The lack of LHCII proteins modulates excitation energy partitioning and PSII charge recombination in *Chlorina F2* mutant of barley

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ABSTRACT

Analysis of the partitioning of absorbed light energy within PSII into fractions utilized by PSII photochemistry (Φ_{PSII}), thermally dissipated via ΔpH - and zeaxanthin-dependent energy quenching (Φ_{NPQ}) and constitutive non-photochemical energy losses (Φ_{NO}) was performed in wild type and *F2* mutant of barley. The estimated energy partitioning of absorbed light to various pathways indicated that the fraction of Φ_{PSII} was slightly higher, while the proportion of thermally dissipated energy through Φ_{NPQ} was 38% lower in *F2* mutant than in WT. In contrast, Φ_{NO} , i.e. the fraction of absorbed light energy dissipated by additional quenching mechanism(s) was 34% higher in *F2* mutant. The increased proportion of Φ_{NO} correlated with narrowing the temperature gap (ΔT_{M}) between $\text{S}_{2/3}\text{Q}_{\text{B}}^-$ and $\text{S}_2\text{Q}_{\text{A}}^-$ charge recombinations in *F2* mutant as revealed by thermoluminescence measurements. We suggest that this would result in increased probability for an alternative non-radiative $\text{P680}^+\text{Q}_{\text{A}}^-$ radical pair recombination pathway for energy dissipation within the reaction centre of PSII (reaction center quenching) and that this additional quenching mechanism might play an important role in photoprotection when the capacity for the primary, zeaxanthin-dependent non-photochemical quenching (Φ_{NPQ}) and state transitions pathways are restricted in the absence of LHCII polypeptides in *F2* mutant. [Physiol. Mol. Biol. Plants 2008; 14(3) : 205-215] E-mail : nhuner@uwo.ca

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Abbreviations : LHCII, the major Chl *a/b* pigment-protein complex associated with PSII; PSI, photosystem I; PSII, photosystem II; Q_{A} , primary electron-accepting quinone in PSII reaction centres; Q_{B} , secondary electron-accepting quinone in PSII reaction centers; NPQ, non-photochemical quenching; qL, photochemical quenching; TL, thermoluminescence; T_{M} , temperature maximum of the TL emission peak.

Changes in irradiance, temperature, nutrient and water availability result in imbalances between the light energy absorbed through photochemistry and energy utilization through photosynthetic electron transport coupled to carbon, nitrogen and sulphur reduction. Such an imbalance caused by changes in irradiance and/or temperature, nutrient and water availability leads to photoinhibition of photosynthesis that may result in photodamage to the D1 reaction centre polypeptide of PSII (Krause, 1988; Aro *et al.*, 1993). The major mechanism for thermal de-excitation of excess light energy in higher plants is currently considered to be

the ΔpH - and xanthophyll-cycle dependent non-photochemical quenching (NPQ) occurring within LHCII antenna pigment bed of PSII (Demmig-Adams and Adams, 1992; Horton *et al.*, 1996). The role of pH- and zeaxanthin-dependent shifts in the oligomerization state of LHCII in developing the rapidly relaxing energy dependent component (qE) of NPQ is well established with qE representing the major protective mechanism against photoinhibitory damage of PSII (Horton *et al.*, 1996; Niyogi, 1999). It has been demonstrated that trimers of LHCII exhibit the optimum level of non-photochemical energy dissipation by modulating the development of the quenched state of the complex (Wentworth *et al.*, 2004).

The redox-dependent reversible phosphorylation of light-harvesting Chl *a/b*-binding proteins (LHCII) is the

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major mechanism controlling the distribution of excitation energy between PSII and PSI through a process known as state transitions (Fork and Satoh, 1986; Allen, 1995). The phosphorylation of LHCII induces lateral migration of P-LHCII from PSII towards PSI increasing the antenna cross-section of PSI at the expense of PSII (Anderson, 1986; Allen, 1995) resulting in a redistribution of the energy in favour of PSI. In addition to non-photochemical quenching, state transitions have been also well recognized as an adaptive mechanism for the short-term redistribution of excitation energy between the two photosystems. State transitions take place in response to fluctuations in excitation light, and may play a substantial role in protecting PSII from over excitation and photodamage (Anderson, 1986; Allen, 1995; Haldrup *et al.*, 2001).

Interestingly, both photoprotective processes (non-photochemical quenching and state transitions) involve the presence and certain structural rearrangements of Lhcb proteins. However, it has been suggested that the presence of LHCII is not an absolute requirement for the energy dependent (qE) non-photochemical quenching of excess light (Falk *et al.*, 1994). Further, xanthophyll cycle-dependent energy dissipation (qN) appears to be independent of the peripheral antenna size in *Chlorina* barley mutants (Gilmore *et al.*, 1996).

The *Chlorina F2* barley mutant (*Hordeum vulgare* L. cv, Dornaria, *Chlorina f2*²⁸⁰⁰) is a homozygous single nuclear gene mutant, recessive for the accumulation of Chl *b* (Thorner and Highkin, 1974; Leverenz *et al.*, 1992). The mutant is deficient in Lhcb1, Lhcb4, and Lhcb6 polypeptides and has reduced amounts of Lhcb2, Lhcb3 and Lhcb5 proteins (Bossmann *et al.*, 1997; Krol *et al.*, 1995). The reduced amount of Lhcb proteins in the *F2* mutant, which is not compensated by extra chlorophylls, results in 20 and 80% reduction of the effective antennae sizes of its PSI and PSII, respectively (Harrison *et al.*, 1993). In addition, the *F2* mutant exhibited a decrease *in vivo* photostability (Leverenz *et al.*, 1992; Havaux and Tardy, 1997) and thermostability (Havaux and Tardy, 1997) of PSII photochemistry compared to WT barley leaves.

Considering the suggested role of LHCII, its oligomeric state and structural alterations in photoprotection of PSII through non-photochemical quenching and state transitions, the *F2* barley mutant was used in this study as a model system for studying the mechanisms and possible additional pathways of efficient light energy utilization and dissipation in the absence of LHCII proteins.

MATERIALS AND METHODS

Plant material

Wild type barley (*Hordeum vulgare* L.) and the Chl-*b* less (*Chlorina F2*) mutant were germinated from seeds in coarse vermiculite with 16 h light/dark period in controlled environment growth chambers (Convion, Winnipeg, MB, Canada) fitted with fluorescent tubes (Cool White, 160W, F72T12/CW/VHO, SylvaNnia, Drummondville, QC, Canada) to provide 250 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Day/night temperatures were 20°/16°C. The relative humidity was maintained at 50%. Fully expanded second and third leaves harvested 2 to 3 hours after the beginning of the light cycle were used in all experiments. High light treatment of fully expanded WT and *F2* leaves was performed at 20°C and PPFD of 1200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$.

SDS-PAGE and immunoblotting

Thylakoid membranes for SDS-PAGE from wild type and the *F2* mutant were isolated as described earlier (Krol *et al.*, 1999). Benzamidine and aminocaproic acid were present in the homogenization buffer at concentrations of 2 mM. Thylakoid preparations were solubilized in a 60 mM Tris-HCl (pH 7.8) buffer containing 1 mM EDTA, 12% (w/v) sucrose and 2% (w/v) SDS to achieve a SDS:Chl ratio of 20:1. Solubilized samples containing equal amount of protein (20 $\mu\text{g lane}^{-1}$) were separated on a 15% (w/v) linear polyacrylamide gel using a Mini-Protean II apparatus (Bio-Rad).

Immunoblotting was performed by transferring the proteins from SDS-PAGE to nitrocellulose membranes (0.2 μm pore size, Bio-Rad). The membranes were probed with antibodies raised against PsbA(D1), and Lhcb1 (Agrisera Sweden). Dilutions used were 1:2500 for D1 and 1:5000 for Lhcb1.

Pigment analysis

Pigments from WT and the *F2* mutant barley leaves were extracted, separated and quantified by high-performance liquid chromatography (HPLC) as described previously (Ivanov *et al.*, 1995). The system composed of a Beckman System Gold programmable solvent module 126, a diode array detector module 168 (Beckman Instruments, San Ramon, CA, USA) and a CSC-Spherisorb ODS-1 reverse phase column (5 μm particle size, 25 x 0.46 cm I.D.) with an Upchurch Perisorb guard column (both columns from Chromatographic Specialties Inc., Concord, ON, Canada). Pigments were eluted isocratically for 6 min with a solvent system of

acetonitrile:methanol:0.1 M Tris-HCl (pH 8.0), (72:8:3.5, v/v/v), followed by a 2 min linear gradient to 100% methanol:hexane (4:1, v/v) which continued isocratically for 4 min.

Chlorophyll fluorescence

Low temperature (77K) fluorescence emission spectra of isolated chloroplasts from WT and *F2* mutant were measured using a PTI LS-100 spectrofluorometer (Photon Technology International, South Brunswick, NJ, USA) as described previously (Morgan *et al.*, 1998). Samples with Chl concentration of 5 mg ml⁻¹ were dark adapted at room temperature for 30 min and frozen in the presence of 30% glycerol before the measurements. Chl *a* fluorescence was excited at 436 nm. Corrected fluorescence spectra were recorded from 660 nm to 780 nm. Exciting and measuring slits were 4 nm. All fluorescence spectra were corrected by subtracting the medium blank and were normalised to the PSII peak at 695 nm. Decomposition analysis of the spectra in terms of Gaussian bands was carried out by a non-linear least squares algorithm that minimises the chi-square function using a MicrocalTM OriginTM Version 6.0 software package (Microcal Software Inc., Northampton, MA, USA). The fitting parameters for the six Gaussian components, that is, position, area and full width at the half maximum (FWHM), were free-running parameters.

Chlorophyll *a* fluorescence of a dark adapted (15 min) leaves of wild type and *F2* mutant barley leaves was measured at the growth temperature of 20°C under ambient CO₂ conditions using a PAM 101 chlorophyll fluorescence measuring system (Heinz Walz GmbH, Effeltrich, Germany) (Schreiber *et al.*, 1986) as described previously (Ivanov *et al.*, 2005). The photochemical fluorescence quenching (qL) and F_v'/F_m' parameters were calculated when the steady state F_s level was reached. The nomenclature of van Kooten and Snel (1990) was used for the parameters of Chl fluorescence. PSII "excitation pressure" or the relative reduction state of PSII at the growth temperature and growth irradiance was measured as 1-qL (Kramer *et al.*, 2004). Partitioning of absorbed light energy was estimated according to the model proposed by Kramer *et al.* (2004). The fraction of excitation energy that is utilized for PSII photochemistry (Φ_{PSII}) was estimated as $\Phi_{PSII} = (F_m' - F_s)/F_m'$. The yield of non-photochemical dissipation within the PSII antennae (Φ_{NPQ}) was calculated as $\Phi_{NPQ} = 1 - \Phi_{PSII} - \Phi_{NO}$, where Φ_{NO} is the yield of all other processes involved in non-photochemical energy losses and was calculated as $\Phi_{NO} = 1/[NPQ + 1 + qL (F_m/F_o - 1)]$.

Thermoluminescence measurements

Thermoluminescence (TL) measurements of intact WT and *F2* mutant barley leaves were performed on a personal-computer-based TL data acquisition and analysis system as described earlier (Ivanov *et al.*, 2001). A xenon-discharge flash lamp (XST103, Heinz Walz GmbH, Effeltrich, Germany) was used to expose the samples to a single turnover flash (1.5 ms peak width at 50% of maximum). Dark adapted leaves (20 minutes at 20°C) were cooled to 2°C prior to exposing to the flashes. After the flash exposure, the samples were measured immediately or quickly cooled in liquid nitrogen. For S₂Q_A⁻ recombination studies, leaves were vacuum infiltrated with DCMU (20 mM) in darkness before the flash illumination. The nomenclature of Sane and Rutherford (1986) and Vass and Govindjee (1996) were used for characterization of the TL glow peaks. Experiments were performed at a heating rate of 0.6°C s⁻¹.

RESULTS

Consistent with previous reports the quantitative analysis of pigment composition in WT and *Chlorina F2* mutant of barley thylakoid membranes clearly indicated the lack of measurable Chl *b* amounts (Table 1) in *F2* mutant (Leverenz *et al.*, 1992). As a consequence, *F2* mutant exhibited a Chl *a/b* ratio higher than 20, as compared to the WT (3.84). This is consistent with the largely reduced amount of the major LHCII polypeptides in *Chlorina F2* thylakoids as revealed from SDS-PAGE analysis (Fig. 2A), drastically reduced amount of Lhcb1 (Fig. 2B) and the lack of immunodetectable Lhcb4, and Lhcb6 polypeptides (Bossmann *et al.*, 1997; Krol *et al.*, 1995, 1999). It is also demonstrated that the content of all individual carotenoid pigments in *F2* mutant chloroplasts is strongly reduced as compared to the WT (Table 1). As expected, since neoxanthin is almost exclusively associated with the Lhcb polypeptides (Bassi *et al.*, 1993), which are severely depleted in the mutant thylakoids, the content of neoxanthin was most affected in *Chlorina F2* in comparison to the content of other pigments. Interestingly, the relative abundance of the PsbA polypeptide (D1 protein of PSII reaction center) was significantly increased in *F2* mutant (Fig. 2B).

Indeed, low temperature (77K) fluorescence emission spectra of thylakoid membranes isolated from *F2* mutant indicated higher intensity of PSII associated emission peaks within the 680-700 nm spectral regions (Krause

Table 1. Photosynthetic pigment content of wild type (WT) and the *Chlorina F2* mutant barley (*Hordeum vulgare* L.) leaves. Pigments were separated and quantified by HPLC, as described in the Materials and Methods section. The results are presented as mg per g fresh weight (FW⁻¹). Mean values $\pm$ S.E. were calculated from three replicate measurements in three independent experiments ($n = 9$). n.d. - not determined.

Pigments (mg gFW ⁻¹)	WT	F2 mutant
Neoxanthin	19.53 $\pm$ 2.28	3.91 $\pm$ 0.45
Violaxanthin	60.68 $\pm$ 1.42	40.27 $\pm$ 1.86
Antheraxanthin	n.d.	n.d.
Lutein	128.15 $\pm$ 2.69	58.64 $\pm$ 3.03
Zeaxanthin	3.07 $\pm$ 0.3	0.15 $\pm$ 0.1
b-Carotene	121.79 $\pm$ 1.75	77.38 $\pm$ 4.48
V+A+Z	62.73 $\pm$ 1.93	40.42 $\pm$ 1.0
Chl <i>a</i>	1506.24 $\pm$ 35.30	882.31 $\pm$ 26.90
Chl <i>b</i>	393.58 $\pm$ 15.40	n.d.
Chl <i>a</i> /Chl <i>b</i>	3.84 $\pm$ 0.22	>20

n.d. – not detected

and Weis, 1984) relative to PSI-related emission peaks at 735 nm as compared to WT (Fig. 2). Decomposition analysis of the fluorescence spectra yielded a best fit with six spectral components (Fig. 2, Table 2) corresponding to the emission of three sub-bands from PSII (678–681, 687–690 and 699–701 nm), two sub-bands from PSI (721–722 and 735–738 nm) (Krause and Weis, 1984) and a spectral component centered around 740–760 nm corresponding to vibrational transitions (F_{vib}) in the near-infrared region (Van Grondelle *et al.*, 1994). Although the overall composition of spectral sub-bands in both WT and *F2* mutant thylakoid membranes was similar, the area ratio of the sum of PSII- and PSI-related components is significantly higher in *F2* mutant (0.71) compared to WT chloroplasts (0.31) (Table 2).

Despite the lack of major Lhcb polypeptides the redox state of the primary stable electron acceptor Q_A measured as $1 - q_L$ (Kramer *et al.*, 2004), which is believed to reflect the excitation pressure of PSII (Huner *et al.*, 1998), was 27.2% lower in *F2* mutant compared to WT (Table 3). Further analysis of the partitioning of absorbed light energy into fractions utilized by PSII photochemistry (F_{PSII}), thermally dissipated *via* ΔpH -

Table 2. Gaussian parameters for the sub-band decomposition of low temperature (77K) fluorescence emission spectra of wild type (WT) barley and the *Chlorina F2* mutant chloroplasts. The percentage areas of the spectral forms have been calculated from the total area given by the sum of all bands. The FWHM of each band is the sum of the left and right HWHM values. FWHM - full width at half maximum; HWHM - half width at half maximum.

Parameters	WT	F2 mutant
1 λ max	678.00	681.06
FWHM	8.28	13.52
Area %	0.70	8.82
2 λ max	687.78	690.14
FWHM	10.56	8.37
Area %	5.27	8.15
3 λ max	701.08	699.52
FWHM	23.50	15.97
Area %	12.53	17.05
4 λ max	721.94	722.08
FWHM	9.52	18.39
Area %	1.52	25.50
5 λ max	738.89	735.38
FWHM	25.29	20.50
Area %	56.45	21.87
6 λ max	763.39	750.56
FWHM	33.09	33.72
Area %	23.50	17.91

and xanthophyll-dependent energy quenching (Φ_{NPQ}) and a fraction of absorbed light neither going to F_{PSII} or F_{NPQ} , i.e. the sum of all other processes involved in non-photochemical energy losses within PSII reaction centers with Q_A in reduced state (Φ_{NO}) was provided according to the model proposed by Kramer *et al.* (2004). The estimated energy partitioning of absorbed light to various pathways indicated that the fraction utilized by photochemistry (Φ_{PSII}) was slightly higher in *F2* mutant than in WT (Table 3). Concomitantly, the proportion of thermally dissipated energy through non-photochemical quenching (Φ_{NPQ}) was 38% lower compared to WT. In contrast, Φ_{NO} , i.e. the fraction of absorbed light energy dissipated by additional quenching mechanism(s) was 34% higher in *F2* mutant (Table 3).

It is generally thought that ΔpH - and zeaxanthin-dependent energy dissipation is the major non-photochemical quenching mechanism reflecting thermal energy dissipation of excess absorbed light within PSII

Fig. 1. A - Polypeptide composition of wild type (WT) and *Chlorina F2* (F2) mutant barley thylakoid membranes. All gels were loaded on an equal chlorophyll basis of 3 μg Chl lane⁻¹ for SDS-PAGE separation. The arrow denotes the band corresponding to the major LHCII polypeptide (25 kDa). Broad range pre-stained SDS-PAGE standards (ST) (Bio-Rad Laboratories, Hercules, CA, USA) were used for estimation of molecular masses. B - Immunoblot analysis of D1 and Lhcb1 proteins abundance in WT and F2 mutant thylakoid membranes.

Table 3. Energy partitioning in wild type (WT) and F2 mutant of barley leaves. Fractions of absorbed irradiance consumed via PSII photochemistry (Φ_{PSII}), ΔpH - and xanthophyll-regulated thermal dissipation (Φ_{NPQ}), and the sum of all other processes involved in non-photochemical energy losses (Φ_{NO}) were estimated according to the model proposed by Kramer *et al.* (2004). All measurements were performed at the corresponding growth temperature and irradiance (20°C and 250 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). Mean values $\pm$ SE were calculated from 3 independent experiments.

Parameters	WT	F2 mutant
1 - qL (qP x Fo'/Fs)	0.460 $\pm$ 0.034	0.335 $\pm$ 0.010
Φ_{PSII}	0.603 $\pm$ 0.018	0.697 $\pm$ 0.011
Φ_{NPQ}	0.293 $\pm$ 0.019	0.164 $\pm$ 0.015
Φ_{NO}	0.103 $\pm$ 0.001	0.138 $\pm$ 0.005

Fig. 2. Low temperature (77K) fluorescence emission spectra and decomposition in Gaussian sub-bands of WT (A) and *Chlorina F2* mutant (B) chloroplast membranes. Fluorescence was excited at 436 nm. Experimental curves represent averages of 3 scans. Spectra were normalized at the PSII peak at 695 nm. Experimental curve (solid line), sum of Gaussian sub-bands (open circles), Gaussian sub-bands (dashed lines). The differences between the sum of the components and the experimental emission curves are shown as residuals in the figure inserts.

antenna bed (Demmig *et al.*, 1987; Demmig-Adams and Adams, 1992). The dynamics in the relative quantitative changes in the amount of zeaxanthin upon high light treatment are presented in Fig. 3. Interestingly, besides the initially lower level of the xanthophyll pool size ($V + A + Z$), in F2 mutant (Table 1), considerably larger fraction of the violaxanthin pool had been converted into zeaxanthin (89%) after 3-h exposure to high light as compared to WT (45.9%). The observed higher amount of Zea in high light treated F2 mutant, reflecting more effective conversion of violaxanthin and/or antheraxanthin to Zea, does not correlate with the lower F_{NPQ} values and implies the involvement of additional quenching mechanism(s) in F2 leaves.

Fig. 3. High light-induced formation of zeaxanthin in wild type (WT) and *Chlorina F2* mutant barley seedlings. For high-light treatments, leaves were exposed to illumination of $1200 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ white light at 20°C . Non-photoinhibited plants were dark-adapted for 30 min. Zeaxanthin contents are presented as percentage of the total xanthophylls (V + A + Z) pool size found in each sample. Mean values $\pm$ S.E. were calculated from three replicate measurements in three independent experiments.

Recently, non-radiative dissipation of excess light energy within the reaction center of PSII (reaction center quenching) occurring through reversible changes in the redox properties of the two quinone acceptors of PSII has been proposed as such additional quenching mechanism supplementing the antenna-based non-photochemical quenching under various environmental conditions (Ivanov *et al.*, 2003, 2006). The redox state of the electron transport components of the PSII acceptor side (Q_A and Q_B) in WT barley and the *F2* mutant was studied by thermoluminescence (TL) measurements providing reliable information on the activation energies associated with the back reactions of electron acceptors (Q_A and Q_B) with the electron donors (S_2 and S_3) and the redox potentials of the participating oxidized and reduced donors of PS II (DeVault and Govindjee, 1990). The temperature maxima (T_M) of the TL emission peaks after excitation with two single turnover flashes of white light in the absence or presence of DCMU to block electron flow between Q_A and Q_B reflect charge recombinations of $S_2Q_A^-$, $S_3Q_A^-$,

Fig. 4. Thermoluminescence glow curves of WT (solid lines) *Chlorina F2* mutant (dashed lines) barley leaves after illumination with two single turnover flashes. A – TL glow curves were recorded immediately after illumination. B – TL curves were recorded after freezing the illuminated samples. The presented glow curves are averages from 3–5 measurements in 3 independent experiments.

$S_2Q_B^-$ and $S_3Q_B^-$ redox pairs (Inoue, 1996; Sane, 2004). It has been well documented that the reliability of TL measurements can be distorted after freezing the samples and some artifacts can occur due to sudden freezing depending on the plant species (Homman, 1999; Janda *et al.*, 2004). For this reason the TL measurements in this study were performed in both frozen and non-frozen samples after flash illumination.

Typical TL emission glow curves obtained following excitation with two successive single turnover flashes in non-frozen samples of WT and *F2* mutant in the absence of DCMU are presented in Fig. 4A. The TL glow curve of WT barley leaves exhibited a peak with characteristic T_M of 25.2°C (Fig. 4A, Table 4). The band at 25.2°C disappeared in DCMU-treated WT leaves and hence represents $S_2/S_3Q_B^-$ charge recombination (Vass and Govindjee, 1996; Sane, 2004). In the presence of DCMU the overall TL emission was much lower (traces not shown) and yielded a new peak exhibiting T_M of

Table 4. Characteristic thermoluminescence (TL) peak emission temperatures (T_M) of $S_2/S_3Q_B^-$ and $S_2Q_A^-$ glow peaks of wild type (WT) and *F2* mutant barley leaves. The samples were cooled to 2°C and illuminated with two single turnover flashes of white light. The TL glow curves were obtained immediately after illumination (non-frozen samples) or frozen after illumination in liquid nitrogen before the measurements (frozen samples). Mean values $\pm$ SE were calculated from 5–8 measurements in 2–3 independent experiments. The measurements were performed in the presence and absence of 20 μ M DCMU. $\Delta T_M = T_M(S_2/S_3Q_B^-) - T_M(S_2Q_A^-)$.

Sample	Frozen samples			Non-frozen samples		
	$S_2/S_3Q_B^- - T_M$ (°C)	$S_2Q_A^- - T_M$ (°C)	ΔT_M (°C)	$S_2/S_3Q_B^- - T_M$ (°C)	$S_2Q_A^- - T_M$ (°C)	ΔT_M (°C)
WT	39.7 $\pm$ 0.3	20.9 $\pm$ 0.1	18.8	25.8 $\pm$ 1.6	9.3 $\pm$ 0.1	16.5
<i>F2</i>	34.7 $\pm$ 0.2	25.3 $\pm$ 1.4	9.4	15.6 $\pm$ 1.5	12.2 $\pm$ 0.3	3.4

9.3°C (Table 4), which was assigned to $S_2Q_A^-$ charge recombination (Vass and Govindjee, 1996; Sane, 2004). The overall TL emission of non-frozen *F2* leaves in the absence of DCMU was significantly lower and the T_M of $S_2/S_3Q_B^-$ charge recombination was down shifted by almost 10°C (Fig. 4A, Table 4) compared to WT. In contrast, $S_2Q_A^-$ peak in non-frozen *F2* leaves appeared at slightly higher temperatures than in WT. Freezing the leaves of both WT and *F2* mutant after the flash illumination had a dramatic effect on the TL glow curves and the charge recombination peaks of $S_2/S_3Q_B^-$ redox pair appeared at much higher temperatures (Fig. 4B, Table 4). However, as in the non-frozen samples of the *F2* mutant $S_2/S_3Q_B^-$ peak was down shifted by 5°C, while $S_2Q_A^-$ peak was up-shifted by 5°C compared to the corresponding peak values in frozen WT samples (Table 4). At present we are not able to fully understand the effect of freezing on the TL patterns, but most probably the redox properties of Q_A and Q_B are extensively altered by exposure to previously compartmentalized damaging solutes during thawing as proposed by Homman (1999). Nevertheless, it is evident that while $S_2/S_3Q_B^-$ peak in *F2* mutant exhibited lower T_M than the corresponding peak in WT, the $S_2Q_A^-$ in the mutant appeared at a higher temperatures than the one in WT (Table 4) and the observed TL peak shifts in *F2* mutant are consistent regardless of the TL measuring protocol (frozen vs. non-frozen samples). As a result of these shifts the temperature gap between $S_2/S_3Q_B^-$ and $S_2Q_A^-$ peaks became much narrower in the *F2* mutant than in WT (Table 4).

DISCUSSION

Several models based on chlorophyll fluorescence parameters have been proposed for quantifying the

partitioning of total absorbed light energy by PS II (Demmig-Adams *et al.*, 1996; Kramer *et al.*, 2004; Hendrickson *et al.*, 2004). Although different approaches, i.e. pure “puddle” (Demmig-Adams *et al.*, 1996) or “lake” antenna models of PSII (Kramer *et al.*, 2004; Hendrickson *et al.*, 2004) were used for estimating the fate of absorbed light energy, all models defined the sum of all energy fluxes via PSII as unity and categorized the partitioning of total absorbed energy by PS II as either photochemical or thermal dissipation processes. In addition, all three models, regardless of their underlying assumptions (“puddle” or “lake” antenna models) were able to recognize two easily distinguishable thermal dissipation processes, the major one originating from the light-harvesting antenna complexes (LHCII) and defined as regulated NPQ (Φ_{NPQ}) and an additional one defined as constitutive thermal dissipation Φ_{NO} (Kramer *et al.*, 2004; Hendrickson *et al.*, 2004) or “excess excitation energy” (E) defined as a fraction of absorbed light neither going to photochemistry or regulated NPQ, i.e. excitation energy trapped and/or dissipated by PSII reaction centers with Q_A in reduced state (Demmig-Adams *et al.*, 1996).

The partitioning of absorbed light to various pathways in WT and the *F2* mutant estimated according to the method of Kramer *et al.* (2004) indicated that the fraction of regulated NPQ (Φ_{NPQ}) measured at the growth conditions is much lower (38%) in the *F2* mutant compared to WT (Table 3). As the regulated ΔpH - and zeaxanthin-dependent NPQ is believed to occur within the LHCII antenna and depend on its oligomerization state (Horton *et al.* 1996; Wentworth *et al.*, 2004) the strongly reduced abundance of Lhcb proteins in the *F2* mutant (Fig. 1) (Bossmann *et al.*, 1997; Krol *et al.*, 1995, 1999) can easily explain the lower NPQ in the *F2* mutant. In addition, an earlier study has demonstrated that the

remaining LHCII proteins in *Chlorina F2* mutant do not form trimers (Allen and Staehelin, 1991).

Concomitantly, the proportion of photochemically dissipated light energy (Φ_{PSII}) was 15% higher, while the “excitation pressure”, measured as 1- qL was 28% lower (Table 3) in the *F2* mutant than in WT. This clearly demonstrates the ability of the *F2* mutant to maximize its photosynthetic performance and keep Q_A in relatively more oxidized state even in the absence (or very limited abundance) of LHCII proteins. Interestingly, the *F2* mutant was able to optimize the energy allocated for growth and reproduction (Φ_{PSII}) while its capacity for regulated NPQ was lower. This implies the existence of an additional quenching mechanism in the *F2* mutant. Moreover, despite the lower Φ_{NPQ} , the *F2* mutant exhibited higher capacity for zeaxanthin formation (Fig. 3). This unexpected result indicates functional uncoupling of the xanthophyll cycle from NPQ in the *F2* mutant and also suggests a greater contribution of an additional quenching process independent of zeaxanthin-regulated NPQ. Indeed, earlier studies have suggested that xanthophyll cycle-dependent NPQ is independent on the peripheral antenna size (Gilmore *et al.*, 1996) and the presence of LHCII is not an absolute requirement for energy dependent NPQ (Falk *et al.*, 1994) in the *F2* barley mutant. The existence of such an additional quenching mechanism is also consistent with previous observations that significant levels of NPQ can occur independent of zeaxanthin (Hurry *et al.*, 1997; Demmig-Adams *et al.*, 1999; Finazzi *et al.*, 2004) and can not be accounted for by antenna quenching (Kramer *et al.*, 2004). The increased Φ_{NO} values in the *F2* mutant (Table 3) demonstrate that up-regulation of constitutive energy dissipation (Φ_{NO}) may compensate for the lower capacity for regulated NPQ (Φ_{NPQ}) and this additional mechanism might play an important role in photoprotection when the capacity for the primary, zeaxanthin-dependent non-photochemical quenching (Φ_{NPQ}) and state transitions (Ivanov *et al.*, 2006) pathways are restricted in the absence of LHCII polypeptides.

It has been proposed recently that dissipation of excess light energy within PSII reaction centre *via* shifts of the redox potentials of the electron acceptors Q_A and Q_B might serve as an additional quenching mechanism in cold acclimated plants (Ivanov *et al.*, 2003). It has been revealed by thermoluminescence measurements that narrowing the temperature gap (ΔT_M) between $S_{2/3}Q_B^-$ and $S_2Q_A^-$ charge recombinations increases the probability for an alternative non-radiative $P680^+Q_A^-$ radical pair recombination pathway for energy dissipation within the reaction centre of PSII (reaction center

quenching) (Sane *et al.*, 2002, 2003; Ivanov *et al.*, 2003, 2005, 2006). Indeed, reaction center quenching of excess light was suggested to play substantial role in supplementing the antenna based NPQ in cold acclimated Scots pine (Ivanov *et al.*, 2001) and cold hardened *Arabidopsis* (Sane *et al.*, 2003) and barley plants (Ivanov *et al.*, 2006) when the enzymatic conversion of violaxanthin to zeaxanthin within the xanthophyll cycle is thermodynamically restricted by low temperatures. In addition, it has been demonstrated that not only shifts to lower temperatures, but exposure to increased “excitation pressure”, i.e. higher growth light can also induce increased probability for reaction center quenching through a shift of the redox potentials of the electron acceptors (Ivanov *et al.*, 2006). Similar reversible changes of the redox potentials of Q_A and Q_B were observed in cold stressed cyanobacteria lacking Lhcb polypeptides and active xanthophylls cycle, thus suggesting that in some cases reaction center quenching can not only supplement, but even replace antenna based NPQ (Sane *et al.*, 2002).

Narrowing the temperature gap (ΔT_M) between $S_{2/3}Q_B^-$ and $S_2Q_A^-$ charge recombinations in *F2* mutant (Fig. 4, Table 4) corresponded with the increased proportion of constitutive non-photochemical quenching (Φ_{NO}) (Table 3) and suggests that dissipation of excess light energy within the reaction center of PSII may well serve as an additional quenching mechanism in the absence of Lhcb polypeptides and a reduced capacity for regulated NPQ. Recent studies demonstrating that decreased oligomerization of LHCII proteins in *Costata 2/133* pea mutant correlates with increased capacity for reaction center quenching (Ivanov *et al.*, 2005) and reaction center-type energy quenching depends on PSII antenna size (Zulfigarov *et al.*, 2007) support this suggestion.

The reduced amount of Lhcb proteins and decreased antenna size of PSII in *F2* mutant (Harrison *et al.*, 1993) is counterbalanced by increased amount of PSII reaction centers (Ghirardi *et al.*, 1986). Similar observation has been also reported earlier in a freeze-fracture electron microscopy study demonstrating decreased unit size and increased density of PSII units in the *Chlorina F2* mutant (Simpson, 1979). Our data also demonstrated a higher abundance of the D1 reaction center protein of PSII in the *F2* mutant compared to WT (Fig. 1B, Fig. 2B). It has been shown that some of these PSII centers are inactive in electron transport and may function as an additional antenna to the fraction of active PSII centers (Terao and Katoh, 1996). Recently, Matsubara and Chow (2004) have suggested that un-active (or

photoinhibited) centers of PSII can receive energy from neighboring functional PSII reaction centers and subsequently dissipates this energy non-photochemically *via* non-radiative charge recombination between Q_A^- and $P680^+$. We suggest that such a mechanism would provide effective photoprotection of the remaining functional PSII units, although the possibility for reaction center quenching in active PSII centers can not be ruled out. More detailed studies are needed to precisely quantify the capacity for reaction center quenching in both non-active and active PSII in the *F2* mutant.

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