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# Temperature dependence of violaxanthin de-epoxidation and non-photochemical fluorescence quenching in intact leaves of *Gossypium hirsutum* L. and *Malva parviflora* L.*

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**Abstract.** The temperature dependence of the rate of de-epoxidation of violaxanthin to zeaxanthin was determined in leaves of chilling-sensitive *Gossypium hirsutum* L. (cotton) and chilling-resistant *Malva parviflora* L. by measurements of the increase in absorbance at 505 nm ($\Delta A_{505}$) and in the contents of antheraxanthin and zeaxanthin that occur upon exposure of predarkened leaves to excessive light. A linear relationship between $\Delta A_{505}$ and the decrease in the epoxidation state of the xanthophyll-cycle pigment pool was obtained over the range 10–40° C. The maximal rate of de-epoxidation was strongly temperature dependent; $Q_{10}$ measured around the temperature at which the leaf had developed was 2.1–2.3 in both species. In field-grown *Malva* the rate of de-epoxidation at any given measurement temperature was two to three times higher in leaves developed at a relatively low temperature in the early spring than in those developed in summer. $Q_{10}$ measured around 15° C was in the range 2.2–2.6 in both kinds of *Malva* leaves, whereas it was as high as 4.6 in cotton leaves developed at a daytime temperature of 30° C. Whereas the maximum (initial) *rate* of de-epoxidation showed a strong *decrease* with decreased temperature the *degree* of de-epoxidation reached in cotton leaves after a 1–2 · h exposure to a constant photon flux density *increased* with decreased temperature as the rate of photosynthesis decrease. The zeaxanthin content rose from 2 mmol · (mol chlorophyll)$^{-1}$ at 30° C to 61 mmol · (mol Chl)$^{-1}$ at 10° C, corresponding to a de-epoxidation of 70% of the violaxanthin pool at 10° C. The degree of de-epoxidation at each temperature was clearly related to the amount of excessive light present at that temperature. The relationship between non-photochemical quenching of chlorophyll fluorescence and zeaxanthin formation at different temperatures was determined for both untreated control leaves and for leaves in which zeaxanthin formation was prevented by dithiothreitol treatment. The rate of development of that portion of non-photochemical quenching which was inhibited by dithiothreitol decreased with decreasing temperature and was linearly related to the rate of zeaxanthin formation over a wide temperature range. In contrast, the rate of development of the dithiothreitol-resistant portion of non-photochemical quenching was remarkably little affected by temperature. Evidently, the kinetics of the development of non-photochemical quenching upon exposure of leaves to excessive light is therefore in large part determined by the rate of zeaxanthin formation. For reasons that remain to be determined the relaxation of dithiothreitol-sensitive quenching that is normally observed upon darkening of illuminated leaves was strongly inhibited at low temperatures.

**Key words:** Chlorophyll fluorescence quenching – Energy dissipation (non-radiative) – *Gossypium* (photosynthesis) – Light (excessive) – *Malva* – Photosynthesis and energy dissipation – Temperature (leaf) – Violaxanthin – Xanthophyll cycle – Zeaxanthin

## Introduction

There is now much evidence that the carotenoid zeaxanthin is involved in "safe" dissipation of light energy excessive to photosynthesis, thereby serving a photoprotective function. Zeaxanthin is formed by de-epoxidation of violaxanthin via antheraxanthin in the "xanthophyll cycle" (Yamamoto 1979; Hager 1980). De-epoxidation takes place when photosynthetic energy consumption can no longer make full use of the light absorbed by the chlorophyll. Conversely, when light be-

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*Abbreviations and symbols:* Chl = chlorophyll, DTT = dithiothreitol; EPS = epoxidation state; NPQ = non-photochemical chlorophyll fluorescence quenching; PFD = photon flux density; PSII = photosystem II; F, $F_m$ = fluorescence emission at the actual, full closure of the PSII centers

comes limiting to photosynthesis zeaxanthin is re-epoxidized to violaxanthin.

Much of the evidence for zeaxanthin-mediated energy dissipation comes from studies which show that there exists a close relationship between zeaxanthin content and non-photochemical quenching (NPQ) of chlorophyll fluorescence under a wide range of conditions; moreover, dithiothreitol (DTT) which inhibits the de-epoxidation of violaxanthin to zeaxanthin, also inhibits a large part of NPQ (for references see Demmig-Adams et al. 1990; Bilger and Björkman 1990). Dithiothreitol treatments did not cause any changes in the photosynthetic gas-exchange characteristics, nor in the efficiency of PSII, as long as the leaves were not subjected to prolonged exposure to excessive light; however such treatments did result in an increase in the reduction state of $Q_A$, the primary acceptor of photosystem II; and also increased the susceptibility to photoinhibtory damage (Bilger et al. 1989; Winter and Königer 1989; Bilger and Björkman 1990; Demmig-Adams et al. 1990).

Unfavorable temperatures that reduce the rate of photosynthetic electron transport potentially also increase the amount of excessive light present at any given incident light and it is well known that chilling causes an increased susceptibility to photodamage, especially in plants of tropical origin. Information on the temperature dependence of zeaxanthin formation could therefore provide further valuable insight into the relationship between zeaxanthin and dissipation of excess excitation energy. So far only one report (Demmig-Adams et al. 1989) has dealt with the relationship between the temperature dependence of zeaxanthin formation and NPQ. These authors found that zeaxanthin formation and the development of NPQ in the tropical mangrove *Rhizophora mangle* were much slower at 5° C than at 20° C and, to lesser extent, also in soybean leaves.

The main objectives of the present study were to determine: (1) the temperature dependence of the maximal rate of de-epoxidation of violaxanthin to zeaxanthin that occurs upon exposure of pre-darkened leaves to excessive light and how this rate may be influenced by the temperature at which a leaf has developed; (2) the temperature dependence of the degree of de-epoxidation reached at steady state when a photosynthesizing leaf is exposed to a constant photon flux density; and (3) the temperature dependence of the relationship between the kinetics of zeaxanthin formation and that of the non-photochemical quenching that occurs when pre-darkened leaves are suddenly exposed to excessive light. We made use of the recent finding that zeaxanthin formation can be readily monitored in intact leaves by measurements of light-induced absorbance changes at 505 nm (Bilger et al. 1989).

## Material and methods

*Plant materials.* Leaves of upland cotton (*Gossypium hirsutum* L. cv. Acala SJC-1) were used for most of the experiments. Seeds were obtained from California Planting Cotton Seed Distributors, Bakersfield, USA. Plants were grown hydroponically in a growth-room in a flowing nutrient solution as described by Schäfer and Björkman (1989). Leaves used in experiments had developed at photon flux densities (PFDs) between 750 and 850 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 14 $h \cdot d^{-1}$. The air temperature was kept at 30° C during the light period and at 25° C during the dark period. Leaf temperatures, measured with an infrared thermometer (Everest Interscience, Tustin, Cal., USA), were near those of the air. In the evening before the experiments plants were shaded such that the PFD was attenuated to 50–100 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The PFD was measured with quantum sensors (Model LI 190 SB; Li-Cor, Lincoln, Neb., USA.

In experiments with DTT-treated plants a plant was transferred in the evening to a continuously aerated nutrient solution containing 1.5 mM oxidized DTT (trans-4,5-dihydroxy-1,2-dithiane, Sigma Chemical Co., St. Louis, Mo., USA). Oxidized DTT was chosen instead of reduced DTT in order to prevent anaerobisis in the root system. (Use of reduced DTT in the root medium resulted in wilting; no wilting was detected when oxidized DTT was used.) Overnight uptake of the oxidized DTT solution was estimated by measuring the loss of solution. Each plant took up 2.5–3 g solution per gram plant fresh weight. That the uptake of DTT was sufficient for complete inhibition of zeaxanthin formation at a PFD of 2000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ was checked by pigment analysis and measurement of light-induced absorbance changes at 505 nm. Both untreated and DTT-treated plants were transferred to darkness 2 h before exposure of the leaves to excessive light.

*Malva parviflora* L., also a member of the Malvaceae, was grown during the months of January–March and May–July 1990 in a field just outside the laboratory at Stanford. The plants belonged to a spontaneous, naturalized population. One set of experiments with *Malva* leaves was conducted during the first week of March 1990. The mean hourly maximal temperature for the 10-d period preceding the experiments was 14.8° C, and the mean hourly minimum temperature was 6.6° C. The extreme hourly mean air temperatures during this period were 2.0° and 19.4° C. Leaf temperatures measured periodically at various times of the day ranged from air temperature to 3° C above air temperature. A second set of experiments on *Malva* leaves was conducted during the last week of July. At this time the mean hourly maximal and minimal temperatures for the preceding ten day period were 25.4° and 14.0° C, respectively, and the extreme air temperatures were 10° and 27° C. In the evening before the day of each experiment, shades were installed above the leaves to be used. In the next morning the leaves were cut under water and those used for DTT treatment were transferred to a small flask to which DTT was added to give a final concentration of 3 mM. The leaves were allowed to take up DTT solution through the cut petiole for 2–4 h at a PFD of 15 $\mu mol \cdot m^{-2} \cdot s^{-1}$ until an amount of solution equal to one to two times the leaf fresh weight had been taken up. The leaves were then kept in darkness for 2–3 h before the start of measurements. Control leaves were treated as described above except that the DTT solution was replaced by water.

*Absorbance and chlorophyll-fluorescence measurements.* For the measurements of the induction of absorbance and fluorescence changes we used the apparatus described in Bilger and Björkman (1990). It incorporates a PAM 101 fluorescence measuring system (H. Walz, Effeltrich, Germany) and a custom-built device for measurement of absorbance. The modulated measuring beams and the beams of the actinic light were directed through two separate fiberoptic systems onto two adjacent spots on the leaf. Absorbance was measured at 505 nm (half-bandwidth = 7.5 nm). The actinic beams for the fluorescence and absorbance measurements were both adjusted to a PFD of 2000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ by means of neutral density filters (FNG series; Melles Griot, Irvine, Cal., USA). For the experiments on leaves of *Malva* grown in the field during summer the actinic PFD was 2100 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Both actinic light beams were filtered through red dichroic filters (OCLI, Santa Rosa, Cal., USA). The maximum PFD of the saturating pulses used in for the fluorescence measurements was 9500 $\mu mol \cdot m^{-2} \cdot s^{-1}$. With cotton leaves at temperatures below 25° C, the PFD of the saturating pulses was reduced in order to obtain maximal fluorescence since the full-pulse PFD induced

fluorescence quenching. The pulse PFD was reduced to 50% at 20° C, 25% at 15° C, and 20% of full beam at 10–12° C.

For control of leaf temperature the leaf was enclosed in a small plastic housing (approx. $10 \cdot 11 \cdot 6$ cm$^3$) open at the leaf side. A heat exchanger, whose temperature was controlled by circulating water from a refrigeration-equipped constant-temperature bath, was mounted on the opposite side, and an air stream was passed through the heat exchanger onto the leaf. Leaf temperature was monitored by a small thermocouple appressed to the lower leaf surface in the center of the "fluorescence" beam. Since the actinic light raised the leaf temperature by approx. 1.6° C the leaf temperature was adjusted prior to illumination such that the temperature was 1.6° C lower than desired. At temperatures above 20° C the leaf temperature tended to fall after 3–6 min in the light, most probably resulting from opening of the stomata. As a result, the leaf temperature was 1.5–2.5° C lower during the first 2–5 min after the light was turned off than during the first minutes of illumination. Measurements were always started at a leaf temperature approximately equal to the daytime growth temperature and was then either raised or lowered. A leaf which had reached one temperature extreme was not used for any further measurements.

*Measurements of the temperature dependence of the degree of violaxanthin de-epoxidation and photosynthetic rate at steady state.* Intact, fully exposed leaves of cotton plants grown as described above were selected to give closely matching leaves as to position, degree of expansion and incident PFD ($800 \pm 50$ µmol $\cdot$ m$^{-2} \cdot$ s$^{-1}$). Plants were transferred to a cylinder containing a continuously aerated nutrient solution which was maintained at 28° C. A leaf was trimmed to an area of 40 cm$^2$ and inserted into the leaf chamber of an open gas-exchange system (Schäfer and Björkman 1989). The PFD incident on the leaf was maintained at 800 µmol $\cdot$ m$^{-2} \cdot$ s$^{-1}$. All experiments were started at a leaf temperature of 30° C, 34 Pa $CO_2$, 21 kPa $O_2$, and a water-vapor pressure deficit of 1.0–1.2 kPa. These conditions were maintained until a constant photosynthetic rate had been reached and then for an additional 30-min period. A 4.0-cm$^2$ strip was then rapidly cut from the leaf for subsequent pigment analysis. The leaf temperature was then either increased or decreased to the desired setpoint and after it had been reached the leaf was maintained at this temperature for 1 h and a leaf sample taken again. (At temperatures $< 16$° C, where the rate of de-epoxidation is slow, samples were again taken after an additional 1-h period). The temperature was then changed to the next setpoint and the process was repeated. No single leaf was subjected to more than two temperature steps. Nine plants were used to obtain the results presented in Fig. 4. In all experiments the $CO_2$ and water-vapor pressure of the incoming air stream was adjusted to maintain a constant partial pressure of these gases in the leaf chamber at all temperatures. Dark respiration rates at the different temperatures were determined in separate experiments on leaves matching those used in the photosynthesis determinations.

*Pigment analyses.* For the pigment analyses made in conjunction with measurements of fluorescence and $\Delta A_{505}$ each disk (0.42 cm$^2$) was punched from the center of the area illuminated by the fluorescence fiberoptics (1.14 cm$^2$). For those made in conjunction with the photosynthesis measurements three disks (each 0.82 cm$^2$) were punched from each of the leaf strips. The disks were frozen in liquid nitrogen within 30 s and stored at −80° C. Carotenoid and chlorophyll (Chl) contents of the disks were determined by extraction in 85% acetone and separation with high-performance liquid chromatography (HPLC) as described in detail by Thayer and Björkman (1990).

*Formulas used to calculate epoxidation state and fluorescence parameters.* The epoxidation state (EPS) was calculated from $EPS = (V + 0.5A)/(V + A + Z)$ where V, A and Z denote the contents of violaxanthin, antheraxanthin and zeaxanthin. Non-photochemical fluorescence quenching (NPQ) was calculated according to the Stern-Volmer equation $NPQ = F_m/F_m' - 1$ and the photon yield of PSII photochemistry in the light ($\Phi_{PSII}$) from the equation $\Phi_{PSII} = (F_m' - F)/F_m'$ where $F_m$ and $F_m'$ denote the fluorescence when all PSII centers are temporarily closed, before and after exposure to light, respectively, and F denotes the fluorescence at the actual redox state of the PSII centers (Butler 1978; Genty et al. 1989; Bilger and Björkman 1990).

## Results

Time courses of the light-induced absorbance change at 505 nm ($\Delta A_{505}$) for cotton leaves at 15°, 20° and 30° C are shown by the traces in Fig. 1. The rate of the absor-

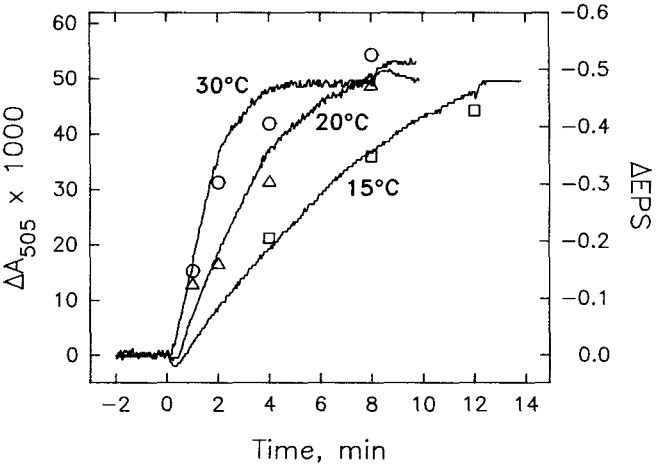


**Fig. 1.** Time course of the light-induced absorbance change at 505 nm ($\Delta A_{505}$) and the change in epoxidation state (ΔEPS) in pre-darkened cotton leaves at 15° (□), 20° (△) and 30° C (○). At time 0 the actinic light (PFD = 2000 µmol $\cdot$ m$^{-2} \cdot$ s$^{-1}$) was turned on. At 8 min (30° and 20° C traces) or at 12 min (15° C trace) the light was switched off. The EPS was determined in disks taken at different times after onset of illumination at spots on the same leaf adjacent to the place where $\Delta A_{505}$ was determined. The EPS in the dark varied from 0.958 to 1.000 and was subtracted from EPS in the illuminated samples to give ΔEPS

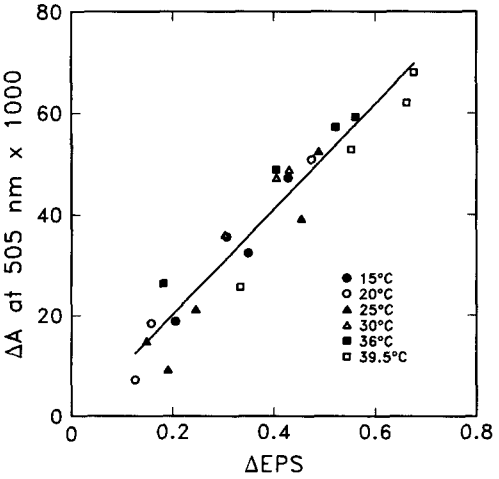


**Fig. 2.** Relationship between $\Delta A_{505}$ and ΔEPS in cotton leaves at different temperatures during the first 1–12 min of exposure to a high PFD of leaves which had been pre-darkened for at least 2 h. The experiments were similar to those shown in Fig. 1. Different symbols denote different temperatures as indicated on the graph. The measurements were made with four different leaves

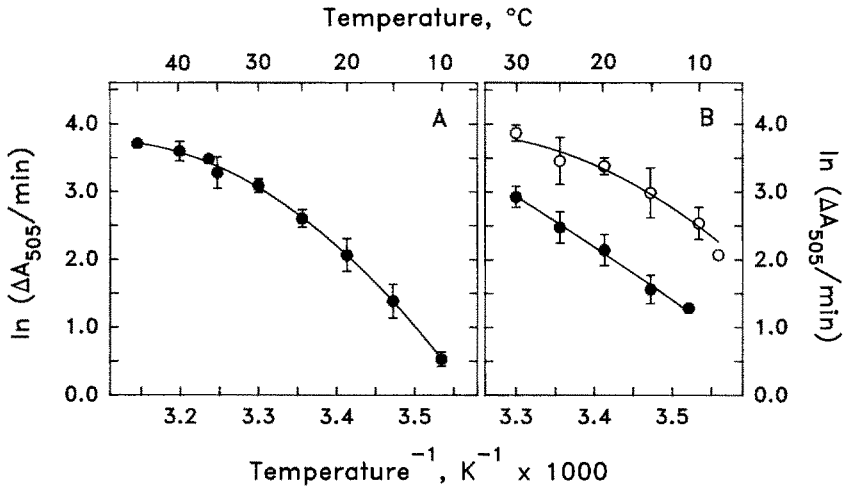


**Fig. 3A, B.** Arrhenius plots of the maximal rate of $\Delta A_{505}$ for cotton (**A**) and *Malva parviflora* (**B**), grown in spring (○) or summer (●). Error bars = SD of two to nine different measurements at each temperature. The curves were fitted by second-order regression analysis

bance increase upon illumination of the predarkened leaves increased strongly with increased leaf temperature. (The small initial decrease in $\Delta A_{505}$ in the 15° trace at first appeared to indicate the presence of an increased lag in the onset of violaxanthin de-epoxidation at low temperature; however, this is unlikely since such an initial decrease was also present in leaves in which de-epoxidation was prevented by DTT. Subtraction of the curves measured in presence of DTT from control curves indicates that the lag was in the order of 5–10 s at both 15° and 30° C.) As shown by the symbols in Fig. 1 the decrease in the epoxidation state (EPS) followed the increase in $A_{505}$ at all three temperatures.

That a close relationship exists between the increase in $A_{505}$ and the decrease in the EPS over the entire range from 15 to 40° C is shown in Fig. 2 which is based on a total of 23 determinations on four different cotton leaves. $\Delta A_{505}$ was a linear function of ΔEPS and the regression line extrapolates to the origin. These results provide the basis for our use of $\Delta A_{505}$ as a measure of ΔEPS in subsequent experiments on the temperature dependence of the rate of de-epoxidation and the relationship between this rate and the development of non-photochemical quenching. A linear relationship between $\Delta A_{505}$ and ΔEPS was also obtained with *Malva* leaves (data not shown) although the slope of the line ($\Delta A_{505}$/ΔEPS) was considerably greater than in the cotton leaves used here, probably because of differences in optical properties between leaves of the two species.

An Arrhenius plot of the maximum (initial) rate of violaxanthin de-epoxidation for cotton leaves developed at a daytime temperature of 30° C is shown in Fig. 3A and plots for *Malva* leaves developed in spring and summer are shown in Fig. 3B. In cotton the relationship between the logarithm of the rate of de-epoxidation and 1/T was non-linear such that the apparent activation energy ($E_a$) decreased as the temperature was increased (see also Table 1). A similar tendency is also apparent in *Malva* leaves developed in the spring whereas an approximately constant $E_a$ was obtained over the 10–30° C range in *Malva* leaves developed in summer. The corresponding $Q_{10}$ for the latter leaves was 2.2–2.6. It is

**Table 1.** Calculated values for apparent activation energy ($E_a$), $Q_{10}$ and net rate of violaxanthin de-epoxidation at different temperatures in leaves of cotton *(Gossypium hirsutum)* grown at a constant daytime temperature of 30° C and of *Malva parviflora*, grown in the early spring and in summer. Data corresponding to the approximate daytime growth temperatures are given in *italics*. $E_a$ and $Q_{10}$ values were obtained by fitting the data points with non-linear regression analysis and calculating the slopes at 15° and 30° C. Net rates of de-epoxidation were calculated from the experimentally determined relationship between ΔEPS and $\Delta A_{505}$ for each leaf type

| | *Gossypium* | | *Malva* | | | |
|---|---|---|---|---|---|---|
| | | | Spring | | Summer | |
| | 15° C | *30° C* | *15° C* | 30° C | 15° C | *30° C* |
| $E_a$, kJ · mol$^{-1}$ | 105 | *57* | *58* | 20 | 66 | *60* |
| $Q_{10}$ | 4.6 | *2.1* | *2.3* | 1.3 | 2.6 | *2.2* |
| Rate of de-epoxidation, μeq. · m$^{-2}$ · s$^{-1}$ | 0.15 | *0.45* | *0.26* | 0.63 | 0.08 | *0.33* |

obvious from the three Arrhenius plots that the $E_a$ or $Q_{10}$ values measured around the respective daytime growth temperatures are similar in the different kinds of leaves ($Q_{10}$ = 2.1–2.2). It is also apparent from Fig. 3B and Table 1 that the rate of de-epoxidation at any given measurement temperature was much higher in *Malva* leaves developed under the lower temperature than those developed at the higher temperature. However, like $Q_{10}$, the *rates* of de-epoxidation measured around the respective daytime growth temperatures were roughly similar in the two kinds of *Malva* leaves (Table 1).

*Temperature dependence of the degree of de-epoxidation at steady state.* Whereas the maximum *rate* of violaxanthin de-epoxidation measured under conditions where pre-darkened leaves were suddenly exposed to a large excess of light energy was greatly decreased at low temperatures, the results in Fig. 4 show that the *degree* of de-epoxidation reached in photosynthesizing leaves at *steady state* was greatly *increased* at low temperatures.

In contrast to the experiments in which wished to impose a sudden, excessive PFD to obtain the maximum rate of de-epoxidation, here the PFD was maintained at the same level as during leaf development (800 μmol · $m^{-2}$ · $s^{-1}$) and care was taken to minimize any transient changes in PFD during the transfer to the leaf chamber. In addition, sufficient time was given to allow the EPS to increase or to decrease until a steady state had been reached at each temperature. The water-vapor pressure deficit was kept low at all temperatures to prevent partial closure of the stomata.

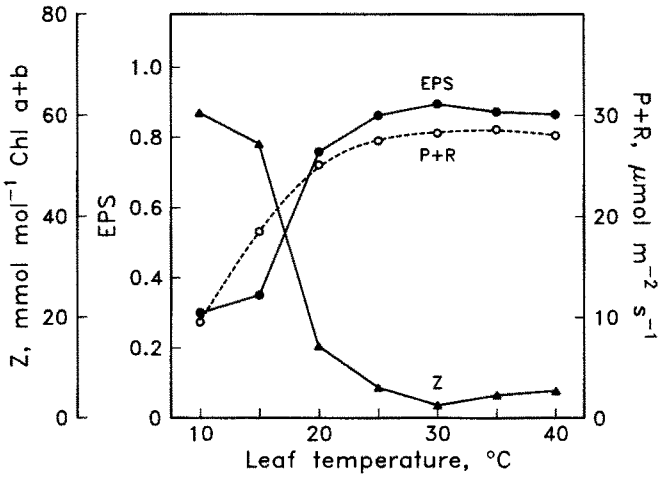


**Fig. 4.** Rate of gross photosynthetic $CO_2$ fixation ($P+R$), epoxidation state (*EPS*) and zeaxanthin content ($Z$) of cotton leaves at steady state as a function of leaf temperature. The PFD was kept constant at 800 μmol · $m^{-2}$ · $s^{-1}$. The $CO_2$ pressure was 34 Pa and the water-vapor pressure deficit 1.0–1.2 kPa. All experiments were begun at 30° C. Each measurement was taken after 1 h at the given temperature. Nine plants were used for these measurements. The mean value (±SD) of P+R at 30° C for these leaves was 28.2±1.0 μmol $CO_2$ · $m^{-2}$ · $s^{-1}$ (see *Material and methods* for details)

The EPS remained high (0.86–0.89) over the temperature range 25–40° C where energy dissipation by photosynthetic $CO_2$ fixation (P+R) was high and essentially constant. However, as photosynthesis became increasingly temperature-limited and thus an increasing amount of excessive excitation energy was created, EPS gradually fell and at 10° C, 70% of the violaxanthin pool had been de-epoxidized and the zeaxanthin content had risen to 61 mmol · (mol Chl)$^{-1}$, compared with 2.5 mmol · (mol Chl)$^{-1}$ at 30° C. Increased duration of the exposure or increased PFD caused little further de-epoxidation at 10° C (data not shown). It is possible therefore that part of the violaxanthin pool is unavailable for de-epoxidation at low temperature in these cotton leaves and that the capacity for de-epoxidation was reached between 10° and 15° C. It may be meaningful that exposure temperatures below 15° C for more than 1 h caused a further gradual decline in photosynthetic rate (data not shown).

*Relationship between the effect of temperature on violaxanthin de-epoxidation and non-photochemical quenching.* In Fig. 5 are shown the development of NPQ (expressed as $F_m/F_m' - 1$) during first 10 min of illumination of pre-darkened leaves at three different temperatures. In the control leaves (left panel) the speed of development of NPQ clearly was much slower at 15° than at 30° C and intermediate at 20° C. In contrast, temperature had little

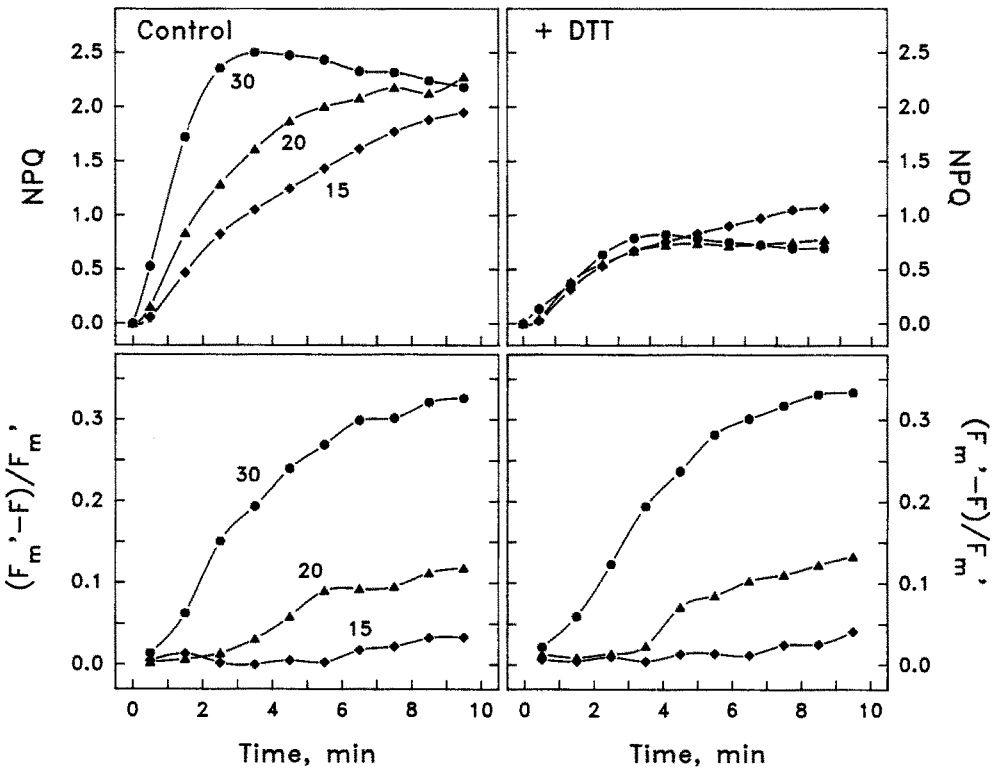


**Fig. 5.** Time courses of non-photochemical quenching (NPQ = $F_m/F_m' - 1$) *(upper panels)* and the relative photon yield of PSII ($\Phi = (F_m' - F)/F_m'$) *(lower panels)* at 15°, 20° and 30° C in control leaves *(left panels)* and DTT-treated *(right panels)* leaves of cotton. At time 0, red light with a PFD of 2000 μmol · $m^{-2}$ · $s^{-1}$ was turned on. The leaf temperatures during the first minutes of illumination are indicated at the control curves. The same leaf was used for the measurements at the three different temperatures

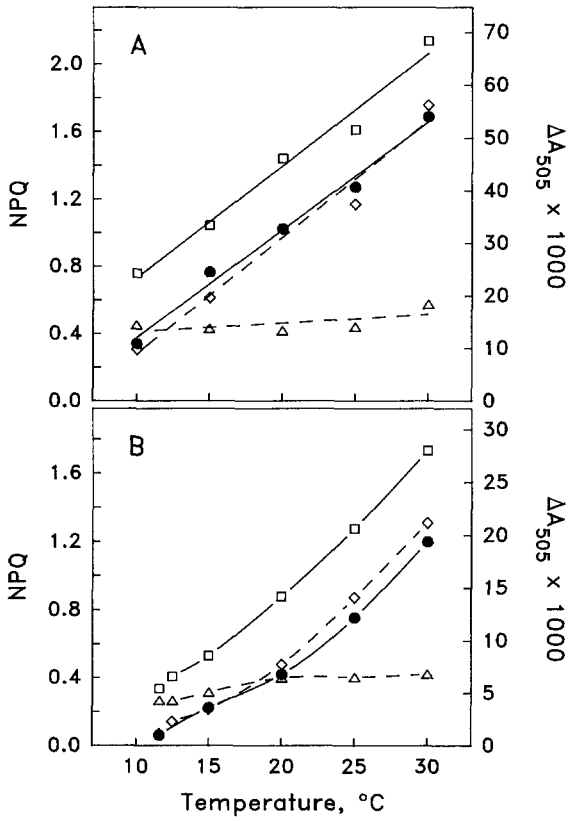


**Fig. 6A, B.** Temperature dependence of violaxanthin de-epoxidation ($\Delta A_{505}$; ●), and NPQ in control leaves (□) and in DTT-treated leaves (△), and DTT-sensitive NPQ (◇) at 1.5 min after onset of illumination at a PFD of 2000 μmol · $m^{-2}$ · $s^{-1}$ in leaves of *Malva parviflora* (**A**) and cotton (**B**). The DTT-sensitive component of NPQ was obtained by subtracting the values of DTT-treated leaves from those of control leaves. The NPQ values are means of two to eight measurements

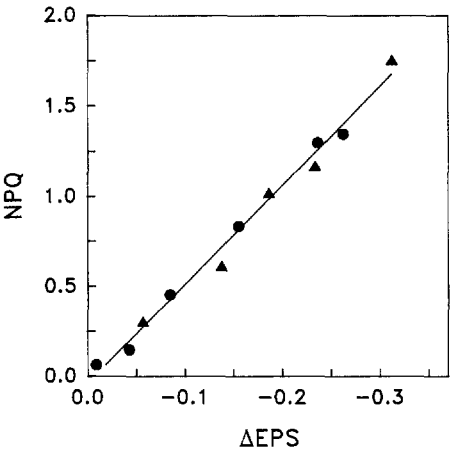


**Fig. 7.** Relationship between DTT-sensitive NPQ and the change in epoxidation state (ΔEPS) at 1.5 min after onset of illumination at different temperatures (10–30° C) for leaves of cotton (●) and *Malva parviflora* (▲). ΔEPS was calculated from the respective relationships between ΔEPS and $\Delta A_{505}$ for the cotton and the *Malva* leaves used in these experiments. *Malva* was grown in the spring. The values for *Malva* are means of two to four measurements on different leaves. The line was fitted by linear regression

effect on NPQ development in leaves in which de-epoxidation was inhibited by DTT treatment (right panel). There was a short lag at 15° and 20° C which was not present at 30° C but the further development of the quenching was very similar and the maximum NPQ reached in DTT-treated leaves was considerably smaller than in the control leaves. Time courses of the photon yield of PSII, calculated from $(F_m' - F)/F_m'$, are shown for the same leaves in the lower panels of Fig. 5. A very strong temperature dependence is obvious, most probably caused by an effect of temperature on the activity of the carbon-reduction cycle. However, the responses of the DTT-treated leaves (lower right panel) were indistinguishable from those of the control leaves (lower left panel). This indicates that the development of NPQ up to 2–4 min after the onset of illumination was strongly dependent on zeaxanthin formation. By contrast, the development of NPQ in leaves in which zeaxanthin formation was inhibited by DTT was essentially independent of temperature (upper right panel).

The finding that the efficiency of PSII photochemistry was not at all affected by DTT treatment at any temperature (Fig. 5, lower panels), corroborates previous results showing that both PSII efficiency and overall photosynthetic activity were unaffected by DTT at concentrations sufficient to fully inhibit zeaxanthin formation (Bilger et al. 1989; Bilger and Björkman 1990; Demmig-Adams et al. 1990). Evidently, the partial inhibition of non-radiative dissipation that occurs in the PSII pigment bed when zeaxanthin formation is prevented caused an increased degree of PSII center closure such that the efficiency of PSII stays the same as in untreated control leaves (Bilger and Björkman 1990).

It seemed probable that the temperature dependence of the DTT-sensitive component of NPQ is a direct consequence of the effect of temperature on zeaxanthin formation (compare Fig. 1 and Fig. 5), i.e., such quenching is governed by the amount of zeaxanthin present in the leaf. To test whether or not this is true we made concurrent measurements of $\Delta A_{505}$ and fluorescence on the same leaves over the 10–30° C range. From the time courses of $\Delta A_{505}$ and NPQ obtained at each temperature we chose the values obtained at an illumination time of 1.5 min for the comparisons shown in Fig. 6 as both $\Delta A_{505}$ and NPQ then were still linear with time.

In Fig. 6, $\Delta A_{505}$ and NPQ of control leaves, NPQ of DTT-treated leaves and the difference between NPQ in control and that in DTT-treated leaves are plotted against leaf temperature. (No de-epoxidation took place in DTT-treated leaves). In both *Malva* (Fig. 6A) and cotton (Fig. 6B), NPQ and the DTT-sensitive component of NPQ (control *minus* DTT-treated) closely followed the temperature dependence of $\Delta A_{505}$. By contrast, the DTT-insensitive component of NPQ was remarkably temperature independent over this range. The ratio between NPQ and $\Delta A_{505}$ was not the same for *Malva* and cotton leaves (note different scales in Fig. 6A and B). However, as shown in Fig. 7 the same linear relationship was obtained for both species when DTT-sensitive NPQ was plotted against ΔEPS. These results provide strong

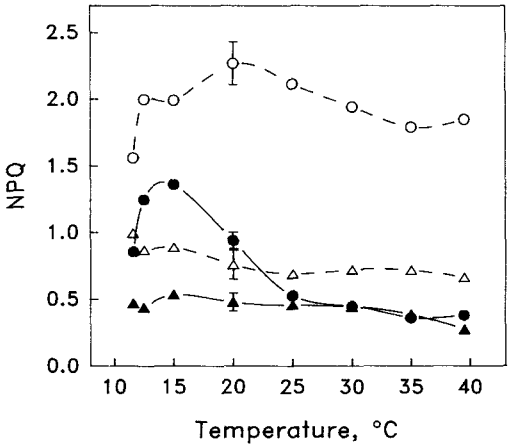


**Fig. 8.** Non-photochemical chlorophyll fluorescence quenching after 9.5 min illumination at a PFD of 2000 μmol · m$^{-2}$ · s$^{-1}$ (○, △) and after a subsequent dark period of 5 min (●, ▲) in control (○, ●) and DTT-treated (△, ▲) cotton leaves as a function of the temperature during the first 9 min of illumination. The actual leaf temperatures during the dark recovery were between 1.5° and 2.5° C below the temperature indicated on the abscissa. The values are means of two to nine measurements on different leaves. Representative error bars are shown for the values obtained at 20° C

evidence that the effect of temperature on the development on NPQ indeed is a direct consequence of its effect on zeaxanthin accumulation.

*Temperature dependence of relaxation of NPQ in darkness.* In Fig. 8 is shown the temperature dependence of NPQ reached after a 9.5-min exposure of cotton leaves to a PFD of 2000 μmol · m$^{-2}$ · s$^{-1}$ and that obtained after a 5-min relaxation period in darkness. In DTT-treated leaves temperature had essentially no effect on NPQ either in the light or after 5 min in the dark but a marked temperature-independent relaxation of NPQ occurred during the 5-min dark period. In control leaves NPQ relaxed to the same level as in DTT-treated leaves within the 5-min dark period above 25° C but the degree of relaxation decreased at temperatures below 25° C. These results indicate that although the relaxation of DTT-sensitive NPQ is temperature dependent this dependence is not related to zeaxanthin re-epoxidation since little or no decrease in $A_{505}$ was observed during the 5-min dark period at any temperature (data not shown).

## Discussion

The present results corroborate earlier findings that the absorbance increase at 505 nm provides an excellent measure of violaxanthin de-epoxidation both in chloroplast suspensions (Yamamoto et al. 1972) and in intact leaves (Bilger et al. 1989). Our results demonstrate that a linear relationship between $\Delta A_{505}$ and de-epoxidation is obtained over a wide temperature range, at widely different de-epoxidation rates and at different times during the course of the de-epoxidation process. The results also indicate that it is unnecessary to adjust the $\Delta A_{505}$ values for the small changes in $\Delta A_{505}$ that are present in leaves in which de-epoxidation has been fully prevented by DTT treatment. It should be noted, however, that the measured $\Delta A_{505}/\Delta$EPS ratio may differ between leaves having different optical properties such as between the cotton and the *Malva* leaves used by us.

In this paper we chose to relate $\Delta A_{505}$ to the change in the number of epoxy groups (ΔEPS) rather than the change in zeaxanthin content alone. This seems justified in view of the fact that de-epoxidation of violaxanthin (5,6,5'6'-diepoxy-zeaxanthin) to the intermediate antheraxanthin (5,6-monoepoxy-zeaxanthin) causes a spectral shift approx. one-half that caused by de-epoxidation of violaxanthin to zeaxanthin (compare Siefermann and Yamamoto 1975). It is yet uncertain whether or not antheraxanthin is effective in mediating energy dissipation; until an answer to this question becomes available it seems reasonable tentatively to assume that antheraxanthin is about half as effective as zeaxanthin. Nevertheless for most purposes the effectiveness of antheraxanthin as a contributor to $\Delta A_{505}$ or as a mediator of NPQ is at most only of secondary importance since usually it accounts for only a small fraction of the xanthophyll cycle pool.

Even the maximum rates of de-epoxidation obtained in this study are very low (< 1 μeq · m$^{-2}$ · s$^{-1}$) in comparison with that of electron transport during $CO_2$ fixation in saturating light (> 100 μeq · m$^{-2}$ · s$^{-1}$). Measurements, not presented in this paper, showed that the maximum rate of re-epoxidation is at least an order of magnitude lower than the maximum rate of de-epoxidation. Therefore, even though both the de-epoxidation and epoxidation processes consume NADPH these results rule out even a transient significant contribution of utilization of reducing equivalents during the turnover of the xanthophyll cycle to the dissipation of excessive light (see also Siefermann-Harms 1977).

The maximum de-epoxidation rate was strongly temperature dependent in both species. In cotton the rate at 10° C was only 5% of the rate at 40° C. The Arrhenius plots of the rate of $\Delta A_{505}$ observed in cotton and cool-grown *Malva* did not have a constant slope. At temperatures roughly equivalent to the respective growth temperature, the apparent activation energy ($E_a$) was approx. 60 kJ · mol$^{-1}$ ($Q_{10}$ = 2.1–2.3) in all leaves examined, a common value for enzyme-catalyzed processes. In cotton, a pronounced increase in $E_a$ (and $Q_{10}$) took place when the temperature was reduced to below 20° C but only a small decrease occurred in *Malva*, irrespective of the temperature regime under which the leaves had developed. It is noteworthy that Terzaghi et al. (1989) found that the temperature at which lateral phase separation of membrane lipids from cotton leaves occurs was 11° C, whereas that for *Malva parviflora* was below 0° C when both plants were grown at 25°/23° C day/night temperature in a greenhouse. Violaxanthin is thought not to be closely associated with violaxanthin de-epoxidase in the membrane (Hager 1980). Therefore some movement of violaxanthin to the enzyme may be necessary to allow de-epoxidation. In addition, monogalactosyldiglyceride is necessary for proper function of violaxanthin de-epoxidase (Yamamoto et al. 1974; Yamamoto and Higashi 1978). For these reasons the fluidity of mem-

brane lipids, especially monogalactosyldiglyceride, may have a strong influence on violaxanthin de-epoxidation and a decreased fluidity could therefore be responsible for the strong increase in $E_a$ for violaxanthin de-epoxidation that occurred at low temperatures in cotton leaves. At temperatures above the growth temperature $Q_{10}$ gradually fell to values below 2 in cotton and the cool-grown *Malva*. Although the cause of this response is not known it is possible that $CO_2$ fixation was induced quite rapidly at these high temperatures so that sufficient energization and-or reduction of the electron-transport chain was not sustained for a long enough time to allow measurement of the true maximal de-epoxidation rate.

Leaves from cool-grown *Malva* had a much higher de-epoxidation rate at any given temperature than leaves from warm-grown plants but the rates were similar when compared at the respective daytime growth temperatures. Such apparent acclimation to growth temperature has also reported for other enzyme-catalyzed processes including photosynthetic carbon fixation (see review by Berry and Björkman 1980).

It is evident from Fig. 4 that a steady state eventually is reached at which the rates of de-epoxidation and re-epoxidation are equal. The EPS then is no longer governed by the kinetics of the these processes but instead is determined by the amount of excessive light, i.e. light which is absorbed by the leaf but cannot be used in photosynthesis because of partial or complete light saturation. This is illustrated by the inverse relationship between EPS and photosynthesis in Fig. 4. It is noteworthy that this decline in EPS with decreasing temperature is remarkably similar to the rise in non-photochemical quenching determined for cotton leaves by Weis and Berry (1988).

Although the rate of de-epoxidation declined with decreasing temperature and was very slow at low temperatures, eventually the leaves were still able to form large amounts of zeaxanthin even at 10° C. After 1 h in the light at this temperature approx. 70% of the violaxanthin pool had been de-epoxidized, largely to zeaxanthin. It seems possible, however, that the slowness of the de-epoxidation at this temperature and also the fact that the available pool of violaxanthin eventually becomes depleted may have the consequence that the zeaxanthin formed is insufficient to mediate the very large excess of excitation energy that would be present at this temperature, even under moderately bright light. Perhaps this may in part explain why low temperature exacerbates, or even induces, photoinhibitory damage in leaves of tropical plants (see review by Powles 1984).

*Relationship between fluorescence quenching and violaxanthin de-epoxidation.* The development of NPQ that took place when pre-darkened control leaves were exposed to a large excess of light was strongly affected by temperature (Fig. 5; see also Adams et al. 1990) as was also the rate of zeaxanthin formation (Fig. 1). The very close and linear relationship obtained between the DTT-sensitive component of NPQ and extent of de-epoxidation (Fig. 7) provides strong evidence that the content of zeaxanthin in the leaf, or at least a constant fraction thereof, actively mediates NPQ during the induction period. Since the development of NPQ in leaves in which zeaxanthin formation was inhibited by DTT was essentially independent of temperature, it follows that the development of total NPQ was in large part also dependent on zeaxanthin formation. That the processes underlying DTT-sensitive quenching is not purely of photochemical nature is indicated by the present finding that the relaxation of this quenching in the dark was strongly temperature dependent. The small temperature dependence of the DTT-insensitive component of NPQ (Figs. 5, 6, 8) points against the possibility that the latter component is caused by a state I to state II shift since the processes underlying such state shifts should have a considerable temperature dependence (Fork and Satoh 1986).

The present results indicate that the influence of the transmembrane $\Delta pH$ on the development of NPQ in the light may in large part be by way of its effect on zeaxanthin formation. It is well known that de-epoxidation of violaxanthin is activated by a low lumen pH both in isolated chloroplasts or thylakoid membranes, and that the optimum occurs around pH 5; this is also true for the activity of violaxanthin de-epoxidase in vitro (Yamamoto 1979; Hager 1980). Incubation of cotton leaves at pH 5.0 in the presence of ascorbate also caused de-epoxidation of violaxanthin to zeaxanthin at a low PFD ($10\ \mu mol \cdot m^{-2} \cdot s^{-1}$) which normally promotes epoxidation of zeaxanthin to violaxanthin (Bilger et al. 1989). Such low-pH induced de-epoxidation also took place in darkness, both in normal, green cotton leaves (present study; data not shown) and in etiolated bean leaves (Pfündel and Strasser 1988). In all of the above cases the low-pH-induced de-epoxidation was blocked in the presence of the violaxanthin de-epoxidase inhibitor, DTT. It is also well known that zeaxanthin formation in the light does not occur when a build-up of a transmembrane $\Delta pH$ and hence the resulting decrease in lumen pH is prevented by presence of uncouplers or electron-transport inhibitors (Yamamoto 1979; Hager 1980).

Since the pre-darkened leaves used in the present study were almost free of zeaxanthin at the onset of the exposures to a high PFD the development of the DTT-sensitive component of NPQ in the light is likely to have been limited by zeaxanthin formation and not by some direct effect of $\Delta pH$. This does not exclude the possibility that when sufficient zeaxanthin is present, another $\Delta pH$-associated factor may limit DTT-sensitive NPQ. Indeed, during the relaxation of NPQ that took place later during the illumination period, and especially after subsequent darkening of the leaves (Fig. 8), the DTT-sensitive component of NPQ showed different kinetics than zeaxanthin re-epoxidation. Previous studies also have shown that presence of zeaxanthin as such does not necessarily result in fluorescence quenching and supports the conclusion that for quenching to occur the thylakoid membrane must additionally be in an "energized" state (Bilger et al. 1989; Demmig-Adams et al. 1989, 1990). It would appear that zeaxanthin-mediated NPQ is dependent on the presence of a specific conformational state of the membrane rather than the transmembrane proton gradient as such.

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