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Rapid Report

Long-wavelength chlorophyll species are associated with amplification of high-energy-state excitation quenching in higher plants

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Thylakoids isolated from light-treated spinach leaves ('light' thylakoids) which contain significant zeaxanthin levels show amplification of high-energy-state chlorophyll fluorescence quenching (qE) with respect to the transthylakoid pH gradient, when compared to thylakoids from dark-adapted leaves ('dark' thylakoids) which contain no zeaxanthin. In 77 K fluorescence emission spectra q_E in 'dark' thylakoids shows maximal quenching with respect to fluorescence yield near 680 nm, whereas in 'light' thylakoids additional quenching occurs near 700 nm, which is associated with the appearance of a chlorophyll species absorbing at long wavelengths. It is suggested that this long-wavelength species is associated with an aggregated state of LHC II.

In higher plants and algae, mechanisms for the dissipation of excess chlorophyll excitation, particularly associated with Photosystem II (PS II), can be observed by measurement of room temperature chlorophyll fluorescence yield. By the kinetics of formation and relaxation, and differential effects of inhibitors, three main mechanisms of dissipation have been identified [1–3]. Under most conditions, the most significant of these, which forms and relaxes in a matter of minutes, is high-energy-state quenching (q_E) which is dependent on the formation of the transthylakoid pH gradient [1–5].

The molecular mechanism of q_E is unknown. Most studies conclude that the dissipation occurs throughout the antenna and light-harvesting complexes [6,7], although a few suggest dissipation at the PS II reaction centre itself [8]. The possible role of the xanthophyll, zeaxanthin, has recently been the subject of a series of investigations [9]. The formation of zeaxanthin from violaxanthin by the enzymes of the xanthophyll cycle [10,11] is favoured by the formation of a transthylakoid pH gradient, such that in vivo a correlation between zeaxanthin levels and the extent of q_E is observed [12–14]. It has thus been hypothesized that zeaxanthin may act as a direct quencher of chlorophyll excitation [15]. Contrary to this, it was observed in vitro that q_E occurred in the absence of zeaxanthin, although at a lower level compared to the magnitude of the pH gradient [16,17]. This led to the alternative hypothesis that zeaxanthin acts as a quenching amplifier.

In order to reconcile the above hypotheses, it has been suggested that the mechanism of q_E quenching may differ depending on whether zeaxanthin is present [9]. In this report we investigate this possibility by comparing the 77 K fluorescence characteristics of q_E in thylakoids isolated from leaves that have been pretreated to manipulate the zeaxanthin levels.

Chloroplasts were isolated as described in Ref. 17, from spinach leaves that had either been light-treated by 50 min exposure to 200 μE m^2 s^{-1} at 25°C under an atmosphere of 98% N_2/2% O_2, or dark-adapted under the same conditions. For both treatments the leaves were floated on water with their cut petioles submerged. These were the same conditions as used previ-

Abbreviations: DTT, dithiothreitol; F_o, dark level chlorophyll fluorescence yield; F_v, variable chlorophyll fluorescence yield; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; LHC II, light harvesting complex associated with Photosystem II; PS I, Photosystem I; PS II, Photosystem II; q_E, high-energy-state quenching.

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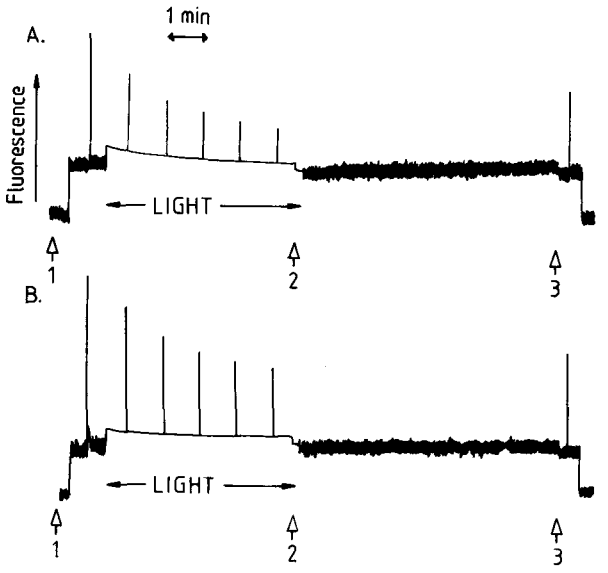


Fig. 1. Room-temperature chlorophyll fluorescence traces obtained from 'light' (A) and 'dark' (B) spinach thylakoids. Samples were taken for the low temperature measurements shown in Fig. 2 where indicated by the numbered arrows. Red actinic light (50 μE m^{-2} s^{-1}) was applied where indicated. Fluorescence spikes were the result of saturating pulses of light to allow resolution of photochemical and nonphotochemical quenching.

ously to obtain 'light' and 'dark' thylakoids that had high and low levels of zeaxanthin, respectively [17]. Thylakoids were obtained immediately prior to each measurement by osmotically shocking chloroplasts by incubation for 30 s in 30 mM $MgCl_2$/0.5 mM EDTA/10 mM Hepes (pH 7.6), after which an equal volume of medium containing sorbitol and Hepes was added to a final concentration of 330 mM and 55 mM, respectively (final concentration of $MgCl_2$; 15 mM). The final chlorophyll concentration was 10 μg chlorophyll ml^{-1} in a volume of 1.2 ml. 0.1 mM NaN_3 and 0.1 mM methylviologen were also added. 10 μM fluorescein was included to act as a standard for normalization of spectra.

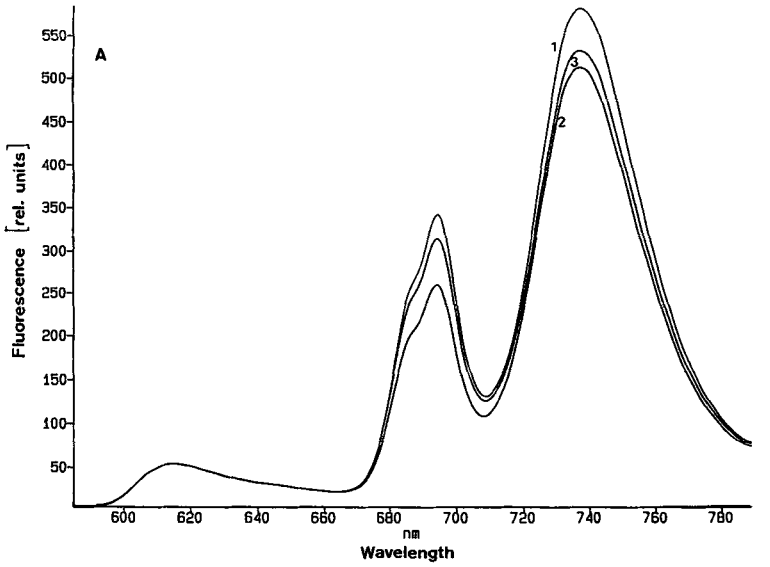
Fig. 1 shows room temperature fluorescence traces measured using a Wall fluorimeter, obtained from both 'light' and 'dark' thylakoids, illuminated in the presence of methylviologen as an electron acceptor. The pulses of light applied at 1 min intervals saturate photochemistry, and so reverse photochemical quenching of fluorescence, leaving only non-photochemical quenching. Thus, any decrease in the maximum level of the fluorescence during the pulse indicates non-photochemical quenching. q_E was resolved from other non-photochemical mechanisms of quenching by the fact that it reverses within a few minutes in the dark. The thylakoids were exposed to light (50 μE m^{-2} s^{-1}) for 5 min, followed by a 7 min dark period, during which time q_E would completely relax. As observed previously [17], at this subsaturating light intensity, there is significantly more q_E in the 'light' thylakoids than in the 'dark' thylakoids.

Fig. 2 shows 77 K fluorescence emission spectra for excitation at 435 nm obtained from 'light' (A) and 'dark' (B) thylakoids at the times indicated in Fig. 1. Experimental details are given in the legend. In each case trace 1 shows the spectrum obtained for thylakoids sampled prior to illumination. Three emission bands from the thylakoids are clearly evident; the band centred at 740 nm arises primarily from PS I with satellite contributions from PS II, while the emission at 695 and the emission at 685 nm which appears as a shoulder, arise from PS II. It is clear that the ratio of PS II to PS I fluorescence is lower in the 'light' thylakoids than in the 'dark' thylakoids. This is due primarily to quenching of PS II fluorescence in 'light' thylakoids which can also be observed as a quenching of variable fluorescence yield at room temperature, resulting in a decrease in the F_v/F_m ratio of 'light' thylakoids (0.732) compared to 'dark' thylakoids (0.788).

Trace 2 shows spectra during quenching, by both q_E and irreversible mechanisms, and trace 3, after recovery, when q_E has relaxed such that only irreversible quenching remains. The difference spectra (trace 3–trace 2), shown in Fig. 2c thus correspond to q_E.

It is difficult to compare the absolute magnitudes of quenching between different thylakoid preparations. However, it can be clearly seen that in 'light' thylakoids q_E results in considerably greater quenching of PS II

Fig. 2. (A) and (B) Fluorescence emission spectra measured at 77 K from 'light' (A) and 'dark' (B) spinach thylakoids. (1) Control spectrum from thylakoids prior to illumination. (2) After 5 min exposure to 50 μE m^{-2} s^{-1}. (3) Alter a subsequent 7 min dark recovery. (C) Difference spectra at 77 K indicating q_E quenching in 'light' (1) and 'dark' (2) thylakoids. q_E was calculated as (trace 3–trace 2) from (A) and (B). 200-μl samples were injected into a sample holder consisting of two flat round pieces of glass (diameter 14 mm) sealed by a metal ring, and rapidly frozen in liquid nitrogen. The whole procedure of taking and freezing samples took less than 15 s. During measurements the sample holder was immersed in liquid nitrogen in a purpose-built cryostat. Excitation of 50 μE m^2 s^{-1} was provided by a Wotan tungsten halogen 150 W lamp and defined by a 4-46 and a 5-57 Corning filter with heat-absorbing glass to give broad band excitation in the Soret band of chlorophyll absorption centered near 435 nm. Fluorescence was detected by a 1024-channel silicon photodiode detector (Model 1455) via a Jarrell-Ash Monospec 27 monochromator and analysed by an EG&G PARC optical multichannel analyser, Model 1461 using EG&G OMA-Vision-PDA data aquisition/analysis software. The resolution of the fluorescence spectra was 0.3 nm, and the signal to noise was greater than 1000:1. Spectra were normalized at 614.81 nm which was the maximum level of fluorescein fluorescence in these experiments due to the lack of sensitivity of measurement below 590 nm for the experimental arrangement used in these studies. The error introduced by normalization was less than 1%, while the total error of processing and calculation of difference spectra was less than 4% of full scale.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Fluorescence [rel. units]
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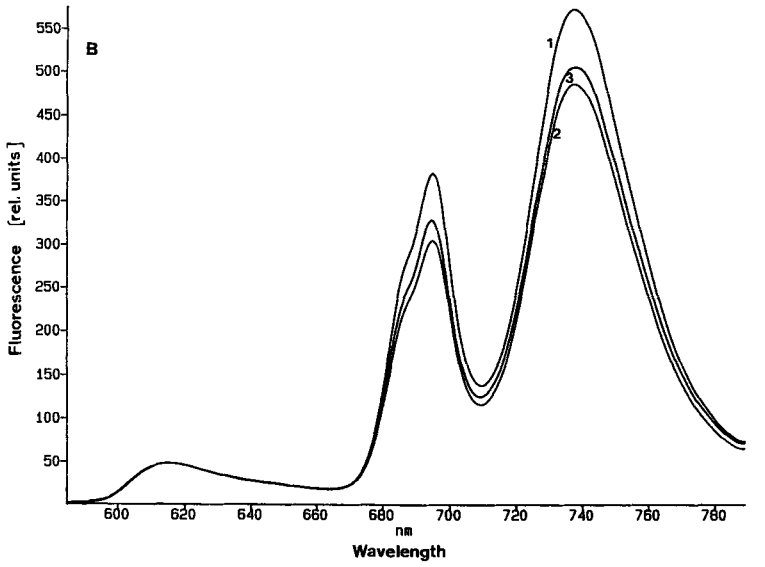
B
Fluorescence [rel. units]
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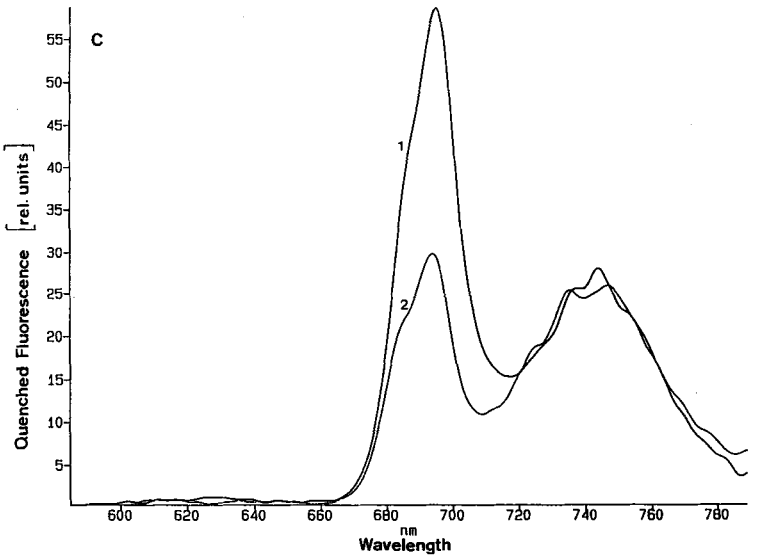
C
Quenched Fluorescence [rel. units]
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Wavelength

fluorescence relative to PS I fluorescence than in 'dark' thylakoids. The additional PS II quenching appears to be preferentially of the longer wavelength bands, resulting in a shift of the PS II band and a less pronounced shoulder for 'light' thylakoids.

The characteristics of q_E quenching and the differences between 'light' and 'dark' thylakoids are shown more clearly when expressed as 'variable' quenching spectra (Fig. 3). These spectra are calculated by normalization of the difference spectra (Fig. 2c) with respect to the quenched spectra (traces 3 in Fig. 2b) and emphasize the extent of quenching with respect to fluorescence yield at each wavelength. In 'light' thylakoids there is clearly preferential quenching at 698 nm (1), whereas in 'dark' thylakoids quenching is preferentially at 680 nm (2) with a shoulder near 698 nm. The spectra of Fig. 3 were obtained in one experiment. In a series of 7 experiments maximal quenching occurred at 700.1 ± 2.6 nm (mean $\pm$ S.D.) in 'light' thylakoids, and, in 15 experiments, at 677.1 ± 2.4 nm in 'dark' thylakoids. At the subsaturating light intensity used to induce quenching in these experiments, the extent of q_E at room temperature was higher in the 'light' thylakoids (0.42) than in the 'dark' thylakoids (0.22). At light intensities saturating for q_E the extent of quenching is similar for the two types of thylakoid. In this case the spectrum of q_E quenching in 'dark' thylakoids is virtually unchanged, while in 'light' thylakoids quenching at 680 mu becomes more significant (spectra not shown). Thus it appears that prominent quenching at long-wavelengths is a characteristic of a mechanism responsible for the amplification of q_E at lower light intensities in 'light' thylakoids. As mentioned above, one of the main differences between the two types of thylakoid preparation is the content of the xanthophyll zeaxanthin. When the thylakoids were analysed for their pigment content by HPLC (as described in Ref. 20) it was found that zeaxanthin comprised 53% and 0% of total xanthophyll cycle components (zeaxanthin + antheraxanthin + violaxanthin) in 'light' and 'dark' thylakoids, respectively.

In order to investigate more fully the involvement of zeaxanthin in the alteration of quenching characteristics, an experiment was also carried out in which leaves were light-treated after DTT feeding to inhibit de-epoxidation, and hence the formation of zeaxanthin [18]. Leaves were placed overnight upright in a beaker containing 1 mM DTT solution. Air was fanned across the leaf surface in order to increase the transpiration rate and aid uptake of DTT. During the subsequent light treatment leaves were also floated on a 1 mM DTT solution. Trace 3 in Fig. 3 shows that the thylakoids isolated from such leaves, which contain no detectable zeaxanthin, show q_E quenching very similar to that in 'dark' thylakoids. The relationship of q_E to the transthylakoid pH gradient was also similar to that in 'dark' thylakoids.

The changes induced by preillumination treatment were further investigated by comparison of the absorption spectra of 'light' and 'dark' thylakoids in the

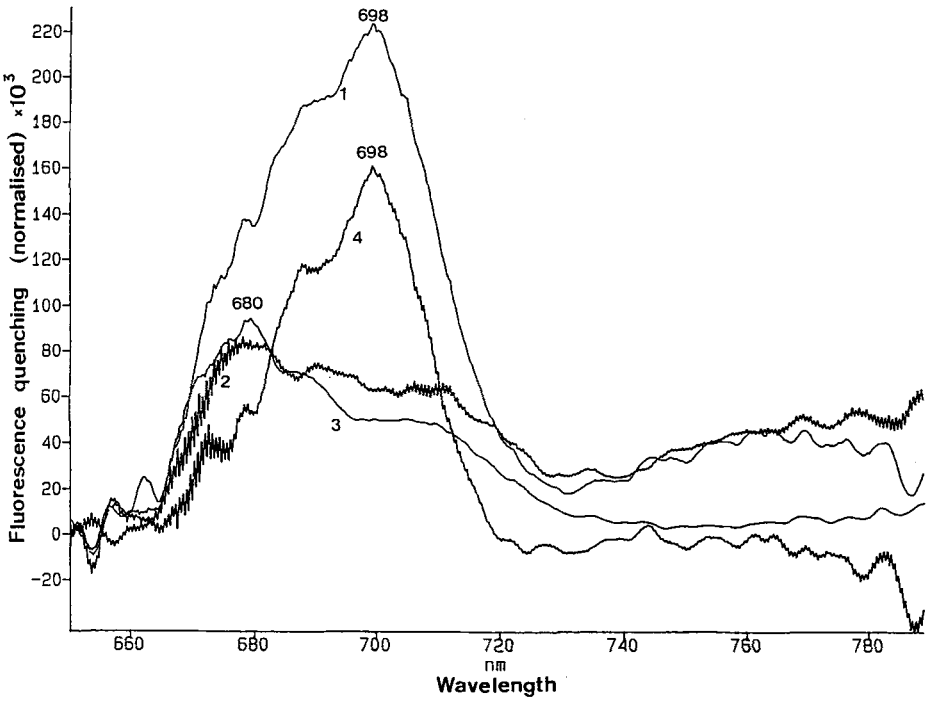

Fig. 3. 77 K 'variable' spectra of q_E quenching calculated by dividing the original difference spectra by the quenched spectra. Trace 1, 'light' thylakoids; 2, 'dark' thylakoids; 3, 'light' thylakoids from DTT-treated leaves; 4, (trace 1–trace 2).

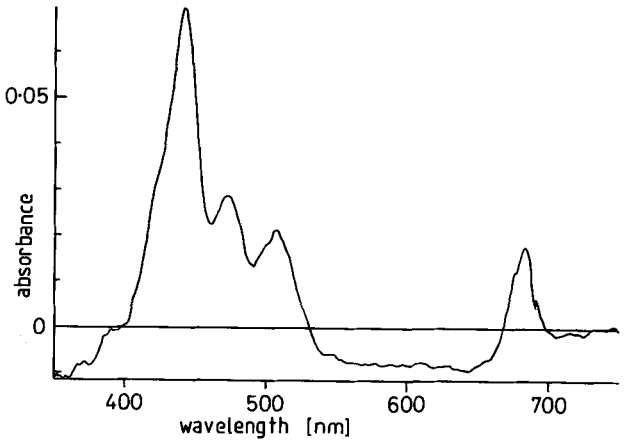

Fig. 4. Difference absorption spectrum obtained by subtracting the absorption spectrum of 'dark' thylakoids from that of 'light' thylakoids. Both spectra were obtained from thylakoids, prior to illumination, suspended at 10 μg chlorophyll ml^{-1} and measured on an Aminco DW2000 dual-wavelength spectrophotometer operating in split-beam mode.

dark-adapted state. A typical difference spectrum of 'light'–'dark' thylakoids is illustrated in Fig. 4, typically four peaks were observed, at 440, 468, 505 and 685 nm. The peaks at 468 and 505 nm are typical of the difference spectrum of zeaxanthin–violaxanthin, and can therefore be explained by the conversion of violaxanthin to zeaxanthin [19]. The peaks at 440 and 685 nm indicate the appearance of a long wavelength chlorophyll *a* species.

It is important to consider the evidence for the origin of the fluorescence bands near 680 nm and near 700 nm, which appear to be important in q_E quenching. As mentioned above, fluorescence bands near 680, 685 and 695 nm (F680, F685, F695) arise from chlorophyll species associated with PS II [20]. F685 and F695 are thought to originate near to the PS II reaction centre. However, F680 is not observed in barley mutants lacking light-harvesting-complex II (LHC II) [20], nor in isolated PS II core complexes [21], and therefore probably arises from LHC II. The major quenching species that we observe in 'light' thylakoids at 700.1 ± 2.1 nm is at a wavelength significantly greater than F695. There are few reports of such long-wavelength fluorescing species in vivo, although a fluorescence emission at 700 nm has been observed in *Chlamydomonas reinhardtii* from an aggregated species of LHC II, that has been termed LHC0 [22]. There have also been interesting reports that long-wavelength fluorescence species can arise in vitro as a result of conformational changes induced by interaction between LHC II complexes [23]. In this respect, it is important to emphasize our observations of the appearance of additional long-wavelength chlorophyll species with absorption maxima (at 685 nm) similar to those observed m the above mentioned complexes [23], in the absorption spectra of 'light' thylakoids (Fig. 4). A proportion of the xanthophyll cycle components have been shown to be located in LHC II [24] (Pascal, A. and Noctor, G., personal communication), which is consistent with the hypothesis that zeaxanthin could be involved in the creation, or stabilization, of an aggregated state of LHC II.

In conclusion, we have identified, for the first time, the chlorophyll species responsible for a significant part of the energy dissipation associated with q_E. This quenching is of a species emitting at 680 nm, the characteristic emission of LHC II. In addition, a species with emission close to 700 nm is involved, and the amplification of q_E in the presence of zeaxanthin results in an enhancement of this band. For reasons discussed above, we suggest that this emission also arises from the LHC II complex and that this modified form of LHC II, absorbing near 685 nm, and emitting near 700 nm, more readily assumes the low pH-induced membrane state that gives rise to q_E.

References

1 Horton, P. and Hague, A. (1988) Biochim. Biophys. Acta 932, 107–115.
2 Quick, W.P. and Stitt, M. (1989) Biochim. Biophys. Acta 977, 287–296.
3 Walters, R.G. and Horton, P. (1991) Photosynth. Res. 27, 121–133.
4 Briantais, J.-M., Vernotte, C., Picaud, M. and Krause, G.H. (1979) Biochim. Biophys. Acta 548, 128–138.
5 Krause, G.H., Vernotte, C. and Briantais, J.-M. (1982) Biochim. Biophys. Acata 679, 116–124.
6 Genty, B., Briantais, J.-M. and Baker, N.R. (1989) Biochim. Biophys. Acta 990, 87–92.
7 Rees, D., Noctor, G.D. and Horton, P. (1990) Photosynth. Res. 25, 199–211.
8 Weis, E. and Berry, J.A. (1987) Biochim. Biophys. Acta 894, 198–208.
9 Demmig-Adams, B. (1990) Biochim. Biophys. Acta 1020, 1–24.
10 Siefermann, D. and Yamamoto, H.Y. (1975) Biochem. Biophys. Res. Commun. 62, 456–461
11 Hager, A. (1967) Planta 89, 224–243.
12 Demmig-Adams, B., Winter, K., Kruger, A. and Czygan, F.-C. (1989) Plant Physiol. 90, 887–893.
13 Demmig-Adams, B., Winter, K., Kruger, A. and Czygan, F.-C. (1989) Plant Physiol. 90, 894–898.
14 Demmig-Adams, B., Winter, K., Kruger, A. and Czygan, F.-C. (1989) Plant Physiol. 90, 881–886.
15 Demmig, B., Winter, K., Kruger, A. and Czygan, F.-C. (1987) Plant Physiol. 84, 218–224.
16 Rees, D., Young, A., Noctor, G., Britton, G. and Horton, P. (1989) FEBS Lett. 256, 85–90.
17 Noctor, G., Rees, D., Young, A. and Horton, P. (1991) Biochim. Biophys. Acta 1057, 320–330.
18 Adams, W.W., Demmig-Adams, B. and Winter, K. (1990) Plant Physiol. 92, 293–301.
19 Bilger, W., Bjorkman, O. and Thayer, S.S. (1989) Plant Physiol. 91, 542–551.

20 Rijgersberg, C.P., Amesz, J., Thielen, A.P.G.M. and Swager, J.A. (1979) Biochim. Biophys. Acta 545, 473–482.
21 Van Dorssen, R.J., Plijter, J.J., Dekker, J.P., Den Ouden, A., Amesz, J. and Van Gorkom, H.J. (1987) Biochim. Biophys. Acta 890, 134–143.
22 Lebedev, N.N., Khatypov, R.A., Ladygin, V.G. and Krasnovskii, A.A. (1988) Photosynthetica 22, 364–370.
23 Mullet, J.E. and Arntzen, C.J. (1980) Biochim. Biophys. Acta 589, 100–117.
24 Siefermann-Harms, D. (1984) Photochem. Photobiol. 40, 507–512.