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The xanthophyll cycle of higher plants: influence of antenna size and membrane organization

Andreas Färber, Peter Jahns *

Heinrich-Heine-Universität Düsseldorf, Institut für Biochemie der Pflanzen, Universitätsstrasse 1, D-40225 Düsseldorf, Germany

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Abstract

The development of the photosynthetic apparatus of intermittent light grown pea plants under continuous illumination has been investigated. We determined the formation of antenna proteins and the synthesis of pigments at different stages of greening and compared the data with the changes in the xanthophyll cycle reactions. The limited convertibility of violaxanthin in the de-epoxidation reactions of the cycle was found to be closely related to the presence of antenna proteins and could be attributed to direct (pigment binding) and indirect (grana formation) functions of antenna proteins. The reduced epoxidation rate in intermittent light plants was found to be accelerated with increasing amounts of antenna proteins. However, the changes in the epoxidation rates were not consistent with the assignment of the epoxidase activity to LHC II, the major light harvesting complex protein of photosystem II. This interpretation was further supported by an unchanged epoxidase activity in – also LHC II depleted – bundle sheath cells of the C_4 plant *Sorghum bicolor* and stroma fractions of isolated spinach thylakoids. We assume that the basic function of antenna proteins in the xanthophyll cycle of higher plants is mainly related to the binding of the substrate and/or to interactions with the de-epoxidase/epoxidase. By that antenna proteins seem to be responsible for the limited violaxanthin convertibility as well as they are required for highest epoxidation rates. © 1998 Elsevier Science B.V.

Keywords: Carotenoid; Chlorophyll *a*/*b* binding protein; Epoxidase; Membrane stacking; Plastid development; Xanthophyll cycle

1. Introduction

The reversible conversion of the carotenoid violaxanthin (Viol) via antheraxanthin (Anth) to zeaxanthin (Zeax) has been termed xanthophyll cycle (for reviews see [1,2]). The role of this cycle is predominantly discussed as a function of Zeax (and possibly of Anth) in thermal dissipation of excess light energy. Recent work has shown that Zeax (and to a lower degree Anth) – in contrast to Viol – is capable to accept energy from an excited singlet Chl [3,4]. This property enables Zeax and Anth to function as a direct quencher of excitation energy in the antenna systems of both photosystems. It is assumed that via this mechanism the xanthophyll cycle may play a key role in photoprotective processes (for recent reviews see [5–7]). Alternatively, an indirect function of Zeax in energy dissipation has been proposed by Horton and co-workers (reviewed in [6]). According to their

Abbreviations: Anth, antheraxanthin; Chl, chlorophyll; ETC, electron transport chain; IML, intermittent light; LHC I, light-harvesting complex of PSI; LHC II, light-harvesting complex of PSII; PFD, photon flux density; Viol, violaxanthin; Zeax, zeaxanthin

* Corresponding author. Fax: +49 (211) 811 3706; E-mail: pjahns@uni-duesseldorf.de

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model, energy dissipation is based on the pH-regulated aggregation of PSII antenna proteins. Zeax (and Anth) formation in these protein complexes is thought to support or amplify this aggregation (see [6]).

The de-epoxidation reactions of the xanthophyll cycle (i.e. the conversion from Viol to Zeax) dominate in high light and are controlled by the lumen pH. The de-epoxidase has been identified as a lumenal protein with an apparent molecular mass of 43 kDa [8–10]. The enzyme requires ascorbate as a cofactor [11] and shows a pH optimum of about 5 [12,13]. Activation of the de-epoxidase by low pH has been suggested to be accompanied by binding to the thylakoid membrane [14–16].

In contrast, the epoxidation reaction (i.e. the reconversion of Zeax to Viol) is regulated by the stromal pH. It shows maximum activity at pH 7.5 [17] and seems to operate mainly under low light or in the dark [1,18]. The epoxidase requires molecular oxygen and NAD(P)H as cofactors [17,19]. Recent work brought evidence that FAD is a further important cofactor for the epoxidation reactions [20,21]. The epoxidase has not been identified up to now. It has been speculated that either Lhcb1/2 (the major Chl *a*/*b* binding proteins of PSII) [22] or Lhcb5 (CP26) [23] may serve as epoxidase. Recently, however, an enzyme of the abscisic acid biosynthesis pathway was found to catalyse the epoxidation of Zeax to Viol [24], but it remained unclear whether this enzyme is also involved in the xanthophyll cycle.

A crucial function of LHC II for the epoxidation reaction was confirmed in studies with intermittent light grown plants (IML plants) and Chl *b* deficient mutants. The reduction of the Chl *a*/*b* antenna in these plants was paralleled by a strong reduction of the epoxidation rates in comparison to normally developed plants [18,25]. However, these results cannot be taken as direct evidence for an epoxidase activity of LHC II itself. Since the xanthophyll cycle pigments seem to be exclusively associated with antenna proteins of both photosystems [26–28], the reduced epoxidation rate in antenna depleted plants may alternatively be related to the missing binding of Zeax to proteins in IML plants [18,25]. The assumption of a large non-protein bound pool of xanthophylls in IML plants was indeed supported by the separation of pigment-binding proteins in CL and IML plants under non-denaturing conditions [29].

A possible function of Lhcb5 as epoxidase was not supported by the studies with antenna depleted plants [18,25], since the Lhcb5 content (per PSII) was unchanged in these plants in contrast to the drastically reduced epoxidation rate. Härtel et al. [25] concluded from their experiments with IML plants and Chl *b* deficient mutants that the reduction of the epoxidation activity in these plants rather corresponds to the reduction of the content in Lhcb4.

Furthermore, the reduced amount of antenna proteins in IML plants is accompanied by the absence of grana formation [30]. Thus an indirect effect of LHC deficiency due to the abolished membrane heterogeneity should also be taken into account.

In the present study, the functions of antenna proteins in the xanthophyll cycle were studied in more detail. The development of antenna proteins in IML plants was induced by continuous illumination of the plants. Changes in the xanthophyll cycle were related to different antenna sizes of both photosystems. The assignment of the epoxidase activity to antenna proteins is discussed.

2. Materials and methods

2.1. Plant material

Pea plants (*Pisum sativum* L. cv. ''Kleine Rheinländerin'') were grown in a climate chamber either under continuous light (14 h light, 10 h darkness; CL plants) or under intermittent light (2 min light, 118 min darkness; IML plants). Under both conditions the photon flux density (PFD) was 100 μmol photons m^{-2} s^{-1}. The development of the photosynthetic apparatus of IML plants under CL conditions was examined at different stages of greening, as indicated. 12 days old IML plants and 12–14 days old CL plants were used for all experiments. Unstacked thylakoids were prepared by EDTA washing and resuspension in low salt media [31]. Re-stacking of thylakoids was obtained by addition of 5 mM $MgCl_2$.

Spinach (*Spinacia oleracea* L.) was cultivated in a greenhouse at 18–20°C and a PFD of up to 300 μmol m^{-2} s^{-1}. Leaves of about 6 weeks old plants were used for the isolation of thylakoids.

Thylakoids were prepared using the method described by Jensen and Bassham [32] with the modifications in [33].

Seeds of Sorghum (*Sorghum bicolor* cv. Tx430; Pioneer Hi-Bred, Plainview, TX, USA) were grown under greenhouse conditions with additional illumination ($300\ \mu mol\ m^{-2}\ s^{-1}$, 14 h light, 10 h darkness). 10–12 days old plants were used for isolation of bundle sheath strands and mesophyll cells. Only the upper two-thirds of the second leaf were used for isolation of bundle sheath and mesophyll cells.

2.2. Fractionation of grana and stroma regions of thylakoid membranes

Grana and stroma fractions from spinach thylakoids were obtained by following a protocol of S. Andrée (Münster, personal communication). Thylakoids ($0.5\ mg\ ml^{-1}$ Chl) were suspended in 7 mM $MgCl_2$, 15 mM NaCl, 10 mM MES/NaOH pH 6.5 and passed twice through a Yeda press at a nitrogen gas pressure of 8 MPa. The broken membranes were layered on a sucrose gradient (10–50%) and centrifuged for 2 h at $120\,000 \times g$ (SW-28, L8-70M ultracentrifuge, Beckman, Munich, Germany). The resulting two bands were collected, washed once in the same medium and pelleted for 30 min at $170\,000 \times g$.

2.3. In vitro de-epoxidation and epoxidation

In vitro de-epoxidation was performed by illuminating thylakoids (Chl concentrations of 50 or $10\ \mu g\ ml^{-1}$ for CL or IML thylakoids, respectively) at 20°C and a PFD of $1\ mmol\ m^{-2}\ s^{-1}$ in a medium containing 0.4 M sorbitol, 50 mM Hepes/NaOH pH 7.5 and 20 mM ascorbate. Methyl viologen (100 μM) served as electron acceptor.

In vitro epoxidation was carried out in the dark with thylakoids (or thylakoid membrane fragments) isolated from pre-illuminated leaves (15 min, PFD $1\ mmol\ m^{-2}\ s^{-1}$, 20°C) in a medium containing 0.4 M sorbitol, 50 mM Hepes/NaOH pH 7.5, $0.3\ mg\ ml^{-1}$ BSA, 5 mM NH_4Cl, 0.5 mM NADH, and 1 μM FAD. Thylakoids were added equivalent to $20\ \mu g$ Chl ml^{-1} (CL thylakoids) or $5\ \mu g$ Chl ml^{-1} (IML thylakoids). The reaction was stopped by rapid cooling of the samples in liquid nitrogen. After thawing, thylakoids were pelleted by 2 min centrifugation at $1000 \times g$ in a Sigma 112 centrifuge (Sigma, Osterode, Germany). Pigments were extracted by resuspension in acetone.

2.4. In vivo epoxidation with Sorghum leaves

Epoxidation experiments with *Sorghum bicolor* were carried out under in vivo conditions. Intact leaves were illuminated for 20 min at a PFD of $1\ mmol\ m^{-2}\ s^{-1}$ yielding a de-epoxidation state (DEPS = (Zeax + 0.5 Anth)/(Viol + Anth + Zeax) $\times 100$) of about 50%. Subsequent epoxidation was induced by transfer of the leaves to low light (PFD of $20\ \mu mol\ m^{-2}\ s^{-1}$). The time course of epoxidation in bundle sheath and mesophyll cells was determined by preparative separation of both cell types after different times of low light illumination.

2.5. Separation of Sorghum bundle sheath and mesophyll cells

Bundle sheath strands from *Sorghum bicolor* were isolated according to [34] with slight modifications. Removal of mesophyll cells from the bundle sheath strands were performed either by enzymatic digestion [34] or simply by mechanical disruption of the strands by extensive grinding (4×1 min) in a Waring blendor. The latter method ensured a fast separation of bundle sheath and mesophyll cells, in order to overcome any problems of continuing Zeax epoxidation during the 30 min of enzymatic incubation at 30°C. This was thought to be a problem since the pH of the digestion medium had to be adjusted to about 7.5 to avoid any pH-induced de-epoxidation of Viol during the digest. Although a pH of 7.5 is known to be the optimum pH for epoxidation, however, no continuing epoxidation was found to occur during 30 min of enzymatic digestion.

Contamination with mesophyll chloroplasts and intactness of bundle sheath strands were assessed in the light microscope for both methods. Mesophyll contamination after enzymatic digestion of bundle sheath strands was not detectable and estimated below 10% after mechanical disruption. Comparative studies revealed no differences in the epoxidation rates of bundle sheath cells after preparation by either method.

2.6. Pigment analysis

Pigment separation was performed by reversed phase HPLC using a Merck LiChrospher 100 RP-18 column with 5 μm particle size (Merck, Darmstadt, Germany). Solvent A was composed of acetonitrile, methanol, and 0.1 M Tris/NaOH pH 8 in a ratio of 87:10:3, solvent B was a 4:1 mixture of methanol and hexane. The gradient from solvent A to solvent B was run from 9 to 12.5 min at a flow rate of 2 ml min^{-1}. Eluted pigments were monitored by their absorption at 440 nm. Conversion factors which allow calculation of pigment concentration from the integrated peak area were determined by calibration with pure pigments. Neoxanthin and Viol were isolated by thin-layer chromatography, Chl *a* and *b* were purchased from Serva (Heidelberg, Germany), lutein from Sigma (Deisenhofen, Germany), β-carotene from Fluka (Neu-Ulm, Germany) and Zeax was a kind gift from Hoffmann-LaRoche (Basel, Switzerland). The factors were 2772 (neoxanthin), 3211 (Viol), 2192 (lutein), 2707 (Zeax), 1609 (Chl *a*), 1298 (Chl *b*) and 2001 (β-carotene) expressed as peak area per pmol of the respective pigment. Anth (not commercially available) was estimated with the conversion factor for lutein according to [46]. This estimation was found to be reasonable since no significant changes in the total amount of xanthophyll cycle pigments (Viol + Anth + Zeax) were found with samples containing low and high amounts of Anth.

2.7. Western blot analysis

Western blot analysis was performed as previously [35], except that binding of antibodies was additionally visualized by luminescence (ECL kit, Amersham, Little Chalfont, UK). The following antibodies were used: Anti-LHC II, against the major 27 kDa protein of LHC II trimers, Lhcb1 (gift from Dr. S. Berg, Minnesota, USA), anti-33, raised against the 33 kDa subunit of the water splitting enzyme of PSII (gift from Dr. B. Andersson, Stockholm, Sweden), and anti-psaC raised against subunit C of PSI (gift from Dr. H. Strotmann, Düsseldorf, Germany). Staining was densitometrically quantified as described previously [35]. To avoid any systematical errors due to the blotting and staining procedure, antibody mixtures (anti-LHC II + anti-33 and anti-psaC + anti-33) were used in all cases.

2.8. Fluorescence measurements

Fluorescence measurements were performed in collaboration with Dr. H.-W. Trissl (University of Osnabrück, Germany) using a self-built apparatus as described in [42]. The Chl *a* antenna size of PSII was derived from quantitative fluorescence induction curves according to the theory described in [41,42]. Thylakoids equivalent to 4 μM Chl were resuspended in a medium containing 50 mM MOPS pH 7.6, 5 mM $MgCl_2$ and 40 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). Samples were excited with a laser diode at 675.5 nm where the molar absorption coefficient of Chl *a* is known [42]. The overall PSII antenna size (Chl *a* + Chl *b*) was subsequently calculated using the Chl *a*/*b* ratio determined by HPLC analysis.

3. Results and discussion

3.1. Influence of thylakoid stacking on the xanthophyll cycle reactions

Between CL and IML plants marked differences in both reactions of the xanthophyll cycle have been observed: In IML plants, the limitation of the conversion of Viol in the de-epoxidation reactions was absent and the epoxidation rate was considerably slowed down in comparison to CL plants [18]. Since IML plants have been shown to contain only Lhcb5 (CP26) out of all Chl *a*/*b* binding proteins in significant amounts [25,36], these differences have been attributed to the lack of most of the antenna proteins in IML plants [18]. However, the possible function of membrane stacking, which is missing in IML plants [30], remained unclear. In order to distinguish between an indirect stacking phenomenon and a direct function of antenna proteins, we investigated the xanthophyll cycle reactions in stacked and unstacked CL thylakoids in comparison with IML thylakoids (Fig. 1). The maximum amount of formed Zeax and the conversion rate of the de-epoxidation reaction were found to be increased upon unstacking of CL thylakoids (Fig. 1(A)). However, this increase was

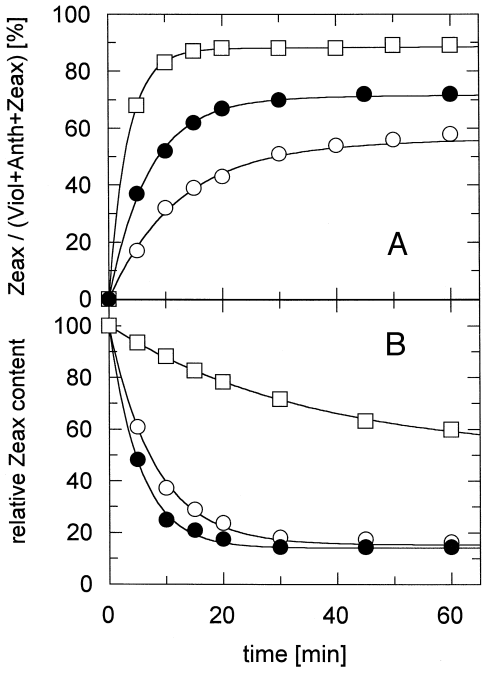

Fig. 1. The influence of membrane stacking on the de-epoxidation (A) and epoxidation (B) reactions of the xanthophyll cycle: ●, unstacked CL thylakoids; ○, stacked CL thylakoids; □, IML thylakoids. For de-epoxidation (A), thylakoids were illuminated at a PFD of 1 mmol m^{-2} s^{-1}. Epoxidation (B) was followed in a separate experiment with thylakoids prepared from preilluminated leaves (15 min, 1 mmol m^{-2} s^{-1}). For a better comparison, thylakoids from the same preparation ± $MgCl_2$ were used for CL thylakoids. For each reaction, one typical experiment out of three is shown. In IML thylakoids, no significant effect of $MgCl_2$ was found for both reactions (data not shown).

less pronounced in comparison with IML thylakoids. Thus, we conclude that the limited conversion of Viol in CL plants can partly be explained by membrane heterogeneity. Additionally, pigment binding to antenna proteins seems to be responsible for this phenomenon.

The influence of membrane stacking on the epoxidation reaction is illustrated in Fig. 1(B). Obviously, unstacking of CL thylakoids induced an acceleration of the epoxidation rate. Hence, the slow epoxidation kinetics in IML plants cannot be explained by the missing grana structure but must be related to a more direct function of antenna proteins. It is tempting to assume that antenna proteins serve as epoxidase [22,23]. However, the reduced epoxidation rate might also be sufficiently explained by the assumption that binding of the pigments to antenna proteins is important for the epoxidation reaction. A differentiation between these two possibilities might be expected from experiments in which the antenna size in IML thylakoids is stepwise increased under continuous illumination. We investigated the development of the photosynthetic apparatus and the xanthophyll cycle reactions under these conditions.

3.2. Development of IML plants under continuous illumination

Fig. 2 shows the development of LHC II and the PSII/PSI ratio in IML thylakoids under continuous illumination. All data were derived from quantitative Western blot analyses. LHC II incorporation (due to Chl *b* synthesis) started immediately in the light, increasing the amount of LHC II per PSII from about 5% in IML plants to about 75% compared to CL plants after 28 h of illumination (14 h light, 10 h dark, 14 h light, 10 h dark). In parallel, the PSII/PSI ratio decreased gradually from 2.5 in IML plants [37,38] to about 1 in CL plants (see also legend to Fig. 2). The formation of antenna proteins was further accompanied by the successive development of grana stacks, visible by electron microscopy (Färber, Kowallik and Jahns, unpublished).

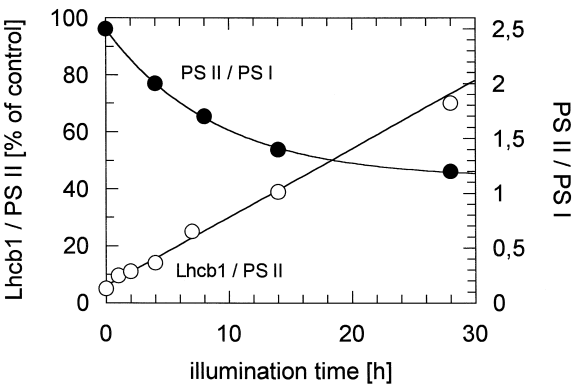

Fig. 2. LHC II content (○) and PSII/PSI ratio (●) in developing IML-plants. The data were derived from densitometric scans of Western blots. Each data point represents the mean of 2–4 independent experiments. SD was below 10% (LHC II) and up to 30% (PSII/PSI), respectively. The data are normalized for both ratios to the values obtained with CL thylakoids. The LHC II/PSII ratio in CL thylakoids was set to 100%, the PSII/PSI ratio in CL thylakoids was assumed to be 1.

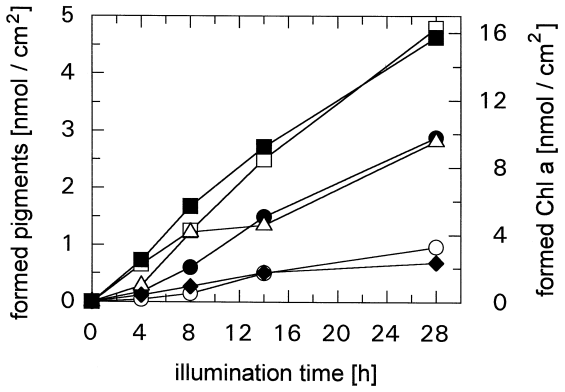

Fig. 3. Pigment formation in IML-plants under continuous illumination. Data are normalized to leaf area (cm^{-2}). Note that the Chl *a* concentration is given at a different ordinate scale. For a better comparison, only the amount of newly synthesized pigments are shown: ○, neoxanthin (initial concentration: 0.19 nmol cm^{-2}); ●, lutein (2.68); □, Chl *b* (0.09); ■, Chl *a* (3.76); △, β-carotene (1.37); ◆, sum of xanthophyll cycle pigments (Viol + Anth + Zeax) (1.64). Mean values of 3 experiments are shown. SD were in the range of 5–20%.

In studies with etioplasts, a simultaneous formation of all antenna proteins of PSII and PSI has been described [39,40]. Therefore the values determined for the LHC II development may be used as a measure for the overall antenna formation.

The pigment synthesis under the same conditions is shown more detailed in Fig. 3. During the first 4 h only Chl (*a* and *b*) was synthesized in significant amounts, whereas the carotenoid content remained nearly constant. Under prolonged illumination, however, also the carotenoid synthesis started. Interestingly, the synthesis of the xanthophyll cycle pigments was less pronounced in comparison to the other carotenoids. With the exception of the xanthophyll cycle pigments, the increase of the antenna associated pigments (lutein, neoxanthin and Chl *b*) roughly correlated with their stoichiometries present in isolated LHC II. Obviously, the incorporation of newly synthesized antenna proteins required parallel synthesis of all carotenoids which are bound to these proteins. Particularly for lutein, it seems to be excluded that pigments, which are already present in IML membranes in very high amounts, can be used for the pigmentation of newly synthesized LHC proteins. Thus the incorporation of lutein and possibly also neoxanthin into antenna proteins seems to be a more critical step than binding of the xanthophyll cycle pigments. This might indicate that the xanthophyll cycle pigments are more loosely bound to LHC proteins than the other carotenoids.

In a separate experiment we determined the absolute antenna size of PSII from analyses of fluorescence induction curves [41,42]. About 40–45 Chl ($a + b$) per PSII were found in IML plants [43] and increased up to 167 in 14 h illuminated IML plants (see Table 1). The antenna size of about 45 Chl ($a + b$) is in good agreement with the antenna size of PSII core (D1/D2/CP43/CP47) and the absence of nearly all Chl a/b binding antenna proteins in IML plants. Assuming an antenna size of about 200 Chl ($a + b$) for PSI in CL plants and a PSI (core) antenna size in IML plants of about 90 Chl ($a + b$) (cf. [44]), we estimated from our pigment and protein data the Chl content per mol PSII, per mol PSI and per mol electron transport chain (ETC) at the respective illumination times (Table 1). The increase in LHC II determined by Western blot analysis was in agreement with the increase of the PSII antenna size derived from fluorescence induction curves. The calculated values are important for the differentiated assessment of the epoxidation rates at the different stages of greening.

Table 1
Changes in the Chl a/b ratio and antenna sizes in developing plastids. IML plants were illuminated for up to 28 h. The Chl a/b ratios were calculated from HPLC pigment analyses. The Chl *a* antenna size of PSII was determined from the kinetics of fluorescence induction curves (cf. [41,42]). From these values the overall PSII antenna sizes were calculated. PSI antenna sizes were estimated with about 90 Chl *a* in IML plants and 200 Chl $a + b$ in CL plants. Intermediate values were calculated under the assumption that the formation of LHC I is similar to the values determined for LHC II (cf. Fig. 2)

Illumination time (h)	Chl a/b ratio	Chl content ($a + b$) per		
		PSII[a]	PS I[b]	ETC
0	45 ± 10	42	90	195
4	11 ± 0.8	85	108	278
8	7.1 ± 0.2	128	122	340
14	5.2 ± 0.3	167	137	371
28	4.1 ± 0.1	235	173	455
CL	3.6 ± 0.1	300	200	500

[a] Derived from fluorescence induction curves (Jahns and Trissl, unpublished).
[b] Estimated.

Table 2
Changes in the xanthophyll cycle pool size in developing plastids. IML plants were illuminated for up to 28 h. All data were derived from HPLC pigment analyses. Mean values ± SD of 2 to 3 independent experiments are shown. The calculations of the amounts of PSII, LHC II and ETC are based on the data from Fig. 2 and Table 1

Illumination time (h)	Xanthophyll cycle pool (Viol + Anth + Zeax) in mmol per				
	mmol PSII	mmol LHC II	mmol ETC	mol Chl	m^{-2} leaf area
0	32.7 ± 1.3	54.5 ± 2.2	81.7 ± 3.2	419 ± 16	16.4 ± 1.8
4	40.4 ± 1.2	24.0 ± 0.7	80.9 ± 2.4	291 ± 8.7	17.6 ± 2.1
8	45.0 ± 0.8	15.0 ± 0.3	76.5 ± 1.4	225 ± 4.0	19.1 ± 1.5
14	40.8 ± 1.1	8.7 ± 0.3	57.1 ± 1.6	154 ± 4.2	21.5 ± 2.6
28	40.6 ± 0.7	4.8 ± 0.1	48.7 ± 0.8	107 ± 1.7	23.3 ± 2.3
CL	37.3 ± 0.7	3.1 ± 0.1	37.3 ± 0.7	75 ± 1.4	22.1 ± 3.9

3.3. Changes in the pool size of xanthophyll cycle pigments

The changes of the overall pool size of the xanthophyll cycle pigments (Viol + Anth + Zeax) are summarized in Table 2. The data are expressed as the ratio of (Viol + Anth + Zeax) to either PSII, LHC II, ETC, Chl or the leaf area, respectively, according to the values given in Table 1. In relation to LHC II, ETC and Chl, the pool size was found to decrease step by step under illumination of IML plants. Normalized to the PSII content of the thylakoids, however, the pool size increased during the first eight hours of illumination and then declined. Since the pool size per ETC and leaf area remains constant in this period, this can be understood as a decrease in the number of PSII centers during the first hours of illumination, in order to adjust the PSII/PSI ratio to the increasing PSII antenna size. The subsequent decline of the pool size per PSII might then indicate that either the pool size itself was reduced or that an additional synthesis of new electron transport chains changes the xanthophyll to PSII ratio. The latter possibility is supported by a large increase of the total Chl content per leaf area within this period (Fig. 3). Thus, in the first hours of illumination, most likely a complementation of present photosystems with newly synthesized antenna proteins dominates the greening process of IML plants. This seems to be accompanied by the degradation of some PSII. At later stages of greening an increase of electron transport chains per leaf area due to synthesis of new protein complexes can be assumed as dominating process.

3.4. Changes in the de-epoxidation reactions

We determined the conversion of the xanthophyll cycle pigments in the de-epoxidation reactions after 15 min illumination of intact leaves at a PFD of

Table 3
Changes of the Viol convertibility in developing plastids. IML plants were illuminated for up to 28 h. De-epoxidation was induced by illumination of isolated thylakoids for 15 min at a PFD of 1 mmol m^{-2} s^{-1}. The relative amounts of the xanthophyll cycle pigments were derived from HPLC pigment analyses. The de-epoxidation state (DEPS) was calculated as (Zeax + 0.5 Anth)/(Viol + Anth + Zeax) × 100. Mean values of 3 independent experiments are shown. SD was below 5% in each case

Illumination time (h)	Pigment content per (Viol + Anth + Zeax) [%]			DEPS
	Viol	Anth	Zeax	[%]
0	8.9	9.4	81.7	86.4
4	9.6	15.6	74.8	82.6
8	11.1	13.9	75.0	82.0
14	15.5	19.0	65.5	75.0
28	26.3	23.6	50.1	61.9
CL	41.4	28.2	30.4	44.5

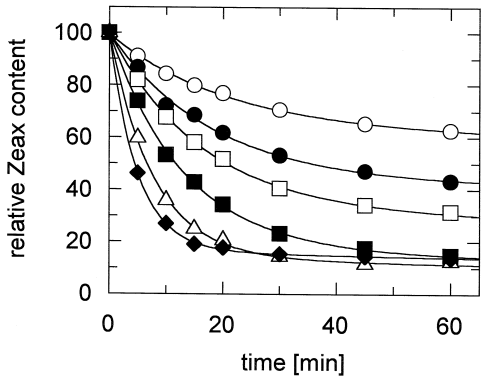

Fig. 4. Epoxidation kinetics in developing IML-plants. Symbols refer to different times of continuous illumination of IML plants: ○, 0 h; ●, 4 h; □, 8 h; ■, 14 h; △, 28 h; ◆, CL plants. All measurements were performed with isolated unstacked thylakoids (cf. Fig. 1). Each data point represents the mean of 2–3 experiments. SD were below 7% in each case.

$1\,mmol\,m^{-2}\,s^{-1}$ (Table 3). In agreement with earlier results ([18,25], cf. Fig. 1(A)), about 90% of Viol was converted to Anth and Zeax in IML plants compared to about 60% in CL plants. Under continuous illumination of IML plants the degree of convertible Viol decreased gradually, reaching 73.7% after 28 h of illumination. In parallel, the amount of Anth increased, after 28 h of illumination with 23.6% near to the value obtained with CL plants (28.2%). Thus the increase of the non-convertible portion of Viol at the different stages of greening correlated with the increase of antenna proteins. This corroborates the earlier assumption that the limited conversion of Viol originates from pigment binding to LHC proteins [18,25] and the presence of grana stacks (Fig. 1(A)). Additionally, also the increase of the relative Anth content seems to be related to the presence of antenna proteins.

3.5. Changes in the epoxidation reactions

Fig. 4 shows the time course of Zeax epoxidation in vitro obtained with thylakoid preparations from developing IML plants. Unstacked thylakoids were used in this experiment in order to overcome any changes of the epoxidation rate due to membrane stacking phenomena (cf. Fig. 1). The data are normalized to the Zeax content after light-induced deepoxidation (cf. Table 3). Kinetic analyses of the curves revealed a clear biphasic decay under all conditions (not shown). The fast kinetics of CL and IML plants (see Fig. 4) were consistent with the kinetics found in in vivo experiments [18,25]. Also the slow phases of epoxidation have been detected in intact leaves. In IML plants, however, the portion of this slower phase, was much less pronounced under in vivo conditions. Therefore, this large portion of slowly epoxidized Zeax might be an in vitro artifact. On the other hand, the extent of the slow phase decreased with increasing illumination time of IML plants (see Fig. 4) and thus seems to be diminished in the presence of antenna proteins. It is conceivable that a stromal component, which might be lost more easily with reduced amounts of antenna proteins, is involved in this phenomenon. Apart from this point, it is clear from our data (Fig. 4) that the relative epoxidation rate successively increases with an increasing antenna size. In principle, this result can be understood in two different ways:

1. The epoxidase in IML plants is only present in drastically reduced amounts and is then formed successively concomitant to LHC protein incorporation. This interpretation would favour the assignment of the epoxidase activity to LHC proteins. It is worth to mention in this context that the reduced epoxidation rate has also been found in the barley Chl *b* mutant *chlorina* 3613 grown under continuous light [25]. Thus it can be excluded that the reduced epoxidase activity in IML plants is due to a secondary effect of the limited illumination time during growth.
2. The epoxidase is present in IML plants in normal amounts but cannot metabolize Zeax in a proper way. There are several possibilities to explain such a disturbed enzyme/substrate interaction. For example, it might be possible either that Zeax has to be bound to antenna proteins for highest epoxidation rates or that the epoxidase is a stroma soluble enzyme which cannot bind to the membrane in IML plants for some reason (e.g. by an altered surface charge of the membrane). The successive incorporation of antenna proteins into IML thylakoid membranes would then increase the epoxidase activity in either case.

3.6. Evidence against the assignment of the epoxidase activity to LHC II

We applied a more detailed analysis of the data in order to distinguish between the two principle interpretations from above. In contrast to the presentation of Fig. 4, we calculated under all conditions the absolute Zeax turnover (i.e. the initial rate of Zeax epoxidation) in relation to the amount of either PSII, LHC II and ETC using the estimations listed in Table 1 and the kinetic parameters. We also took into account the increased PSII/PSI ratio and the higher absolute Zeax content in IML plants, the latter dependent on the increased convertibility of Viol to Zeax and the larger xanthophyll cycle pool (cf. Table 2). The results are summarized in Table 4.

If the epoxidase activity is located in LHC II, one would expect a successive increase of the epoxidation rate under normalization of the initial epoxidation rate to the same PSII content of each sample, with a maximum rate for CL plants. On the other hand, the initial rate of epoxidation normalized to the same LHC II content should remain nearly constant. Indeed, we found that the initial rate of Zeax epoxidation per PSII increased with higher amounts of LHC II (Table 4). Normalized to LHC II, however, the rate decreased gradually in developing IML plants. This is in our hands an argument against the assignment of the epoxidase activity to LHC II.

However, this conclusion would only be justified when all other parameters that influence the epoxidation rate are unchanged in both types of plants. Particularly the higher substrate to enzyme ratio (Zeax/LHC II) in IML plants and the different organisation of the xanthophylls in both types of plants (due to the absence of the pigment binding antenna proteins in IML plants) might be critical. In order to overcome these problems, we additionally investigated the epoxidation in two other systems that differ considerably in the LHCII content: (1) bundle sheath and mesophyll chloroplasts from the C_4 plant *Sorghum bicolor* and (2) grana and stroma fractions of spinach thylakoids.

3.7. Epoxidation in bundle sheath cells

It has been shown earlier that bundle sheath chloroplasts isolated from *Sorghum bicolor* contain nearly no active PSII [45]. We estimated the content of several PSII proteins in bundle sheath chloroplasts (D1, LHC II and the extrinsic 23 kDa protein of the water oxidase) with about 20% (on Chl basis) in comparison with mesophyll chloroplasts (not illustrated). The reduced PSII content of bundle sheath chloroplasts was further confirmed by the high Chl *a*/*b* ratio of about 6–7. Assuming that LHC II (or another PSII antenna protein) is the Zeax epoxidase one should expect a reduced epoxidation rate in bundle sheath chloroplasts. In contrast to that, the epoxidation rate was found to be rather somewhat increased in comparison with mesophyll chloroplasts (Fig. 5). Since the stoichiometries of xanthophyll cycle pigments (28.3 and 27.9 mmol per mol Chl in bundle sheath and mesophyll cells, respectively) and the DEPS (49.3% and 50.4% in bundle sheath and mesophyll cells, respectively) were very similar in

Table 4
Changes in the initial amounts of Zeax and initial rates of Zeax epoxidation (Zeax → Anth) in developing plastids. IML plants were illuminated for up to 28 h. All data were derived from HPLC pigment analyses. The calculations of the amounts of PSII, LHC II and ETC are based on the data from Fig. 2 and Table 1. The initial amount of Zeax was calculated from the data of Tables 2 and 3. Initial epoxidation rates (Zeax → Anth) were derived from the initial amount of Zeax and the kinetic parameters of the fast phase of Zeax epoxidation derived from Fig. 4

Illumination time (h)	Initial amount of Zeax (mol per mol)			Initial epoxidation rate (mol Zeax min^{-1} per mol)]		
	PSII	LHC II	ETC	PSII	LHC II	ETC
0	26.7	44.5	66.7	0.48	0.80	1.20
4	30.2	18.0	60.5	0.95	0.57	1.91
8	33.8	11.3	57.4	1.41	0.47	2.39
14	26.7	5.7	37.4	1.72	0.37	2.41
28	20.3	2.4	24.4	2.29	0.27	2.75
CL	11.3	0.9	11.3	1.92	0.15	1.92

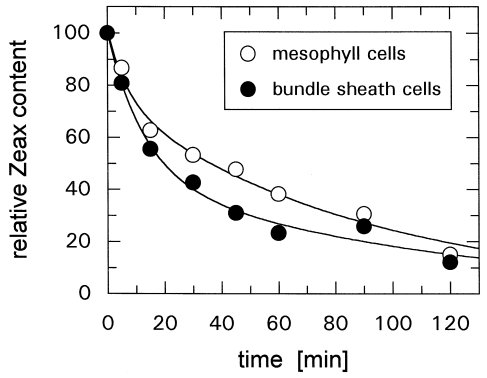

Fig. 5. Epoxidation in mesophyll (○) and bundle sheath (●) cells of *Sorghum bicolor*. Experiments were performed with intact leaves. Leaves were preilluminated for 20 min at a PFD of 1 mmol $m^{-2} s^{-1}$ to induce Z formation. Similar degrees of de-epoxidation were reached in mesophyll and bundle sheath cells. Epoxidation was performed in low light at a PFD of about 15 μmol $m^{-2} s^{-1}$. Mesophyll and bundle sheath cells were separated after different times of epoxidation. No detectable epoxidation of Z occurred during preparation of mesophyll and bundle sheath cells. For further details see Section 2. Each data point represents the mean of 3 independent experiments. SD were below 10% in each case.

both types of chloroplasts, the acceleration of the epoxidation reaction might be best explained by the missing thylakoid stacking in bundle sheath chloroplasts (cf. also Fig. 1). The slightly increased epoxidation rate in bundle sheath chloroplasts is thus a further argument against an epoxidase activity of LHC II (and other PSII antenna proteins).

The validity of the epoxidation rates obtained with C_4 plants might be doubtful, since the availability of the required cofactors (NADPH and O_2) could be limiting the epoxidation reaction in these in vivo experiments. Due to the physiology of bundle sheath and mesophyll chloroplasts, however, a limitation of the cofactors might rather be expected for bundle sheath chloroplasts. Thus, an increased (or similar) epoxidation rate in bundle sheath chloroplasts can be taken seriously.

3.8. Epoxidation in grana and stroma fractions

The heterogenous distribution of both photosystems between grana and stroma regions of the membrane has been used as further experimental approach to get informations about the assignment of the epoxidase activity to LHC II proteins. In agreement with recent data by Jansson et al. [47] the content of PSII proteins (including all PSII antenna proteins) in the stroma regions was found to be reduced to about 30–50% (on Chl basis) of the respective values in intact chloroplasts. Again, the reduced PSII content was confirmed by an increased Chl a/b ratio of about 5–6 in the stroma fractions (in comparison to about 3.2 in intact chloroplasts). The stoichiometries of Zeax (about 90 mmol per mol Chl in both fractions) as well as the DEPS (about 50% in both fractions) were very similar in the grana and stroma regions, which have been separated from spinach thylakoids after preillumination of intact leaves. It is obvious from Fig. 6 that the time course of epoxidation in the stroma fraction was similar in comparison to whole thylakoids – although the PSII and the LHC II content was drastically reduced in this fraction. Compared to the grana fraction, however, epoxidation in the stroma was considerably faster. The decreased epoxidation rate of the grana fraction can be explained by an impeded accessibility of the cofactors (NADPH and FAD) to the epoxidase due to a partial formation of inside-out vesicles in the grana region during the Yeda press treatment.

Thus, this experiment supports not only our suggestion that LHC II is not identical with the epoxidase of the xanthophyll cycle. It further shows that

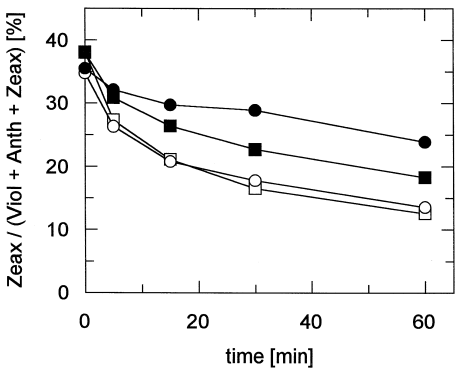

Fig. 6. Epoxidation in separated stroma (○) and grana (●) fractions of spinach thylakoids. Control rates in thylakoids are given for intact membranes (□) and fragmented but not separated membrane fragments (■). Zeax formation was induced by illumination of leaves for 20 min at a PFD of 1 mmol $m^{-2} s^{-1}$. Epoxidation was performed in low light at a PFD of about 15 μmol $m^{-2} s^{-1}$. One typical experiment out of three is shown.

the epoxidase is most likely equally distributed between stroma and grana regions of thylakoid membranes and that the epoxidation reaction does not require an intact membrane.

The unchanged epoxidation rates in bundle sheath chloroplasts (Fig. 5) and the stroma fraction of the membrane (Fig. 6) further indicate that also no other PSII protein serves (exclusively) as Zeax epoxidase. Therefore, both experiments argue also against the function of Lhcb5 as epoxidizing enzyme [23]. Moreover, also an exclusive function of the two other minor Chl *a*/*b* binding PSII proteins, Lhcb4 and Lhcb6, seems not very likely in the light of our experiments.

On the other hand, our data would be in agreement with the recently discovered epoxidase activity of the ABA2 protein which is involved in the abscisic acid biosynthesis pathway [24]. However, preliminary studies of the distribution of ABA2 in chloroplasts showed, that the protein is predominantly (if not exclusively) located in the chloroplast stroma and not associated with the membrane (Färber and Jahns, unpublished). Thus it remains to be proven that this enzyme is identical with the epoxidase of the xanthophyll cycle.

The increasing epoxidation rate in developing IML plants can then easily be explained by an improved substrate accessibility for the epoxidase due to pigment binding to the newly formed antenna proteins. Alternatively, an improved binding to the thylakoid membranes of the epoxidase might be responsible for the increased epoxidation rate. Both possibilities can also be related to each other if the presence of LHC proteins is required for the binding of the epoxidase.

In conclusion, our data support an important function of antenna proteins for the regulation of both reactions of the xanthophyll cycle. They restrict not only the accessibility of Viol in the de-epoxidation reactions but are also important for an efficient epoxidation. The formation of grana (which parallels the antenna protein synthesis) is only partly responsible for the limited Viol convertibility and has nearly no influence on the epoxidation reactions. An epoxidase activity of antenna proteins is not supported by our data. The function of antenna proteins in the xanthophyll cycle might thus be seen in the binding of substrate and/or in interactions with the de-epoxidase and epoxidase.

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