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Picosecond fluorescence decay studies on water-stressed pea leaves: energy transfer and quenching processes in photosystem 2

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Abstract

Detached leaves of pea (*Pisum sativum*) were submitted to water stress at different relative air humidities. The photosynthetic activity of photosystem 2 (PS2) was monitored by time-resolved picosecond chlorophyll (Chl) fluorescence spectroscopy. In the first days the well-known fast Chl fluorescence decay was observed which indicated high PS2 activity. After a few days the average fluorescence decay time τ_m reached a maximum, depending on the wilting conditions, but always at a relative loss of leaf mass of 80 %. After this maximum, τ_m decreased within a few hours, the fluorescence decay became similar to that one of an intact leaf, but an additional fluorescence decay component with a lifetime of 3.6 ns appeared. At first the primary quinone Q_A was reduced due to inhibition of the electron transfer to the secondary quinone Q_B. Simultaneously, water deficiency caused an electron lack at the oxidizing site of PS2. This disabled the primary electron donor of PS2, tyrosine Z, from reducing the oxidized reaction centre of PS2 ($P680^+$). Thus a recombination of $P680^+$-pheophytin-Q_A^- took place, and the energy was lost as heat. With further water stress, Q_A was decoupled from PS2. The new fluorescence decay component could therefore be assigned to energetically decoupled antenna complexes.

Additional key words: chlorophyll; DCMU; exciton-radical pair equilibrium model; NH_2OH; photosynthesis; *Pisum*; quinones; relative air humidity; wilting.

Introduction

Absorption of photons in the light-harvesting complex of PS2 (LHC2) leads to an excited state. The excitation energy transfer to the reaction centre of PS2 ($P680^*$) is a multiphasic process with kinetics that range from some hundreds of femtoseconds to about 30 ps. Charge separation occurs by the transfer of an electron from $P680^*$ to

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Abbrevations: DCMU, 3-(3,4-dichlorphenyl)-1,1-dimethylurea; Pheo, pheophytin; PQ, plastoquinone pool; PS, photosystem; Z, tyrosine Z.

the primary acceptor pheophytin (Pheo) in some hundreds of picoseconds. In intact PS2 this charge separation is stabilized by the reduction of the primary quinone Q_A and an electron transfer to the plastoquinone pool (PQ) (for a review see van Grondelle *et al.* 1994).

In general, energy trapping and transfer in intact PS2 could be well described within the exciton-radical pair equilibrium model (Schatz *et al.* 1988, Roelofs *et al.* 1992). Moreover, the transition from open to closed PS2 (P680-Pheo-Q_A → $P680^+$-Pheo-Q_A^-) and the corresponding fluorescence decay kinetics could be interpreted in this model as well.

In recent studies the influence of several factors such as herbicides or sulfide on the Chl fluorescence decay has been examined and discussed in the exciton-radical pair equilibrium model. For these factors the average fluorescence decay time τ_m was shown to be a suitable parameter to characterize the state of the oxygenic photosynthesis (Maier-Schwartz *et al.* 1992, Pearlstein 1992, Maier *et al.* 1995, Terjung *et al.* 1996).

However, under water stress the situation is more complicated and the different decay parameters have to be investigated. Various methods have been applied and the observed results vary from complete conservation to irreversible damage of PS2 and PS1.

The CO_2 exchange rate decreases due to stomatal closure in water-stressed plants. In addition, the activity of nitrate reductase and sucrose-phosphate synthase is reduced (Sharkey 1990). Meyer *et al.* (1992) reported a dramatic drop of the lipid content of thylakoids submitted to water stress. These lipids are related to the transport of the proteins in the cells. Nevertheless, they found a preserved thylakoid structure even after severe water stress. Moran *et al.* (1994) showed a nearly doubling of damaged proteins in water-stressed pea plants. De las Rivas *et al.* (1993) and Kyle (1987) reported a higher susceptibility of water-stressed photosynthetic systems to photoinhibition even at very low irradiances. Photoinhibition causes degradation of D1 proteins (Mattoo *et al.* 1989). Up to now an effect on the water splitting complex itself has not been reported.

In this study we measured the Chl fluorescence decay of water-stressed pea leaves in the ps and ns ranges. The exciton-radical pair equilibrium model enabled to reveal a damage of different parts of PS2, the electron transfer chain, and the water splitting complex in its chronological development.

Materials and methods

Pea (*Pisum sativum* L.) plants were grown for three weeks at 20 °C under natural irradiances. Leaves were detached from the plant and wilted at different relative air humidities (R.H.) in darkness to avoid activation of the xanthophyll cycle (Demmig-Adams 1990, Horton *et al.* 1994). The relative loss of leaf mass Δm_{rel} was calculated as

$$(m_{fr} - m_m)/m_{fr}$$

where m_{fr} is the mass of the intact leaf, and m_m the mass of the wilted leaf.

The Chl fluorescence decay measurements were carried out using the mobile picosecond fluorimeter (Maier *et al.* 1995) in a modified version. The mobile picosecond fluorimeter is based on the technique of time correlated single photon counting. The excitation pulses (excitation wavelength: λ_e = 651 nm, full width at half maximum: FWHM = 80 ps, repetition rate: f = 800 kHz) were generated by a laser diode *Philips CQL 820/D*. Only weak irradiance of 2×10^{17}(photon) pulse^{-1} m^{-2} was applied to avoid any closure of PS2. The leaves were adapted to the measuring radiation for 15 min before every measurement. Fluorescence was detected at the wavelength of 695 nm by a *MCP Hamamatsu R1645U-01*.

The apparatus function was collected by measuring the well-known decay of the fluorescence scatterer DQTCI in ethanol (10^{-5} M, τ_f = 40 ps). After deconvolution the decay curves were analysed by a sum of exponential functions with the amplitudes α_i and the decay times τ_i using the Marquardt algorithm. The average fluorescence decay time τ_m was calculated from these values:

$$\tau_m = \frac{\int t\,F(t)\,dt}{\int F(t)\,dt} = \frac{\Sigma_i\,\alpha_i\,\tau_i^2}{\Sigma_i\,\alpha_i\,\tau_i}$$

The quality of the fits was tested by the χ_{red}^2 criterion and the distribution of the weighted residuals (O'Connor and Phillips 1984).

The relative fluorescence yields Φ_i were calculated as

$$\Phi_i = \alpha_i\,\tau_i$$

The fastest decay component τ_1 of 80 ps was assigned to PS1 and fixed during all fits, because the PS1 fluorescence was constant under both normal and water-stressed conditions (Canaani *et al.* 1986, Roelofs *et al.* 1992). The other parameters went free. After measurements on different leaves, the averages of the single decay times τ_i (i = 2, 3, 4) of leaves with the same Δm_{rel} were selected to fit the decay curves again with these average decay times as fixed parameters. The decay times τ_2 and τ_3 were assigned to PS2. They were interpreted as mixed components of $PS2_\alpha$ and $PS2_\beta$, because the exciton-radical pair equilibrium model demanded a biexponential decay for each modification of PS2 (Schatz *et al.* 1988, Roelofs *et al.* 1992, Trissl *et al.* 1993). As the fits were excellent with $0.9 \le \chi_{red}^2 \le 1.1$ and with randomly distributed weighted residuals between +4 and -4, just two exponential functions were used to describe the contribution of PS2 to the total fluorescence in the two extreme cases of photosynthetic activity, *i.e.*, with either all PS2 open or all PS2 closed.

For the biochemical calibration of the totally blocked electron transfer out of PS2, the fluorescence decay of DCMU treated leaves was measured (Jansen *et al.* 1993). The leaves were put into an aqueous solution of DCMU (10^{-4} M) for 5 min. In the measurements with an additional electron donor, the leaves were put into an aqueous solution of hydroxylamine (10^{-2} M NH_2OH, Canaani *et al.* 1986) for 10 min.

Results

The maximum and minimum of the photosynthetic activity of PS2 were represented by the fluorescence decay curves of an intact and a DCMU-treated leaf (Fig. 1). The fit parameters in Table 1 show the well-known fluorescence decay components for an optimal and a totally blocked electron transport (reduced Q_A) out of PS2 (Roelofs *et al.* 1992, Maier *et al.* 1995).

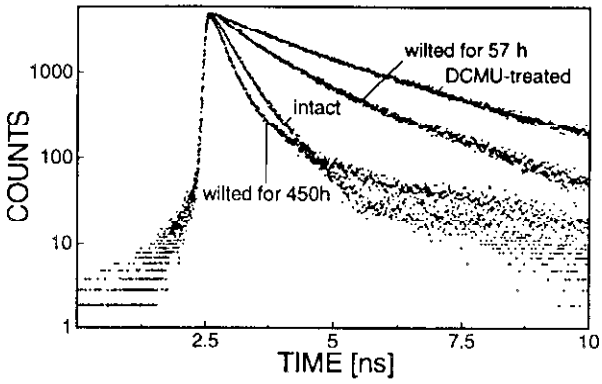

Fig. 1. Chlorophyll fluorescence decay curves of intact, DCMU-treated and detached (wilted for 57 and 450 h at 22 % R.H.) pea leaves. The curve of the 57 h wilted leaf shows the maximum of τ_m during wilting.

After a few days, depending on the wilting conditions, the average fluorescence decay time τ_m reached a maximum (Fig. 2). The decay times τ_2, τ_3, and the relative yield Φ_3 increased, while Φ_2 decreased in comparison to those an intact leaf. Up to the maximum, all decay curves could be described by three exponential functions.

Table 1. Parameters of the chlorophyll fluorescence decay curves of intact, DCMU-treated, detached (wilted for 57 and 450 h at 22 % R.H.), and NH_2OH-treated (wilted for 450 h) pea leaves obtained from the Marquardt fit. The curve of the 57 h wilted leaf shows the maximum of τ_m during wilting. τ_1 and τ_4 were fixed during the fit. χ_{red}^2 was between 0.9 and 1.1.

	Φ_1 [%]	Φ_2 [%]	Φ_3 [%]	Φ_4 [%]	τ_1 [ps]	τ_2 [ps]	τ_3 [ps]	τ_4 [ps]	τ_m [ps]	Δm_{rel}
intact	16.3	54.8	28.9	-	80	362	660	-	402	0.00
intact+DCMU	2.4	12.3	85.3	-	80	910	2375	-	2140	-
wilted for 57 h	4.4	19.5	65.6	10.5	80	488	1306	3600	1337	0.81
wilted for 450 h	23.9	43.0	20.1	13.0	80	238	620	3600	714	0.86
wilted+NH_2OH	3.9	14.8	30.0	51.3	80	289	1054	3600	2208	-

After the maximum, a new fluorescence decay component with a lifetime of τ_4 = 3.6 ns appeared (Table 1). Within a few hours, τ_m decreased (Fig. 2), and the fluorescence decay became similar to that one of an intact leaf (values not shown).

After severe water stress (about 300 h), the scaled decay curve (Fig. 1) was characterized by a faster fluorescence decay in the first nanoseconds, but it was slower subsequently, compared with the fluorescence decay of an intact leaf. The relative fluorescence yields of PS1 (Φ_1) and of the fourth component (Φ_4) increased, while the yields of the other PS2 components decreased (Table 1).

Unless the dependence of τ_m on the wilting time varied strongly with the wilting conditions (Fig. 2), the variations of the average fluorescence decay time as function of the relative loss of leaf mass Δm_{rel} did not depend on the wilting conditions, *i.e.*, on the relative humidity of the air: the maximum of τ_m was always detected at $\Delta m_{rel} = 0.8$ (Fig. 3).

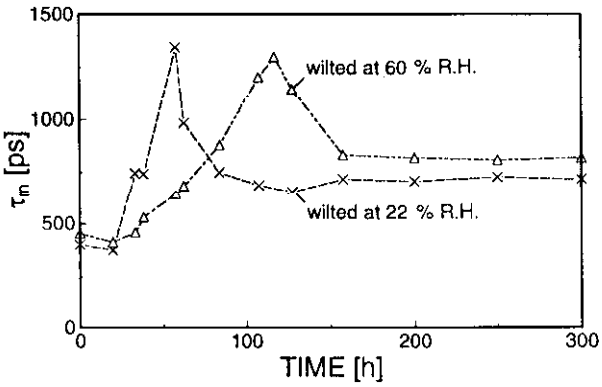

Fig. 2. Average chlorophyll fluorescence decay time τ_m of two pea leaves wilted at different relative humidities (R.H., 60 or 22 %) as a function of the wilting time.

If wilted leaves with $\Delta m_{rel} > 0.8$ were rehydrated in water for 10 min, the maximum of τ_m could be restored. Only after severe water stress (more than 100 h after reaching the maximum of τ_m), rehydration had no effect on the fluorescence decay (results not shown). Treatment of such a leaf with the artificial electron donor NH_2OH caused a dramatic increase in the relative yield of the longest decay component Φ_4 (Table 1).

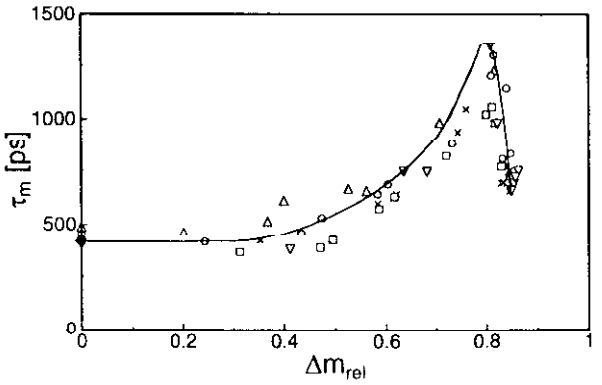

Fig. 3. Average chlorophyll fluorescence decay time τ_m of pea leaves wilted at relative humidity of 22 % (*crosses and triangles*) or 60 % (*circles and squares*) as a function of the relative loss of the leaf mass, Δm_{rel}.

Experiments on whole plants of *Arabidopsis thaliana* led to the same qualitative results, but the effects could be observed within just a few hours.

Discussion

In the analysis of our measurements we obtained an excellent fit with a sum of just three exponential functions for the extremes of photosynthetic activity of PS2. The two fluorescence decay components attributed to PS2, τ_2 and τ_3, increased and the ratio of the amplitudes α_2/α_3 decreased during the transition from open to closed PS2. This corresponds to the solutions of the differential equations based on the exciton-radical pair equilibrium model (for details see, *e.g.*, Schatz *et al.* 1988, Roelofs *et al.* 1991, Liu *et al.* 1993).

Though five fluorescence decay components are demanded by the exciton-radical pair equilibrium model (one for PS1, two for $PS2_\alpha$, and two for $PS2_\beta$ - Roelofs *et al.* 1992), we fit with as few components as possible. If there are many (up to ten) free running parameters, a very good Marquardt fit could be obtained but the significance of the single calculated parameters is doubtful (O'Connor and Phillips 1984). Therefore the obtained decay components τ_2 and τ_3 must be interpreted as mixtures of the decay components for the two modifications, $PS2_\alpha$ and $PS2_\beta$.

According to the exciton radical pair equilibrium model, the development of the fluorescence decay times and relative yields in the region $0.5 \leq \Delta m_{rel}$ 0.8 indicates a partial blockade of the electron transfer out of PS2. This lengthening of the fluorescence decay is caused by a reduced Q_A resulting in a decrease in the charge stabilization rate (k_2) and an increase in the charge recombination rate (k_{-1}) (Schatz *et al.* 1988, Roelofs *et al.* 1992). We believe that this effect is caused by decoupling of the Q_B as a consequence of degradation of D1 proteins.

This decrease is confirmed by several investigations of other groups: De las Rivas *et al.* (1993) and Kyle (1987) show that water-stressed photosynthetic systems are susceptible to photoinhibition even at very low irradiances. Photoinhibition causes an increase of the amount of damaged D1 proteins (Mattoo *et al.* 1989). The PS2 with a damaged Q_B-binding site shows the same fluorescence decay as PS2 closed due to a reduced Q_A. Moran *et al.* (1994) found a nearly doubling of damaged proteins in water-stressed pea plants. In an experiment with a mutant of a green alga, that is inactive in water-splitting, Bowyer *et al.* (1992) report that these PS2 centres are far more susceptible to photoinhibition. Thus the D1 protein must be damaged to a high degree even under low irradiances.

But the fluorescence decay parameters did not reach values similar to those obtained when all PS2 were blocked (Table 1). Another fluorescence quenching process must take place. Fluorescence quenching in the antennas due to activation of the xanthophyll cycle could be excluded, because the leaves were wilted in darkness (Demmig-Adams 1990, Horton *et al.* 1994).

Krieger *et al.* (1992a,b) present a recombination model that considers an electron deficiency at the oxidizing site of PS2 (Fig. 4): Charge separation (state *C*) and charge stabilization (state *D*) take place, but the oxidized $P680^+$ cannot be reduced by tyrosine Z. The electron from Q_A^- is transferred back to $P680^+$ *via* an unknown route, but the recombination energy is not sufficient to create excited states of the reaction centre, and thus the energy is lost as heat.

This cannot be the only effect that causes the fluorescence quenching, because it does not explain the new fluorescence decay component τ_4. Furthermore, the addition of an electron donor (water or NH_2OH) cannot establish the fluorescence decay of closed PS2 (Table 1). If an electron deficiency at the donor site of PS2 was the only effect, electron donation should have led to the same decay parameters obtained from measurements on a DCMU-treated leaf.

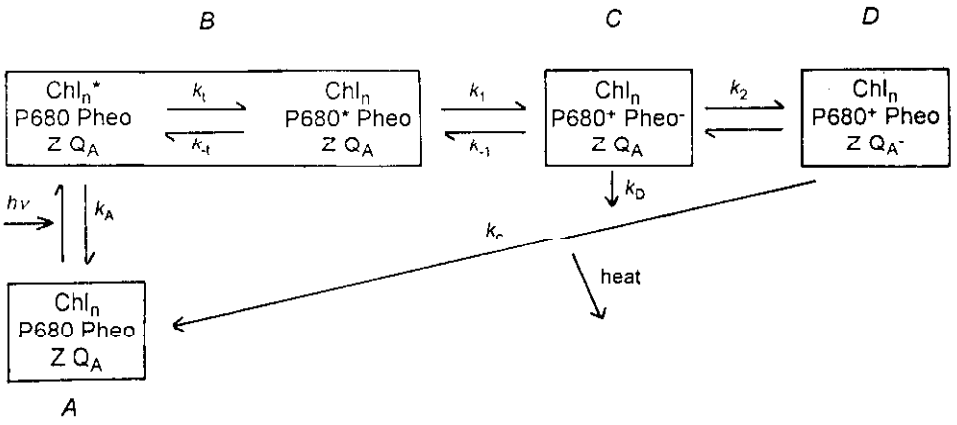

Fig. 4. Exciton-radical pair equilibrium model with an electron circle (according to Krieger *et al.* 1992a). Chl_n describes the antennas, * the excited states.

We believe that the Q_A is decoupled from PS2 after severe water stress due to a damage of the D2 protein at the Q_A-binding site. The D2 protein is one order of magnitude more stable than the D1 protein (Mattoo *et al.* 1989), and thus the fluorescence quenching starts with a time delay. If the Q_A is decoupled, absorption and charge separation take place, but the charge stabilization (Fig. 4, state *D*) is not possible any longer. The primary radical pair $P680^+Pheo^-$ (state *C*) is stabilized because of the absence of the strong electrostatic interaction between $Pheo^-$ and Q_A^-

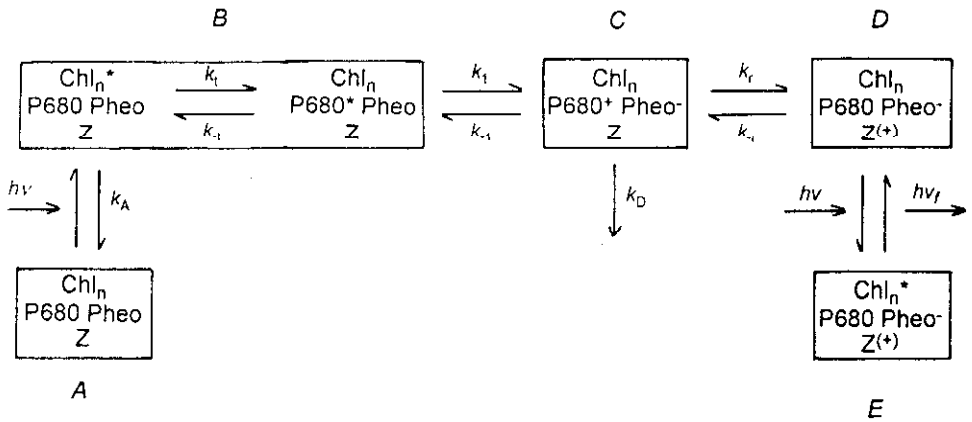

Fig. 5. Modified exciton-radical pair equilibrium model after decoupling of the primary quinone Q_A.

(Fig. 5), and recombines with thermal energy dissipation (rate k_D), while the rate k_{-1} decreases (*i.e.*, Φ_2 and Φ_3 decrease, Table 1).

With a low probability, the reaction centre can be re-reduced. In that case cytochrome b_{559} or tyrosine Z can act as an electron donor (Kyle *et al.* 1984), and the state *D* (see Fig. 5) is occupied. If a photon is absorbed in the antenna (state *E*), no charge separation occurs, and the energy is emitted as fluorescence. A new fluorescence decay component with a lifetime comparable to that of energetically decoupled antennas should arise. Depending on the preparation, lifetimes between 3 and 7 ns have been observed for decoupled antennas (Hansson *et al.* 1988, Bassi *et al.* 1991, Roelofs *et al.* 1991, van Grondelle *et al.* 1994).

This explains the fluorescence decay of water-stressed leaves after adding the electron donor hydroxylamine as well: Through electron donation to $P680^+$ the state *D* (Fig. 5) becomes more populated, and thus the relative amount of the fourth decay component (Φ_4) to the fluorescence increases. Thus water stress does not affect the capability of PS2 for radiant energy absorption in the antennas, for energy transfer to the reaction centre, and for charge separation.

If a component with a lifetime of about 3 ns would arise from a relaxed state of the radical pair as suggested elsewhere (De las Rivas *et al.* 1992, Vass *et al.* 1993), electron donation to PS2 should not increase the relative yield of the fourth component.

Severe water stress inhibits the water splitting complex itself: water cannot act as electron donor to PS2 any longer. Obviously the Y-enzyme could not pass through the water-splitting cycle, while hydroxylamine could spend electrons directly to the $P680^+$ (Canaani *et al.* 1986).

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