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Photoprotective energy dissipation in higher plant leaves investigated by chlorophyll fluorescence decay measurements with additional radiation pulses

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Abstract

Chlorophyll (Chl) fluorescence decay measurements were performed on higher plant leaves to investigate the photoprotective mechanisms under *in vivo* conditions. Measurements on leaves with different amounts of zeaxanthin pointed out that zeaxanthin is necessary for most of the observed nonphotochemical energy quenching, that has to be activated by a transthylakoid ΔpH. An additional sustained energy quenching component was clearly resolved in leaves with high amounts of zeaxanthin. The changes of the Chl fluorescence decay parameters did not correlate with a photoprotective energy dissipation in the reaction centre of photosystem 2 (P680), nor with a ΔpH-mediated, zeaxanthin-independent aggregation of the antenna complexes; no indications for a state 1/state 2 transition of the main light-harvesting complex LHC2 were found.

Additional key words: energy quenching; *Pisum sativum*; state 1/state 2 transition; sustained energy quenching; xanthophyll cycle; zeaxanthin.

Introduction

Higher plant leaves are able to dissipate part of the absorbed energy under increased irradiances to avoid photoinhibitory damages. Most of this so-called nonphotochemical energy quenching (q_E) occurs in photosystem (PS) 2 and can be assessed by changes in the Chl fluorescence decay parameters. Different mechanisms of q_E were proposed: (*1*) quenching in the reaction centre of PS2 (Weis and Berry 1987, Krieger *et al.* 1992), (*2*) quenching in the antenna complexes of PS2 in the

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Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DTT, dithiothreitol; D1, the 32 kDa protein of the PS2 core; FWHM, full width at half maximum; LHC2, main light-harvesting complex of PS2; PS, photosystem; P680, reaction centre of PS2; Q_A, primary quinone in PS2; τ_m, average chlorophyll fluorescence decay time; TLC, thin layer chromatography.
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presence of zeaxanthin (and perhaps antheraxanthin) and a transthylakoid ΔpH (Demmig-Adams *et al.* 1996, Gilmore 1997), and (*3*) quenching by a ΔpH-mediated, zeaxanthin-independent aggregation of the antenna complexes of PS2 (Ruban and Horton 1992, Mullineaux *et al.* 1993). Another energy dissipation process is the so-called sustained or inhibitory energy quenching (q_I). Two different mechanisms are under discussion: (*a*) a stable quenching configuration in the presence of high amounts of zeaxanthin (Adams *et al.* 1995, Demmig-Adams *et al.* 1995, Thiele *et al.* 1996), and (*b*) a quenching due to damaged D1 proteins (Aro *et al.* 1993, Anderson *et al.* 1997). The slow deactivation of q_I was proposed to correlate with the epoxidation of zeaxanthin and antheraxanthin to violaxanthin or the insertion of new D1 proteins, respectively. Another photoprotective mechanism is the so-called state 1/state 2 transition (Bennett 1991, Allen 1995). Reduction of the plastoquinone pool leads to the activation of a thylakoid protein kinase; the main light-harvesting complex LHC2 is phosphorylated by this kinase, detached from PS2, and shifted to PS1. Increased excitation energy transfer to PS1 was observed by increased Chl fluorescence intensities around 740 nm (Saito *et al.* 1983, Larsson *et al.* 1986), but not observed in all investigations (Harrison and Allen 1992). The significance and molecular mechanism of this adaptation process is still unknown (Horton *et al.* 1996, Gal *et al.* 1997). The following points were investigated by Chl fluorescence decay measurements under different irradiances: what is the origin of q_E in higher plant leaves, is a sustained energy quenching component (q_I) present, and are there any indications for a state 1/state 2 transition in higher plant leaves?

Materials and methods

Fully developed leaves of pea (*Pisum sativum* L. cv. Lancet) were investigated in three different photosynthetic states: (*1*) dithiothreitol (DTT)-treated: DTT that blocks the formation of zeaxanthin and antheraxanthin (Bilger and Björkman 1990) was provided through the cut petiole. Leaves were set into a 3 mM DTT solution and kept in darkness for a minimum of 12 h to allow uptake and epoxidation of zeaxanthin and antheraxanthin to violaxanthin (Demmig-Adams *et al.* 1990, Ruban *et al.* 1991). The high amount of violaxanthin was also checked by thin layer chromatography (TLC) following the procedure of Lichtenthaler and Pfister (1978). The molar ratio of Chl *a*/violaxanthin was about 10.5/1.0. Prior to the first measurement, the DTT-treated leaves were submitted to weak "white light" (about 50 μmol m^{-2} s^{-1}) for *ca.* 20 min to induce photosynthetic activity. (*2*) Low irradiance-adapted: Attached leaves were adapted to low irradiances by exposing them to 50 μmol m^{-2} s^{-1} of "white light". The leaves were submitted to this irradiance for about 2 h to allow the epoxidation of zeaxanthin and antheraxanthin to violaxanthin; the reverse step of de-epoxidation was strongly reduced during that period of time (Härtel *et al.* 1996, Jahns and Miehe 1996). The large amount of violaxanthin was also confirmed in TLC measurements with a Chl *a*/violaxanthin ratio of 9.5/1.0. (*3*) High irradiance-adapted: Attached leaves were adapted to high irradiances by exposing them to 1400 μmol m^{-2} s^{-1} of a *Schott KL1500* lamp. Minimal exposure

time was 1 h to allow the de-epoxidation of most of violaxanthin to zeaxanthin (Härtel *et al.* 1996, Jahns and Miehe 1996); hardly any violaxanthin was observed in TLC measurements. The applied irradiance was also weak enough to avoid photoinhibitory damages (Terjung and Maier 1998).

3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was used to block the electron transfer after the primary quinone, Q_A (Velthuys 1981). Leaves were put into a 1 mM solution for about 15 min, long enough to block all PS2 centres.

Chl fluorescence decay measurements were based on the technique of inverse time-correlated single photon counting (O'Connor and Phillips 1984). Excitation pulses (about 15 ps FWHM) were generated by a synchronously pumped and cavity-dumped dye laser-system (*Spectra Physics*) with a repetition rate of 800 kHz. The selected excitation wavelength was 650 nm. Additional radiation pulses were generated by a light-emitting diode (selected *LED, Hewlett Packard HLMP8103*, with a red-shifted peak-wavelength of 650 nm, top LED in Fig. 1). The LED was pulsed by a pulse generator (*Hewlett Packard 8013B*) that was triggered by the laser system. The additional radiation pulses had a width of 800 ns, the same repetition rate as the laser system, and an irradiance of 110 μmol m^{-2} s^{-1}. The additional radiation pulses were positioned between two measuring laser pulses (timing scheme see Fig. 2) and used to excite the leaf area where the Chl fluorescence decay measurements were made. It was possible to irradiate other parts of the leaf by an LED-array with LEDs of the same type and intensity (see Fig. 1).

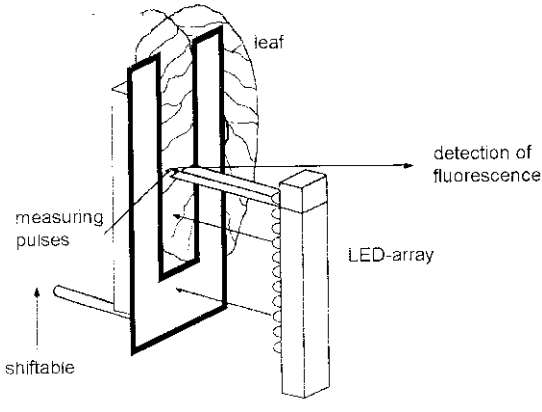

Fig. 1. Experimental set-up of the different radiation sources.

The irradiance of the pulsed LED (top LED in Fig. 1) could be increased to such values that the additional radiation pulses were able to close all PS2 centres for a short time. These so-called saturating radiation pulses were of 1100 μmol m^{-2} s^{-1}. The leaf was moved to avoid adaptation processes and to get a better statistic of the Chl fluorescence decay under this condition (Terjung *et al.* 1997). Only a very weak adaptation of the photosynthetic apparatus occurred.

The Chl fluorescence decay was measured at the wavelength of 685 nm. The fluorescence detection system consisted of a double monochromator (*Jobin Yvon*

H25) in substractive mode and a multichannel plate-photomultiplier (*Hamamatsu R1564u-07*). The time response of the setup was about 70 ps (FWHM). An electronic gating of the constant fraction discriminator (*Tennelec TC 454*) was applied to

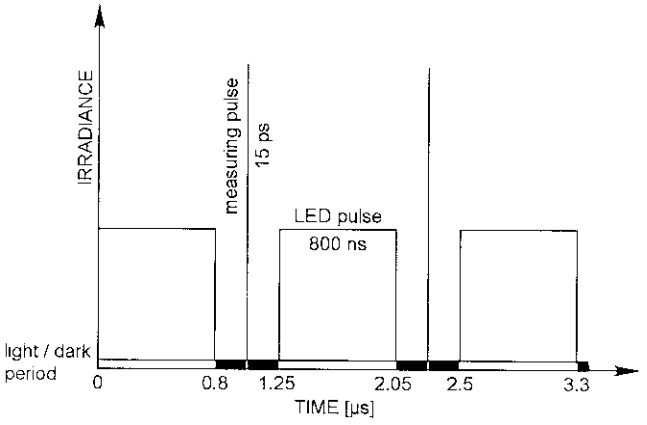

Fig.2. The timing scheme of the measuring laser pulses and the additional LED pulses; the irradiances depend on the selected condition (see Table 1).

process only the fluorescence photons induced by the measuring laser pulses. Fluorescence decay curves were analysed using iterative deconvolution techniques (Marquardt algorithm). The quality of the fits was controlled by the reduced chi-square criterium χ_{red}^2 (between 1.0 and 1.1 for all decay curves) and the random distribution of the weighted residuals. The obtained amplitudes α_i and lifetimes τ_i were used to calculate the average Chl fluorescence decay time τ_m:

$$\tau_m = \Sigma\, \alpha_i\, \tau_i^2 / \Sigma\, \alpha_i\, \tau_i$$

A decay time of 80 ps, characteristic for PS1 centres (Holzwarth 1991, Trissl *et al.* 1993), was observed in all Chl fluorescence decay measurements; no variations of this decay time were found. Both other decay components were assigned to PS2; they varied in decay time and amplitude in dependence on the photosynthetic state.

Results

Chl fluorescence decay measurements (repeated at least on three other leaves in the same photosynthetic state) were performed under four different conditions of irradiation (Table 1). The laser intensity in conditions *A* and *C* was weak enough to avoid the presence of reduced Q_A (closure of PS2 centres). The saturating radiation pulses in conditions *B* and *D* closed all PS2 centres, so the increased laser intensity under these conditions had no effects on the photosynthetic state but enabled a better statistic of the Chl fluorescence decay.

In a DTT-treated leaf (Table 2), a fast Chl fluorescence decay was observed in condition *A*, characteristic for open PS2 centres. The saturating radiation pulses

(condition *B*) closed all PS2 centres and caused the typical slow Chl fluorescence decay. The leaf was adapted to the additional LED irradiance of 110 μmol m^{-2} s^{-1} (condition *C*) for 20 min before the Chl fluorescence decay was measured again. Some PS2 centres were closed by the additional LED irradiance and caused the slower Chl fluorescence decay in comparison to condition *A*. Addition of the saturating radiation pulses closed all PS2 centres (condition *D*) and led to the slow Chl fluorescence decay, that was slightly faster in comparison to condition *B*. The final treatment with DCMU led to a Chl fluorescence decay similar to the one observed in condition *B*.

Table 1. The applied irradiances [μmol m^{-2} s^{-1}] in the four measuring conditions (for experimental set-up see Fig. 1, for timing scheme see Fig. 2).

Condition	Irradiance by measuring laser	additional LED pulses	Measuring conditions
A	3	-	Measurement with laser pulses
B	22	1100	Exposure to additional LED pulses with saturating irradiance. The leaf was shifted to avoid adaptation processes
C	3	110	Exposure to additional LED pulses for 20 min to allow photosynthetic adaptation
D	22	1100 + 110	Exposure of whole leaf to additional LED irradiance (LED-array) of 110 μmol m^{-2} s^{-1} for 20 min to allow photosynthetic adaptation. The leaf was shifted and measurements were done in presence of saturating LED pulses (top LED in Fig. 1) with 1100 μmol m^{-2} s^{-1}

The same measuring protocol was applied to investigate the adaptation processes of a low irradiance-adapted leaf (Table 2). The Chl fluorescence decay in the conditions *A* and *B* was similar to the DTT-treated leaf. The Chl fluorescence decay in condition *C* was slower in comparison to condition *A*, due to the closure of PS2 centres, but faster in comparison to the DTT-treated leaf, probably due to energy quenching processes. This energy quenching was clearly resolved in condition *D*: the Chl fluorescence decay was significantly faster in comparison to condition *B*, and also to condition *D* of the DTT-treated leaf. An increase of the saturating irradiance had no effect on the Chl fluorescence decay (values not shown), indicating the closure of all PS2 centres by the applied irradiance. The final treatment with DCMU led to a Chl fluorescence decay that was comparable to the Chl fluorescence decay in condition *B*. These measurements indicate that the additional LED irradiance of 110 μmol m^{-2} s^{-1} was able to provide a ΔpH and to "switch on" q_E in the presence of low amounts of zeaxanthin (and perhaps antheraxanthin).

In a high irradiance-adapted leaf (Table 2), the first measurement of Chl fluorescence decay parameters was made in condition *C* to maintain the high irradiance-adapted state, followed by the measurement in the presence of the saturating radiation pulses (condition *D*). The leaf was adapted to the weak measuring laser pulses

for *ca.* 20 min and the Chl fluorescence decay was measured again (condition *A*), followed by the measurement with the saturating radiation pulses (condition *B*).

Table 2. The relative amplitudes α_i, the decay times τ_i, and the average chlorophyll fluorescence decay time τ_m of the fluorescence decay of a DTT-treated, low and high irradiance-adapted leaves under different measuring conditions.

Leaf	Condition	α_1 [%]	α_2	α_3	τ_1 [ps]	τ_2	τ_3	τ_m
DTT-treated	*A*	47	34	19	80	413	943	644
	B	42	11	47	80	1100	2372	2190
	C	45	32	23	80	599	1281	948
	D	42	23	35	80	1091	2135	1814
	+DCMU	45	19	36	80	1076	2591	2250
Low irradiance-adapted	*A*	47	29	24	80	312	827	598
	B	43	12	45	80	1101	2353	2154
	C	42	32	26	80	437	982	734
	D	47	33	20	80	681	1550	1112
	+DCMU	44	19	37	80	1013	2692	2353
High irradiance-adapted	*A*	46	33	21	80	363	851	592
	B	43	37	20	80	868	1827	1316
	C	46	32	22	80	316	654	458
	D	47	37	16	80	570	1184	794
	+DCMU	42	37	21	80	1002	2183	1594

A typical fast Chl fluorescence decay was observed in condition *A*, comparable to the DTT-treated leaf and the low irradiance-adapted leaf. The Chl fluorescence decay in the presence of the saturating radiation pulses (condition *B*) was faster compared with the DTT-treated leaf and the low irradiance-adapted leaf, due to a sustained energy quenching process (q_I). The Chl fluorescence decay in condition *C* was faster in comparison to condition *A*, in contrast to the DTT-treated leaf and the low irradiance-adapted leaf; this indicates the dependence of the "switched on" q_E on the amount of zeaxanthin. The Chl fluorescence decay in condition *D* was only slightly slower in comparison to condition *C*, and significantly faster in comparison to the DTT-treated leaf and the low irradiance-adapted leaf. Separate measurements on other leaves indicated that all PS2 centres were closed by these saturating radiation pulses (values not shown). The final treatment with DCMU caused a Chl fluorescence decay similar to condition *B*; the slightly slower Chl fluorescence decay is probably due to the slow relaxation of q_I.

Discussion

All investigated leaves showed a fast Chl fluorescence decay in the irradiation condition *A*, indicating a high electron transfer activity in PS2. The saturating

radiation pulses closed all PS2 centres (condition *B*) and clearly indicated a sustained energy quenching process (q_I) in the high irradiance-adapted leaf (Table 2). No damages on the donor- and acceptor-site of PS2 were observed in delayed fluorescence decay measurements, made on leaves in the same photosynthetic states (Terjung and Maier 1998); the observed q_I seems to be correlated with the amount of zeaxanthin and a stable energy quenching configuration, as also proposed by Demmig-Adams *et al.* (1995), Adams *et al.* (1995), and Thiele *et al.* (1996). The damage of the D1 proteins by high irradiances, as observed by Aro *et al.* (1993) or Anderson *et al.* (1997), played obviously no role. The additional LED irradiance of 110 μmol m^{-2} s^{-1} (condition *C*) provided a transthylakoid ΔpH and was able to "switch on" q_E. The extent of q_E depended strongly on the amount of zeaxanthin (and perhaps antheraxanthin). The ability of energy quenching was also observed in measurements with the additional LED irradiance and the saturating radiation pulses (condition *D*). Energy quenching was clearly observed in the high irradiance-adapted leaf, but also in a lesser extent in the low irradiance-adapted leaf, due to the low amounts of zeaxanthin (and perhaps antheraxanthin). Hardly any energy quenching was observed in measurements on the DTT-treated leaf, due to the absence (or very low amounts of) zeaxanthin. The slightly faster Chl fluorescence decay of the DTT-treated leaf in condition *D* in comparison to condition *B* can have many reasons, like the energy quenching by some retained zeaxanthin in the presence of the ΔpH, quenching in the reaction centre of PS2 (Weis and Berry 1987, Krieger *et al.* 1992) or quenching by the ΔpH-mediated, zeaxanthin-independent aggregation of antenna complexes (Ruban and Horton 1992, Mullineaux *et al.* 1993). But this energy quenching is minimal in comparison to the effects in the presence of zeaxanthin and the ΔpH, so the energy quenching in the reaction centre P680 and the ΔpH-mediated, zeaxanthin-independent aggregation of the antenna complexes do not play an important role in photoprotection of higher plant leaves.

The additional LED irradiance of 110 μmol m^{-2} s^{-1} was high enough to reduce the plastoquinone pool and activate the thylakoid protein kinase (conditions *C* and *D*). A state 1/state 2 transition would have been correlated with an increase in the relative amplitude of the 80 ps decay component; this was not observed (Table 2). Separate measurements on the spectral distribution of the Chl fluorescence on leaves in the same photosynthetic states did not give any indications for a state transition, too (to be published).

In conclusion, Chl fluorescence decay measurements with additional radiation pulses made it possible to take into consideration all photoprotective mechanisms discussed in the literature under *in vivo* conditions. The extent of q_E was correlated with the amount of zeaxanthin in the presence of the activating ΔpH. Sustained energy quenching (q_I) was clearly resolved in high irradiance-adapted leaves, correlating with a stable energy quenching configuration in the presence of zeaxanthin. Reaction centre-quenching, ΔpH-mediated, zeaxanthin-independent aggregation of antenna complexes, and the state 1/state 2 transition played only a minor (if any) role in photoprotection of higher plant leaves.

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