0.05$ were considered as significantly different between the temperature-treated samples and the control sample (at 20 °C).

3. Results

In our previous study, we reported that the treatment of pea plants with 10^{-8} M fluridone (LF) and 10^{-7} M fluridone (HF) leads to a decrease in carotenoid content by 25% and 40% of the control values, respectively. The decrease in Chl content was 10% and 35%, respectively (Dankov et al., 2009). The effect of temperature on the thylakoids isolated from LF, HF, and untreated (control) leaves was studied.

3.1. Effect of temperature treatment on the photochemistry of PSII

Figures 1A–1C show the temperature-induced changes in the parameters of the PAM chlorophyll fluorescence (F_v/F_m, F_v/F_0, and F_v'/F_m'). These ratios decrease with an increase in incubation temperature. Changes in the

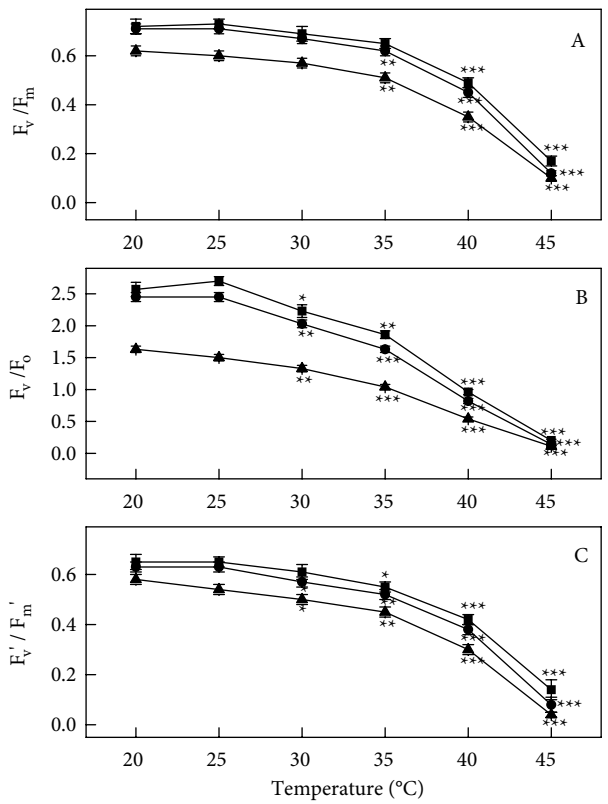

Figure 1. A- Changes in the maximum quantum yield of primary photochemistry, F_v/F_m, **B**- maximum ratio of quantum yields of photochemical and concurrent nonphotochemical processes in PSII, F_v/F_0; **C**- effective quantum yield of photosystem II photochemistry, F_v'/F_m'. The thylakoid membranes were subjected to temperature treatment for 5 min at different temperatures. Control thylakoid membranes (■), treatment with 10^{-8} M fluridone (LF concentration) (●), and treatment with 10^{-7} M fluridone (HF concentration) (▲). Mean values ± SE were calculated from 5 independent experiments. Asterisks indicate statistically significant differences of the corresponding type of thylakoid membranes at 20 °C and treated with different temperatures (*, **, ***: $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively).

F_v/F_m and F_v'/F_m' ratios between 20 and 30 °C were not significant, neither in the control (untreated) nor in the fluridone-treated (LF or HF) thylakoid membranes. A strong decrease in these parameters was recorded in the temperature range of 35–45 °C. In this range, the inhibition of the primary photochemistry in DAS and LAS is strongest in thylakoid membranes treated with the HF concentration (Figure 1). Moreover, the changes in the parameter F_v'/F_m' in fluridone-treated thylakoid membranes were still observed at 30 °C. The F_v/F_0 ratio also decreased at 30 °C (Figure 1). Temperature above 45 °C leads to almost complete inactivation of the PSII photochemistry in all the thylakoid membranes.

3.2. Effect of temperature on photosynthetic oxygen evolution

In order to assess the effect of high temperature on the oxygen-evolving complex, we measured the oxygen evolution with a Joliot-type polarographic oxygen rate electrode. The changes in oxygen evolution under continuous illumination at different temperatures for untreated and fluridone (LF)-treated membranes are shown in Figure 2. The amplitude (A) of the initial oxygen burst under continuous illumination gives information about the amount of functionally active PSII centers (PSIIα and PSIIβ) (Zeinalov, 2010). Parameter A decreased with an increase in temperature for both untreated and fluridone-treated (LF and HF) thylakoid membranes. The changes were significant at 40–45 °C for the control and 30–45 °C for the membranes after fluridone treatment (Figure 3). This decrease in oxygen evolution parameter A was accentuated in fluridone-treated (LF and HF) thylakoid membranes compared to the untreated ones (Figure 3). These observations suggest that a higher temperature induces faster inactivation of the functionally active PSII centers in the thylakoid membranes after fluridone treatment in comparison to the untreated membranes. The oxygen-evolving activity was completely blocked at 50 °C.

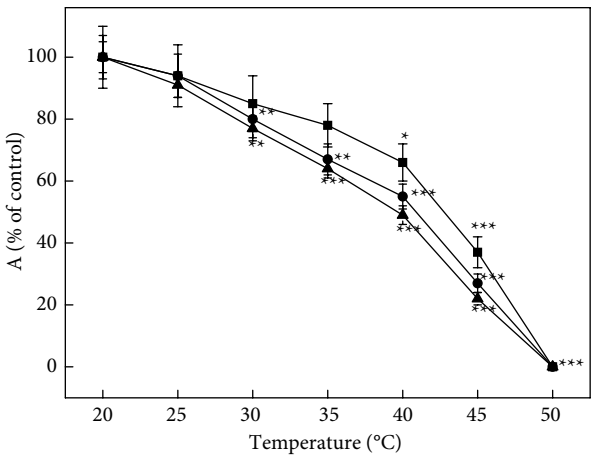

Figure 3. Amplitudes of the oxygen burst (A: % of the respective thylakoid membranes treated or untreated) at 20 °C at continuous illumination (450 μmol photon m^{-2} s^{-1}) of thylakoid membranes subjected to temperature treatment for 5 min at different temperatures. Untreated thylakoid membranes (■), thylakoid membranes from plants treated with 10^{-8} M fluridone (●), and thylakoid membranes from plants treated with 10^{-7} M fluridone (▲). Mean values ± SE were calculated from 5 independent experiments. The chlorophyll concentration was 150 μg Chl mL^{-1}. Polarographic sensitivity is 1.5 V/μA. Asterisks indicate statistically significant differences of the corresponding type of thylakoid membranes at 20 °C and treated with different temperatures (*, **, ***: $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively).

The decrease in the amplitude of the initial oxygen burst (A) is accompanied by changes in the kinetics of the oxygen burst decay (Table). The oxygen burst decay of the

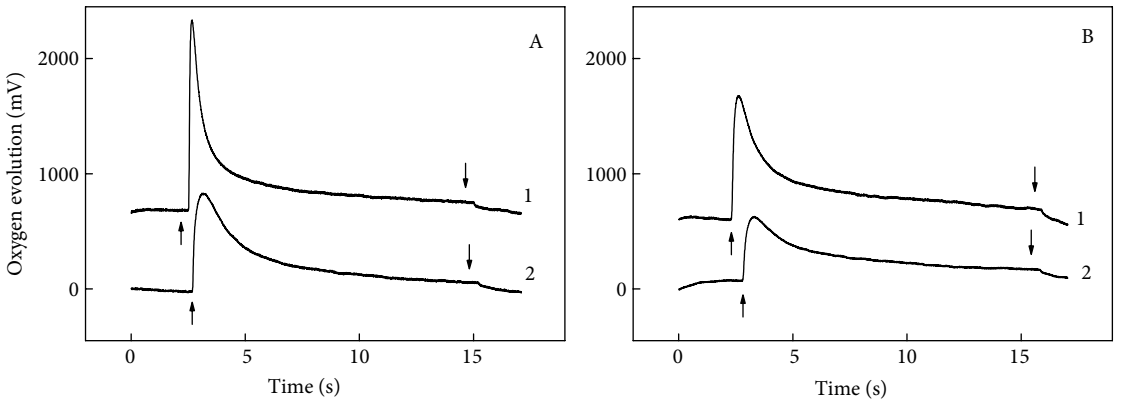

Figure 2. Time course of the induction curve of the oxygen evolution under continuous illumination of 450 μmol photon m^{-2} s^{-1} (**A**- control, **B**- thylakoid membranes isolated from plants treated with 10^{-8} M fluridone) of dark-adapted thylakoid membranes subjected to temperature treatment for 5 min: (1) 20 °C, (2) 35 °C. Time constant of the electrode is less than 2 ms. Polarographic sensitivity is 1.5 V/μA for oxygen evolution under continuous illumination. Arrows indicate switching the light on and off.

Table. Kinetic parameters of the initial oxygen burst under continuous illumination and oxygen flash yields of thylakoid membranes from plants treated with fluridone. (10^{-8} M, LF and 10^{-7} M, HF). The thylakoid membranes were incubated for 5 min at different temperatures (20–50 °C). A_1 and A_2 are the amplitudes and k_1 and k_2 are the rate constants of the fast and slow components of the oxygen burst decay, respectively. Initial dark S_0 state of PSII in percentage of the total S states ($S_1 = 100 - S_0$), values of misses (α), and double hits (β) according to Kok's model. x: The curve of the oxygen evolution under continuous illumination has only slow component (A_2), --: there are no oxygen flash yields. Asterisks indicate statistically significant differences of the corresponding type of thylakoid membranes at 20 °C and treated with different temperatures (*, **, ***: $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively).

Concentration (M)	Temperature (°C)	A_1/A_2	k_1 (s^{-1})	k_2 (s^{-1})	S_0 (%)	α (%)	β (%)
Untreated	20 °C	2.45 ± 0.10	4.55 ± 0.22	0.61 ± 0.04	24 ± 1	25 ± 1	3.70 ± 0.2
10^{-8} [LF]		1.97 ± 0.09	4.12 ± 0.20	0.58 ± 0.03	25 ± 1	27 ± 1	4.20 ± 0.2
10^{-7} [HF]		1.54 ± 0.06	3.87 ± 0.19	0.54 ± 0.03	27 ± 2	29 ± 2	4.50 ± 0.2
Untreated	25 °C	2.33 ± 0.07	4.14 ± 0.22	0.58 ± 0.04	25 ± 2	25 ± 1	3.80 ± 0.2
10^{-8} [LF]		1.65 ± 0.07*	3.65 ± 0.17	0.55 ± 0.04	26 ± 1	28 ± 1	4.30 ± 0.2
10^{-7} [HF]		1.42 ± 0.06	3.43 ± 0.18	0.50 ± 0.03	28 ± 2	30 ± 2	4.80 ± 0.3
Untreated	30 °C	2.17 ± 0.09	4.03 ± 0.21	0.55 ± 0.03	26 ± 2	25 ± 1	3.80 ± 0.2
10^{-8} [LF]		1.45 ± 0.07**	3.17 ± 0.16**	0.53 ± 0.04	27 ± 1	28 ± 2	4.40 ± 0.3
10^{-7} [HF]		1.28 ± 0.05**	2.89 ± 0.14**	0.49 ± 0.03	29 ± 2	32 ± 2	4.90 ± 0.3
Untreated	35 °C	2.05 ± 0.07**	3.98 ± 0.20	0.53 ± 0.03	26 ± 2	26 ± 2	4.00 ± 0.2
10^{-8} [LF]		1.34 ± 0.05***	3.01 ± 0.15**	0.52 ± 0.02	28 ± 2	29 ± 2	4.40 ± 0.2
10^{-7} [HF]		1.16 ± 0.07**	2.55 ± 0.13***	0.49 ± 0.03	--	--	--
Untreated	40 °C	1.34 ± 0.07***	3.81 ± 0.20*	0.55 ± 0.03	28 ± 2	29 ± 2	4.20 ± 0.3
10^{-8} [LF]		0.87 ± 0.05***	2.56 ± 0.13***	0.54 ± 0.04	32 ± 3*	33 ± 3*	4.80 ± 0.3*
10^{-7} [HF]		x	x	x	--	--	--
Untreated	45 °C	0.85 ± 0.06***	3.54 ± 0.24*	0.57 ± 0.04	31 ± 3*	33 ± 3*	4.50 ± 0.3*
10^{-8} [LF]		0.53 ± 0.05***	2.11 ± 0.16***	0.56 ± 0.05	--	--	--
10^{-7} [HF]		x	x	x	--	--	--

untreated and the fluridone-treated (20 °C) membranes shows biphasic kinetics (Figure 2). The ratio of the fast (A_1) and slow (A_2) components (with rate constants k_1 and k_2) corresponds to the proportion of the oxygen-evolving centers, evolving oxygen by the noncooperative (A_1) and cooperative (A_2) mechanisms, respectively, i.e. the ratio of the functionally active PSIIα centers in grana to PSIIβ centers in stroma lamellae (Zeinalov, 2010). The A_1/A_2 ratio decreases with the rise in temperature, as the changes are more pronounced for the fluridone-treated thylakoid membranes, i.e. membranes with decreased Car content (Table). The changes in the A_1/A_2 ratio are mostly a result of decrease in the fast component A_1 (Table), which suggests a decrease in the functionally active grana domain centers (i.e. PSIIα) with higher temperature treatments. A monoexponential decay (lacking a fast A_1 component) of the curve of the oxygen evolution is observed at 40 °C and above for the thylakoid membranes from plants treated with HF, indicating absence of the functionally active PSIIα centers in HF-treated plants at these temperatures. The rate constant of the fast component of the oxygen evolution (k_1) decreases after temperature treatment, and the decrease in this constant is further observed in the fluridone-treated thylakoid membranes (i.e. Car-depleted membranes) than in the control membranes (Table). In contrast to k_1, the rate constant of the slow component of oxygen evolution (k_2) is almost unaffected by temperature treatment (Table).

The influences of temperature stress on the oxygen flash yields of the untreated membranes and those treated with the LF concentration are presented in Figure 4. Characteristic oscillations with a period of 4 are observed for both the control and thylakoid membranes from fluridone-treated plants. The Y_3 value (oxygen yield after the third flash), which is used to assess the influence of temperature on the flash oxygen evolution, decreased with an increase in temperature (Figure 5). The oxygen flash yields, which are produced by the PSIIα centers (Zeinalov, 2010), are strongly inhibited in comparison to the oxygen evolution under continuous illumination for both control and fluridone-treated samples (Figures 3 and 5). The flash oxygen yield was recorded at up to 45 °C, 40

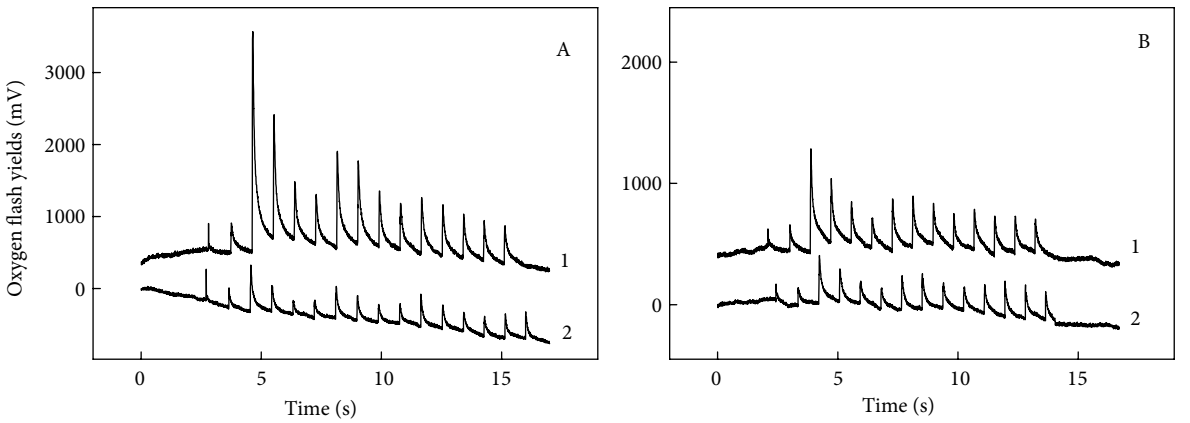

Figure 4. Time course of the oxygen flash yields of dark-adapted thylakoid membranes (**A**- control, **B**- 10^{-8} M fluridone-treated plants) subjected to temperature treatment for 5 min at 20 °C (1) and 35 °C (2). Time constant of the electrode is less than 2 ms. Polarographic sensitivity is 3 V/μA for oxygen flash yield determination.

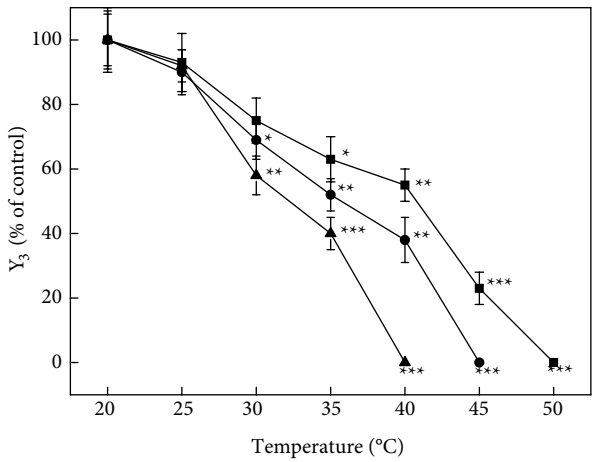

Figure 5. Oxygen flash yields after third flash of thylakoid membranes (Y_3, % of the respective control; thylakoid membranes at 20 °C) subjected to temperature treatment for 5 min at different temperatures. Control thylakoid membranes (■), thylakoid membranes from plants treated with 10^{-8} M fluridone (25 CarD) (●), and thylakoid membranes from plants treated with 10^{-7} M fluridone (40 CarD) (▲). Mean values ± SE were calculated from 5 independent experiments. The chlorophyll concentration was 150 μg Chl mL^{-1}. Polarographic sensitivity is 3 V/μA. Asterisks indicate statistically significant differences of the corresponding type thylakoid membranes at 20 °C and treated with different temperatures (*, **, ***: $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively).

°C, and 35 °C for untreated, LF-treated, and HF-treated plants, respectively (Figure 5). Higher temperatures were inhibitory for the samples, as the fluridone treatment makes the thylakoid membrane more susceptible to temperature treatments.

The values of the initial population of the redox states S_0 in percentages ($S_1 = 100 - S_0$) in the oxygen-evolving complex of the experimental samples were calculated as described by Kok's model (Kok et al., 1970) and are given in the Table. The temperature-induced inhibition of the flash oxygen evolution (Table) is related to the influence of the S_0–S_1 dark distribution, as well as to an increase in the misses (α) and double hits (β). The temperature stress leads to an increase in the most reduced S_0 state population of PSII in the DAS, as the increase is greater in fluridone-treated membranes (i.e. carotenoid-depleted thylakoid membranes) in comparison to the control membranes (Table).

4. Discussion

In this study, we present evidence showing the role of carotenoid depletion after fluridone treatment in high-temperature inactivation of the PSII functions. The data reveal an increased inhibition of oxygen evolution and primary photochemistry of PSII after fluridone treatment. This effect could be due to a decrease in the amount of carotenoids in the thylakoid membranes from fluridone-treated plants (Dankov et al., 2009). In addition, fluridone treatment also leads to a decrease in chlorophyll content (Dankov et al., 2009). A simultaneous decrease in chlorophyll and carotenoid could be attributed to photooxidation as a consequence of the decrease in carotenoids, which can, in turn, alter the PSII stability of fluridone-treated thylakoid membranes and their susceptibility to temperature stress.

PAM chlorophyll fluorescence measurements revealed a high temperature-induced inhibition of the primary photochemistry of PSII in DAS and LAS (ratios F_v/F_m and F_v'/F_m', Figure 1) in thylakoid membranes from untreated or fluridone-treated leaves, which is a result of changes in the F_v/F_0 ratio of the photochemical and nonphotochemical processes (Figure 1B). Taking into account the strong influence of the redox state of cyt. b559 on the F_m fluorescence level (Lazar et al., 2005), as

well as the conversion of its high-potential form into its low-potential form during high temperature (Tiwari et al., 2008), it could be suggested that this is a reason for the decrease of F_m under heat stress. The inhibition of Q_A–Q_B electron transfer as a result of structural modification in D1 and D2 proteins under high temperature (Misra et al., 1998, 2001a, 2001b) could be another reason for the decrease in the F_v/F_m ratio. In addition, our data revealed that the high temperature inhibition of the maximal quantum yield of primary photochemistry (F_v/F_m) is more pronounced for the fluridone-treated membranes and is concentration-dependent (Figure 1). These results suggest an influence of the amount of carotenoids on the temperature stability of the photosynthetic apparatus under high-temperature treatment (Figure 1). Bearing in mind that carotenoid depletion corresponds to an increased fraction of reduced Q_A, an alteration in the PSII antenna complex organization and an influence on the assembly and maintenance of the PSII reaction center (Trebst, 2003; Dankov et al., 2009; Misra and Apostolova, 2014) could suggest that all these alterations lead to a stronger influence of high temperature on the primary photochemistry in thylakoid membranes with decreased Car content in the fluridone-treated membranes compared to that of the control membranes.

Earlier observations showed that heat stress leads to damage in the PSII reaction center (Misra et al., 1998; Pospíšil and Tyystjärvi, 1999) and to disorganization of the Mn clusters (Enami et al., 1994) as a result of the detachment of the Mn-stabilizing 33-kDa protein and Mn atoms from the PSII core complex (Havaux and Tardy, 1997). All these structural changes in temperature treatment could lead to a decrease of the functionally active PSII centers (parameter A, Figure 3), resulting in an inhibition of oxygen evolution. On the other hand, the analysis of the oxygen evolution under continuous illumination shows a decrease in the A_1/A_2 ratio after high temperature treatment and depends on the concentration of fluridone or the amount of Car in the membranes (Table). The decrease in the A_1/A_2 ratio is a result of the decrease in the fast component A_1, which is associated with functionally active PSII centers evolving oxygen by the noncooperative Kok mechanism (Zeinalov, 2010). This mechanism of oxygen evolution (or PSIIα) is strongly influenced in Car-depleted membranes in comparison to the control membranes and was completely inhibited in the thylakoid membranes of plants treated with the HF concentration at 40 °C (Table). High temperature damage of these centers is associated with alteration in their rate constant (k_1). To the contrary, the constant k_2, which characterized PSIIβ centers, is least affected by temperature treatment in thylakoid membranes from plants with or without fluridone treatment (Table). The change in the rate constant k_1 could be a consequence of the high temperature-induced damage of the oxygen-evolving complex and/or changes in the acceptor side of PSII, which modify the interaction between Q_B and plastoquinone. Our previous investigation showed that the structural organization of the PSII supercomplex (in particular, the degree of LHCII oligomerization) strongly influences oxygen evolution (Apostolova et al., 2006). In conformity with these observations and with the decreased antenna size of PSII in Car-depleted membranes reported previously by Dankov et al. (2009), it is proposed that alteration of the PSII supercomplex organization could be one of the reasons for increased sensitivity of the thylakoid membranes with decreased Car content after fluridone treatment. A cyclic electron flow around PSII prevents photochemical activity of PSII at high temperature and plays a protective role in the substitution of the thermally damaged oxygen-evolving complex (Prasil et al., 1996). This process operates when the lifespan of $P680^+$ is increased and involves a carotenoid (Faller et al., 2001). The influence of the altered structural organization of thylakoid membranes on this process cannot be excluded, as it could lead to decreased thermal stability for the fluridone-treated thylakoid membranes (i.e. Car-depleted thylakoid membranes). The alteration of the physical properties of the lipid phase of the membranes as a result of changes in Car content (Gruszecki and Strzałka, 2005; Kłodawska et al., 2012), which influences the interaction between Q_B and plastoquinone, might be an additional reason for the thermolability of the fluridone-treated thylakoid membranes.

The statement that temperature stress strongly influenced the PSIIα centers is also confirmed by flash oxygen measurements (Figures 4 and 5). The oxygen flash yields, produced mainly by the PSIIα centers (Zeinalov, 2010), are strongly inhibited in comparison to the oxygen evolution under continuous illumination for all studied thylakoid membranes (Figures 3 and 5). The kinetic parameters of the flash oxygen evolution after heat stress revealed a stronger influence on the S_0–S_1 state dark distribution and an increase of misses in Car-depleted membranes in comparison to the control membranes (Table). An increase in the S_0 state population after heat treatment could be attributed to high temperature-induced structural changes in the oxygen-evolving complex. The alterations in the S_0–S_1 dark distribution also suggest modification of the function in the still-active PSII centers, as the effect is stronger in Car-depleted membranes.

In conclusion, fluridone treatment, which decreases the carotenoid content of thylakoid membranes, increases the temperature sensitivity of PSII by increasing the heat-induced damage of the PSIIα centers and the changes in the donor side of PSII.

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