

Activation of non-photochemical quenching in thylakoids and leaves

Giles N. Johnson^{1*}, Andrew J. Young², Peter Horton¹

¹ Robert Hill Institute, Department of Molecular Biology and Biotechnology, University of Sheffield, P.O. Box 594, Firth Court, Western Bank, Sheffield S10 2UH, UK

² School of Biological and Earth Sciences, John Moores University Liverpool, Byrom Street, Liverpool L3 3AF, UK

Received: 20 January 1994 / Accepted: 17 February 1994

Abstract. The mechanism of rapidly-relaxing non-photochemical quenching in two plant species, *Chenopodium album* L. and *Digitalis purpurea* L., that differ considerably in their capacity for such quenching has been investigated (Johnson G.N. et al. 1993, Plant Cell Environ. **16**, 673–679). Illumination of leaves of both species in the presence of 2% O₂ balance N₂ led to the formation of zeaxanthin. When thylakoids were isolated from leaves of each species that had been so treated it was found that in *D. purpurea* non-photochemical quenching was “activated” relative to the control; a higher level of quenching was found for a given trans-thylakoid pH gradient. No such activation of non-photochemical quenching was observed in *C. album*. Similar conclusions were drawn when comparing quenching in intact leaves. It is concluded that light activation of quenching is a process that cannot readily be induced in *C. album*. Measurement of the sensitivity of non-photochemical quenching in leaves of *C. album* and *D. purpurea* to dithiothreitol (DTT; a reagent that inhibits formation of zeaxanthin) showed differences between the two species. In both cases, feeding leaves with DTT inhibited the light-induced formation of zeaxanthin. In *C. album* this was accompanied by complete inhibition of reversible non-photochemical quenching, whereas in *D. purpurea* this inhibition was only partial. Data are discussed in relation to studies on the mechanism of quenching and the role of zeaxanthin in this process.

Key words: Chlorophyll fluorescence – *Chenopodium* – *Digitalis* – Non-photochemical quenching – Photosynthesis – Xanthophyll cycle – Zeaxanthin

* Present address: Institut für Botanik, Westfälische Wilhelms-Universität, D-48149, Münster, Germany

Abbreviations: DTT = dithiothreitol; $\Delta F_m/F_m'$ = non-photochemical quenching of chlorophyll fluorescence; F_v/F_m = ratio of variable to maximal fluorescence quenching; LHCII = light harvesting complex II; ΔpH = trans-thylakoid pH gradient; PFD = photon flux density; q9AA = quenching of 9-amino acridine fluorescence

Correspondence to: P. Horton; FAX: 44 (742) 728 697

Introduction

Under conditions where light is saturating for photosynthesis, excess light energy can be dissipated by non-photochemical routes (For a review, see Krause and Weis 1991). Such energy dissipation is reflected in the yield of fluorescence from the chlorophyll associated with photosystem II and can be quantified, both in vitro and in vivo, using a procedure termed “quenching analysis” (see Horton and Bowyer 1990).

A number of different processes are known to be involved in non-photochemical quenching of chlorophyll fluorescence and these different processes can be distinguished by the kinetics with which they relax (Demmig and Winter 1988; Horton and Hague 1988; Quick and Stitt 1989; Walters and Horton 1991). In leaves, under high light conditions the major component of non-photochemical quenching relaxes within seconds to minutes after illumination (Walters and Horton 1991). This form of quenching has been associated with a form of quenching occurring in vitro in the presence of a pH gradient (ΔpH) across the thylakoid membrane (“high energy-state” quenching) (Briantais et al. 1979). Such quenching has been shown to protect isolated thylakoids from light-induced damage (Krause and Behrend 1986).

In recent years there has been a considerable amount of work conducted attempting to determine the mechanism of high-energy-state quenching, leading to the proposal of several models for this process (Horton and Ruban 1992). In vitro, at least two different mechanisms of low-pH-induced quenching occur and can be distinguished by their differential effects on the F_0 level of fluorescence (the minimum level of fluorescence, measured at very low light intensities) and by their sensitivity to reducing agents (Rees et al. 1992). “Reaction centre” quenching is sensitive to ascorbate and does not lead to significant quenching of F_0 (Crofts and Horton 1991; Krieger and Weis 1992). “Antenna” quenching is redox insensitive and leads to significant quenching of F_0 (Rees et al. 1990a). Krieger and Weis (1992) have shown that reaction-centre quenching can result from the release of

Ca^{2+} from the donor side of photosystem II. Release of Ca^{2+} inhibits the water-splitting complex, so slowing electron donation to the oxidised primary donor P_{680}^{+} . It is probably this species that is responsible for quenching. The relevance of this type of quenching to physiological conditions is still a matter of debate.

Demmig-Adams has suggested that quenching in the antenna involves the formation of zeaxanthin from violaxanthin, in a two-step de-epoxidation reaction (see Demmig-Adams 1990). Correlations have been observed between the leaf zeaxanthin content and the extent of non-photochemical quenching, measured under a wide variety of conditions, and this has led Demmig-Adams (1990) to suggest that zeaxanthin, in the presence of a low pH in the thylakoid lumen, acts as a quencher of fluorescence. To date, no results have been published that demonstrate that zeaxanthin is able to quench excited chlorophyll directly.

The correlation between zeaxanthin formation and ΔpH -dependent fluorescence quenching is not surprising since both processes are induced by a low lumen pH. Circumstantial evidence for a role of zeaxanthin in quenching has come from comparison of quenching in lichens containing either green algae or cyanobacteria (Demmig-Adams et al. 1990a). Whilst the former produce zeaxanthin and show quenching, the latter do not possess a xanthophyll cycle and do not perform pH-dependent quenching. Since, however, green algae and cyanobacteria have completely different light-harvesting systems this evidence is not compelling. The only argument that *directly* indicates a role for zeaxanthin in pH-dependent quenching is the effect of dithiothreitol (DTT).

Dithiothreitol inhibits the enzyme violaxanthin deepoxidase (Yamamoto and Kamite 1972). In leaves that have been fed with DTT, the formation of zeaxanthin is inhibited (Bilger et al. 1989). At the same time, non-photochemical quenching is also inhibited, to a variable extent, in different plant species (Bilger and Björkman 1990; Demmig-Adams et al. 1990b). This suggests that inhibition of zeaxanthin formation may bring about inhibition of non-photochemical quenching, giving support for the idea that zeaxanthin is involved in that quenching. This evidence is not, however, conclusive since DTT is not a specific inhibitor of violaxanthin deepoxidase and is known to affect many different enzymes, including certain Calvin-cycle enzymes and the thylakoid ATP synthase. Hence, DTT may influence the extent of the ΔpH . Schreiber and Neubauer (1990) have demonstrated that, *in vitro*, DTT inhibits electron transport via the Mehler reaction and so inhibits ΔpH formation. The concentration of DTT required to induce this effect is, however, higher than that required to inhibit non-photochemical quenching (Neubauer 1993) so cannot alone explain the effect of DTT on non-photochemical quenching.

A recent model, also implicating zeaxanthin, involves energy dissipation in LHCII, the light-harvesting complex of photosystem II (Horton et al. 1991; Horton and Ruban 1992). It is proposed that a low pH in the thylakoid lumen leads to the formation of a quenching state of LHCII and that this state is more readily attained if

the complex is in an aggregated form. Evidence for this model includes the similarity between spectral changes accompanying quenching *in vivo* and in isolated LHCII (Ruban et al. 1992). An important aspect of this model is the notion of light activation of non-photochemical quenching; the initial observations that led to this idea included experiments in which leaves of spinach were illuminated in the presence of 2% O_2 , balance N_2 (Noctor et al. 1991; Rees et al. 1990b). When thylakoids were isolated from such leaves it was found that activation of high-energy-state quenching had occurred, relative to untreated material, such that higher levels of quenching were seen for a given ΔpH . Activation of quenching was found to be inhibited if leaves were pre-fed with DTT, so implying that this activation may require formation of zeaxanthin (Noctor et al. 1991). Importantly, zeaxanthin is seen to be acting as a facilitator of quenching but is not absolutely required for quenching to occur. Recently, evidence for zeaxanthin-dependent light activation of quenching in leaves has been obtained (Ruban et al. 1993).

The interpretation of Noctor et al.'s (1991) data has been questioned by Demmig-Adams (1990) who has suggested that what is in fact being seen is two forms of quenching, one of which involves zeaxanthin, the second being zeaxanthin-independent. Non-photochemical quenching observed in leaves treated with DTT does not result in measurable quenching of F_0 and is therefore presumed to be reaction-centre quenching (Bilger and Björkman 1990). Demmig-Adams (1990) has suggested that quenching observed in thylakoids in the absence of zeaxanthin is reaction-centre quenching. Against this, Noctor et al. (1991) reported that in zeaxanthin-containing and zeaxanthin-lacking thylakoids the relationship between quenching of F_m (maximal fluorescence) and F_0 is the same, implying that in both cases quenching is occurring in the antenna. Also, Rees et al. (1992) have shown that quenching can be induced by low pH in thylakoids lacking zeaxanthin, even in the presence of sodium ascorbate, which inhibits reaction-centre quenching.

Activation of non-photochemical quenching by pre-illumination has, to date, only been reported in spinach thylakoids and there is no evidence that it is a phenomenon that occurs in other species. In addition, there is little evidence that this process is relevant to events occurring within the intact leaf. The activation of quenching observed may simply be an artifact of the *in vitro* conditions.

Recently, it has been demonstrated that plants possess a finite capacity for non-photochemical quenching of chlorophyll fluorescence ($\Delta F_m/F_m'$) and that the capacity varies considerably between species (Johnson et al. 1993). Two extreme species included *Chenopodium album* (saturated $\Delta F_m/F_m' = 2.59 \pm 0.06$) and *Digitalis purpurea* (saturated $\Delta F_m/F_m' = 4.36 \pm 0.08$). What is not known is what determines the capacity of quenching in these plants. In the present study we compare fluorescence quenching characteristics from these two species in order to investigate further the nature of species differences and to relate them to previous observations on activation of quenching.

Materials and methods

Plants of *Chenopodium album* L. and *Digitalis purpurea* L. were grown from seeds collected in the region of Sheffield and grown for four to six weeks in Levington M2 compost under metal-halide lights, photon flux density (PFD) $350 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ with a 12-h photoperiod at an average temperature of 25°C .

For experiments on isolated thylakoids, leaves were either dark- or light-treated as described by Noctor et al. (1991). This treatment involved exposing leaves to a CO_2 -free atmosphere (with 0, 2 or 20% O_2 , balance N_2 , as indicated in the text) for 50 min in the absence or presence of $200 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ illumination. Pretreated leaves were then coarsely cut (*D. purpurea*) or used whole (*C. album*) before being ground in 300 ml of a partially frozen grinding medium (300 mM sorbitol; 2% w/v polyvinylpyrrolidone; 0.5% w/v bovine serum albumin; 5 mM EDTA; 100 mM Hepes; 5 mM MgCl_2 ; pH 7.6) by giving two to three short bursts of a polytron. The homogenate was filtered through a double layer of muslin and then through eight layers of muslin and one layer of absorbent cotton wool. The filtrate was centrifuged at $2000 \times g$ for 30 s and the pellet washed twice and resuspended in a solution containing 300 mM sorbitol, 5 mM EDTA, 100 mM Hepes, 5 mM MgCl_2 (pH 7.3) using a soft paint brush. Resuspended chloroplasts were stored on ice until subsequent use.

The apparatus used to measure O_2 evolution and chlorophyll and 9-amino acridine (9-AA) fluorescence was as described by Noctor and Horton (1990). Thylakoids were used at a final concentration of $35 \mu\text{g}\cdot\text{ml}^{-1}$. Immediately prior to each measurement, an aliquot of chloroplast suspension was osmotically shocked in a solution containing 0.5 mM EDTA, 10 mM Hepes, 30 mM MgCl_2 (pH 7.3). After 30 s, an equal volume of a solution containing 660 mM sorbitol and 90 mM Hepes was added. Methyl viologen ($100 \mu\text{M}$) was added as an electron acceptor. 9-Amino acridine ($2 \mu\text{M}$) was added after establishing the zero level of the 9-AA fluorescence measuring system. Reversible quenching of chlorophyll fluorescence was estimated as $\Delta F_m/F_m'$ (Johnson et al. 1993) and quenching of 9-AA fluorescence (q9AA) as $(F_{\text{dark}} - F_{\text{light}})/F_{\text{dark}}$. Variation in the level of q9AA was achieved by illuminating thylakoids at PFDs (in the range 0 – $1000 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) for a period of 2 min. Quenching of chlorophyll fluorescence was measured relative to the F_m 2 min after the end of the actinic illumination. The ratio F_v/F_m is as defined in van Kooten and Snel (1990).

In experiments on intact leaves, dark-adapted leaves were held in an ADC detached leaf chamber (ADC, Hoddesdon, UK) with the cut edges being supplied with circulating water or a solution of DTT, as appropriate. Chlorophyll fluorescence was measured with a PAM 101 fluorimeter (Walz, Effeltrich, Germany) as described in Johnson et al. (1993).

In experiments where leaves were pre-fed with DTT, this was achieved by either placing the leaves with their petioles in (*D. purpurea*) or floating the leaves on the surface of (*C. album*) a 3 mM

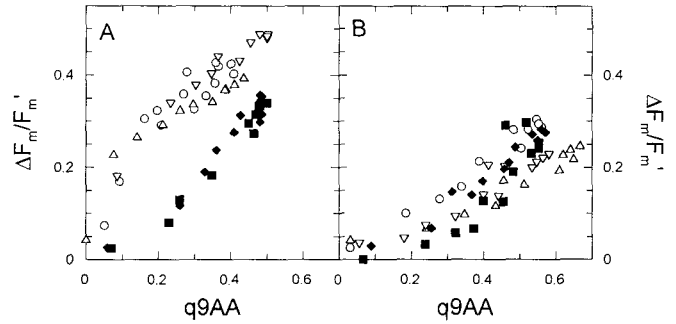


Fig. 1A, B. The relationship between 9-amino acridine quenching (q9AA) and fluorescence quenching ($\Delta F_m/F_m'$) in thylakoids isolated from *Digitalis purpurea* (A) and *Chenopodium album* (B). Open symbols refer to thylakoids prepared from light-treated leaves, filled symbols, dark-treated thylakoids. Circles refer to leaves that were treated in the presence of 20% O_2 balance N_2 (A) and O_2 -free N_2 (B). All other samples were treated in the presence of 2% O_2 balance N_2 . Zeaxanthin formation for each sample is indicated in Table 1

solution of DTT. Leaves were left for a minimum of 18 h to allow uptake of the DTT.

Results

Figure 1 shows the relationship between quenching of 9-AA fluorescence and of chlorophyll fluorescence in thylakoids of *C. album* and *D. purpurea* that were either dark- or light-treated. Data are shown from a total of five thylakoid isolations for each species. Table 1 shows the zeaxanthin formation and the dark-level F_v/F_m ratio of the thylakoids in each of these isolations. Data for *D. purpurea* are consistent with previous observations for spinach thylakoids (Noctor et al. 1991; Rees et al. 1990b). Following light pretreatment there was found to be a shift in the relationship between $\Delta F_m/F_m'$ and q9AA, such that high levels of chlorophyll fluorescence quenching were formed at a lower ΔpH . Such activation of quenching has been suggested to result from a facilitation of aggregation of light-harvesting complex induced by the formation of zeaxanthin (Horton et al. 1991). For thylakoids of *C. album* no activation of $\Delta F_m/F_m'$ is seen.

The level of zeaxanthin formation in thylakoids of *D. purpurea* pretreated in the presence of 2% O_2 is higher

Table 1. Zeaxanthin formation and dark-level F_v/F_m ratio in thylakoids of *Digitalis purpurea* and *Chenopodium album* exposed to different treatments. Symbols refer to those used in Fig. 1. Data for F_v/F_m are means \pm SE

Preparation	Treatment	Zeaxanthin formation (% total xanthophyll cycle)	F_v/F_m
<i>Digitalis purpurea</i> :			
(Upward triangle)	light 2% O_2 bal. N_2	60%	0.69 ± 0.002
(Downward triangle)	light 2% O_2 bal. N_2	59%	0.73 ± 0.001
(Circle)	light 20% O_2 bal. N_2	39%	0.75 ± 0.002
(Squares)	dark 2% O_2 bal. N_2	0%	0.73 ± 0.002
(Diamonds)	dark 2% O_2 bal. N_2	0%	0.75 ± 0.002
<i>Chenopodium album</i> :			
(Upward triangle)	light 2% O_2 bal. N_2	46%	0.73 ± 0.003
(Downward triangle)	light 2% O_2 bal. N_2	20%	0.70 ± 0.003
(Circle)	light O_2 free N_2	32%	0.66 ± 0.004
(Squares)	dark 2% O_2 bal. N_2	0%	0.80 ± 0.001
(Diamonds)	dark 2% O_2 bal. N_2	0%	0.79 ± 0.001

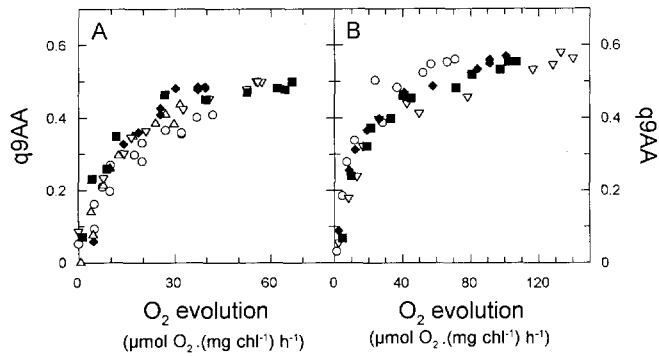


Fig. 2A, B. Relationship between 9-amino acridine fluorescence quenching (*q9AA*) and the rate of oxygen evolution per unit chlorophyll for different preparations of thylakoids of *Digitalis purpurea* (A) and *Chenopodium album* (B). Different symbols correspond to the different preparations of thylakoids as in Fig. 1 and Table 1

than in *C. album* (60%/59% cf. 46%/20% for data shown). It is therefore possible that the absence of light activation in *C. album* reflects a failure to attain a threshold level of zeaxanthin in these preparations. In an attempt to induce higher levels of zeaxanthin in this plant, leaves were treated in an atmosphere of O₂-free N₂ (circles in Fig. 1B). This treatment resulted in the conversion of only 32% of violaxanthin to zeaxanthin and did not lead to any activation of non-photochemical fluorescence quenching. In order, therefore, to obtain thylakoids from *D. purpurea* that had a lower zeaxanthin content, leaves of this species were illuminated in the presence of 20% O₂ balance N₂ (circles, Fig. 1A). This treatment resulted in the conversion of 39% violaxanthin to zeaxanthin, a level in the range obtained for *C. album*. Data from such thylakoids are indistinguishable from those with higher zeaxanthin contents. The lack of a direct correlation between zeaxanthin content and the extent of enhancement is inconsistent with the additional quenching observed being due to quenching directly by zeaxanthin, which is likely to require a quantitative relationship between the two parameters.

The measured level of non-photochemical quenching of chlorophyll fluorescence is sensitive to the dark level of the rate constant for thermal dissipation, k_D (Johnson et al. 1993). This parameter is reflected in the ratio F_v/F_m . A decrease in F_v/F_m will be induced by an increase in the dark level of k_D . In thylakoids of *D. purpurea* the level of F_v/F_m in light- and dark-treated thylakoids did not differ markedly (Table 1), indicating that this could not affect the relationship between changes in k_D and the value of $\Delta F_m/F_m'$. This was not the case for *C. album* where there was found to be a decrease in F_v/F_m following light treatment (Table 1). Such a difference would result in a lower value of $\Delta F_m/F_m'$ for a given light-induced increase in thermal dissipation. In Fig. 1B it appears that the relationship between $\Delta F_m/F_m'$ and *q9AA* differs slightly in gradient between dark- and light-treated thylakoids. This difference can be explained by the decrease in F_v/F_m .

The relationship between $\Delta F_m/F_m'$ and *q9AA* will also be altered if light treatment affected the relationship between ΔpH and *q9AA*. Such an effect alone is unlikely to explain the different behaviour seen in the titrations in

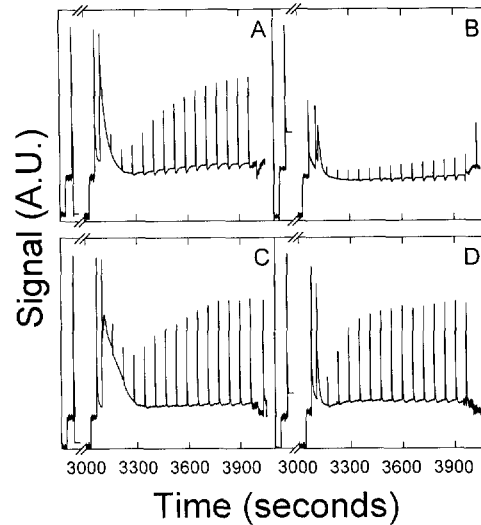


Fig. 3A–D. Chlorophyll-fluorescence induction in leaves of *Digitalis purpurea* (A, B) and *Chenopodium album* (C, D) following 50 min in an atmosphere of 2% O₂ balance N₂ either illuminated at a PFD of 200 μmol·m⁻²·s⁻¹ (B, D) or in the dark (A, C). Leaves were left darkened for 10 min in the presence of normal air (350 μl·l⁻¹ CO₂) after treatment, prior to measurement of chlorophyll fluorescence. The break in the x-axis represents the period of treatment. Zeaxanthin formation and quenching under each set of conditions are indicated in Table 2

Fig. 1A, however it should be considered as a possible factor affecting the data. Figure 2 shows the relationship between *q9AA* and O₂ evolution recorded at a variety of different PFDs in thylakoid preparations included in Fig. 1. At any given irradiance the level of the ΔpH will be a function of the rate at which protons are translocated across the thylakoid membrane (indicated by the rate of electron transport) and the rate of leakage from the lumen. Hence, if leakage is assumed to be constant, the rate of O₂ evolution will, to some extent, be indicative of the ΔpH . For both *D. purpurea* and *C. album* light treatment does not affect the relationship between O₂ evolution and *q9AA*. Indeed the relationship is almost identical when comparing the two species, suggesting that it maybe reasonable to compare levels of *q9AA* between these species.

The highest levels of $\Delta F_m/F_m'$ obtained in *D. purpurea* were found to be significantly higher than in *C. album* (0.5 cf. 0.3). Although making direct comparisons between levels of quenching in thylakoids of different species should be approached with caution, this difference is consistent with the differences observed in vivo.

The failure to induce activation of high-energy-state quenching in thylakoids from *C. album* may reflect a low tendency for, or complete absence of, such activation in vivo. Alternatively, the lack of activation may simply reflect a response to the procedure used to isolate the thylakoids. Similarly, the activation response seen here in thylakoids of *D. purpurea* and previously in spinach may represent a process that occurs in an intact leaf or may simply reflect the maintenance of a state in vitro that is ordinarily present in vivo. In an intact leaf it is not possible to measure ΔpH and hence to establish the relationship between ΔpH and non-photochemical quenching in

Table 2. Effect of light treatment on reversible non-photochemical quenching in leaves of *Chenopodium album* and *Digitalis purpurea* at a PFD of $200 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Measurements were taken after 15 min illumination and quenching estimated relative to the F_m before light treatment, correcting for irreversible quenching. Zeaxanthin formation prior to the measurement of quenching in leaves that were either dark-adapted or light-treated is also indicated. Data are means \pm SE

	<i>C. album</i>	<i>D. purpurea</i>
$\Delta F_m/F_m'$ (reversible):		
Dark-adapted	0.27 ± 0.04	0.38 ± 0.04
Light-treated	0.41 ± 0.19	1.16 ± 0.45
Zeaxanthin Formation (% total xanthophyll cycle):		
Dark-adapted	0 ± 0	0 ± 0
Light-treated	57 ± 6.6	65 ± 5.3

vivo. Comparison between the extent of quenching at limiting light intensities in leaves that have been either dark-adapted or have been pretreated to form zeaxanthin should, however, indicate whether activation of quenching has occurred. Figure 3 shows fluorescence-induction curves for intact leaves of *C. album* and *D. purpurea* that had been either dark- or light-treated. Leaves were maintained in an atmosphere of 2% O_2 balance N_2 (CO_2 free) for 10 min prior to being illuminated (or not) for 50 min. The sample chamber was then purged with normal air for 10 min in the dark prior to measurement of fluorescence. The levels of reversible non-photochemical quenching and zeaxanthin formation in leaves treated in this way are indicated in Table 2. There are clear quantitative and qualitative differences in the responses of these plants to light treatment. In *D. purpurea* there is a clear indication that activation of non-photochemical quenching is occurring, with $\Delta F_m/F_m'$ increasing significantly (Table 2). No such activation of quenching is apparent in *C. album*. In addition, light treatment causes a depression of the ratio F_v/F_m in *D. purpurea*, contrasting with the behaviour of isolated thylakoids (Table 1). This depression is less marked in *C. album*. The only obvious effect of light treatment on *C. album* is an acceleration in the rate of quenching induced upon illumination.

The activation of non-photochemical fluorescence quenching seen in thylakoids can be inhibited by prefeeding of leaves with DTT (Noctor et al. 1991). Hence, from the above results, DTT would be expected to have differential effects on *C. album* and *D. purpurea*. Since activation of quenching cannot be detected in *C. album*, it would be predicted that in this species DTT would exert little or no effect on quenching. Figure 4 shows the effect of DTT feeding on fluorescence induction in both *C. album* and *D. purpurea*. Table 3 indicates the effect of such treatment on both the steady-state level of quenching and on zeaxanthin formation. In both plant species, feeding of leaves with DTT completely inhibits zeaxanthin formation. In *D. purpurea* this results in 63% inhibition of reversible non-photochemical quenching. In *C. album* this inhibition is 92%. In *C. album* there is a striking difference in the overall pattern of quenching following treatment with DTT. An initial fast phase in quenching is still

Table 3. Effect of infusion of leaves with dithiothreitol on rapidly reversible non-photochemical quenching and on zeaxanthin formation in *Chenopodium album* and *Digitalis purpurea* following illumination for 15 min at a PFD of $1000 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Quenching was calculated relative to F_m recorded 5 min after the end of illumination. Control data represent the mean \pm SE of three samples (*C. album*), treated the mean \pm SE of six (*C. album*) or four (*D. purpurea*) samples. *The control value for *C. album* is for a single determination

	<i>C. album</i>	<i>D. purpurea</i>
$\Delta F_m/F_m'$ (reversible):		
Treated	0.10 ± 0.03	1.68 ± 0.43
Control	1.98 ± 0.33	3.11 ± 0.14
% Inhibition	92 ± 2	63 ± 10
Zeaxanthin formation (% xanthophyll cycle):		
Control	44.9^*	62.2 ± 3.7
Treated	0 ± 0	0 ± 0

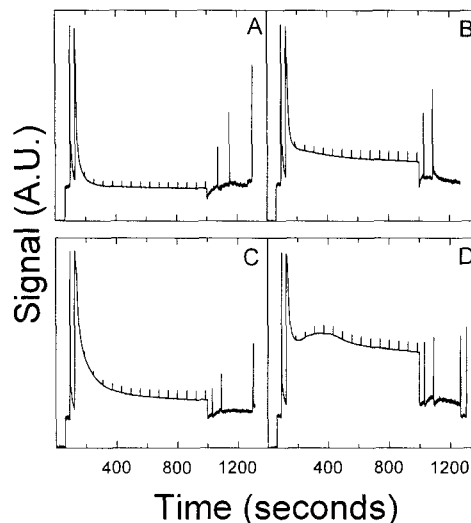


Fig. 4A–D. Effect of DTT on leaves of *Digitalis purpurea* (A, B) and *Chenopodium album* (C, D). Leaves were infused overnight with either 3 mM DTT (B, D) or water (A, C). Fluorescence quenching was recorded during illumination at a PFD of $1000 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The proportion of non-photochemical fluorescence quenching that recovered from this treatment was estimated by application of a high-intensity light pulse 5 min after the actinic light was removed. Zeaxanthin content and quenching after such treatments are indicated in Table 3

seen but the slower component is largely absent. The non-photochemical quenching that is observed does not reverse significantly over a period of 10 min. Although similar effects are seen in *D. purpurea*, they are less marked than in *C. album* and a significant component of reversible quenching is still present.

Discussion

In this paper we have demonstrated that activation of high energy-state quenching in thylakoids by pre-illumination of leaves in a CO_2 -free atmosphere is a phenomenon that is not exclusive to spinach (Fig. 1). The

presence of such activation in *D. purpurea* and its absence in *C. album* suggests that the molecular processes underlying this may be of importance in the adaptation of plants to different environments. Measurements of non-photochemical quenching in intact leaves (Fig. 3, Table 2) are consistent with the behaviour of thylakoids isolated from the same plants, giving confidence to the assertion that in vitro observations are relevant to the in vivo system.

A physiological role for the activation of quenching is easy to envisage. For a plant growing in a semi-shaded situation, exposure to short periods of high light ("sunflecks") is unlikely to lead to severe stress. Indeed sunflecks have been proposed to play an important role in the photosynthesis of shade plants (Pearcy 1990). If however such a plant is exposed to high light for longer periods the risk of photo-damage is far greater. Activation of quenching processes will allow the plant to down-regulate photosynthesis, limiting the rate of electron flow and preventing over-reduction of intermediates in the photosynthetic pathway, whilst ensuring that excess excitation energy is dissipated harmlessly. In the above context, the apparent lack of activation of non-photochemical quenching in *C. album* may represent the adoption of an ecological strategy different from that seen in *D. purpurea*. The maximum rate of photosynthesis in *C. album* is far higher than that in *D. purpurea*. Measurements at saturating CO₂ concentrations showed the maximal rate of photosynthesis per unit area in *C. album* to be approximately twice that in *D. purpurea* (30 $\mu\text{mol O}_2\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ for *C. album* cf. 15 $\mu\text{mol O}_2\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ for *D. purpurea*; Johnson 1992) This high photosynthetic rate will enable absorbed energy to be dissipated far more efficiently by photochemical routes. Hence, under most natural circumstances, thermal dissipation may be of little significance. Such a strategy is only practical, however, for plant species that are adapted to habitats where nutrient supply is not limiting to growth. *Chenopodium album* is a classic example of a ruderal species that grows only in fertile soils (Grime et al. 1988). Examination of a wider range of species in an ecological context is clearly required to understand better the different strategies that plants adopt in dealing with excess light.

The similarity in the behaviour seen here for *D. purpurea* and that seen previously for spinach might lead to the expectation that spinach and *D. purpurea* are similar in other respects. However, arguments based on plant ecology are difficult to apply to species such as spinach that have been selected for agriculture and are not subject to the restraints imposed by competing in a natural habitat. Previous studies have demonstrated similarities between spinach and *C. album* (Johnson 1992; Johnson et al. 1993), and in some respects spinach might be thought of as showing behaviour that is intermediate between that of the two species studied here.

Differences in the relationship between high-energy-state quenching and ΔpH have been explained in the literature in at least two ways. Rees et al. (1990b) and Noctor et al. (1991) interpreted their data as indicating that treatments that induce zeaxanthin formation result in an activation of quenching such that high levels of quench-

ing are attained at low ΔpH s. Demmig-Adams (1990) interpreted these same data as representing two forms of quenching, one being zeaxanthin-dependent and the other being zeaxanthin-independent. Whichever of these explanations is correct, it is clear from the data we present here that the presence of zeaxanthin per se is not sufficient to induce this phenomenon. Nor, where activation is seen, is there a direct quantitative relationship between the extent of quenching and the concentration of zeaxanthin.

The sensitivity of *C. album* to DTT is an anomaly that is not readily explained in the light of current models of quenching. The failure of this species to show any activation of quenching, whilst still being sensitive to DTT, implies that, in this plant at least, DTT does not inhibit quenching by preventing activation. It is possible, therefore, that any role of zeaxanthin in quenching does not necessarily involve light activation. However, the effect of DTT is by no means confined to inhibiting violaxanthin de-epoxidation and may therefore be inhibiting quenching in a way that is unrelated to zeaxanthin formation. The high level of inhibition of quenching in *C. album* may, for example, be the result of inhibition of ΔpH formation, as suggested by Schreiber and Neubauer (1990), combined with the absence of a second quenching process. The concentration of DTT used in our experiments is sufficient to cause inhibition of Mehler reaction, should that be occurring (Neubauer 1993). Alternatively, activation of the thylakoid ATPase may lead to the maintenance of a significant ΔpH following illumination, so giving low apparent levels of relaxation of quenching. Differences in the sensitivity of various species to DTT feeding have been observed previously (Bilger and Björkman 1990; Demmig-Adams et al. 1990b).

The disparity between observations of DTT-insensitive quenching of F_o in vivo (Bilger and Björkman 1989) and in vitro (Noctor et al. 1992) implies that different processes may be occurring in the different conditions. It is seen in Figure 1 that quenching in dark-adapted thylakoids requires a certain threshold ΔpH to be attained. Hence, in vivo, in the presence of DTT, the ΔpH may not reach the levels required to cause quenching of the type seen in isolated thylakoids (Schreiber and Neubauer 1990; Neubauer 1993). A second form of quenching may therefore be present in intact leaves of certain species (for example *D. purpurea*) that is not observed under the experimental conditions used for the in-vitro experiments.

The data presented in this paper suggest that the mechanisms involved in regulation of non-photochemical quenching in *C. album* and *D. purpurea* are, in some fundamental respects, different. These differences probably reflect different ecological strategies in dealing with excess light. More work is required both to elucidate the mechanisms of non-photochemical quenching and to understand fully the significance of the differences observed between species.

We would like to thank Dr. Debbie Rees for her help with the chloroplast experiments and for her invaluable support. We would also like to thank Dr. Robin Walters, Dr. Sasha Ruban and other colleagues at Sheffield for many useful discussions and Dr. Anja Krieger (C.E.-Saclay) for her critical reading of the manuscript.

G.N.J. was in receipt of an Natural Environment Research Council studentship.

References

- Bilger, W., Bjorkman, O. (1990) Role of the xanthophyll cycle in photoprotection elucidated by measurements of light induced absorbance changes, fluorescence and photosynthesis in leaves of *Hedera canariensis*. *Photosynth. Res.* **25**, 173–185
- Bilger, W., Bjorkman, O., Thayer, S.S. (1989) Light-induced spectral absorbance changes in relation to photosynthesis and the epoxidation state of xanthophyll cycle components in cotton leaves. *Plant Physiol.* **91**, 542–551
- Briantais, J.M., Verrotte, C., Picaud, M., Krause, H. (1979) A quantitative study of the slow decline of chlorophyll *a* fluorescence in isolated chloroplasts. *Biochim. Biophys. Acta* **548**, 128–138
- Crofts, J., Horton, P. (1991) Dissipation of excitation energy by photosystem II particles at low pH. *Biochim. Biophys. Acta* **1058**, 187–193
- Demmig, B., Winter, K. (1988) Characterisation of three components of non-photochemical fluorescence quenching and their response to photoinhibition. *Aust. J. Plant Physiol.* **15**, 163–177
- Demmig-Adams, B. (1990) Carotenoids and photoprotection: A role for the xanthophyll zeaxanthin. *Biochim. Biophys. Acta* **1020**, 1–24
- Demmig-Adams, B., Adams, W.W., Czygan, F.-C., Schreiber, U., Lange, O.L. (1990a) Differences in the capacity for radiationless energy dissipation in the photochemical apparatus of green and blue-green algal lichens associated with differences in carotenoid composition. *Planta* **180**, 582–589
- Demmig-Adams, B., Adams, W.W., Heber, U., Neimanis, S., Kruger, A., Czygan, F.-C., Bilger, W., Bjorkman, O. (1990b) Inhibition of zeaxanthin formation and of rapid radiationless energy dissipation by dithiothreitol in spinach leaves and chloroplasts. *Plant Physiol.* **92**, 293–301
- Grime, J.P., Hodgson, J.G., Hunt, R. (1988) *Comparative Plant Ecology*. Unwin Hyman, London
- Horton, P., Bowyer, J.R. (1990) Chlorophyll fluorescence transients. In: *Methods in plant biochemistry*, vol. 4, pp. 259–296, Bowyer, J.R., Harwood, J., eds. Academic Press, London
- Horton, P., Hague, A. (1988) Studies on the induction of chlorophyll fluorescence in isolated barley protoplasts: IV. Resolution of non-photochemical quenching. *Biochim. Biophys. Acta* **932**, 107–115
- Horton P., Ruban, A.V. (1992) Regulation of photosystem II. *Photosynth. Res.* **34**, 375–385
- Horton, P., Ruban, A.V., Rees, D., Pascal, A.A., Noctor, G., Young, A.J. (1991) Control of the light-harvesting function of chloroplast membranes by aggregation of the LHClI chlorophyll-protein complex. *FEBS Lett.* **292**, 1–4
- Johnson, G.N. (1992) Dissipation of excitation energy in ecologically contrasted plant species. PhD thesis, University of Sheffield
- Johnson, G.N., Young, A.J., Scholes, J.D., Horton, P. (1993) The dissipation of excess excitation energy in British plant species. *Plant Cell Environ.* **16**, 673–679
- Krause G.H., Behrend, U. (1986) Δ pH-dependent chlorophyll fluorescence quenching indicating a mechanism of protection against photoinhibition of chloroplasts. *FEBS Lett.* **200**, 298–302
- Krause, G.H., Weis, E. (1991) Chlorophyll fluorescence and photosynthesis: the basics. *Annu. Rev. Plant Phys. Plant Mol. Biol.* **42**, 313–349
- Krieger, A., Weis, E. (1992) Energy-dependent quenching of chlorophyll-*a* fluorescence: The involvement of proton-calcium exchange at photosystem 2. *Photosynthetica* **27**, 89–98
- Neubauer, C. (1993) Multiple effects of dithiothreitol on non-photochemical fluorescence quenching in intact chloroplasts. *Plant Physiol.* **103**, 575–583
- Noctor, G., Horton, P. (1990) Uncoupler titration of energy dependent chlorophyll fluorescence quenching and photosystem II photochemical yield in intact pea chloroplasts. *Biochim. Biophys. Acta* **1016**, 228–234
- Noctor, G., Rees, D., Young, A.J., Horton, P. (1991) The relationship between zeaxanthin, energy-dependent quenching of chlorophyll fluorescence and trans-thylakoid pH gradient in isolated chloroplasts. *Biochim. Biophys. Acta* **1057**, 320–330
- Pearcy, R.W. (1990) Sunflecks and photosynthesis in plant canopies. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **41**, 421–453
- Quick, W.P., Stitt, M. (1989) An examination of the factors contributing to non-photochemical quenching of chlorophyll fluorescence quenching in barley leaves. *Biochim. Biophys. Acta* **977**, 287–296
- Rees, D., Noctor, G., Horton, P. (1990a) The effect of high energy-state excitation quenching on maximum and dark level fluorescence yield. *Photosynth. Res.* **25**, 199–211
- Rees, D., Noctor, G., Young, A.J., Britton, G., Horton, P. (1990b) Enhancement of the Δ pH dependent dissipation of excitation energy in a spinach chloroplasts by light activation: correlation with the synthesis of zeaxanthin. *FEBS Lett.* **256**, 85–90
- Rees, D., Noctor, G., Ruban, A.V., Crofts, J., Young, A.J., Horton, P. (1992) pH-dependent chlorophyll fluorescence quenching in spinach thylakoids from light-treated and dark-adapted leaves. *Photosynth. Res.* **31**, 11–19
- Ruban, A.V., Rees, D., Pascal, A.A., Horton, P. (1992) Mechanism of Δ pH-dependent quenching of the F_0 level of chlorophyll fluorescence in spinach leaves. *Biochim. Biophys. Acta* **1102**, 39–44
- Ruban, A.V., Young, A.J., Horton, P. (1993) Induction of non-photochemical energy dissipation and absorbance changes in leaves; evidence for changes in the state of the light harvesting system of photosystem II in vivo. *Plant Physiol.* **102**, 741–750
- Schreiber, U., Neubauer, C. (1990) O_2 -dependent electron flow membrane energisation and the mechanism of non-photochemical quenching of chlorophyll fluorescence. *Photosynth. Res.* **25**, 279–293
- van Kooten, O., Snel, J.F.H. (1990) The use of chlorophyll fluorescence nomenclature in plant stress physiology. *Photosynth. Res.* **25**, 147–150
- Walters, R.G., Horton, P. (1991) Resolution of components of non-photochemical chlorophyll fluorescence quenching in barley leaves. *Photosynth. Res.* **27**, 121–133
- Walters, R.G., Horton, P. (1993) Theoretical assessment of alternative mechanisms for non-photochemical chlorophyll quenching in barley leaves. *Photosynth. Res.* **36**, 741–750
- Yamamoto, H.Y., Kamite, L. (1972) The effects of dithiothreitol on violaxanthin de-epoxidation and absorbance changes in the 500 nm region. *Biochim. Biophys. Acta* **267**, 538–543