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The Xanthophyll Cycle in Intermittent Light-Grown Pea Plants[1]

Possible Functions of Chlorophyll *a/b*-Binding Proteins

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The xanthophyll cycle in pea (*Pisum sativum* L. cv Kleine Rheinländerin) plants has been investigated in vivo. Control plants were compared with those grown under intermittent light (IML plants). IML plants are particularly characterized by the absence of nearly all chlorophyll *a/b*-binding proteins. The rates of de-epoxidation during 30 min of illumination and their dependence on the incident photon flux density (PFD) have been determined. They were very similar in both types of plants, with the exception that IML plants contained, at any PFD, much higher zeaxanthin concentrations in the steady state (reached after about 15 min of illumination) than control plants. This indicates that the amount of convertible violaxanthin under illumination is dependent on the presence of chlorophyll *a/b*-binding proteins. The epoxidation rate (examined at a PFD of 15 μE m^{-2} s^{-1}, after 15 min of preillumination with different PFDs) showed significant differences between the two types of plants. It was about 5-fold slower in IML plants. On the other hand, in both types of plants, the epoxidation rate decreased with increasing PFD during preillumination. Prolonged preillumination at high PFDs resulted in a decrease of the epoxidation rate without a further increase of the zeaxanthin concentration in both continuous light and IML plants. This result argues against a permanent turnover of the xanthophylls under illumination, at least at high PFDs.

The xanthophyll cycle describes the light-dependent and reversible conversion of Vio to Zea via the intermediate Ant (for a review, see Siefermann-Harms, 1977; Yamamoto, 1979). The de-epoxidation of Vio to Zea is catalyzed by a water-soluble enzyme with a molecular mass of about 54 kD, which is probably located in the thylakoid lumen (Hager and Perz, 1970). The de-epoxidase requires ascorbic acid as a reducing cofactor (Hager, 1966) and shows maximum activity at a about pH 5 (Hager, 1969; Siefermann-Harms, 1977), under conditions of strong illumination. The portion of Vio that can be de-epoxidized is controlled by an unknown factor (Siefermann-Harms, 1977). It has been speculated that the Vio availability might be related to structural changes of the thylakoid membrane or to the redox state of electron carriers between the two photosystems (Siefermann and Yamamoto, 1974). A recent study suggested that acidification of the thylakoid lumen is accompanied by binding of the de-epoxidase to the membrane, which is reversed upon alkalization (Hager and Holocher, 1994).

The epoxidase is known to require molecular oxygen and NADPH (Takeguchi and Yamamoto, 1968; Siefermann-Harms and Yamamoto, 1975) as co-substrates. It has a pH optimum of about 7.5 (Siefermann-Harms and Yamamoto, 1975), implying a location on the stroma side of the thylakoid membrane. It has recently been suggested that LHCs of PSII exhibit epoxidase activity in vitro (Gruszecki and Krupa, 1993).

Until now it was not clear whether the two reactions, the de-epoxidation and the epoxidation, occur simultaneously under illumination. Schubert et al. (1994) proposed a nonenzymatic epoxidation of Zea in the light and an enzymatic epoxidation only in the dark. Gilmore and Björkman (1994a) suggested that the epoxidation state in the dark following a light treatment may be influenced by sustained ATPase activity, maintaining the light-induced ΔpH and thus reducing the availability of Zea to the epoxidase.

The physiological relevance of the xanthophyll cycle is based on the function of Zea in thermal energy dissipation, thereby decreasing PSII efficiency. During the last years several reports have postulated a function of Zea in the energy- or pH-dependent mechanism of fluorescence quenching (Gilmore and Yamamoto, 1993) or a protective role of Zea in photoinhibitory processes (Demmig-Adams and Adams, 1992).

The aim of this study was to investigate the function of CAB proteins in the xanthophyll cycle. The results seem to corroborate the recent hypothesis that CAB proteins of PSII bear epoxidase activity (Gruszecki and Krupa, 1993) and imply an important function of CAB proteins for controlling the steady-state concentrations of the xanthophyll-cycle pigments under illumination.

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Abbreviations: Ant, antheraxanthin; CAB, Chl *a/b* binding; CL, continuous light; ΔpH, transmembrane pH gradient; ELIP, early light-induced proteins; IML, intermittent light; LHC, light-harvesting complex; PFD, photon flux density; Vio, violaxanthin; Zea, zeaxanthin.

MATERIALS AND METHODS

Plant Material

Pea seedlings (*Pisum sativum* L. cv Kleine Rheinländerin) were grown either under intermittent light (40 μmol m^{-2} s^{-1}, 2 min of light, 118 min of darkness: IML plants) or under continuous light (100 μmol m^{-2} s^{-1}, 14 h of light, 10 h of darkness, CL plants). Intact leaves from 11- to 12-d-old IML plants or 12- to 14-d-old CL plants were used for all experiments.

Illumination Conditions

Leaflets were illuminated with a 250-W halogen light source. During illumination with high PFDs (100–2200 μmol m^{-2} s^{-1}) leaflets were floated on distilled water in a temperature-controlled cuvette (20°C). Illumination at low PFDs (15 μmol m^{-2} s^{-1}) was performed at room temperature in Petri dishes (containing distilled water) placed under ceiling lamps.

Pigment Analysis

For pigment analysis, leaflets (about 80 mg from CL plants and 120 mg from IML plants) were frozen in liquid nitrogen and disrupted very carefully in a mortar for 1 min. Pigments were extracted by addition of acetone (1.2 mL for CL leaves and 2 mL for IML leaves) and continued grinding for 1 min. The pigment extracts were filtered through a 0.2-μm membrane filter and applied immediately to an HPLC column. Pigments were separated in a HPLC system (Pharmacia) using a reversed phase column. Most of the experiments were done with a Nucleosil column (ET 250/8/4, Nucleosil 120–5 C_{18}; Macherey-Nagel, Düren, Germany) as described by Jahns and Krause (1994). Additionally a Spherisorb ODS-1 column (5 μm particle size, 250 × 4.6 mm i.d.; Schambeck SFD GmbH, Bad Honnef, Germany) was used based on the method by Gilmore and Yamamoto (1991) with the following modifications: the acetonitrile:methanol:Tris ratio of solvent A was changed to 85:25:8 and the gradient from solvent A to solvent B was run from 5 to 7 min. Eluted pigments were monitored at 440 nm. Peak areas were calculated by an integrator (LKB 2221, Pharmacia). Relative concentrations of the xanthophyll-cycle pigments (Vio, Ant, and Zea) were determined by using the conversion factors for peak area to nanomoles given by Gilmore and Yamamoto (1991). These conversion factors were thought to be useful because very similar systems were used in this study and because the pool size of the total xanthophyll-cycle pigments was unchanged when comparing very low and very high epoxidation states of the xanthophyll-cycle pigments.

RESULTS AND DISCUSSION

Pigment Composition

The pigment compositions of both types of plants used in this study are summarized in Table I. As was shown earlier (Jahns and Krause, 1994), IML plants showed a drastically increased carotenoid:Chl ratio. However, these changes were mainly due to the reduced antenna size of both photosystems in IML plants. For a better comparison, the data have also been normalized to the amount of pigment per photosynthetic reaction center. This calculation was based on the (reasonable) assumption of an antenna size of 200 to 300 Chl in Cl plants and 70 to 100 Chl in IML plants, which has been estimated from flash-spectrophotometric data (Jahns and Junge, 1992). The normalized data revealed similar amounts of β-carotene and lutein for both types of plants. Clear differences, however, were obvious for neoxanthin, which was reduced in IML plants to 30% of the amount in CL plants and for the content of the xanthophyll-cycle pigments (Vio plus Ant plus Zea), which was doubled in IML plants. The reduction of the neoxanthin content in IML plants can be understood by the absence of nearly all CAB proteins, which were found to bind all neoxanthin in PSII (Bassi et al., 1993). The increase of the xanthophyll-cycle pigments, on the other hand, cannot be explained by the reduction of antenna proteins. Bassi et al. (1993) found Vio to be associated mainly with the inner CAB proteins (CP24, CP26, and CP29), whereas Peter and Thornber (1991) and Ruban et al. (1994) additionally determined Vio associated with the major LHCII proteins. The stoichiometries of Vio bound to CAB proteins have been determined by the above-cited authors to be about 1 each per CP24, CP26, and CP29 and about 0.3 to 0.5 per LHCII monomer. No Vio was found to be bound to the PSII core (Bassi et al., 1993). Since IML plants contain only CP26 of all CAB proteins (Jahns and Krause, 1994), it must be concluded that nearly all xanthophyll-cycle pigments in these plants are either free in the membrane or must be associated with other proteins. The high stoichiometry of about 40 Vio per reaction center, however, makes an association of Vio with reaction center proteins very unlikely. Possibly, other proteins are involved in carotenoid binding in IML plants. A very recent study reported evidence that ELIPs are present in IML plants from barley (Król et al., 1995). The authors hypothesized that ELIPs might be involved in binding of photoconvertible xanthophylls. This

Table I. *Pigment content of leaves from CL and IML plants*

Pigments of whole leaves were extracted with acetone and separated by HPLC. Pigment concentrations were calculated from the respective peak areas at 440 nm using the conversion factors given by Gilmore and Yamamoto (1991). Chl *a/b* ratios were in the range of 2.5 to 2.7 for CL plants and 30 to 40 for IML plants. The total Chl content of the leaves per gram fresh weight was 2.2 to 2.5 mg (CL plants) and 120 to 180 μg (IML plants).

Pigment	mmol per mol Chl		Per Reaction Center[a]	
	Cl plants	IML plants	CL plants	IML plants
Neoxanthin	55.8	61.3	11–17	4–6
Vio + Ant + Zea	88.3	526.6	18–26	37–53
Lutein	227.5	784.1	45–69	56–78
β-Carotene	88.1	232.1	18–26	17–23
Chl(*a* + *b*)	–	–	200–300	70–100

[a] The reduction of the antenna size of IML plants was estimated from earlier flash-spectrophotometric measurements (Jahns and Junge, 1992).

could indeed also be the case for pea IML plants and has to be investigated.

The stoichiometry of about 20 Vio per reaction center in CL plants can be sufficiently explained by an association with CAB proteins only under the assumption that each polypeptide of the CAB proteins binds at least one Vio. Thus, either these carotenoids are only loosely bound to proteins and are therefore lost during isolation of the polypeptides or one has to propose that at least some of the xanthophyll-cycle pigments are generally not associated with reaction center or antenna proteins. A function of ELIPs in Vio binding in CL plants is not likely, since these proteins were not detectable in CL plants from barley (Król et al., 1995).

The unchanged content of β-carotene per reaction center in IML plants coincides with the common picture that these carotenoids are associated with the reaction center core of both photosystems. The predominant binding of β-carotene by PSI (Thayer and Björkman, 1992), however, seems not to be valid in IML plants. These CAB protein-deficient plants have been shown to have a much lower PSI:PSII ratio (Tzinas et al., 1987; Jahns and Junge, 1992). Thus, in IML pants, β-carotene must also be associated in equal amounts with PSII or a drastically increased β-carotene content of PSI must be postulated. The latter possibility could be understood as compensation for the absence of PSI CAB proteins, suggesting a photoprotective role of CAB proteins in PSI.

A surprising result was also the high lutein content of IML plants. It seems to be established that this pigment is associated exclusively with CAB proteins (Thayer and Björkman, 1992; Bassi et al., 1993; Ruban et al., 1994), mainly with those from PSII (Thayer and Björkman, 1992). With respect to the high amount of lutein per reaction center, it looks reasonable to assume that in IML plants lutein is also either bound to other proteins or present as free pigment to a large extent.

De-epoxidation in CL Plants

Figure 1 shows the time dependence of the de-epoxidation of Vio into Ant and Zea under illumination of dark-adapted leaves from CL plants. The de-epoxidation was followed for 30 min at six different PFDs varying from 100 to 2,200 μmol m^{-2} s^{-1}. The following results are obvious from Figure 1: (a) After 10 to 15 min of illumination maximum values of Zea and minimum values of Vio concentrations were reached at each PFD; (b) with increasing PFDs the maximum (minimum) concentrations of Zea (Vio) in the steady state increased (decreased); (c) at low PFDs of 300 and 100 μmol m^{-2} s^{-1} (Fig. 1, E and F) the Zea (Vio) concentration decreased (increased) after reaching the maximum (minimum); and (d) the Ant concentrations remained constant after reaching the maximum value at each PFD. The steady-state Ant content was about 20% at all PFDs and slightly lower at the two highest PFDs.

The steady-state levels of Zea and Vio should depend on (a) the availability of Vio to the de-epoxidase and (b) the ratio of the rates of the de-epoxidation and epoxidation reaction at each PFD. Changes in the steady-state concentrations of Zea and Vio must therefore be explained by changes of one (or more) of these parameters. The rate of de-epoxidation is known to depend on the luminal pH and on the availability of reducing equivalents. The steady-state levels of Zea and Vio are therefore determined by the electron transport rate (i.e. the generation of the ΔpH) and by the rate of carbon fixation (i.e. the consumption of reducing equivalents and utilization of the ΔpH by ATP synthesis). At PFDs that are insufficient to saturate photosynthesis (100 and 300 μmol m^{-2} s^{-1}, Fig. 1, F and E, respectively), the electron transport is limiting. Therefore, under these conditions, it can be expected that the de-epoxidation rate will (after an initial increase) decrease when, under continuing illumination, the Calvin cycle becomes activated. This is verified in this experiment by a

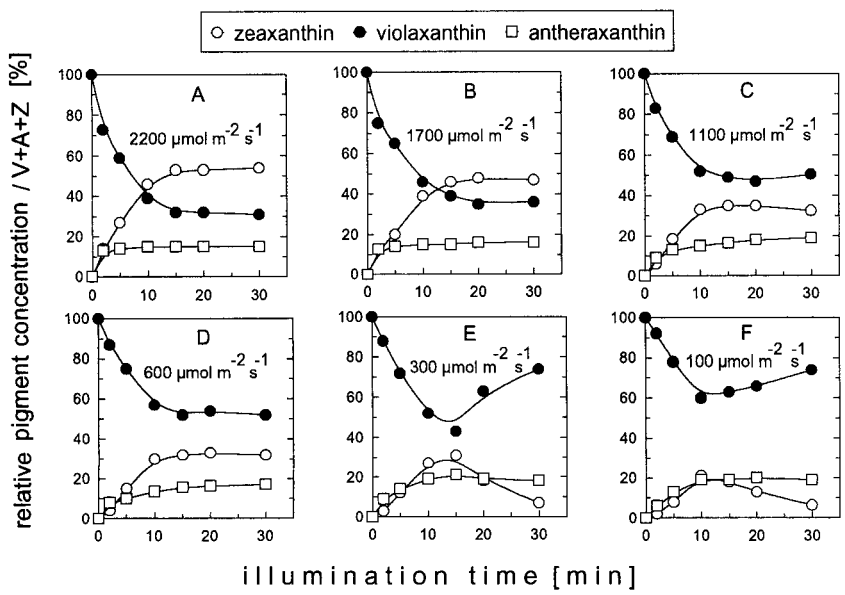

Figure 1. Time course of the de-epoxidation reactions in CL plants. Leaflets from 10-h dark-adapted plants were illuminated at different PFDs as indicated. Reactions were stopped by freezing of the leaflets in liquid nitrogen. Pigment concentrations were calculated from peak areas using the conversion factors given by Gilmore and Yamamoto (1991). Each value represents the mean of two to four measurements. SE was in the range of 1 to 4%. V, Vio; A, Ant; Z, Zea.

decrease in the Zea and an increase in the Vio concentrations after about 10 to 15 min of continuous illumination at these low PFDs. Obviously, the epoxidation rate exceeds the de-epoxidation rate under these conditions. It can be concluded that at these low PFDs the two reactions, the de-epoxidation and the epoxidation, occur simultaneously.

A different picture was seen at moderately high PFDs (600 and 1100 μmol m^{-2} s^{-1}, Fig. 1, D and C, respectively). At these PFDs, rates of electron transport (and thus the ΔpH) and of concomitant carbon fixation (and thus of the consumption of reducing equivalents) are saturated. Thus, the de-epoxidase should operate with its maximum activity under these illumination conditions. Indeed, this was confirmed by (a) the constant steady-state level of Zea and Vio concentrations and (b) by the identical values of Zea and Vio concentrations of about 30% and 50%, respectively (Fig. 1, C and D). The constant steady-state levels of all pigments after about 15 min of illumination may be interpreted as an equilibrium of the de-epoxidation and epoxidation reactions or, alternatively, by a saturation of the de-epoxidation due to a limited availability of Vio.

At higher PFDs, however, a further decrease of the luminal pH seems unlikely. This can be concluded from the saturation of the pH-dependent fluorescence quenching between 600 and 1100 μmol m^{-2} s^{-1} (Jahns and Krause, 1994). Nevertheless, a significant further increase of Zea and decrease of Vio were observed at 1700 and 2200 μmol m^{-2} s^{-1} (Fig. 1, B and A, respectively). Again, at these highest PFDs, further changes of the Zea and Vio concentrations might be explained either by an increased availability of Vio or by a decelerated rate of the epoxidation.

The constant steady-state concentration of Ant at all saturating PFDs (Fig. 1, A–D) implies that either (a) essentially no further pigment conversion occurs or (b) the formation of Ant (from either Vio or Zea) and the reconversion of Ant (to either Zea or Vio) are in equilibrium. The first possibility might be related somehow to the limited Vio conversion; the latter possibility would again imply a real equilibrium of de-epoxidation and epoxidation.

De-epoxidation in IML Plants

The conversion of the xanthophyll-cycle pigments in IML plants is shown in Figure 2. Compared to CL plants the following important differences were observed: (a) At each PFD, much more Vio was converted into Zea; (b) at highest PFDs, nearly 100% of the xanthophyll-cycle pigments were converted into Zea; (c) the decrease in levels of Zea and increase in levels of Vio during longer illumination (15–30 min) at low PFDs were much less pronounced; and (d) the Ant formation and degradation showed clear differences in dependence on the PFD.

The conversion of nearly all Vio into Zea argues against an essentially unavailable pool of Vio in IML plants. Most likely, the absence of CAB proteins made all Vio available to the de-epoxidase in these plants. How can these differences be explained? It is conceivable that xanthophyll binding by CAB proteins influences the de-epoxidase activity and substrate availability in CL plants. Another possibility is that the recently proposed epoxidase activity of CAB proteins (Gruszecki and Krupa, 1993) is responsible for the differences. Under the assumption that the epoxidation reaction occurs simultaneously under illumination, the nearly complete conversion of Vio to Zea in IML plants can be well understood as a result of a slowed-down epoxidation rate in IML plants. All steady-state concentrations of the xanthophylls would then strongly depend on the respective epoxidation rates at the different PFDs. At PFDs that saturate photosynthesis (Figs. 1, A–C, and 2, A–C), the increase in Zea formation with increasing PFD could then be explained by a slowed-down epoxidation rate. The epoxidation kinetics and its light dependence will be presented later in more detail (see below). Finally, other structural changes in IML plants (e.g. the missing grana structure of the membrane) could have changed the Vio availability to the de-epoxidase.

Another important feature of Figures 1 and 2 are the different kinetics of Ant formation and degradation in IML and CL plants. At high PFDs (Fig. 2, A–D), a rapid forma-

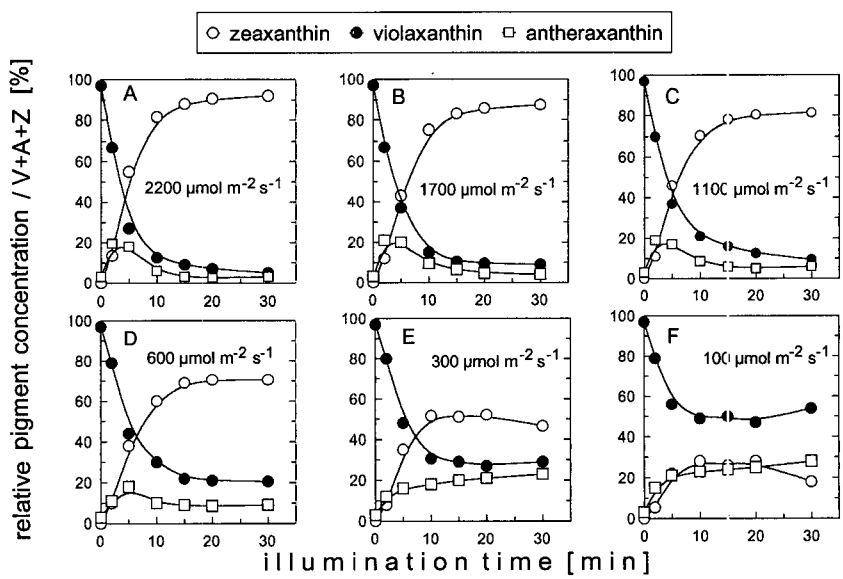

Figure 2. Time course of the de-epoxidation reactions in IML plants. Leaflets from 3-h dark-adapted plants were illuminated at different PFDs as indicated. Reactions were stopped by freezing the leaflets in liquid nitrogen. Pigment concentrations were calculated from peak areas using the conversion factors given by Gilmore and Yamamoto (1991). Each value represents the mean of two to six measurements. SE was in the range of 1 to 8%. V, Vio; A, Ant; Z, Zea.

tion of Ant during the first 5 min is followed by a decline in Ant levels upon prolonged illumination in IML plants. This was not seen in CL plants (Fig. 1, A–D) and can be attributed to a slower epoxidation reaction in IML plants. Under the assumption that no significant epoxidation of newly formed Zea occurs in IML plants within the first 10 min of illumination, the kinetics of Ant conversion in these plants are determined only by the two steps of de-epoxidation from Vio to Ant and from Ant to Zea. In CL plants, however, the fact that the value of Ant remains constant can be best explained by a superimposed epoxidation.

At low PFDs (Fig. 2, E and F) the transient accumulation of Ant disappears in IML plants and the curves are similar to those from CL plants, indicating a decelerated rate of Zea formation due to a higher luminal pH under nonsaturating light conditions.

These data indicate that the de-epoxidation rates of Vio to Ant and Zea are similar in the two types of plants. All differences between CL plants and IML plants can be equally explained by either an increased Vio availability or a slowed-down epoxidation of Zea to Ant and Vio. The epoxidation reactions have been analyzed more directly in a second set of experiments for both plants: Leaves were illuminated for 15 min at the same PFDs used before (Figs. 1 and 2) so that under all conditions the maximum level of Zea was reached. Subsequently, leaves were transferred to low light (15 μmol m^{-2} s^{-1}) and the changes in pigment composition were followed for up to 4 h. This experimental setup should provide information about any dependence of the epoxidation rate on the PFD of preillumination, although one should keep in mind that it cannot give reliable information about the epoxidase enzyme activity.

Epoxidation in CL Plants

Figure 3 shows the results of these experiments with leaves from CL plants. The most important aspects are: (a) At any PFD, the epoxidation of Zea to Ant was faster than that of Ant to Vio by about a factor of 2; (b) the net decrease of the Zea content was delayed by about 2 min after transfer to low light, and the net formation of Vio was delayed by about 5 min after transfer to low light; and (c) the epoxidation rates increased with decreasing PFDs (Fig. 3).

The delay in the decrease in Zea and the increase in Vio can be explained by the continuing de-epoxidation reactions during the first minutes after transfer of the leaves to low light (see also Gilmore and Björkman, 1994a). The de-epoxidation rate depends mainly on the luminal pH and on the ascorbate availability. The delay may therefore be interpreted as a transient increase of the ascorbate pool (as reported by Foyer et al., 1989) in combination with a delayed breakdown of the ΔpH due to ATPase activity (Gilmore and Björkman, 1994a). The somewhat longer delay for the Vio formation in comparison to the Zea decrease correlates with the slower rate for the Ant to Vio reaction. The most interesting finding of these experiments, however, is the dependence of the epoxidation rate on the PFD of the preillumination. This result seems to confirm the above assumption deduced from the experiments shown in Figures 1 and 2 that the increase in the steady-state level of Zea and the decrease in the steady-state level of Vio during illumination at higher PFDs were caused by a slowed-down epoxidation rate rather than by an increased Vio availability. However, this correlation did not hold true when illumination times longer than 15 min were applied to the leaves prior to low light illumination, as illustrated in Figure 4. Prolonged illumination (e.g. 90 min) led to a strong reduction of the epoxidation rate without any influence on the steady-state level of Zea. This effect was more pronounced when illumination was performed at lower temperature (2°C) (Fig. 4). Thus, the increase in the amount of convertible Vio with increasing PFDs during illumination of CL plants (Fig. 1) was probably not caused by a reduction of the epoxidation rate. Similar results were published very recently by Gilmore and Björkman (1994a, 1994b). These authors assigned the slowed-down Zea re-

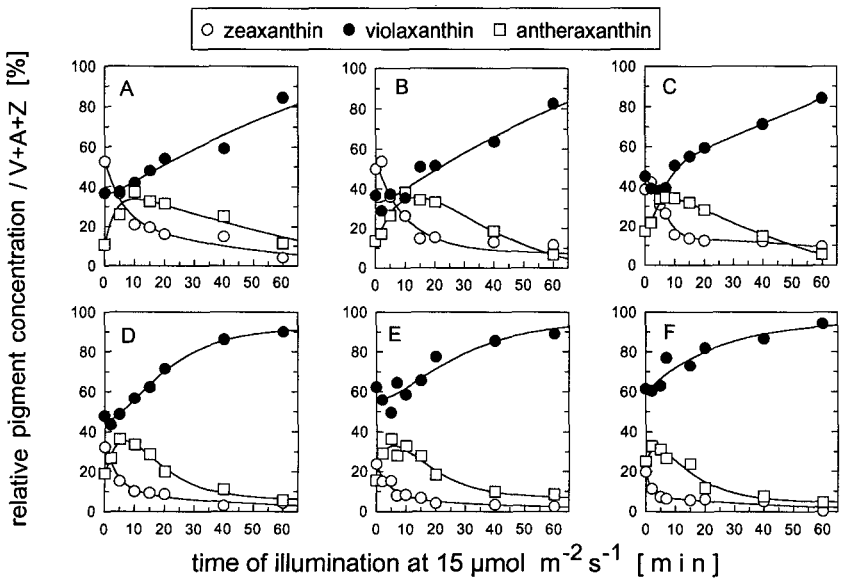

Figure 3. Time course of the epoxidation reactions in CL plants. Leaflets from 10-h dark-adapted CL plants were preilluminated for 15 min at different PFDs as shown in Figure 1. A, 2200 μmol m^{-2} s^{-1}; B, 1700 μmol m^{-2} s^{-1}; C, 1100 μmol m^{-2} s^{-1}; D, 600 μmol m^{-2} s^{-1}; E, 300 μmol m^{-2} s^{-1}; F, 100 μmol m^{-2} s^{-1}. Subsequently, leaflets were transferred to low light (15 μmol m^{-2} s^{-1}) and the dependence of the pigment content on illumination time was analyzed. V, Vio; A, Ant; Z, Zea. A and D represent single experiments. Each value in B, C, E, and F represents the mean of two measurements. SE was in the range of 1 to 7%.

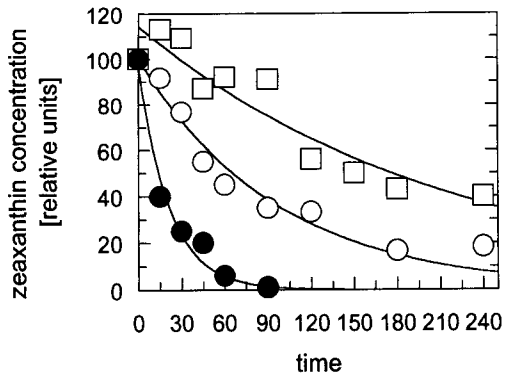

Figure 4. Zea epoxidation in CL plants after prolonged illumination. Leaflets were illuminated at a high PFD (2.500 μE m^{-2} s^{-1}) either at 2°C for 180 min (□) or at 20°C for either 90 min (○) or 15 min (●), yielding relative Zea concentrations of 51, 59, and 57%, respectively. Changes in Zea concentrations were determined after transfer of the leaflets to low light (15 μE m^{-2} s^{-1}) at room temperature. For better comparison of the kinetics of Zea reconversion, the Zea concentrations after high-light treatment were normalized to 100 for all samples. The data represent a single experiment.

epoxidation to a maintenance of ΔpH by prolonged ATPase activity, either after longer illumination times or after low-temperature illumination. This could indeed also be the case for the changes shown above (Fig. 4) and might be related somehow to an altered availability of Zea to the epoxidase under prolonged illumination, possibly by changes in the binding site of Zea or by alterations of the protein environment. In any event, the results indicate that after prolonged illumination or illumination at low temperatures the re-epoxidation of Zea is slowed down. Since this deceleration was not paralleled by changes in the steady-state level of Zea during the illumination period, it can be concluded that the steady-state level of Zea is not influenced by the epoxidation rate in the light. This would exclude a permanent turnover of the xanthophyll cycle under illumination at high PFDs.

Epoxidation in IML Plants

Very similar results were obtained in the respective experiments with IML plants (Fig. 5). In particular, the dependence of the epoxidation rate on the PFD during preillumination was obvious in IML plants, although with one very important difference: At all PFDs the epoxidation rates were significantly slower than in CL plants. This could be interpreted as evidence for a reduced epoxidase activity in IML plants and would then support the proposed epoxidase function of LHCII proteins (Gruszecki and Krupa, 1993). Alternatively, a limitation of the availability of Zea in IML plants could explain the slowed-down re-epoxidation of Zea. The latter possibility, however, seems at first sight more unlikely. Under the assumption that the epoxidase is located in the chloroplast stroma, one can expect that membrane-associated Zea (whether or not bound to proteins) should be rather better available to the epoxidase enzyme in an unstacked membrane system such as that in IML plants. This point of view is supported by the more efficient de-epoxidation in IML plants.

On the other hand, the generally slower epoxidation in IML plants cannot be explained by the maintenance of ΔpH by ATPase activity, since the differences were also obvious at low PFDs, which were insufficient to build up a high ΔpH (cf. Fig. 1, E and F, and Fig. 2, E and F).

In contrast to what was seen in CL plants, a further deceleration of the epoxidation rate upon prolonged illumination was apparent only after illumination at lower temperatures (see Fig. 6). As in CL plants, however, the slower epoxidation was not accompanied by an increase in the steady-state concentrations of Zea.

The following conclusions can be drawn from the above

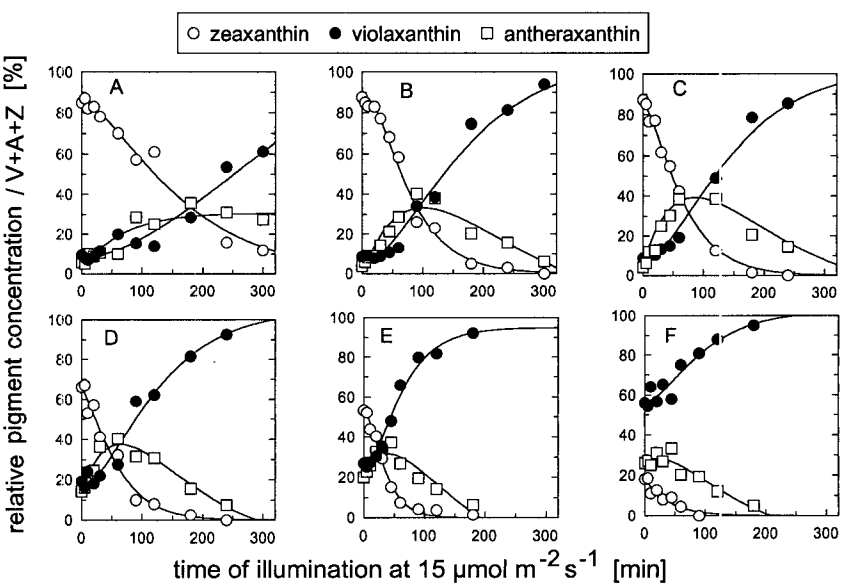

Figure 5. Time course of the epoxidation reactions in IML plants. Leaflets from 3-h dark-adapted IML plants were preilluminated for 15 min at different PFDs as shown in Figure 2. A, 2200 μmol m^{-2} s^{-1}; B, 1700 μmol m^{-2} s^{-1}; C, 1100 μmol m^{-2} s^{-1}; D, 600 μmol m^{-2} s^{-1}; E, 300 μmol m^{-2} s^{-1}; F, 100 μmol m^{-2} s^{-1}. Subsequently, leaflets were transferred to low light (15 μmol m^{-2} s^{-1}) and the dependence of the pigment content on illumination time was analyzed. V, Vio; A, Ant; Z, Zea. Each value represents the mean of two measurements. SE was in the range of 1 to 7%.

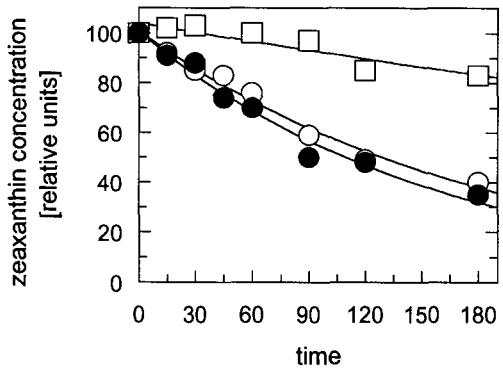

Figure 6. Zea epoxidation in IML plants after prolonged illumination. Leaflets were illuminated at a high PFD (2500 μE m^{-2} s^{-1}) either at 2°C for 60 min (□) or at 20°C for either 50 min (●) or 15 min (○), yielding relative Zea concentrations of 85, 89, and 90%, respectively. Changes in Zea concentrations were determined after transfer of the leaflets to low light (15 μE m^{-2} s^{-1}) at room temperature. For better comparison of the kinetics of Zea reconversion, the Zea concentrations after high-light treatment were normalized to 100 for all samples. The data represent a single experiment.

results:

1. At PFDs insufficient to saturate photosynthesis (and thus to build up the maximum ΔpH), the xanthophyll cycle seems to operate in both directions (de-epoxidation and epoxidation) under illumination.
2. At higher (saturating) PFDs, epoxidation reactions during illumination seem unlikely, if a concerted down-regulation of de-epoxidase and epoxidase activity is excluded.
3. The rate of de-epoxidation is very similar in CL plants and IML plants.
4. In comparison to CL plants, the rate of epoxidation is strongly reduced in IML plants.
5. The epoxidation rate depends (a) on the PFD and the time of illumination and (b) on the temperature during illumination. It is independent of the steady-state level of Zea.
6. The steady-state level of the xanthophyll-cycle pigments (and thus the Vio availability) is not determined by the epoxidation rate.
7. The limited availability of Vio to the de-epoxidase in CL plants seems to depend on the presence of CAB proteins.

The results point to an important (direct or indirect) function of CAB proteins in the xanthophyll cycle. Not only is the epoxidation rate influenced by CAB proteins but the unavailability of parts of Vio to the de-epoxidase (in CL plants) also seems to be determined by CAB proteins. Both may indicate the binding of xanthophyll-cycle pigments by CAB proteins and would be in agreement with other studies (Peter and Thornber, 1991; Bassi et al., 1993; Ruban et al., 1994) that showed both Vio and Zea to be associated with CAB proteins of PSII. As pointed out above (cf. Table I), however, the binding stoichiometries of either Vio or Zea to CAB proteins found in vitro are insufficient to justify the assumption that all Vio is associated with antenna proteins, implying either a loss of xanthophylls during preparation or a non-protein-associated pool of Vio. Although a possible function of ELIPs in xanthophyll binding (Król et al., 1995) has to be further examined, it cannot be excluded that the de-epoxidase enzyme can use free Vio/Ant as substrate.

It has further been shown that at least some of the Zea formed is bound to CAB proteins (Thayer and Björkman, 1992; Ruban et al., 1994). To explain the increased availability of Vio to the de-epoxidase in IML plants, it is then conceivable that (in CL plants) the de-epoxidation could be limited by an interaction of the de-epoxidase enzyme with CAB proteins. Possibly, the binding capacity of CAB proteins for Zea/Ant is involved in this limitation. If all Vio in dark-adapted leaves is bound to CAB proteins, this would imply that the binding capacities of CAB proteins for Vio and Zea are different. Either the altered polarity of the xanthophylls due to the de-epoxidation or possibly the existence of different binding sites for Vio and Zea could then explain these changes.

Alternatively, the absence of grana in IML plants, and thus structural differences between the thylakoid membranes of the two types of plants, could be responsible for the increased conversion of Vio (in agreement with earlier speculations by Siefermann and Yamamoto, 1974). A strong argument for the former possibility, however, is the constant level of Ant in the steady state at higher PFDs (see Fig. 1). This indicates that there is a down-regulated de-epoxidation in the steady state rather than a restriction of the de-epoxidation by a limited Vio availability. The latter possibility would imply that the Ant de-epoxidation should proceed under prolonged illumination, as given to IML plants at high PFDs (Fig. 2, A and B). This was definitely not the case in CL plants, so control of the xanthophyll-pigment conversion by binding of Zea/Ant to CAB proteins is much more likely.

The deceleration of the re-epoxidation with increasing PFD, under prolonged illumination and under illumination at lower temperatures in both types of plants, may best be explained by a decreased availability of Zea/Ant to the epoxidase enzyme. This may possibly also be influenced by prolonged ATPase activity after light treatment, according to Gilmore and Björkman (1994a, 1994b). An altered availability of Zea/Ant to the epoxidase might also simply originate from changes in the binding site of Zea under prolonged or high-light illumination. It is conceivable that more Zea is bound to inner parts of the antenna (i.e. closer to the reaction center). It can be speculated that Zea might be involved in thermal energy dissipation at these binding sites, possibly also independent of a ΔpH. The efficiency of this process should be increased at sites that are closer to the reaction center. This could then explain an increase in a possibly pH-independent thermal energy dissipation that, on the other hand, might be paralleled by a decreased availability of Zea to the epoxidase enzyme. In further studies with IML plants (P. Jahns and B. Miehe, unpublished data), we found a very close kinetical correlation between the slowly relaxing nonphotochemical quenching and the epoxidation of Zea. This was also found for CL plants, but a close correlation was only obvious after long

periods of illumination at high PFDs. Thus, it seems very reasonable to propose a general function of Zea in the "inactivation" of PSII related to thermal energy dissipation, which might be based on two mechanisms, one pH dependent and one pH independent, which could differ mainly in the binding sites of Zea.

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