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Gradual disassembly of photosystem 2 *in vivo* induced by excess irradiance. A hypothesis based on changes in 77 K fluorescence spectra of chlorophyll *a* in barley leaves

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Abstract

Effects of short-term exposure to different irradiances on the function of photosystem 2 (PS2) were studied for barley grown at low (LI; 50 μmol m^{-2} s^{-1}) and high (HI; 1100 μmol m^{-2} s^{-1}) irradiances. HI barley revealed higher ability to down-regulate the light-harvesting within PS2 after exposure to high irradiance as compared to LI plants. This ability was estimated from the light-induced decreases of F685/F742 and E476/E436 in emission and excitation spectra of 77 K chlorophyll (Chl) *a* fluorescence *in vivo* which was 65 and 10 % for HI plants as compared to 30 and 2 % for LI plants, respectively. For LI plants this protective down-regulation of the light-harvesting of PS2 was saturated at 430 μmol m^{-2} s^{-1}, and progressive PS2 photodamage was induced at higher irradiances. After exposure of LI segments to 2 200 μmol m^{-2} s^{-1} a pronounced maximum at 700 nm appeared in emission spectrum of 77 K Chl *a* fluorescence. Based on complementary analysis of 77 K excitation spectra measured at the emission wavelength 685 nm we suggest that this emission maximum may be attributed to the formation of aggregates of light-harvesting complexes of PS2 (LHC2) with part of PS2 core during progressive PS2

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Abbreviations: Car (x+c) – total carotenoids and xanthophylls; Chl – chlorophyll; E476/E436 – ratio of excitation maxima of Chl *b* and Chl *a* in the excitation spectrum of Chl *a* fluorescence at 77 K; F_0 and F_M – minimum and maximum levels of Chl *a* fluorescence of dark-adapted leaves; F_0' and F_M' – minimum and maximum levels of Chl *a* fluorescence of light-adapted leaves; F685, F700 – maxima in emission spectrum of Chl *a* fluorescence at 77 K specified according to the emission wavelength; LHC2 – light-harvesting Chl *a/b* complexes of photosystem 2; NPQ – nonphotochemical quenching of maximum Chl *a* fluorescence; NRD – nonradiative dissipation of absorbed excitation energy; PPFD – photosynthetic photon flux density; PS – photosystem; RC – reaction centre; SV_0 – nonphotochemical quenching of minimum Chl *a* fluorescence.

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photodamage. Our results can be explained assuming different contributions of LHC2 and PS2 core to the total nonradiative dissipation of absorbed excitation energy for the LI and HI barley.

Additional key words: carotenoids; *Hordeum vulgare*; photodamage.

Introduction

The photosynthetic apparatus of higher plants and algae has to cope with excess irradiance and prevent a massive photodamage of pigments, proteins, and lipids of the thylakoid membranes. Hence, a great attention is focussed on the mechanisms of the acclimation of the assimilatory apparatus to the different growth irradiances (Demmig-Adams *et al.* 1997, Huner *et al.* 1998, Špunda *et al.* 1998). Generally, the adaptation of the thylakoid membranes to high irradiances results in increased amount of reaction centres (RC's) and other components of electron transport chain of photosystem 2 (PS2) on the Chl basis and in a reduced content of light-harvesting complexes of PS2 (LHC2) (Lichtenthaler *et al.* 1982a,b, Chow *et al.* 1988, Lindahl *et al.* 1997). This acclimative strategy of the plants grown at high irradiances usually provides more efficient protection against progressive photodamage to PS2 as compared with the plants grown at low irradiances (Demmig-Adams and Adams 1996, Gray *et al.* 1996, Horton *et al.* 1996, Demmig-Adams 1998).

In addition to acclimative responses, the protection of the photosynthetic apparatus against PS2 photodamage involves several prompt mechanisms. Among them the decisive importance is attributed to the down-regulation of utilization of absorbed excitation energy in PS2 photochemistry (Genty and Harbinson 1996, Horton *et al.* 1996, Gilmore 1997, Špunda *et al.* 1997b, 1998). It is generally accepted that high irradiance induces rapid structural and functional changes within both LHC2 and the PS2 core (Ruban and Horton 1994, 1995, Jahns and Miehe 1996, Färber *et al.* 1997, Depka *et al.* 1998). Particularly the ΔpH dependent and zeaxanthin mediated structural changes of LHC2 (formation of LHC2 aggregates, Ruban *et al.* 1993) result in a decrease of excitation energy transfer to the PS2 core and in a related increase of nonradiative dissipation (NRD) of excitation energy within LHC2 (Horton *et al.* 1996, Špunda *et al.* 1998).

The measurements of Chl *a* fluorescence parameters provide nondirect but invaluable information about light-induced NRD *in vivo*. Generally, the total NRD is monitored through the nonphotochemical quenching of maximum Chl *a* fluorescence yield (NPQ), whereas the light-induced quenching of minimum Chl *a* fluorescence (SV_0) corresponds to the relative contribution of the NRD localized within LHC2 (Demmig-Adams and Adams 1994, 1996, Härtel and Lokstein 1995, Pospíšil 1997, Špunda *et al.* 1997b, 1998). Recently, the low temperature spectra of Chl *a* fluorescence were used to characterize the light-induced functional changes of LHC2 in the intact leaves. We have shown that increased NRD localized within LHC2 is related not only to the pronounced SV_0 but also to the decrease of excitation energy transfer from LHC2 to the PS2 core as judged from the relative decrease of the excitation bands of the supplementary pigments in the Soret region of the 77 K

excitation spectra of Chl *a* fluorescence (Špunda *et al.* 1998). Ruban *et al.* (1993) observed with the irradiated leaves of *Guzmania* the changes in 77 K excitation spectra of Chl *a* fluorescence similar to those previously found for the chemically induced aggregation of isolated LHC2 (Horton *et al.* 1991). Also the light-induced appearance of minor shoulder at 700 nm in the Chl *a* fluorescence emission spectrum of the same plant material probably corresponds to the LHC2 aggregation *in vivo* (Ruban and Horton 1994). Šiffel and Vácha (1998) observed the pronounced Chl *a* emission maximum at 699 nm for the intact tobacco leaves grown under prolonged CO_2 deficit. They attributed this maximum in agreement with Ruban and Horton (1994) to the LHC2 aggregation, but mentioned that the leaves revealed clear signs of PS2 photodamage.

The 77 K spectra of Chl *a* fluorescence were applied mainly to the analysis of regulation of light-harvesting function within PS2. The studies on the spectral changes of 77 K Chl *a* fluorescence related to the progressive photoinactivation of PS2 RC's *in vivo* are still lacking. Here we have studied the effects of short-term exposure of barley leaves to different high irradiance stresses on the PS2 function for the plants grown at low, LI (50 μmol m^{-2} s^{-1}) and high, HI (1100 μmol m^{-2} s^{-1}) irradiances using 77 K emission and excitation spectra of Chl *a* fluorescence and analysis of fluorescence quenching at room temperature. The attention was focused on the attribution of spectral changes to the structural changes of the PS2 pigment-protein complexes reflecting either down-regulation of LHC2 function and/or progressive photodamage to the PS2 core.

Materials and methods

Plants: The spring barley (*Hordeum vulgare* L. cv. Akcent) was grown under controlled climate (growth chamber *HB 1014 Bioline-Heraeus*, Germany; temperature 20 °C, relative humidity 65 %, 16/8 h day/night regime) at irradiances (PPFD) of 50 (LI) and 1100 (HI) μmol m^{-2} s^{-1}. All measurements were made on the primary leaves of 8-d-old plants.

Room temperature Chl *a* fluorescence: Parameters of the room temperature modulated Chl *a* fluorescence were measured using a *PAM 101, 103* fluorometer (*Heinz Walz*, Effeltrich, Germany). The dark-adapted leaf segments were placed inside the leaf disc chamber (*LD2/2*; *Hansatech*, UK) with the adaxial leaf surface up. Afterwards, minimum (F_0 – all PS2 RC's in open state) and maximum (F_M - all PS2 RC's in close state) Chl *a* fluorescence levels were measured. To close all the PS2 RC's, a saturating pulse of "white light" (1 s duration, PPFD 4000 μmol m^{-2} s^{-1} at the leaf surface) was applied. Measurements of the maximum (F_M') and minimum (F_0') Chl *a* fluorescence in the light-adapted state were carried out at steady state conditions 10 min after the begin of irradiation at the given incident irradiances on the surface of leaf segments: 36, 51, 77, 115, 263, 557, 805, and 1560 μmol m^{-2} s^{-1}. The F_0' was estimated as the lowest fluorescence level during 10 s of darkness following the irradiation period at the given irradiance. The determination of F_0' was not influenced by additional far-red radiation (values not shown). The nonphotochemical

quenching of F_M (NPQ) and F_0 (SV_0) were calculated using Stern-Volmer formalism as follows: $NPQ = F_M/F_M' - 1$, $SV_0 = F_0/F_0' - 1$ (Härtel and Lokstein 1995, Špunda *et al.* 1998).

77 K Chl *a* fluorescence excitation and emission spectra: The dark-adapted leaf segments were exposed for 10 min to irradiances of 90, 200, 430, 660, 880, 1570, and 2 200 μmol m^{-2} s^{-1}. They were fixed on a sample holder and immediately frozen in liquid nitrogen. Chl *a* fluorescence excitation and emission spectra at 77 K were measured on the frozen leaf segments in the custom-made Dewar-type optical cryostat using a luminescence spectrophotometer *LS 50B* (*Perkin Elmer*, UK). Emission spectra were recorded at the excitation wavelength 436 nm (preferentially Chl *a* excited). The slit widths of the excitation and emission monochromators were 10 and 5 nm, respectively. Excitation spectra were detected at the emission wavelength 685 nm (preferentially PS2 emission). The relative efficiency of excitation energy transfer from LHC2 to the PS2 core was estimated as E476/E436 (Špunda *et al.* 1998). In order to obtain better legibility of spectral changes only the values of selected irradiances are presented.

Pigment analysis: Estimation of the pigment contents (Chl *a*, Chl *b*, and carotenoids) was performed spectrophotometrically (*Specord M400, Carl Zeiss,* Jena, Germany) from pigment extracts in 80 % acetone with a small amount of $MgCO_3$ according to Lichtenthaler (1987).

Results and discussion

Acclimation of pigment contents and composition to growth irradiance: The values of pigment content and composition of the LI and HI barley are shown both for the dark-adapted leaf segments and immediately after short exposure of the leaf segments to the extremely high irradiance (2 200 μmol m^{-2} s^{-1}) (Table 1). The contents of Chl *a*, Chl *b*, and Car (x+c) per dry matter were by 72, 85, and 58 % higher for LI barley

Table 1. Total chlorophyll (Chl) *a* and *b*, and carotenoid (Car x+c) contents as well as pigment ratios Chl *a/b* and Chl (*a+b*)/Car (x+c) in barley leaves under following conditions: HI - d: dark adapted HI plants; HI - i: HI plants exposed for 10 min to 2 200 μmol m^{-2} s^{-1}; LI - d: dark adapted LI plants; LI - i: LI plants exposed for 10 min to 2 200 μmol m^{-2} s^{-1}. The mean values from six measurements ± standard deviation are presented.

Sample	HI - d	HI - i	LI - d	LI - i
Chl *a* [g kg^{-1}]	6.75 ± 0.11	6.65 ± 0.38	11.52 ± 0.88	11.44 ± 0.60
Chl *b* [g kg^{-1}]	1.94 ± 0.03	1.91 ± 0.30	3.58 ± 0.24	3.64 ± 0.22
Car x+c [g kg^{-1}]	1.88 ± 0.05	1.72 ± 0.12	2.98 ± 0.22	2.84 ± 0.09
Chl *a/b*	3.48 ± 0.05	3.40 ± 0.25	3.21 ± 0.06	3.15 ± 0.03
Chl (*a+b*)/Car x+c	4.62 ± 0.17	4.86 ± 0.24	5.08 ± 0.10	5.32 ± 0.27

leaves as compared with HI plants (Table 1). Moreover, the Chl *a*/*b* ratio was by 8 % lower for LI leaves, whereas the ratio of Chl (*a*+*b*) to Car (x+c) was by 10 % higher for LI plants as compared with the HI ones. Hence, the differences in pigment composition between LI and HI barley observed in the present experiments were similar to those for barley and similar crop species (Lichtenthaler *et al.* 1981, Falbel *et al.* 1996, Gray *et al.* 1996, Melis 1998). Only insignificant changes in pigment contents and ratios were observed after a 10 min exposure of LI and HI plants to 2 200 μmol m^{-2} s^{-1} (Table 1). Hence, the below reported changes in Chl *a* fluorescence parameters of the irradiated barley leaf segments were not caused by a rapid pigment photobleaching, neither for HI nor for LI plants.

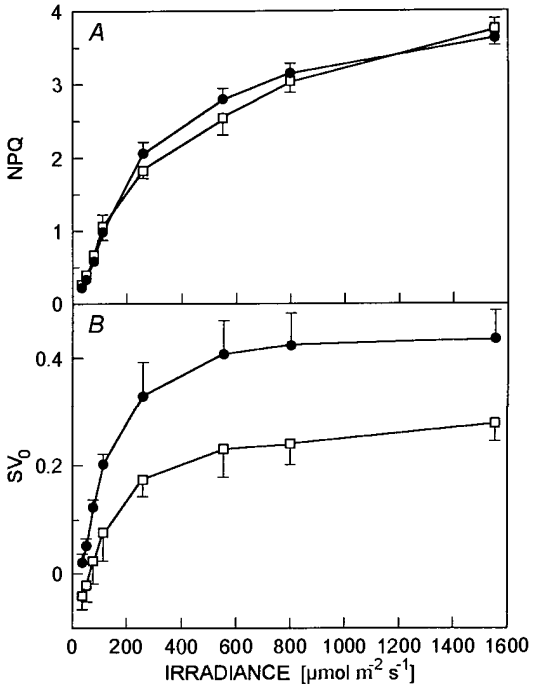

Fig. 1. Irradiance responses of nonphotochemical quenching of F_M (NPQ; *A*) and F_0 (SV_0; *B*) chlorophyll (Chl) *a* fluorescence levels for barley leaves grown at low (LI; *open squares*) and high (HI; *closed circles*) irradiances. Mean values of Chl *a* fluorescence quenching parameters and standard deviation from nine measurements.

Dependence of nonphotochemical fluorescence quenching on irradiance: The increased nonradiative dissipation originating preferentially from structure-functional modification of PS2 is monitored by the NPQ. Usually the plants grown at high irradiance possess a higher capacity of NRD as judged from higher values of irradiance-saturated NPQ values (NPQ_{max}) (Demmig-Adams 1998, Špunda *et al.* 1998). However, the HI barley leaf segments are not characterized by elevated NPQ at high irradiances in comparison to the LI ones (Fig. 1*A*). Hence, we did not find an increased capacity of NRD for HI-grown barley (Fig. 1*A*). On the contrary, in

agreement with typical adaptive response to the high irradiance, the SV_0 quenching related to the part of NRD localized within LHC2 was much more pronounced for the HI plants (Fig. 1*B*). Hence, the LI barley revealed the reduced ability to down-regulate the light-harvesting function within PS2, but the same capacity of total NRD as did the HI one. Therefore, at least a partly different mechanism of NRD is expected for the LI plants characterized with preferential localization of the dissipative centres within the PS2 core.

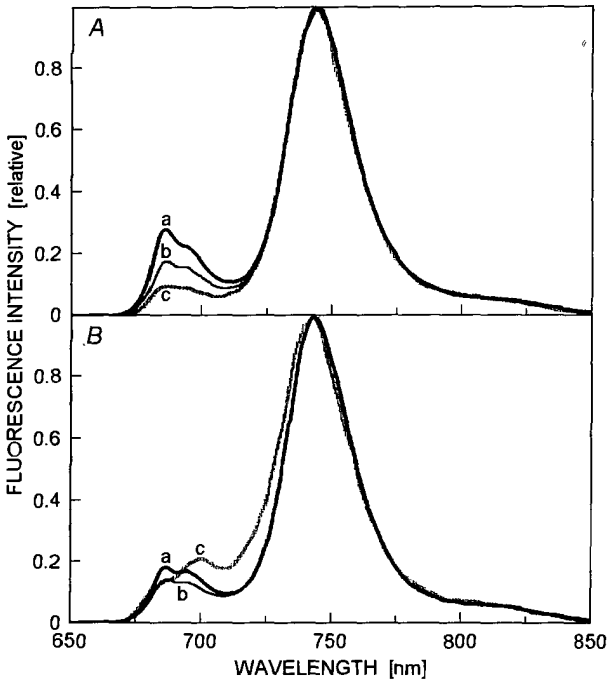

Fig. 2. Chlorophyll *a* fluorescence emission spectra at 77 K of barley grown at high (HI; *A*) and low (LI; *B*) irradiances. The leaf segments used for measurements were pre-treated as follows: before cooling in liquid nitrogen the leaves were kept in darkness (*a*), leaves exposed for 10 min to 430 μmol m^{-2} s^{-1} (*b*), and leaves exposed for 10 min to 2 200 μmol m^{-2} s^{-1} (*c*). Fluorescence emission was excited at 436 nm. The spectra are normalized at the maximum of fluorescence emission. The typical spectra selected from measurements of five leaf segments are presented. The relative changes of the fluorescence emission in the red region of spectra (680-705 nm) are significant (values not shown).

Light induced decrease of PS2 Chl *a* fluorescence at 77 K: The effect of selected irradiances on the emission spectrum of 77 K Chl *a* fluorescence in HI and LI barley is shown in Fig. 2. For HI leaf segments the pronounced relative depression of PS2 fluorescence was observed upon increasing irradiance (by 40 % at 430 μmol m^{-2} s^{-1} and by 65 % at 2 200 μmol m^{-2} s^{-1} compared with dark-adapted leaves) (Fig. 2*A*). This relative decrease of fluorescence emission originating from PS2 probably reflects the decreased absorption cross-section of PS2 core, which emits fluorescence at 685 (CP 43) and 695 (CP 47) nm. Because we did not observe any significant

light-induced changes in pigment contents (Table 1), we suggest that this decrease of the PS2 absorption cross-section is mainly due to the decrease of excitation energy transfer from LHC2 to the PS2 core. The changes in fluorescence spectra for HI barley leaves exposed to increased irradiances were similar to those reported by Ruban *et al.* (1993) for *Guzmania* leaves in the state with aggregated LHC2 *in vivo*. For LI plants, the relative depression of PS2 fluorescence was saturated already at 430 μmol m^{-2} s^{-1} and did not exceed 30 % compared with dark-adapted leaves (Fig. 2*B*). We suggest that this limitation of relative depression of PS2 fluorescence emission is related particularly to the decreased ability of photosynthetic apparatus of LI barley to down-regulate the efficiency of excitation energy transfer from LHC2 to the PS2 core. This is in agreement with the lowered capacity to quench the F_0 fluorescence level at room temperature (Fig. 1*B*).

Pronounced changes in the shape of Chl *a* fluorescence emission spectrum at 77 K: A pronounced maximum at 700 nm (F700) appeared in 77 K Chl *a* fluorescence emission spectrum for LI plants exposed to 2 200 μmol m^{-2} s^{-1} (Fig. 2*B*). This maximum corresponds to results of Šiffel and Vácha (1998) who observed a pronounced maximum at 699 nm for tobacco leaves stressed by CO_2 deficit. Even the short-term exposure of LI leaves to 2 200 μmol m^{-2} s^{-1} caused the progressive photoinactivation of PS2 RC´s as judged from complete cessation of variable Chl *a* fluorescence and significantly limited dark-relaxation of the quantum yield of PS2 photochemical reaction ($F_M' - F_0'/F_M'$) (values not shown). Hence, although the pronounced manifestation of F700 in 77 K emission spectrum of Chl *a* fluorescence was induced in different way than reported by Šiffel and Vácha (1998), our results support the notion that this spectral change *in vivo* reflects a pronounced photodamage to PS2.

Down-regulation of light-harvesting within PS2: In order to estimate the relation between the observed changes in Chl *a* fluorescence emission spectra and the efficiency of excitation energy transfer from the supplementary pigments of LHC2 complexes to the Chl *a* fluorescing spectral forms within the PS2 core, we analyzed the 77 K excitation spectra of Chl *a* fluorescence (Fig. 3). The excitation spectra provide qualitative information about the content of photosynthetic pigments and individual spectral forms, and concomitantly about excitation energy transfer from excited pigments to the fluorescing forms of Chl *a* (Šiffel *et al.* 1985, Šesták and Šiffel 1997, Špunda *et al.* 1997a, 1998, Šiffel and Vácha 1998). As already mentioned, a significant pigment photobleaching did not occur after exposure of leaf segments to 2 200 μmol m^{-2} s^{-1} (Table 1). Hence, the light-induced relative changes in the shape of excitation spectra measured at emission wavelength 685 nm qualitatively reflect the excitation energy transfer from individual pigments to Chl *a* emission form located in the PS2 core (Špunda *et al.* 1998). Whereas the first excitation maximum in the Soret region of the spectrum around 436 nm (E436) corresponds to energy transfer from Chl *a* molecules, the structure of the second broad band within 460-490 nm is more complicated and reflects the transfer of excitation energy from Chl *b* and other accessory pigments (Šiffel *et al.* 1993,

Špunda *et al.* 1998). Nevertheless, at 474-476 nm (E476) preferentially the excitation energy transfer from Chl *b* is monitored (Day *et al.* 1984, Špunda *et al.* 1997a). As Chl *b* is present only in LHC's, whereas Chl *a* is the main pigment of RC's, core antennae, and LHC's, the light-induced changes of E476/E436 ratio may be taken as the measure of the regulation of excitation energy transfer from LHC2 to the PS2 core (mainly CP43). For HI plants, a slight gradual decrease of E476 was observed

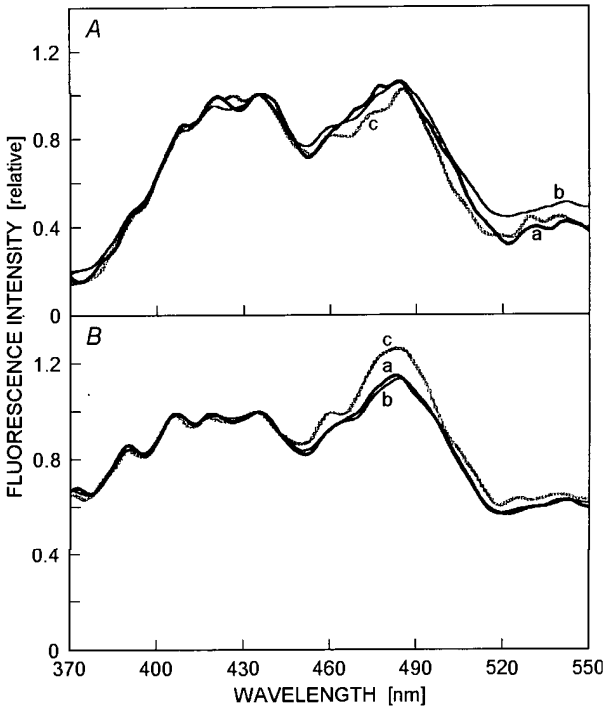

Fig. 3. Chlorophyll (Chl) *a* 77 K fluorescence excitation spectra in the Soret region of barley grown at high (HI; *A*) and low (LI; *B*) irradiances. The spectra of dark adapted leaf segments (*a*), leaves exposed for 10 min to 430 μmol m^{-2} s^{-1} (*b*), and leaves exposed for 10 min to 2 200 μmol m^{-2} s^{-1} (*c*) are presented. Excitation spectra were detected at emission wavelength 685 nm. The spectra are normalized at the excitation maximum of Chl *a* in the Soret region. The typical spectra selected from measurements of five leaf segments are presented. The relative changes of the excitation maximum E476 observed for the segments exposed to 2 200 μmol m^{-2} s^{-1} (*c*) are significant as compared to the dark adapted segments (*a*) (values not shown).

following the exposure of leaf segments to the increasing irradiances (Fig. 3*A*). The most pronounced decrease of E476/E436 ratio (by 10 %) was found for HI leaf segments exposed to 2 200 μmol m^{-2} s^{-1} if compared with the dark-adapted leaves (Fig. 3*A*). This relative decrease of E476 indicates the decreased efficiency of excitation energy transfer from the LHC2 to the PS2 core which is related to the nonradiative dissipation of excess excitation energy within LHC2 (Špunda *et al.* 1998). For LI segments, the relative decrease of E476 was significantly limited (Fig. 3*B*). Maximum decrease of E476/E436 was observed for LI leaf segments exposed to

430 μmol m^{-2} s^{-1} and did not exceed 2-3 % if compared with dark-adapted leaves. The reduced ability to down-regulate the efficiency of excitation energy transfer from the LHC2 to the PS2 core for LI barley is consistent with limitation of light-induced changes in the 77 K emission spectra of Chl *a* fluorescence (Fig. 2*B*) and quenching of F_0' level (Fig. 1*B*). These independent results confirmed that LI barley possesses a limited capacity to regulate the light-harvesting function.

Disassembly of photodamaged PS2: The irradiances higher than 880 μmol m^{-2} s^{-1} caused even opposite effect on the shape of 77 K excitation spectra of LI leaves measured at 685 nm and characterized by the slight increase of E476 (values not shown). For the LI segments exposed to 2200 μmol m^{-2} s^{-1} a pronounced increase of E476/E436 by 10 % was observed as compared with the dark-adapted LI leaves (Fig. 3*B*). Hence, the appearance of the pronounced emission maximum at 700 nm in the Chl *a* fluorescence spectrum of LI leaf segments exposed to 2200 μmol m^{-2} s^{-1} was related to the increased efficiency of excitation energy transfer from LHC2 to the PS2 core as compared both to the dark adapted and moderately irradiated leaves. This finding does not confirm the attribution of pronounced emission band at 700 nm entirely to the LHC2 aggregation as suggested by Šiffel and Vácha (1998). The formation of LHC2 aggregates itself should be accompanied by further decrease of excitation energy transfer from LHC2 to the PS2 core. We suggest that more severe high irradiance stress may induce a structural disassembly of PS2 core and aggregation of LHC2 with part of PS2 core antenna (probably CP 43 with fluorescence emission at 685 nm). This could explain both the pronounced decrease of F685 and increase of F700 (Fig. 2*B*) and the increase of excitation transfer within LHC2 and CP43 (Fig. 3*B*). This process seems to be related to the progressive PS2 degradation *in vivo*, in agreement with the fact that the degraded monomers of PS2 RC's loose the CP 43 before D1 protein is exchanged (Barbato *et al.* 1992, Aro *et al.* 1993).

Conclusion: The resistance of PS2 against photodamage is provided particularly *via* an adaptive reduction of the LHC2 size and efficient nonradiative dissipation within LHC2 (Demmig-Adams and Adams 1996, Horton *et al.* 1996, Špunda *et al.* 1998). Compared to LI barley, our HI seedlings revealed a significantly reduced size of LHC2 (Table 1) and a higher capacity of NRD within LHC2 (Fig. 1*B*) accompanied by pronounced functional disconnection of LHC2 (Fig. 3). Hence, as expected, HI plants were more resistant to PS2 photodamage induced by short-term exposure to high irradiances then the LI ones. However, the resistance of HI barley to photodamage was not accompanied with higher NPQ levels. This discrepancy may be explained assuming different contribution of LHC2 and PS2 core to the total NRD. Jahns and Miehe (1996) proved that NRD within LHC2 prevailed under moderate high irradiance stress, whereas under severe stress the photodamaged PS2 core contributed significantly to the NRD. Thus we can summarize the following scenario of light-induced effects on the functional state of PS2 for the barley grown at HI or LI: For the HI plants, the reduced amount of LHC2 and efficient down-regulation of light-harvesting function within PS2 provide sufficient protection up to

2200 μmol m^{-2} s^{-1}. The changes in emission and excitation spectra of 77 K Chl *a* fluorescence *in vivo* confirmed that the NRD was localized preferentially within LHC2 for the whole range of applied irradiances. On the contrary, the limited down-regulation of light-harvesting within PS2 observed for LI plants led to a gradual photodamage of the PS2 core. This was accompanied with a disassembly of PS2 core, which may be attributed to the formation of mixed aggregates of the LHC2 and part of PS2 core. Hence, at the highest applied irradiance the NRD was preferentially associated with the disassembly of the PS2 core.

The 77 K Chl *a* fluorescence excitation spectra may be used as an indicator of the prevailing localization of NRD and as a measure of the PS2 photodamage. The fluorescence is preferentially excited from the upper leaf layer. Hence, the effect of high irradiance exposure on various Chl *a* fluorescence parameters *in vivo*, as reported here, may differ from the effects on the leaf photosynthetic activities related to the whole leaf.

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