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Temperature-dependent adjustment of the thermal stability of photosystem II in vivo: possible involvement of xanthophyll-cycle pigments

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Abstract. Moderately elevated temperatures induce a rapid increase in the heat and light resistance of photosystem II (PSII) in higher-plant leaves. This phenomenon was studied in intact potato leaves exposed to 35 °C for 2 h, using chlorophyll fluorometry, kinetic and difference spectrophotometry and photoacoustics. The 35 °C treatment was observed to cause energetic uncoupling between carotenoids and chlorophylls: (i) the steady-state chlorophyll fluorescence emission excited by a blue light beam (490 nm) was noticeably reduced as compared to fluorescence elicited by orange light (590 nm) and (ii) the quantum yield for photosynthetic oxygen evolution in blue light (400–500 nm) was preferentially reduced relative to the quantum yield measured in red light (590–710 nm). Analysis of the chlorophyll-fluorescence and light-absorption characteristics of the heated leaves showed numerous analogies with the fluorescence and absorption changes associated with the light-induced xanthophyll cycle activity, indicating that the carotenoid species involved in the heat-induced pigment uncoupling could be the xanthophyll violaxanthin. More precisely, the 35 °C treatment was observed to accelerate and amplify the non-photochemical quenching of chlorophyll fluorescence (in both moderate red light and strong white light) and to cause an increase in leaf absorbance in the blue-green spectral region near 520 nm, as do strong light treatments which induce the massive conversion of violaxanthin to zeaxanthin. Interestingly, short exposure of potato leaves to strong light also provoked a significant increase in the stability of PSII to heat stress. It was also observed that photosynthetic electron transport was considerably more inhibited by chilling temperatures in 35 °C-treated leaves than in untreated leaves. Further, pre-exposure of potato leaves to 35 °C markedly increased the amplitude and the rate of light-induced changes in leaf absorbance at 505 nm (indicative of xanthophyll cycle activity), suggesting the possibility that moderately elevated temperature increased the accessibility of violaxanthin to the membrane-located de-epoxidase. This was supported by the quantitative analysis of the xanthophyll-cycle pigments before and after the 35 °C treatment, showing light-independent accumulation of zeaxanthin during mild heat stress. Based on these results, we propose that the rapid adjustment of the heat resistance of PSII may involve a modification of the interaction between violaxanthin and the light-harvesting complexes of PSII. As a consequence, the thermoresistance of PSII could be enhanced either directly through a conformational change of PSII or indirectly via a carotenoid-dependent modulation of membrane lipid fluidity.

Key words: Heat resistance – Photosystem II – Xanthophyll cycle

Introduction

Photosynthesis is one of the most heat-sensitive functions in plant cells (Berry and Björkman 1980). Exposure of green plants or algae to high temperatures in the range 35–45 °C (depending on the species, growth conditions and environmental conditions during heat stress) has been shown to result in a rapid and dramatic inhibition of their oxygen evolution, carbon-dioxide fixation and photophosphorylation capability (Berry and Björkman 1980; Quinn and Williams 1985). There is general agreement in the literature that the primary site of thermal damage is associated with some components of the photosynthetic system located in thylakoid membranes, most probably PS II. Heat-induced deactivation of PSII involves a physical separation of the peripheral light-harvesting pigments (LHCII) from the PSII complexes (Schreiber and Berry

Abbreviations and symbols: Fo and Fm = initial and maximal level of chlorophyll fluorescence, respectively; Fv = Fm − Fo = variable chlorophyll fluorescence; LHC(II) = light-harvesting chlorophyll *a*/*b*-protein complexes (of PSII); Φ = photoacoustically measured quantum yield of photosynthetic oxygen evolution (in relative values); Φ_P = fluorimetrically measured quantum yield of PSII photochemistry in the light; PFD = photon flux density; q_E = ΔpH-dependent quenching of chlorophyll fluorescence

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1977; Armond et al. 1978; Gounaris et al. 1984; Sundby et al. 1986) and a disruption of the water-splitting/oxygen-evolving system with functional manganese ions and extrinsic proteins being released (Nash et al. 1985; Enami et al. 1994). It is assumed that these denaturation events result from changes in lipid-protein interactions associated with increased lipid fluidity at high temperature, causing disruption of the supramolecular organization of PSII (Berry and Björkman 1980). In agreement with this idea, there are the observations that the close lipidic environment of PSII is of structural importance (Webb and Green 1991) and that changes in membrane lipid composition are associated with changes in PSII thermostability (Thomas et al. 1986; Kunst et al. 1989). In 23 °C-grown potato leaves, PSII denaturation was observed to occur at temperatures higher than around 38 °C, with a complete loss of PSII photochemistry at ca. 42 °C (Havaux 1993b); no significant inhibition of PSI activity was detected in this temperature range.

Recently, Havaux (1993a, 1994, 1995) has shown that chloroplasts possess an adaptive mechanism that senses a moderate elevation of environmental temperature and triggers the rapid conversion of PSII from its "normal" heat-sensitive state to a heat-resistant state. When leaves of the Haig cultivar of potato were exposed to 35 °C for 2 h, the temperature at which PSII denatures was shifted by about + 5 °C. Interestingly, this treatment also resulted in an increase in the resistance of photosynthesis to strong light at high temperature (Havaux 1994). The molecular bases of this short-term adaptation to heat and light stresses are unknown. The phenomenon does not seem to involve de-novo protein syntheses since it is not perturbed by chloramphenicol or cycloheximide (Havaux 1994). Significant changes in the lipid composition of thylakoid membranes are also improbable because they are known to occur much more slowly (> 24 h in spinach, Santarius and Müller 1979) than the aforementioned increase in PSII thermostability induced by moderately elevated temperatures. Consequently, the rapid adjustment of PSII thermostability has been interpreted in terms of temperature-induced conformational changes of PSII (Havaux 1994). Using spinach leaves treated at 35 °C, Weis (1984) observed a similar increase in the thermal stability of the PSII pigment system and also suggested that this phenomenon could involve a reversible transition in membrane conformation.

Some oxygenated carotenoids (xanthophylls) are known to be important factors controlling and stabilizing the conformation of LHCII which contains about 14 chlorophyll molecules per polypeptide of the apoprotein and three to four xanthophylls (Thornber et al. 1993). In-vitro reconstitution experiments have shown that the presence of the xanthophylls lutein and neoxanthin is a prerequisite for obtaining light-harvesting complexes with native spectroscopic properties (Paulsen et al. 1990, 1993). This is consistent with the central position attributed to lutein in LHCII (Kühlbrandt et al. 1994). In contrast to lutein and neoxanthin, violaxanthin (a di-epoxy xanthophyll) is weakly bound to LHCII (Lichtenthaler 1987; Lee and Thornber 1995) and its omission in reconstitution experiments has relatively little effect on the amount of pigment protein complexes detected (Paulsen et al. 1990). A large fraction of violaxanthin is believed to be located at the surface of the LHC (Lehmann-Kirk et al. 1979; Kühlbrandt et al. 1994), possibly in equilibrium with a pool of free violaxanthin (Pfündel and Dilley 1993; Jahns and Krause 1994). Such a location is consistent with the functioning of the so-called xanthophyll cycle, i.e. the reversible photoconversion of violaxanthin to zeaxanthin by a transmembranous enzymic system (Yamamoto 1979; Pfündel and Bilger 1994). In strong light that is saturating for photosynthetic electron transport, violaxanthin is rapidly converted to zeaxanthin via antheraxanthin on the lumen-exposed side of the membrane whereas zeaxanthin is continuously reconverted to violaxanthin in a light-independent reaction on the other side of the membrane, hence implying a certain freedom of movement of these carotenoids in the membrane lipid phase. Recently, in a study of excitation spectra of in-vivo chlorophyll fluorescence from intact leaves, Gruszecki and Krupa (1993) have observed an energetic uncoupling of xanthophyll-pigments and chlorophylls during a dark-to-light transition. These authors have related this phenomenon with the process of making violaxanthin available to the membrane-anchored de-epoxidase, understood as detaching of the pigment molecule from the protein environment and migration towards the membrane where the de-epoxidase is located. Light-induced physical desorption of violaxanthin from LHCII has also been observed in vitro (Gruszecki et al. 1994). In-vivo interaction of the xanthophyll-cycle pigments with the membrane lipid matrix is supported by a series of experimental facts (reviewed by Sarry et al. 1994). For instance, accumulation of zeaxanthin in light-treated chloroplasts was accompanied by a decrease in thylakoid membrane fluidity (Gruszecki and Strzalka 1991). On the other hand, block of the violaxanthin-to-zeaxanthin transformation during light stress has been shown to result in pronounced peroxidative damage of membrane lipids (Sarry et al. 1994). Concomitant with the violaxanthin-to-zeaxanthin conversion, marked changes in LHCII conformation take place in high-light-treated leaves, as reflected by the modification of the yield and the excitation/emission spectra of PSII-chlorophyll fluorescence (Demmig-Adams 1990; Ruban et al. 1991, 1993; Gruszecki and Krupa 1993). Those fluorescence changes have been attributed by Horton and co-workers to a proton-induced aggregation of the LHC, presumably facilitated by the synthesis of zeaxanthin and/or the release of violaxanthin to the lipid bilayer (Horton et al. 1994). To sum up, one can see the xanthophyll cycle as a protective system with two simultaneous effects in strong light: (i) it provides thylakoid membrane lipids with efficient photoprotectors/stabilizators (zeaxanthin, antheraxanthin) and (ii) it converts PSII to a state of high thermal energy dissipation, thus decreasing excitation-energy delivery to the sensitive reaction center.

Interestingly, the synthesis of zeaxanthin in the dark in ascorbate-treated leaves has been observed to result in an increased thermostability of PSII and a slow down of the plastoquinone reoxidation at chilling temperatures (Havaux and Gruszecki 1993) – both effects which were also observed in 35 °C-treated leaves (Havaux 1995). The results presented in this paper show a number of other

analogies between the temperature-dependent modulation of PSII thermal sensitivity and the light-induced violaxanthin transformation, and suggest that the former phenomenon could involve a modification of the interaction between xanthophyll carotenoids and PSII. More precisely, the presented data suggest that moderately elevated temperatures trigger the first step of the cyclic photoconversion of violaxanthin to zeaxanthin, namely the increased availability of violaxanthin to the de-epoxidase system through detachment from LHCII and solubilisation in the lipid phase of thylakoid membrane. As a consequence, partial de-epoxidation of violaxanthin in darkness was observed during mild heat treatment.

Material and methods

Plant material and treatments. Mature leaves were picked from five-week-old potato (*Solanum tuberosum* L.) plants grown from in-vitro plantlets in a growth chamber or in a greenhouse as previously described (Havaux 1993a, 1994). The in-vitro plants were originally provided by Dr. P. Chagvardieff (CEA-Cadarache). Growth temperature was 25 °C/23 °C (day/night) in the growth chamber and 25 °C/15 °C (day/night) on average in the greenhouse. Detached leaves placed on moist filter paper were treated at 35 °C in darkness as described elsewhere (Havaux 1993a, 1994). Some experiments were also done in very weak light at a photon flux density (PFD) of $< 2\ \mu mol \cdot m^{-2} \cdot s^{-1}$; it was checked that total darkness and very weak light gave similar results. No decrease in the relative water content of the leaf samples was observed during the 35 °C treatment. For purposes of comparison, other plant species were examined: barley (*Hordeum vulgare* L.), tobacco (*Nicotiana tabacum* L.), tomato (*Lycopersicon esculentum* Mill.), pea (*Pisum sàtivum* L.), bean (*Phaseolus vulgaris* L.), black nightshade (*Solanum nigrum* L.) and maize (*Zea mays* L.).

Chlorophyll fluorescence measurements. Modulated chlorophyll fluorescence emission from the upper surface of the leaves was measured at 25 °C with a PAM-2000 fluorometer (Walz, Effeltrich, Germany). The initial level of chlorophyll fluorescence (Fo) was excited by a dim red light (centered at 655 nm) modulated at 600 Hz and was measured with a photodiode at wavelengths higher than 700 nm. The temperature-dependence of the apparent Fo level was measured by increasing the leaf temperature at a rate of $1\ °C \cdot min^{-1}$, as previously described (Havaux 1993a). Variable chlorophyll fluorescence (Fv) was induced by a moderate red light ($PFD = 200\ \mu mol \cdot m^{-2} \cdot s^{-1}$) produced by an array of light-emitting diodes (LEDs) (maximal emission at 655 nm) or by a strong white light (< 710 nm, $PFD = 1000\ \mu mol \cdot m^{-2} \cdot s^{-1}$) produced by a 20-W halogen lamp. The maximal level (Fm) of chlorophyll fluorescence was induced by a 800-ms pulse of strong white light ($PFD > 4000\ \mu mol \cdot m^{-2} \cdot s^{-1}$). The actual quantum yield Φ_P of PSII photochemistry in illuminated leaves was calculated as (Fm − Ft)/Fm where Ft is the fluorescence level in the light (Genty et al. 1989).

The maximal quantum yield of PSII photochemistry was estimated in dark-adapted leaves in two different wavelength regions (between 660 and 710 nm; above 710 nm) as (Fm − Fo)/Fm = Fv/Fm, using a PAM-101 fluorometer (Walz) and an ED-101BL emitter/detector unit (Walz) allowing excitation of chlorophylls with modulated blue light (1.6 kHz, maximal emission at 450 nm).

Steady-state fluorescence emission at 680 nm was excited at 25 °C by a blue (490 nm) or orange (590 nm) light beam in a Jobin-Yvon JY3D spectrofluorometer (I.S.A. Jobin Yvon, Longjumeau, France). The bandwidth of the excitation beam was 2 nm.

Photoacoustic measurements of photosynthetic oxygen evolution. Leaf discs of 1 cm diameter were placed in the hermetically closed cell of a custom-built photoacoustic apparatus that has previously been described in detail (Havaux 1993b). The samples were illuminated with a (white, blue or red) light modulated at 17 Hz. An Oriel 57530 or an Oriel 57610 broadband interference filter (Oriel, Stratford, Conn., USA) was placed in front of the modulated light source to obtain the blue (peak wavelength, 445 nm; bandwith, 50 nm) or the red (peak wavelength, 650 nm; bandwith, 70 nm) exciting light beams, respectively. The PFD of the blue light was $10\ \mu mol \cdot m^{-2} \cdot s^{-1}$ (at the leaf surface) and that of the red light was $23\ \mu mol \cdot m^{-2} \cdot s^{-1}$. Using these PFDs, the levels of light absorption by the leaves in the red and blue light domains were comparable as indicated by the amplitudes (Apt) of the photothermal signals measured in the presence of a strong (photosynthetically saturating) background light. When no filter was used, a modulated white light of PFD $175\ \mu mol \cdot m^{-2} \cdot s^{-1}$ was obtained. The modulated oxygen-evolution component of the photoacoustic signal (amplitude, Aox) was separated from the photothermal component using the light saturation and phase adjustment method developed by Poulet et al. (1983). Photochemical energy storage was estimated at a high modulation frequency of 570 Hz. The (relative) quantum yield Φ of oxygen evolution was calculated as Aox/Apt (Poulet et al. 1983).

Leaf absorbance measurements. Apparent leaf absorbance was measured at 25 °C in an Aminco-Chance DW-2 spectrophotometer (Aminco, Silver Spring, Md., USA) in the dual-wavelength mode. The reference wavelength was 630 nm and the width of the monochromator slit openings was 2 nm. The leaf sample, stuck on a glass slide, was positioned diagonally in the cuvette filled with distilled water.

The kinetics of light-induced changes in leaf absorbance at 505 nm were measured in the transmission mode with a Walz PAM-101 system driving a blue LED (Walz ED-101BL) pulsed at 100 kHz and shielded with a Corion 505-nm interference filter (Corion, Holliston, Mass., USA; bandwidth, 2 nm). The leaf sample was placed directly on the light emitter and the light guide used to direct the measuring light to the detector was placed at a distance of 1 cm from the leaf surface. Leaves were illuminated with a red light of PFD $200\ \mu mol \cdot m^{-2} \cdot s^{-1}$ produced by a 1000-W halogen lamp (Oriel) and an RG665 filter (Schott, Mainz, Germany). The light detector was protected from the actinic red light by a broadband 400- to 600-nm filter. It was checked in preliminary experiments that the 505-nm absorbance changes were blocked by pre-infiltrating detached leaves with 3 mM dithiothreitol for 4 h. The PFDs were measured with a Li-Cor Li-185B/Li-190SB quantum-meter (Li-Cor Inc., Lincoln, Neb., USA).

Carotenoid pigment determinations. Leaf discs of 1 cm diameter were frozen in liquid nitrogen before pigment analysis. Pigments were extracted in methanol. After centrifugation and filtration, pigments were separated and quantified by the HPLC method of Gilmore and Yamamoto (1991) with some modifications. The pigments were separated at 30 °C on a 5-µm Spherisorb ODS1 column (Alltech, Deerfield, Ill., USA), protected by a C18 Adsorbosphere guard column (Alltech), at a flow rate of $1\ ml \cdot min^{-1}$ and with a 50-µl injection volume. A 20-min linear gradient to 40% solvent C [methanol:ethyl acetate, 68:32 (v/v)] in solvent A [acetonitrile:methanol: water, 72:8:3 by vol.)] was followed by 10 min with solvent A. Pigment concentrations were calculated using standards and published extinction coefficients. Purified zeaxanthin was obtained from Extrasynthèse (Genay, France). Violaxanthin and antheraxanthin were prepared by TLC with *n*-hexane:isopropanol [100:10 (v/v)] as solvent system.

Results

Effects of moderately elevated temperatures on PSII thermostability. When a potato leaf was slowly heated (Fig. 1), an abrupt rise in the chlorophyll fluorescence emission excited by a weak red light was observed at

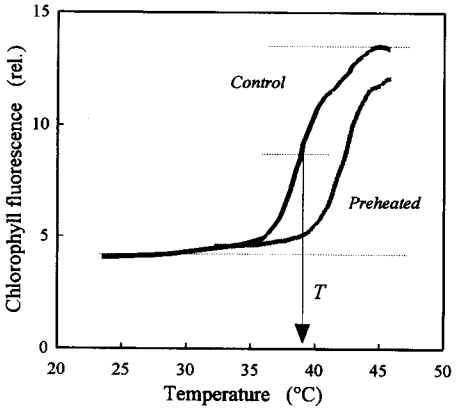

Fig. 1. Changes in the chlorophyll fluorescence emission from potato leaves excited with a dim red light and slowly heated at a rate of 1 °C·min^{-1}. *T* is the temperature corresponding to 50% of the maximal heat-induced fluorescence rise. *Continuous black line*, control potato leaf; *dotted grey line*, leaf pretreated at 35 °C for 2 h

a threshold temperature of around 37 °C, presumably corresponding to the temperature at which LHCII separates from the PSII core complex (Schreiber and Berry 1977; Armond et al. 1978). As the heat-induced fluorescence rise is sometimes biphasic (Havaux 1993b), the most convenient way to characterize this phenomenon is to measure the temperature corresponding to half of the maximal fluorescence increase (denoted T in Fig. 1). The T values for a variety of plant species grown under similar conditions are given in Table 1, showing that T varied from around 39 °C [in potato and in the weed *Solanum nigrum* (black nightshade)] to more than 47 °C (in maize). As previously reported (Havaux 1993a), brief pre-exposure (2 h) of potato leaves to a moderately elevated temperature of 35 °C resulted in a significant upward shift of the heat-induced fluorescence increase (Fig. 1), indicating enhanced thermal stability of the PSII supramolecular organization. A qualitatively similar response was observed in all the plant species examined in Table 1, with barley leaves exhibiting the most pronounced increase in PSII thermoresistance (+ 3.7 °C). Considering the sharp temperature-dependence of photosynthesis, a gain of a few degrees in the PSII thermoresistance is probably of great ecophysiological significance. Increased thermostability of PSII at moderately elevated temperatures has been reported to be slowly reversible upon lowering leaf temperature (Havaux 1993a).

Effects of moderately elevated temperatures on photosynthetic oxygen evolution and chlorophyll fluorescence. Pretreatment of potato leaves at 35°C slightly reduced the quantum yield of PSII photochemistry as measured in the dark by the chlorophyll fluorescence ratio Fv/Fm (Table 2). The decrease in Fv/Fm appeared to be a little bit more pronounced when fluorescence was measured between 660 and 710 nm (about − 5.6%) than when fluorescence was measured at wavelengths higher than 710 nm (− 3.2%). The relative quantum yield, Φ, of oxygen evolution (as measured in white light by the photoacoustic method) was also reduced by the 35 °C-treatment (Table 2). However, the decrease in Φ was much more marked

Table 1. Increase in the PSII thermotolerance induced in leaves of various plant species by 2 h exposure to 35 °C. The thermal stability of PSII was estimated by the temperature (T) at which 50% of the maximal heat-induced chlorophyll fluorescence rise was obtained (cf. Fig. 1)

	T (°C)		ΔT (°C)
	Before	After exposure to 35 °C	
Tomato	43.2	44.5	+ 1.3
Potato	38.9	42.4	+ 3.5
Bean	41.7	43.9	+ 2.2
Tobacco	41.6	43.3	+ 1.7
Barley	41.6	45.3	+ 3.7
Pea	42.3	45.2	+ 2.9
Black nightshade	38.8	42.0	+ 3.2
Maize	47.6	49.0	+ 1.4

Table 2. Relative quantum yield, Φ, of photosynthetic oxygen evolution (photoacoustically measured in white light), ratio (Φ_{445}/Φ_{650}) between Φ measured in blue light (445 nm) and in red light (650 nm), quantum yield of PSII photochemistry (Fv/Fm measured in two wavelength regions, 600–710 nm and > 710 nm) and ratio (F_{490}/F_{590}) between the chlorophyll fluorescence emission (at 680 nm) excited by a blue light (490 nm) and an orange light (590 nm) in control potato leaves and in leaves pretreated for 2 h at 35 °C. Data are mean values of *n* separate experiments ± SD

	Before	After exposure to 35 °C
Φ in white light	2.27 ± 0.22 ($n = 4$)	1.94 ± 0.10 ($n = 3$)
Φ_{445}/Φ_{650}	0.471 ± 0.037 ($n = 15$)	0.383 ± 0.052 ($n = 17$)
Fv/Fm at 660–710 nm	0.820 ± 0.006 ($n = 3$)	0.774 ± 0.008 ($n = 4$)
Fv/Fm above 710 nm	0.778 ± 0.010 ($n = 3$)	0.753 ± 0.009 ($n = 4$)
F_{490}/F_{590}	1.30 ± 0.10 ($n = 23$)	1.12 ± 0.09 ($n = 20$)

when measured in weak blue light (445 nm) than in red light (650 nm): the ratio Φ_{445}/Φ_{650} significantly decreased from 0.47 to 0.38. Similarly, the intensity of steady-state chlorophyll fluorescence emission (at 680 nm) excited by a blue light beam (490 nm) was reduced as compared to the fluorescence intensity measured in orange light (590 nm). Blue light is absorbed by both chlorophyll and carotenoid molecules whereas only chlorophylls participate in light absorption in the orange/red spectral region. Consequently, loss of efficiency of the blue light as compared to the red/orange light for both light-limited oxygen evolution and chlorophyll fluorescence emission suggests that the 35 °C treatment affected excitation-energy transfer from a certain pool of carotenoid pigments to fluorescent chlorophylls.

Effects of moderately elevated temperatures on leaf absorbance. Figure 2A shows the absorption spectra of potato leaves between 400 nm and 630 nm. Leaves pre-exposed to 35°C for 2 h exhibited a significant enhancement of

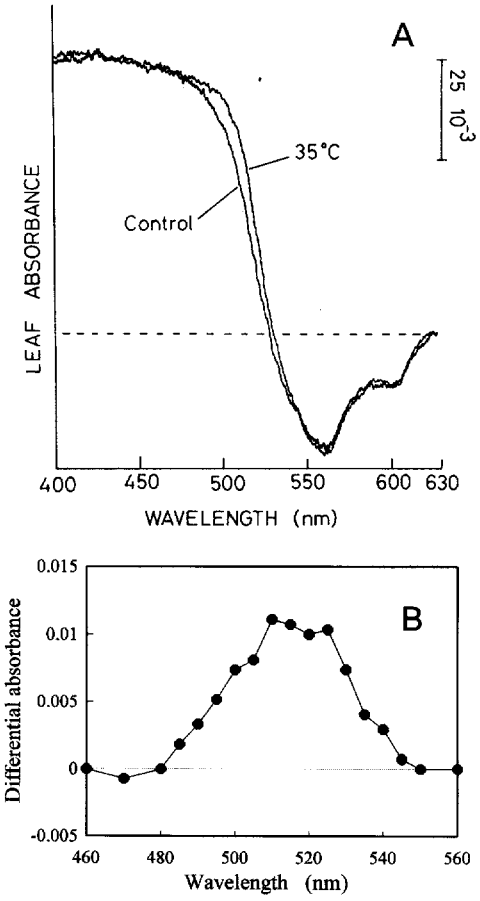

Fig. 2A. Absorption spectra of a control potato leaf and a leaf pretreated at 35 °C for 1.5 h. Leaf absorbance was measured at 25 °C with 630 nm as reference wavelength. **B** Heated-minus-control difference absorbance spectrum

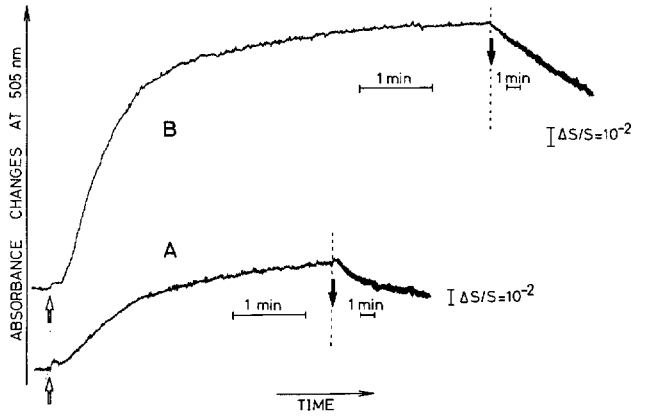

Fig. 3A, B. Light-induced changes in leaf absorbance at 505 nm before (**A**) and after (**B**) 2 h exposure at 35 °C. At the time indicated by the upward pointing arrow (↑), potato leaves were suddenly illuminated with a red light of PFD 200 μmol · m^{-2} · s^{-1}. ↓, light off

their apparent absorbance in the blue-green spectral region from approx. 480 nm to 540 nm, with the maximal absorbance change being in the range 510–530 nm (Fig. 2B). It is interesting to note that absorbance changes in the same wavelength domain were reported in leaves or chloroplasts during strong illumination (Ruban et al. 1992, 1993; Bilger and Björkman 1994). Although the exact origin of this signal is unknown, there is evidence that it results from a structural change in LHCII possibly involving a xanthophyll (Ruban et al. 1993; Horton et al. 1994).

In Fig. 3, light absorption of potato leaves at 505 nm was monitored during a transition from darkness to red light (PFD = 200 μmol · m^{-2} · s^{-1}). Upon illumination, the 505-nm apparent absorbance of control leaves rapidly increased after a lag period of a few seconds, finally reaching a plateau after around 4 min. When the red light was turned off, leaf absorbance decreased back to the initial level at a much slower rate than the light-induced increase. The 505-nm absorbance changes reported here were cancelled if leaves were pre-fed with 3 mM dithiothreitol through the cut petiole (data not shown). A PFD of 200 μmol · m^{-2} · s^{-1} was not sufficient to saturate the 505-nm changes; the plateau level was increased by about 35% when the PFD was increased to the saturating PFD (ca. 600 μmol · m^{-2} · s^{-1}; data not shown). Numerous previous works have related the photoinduced absorbance changes at around 505 nm to the xanthophyll cycle activity (see e.g., Bilger et al. 1989; Bilger and Björkman 1994), with the 505-nm change being linearily correlated with the decrease in violaxanthin content (Siefermann and Yamamoto 1974). The first-order rate constant and the final extent of the 505-nm change are a measure of de-epoxidase activity and variable availability of violaxanthin, respectively (Siefermann and Yamamoto 1974). Clearly, pretreatement of the leaves at 35°C stimulated the light-induced 505-nm absorbance changes, with both the initial rate and the maximal amplitude of the changes being markedly increased (Fig. 3). A possible explanation of this effect could be that violaxanthin became more easily and rapidly accessible to the de-epoxidation enzymic system after mild heat stress.

Pigments were extracted from leaves and separated by HPLC. The results are summarized in Table 3. Very little zeaxanthin or antheraxanthin was detected in control potato leaves. Exposing control leaves to moderate red light for 6 min resulted, as expected, in a limited conversion of violaxanthin (V) to antheraxanthin (A) and zeaxanthin (Z), with the (A + Z)/V ratio increasing from 0.08 to around 0.2. Interestingly, mild heat stress in darkness caused a partial de-epoxidation of violaxanthin, with (A + Z)/V reaching 0.3. Red light (imposed at 25 °C) further increased the leaf content in both zeaxanthin and antheraxanthin, with the (A + Z)/V ratio reaching a rather high value of 0.52. Incidentally, maximal conversion of violaxanthin to zeaxanthin and antheraxanthin in potato leaves under strong light stress (1 h at 2000 μmol · m^{-2} · s^{-1}) yielded a (A + Z)/V ratio of around 1 (Sarry et al. 1994).

Effects of moderate heat on non-photochemical quenching of chlorophyll fluorescence. Dark-adapted potato leaves were suddenly illuminated with a red light beam of 200 μmol photons · m^{-2} · s^{-1} and Fm (determined by repetitively applying 800-ms pulses of intense white light) was monitored during illumination (Fig. 4A). The

Table 3. Violaxanthin (V), antheraxanthin (A) and zeaxanthin (Z) contents of control and preheated (2 h at 35 °C) potato leaves before and after 6 min exposure to red light (PFD = 200 μmol·m^{-2}·s^{-1}) at 25 °C. Data are expressed in nanograms per leaf disc of 1 cm diameter (area = 0.785 cm^2) and are mean values of three separate experiments ± SD. The epoxidation state, EPS, is calculated as [(V + 0.5 A)/(A + Z + V)]*100

	V	A	Z	(A + Z)/V	EPS
Control, dark	133 ± 17	3 ± 1	7 ± 3	0.08	94
Control, light	101 ± 25	10 ± 4	8 ± 3	0.19	89
Heated, dark	103 ± 12	14 ± 2	14 ± 5	0.27	84
Heated, light	84 ± 16	18 ± 5	20 ± 9	0.52	76

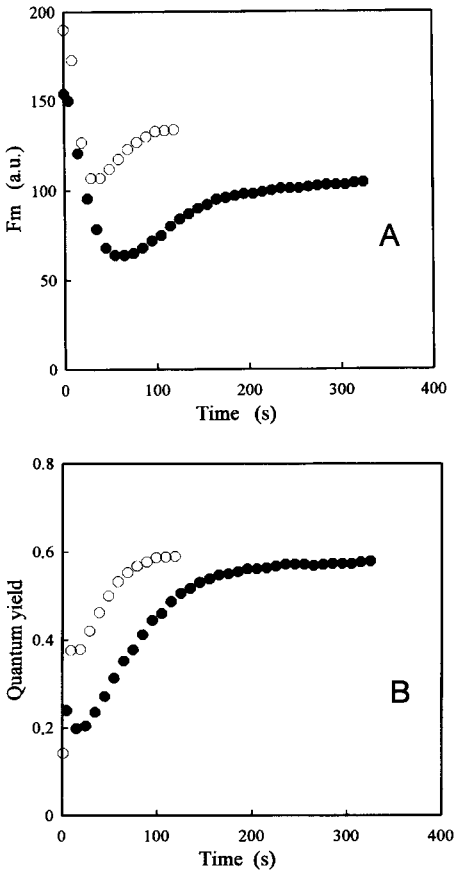

Fig. 4A, B. Changes in the maximal level (Fm) of chlorophyll fluorescence (**A**) and in the actual quantum yield, Φ_P, of PSII photochemistry (**B**) in potato leaves upon an transition from darkness to red light of moderate PFD (200 μmol·m^{-2}·s^{-1}). *Open circles*, control potato leaf; *closed circle*, leaf pretreated at 35 °C for 1.5 h

light-induced decrease in Fm is indicative of non-photochemical quenching processes, the most prominent one being, in this PFD range, the ΔpH-dependent quenching q_E (Horton and Hague 1988). Again, a clear difference was observed between 35 °C-treated leaves and untreated leaves: the non-photochemical quenching of Fm was much more pronounced in the prewarmed leaves. Prolonged illumination with red light (> 2 min) resulted, however, in almost similar quantum yields for linear electron transport (Φ_P) in the two types of leaves (Fig. 4B).

The quantum yield, Φ_P, for photosynthetic electron transport was observed to be strongly dependent on leaf temperature (Fig. 5), as expected. In control potato leaves, Φ_P slightly decreased at chilling temperatures lower than 10 °C whereas, in preheated leaves, a dramatic reduction of Φ_P was observed at temperatures lower than about 18 °C. Conversely, Φ_P of control leaves was more rapidly inhibited by high temperatures than Φ_P of 35 °C-treated leaves, thus confirming the enhancement of the PSII thermoresistance observed in Fig. 1 and Table 1.

The high-energy-state quenching, q_E, of chlorophyll fluorescence can be activated by preilluminating leaves with a strong light (Ruban et al. 1993). This is attributed to the violaxanthin-to-zeaxanthin conversion which presumably results in an increased sensitivity of PSII to low pH. More precisely, the q_E-quenching has been ascribed to an aggregation of LHCII which is favoured in the absence of violaxanthin (Ruban et al. 1993; Horton et al. 1994). The light-activation of q_E is illustrated in Fig. 6A: the Fm quenching in potato leaves suddenly exposed to a white light of high PFD (900 μmol·m^{-2}·s^{-1}) was much more rapid after pre-illumination (3 min at 900 μmol·m^{-2}·s^{-1} followed by 9 min in darkness) than after dark-adaptation (> 30 min). Comparison of Fig. 6A and 6B clearly shows that leaves pretreated at 35 °C for 2 h in darkness (Fig. 6B) were already in an activated state.

Effects of preillumination on PSII thermostability. Figure 7 shows that briefly exposing potato leaves to a strong white light (PFD = 1000 μmol·m^{-2}·s^{-1} for 4 min) significantly increased the thermostability of PSII as measured subsequently by the fluorescence-versus-temperature curves. Thus, strong light and mild heat induced a qualitatively similar adaptation of PSII to subsequent heat stress.

Discussion

Singlet-singlet energy transfer from excited LHC-bound carotenoids to chlorophyll *a* requires a close contiguity of the pigments (Siefermann-Harms 1985) and, consequently, slight modifications of the LHCs can affect this short-distance energy transfer. Accordingly, incubation of isolated LHCII in low concentrations of detergent or urea has been demonstrated to interrupt energy transfer from carotenoids to chlorophyll *a* but not from chlorophyll *b* to chlorophyll *a* (Siefermann-Harms and Ninnemann 1982; Siefermann-Harms 1990). Lowered rate of excitation energy transfer from accessory carotenoid pigments to antenna chlorophylls has been shown also in leaves submitted to stressful environmental or developmental conditions such as strong light stress (Gruszecki et al. 1991a; Havaux et al. 1991) or leaf senescence (Gruszecki et al. 1991b). Recently, a similar phenomenon has been reported in rye leaves suddenly transferred from darkness to moderate light (Gruszecki and Krupa 1993), suggesting the possibility of a regulatory function. This possibility is confirmed in the present work which has shown that the carotenoid-chlorophyll energetic coupling is also modulated by temperature (Table 2). This finding confirms previous in-vitro data on the selective block of

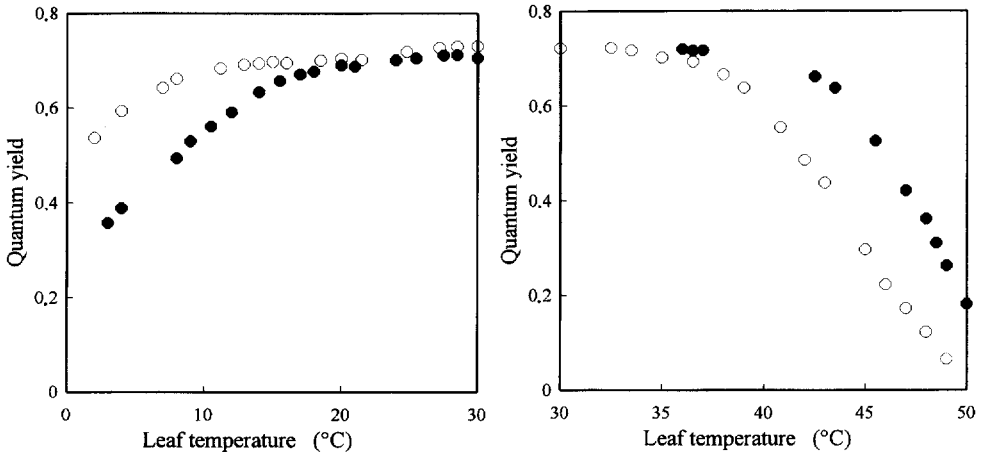

Fig. 5. Temperature dependence of the actual quantum yield, Φ_P, of PSII photochemistry in potato leaves pre-treated at 35 °C for 0 h (*open circles*) and 2 h (*closed circles*). Leaves were heated in red light (PED = 150 μmol·m^{-2}·s^{-1}) from 2 °C to 30 °C and from 30 °C to 50 °C at a rate of approximately 1 °C·min^{-1}

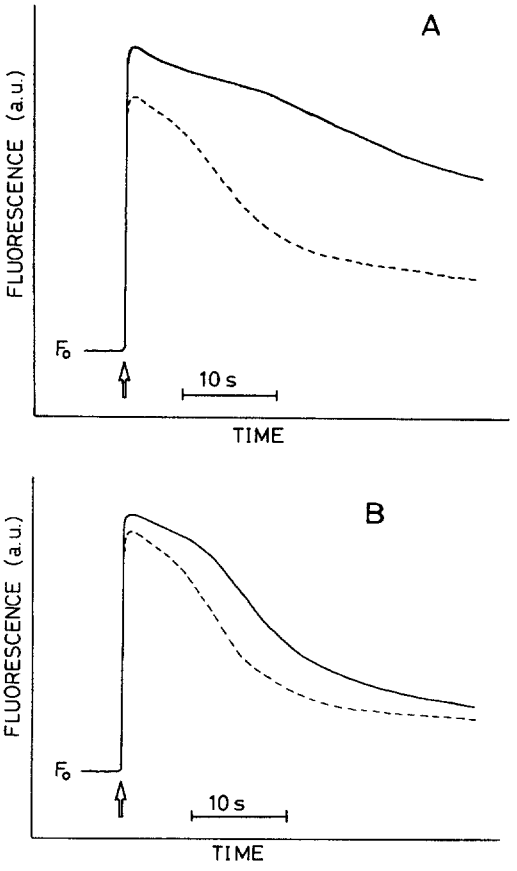

Fig. 6A, B. Quenching of variable chlorophyll fluorescence induced by a white light of PFD 900 μmol·m^{-2}·s^{-1} after dark adaptation (*continuous line*) or after preillumination (900 μmol·m^{-2}·s^{-1} for 3 min followed by 9 min in darkness, *dotted line*). **A** Control potato leaf; **B** leaf pretreated at 35 °C for 1.5 h. ↑, white light on. In these experiments, the fluorescence level was close to Fm

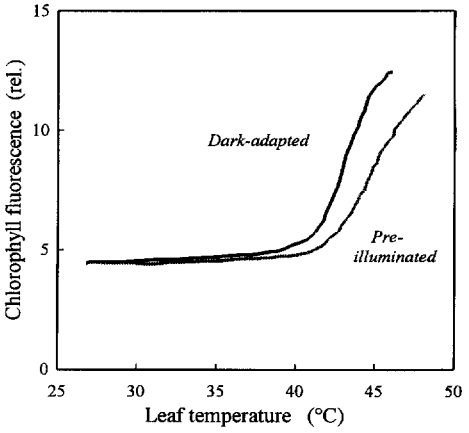

Fig. 7. Changes in the chlorophyll fluorescence emission from potato leaves excited with a dim red modulated light and slowly heated at a rate of 1 °C·min^{-1}. *Continuous line*, control leaf; *dotted line*, leaf pre-exposed for 4 min to a white light of PFD 1000 μmol·m^{-2}·s^{-1} at 25 °C

energy transfer from carotenoids to chlorophylls when isolated LHCII are submitted to elevated temperatures (Siefermann-Harms and Ninnemann 1982).

The main carotenoids present in (dark-adapted) LHCII are the xanthophylls, neoxanthin, violaxanthin and lutein (Lichtenthaler 1987; Thayer and Björkman 1992; Ruban et al. 1994) in the following molar ratios: 0.3:0.5:1:4 (neoxanthin:violaxanthin:lutein:chlorophyll *a*; Lee and Thornber 1995). From the analysis of absorption and chlorophyll fluorescence excitation/emission spectra of illuminated leaves (Gruszecki et al. 1991a; Gruszecki and Krupa 1993), the carotenoid pigment that is decoupled from chlorophylls in light-treated leaves has been identified as being violaxanthin. This is consistent with biochemical analyses of the pigment composition of the photosystems which have pointed to the exceptionally weak binding of violaxanthin to the thylakoid pigment-proteins (Lichtenthaler 1987; Lee and Thornber 1995). Additionally, energetic coupling of exogenous violaxanthin to purified LHCII in darkness (Gruszecki and Krupa 1993) and desorption of native violaxanthin from isolated LHCII under illumination (Gruszecki et al. 1994) have been demonstrated in vitro. As discussed in the *Introduction*, reduced direct contact between the antenna system of PSII and the violaxanthin pool is supported by studies on the xanthophyll cycle. Indeed, there exists a light-dependent limitation in violaxanthin de-epoxidation, termed violaxanthin availability (Pfündel and Bilger 1994). Light-induced energetic uncoupling of xanthophyll pigments from photosynthetic processes may be understood as the

manifestation of the process of making violaxanthin available to de-epoxidation which precedes zeaxanthin formation and provides the protein-bound in-situ pigment molecules with a more diffusional freedom in order to facilitate direct contact with the membrane-anchored de-epoxidase enzyme (Gruszecki and Krupa 1993). Actually, in-vivo interaction between zeaxanthin and thylakoid membrane lipids has been inferred from previous studies where the effects of strong light stress on the lipid fluidity and peroxidation status were examined in the presence or absence of the violaxanthin-cycle inhibitor, dithiothreitol (Gruszecki and Strzalka 1991; Sarry et al. 1994).

Our data suggest that violaxanthin detaching from LHCII and solubilization of this pigment within the lipid phase of thylakoid membranes is triggered by elevated temperatures too. Analysis of the kinetics of light-induced 505-nm changes in potato leaves could suggest an increased availability of violaxanthin to de-epoxidation after mild heat treatment (Fig. 3). Although red light of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ was not saturating for the 505-nm changes, the maximal extent of the 505-nm absorbance change in control leaves remains noticeably lower than the plateau level measured in preheated leaves. Likely, the increased accessibility of violaxanthin to the de-epoxidase explains the partial conversion of violaxanthin to zeaxanthin observed in leaves treated at 35 °C in the absence of light (Table 3). Alternatively, stimulated conversion of violaxanthin to zeaxanthin could also result from a certain reduction of photosynthetic activity, possibly in the dark reactions. However, the 35 °C-induced reduction of photosynthetic electron transport in red light (Fig. 4B) appeared to be too small to fully account for the marked changes observed in the kinetics of violaxanthin phototransformation (Fig. 3). Additionally, the light-absorption and chlorophyll-fluorescence characteristics of the prewarmed leaves resemble very much those of leaves where violaxanthin has been massively converted to zeaxanthin by strong light or ascorbate infiltration. Indeed, pre-exposing leaves to 35 °C modified their absorption in the blue-green spectral region near 510–530 nm (Fig. 2), activated the high-energy-state quenching of chlorophyll fluorescence (Figs. 4, 6), blocked photosynthetic electron transport at chilling temperature (Fig. 5), protected PSII against heat denaturation (Figs. 1 and 5, Table 1) and preferentially quenched fluorescence emission at wavelengths lower than 710 nm (Table 2). All these phenomena were reported to occur in light- or ascorbate-treated leaves. For instance, conversion of violaxanthin to zeaxanthin in potato leaves infiltrated with ascorbate at pH 5 was associated with a decreased PSII thermolability and a block of electron transport at chilling temperature (Havaux and Gruszecki 1993). Furthermore, this work (Fig. 7) has shown that pre-exposing potato leaves to strong white light brought about a significant increase in the thermotolerance of PSII. On the other hand, an impressive number of studies have correlated the non-photochemical quenching, q_E, of chlorophyll fluorescence with the xanthophyll cycle activity (Demmig-Adams 1990). In reality, synthesis of zeaxanthin is not required for q_E but rather potentiates or activates its formation (Horton et al. 1994). Recently, a molecular explanation of q_E has been proposed by Horton and collaborators who have attributed q_E-quenching to an increased rate of non-radiative de-excitation associated with a proton-induced structural change in LHC (Horton et al. 1994). This structural change is presumably favoured or amplified by the synthesis of zeaxanthin and/or the release of violaxanthin from the LHC. The q_E-related increase in energy dissipation has also been correlated with absorbance changes with λ_{max} at 520 nm to 540 nm (Ruban et al. 1992, 1993; Bilger and Björkman 1994). Interestingly, the maximal quenching of chlorophyll fluorescence due to the q_E process occurs near 700 nm (Ruban et al. 1991). One can suggest from the strong analogy observed in this study between the responses of the photochemical apparatus of leaves to mild heating and to strong illumination that similar phenomena underly the short-term adaptation of photosynthesis to heat and to light. In support of this idea, there is the observation that mild heat stress simultaneously caused an increase in the PSII tolerance to heat (this study) and to strong light at high temperature (Havaux 1994). In this context, it is worth mentioning that atrazine-resistant weed mutants have been reported to be, at the same time, impaired in their xanthophyll cycle activity (Varadi et al. 1994) and much more vulnerable to heat than their wild counterpart (Havaux 1989).

Violaxanthin could adjust the PSII thermostability in two different ways. Conceivably, PSII with aggregated LHC is more heat stable. Violaxanthin desorption from LHCII could facilitate protein unfolding (Paulsen et al. 1993), possibly leading to a more heat-stable configuration (Haltia and Freire 1995). Alternatively, violaxanthin can affect indirectly the PSII response to elevated temperatures via changes in the lipid properties of the thylakoid membrane. Indeed, when incorporated into artificial membranes, xanthophylls are known to strongly interact with lipids, with membrane reinforcement, decreased lipid fluidity and reduced membrane permeability being the major effects (Lazrak et al. 1987; Ourisson and Nakatani 1990; Gruszecki and Sielewiesiuk 1991; Subczynski et al. 1991). There are in-vivo data suggesting that such a carotenoid-mediated modification of the lipid phase occur also in higher-plants thylakoids (Gruszecki and Strzalka 1991; Havaux and Gruszecki 1993) and in bacterial membranes (Ourisson and Nakatani 1990). Seemingly, dihydroxy carotenoids span, like rivets, the entire membrane bilayer, to which they confer the desirable mechanical properties (Lazrak et al. 1987; Ourisson and Nakatani 1990; Gruszecki and Sielewiesiuk 1991; Subczynski et al. 1991). Such an effect is likely to counterbalance (at least partially) the heat-induced increase in membrane fluidity, thus protecting PSII from denaturation. In fact, we have at least two arguments in favor of an indirect effect of violaxanthin/zeaxanthin on the PSII thermostability. First, from the analysis of light-induced absorption changes around 518 nm (electrochromic shift), Weis (1981) reported that thylakoid membranes become leaky to ions after heat stress. However, this heat-induced membrane damage disappeared after prolonged incubation of leaves at 34 °C, indicating stabilization of the thylakoid membrane structure. This effect was also observed in our plant material exposed for 2 h to 35 °C (data not shown). Secondly, the rate-limiting step of photosynthetic electron transport is the reoxidation of reduced plastoquinone, which involves

diffusion of plastoquinone molecules within the lipid matrix (Haehnel 1984). At low temperature when membrane fluidity is drastically lowered, lateral migration of plastoquinone is believed to determine the rate of electron transport. The fact that the 35 °C treatment markedly inhibited both the plastoquinone reoxidation (Havaux 1995) and the photosynthetic electron transport (Fig. 5) at chilling temperatures could suggest a decreased membrane fluidity. It is clear that further topological and functional studies will have to confirm our interpretations and to determine the exact mode of action of xanthophylls in the PSII adaptation to high temperature stress.

In summary, this paper provides data that suggest, for the first time, the implication of carotenoids in the short-term adaptive response of the photosynthetic apparatus to an elevation of leaf temperature. Taken together with other published evidence, our data indicate that moderately elevated temperatures in darkness trigger the first step of the violaxanthin-to-zeaxanthin photoconversion, namely the process of making violaxanthin available to de-epoxidation. As a consequence, a significant light-independent synthesis of zeaxanthin was noticed in potato leaves exposed for 2 h at 35 °C. Those carotenoid changes were associated with an appreciable increase in the stability of PSII to heat stress. Thus, the xanthophyll cycle seems to be important in the response of the photochemical apparatus of photosynthesis not only to strong light but also to high temperature. The ecophysiological significance of a simultaneous adaptation to temperature and light stresses is evident since high solar irradiance and heat are usually combined in the field.

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