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Correlation between photoinhibition sensitivity and the rates and relative extents of xanthophyll cycle de-epoxidation in *chlorina* mutants of barley (*Hordeum vulgare* L.)

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Abstract

We compared photoinhibition sensitivity to high irradiance (HI) in wild-type barley (wt) and both its *chlorina* f_{104}-nuclear gene mutant, that restricts chlorophyll (Chl) *a* and Chl *b* synthesis, and its f_2-nuclear gene mutant, that inhibits all Chl *b* synthesis. Both F_v/F_m and Φ_{PS2} decreased more significantly in f_2 than f_{104} and wt with duration of HI exposure. Chl degraded more rapidly in the f_2 than in either f_{104} or wt. Most sensitivity to photoinhibition was exhibited for f_2, whereas there was little difference in response to HI between the f_{104} and wt. The highest de-epoxidation (DES) value at every time point of exposure to HI was measured for f_2, whereas the wt had the lowest value among the three strains. There were two lifetime components resolved for the conversion of violaxanthin (V) to zeaxanthin plus antheraxanthin (Z + A). The most rapid lifetime was around 6 min and the slower lifetime was >140 min, in both the mutants and wt. However, the wt and f_{104} both displayed larger amplitudes of both de-epoxidation lifetimes than f_2. The difference between the final de-epoxidation state (DES = [Z + A]/[V + A + Z]) in the light compared to the dark expressed as ΔDES for wt, f_{104}, and f_2 was 0.630, 0.623, and 0.420, respectively. The slow lifetime component and overall larger ΔDES in the wt and f_{104} correlated with more photoprotection, as indicated by relatively higher F_v/F_m and Φ_{PS2}, compared to the f_2. Hence the photoprotection against photoinhibition has no relationship with the absolute DES value, but there is a strong relationship with de-epoxidation rate and relative extent or ΔDES.

Additional key words: antheraxanthin; chlorophyll *a* fluorescence; chlorosis; global kinetic analysis; non-photochemical quenching; photoprotection; violaxanthin; zeaxanthin.

Introduction

When plants are exposed to strong excessive irradiation, the absorbed photon energy can damage and reduce the capacity of the photosynthetic system, resulting in the phenomenon termed photoinhibition (Demmig-Adams and Adams 1992). Measuring electron transport and carbon assimilation, chlorophyll fluorescence (Krause and Weis 1991), or thermal de-excitation (Buschmann 1987) can detect photoinhibition. Photosystem 2 (PS2) is the major site of photoinhibtion even though the direct location of the primary target within PS2 and the mechanism of inhibition are still under discussion. Photoinhibition can be manifested as a photodegradation of both pigments and proteins, if the plants are not able to protection themselves against photoinhibitory damage (Lin *et al.* 1998). Thermal dissipation of excess excitation energy in the PS2 antenna depends on the xanthophyll cycle and is one of the main photoprotective mechanisms against photoinhibition in PS2 (Demmig-Adams and Adams 1992, Gilmore and Yamamoto 1993, Govindjee 1995, Gilmore and Govindjee 1999, Choudhury and Behera 2001, Müller *et al.* 2001). Thermal energy dissipation, often measured as a non-photochemical quenching of PS2

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Abbreviations: A – antheraxanthin; Chl – chlorophyll; DES – de-epoxidation state; f_2 – nuclear gene mutant of barley, that inhibits all Chl *b* synthesis; f_{104} – nuclear gene mutant of barley, that restricts Chl *a* and Chl *b* synthesis; HI – high irradiance; NPQ – non-photochemical quenching; PS – photosystem; V – violaxanthin; [VAZ] – summed concentration of pigments of the xanthophyll cycle; wt – wild phenotype of barley; Z – zeaxanthin.
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fluorescence (NPQ), protects PS2 by preventing excessive photon energy from reaching the PS2 reaction centres. NPQ prevents over-excitation and potentially harmful over-reduction of the primary electron acceptor components of PS2 that are associated with the formation of dangerous active oxygen species (Müller *et al.* 2001).

Chl-deficient mutants offer a potentially useful system with which to examine the factors that determine the photosynthetic performance of leaves. Reduction of Chl content reduces the ability of leaves to absorb photons. However, changes in the components and organisation of the light-harvesting apparatus could also change the efficiency with which absorbed photons are subsequently used in photosynthesis. Chl-deficient mutants of barley (Nielsen *et al.* 1979), maize (Greene *et al.* 1988), pea (Highkin *et al.* 1969), soybean (Keck *et al.* 1970), and tobacco (Okabe *et al.* 1977) have increased both Chl *a/b* ratios and rates of electron transport per unit Chl compared to the wild types. The latter symptom is indicative of a decreased PS2 antenna size. However, few investigations have been made to compare the sensitivity of such Chl deficiencies to photoinhibition or photoprotective xanthophyll cycle activity. In this study we compared the responses of leaves of two well-characterised light-harvesting barley mutants known as *chlorina* f_{104} (f_{104}) and *chlorina* f_2 (f_2) to HI stress. These mutants are distinguished by unique changes in the content and composition of the light-harvesting pigment-protein complexes associated with both PS2 and PS1. The Chl *b*-less f_2 mutant primarily lacks the major complement of Lhcb1 and Lhcb6 proteins associated with PS2, in addition to lacking the Lhca4, protein associated with PS1. The f_{104} mutant lacks a large complement of Lhcb1 in addition to lacking the 23 kD Lhca2 protein of PS1 (Bossmann *et al.* 1997).

Materials and methods

Plants: Seeds of wild-type (wt) barley (*Hordeum vulgare L.*) and the nuclear gene mutants f_2 and f_{104} were obtained from Prof. D. Simpson of the Carlsberg Research Laboratories (Denmark). Plants were grown from seeds for 3 to 4 weeks in a growth chamber under 16-h irradiation (230 μmol m^{-2} s^{-1}) and a day/night temperature of 22/17 °C.

HI treatment: Mature leaf segments (20 mm long and *ca.* 5 mm wide) were floated adaxial side up on distilled water in a container kept in a water bath (25 °C). Irradiation of 2 000 μmol(photon) m^{-2} s^{-1} was provided by a *HMI* Universal Spotlight (model *HMI 575W/GS*; *Osram*) behind a heat filter (*Schott 115*; *Tempax*) and a piece of glass. The HI-exposed leaves were then used for determinations of F_v/F_m, Φ_{PS2}, pigment contents, and 77 K fluorescence emission spectra as described below. F_v/F_m and Φ_{PS2} were measured at different time points (20, 40, 60, 80, and 100 min). The treated leaves were recovered at low irradiance (20 μmol m^{-2} s^{-1}) for 2 h at room temperature (25 °C) during which time the recovery of F_v/F_m was also determined. All the data are means ± s.d. of three replicates.

Modulated Chl fluorescence measurements were carried out with a *Walz PAM 101* fluorometer (*H. Walz*, Effeltrich, Germany) (Schreiber *et al.* 1986). The treated detached leaves were dark-adapted (<10 μmol m^{-2} s^{-1}) for 20 min prior to fluorescence determinations. The F_0 level of fluorescence was determined with the low intensity modulated beam (1.6 KHz, <0.15 μmol m^{-2} s^{-1}, 440 nm) and the emission was monitored with a filter passing all radiation >660 nm. The initial F_m level was monitored with a saturating 2 s pulse of "white light" (>10 000 μmol m^{-2} s^{-1}) passed through an infrared heat filter (*DT Cyan*, *Walz*), while the *PAM* modulated measuring beam automatically switched to 100 kHz. The "white actinic light" (500 μmol m^{-2} s^{-1}), passed through a heat filter (*DT Cyan*, *Walz*), lasted 15 min at each min of which F'_m was determined during 2-s saturating pulse. All fluorescence intensity parameters were used as defined by van Kooten and Snel (1990). The irradiance-limited PS2 quantum efficiency was calculated as the ratio F_v/F_m, where the variable fluorescence, $F_v = F_m - F_0$. The quantum yield of PS2 dependent non-cyclic electron transport (Φ_{PS2}) was given by the equation $(F_m' - F_s)/F'_m$ (Genty *et al.* 1989).

Photosynthetic pigments were extracted and assayed by HPLC as described by Gilmore and Yamamoto (1991). Absorption at 440 nm was measured with a *Waters 490* (*Waters*, Milford, USA) variable wavelength detector.

De-epoxidation (DES) increasing rates in mutants and wt were expressed by the ratio (×100 %) of DES values at different time points of HI exposure (20, 40, 60, 80, and 100 min) to pre-treatment. At the same time the decrease of F_v/F_m or Φ_{PS2} at the corresponding time point was calculated as the percentage of the pre-treatment control. The linear relationship between the degree of photoinhibition and the increasing rate of DES was obtained by plotting the decrease rate of F_v/F_m or Φ_{PS2} [% of pre-treatment] against the increase rate of DES [% of pre-treatment] for the two mutants and wild-type exposed to HI for different time.

Results

Effect of HI on F_v/F_m and Φ_{PS2} in leaves of barley wild type and *chlorina* mutants: When detached leaves of mutants and wt were exposed to 2 000 μmol m^{-2} s^{-1} PPFD at room temperature (25 °C) for the different time intervals shown in Fig. 1, F_v/F_m decreased with the duration of treatment as an indicator of PS2 photoinhibition (Fig.1*A*). Leaves of f_2 exhibited the largest and most rapid declines in F_v/F_m, whereas there was little difference between f_{104} and wt. The inhibition of F_v/F_m could be partly recovered in all three strains of barley at all treatment time points (Fig. 1*B*). Consistent with the relative degree of photoinhibition, the recovery from photoinhibition was most obvious in f_{104} and wt, being least and slowest in the f_2.

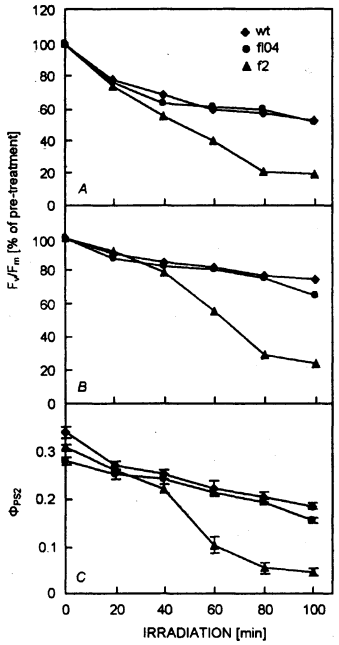

Fig. 1. Changes in quantum efficiency of PS2, measured as the ratio of variable to maximal Chl *a* fluorescence (F_v/F_m) (*A, B*) or Φ_{PS2} (*C*) during exposure of barley *chlorina* mutant and wild type leaves to high irradiance (*A, C*) or during a subsequent dark recovery period (*B*). Irradiance was 2 000 (*A*) or 20 (*B*) μmol m^{-2} s^{-1} (PPFD) at 25 °C. The time scale in *B* refers to the length of time the samples were exposed to high irradiance in *A*. All samples underwent the same 2-h recovery period.

Fig. 1*C* shows the time course for the changes in the quantum yield of electron flow through PS2 (Φ_{PS2}) during HI exposure. Φ_{PS2} depends on both the efficiency of excitation capture by PS2 open centres (F_s/F_m') and the proportion of PS2 open centres (q_P). Therefore Φ_{PS2} is positively related to PS2 activity (Krall and Edwards 1992, Wullschleger 1993). After 100 min treatment with HI, the Φ_{PS2} decreased by 47.06 (wt), 46.43 (f_{104}), and 87.10 (f_2) % compared to the pre-treated controls. Clearly there was more reduction of Φ_{PS2} in the f_2 than in f_{104} or wt, which was of course consistent with the changes observed for F_v/F_m in Fig. 1.

Changes in photosynthetic pigments in mutants and wt exposed to HI: The pre-irradiation control Chl content was the highest in leaves of wt and lowest in f_2 (Fig. 2*A*). The starting contents of Chl in f_{104} and f_2 were 59.7 and 43.4 % of wt, respectively. During the HI exposure there was a slight decrease in Chl content in f_{104} and wt, and significant decrease in f_2. In comparison to pre-treatment controls, Chl contents were reduced by 47, 27, and 22 % in the f_2, f_{104}, and wt after 100 min of HI. Fig. 2*B* illustrates no marked change of Chl *a/b* with irradiation time in f_{104} and wt treated with HI. f_{104} had higher values of Chl *a/b* than wt and of course the f_2 mutant never contained Chl *b*. Thus extended HI treatments induced pigment photooxidation or bleaching in leaves of both mutants and wt.

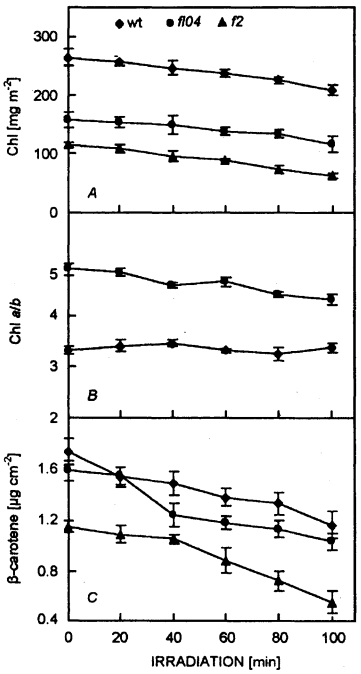

Fig. 2. Changes in leaf area-based chlorophyll (Chl) *a+b* (*A*) and β-carotene (*C*) contents and Chl *a/b* ratio (*B*) in leaves of barley *chlorina* mutants (f_{104}, f_2) and wt exposed to high irradiance for the indicated times. s.d. of three replicates.

β-carotene acts as an important photoprotective agent against photoinhibition by quenching active oxygen molecules (Gilmore and Govindjee 1999, Pogson and Rissler 2000). The starting contents of β-carotene in f_2 and f_{104} were 66.1 and 91.4 % of wt, respectively (Fig. 2*C*). After 100 min of HI, the β-carotene content decreased by 52.5, 35.2, and 33.3 % in the f_2, f_{104}, and wt, respectively. Hence (Figs. 3 and 4) f_2 suffered the most severe photoinhibition reaction manifested as carotenoid pigment bleaching and chlorosis under the HI treatment.

Changes in the de-epoxidation state (DES) in mutants and wt exposed to HI: The photon-driven de-epoxidation of V to yield Z *via* A was characterised using the de-epoxidation state or DES = [Z + A]/[Z + A + V] (Gilmore and Björkman 1994). The changes of DES and V in the two *chlorina* mutants and wt increased during exposure to HI (Fig. 3). Kinetic analysis showed two phases

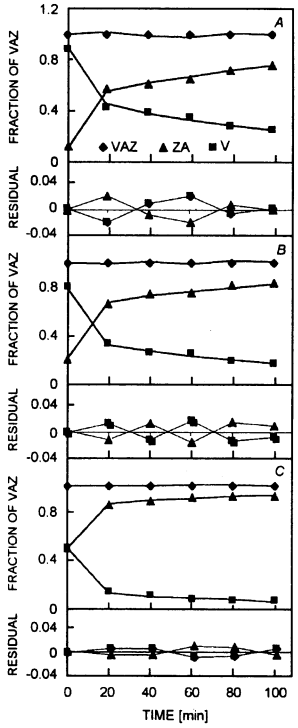

Fig. 3. Global kinetic analysis of the de-epoxidation of violaxanthin to zeaxanthin and antheraxanthin in leaves of wild-type barley and *chlorina* mutants during high irradiance treatment. The main plots in *A* (wt), *B* (f_{104}), and *C* (f_2) show values (*symbols*) and model fits (*lines*) for the relative concentrations of [V]/[V + A + Z], [Z + A]/[V + A + Z] and [V + A + Z]. The sub-panels illustrate the un-weighted residual errors (data-model) for each of the three parameters at each corresponding time point. The coefficient of determination for the model fit was $r^2 = 0.999$.

of de-epoxidation. Global analysis indicated the most rapid phase exhibited a lifetime around 5 min and the slower lifetime component was around 140 min; all three lines could be reasonably well fit assuming the V de-epoxidation kinetics were described by varying amplitudes of the same two components. The largest increase in DES occurred in the first measurement at 20 min for all three strains. f_2 leaves exhibited the highest DES value at every time point of exposure to HI, whereas wt had the lowest values among the three strains. The wt and f_{104} displayed larger amplitudes of both V de-epoxidation lifetimes than f_2 (Fig. 4*A*). The fact that the total sum of the amplitudes was less in f_2 than in wt and f_{104} indicated that the total amount of V converted to Z+A in f_2 was less in the 100-min time frame. The relative light-dark change in DES or ΔDES is consistent with the relative de-epoxidation amplitudes (Fig. 4). The ΔDES in the wt, f_{104}, and f_2 was 0.630, 0.623, and 0.420, respectively, which was consistent with the relative degrees of photoinhibition. Moreover, the scant differences in ΔDES found between wt and f_{104} were also consistent with them showing similar photoprotective capacities.

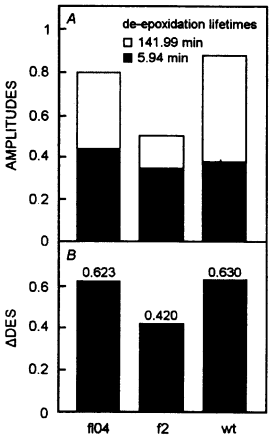

Fig. 4. Changes in amplitudes of the violaxanthin de-epoxidation lifetime component [min] corresponding to the model fit shown in Fig. 3 (*A*) and the relative changes in the de-epoxidation state (*B*). The ΔDES was calculated as the difference between the DES measured in the dark-adapted leaves before irradiation to the DES measured after 100 min of irradiation.

Relationships between the increasing rates of photoinhibition and increasing rates of the de-epoxidation state: The extent of photoinhibition rate expressed as decreasing of F_v/F_m or Φ_{PS2} (% of pre-treatment) was plotted against the increasing rate of DES (% of pre-treatment) for the two mutants and wt exposed to HI for different time (Fig. 5). In this way near-linear correlations were obtained between decreasing F_v/F_m (Fig. 5*A*) or Φ_{PS2} (Fig. 5*B*) and changes of DES. The linear regres-sion statistics of the model lines shown in Fig. 5 were tabulated (Table 1) to show that the f_2 mutant exhibited almost 1 order of magnitude lower slope parameter for both the DES *versus* F_v/F_m and Φ_{PS2} plots. The significance of all the statistical parameters was evaluated with *p*-values less than or equal to 0.05 and the uncorrected coefficients of determination were all better than 85 %. The slope parameters for the plots in Fig. 5 and Table 1 were most sensitive to the slow >140 min DES component, compared to the rapid 6 min component. Thus we surmise that the resistance to photoinhibition in wt and f_{104} is strongly correlated with the larger amplitudes of the slower lifetime component of DES described in Figs. 3 and 4. We conclude that it is the relative extent and amplitudes of DES, especially the slower components, not the absolute value of DES, that correlate most strongly with resistance to photoinhibition when comparing the barley *chlorina* mutants and wt.

Table 1. Linear regression model and statistical parameters defining percent increase in DES as a function of percent decrease in photosystem 2 (PS2) quantum efficiency (F_v/F_m) and as quantum yield of electron transport through PS2 (Φ_{PS2}). P values denote the probability for the null hypothesis of the slope or the intercept R^2 is the coefficient of determination. $n = 5$.

	Slope (P)	Intercept (P)	R^2	Adjusted R^2
	Model equation: % change DES = slope × (percent change of F_v/F_m) + intercept			
wt	0.0592±0.0114 (≤0.05)	3.2850±0.0114 (≤0.01)	0.8993	0.8657±0.2287
f_{104}	0.0358±0.0057 (≤0.01)	2.4220±0.2239 (≤0.01)	0.9285	0.9047±0.1031
f_2	0.0025±0.0003 (?0.01)	1.6434±0.0010 (≤0.001)	0.9493	0.9325±0.0159
	Model equation: % change DES = slope × (percent change of Φ_{PS2}) + intercept			
wt	0.0650±0.0126 (≤0.05)	2.8479±0.5232 (≤0.05)	0.8991	0.8654±0.2289
f_{104}	0.0212±0.0055 (≤0.05)	3.2442±0.1581 (≤0.001)	0.8325	0.7766±0.1580
f_2	0.0018±0.0003 (≤0.01)	1.6880±0.0159 (≤0.001)	0.9472	0.9296±0.0160

Discussion

Similar to other Chl mutants (Highkin *et al.* 1969, Keck *et al.* 1970, Okabe *et al.* 1977, Greene *et al.* 1988), significant reduction in total Chl content per leaf area was found in the Chl *b*-less mutant f_{104} (40.3 %) and another Chl *b*-deficient mutant f_2 (56.4 %) in comparison with wt of barley (Fig. 2*B*). These two mutants also showed different responses to photoinhibtion induced by HI. The f_2 mutant was most susceptible to photoinhibition expressed as the decrease in F_v/F_m or Φ_{PS2} (Fig. 1). This result is consistent with that of Leverenz *et al.* (1992). However, little difference was observed in relative susceptibility to photoinhibition between the f_{104} and wt. The wt and f_{104} also exhibited similar and lower degrees of Chl loss compared to f_2 (Fig. 2*A*). Hence for f_{104} we found a reduction of total Chl content to 40-56 % of the wt value and the elevated Chl *a/b* ratio (4.3-4.9) had little effect on its resistance to photoinhibition. We suggest that the photoinhibition sensitivity in f_{104} may be influenced by its decreased cross section of photon absorption and PS2 antenna size.

Non-photochemical quenching (NPQ) that depends on the xanthophyll cycle is one of the most important regulation mechanisms in photoprotection. Many studies found a linear relationship between NPQ and Z content in leaves or chloroplasts (Demmig-Adams 1990). Gilmore and Yamamoto (1993) showed that A can also act as a quencher of excess energy like Z, and (Z+A) had a better correlation to NPQ than Z. In this study, however, the different photoinhibition sensitivity to HI in leaves of wt, f_{104}, and f_2 obviously cannot be simply explained by the Z or (Z+A) content alone. f_2 had the highest value of Z (values not shown) or DES before treatment and at every time point of HI treatment, whereas wt had the lowest value (Fig. 3). The highest value of Z content and DES in f_2 seems to contradict its highest sensitivity to photoinhibition. This apparent contradiction also existed in other reports (Leverenz *et al.* 1992, Schindler *et al.* 1994), but there is no reasonable explanation for it till now. Xu *et al.* (2000) showed that the ability of protection against photoinhibition in different chilling-sensitive plants was not correlated to their Z content under chilling-induced photoinhibition, but displayed some relationship to the rate of Z formation.

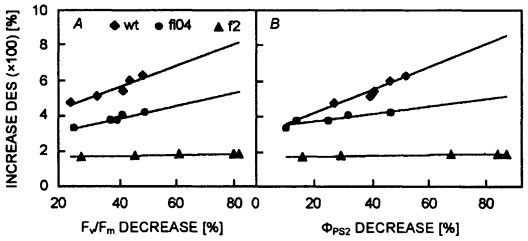

Fig. 5. Relationship between photoinhibition degree [% of quantum efficiency of PS2] measured as the ratio of variable to maximal chlorophyll *a* fluorescence (F_v/F_m) (*A*) or quantum efficiency of electron transport through photosystem 2 (Φ_{PS2}) (*B*) and changes of DES in mutants and wild-type under high irradiance treatment.

Our results showed that the photoinhibition degree had no apparent relationship with Z contents or DES values. On the other hand, there was a strong correlation between photoprotection and the relative changes of DES (Figs. 4 and 5). The f_2 clearly exhibited the lowest amplitudes for both the rapid and slow V de-epoxidation components and ΔDES (Fig. 4). Furthermore, a large pool of Z + A persists in the dark and it is possible this pool may not be part of the active xanthophyll cycle or NPQ mechanism and hence may not directly play a part in photoprotection (Pogson *et al.* 1998, Gilmore 2001).

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