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# The *aba* Mutant of *Arabidopsis thaliana* (L.) Heynh. Has Reduced Chlorophyll Fluorescence Yields and Reduced Thylakoid Stacking[1]

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## ABSTRACT

**It has been shown that the *aba* mutant of *Arabidopsis thaliana* (L.) Heynh. is impaired in epoxy-carotenoid biosynthesis and accumulates the epoxy-carotenoid precursor, zeaxanthin (C.D. Rock, J.A.D. Zeevaart [1991] Proc Natl Acad Sci USA 88: 7496–7499). In addition to providing conclusive evidence for the indirect pathway of abscisic acid biosynthesis from epoxy-carotenoids, the *aba* mutation offers a powerful means to study the function of xanthophylls (oxygenated carotenoids) in photosynthesis. We measured in vivo the chlorophyll (Chl) fluorescence parameters $F_o$ (initial), $F_m$ (maximum), $F_v$ (variable = $F_m - F_o$), and $t_{½}$ (half-rise time of fluorescence induction) of wild-type (WT) and three allelic *aba* mutants. The mutant genotypes had significantly lower $F_o$ and $F_m$ values relative to those of WT. The $F_v/F_m$ ratio and $t_{½}$, which are parameters affected by photochemical efficiency, photosystem II (PSII), and plastoquinone pool sizes, were similar in the *aba* alleles and WT. Because the *aba* genotypes accumulate high levels of zeaxanthin, which is involved in nonphotochemical quenching of Chl fluorescence, we propose that the reduced fluorescence yields in the *aba* genotypes are a consequence of the accumulated zeaxanthin. Measurement of PSII oxygen evolution rates in isolated thylakoid membranes of WT and *aba-4* confirmed that quantum efficiency was not altered in *aba-4* but indicated that the mutant had reduced PSII activity in vitro. Electron microscopy revealed an abnormal chloroplast ultrastructure in the *aba* plants: the mutants had significantly fewer thylakoid lamellae per granum stack but significantly more grana per chloroplast, as well as more chloroplasts per cell than WT. Immunoblot analysis established that *aba-4* had normal levels of the Chl *a*/*b*-binding core polypeptide of PSII (CP29) and the PSII light-harvesting Chl *a*/*b*-binding complex. These results provide evidence for the role of zeaxanthin in nonphotochemical fluorescence quenching and suggest involvement of epoxy-carotenoids and/or zeaxanthin in thylakoid stacking and PSII activity.**

Carotenoids are essential components of the photosynthetic apparatus. β-Carotene functions in the reaction centers of PSI and PSII, and xanthophylls, such as zeaxanthin, violaxanthin, and neoxanthin, are associated with the light-harvesting complexes (25, 26). Yet, little is known of the functions of xanthophylls in plants. Proposed functions include photosynthetic light harvesting, $O_2$ radical scavenging, assembly of photosynthetic complexes, and radiationless energy dissipation (5–7, 14, 19, 25, 26). The xanthophyll cycle is a light-inducible, dark-reversible deepoxidation of violaxanthin to zeaxanthin involved in protection of the photosynthetic apparatus from photoinhibition. Zeaxanthin accumulates during exposure of leaves to high PPFDs and plays a role in increased Chl fluorescence quenching and protection of the photosynthetic apparatus (5–7). Photoinhibition can be due to effects at different sites in photosynthetic electron transport, but the major source is inactivation of PSII and degradation of the D1 reaction center protein (16, 18, 27).

It has been shown that the *aba* mutant of *Arabidopsis thaliana* (12) is deficient in epoxy-carotenoids and accumulates zeaxanthin (21) but has normal Chl levels. This finding provides conclusive evidence for the indirect pathway of ABA biosynthesis from epoxy-carotenoids (20, 21). Because the *aba* mutant greens normally and grows well when sprayed with ABA (12), we exploited the xanthophyll imbalances associated with the mutant alleles to study the role of xanthophylls in photosynthesis. The results presented here support the hypothesis that zeaxanthin is involved in nonphotochemical fluorescence quenching and suggest that violaxanthin, neoxanthin, and/or zeaxanthin are involved in thylakoid stacking and PSII function.

## MATERIALS AND METHODS

### Plant Material

Seeds of *Arabidopsis thaliana* (L.) Heynh., ecotype Landsberg *erecta* (WT; collection No. W20) and the mutant genotypes *aba-3* (W122), *aba-1* (W21), and *aba-4* (W123) were obtained from Dr. M. Koornneef, Agricultural University, Wageningen, The Netherlands. The *aba-1* genotype also carried the recessive markers *ttg* (transparent testa, glabra) and *yi* (yellow inflorescence). Mutant and WT[3] plants were grown together in the same trays as described in ref. 21 under 300 $\mu E \cdot m^{-2} \cdot s^{-1}$ fluorescent light. For fluorescence experiments,

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[3] Abbreviations: WT, wild type; LHCII, PSII light-harvesting Chl *a*/*b*-binding complex; $F_o$, initial fluorescence; $F_m$, maximal fluorescence; $F_v$, variable fluorescence, $F_v = F_m - F_o$; $t_{½}$, half-rise time constant for fluorescence induction; CP29, 29-kD Chl *a*/*b*-binding PSII core polypeptide.

plants were sprayed on alternate days with 10 μM of the ABA analog LAB 173711 (11), containing 0.05% (v/v) Tween 20. This treatment resulted in partial reversion of the mutant phenotype (data not shown), which is characterized by wilting and slow growth; these symptoms are more severe in *aba-1* and *aba-4* than in *aba-3* plants (12). The application of LAB 173711 did not cause any gross changes in the carotenoid profiles or the Chl levels of the mutants (data not shown). Because the mutant plants were phenotypically WT, we assume that differences in light gradients in WT and mutant leaves were negligible.

### Fluorescence Induction Measurements

Fluorescence induction was measured in leaves of WT and mutant plants using a Morgan CF-1000 Chl fluorescence measurement system (P.K. Morgan Instruments, Inc., Andover, MA). Experiments were performed under room light using specially designed cuvettes that allowed for dark adaptation (5–10 min) of the sample before measurement. Fully expanded leaves were chosen so that the 1-cm diameter circular sampling area provided by the cuvette was completely filled. A different leaf was sampled for each fluorescence transient acquired, resulting in the use of several plants from each genotype. The results from at least two and as many as five transients were averaged to minimize small differences due to sampling geometry. Following illumination, the instrument provided estimates of the relevant fluorescence parameters. It should be pointed out that the fluorescence parameters reported by the instrument are obtained with continuous illumination rather than the pulse-saturation technique and, therefore, do not conform to convention (28). However, the time resolution of the instrument during the onset of induction (20 μs/point) gives an accurate estimate of $F_o$, and at the higher PPFDs (500 and 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$), the actinic light is expected to fully reduce the primary quinone acceptor, $Q_A$, providing an accurate estimate of the actual value of $F_m$.

### Thylakoid Membrane Preparation and $O_2$ Evolution Measurements

Thylakoid membranes of WT and the *aba-4* genotype were prepared by flotation in a 1.8 M sucrose layer as described in ref. 3, except that the tissue homogenates were filtered through Miracloth before centrifugation.

Steady-state $O_2$ evolution was measured in a Clark-type electrode (Rank Bros.) maintained at 27°C. The cuvette (1 mL) contained Hepes/NaOH (20 mM) buffer at pH 7.5, NaCl (10 mM), $MgCl_2$ (5 mM), and thylakoids equivalent to 10 μg of Chl. Potassium ferricyanide (1 mM) and 2,6-dichloro-*p*-benzoquinone (0.25 mM) were used as electron acceptors. Illumination was provided by two 250-W lamps and was filtered by a $CuSO_4$ solution (to remove IR radiation) and two layers of red acetate cellophane (half-transmittance at 650 nm). The PPFD was attenuated by use of a Variac transformer. Actinic light intensity was measured using a Li-Cor LI-185A photometer fitted with a quantum probe. The water jacket and cuvette were removed, and the probe was placed directly above the electrode facing each illuminator separately. The PPFDs were then summed to give the values reported here. The actual PPFD reaching the sample was likely much lower because of lensing effects of the cylindrical water jacket and cuvette.

### EM

Rosette leaves from 5- to 7-week-old plants were fixed in 2% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), followed by a secondary fixation in 1% $OsO_4$ in the same buffer. Both fixation steps were performed at 4°C. The specimens were dehydrated in a graded ethanol series and embedded in Spurr's epoxy resin at room temperature. Ultrathin sections were stained with uranyl acetate and lead citrate and examined under a Philips 201 transmission electron microscope. Numbers of chloroplasts and number and size of grana were evaluated on prints of two independent preparations. Chloroplasts were counted in the spongy parenchyma.

### Immunoblot Analysis

Purified thylakoid membrane aliquots containing 30 μg of Chl were electrophoresed in a 7.5 to 15% linear gradient SDS-polyacrylamide gel (2). Gels were stained with Coomassie brilliant blue R-250 or electrophoretically transferred to nitrocellulose filters and probed by immunodetection (30) with monoclonal antibodies directed against pea CP29 (MLH2) or LHCII (MLH1) (4, 9).

## RESULTS AND DISCUSSION

### Chl Fluorescence and Photosynthesis in WT and *aba* Leaves

The photosynthetic activities of WT plants and plants homozygous for one of the three *aba* alleles were studied by measuring Chl fluorescence in vivo. Although interpretation of the complex fluorescence signals from leaves is not straightforward, comparison of $F_o$, $F_m$, $F_v/F_m$, and $t_{1/2}$ allows a relative assessment of the effect of the mutations on the primary photochemical reactions as well as on photosynthetic electron transport. Figure 1 shows the fluorescence parameters $F_o$, $F_m$, $F_v$, $F_v/F_m$, and $t_{1/2}$ for dark-adapted leaves of WT and the three *aba* genotypes subjected to different PPFDs. The data show the expected pattern for the type of instrument used in these experiments, i.e. fluorescence yields increased with increasing PPFD, and there was a slight decrease in the $F_v/F_m$ ratio at high PPFDs (8). At a PPFD of 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$, fluorescence yields ($F_o$ and $F_m$) of each *aba* genotype were significantly lower than those of WT plants ($P < 0.04$, one-sided *t* test) and were lower than WT yields at each PPFD tested (Fig. 1A). These results establish a correlation between the elevated levels of zeaxanthin in the *aba* mutant leaves (21) and the putative nonphotochemical fluorescence quenching by zeaxanthin (5–7). However, the lower fluorescence yield in the *aba* genotypes could also result from increased photochemical efficiency, decreased antenna size, or a decrease in the amount of PSII per unit of leaf area (13).

Several factors that affect PSII efficiency can be conveniently analyzed by the $F_v/F_m$ ratio. Figure 1B shows that WT and *aba* genotypes did not have different $F_v/F_m$ ratios from

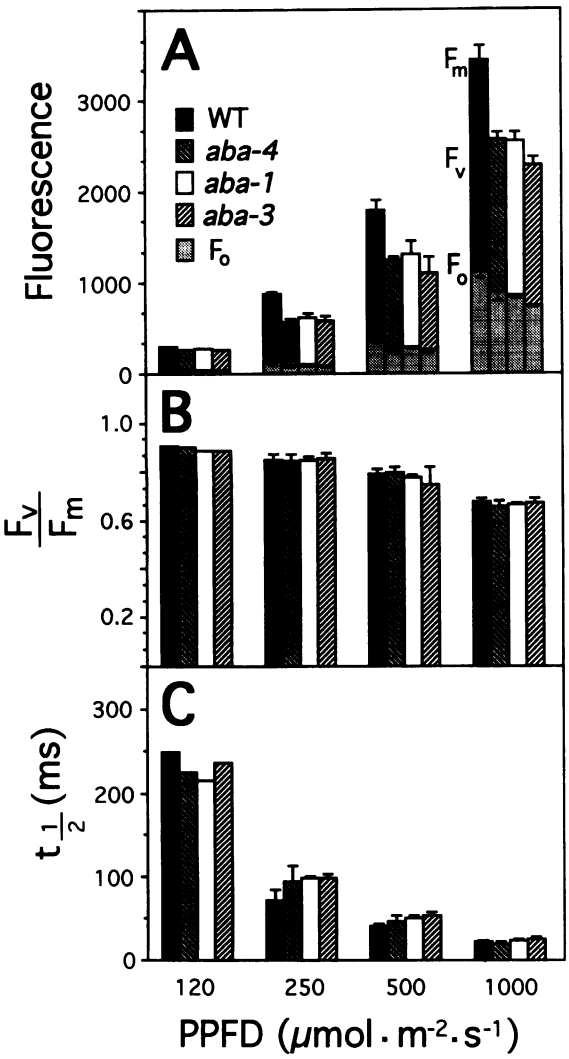


**Figure 1.** In vivo fluorescence induction parameters in Landsberg *erecta* (WT) and the three *aba* genotypes measured at various PPFDs. A, $F_o$, $F_v$, and $F_m$; B, $F_v = F_m - F_o)/F_m$; C, $t_{½}$. Fluorescence units are arbitrary. Measurements at a PPFD of 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$ are the average of two experiments; at other PPFDs, averages ± SE, $n$ = three to five.

each other over a range of PPFDs. This result suggests that the reduced fluorescence yield in the *aba* genotypes was not due to altered PSII efficiency, a conclusion that is further supported by the observed similarity of $t_{½}$ between WT and mutant genotypes (Fig. 1C). Thus, the fluorescence data demonstrate that the rates of photosynthetic electron transfer are similar in the WT and *aba* genotypes and suggest that, at most, subtle changes have occurred in the photosynthetic apparatus as a result of zeaxanthin accumulation and epoxycarotenoid deficiency.

To confirm the results obtained from in vivo fluorescence measurements, thylakoid membranes were prepared from WT and *aba-4* leaves (not treated with LAB 173711) to measure $O_2$ evolution rates as a function of PPFD. As shown in Figure 2A, the *aba-4* thylakoid membranes had less PSII activity than WT membranes. When the data were analyzed by linear regression in a Lineweaver-Burk reciprocal plot, the calculated Michaelis constants for half-maximal $O_2$ evolution activity for the photochemical reactions were essentially identical for *aba-4* and WT (Fig. 2B, $K_m$ values). These results support the conclusion that the quantum efficiency of PSII is not altered in the *aba* genotypes. The *aba-4* mutant thylakoids, however, exhibited a lower rate of $O_2$ evolution at the highest PPFD tested relative to the maximum rate at the next lower PPFD (Fig. 2A), suggesting a greater sensitivity to inactivation at high PPFDs. Because the reduced Chl fluorescence in the mutants is not due to altered PSII activity or PSII abundance (see further), we infer that the decreased fluorescence yields in the *aba* mutants are due to elevated levels of zeaxanthin and the radiationless energy dissipation associated with zeaxanthin accumulation (5).

## Chloroplast Ultrastructure, PSII, and LHCII Abundance in WT and *aba* Mutants

To determine whether the xanthophyll imbalances in the *aba* mutant plants affect the supramolecular architecture of the photosynthetic apparatus, we visualized chloroplasts, grana, and grana lamellae in WT and mutant mesophyll cells by transmission EM. We observed differences in the extent of the grana stacking between WT and the mutant genotypes (Fig. 3). A statistical analysis of the data is presented in Table I. The mutants had significantly more chloroplasts per cell than WT, as well as significantly more grana stacks per chloroplast, but fewer lamellae per granum (Table I). Furthermore, these quantitative differences were correlated with the levels of xanthophylls in the mutants: the *aba-4* genotype had the highest zeaxanthin accumulation (52.2 $\mu g \cdot g^{-1}$ fresh

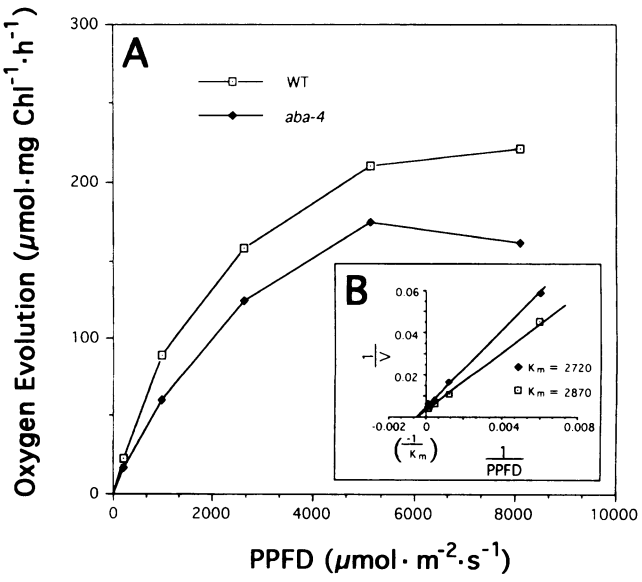


**Figure 2.** A, PSII activity in Landsberg *erecta* (WT) and *aba-4* thylakoids as a function of PPFD. B, Lineweaver-Burk reciprocal plot of $O_2$ evolution rates (V) and PPFD to calculate $K_m$ values for the photochemical reactions of PSII. Data are from one of two experiments with similar results. PPFDs were overestimated (see "Materials and Methods").

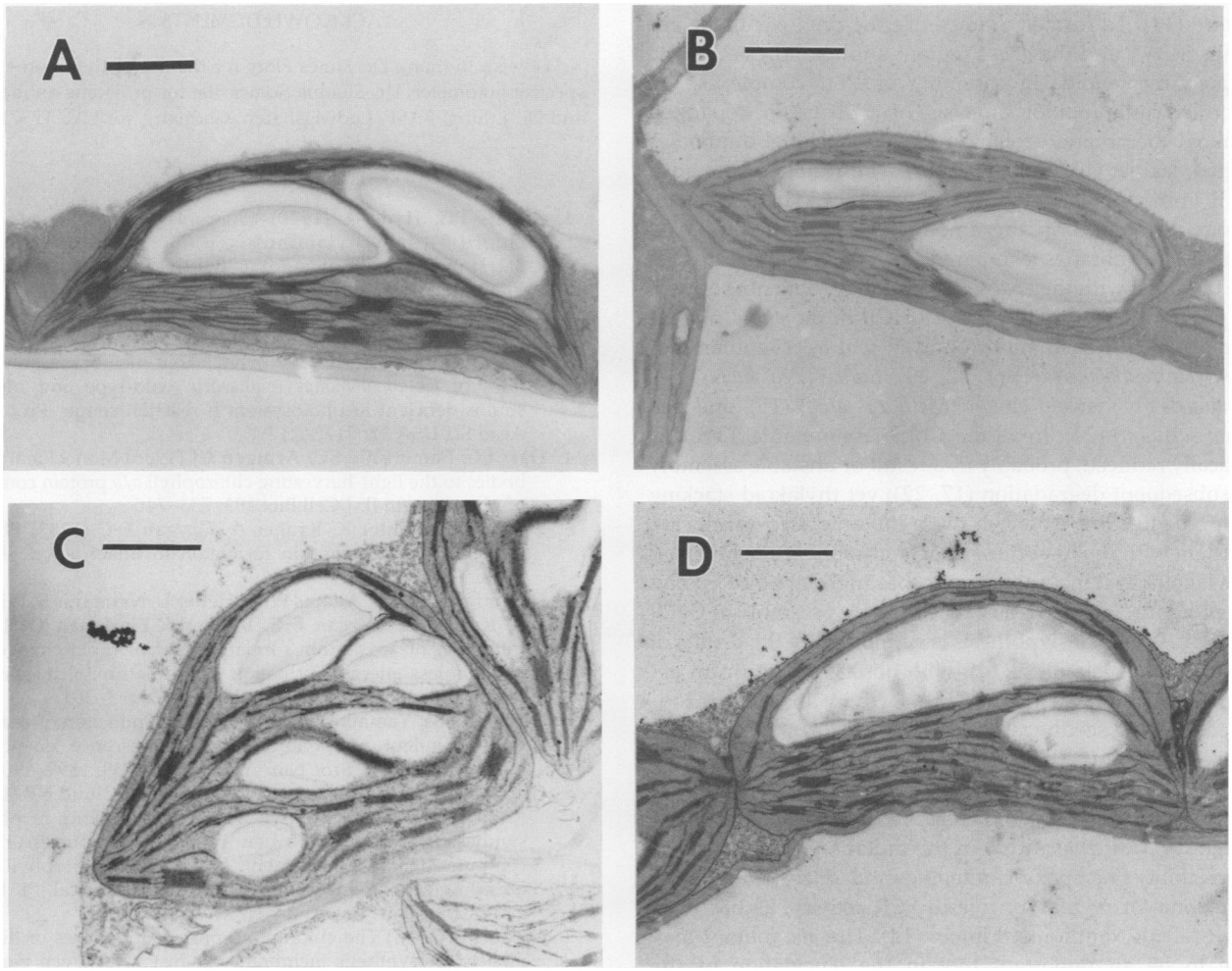


**Figure 3.** Transmission electron micrographs of chloroplasts in mesophyll cells of Landsberg *erecta* and *aba* plants. A, Landsberg *erecta* (WT); B, *aba-1*; C, *aba-3*; D, *aba-4*. The bars represent 1 μm.

weight compared with 1.2 $\mu g \cdot g^{-1}$ fresh weight in WT [21]) and exhibited the largest increase in numbers of chloroplasts and grana and the largest decrease in thylakoid stacking (Table I). The *aba-1* and *aba-3* genotypes are less extreme in xanthophyll imbalance (44.7 and 40.0 μg of zeaxanthin $g^{-1}$ fresh weight, respectively [21]) and also showed less extreme alterations in chloroplast ultrastructure (Table I). Pea and wheat chloroplasts depleted of carotenoids by inhibitors of carotenoid biosynthesis also showed reduced thylakoid stacking (1, 23). We interpret these data to mean that epoxycarotenoids and/or zeaxanthin are involved in the supramolecular assembly of the thylakoid membrane. We speculate that the mutants may compensate for decreased stacking efficiency by increasing the number of grana and chloroplasts. The altered chloroplast ultrastructure in the *aba* genotypes supports the hypothesis that the photosynthetic apparatus is adversely affected by the xanthophyll imbalance in the *aba* mutants.

Assembly of functional LHCII complexes in vitro requires the presence of violaxanthin or neoxanthin (19). CP29 also contains xanthophylls (9, 25). It has recently been shown that the xanthophyll lutein is required for PSII activity in

**Table I.** *Differences in Chloroplast Ultrastructure in WT and aba Genotypes of A. thaliana*

Mutants are listed in order of increasing phenotypic severity (12, 21). Means ± SE in each line that are followed by a different letter are significantly different at $P < 0.05$.

| | WT | *aba-3* | *aba-1* | *aba-4* |
|---|---|---|---|---|
| No. of chloroplasts/cell | 8.7 ± 0.5 a | 10.2 ± 0.6 ab | 10.9 ± 0.6 b | 13.4 ± 1.0 c |
| No. of grana/chloroplast | 34.0 ± 1.6 a | 50.1 ± 2.7 b | 50.3 ± 2.7 b | 55.7 ± 2.1 b |
| No. of thylakoids/granum | 8.1 ± 0.4 a | 4.1 ± 0.2 bc | 4.9 ± 0.2 b | 3.5 ± 0.2 c |

mustard (14). To further characterize the photosynthetic apparatus in WT and the *aba-4* mutant and to address the role of epoxy-carotenoids in assembly of PSII complexes, we performed immunoblot analysis of SDS-PAGE-separated thylakoid membrane proteins using monoclonal antibodies directed against CP29 or LHCII (4). The results of the anti-LHCII immunoblot are shown in Figure 4. There were no obvious differences in the amount of LHCII in the *aba-4* thylakoid membranes compared with WT. Similar results were obtained with the anti-CP29 antibody (data not shown). The presence of normal levels of LHCII in the *aba-4* mutant contrasts with the situation found in Chl *b*-less mutants of barley (*Hordeum vulgare*) (4, 22, 29), maize (*Zea mays*) (10), *Arabidopsis* (17), sweet clover (*Melilotus alba*) (15), and pea (*Pisum sativum*) (24). In all the Chl *b*-less mutants, LHCII is drastically reduced, probably as a result of unstable assembly and subsequent degradation (17, 22); yet thylakoid stacking does occur to some extent in these mutants. Our results are consistent with the hypothesis that xanthophylls are involved in thylakoid stacking and suggest that high levels of epoxy-carotenoids are not necessary for the stable assembly of CP29 or LHCII. The latter hypothesis is supported by the results of Markgraf and Oelmüller (14), who observed significant accumulation of LHCII but no PSII activity in norflurazon-treated mustard seedlings.

In conclusion, we have shown that the *aba* mutant of *A. thaliana* has reduced fluorescence yields (Fig. 1), and we propose that this is due to increased fluorescence quenching by high levels of zeaxanthin in the mutant. Our results raise the possibility that epoxy-carotenoids and/or zeaxanthin play a functional or regulatory role in PSII activity, as has been shown for the xanthophyll lutein (14). The *aba* mutant may prove to be an important tool in the elucidation of the complex processes of photosynthesis, multisubunit assembly, energy transfer, and nonphotochemical fluorescence quenching.

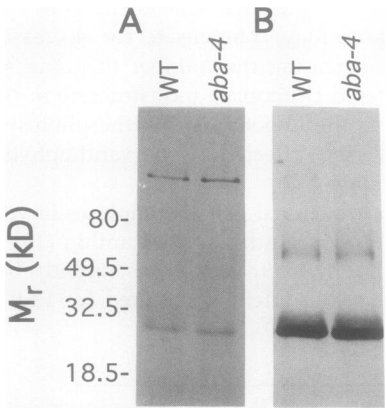


**Figure 4.** Immunoblot analysis of LHCII abundance in Landsberg *erecta* (WT) and *aba-4* thylakoids. Proteins from thylakoid samples containing 30 μg of Chl were subjected to electrophoresis in SDS-PAGE (7.5–15% gradient) and stained with Coomassie blue (A) or electroblotted to nitrocellulose and probed with a monoclonal antibody (MHL-1) against LHCII (B).

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