Plant Physiol. (1989) 90, 894–898
0032-0889/89/90/0894/05/$01.00/0

Received for publication May 5, 1988
and in revised form February 12, 1989

Zeaxanthin Synthesis, Energy Dissipation, and Photoprotection of Photosystem II at Chilling Temperatures[1]

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ABSTRACT

When leaves of a mangrove, *Rhizophora mangle*, were exposed to an excess of light at chilling temperatures, synthesis of zeaxanthin through violaxanthin de-epoxidation as well as nonphotochemical fluorescence quenching were markedly reduced. The results suggest a protective role of energy dissipation against the adverse effects of high light and chilling temperatures: leaves of *R. mangle* that had been preilluminated in 2% O_2, 0% CO_2 at low photon flux density and showed a high level of zeaxanthin, and leaves that had been kept in the dark and contained no zeaxanthin, were both exposed to high light and chilling temperatures (5°C leaf temperature) in air and then held under control conditions in low light in air at 25°C. Measurements of chlorophyll *a* fluorescence at room temperature showed that the photochemical efficiency of PSII and the yield of maximum fluorescence of the preilluminated leaf recovered completely within 1 to 3 hours under the control conditions. In contrast, the fluorescence responses of the predarkened leaf in high light at 5°C did not recover at all. During a dark/light transient in 2% O_2, 0% CO_2 in low light at 5°C, nonphotochemical fluorescence quenching increased linearly with an increase in the zeaxanthin content in leaves of *R. mangle*. In soybean (*Glycine max*) leaves, which contained a background level of zeaxanthin in the dark, a similar treatment with excess light induced a level of nonphotochemical fluorescence quenching that was not paralleled by an increase in the zeaxanthin content.

Exposure of many plants to temperatures in the chilling range (0–12°C) increases the sensitivity of PSII to photoinhibition (1, 10, 23–25) because a given light level becomes increasingly excessive when rates of photosynthesis decrease as a consequence of the low leaf temperatures. Furthermore, specific effects have been reported such as an inactivation of photophosphorylation (9, 14). There is also a possibility that certain protective responses may not be fully effective at low leaf temperatures. Increased radiationless energy dissipation, reflected by the quenching of Chl fluorescence, is thought to protect the photochemical apparatus against the destructive effects of excess light (2, 16, 27). A possible role of the carotenoid zeaxanthin in the dissipation of excess excitation energy has been suggested (4–7). In this paper, we show that the formation of zeaxanthin and the development of nonphotochemical fluorescence quenching are much slower at 5°C than at 20°C. Leaves were pretreated to contain either high or low levels of zeaxanthin and were then subjected to a combination of high light and chilling temperatures. The experiments show that leaves which contain high zeaxanthin levels and which are in a low fluorescence state exhibit a decreased sensitivity to sustained reductions in the photochemical efficiency of PSII. Furthermore, the decreased rapidity of the development of fluorescence quenching at 5°C is used to separate two components of fluorescence quenching with different induction kinetics.

MATERIALS AND METHODS

Plant material, growth conditions, analysis of pigments as well as continuous recording of Chl *a* fluorescence with a pulse amplitude modulation fluorometer (26) in leaves were as described earlier (6), with the exception that soybean (*Glycine max*) plants were taken straight from the growth cabinet. To determine fluorescence in the light, leaves were sealed into a gas exchange chamber in darkness and leaf temperature was either kept at 20°C or was brought to 5°C within 15 to 20 min. The dew points of the incoming gas streams (2% O_2, 0% CO_2) were 15 and 0°C, respectively. After 25 min at 20°C or 5 min at 5°C leaf temperature in the dark, the (control) fluorescence yield was determined and control samples for pigment analysis were taken. Subsequently, illumination with 120 μmol photons m^{-2} s^{-1} was begun.

For the determination of the effect of a combination of high light and chilling temperature on PSII photochemistry, leaf discs were removed from the treated leaves and fluorescence (F_O[3] and F_M) determined at room temperature with a modulation fluorometer at a constant distance between sample and fiber optics. These fluorescence measurements were preceded by a 5 min period of complete darkness at room temperature.

RESULTS

Time course of Zeaxanthin Formation and Fluorescence Quenching at 20 and 5°C

Figure 1 shows the time course of the formation of zeaxanthin and the decrease of maximum fluorescence, F_M,

[1] Supported by the Deutsche Forschungsgemeinschaft and by the Fonds der Chemischen Industrie.

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[3] Abbreviations: F_O, yield of instantaneous Chl fluorescence; F_M, yield of maximum Chl fluorescence; F_V, yield of variable fluorescence; F_V/F_M, PSII photochemical efficiency; k_D, rate constant for radiationless energy dissipation in the antenna Chl.

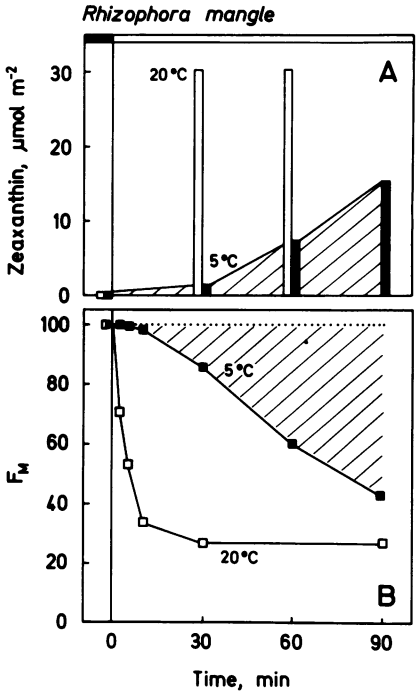

Figure 1. Time course of (A) zeaxanthin content and (B) the decrease of maximum fluorescence F_M in leaves of *R. mangle* exposed to a PFD of 120 μmol m^{-2} s^{-1} in 2% O_2, 0% CO_2 at leaf temperatures of 20 or 5°C. Steady state fluorescence was recorded continuously in the light (not shown) and pulses of saturating light to determine F_M during illumination with photosynthetically active radiation were given at the respective time points. Samples for carotenoid analysis were taken during a corresponding treatment on the leaf opposite to the one used for the fluorescence measurements. Leaf discs were punched and immersed into liquid N_2 within no more than 3 s.

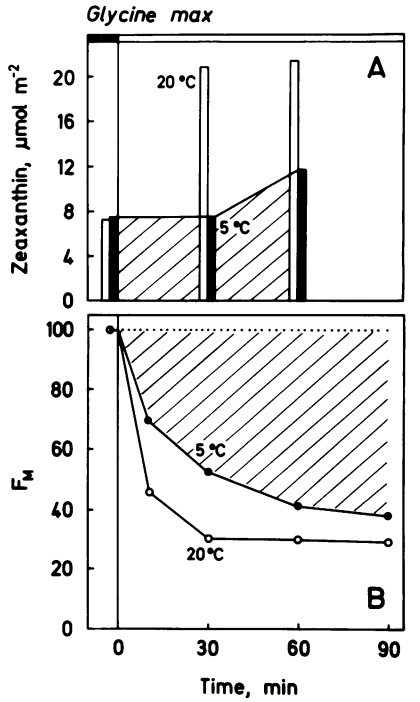

Figure 2. Time course of (A) zeaxanthin content and (B) the decrease of maximum fluorescence F_M in leaves of *G. max*, exposed to a PFD of 120 μmol m^{-2} s^{-1} in 2% O_2, 0% CO_2. Procedures were as in the legend of Figure 1. Fluorescence was recorded on one leaflet of a trifoliate leaf and samples for carotenoid analysis were taken during a corresponding treatment on the remaining two leaflets.

determined in a saturating flash in the light in a leaf of *Rhizophora mangle* subjected to a dark/light (120 μmol photons m^{-2} s^{-1}) transition in 2% O_2, 0% CO_2 at leaf temperatures of 20 and 5°C. At 5°C virtually no nonphotochemical fluorescence quenching occurred over a period of 10 min, and thereafter fluorescence (F_M) declined very slowly. There was almost no formation of zeaxanthin over a period of 30 min. In contrast, at 20°C, a steady state level of zeaxanthin had already been reached when the first measurement was taken after 30 min at 120 μmol photons m^{-2} s^{-1} in 2% O_2, 0% CO_2, and when F_M had reached a minimum value. At 5°C, F_M was still declining even after 90 min at 120 μmol m^{-2} s^{-1} in 2% O_2, 0% CO_2, and the zeaxanthin content had not reached the level in the leaf kept at 20°C for 30 to 60 min. Figure 2 shows zeaxanthin content and fluorescence (F_M) in leaves of *G. max* subjected to the same treatment as *R. mangle*. In *G. max* there was also virtually no increase in zeaxanthin content at a leaf temperature of 5°C over a period of 30 min at 120 μmol photons m^{-2} s^{-1} in 2% O_2, 0% CO_2. In *G. max*, however, there was a background level of zeaxanthin present even under control conditions, *i.e.* in leaves which had been kept in the dark for 12 h. This background level was as high as about 35% of the steady state level after 60 min at 20°C and 120 μmol photons m^{-2} s^{-1}, 2% O_2, 0% CO_2. In these *G. max* leaves some degree of nonphotochemical fluorescence quenching was observed between 0 and 10 min at 5°C (Fig. 2), where there had been no quenching detectable in *R. mangle* (Fig. 1). Further fluorescence quenching between 0 and 30 min proceeded more slowly at 5°C than at 20°C but clearly was not related to any changes in the zeaxanthin content of the leaves. This also becomes clear when the zeaxanthin content is compared with the rate constant for radiationless dissipation, k_D, as calculated from the decrease in F_M (Fig. 3). This was done according to simple theory where it is assumed that radiationless energy dissipation occurs in the antenna Chl (15; *cf.* also ref. 6). In *R. mangle* the increase in zeaxanthin content was linearly related to the increase in k_D over the entire range of values. In *G. max*, however, k_D increased from 12.5 (the control level for a nonphotoinhibited leaf with F_V/F_M = 0.856; *cf* 6) to 25 over a period of 30 min while the zeaxanthin content remained constant at 35% of the steady state level. Thereafter, all further (strong) increases in k_D were accompanied by pronounced increases in zeaxanthin content with the relationships between k_D and zeaxanthin being very similar in *G. max* and in *R. mangle*.

Photochemical Efficiency and Fluorescence Yield in Preilluminated and Predarkened Leaves Differing in Their Carotenoid Composition during a High Light Treatment at Chilling Temperatures followed by a Recovery Period at 25°C in Low Light

Figure 4 shows the level of the three components of the xanthophyll cycle, zeaxanthin, antheraxanthin, and violaxanthin, in a leaf of the mangrove *R. mangle* prior to and

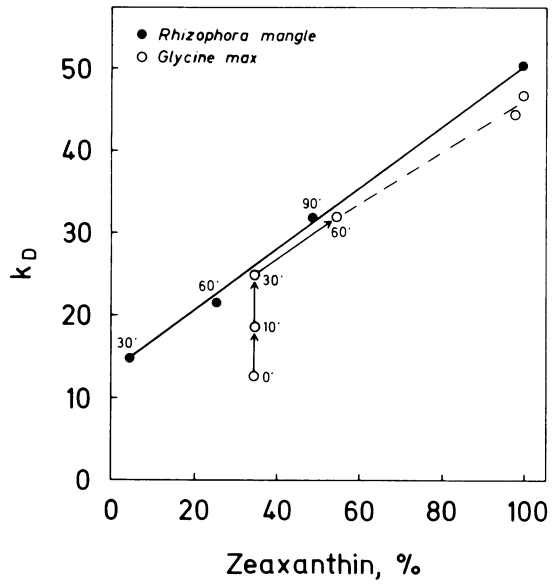

Figure 3. Relationship between the rate constant for radiationless energy dissipation k_D and the zeaxanthin content of *R. mangle* and *G. max* leaves kept at 5°C. The zeaxanthin content is given in percent of the maximum zeaxanthin content of leaves kept at 20°C and 120 μmol photons m^{-2} s^{-1} in 2% O_2, 0% CO_2. Data are from Figures 1 and 2. At 20°C, maximum zeaxanthin content (100%) was reached after 30 min in *R. mangle* and after 60 min in *G. max* (points in the upper right hand). Values of k_D were calculated from F_M, induced by pulses of saturating light during illumination with photosynthetically active radiation. The numerals indicate the duration (in min) of the exposure to 120 μmol photons m^{-2} s^{-1} in 2% O_2, 0% CO_2 at 5°C.

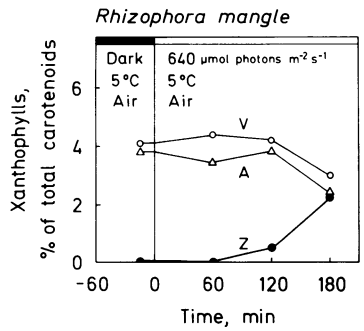

Figure 4. Time course of changes in the level of the three xanthophylls, zeaxanthin (Z), antheraxanthin (A), and violaxanthin (V), in a leaf of the mangrove *R. mangle* exposed to a PFD of 640 μmol m^{-2} s^{-1} at 5°C leaf temperature for 180 min. The leaf was sealed into a gas exchange chamber and the leaf temperature brought to 5°C in the dark. After 5 min at 5°C control samples were taken. Samples were kept in the dark at room temperature for 5 min before they were frozen.

during exposure to a PFD of 640 μmol m^{-2} s^{-1} at a leaf temperature of 5°C. The leaf was kept in air in the dark while the leaf temperature was lowered from 25 to 5°C. Thereafter, the leaf was exposed to the high PFD for 180 min in air. In spite of the fact that this PFD represents greatly excessive light at these low leaf temperatures, zeaxanthin levels remained zero for at least 60 min and then began to increase gradually. The levels of zeaxanthin, antheraxanthin, and violaxanthin in a leaf which was pretreated in 2% O_2, 0% CO_2 under low light and leaf temperatures of 25°C are shown in Figure 5. This treatment resulted in an almost complete conversion of violaxanthin and antheraxanthin to zeaxanthin. During the subsequent exposure to 640 μmol photons m^{-2} s^{-1} at leaf temperatures of 5°C in air, the levels of zeaxanthin, antheraxanthin, and violaxanthin remained virtually unchanged. These two kinds of *R. mangle* leaves, predarkened and containing no zeaxanthin or pretreated such as to contain high levels of zeaxanthin, were used to study the effects of a high light treatment at 5°C on fluorescence characteristics (Fig. 6). The leaf which was exposed to high light at 5°C while in a high fluorescence state (*i.e.* dark pretreatment) showed decreases in the yield of maximum fluorescence, F_M, and PSII photochemical efficiency, F_V/F_M, which were essentially irreversible during a subsequent period of 6 h at 25°C in low light (100 μmol photons m^{-2} s^{-1}). The leaf which was exposed to the high PFD at 5°C while in the low fluorescence state (*i.e.* pretreated with 2% O_2, 0% CO_2 at 100 μmol photons m^{-2}

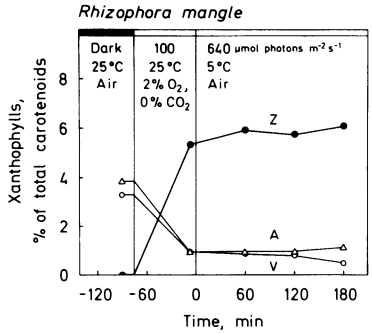

Figure 5. Time course of changes in the level of the three xanthophylls, zeaxanthin (Z), antheraxanthin (A), and violaxanthin (V), in a leaf of *R. mangle* preilluminated with 100 μmol photons m^{-2} s^{-1} at 25°C in 2% O_2, 0% CO_2 and then exposed to a PFD of 640 μmol m^{-2} s^{-1} for 180 min. For other procedures see legend of Figure 4.

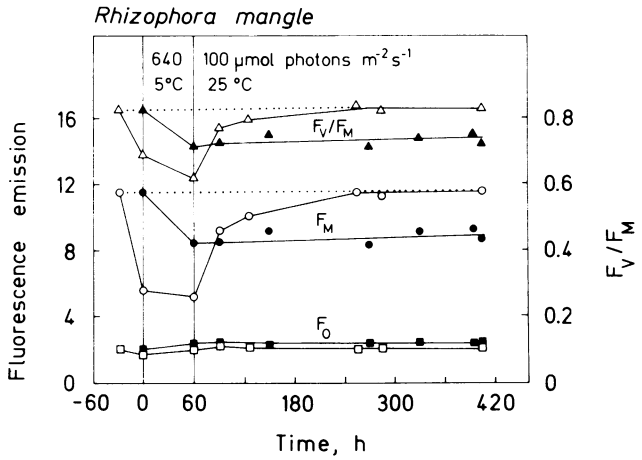

Figure 6. Time course of changes in Chl fluorescence characteristics, F_O, F_M, and F_V/F_M, in a predarkened leaf (closed symbols) and a preilluminated leaf (open symbols) of *R. mangle*, both exposed to a PFD of 640 μmol m^{-2} s^{-1} at leaf temperatures of 5°C for 60 min. Preillumination was with 100 μmol photons m^{-2} s^{-1} at 25°C in 2% O_2, 0% CO_2. Subsequent to the high light treatment both leaves were taken back to 100 μmol photons m^{-2} s^{-1} and 25°C leaf temperature. Measurements were done after 5 min of darkness.

s^{-1}) also exhibited some further reduction in photochemical efficiency F_V/F_M over the period of 60 min at the high PFD and 5°C. However, in the latter case, both fluorescence yield and PSII photochemical efficiency recovered within a few hours at 25°C in low light. During the chilling treatment at high PFD, no loss of either Chl *a* or *b* was detectable in either the predarkened or the preilluminated *R. mangle* leaves, and there also were no changes in the levels of β-carotene and lutein during the treatment (not shown).

DISCUSSION

When leaves are exposed to an excess of light in 2% O_2, 0% CO_2, for example 100 to 200 μmol photons m^{-2} s^{-1}, at chilling temperatures, both the development of nonphotochemical fluorescence quenching which is indicative of an increase in the rate of radiationless dissipation of excess excitation energy and the formation of zeaxanthin through violaxanthin de-epoxidation are greatly retarded (Figs. 1 and 2). We used this condition to study the possible involvement of zeaxanthin and radiationless energy dissipation in the protection of the photochemical apparatus and to investigate the relationship between zeaxanthin and two components of nonphotochemical fluorescence quenching with different induction kinetics. The strong decrease in the rate of the development of fluorescence quenching at 5°C compared to 20°C allows one to distinguish between these two components.

Relationship between Zeaxanthin and Two Different Components of Nonphotochemical Fluorescence Quenching Indicative of Radiationless Energy Dissipation

The close correlation between the rate constant for radiationless energy dissipation, k_D, and zeaxanthin content in the *Rhizophora mangle* leaf that did not contain any zeaxanthin prior to the treatment with 2% O_2, 0% CO_2 at low PFD at 5°C (Fig. 3) is similar to that reported previously for various conditions representing excess light (4–7). On the grounds of this correlation we have suggested that zeaxanthin may act as a fluorescence quencher and thus mediate this dissipation process. In leaves such as the soybean leaf which did contain some background level of zeaxanthin, a certain degree of fluorescence quenching developed without any changes in the bulk zeaxanthin content (Fig. 2). This finding would be consistent with zeaxanthin acting as a fluorescence quencher only if the activity of zeaxanthin as a quencher was controlled not only biochemically through the regulation of violaxanthin de-epoxidation, but also by an additional biophysical activation/deactivation step working on a shorter time scale. Upon exposure to excess light, zeaxanthin present in the antenna Chl complexes may be rendered effective as a quencher of fluorescence by a condition related to the high energy state of the membrane. Under control conditions, *i.e.* in limiting light, the physical arrangement between zeaxanthin and chlorophyll molecules in the antennae may not allow any such interaction between them. A relationship between zeaxanthin and the component of fluorescence quenching which develops very rapidly is suggested by the following findings: (a) The rapidly developing component of quenching was never observed in the absence of zeaxanthin (Fig. 1). (b) In soybean, the extent of radiationless energy dissipation did not exceed the level which would be expected to occur at the actual zeaxanthin content of the leaves (Fig. 3), *i.e.* there were no k_D values above the straight line which marks the relationship between k_D and zeaxanthin in the mangrove leaf. This means that the degree of fluorescence quenching which occurred without further increases in the bulk zeaxanthin content in soybean was exactly as great as the degree of fluorescence quenching which was observed in *R. mangle* when the zeaxanthin content in the mangrove leaf had risen to that present in soybean under control conditions (Fig. 3). The time course of the development of the slower type of fluorescence quenching matches the time course of the formation of zeaxanthin via violaxanthin de-epoxidation (13, 29).

Effect of High PFD at Chilling Temperatures on PSII Photochemical Efficiency in Zeaxanthin-Containing and Zeaxanthin-Free Leaves

A combination of chilling temperatures and strong light is known to result in sustained reductions in photochemical efficiency and electron transport as well as in net CO_2 uptake by leaves of chilling-sensitive plants (8, 10, 19, 22, 23, 25). Here we show that during a high light treatment at chilling leaf temperatures sustained reductions of F_V/F_M do indeed occur in leaves which had been kept in darkness prior to the chilling treatment, *i.e.* leaves which contained no zeaxanthin. Pretreatment of leaves under conditions (2% O_2, 0% CO_2 at low PFD) which greatly increase the zeaxanthin content (Fig. 2) and promote strong nonphotochemical fluorescence quenching proved to diminish the extent of sustained inactivation of photochemistry resulting from a subsequent exposure to chilling temperatures and high PFD in air (Fig. 6). This became evident during a recovery period at 25°C in low light in air.

The irreversible reductions in PSII photochemical efficiency observed here were not accompanied by any decrease in the Chl content and are therefore probably not related to photooxidative processes. Baker *et al.* (1) also reported a 45% reduction in the apparent photon yield of O_2 evolution with no significant decrease in the bulk Chl content of *Zea mays* leaves subjected to a chilling treatment.

Since the present study demonstrates that the inhibition of controlled energy dissipation and zeaxanthin synthesis is related to an increased susceptibility of the photochemical apparatus to photoinhibitory inactivation at chilling temperatures, it is interesting to compare these conditions with other conditions under which controlled energy dissipation is prevented. A protective role of high-energy-state quenching in isolated chloroplasts has previously been demonstrated by inhibiting the development of the quenching by uncouplers removing the transthylakoid pH gradient (16). Such a gradient is a prerequisite for the development of strong nonphotochemical fluorescence quenching (17, 18) and also for zeaxanthin formation (11–13). Under the conditions used here, the rates of zeaxanthin formation and of radiationless energy dissipation are probably lowered as a direct consequence of low temperature on the enzymatic conversion of violaxanthin to zeaxanthin and not by the absence of the ΔpH.

Preillumination of leaves prior to a chilling treatment in high light has also been reported to prevent sustained decreases in their CO_2 uptake (20). In these studies, CO_2 uptake

of predarkened and preilluminated leaves was determined after a night at room temperature and preillumination was at high PFD in air. It has previously been suggested that phosphorylation of the light-harvesting complex of PSII and a resulting change in the energy distribution between PSII and PSI may be involved in protection against photoinhibitory damage of PSII (21). Both at high PFD in air and under the conditions used by us (2% O_2, 0% CO_2, low PFD) the light-harvesting complex was shown to be dephosphorylated (3). Thus, movements of the LHC-II from PSII to PSI are probably not involved in the protection demonstrated here. The strong nonphotochemical fluorescence quenching observed in the current study is indicative of increased radiationless energy dissipation. Our results are consistent with a causal relationship between the dissipation process and zeaxanthin, but further experiments are required to determine whether zeaxanthin is involved in photoprotection via an interaction with the singlet excited state of Chl or, alternatively, by leading to deexcitation of other excited species.

In view of the suggested function of zeaxanthin in photoprotection it is noteworthy that chilling resistant plants were reported to show increased levels of ascorbate and glutathione (28). Both substances are thought to be effective as antioxidants. However, both are also required for the operation of the xanthophyll cycle (13, 29) where ascorbate acts as the endogenous reductant for the de-epoxidation of violaxanthin (13). Despite the fact that, initially, chilling damage appears to affect sites other than PSII (*e.g.* 9, 14), the formation of large amounts of zeaxanthin in the xanthophyll cycle may be an important factor in the acclimation of plants to chilling temperatures.

ACKNOWLEDGMENTS

We thank Profs. Marianne Popp and Miriam Diaz for the collection of *Rhizophora mangle* propagules, Maria Lesch for the cultivation of the mangrove plants, and Drs. William W. Adams III and Ulrich Schreiber for reading the manuscript.

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