Plasticity in the Composition of the Light Harvesting Antenna of Higher Plants Preserves Structural Integrity and Biological Function*

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Arabidopsis plants in which the major trimeric light harvesting complex (LHCIIb) is eliminated by antisense expression still exhibit the typical macrostructure of photosystem II in the granal membranes. Here the detailed analysis of the composition and the functional state of the light harvesting antennae of both photosystem I and II of these plants is presented. Two new populations of trimers were found, both functional in energy transfer to the PSII reaction center, a homotrimer of CP26 and a heterotrimer of CP26 and Lhcb3. These trimers possess characteristic features thought to be specific for the native LHCIIb trimers they are replacing: the long wavelength form of lutein and at least one extra chlorophyll b, but they were less stable. A new population of loosely bound LHCII was also found, contributing to an increased antenna size for photosystem I, which may in part compensate for the loss of the phosphorylated LHCIIb that can associate with this photosystem. Thus, the loss of LHCIIb has triggered concerted compensatory responses in the composition of antennae of both photosystems. These responses clearly show the importance of LHCIIb in the structure and assembly of the photosynthetic membrane and illustrate the extreme plasticity at the level of the composition of the light harvesting system.

The light harvesting antenna of higher plants displays a highly conserved complexity in terms of genetic make-up, protein composition, oligomerization, and macrostructure that is mostly poorly understood (1). In the case of PSII there are at least six different polypeptides that are assembled into a mixture of monomeric and trimeric chl a/b2 xanthophyll protein complexes, collectively referred to as LHCII. The major complex, LHCIIb is composed of three types of polypeptides, products of the Lhcb1, 2, and 3 genes. The Lhcb1 and 2 are the dominating proteins. The minor complexes, CP29, CP26, and CP24, consist of the Lhcb4, Lhcb5, and Lhcb6 polypeptides and contain ~15% of PSII chlorophyll. LHCIIb carries up to 60% of the pigments of the PSII antenna (2, 3), with efficient energy transfer between chls and with a long chl singlet state excitation lifetime (4, 5); this provides a large and highly efficient light harvesting system. LHCIIb is also involved in a crucial low light adaptation strategy of plants, the state transitions, which control the relative PSI and PSII cross-sections (6). Equally, under light stress, excess light energy in LHCIIb can be safely dissipated into heat, an important photoprotective process, nonphotochemical quenching (7). The ability of LHCIIb to exist in different conformations with a range of excited state lifetimes seems to be an important aspect of this latter process (8, 9).

The native LHCIIb state is a trimer of monomeric pigment-protein complexes. These trimers possess certain unexplained characteristics not found in monomers (e.g. extra chl b, a long wavelength lutein, bound phospholipid). On the other hand, the minor LHCII components, CP26, CP24, and CP29, are always monomeric. Trimeric LHCIIb and the monomeric complexes are associated with the dimeric PSII core to form the LHCII-PSII supercomplexes found in the granal membranes of the chloroplast (10). The role of the trimeric state or indeed of the highly conserved macromolecular organization of the entire granum is not understood, despite the recent advances in structure determination (3, 10, 11).

Genetic manipulation of the levels of specific pigments and polypeptides is proving a powerful approach to understanding the structure and function of the photosynthetic antennae (12–15). Lhcb2 antisense Arabidopsis thaliana plants have been constructed by introducing the Lhcb2.1 coding region in the antisense orientation into the plant genome (16). This resulted in not only the complete absence of Lhcb2 but also Lhcb1 polypeptides. These plants, despite having a pale green appearance, were able to develop, grow, and produce seeds. Photosynthetic quantum yield was only slightly reduced, and despite the absence of the key LHCII polypeptides, chloroplasts of antisense plants possess grana. However, the vital adaptive functions of the photosynthetic membrane where LHCIIb plays a key role were found to be significantly inhibited (16); the state transitions were completely absent in the antisense plants, and nonphotochemical chlorophyll fluorescence quenching was reduced by over 30%. Remarkably, despite the absence of LHCIIb, trimeric structures were still found in the antenna, and photosystem II retained its characteristic macro-organization, the supercomplex structure being virtually indistinguishable from that of the wild type (17). Biochemical analysis showed that the levels of other antenna proteins increase in the antisense plants, and we suggested that the trimers are composed of the greatly increased amounts of Lhcb5, which is normally only found in the monomeric CP26 complex.

These observations led to the hypothesis that the increases in expression of other light harvesting genes may be compensatory changes that allow the thylakoid membrane structure to be preserved and photosynthetic performance to be maintained. The oligomeric structure of the antenna, particularly LHCIIb trimerization, is therefore seen as a key...
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feature of the molecular design of the functional thylakoid membrane. If this hypothesis is correct, these responses indicate the presence of a remarkable level of plasticity and robustness, which must result from a complex response of the altered LHC genome in the form of interplay between the expression of both Lhcb and Lhca genes and in the processes of assembly of supramolecular structure. Therefore, the aim of the current work was to test this hypothesis, first by carrying out a systematic analysis of the composition of the light harvesting antenna in the antisense plants and second by determination of the functional competence of the new proteins. The data obtained also allowed us to assess the effectiveness of the compensatory response of the whole antenna system and to address the question of the reason for the trim- 

For sucrose gradient separation, unstacked thylakoids were treated with 1% n-dodecyl β-D-maltoside with a ratio of β-DM/chl of 10 on ice for 30 min. Sucrose gradients were seven-step exponential gradients from 0.15 to 1.0 M sucrose dissolved in 20 mM HEPES buffer containing 20 mM β-DM. The run time was 18 h at 200,000 × g in a SW41 rotor at 4 °C. LHCl was prepared according to the method of Croce et al. (20).

To separate LHClI components by non-denaturing isoelectric focusing (IEF), a procedure modified from that described before (18, 21) was used with an Amersham Biosciences Multiphor II electrophoresis system protocol. A slurry of volume 100 ml containing 4% Ultradex (Amersham Biosciences), 2% ampholine carrier ampholites (pH 3.5–9.0), 1% glycerine, and 0.06%/n-dodecyl β-D-maltoside (Sigma) was prepared and poured into the 24.5 × 11.0-cm tray to form a homogeneous layer. After carefully removing air bubbles, the tray was placed 70 cm below a small fan on a balance to allow the monitoring of the weight of evaporated water; 30 g of water was evaporated in ~2 h. Freshly prepared PSI particles or unstacked thylakoids with a total chl concentration of 2.5 mg/ml were resuspended in 1 ml of deionized water, and 0.5 ml of 3% n-dodecyl β-D-maltoside was added on ice. Incubation with occasional stirring lasted for 30 min. The samples were centrifuged at 35,000 × g, and the supernatants were applied 2 cm from the cathode of the precooled and prefocused gel (1 h of prefocusing at 8 W) using a sample applicator (10 × 2 cm). After the samples had been applied, the gel was allowed to equilibrate for 3 min before the start of focusing. The focusing procedure was carried out for 18 h at a constant power of 8 W at 4 °C. The initial and final current values were normally ~15 and 5 mA, respectively. After measuring pH values, each green band was carefully collected using a spatula and eluted using columns with a minimum volume of a solution containing 100 mM HEPES (pH 7.6) and 0.01% n-dodecyl β-D-maltoside.

For electrophoresis and Western blot analysis, the protein samples were solubilized and separated by 15% denaturing SDS-PAGE. Roughly equal amounts of chlorophyll were loaded, ~2 μg/lane for PSI membranes and 0.5 μg for isolated supercomplexes and trimers. The proteins were transferred to Hybond-P polyvinylidene difluoride membrane (Amersham Biosciences) in a Mini-Trans-Blot transfer cell (Bio-Rad) at 30 mA for 12 h. The membranes were detected with specific antibodies against the proteins Lhcb1–Lhcb6. The primary antibody was detected by a horseradish peroxidase-labeled secondary antibody using an ECL Plus kit (Amersham Biosciences). Chemiluminescence was detected on Hyperfilm ECL (Amersham Biosciences) photographic film. For the antibodies against Lhcb1, Lhcb2, and Lhcb5, linear densitometric responses were found in the range of 0.5–10 μg of chl/lane for PSI membranes. For quantification of PSI, 1 μg of chl was separated on SDS-PAGE and analyzed as described by Andersson et al. (13). PsAD and Lhca proteins were measured by densitometry of the immunoblots.

Total RNA was prepared from six-week-old plants as described in Ganeteg et al. (22). For Lhca4, the same primers as in Ganeteg et al. (22) and...
were used, whereas for Lhcb5 the following primers were used: b5FW, 5'-GGAGGACGCTGGTTCTATCTTGGCG, and b5RV, 5'-GAGAA-CAACCTCATGACTACCCG. The primers were constructed so that the amplicon spanned an intron. cDNA was synthesized from 150 ng of RNA using a first strand cDNA synthesis kit (Amersham Biosciences). Quantum RNA 18 S internal standards (Ambion, Austin, TX) were used. The linear range of the primers and the optimal ratio of 18 S primers:competitors were determined, as recommended by the vendor. PCRs were performed using AmpliTaq Taq polymerase (Applied Biosystems, Foster City, CA). Both the cDNA synthesis and the PCRs were performed in triplicate, giving a total of nine reactions.

For microarray analysis, total RNA (1 μg) from three separately grown batches of wild type (WT1–3) and asLhcb2 (asLhcb21–3) plants was amplified as described by Allemeersch et al. (23), using the MessageAmp II aRNA kit (Ambion). cDNA synthesis, labeling, microarray hybridization, and scanning were performed according to Sjödin et al. (24) and stored in the public data base UPSC-BASE (www.upscbase.umu.se) as experiment number UMA-0061. Statistical analysis was conducted using UPSC-BASE and was based on three dye swaps (i.e. six microarrays). The raw data were transformed (25), and print tip loss was normalized (26) and analyzed further in UPSC-BASE.

RESULTS

Photosystem II Antenna Function in the Absence of LHCIIb—Low temperature absorption and fluorescence spectroscopy were used to analyze the antenna in the asLhcb2 plants, to provide information on the organization of the pigments as well as their efficiency in supplying energy to the reaction center complexes. Fig. 1A shows 77 K absorption spectra of thylakoid membranes and their second derivatives. The latter was used to resolve the complex spectral structure to identify different chlorophyll species and assign them to various pigment proteins (27). chl b (650 nm) and the short wavelength chl a at 661 nm are strongly reduced in the spectrum of asLhcb2 thylakoids, indicating the disappearance of typical LHCIIb spectral forms. The major maximum at 675 nm in wt is 3 nm red-shifted to 678 nm in asLhcb2, and the form at 683 nm, attributed to the PSII core antenna complex, CP47 (28), is enhanced. The bands above 690 nm that belong to PSII (29) remains unchanged, because the spectra were normalized in this region. This picture is consistent with the fact that in asLhcb2 plants the amount of LHCII is reduced and the PSII to PSI ratio is strongly enhanced, i.e. there is a larger number of PSII units with a smaller antenna size (16). Fig. 1B displays the 77 K fluorescence spectra of the wt and asLhcb2 thylakoids. The PSII fluorescence region from 670 to 700 nm was the most affected by the absence of LHCIIb. The difference spectrum, wt-minus-asLhcb2, exhibits a PSII core band at 685 nm with strong shoulders at 681 and 699 nm, both attributed to LHCIIb (30). This difference spectrum is explained by the reduction in content of LHCII, reducing the amount of energy transferred to the core and also the amount of direct emission. There is no indication of increase in fluorescence in the asLhcb2 plants that would arise from disconnected or nonfunctional antenna.

Fig. 1C displays the Soret band absorption spectra and PSII fluorescence excitation spectra used to find whether the chl and xanthophyll species are transferring excitation energy to the PSII core in the asLhcb2 plants. For both wt and asLhcb2 thylakoids, the features in the absorption spectra closely matched those in the excitation spectra. The second derivative analysis of the wt and asLhcb2 absorption spectra revealed very similar chl a profiles and reduced participation of chl b (471 nm band) and efficient involvement of xanthophylls absorbing at 485 nm (neoxanthin) and 495 nm (lutein). The most red-shifted Soret band at 510 nm is present in the spectrum of both the wt and asLhcb2 thylakoids. This band is characteristic of the trimeric state of LHCIIb (31). A very similar picture was obtained using detection at 678 nm, the LHCII
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fluorescence band (not shown). Therefore these data reveal the characteristics of a new type of PSII antenna in the asLhcb2 plants in which antenna chls and xanthophylls are effectively transferring energy to the chl of PSII core complexes and which is likely to be trimeric.

Polypeptide and Pigment Composition of LHCII—Thylakoid membranes from wt and asLhcb2 plants were solubilized with detergent and subjected to sucrose gradient centrifugation, which separates three fractions enriched in PSI, PSII cores, and LHCII (Fig. 2A). In agreement with previous work (16), measurement of the amount of chlorophyll in each fraction shows that the amount of PSI is very similar in both the wild type and antisense plants, but the amount of LHCII is approximately two times reduced and the PSII core complexes are twice enhanced in the asLhcb2 plants. As expected, the polypeptide composition of the LHCII fraction was also different. In the wt, this fraction shows the expected profile of LHCII polypeptides (Fig. 2B). In the preparation from the asLhcb2 plants, Lhcb4, Lhcb5, and Lhcb6 were found (arrows), with a significant increase in the relative content of Lhcb5, identified by the Western blotting, consistent with previous results (16, 17). However, in addition to the Lhcb polypeptides, a group of four smaller polypeptides from 20 to 23 kDa was clearly seen (bracketed). Western blotting using Lhca1–4 antibodies positively identified these as being from LHCI (Fig. 2C). It should be noted that the LHCII fraction from asLhcb2 did not contain the PSI core polypeptides, which are signified by the high molecular mass bands of the reaction center and the number of smaller bands ~10 kDa. Normally, the Lhca proteins always stay very tightly attached to the PSI core under the solubilization conditions/treatments used here, and special treatments using a combination of nonionic and ionic detergents are needed to remove them. This suggests that the asLhcb2 plants contain a population of loosely bound LHCI not found in the wt.

The pigment compositions of the wt and asLhcb2 thylakoids were different and reflected the reduction in LHCII and the increase in PSII content (Table 1). Thus, there were decreases in neoxanthin and chl b, which are enriched in LHCIIb, but an increase in chl b, which is predominant in reaction center cores. It is interesting to note that the content of the xanthophyll cycle carotenoids is higher in the asLhcb2 thylakoids, probably because of the increased content of minor LHCII. The carotenoid compositions of the LHCII fractions also differed. Again the xanthophyll cycle content is higher, whereas percentages of neoxanthin and lutein are reduced, as expected from the Lhcb compositions of these fractions and consistent with a fact that the xanthophyll cycle carotenoids have a stronger affinity for binding to the minor LHCII complexes than to LHCIIb. Also the amount of b-carotene is enhanced in the asLhcb2 preparation. This is likely to be a result of the presence of LHCI that is reported to bind this carotenoid (29) and also the CP47 and CP43 that are found in this fraction (Fig. 2B). The chl a/b ratio of the asLhcb2 LHCI preparation was higher than that for the wild type (2.0 versus 1.6). This is consistent with the fact that the chl b-enriched polypeptides, Lhcb1 and Lhcb2, were absent.

To get information on the levels at which the amounts of the LHC proteins were regulated, the expression of the genes coding for the different Lhc gene products was examined. When RNA prepared from wt and asLhcb2 leaves were analyzed on CATMA whole genome DNA microarrays (32), no differential expression of any Lhca or Lhcb gene could be observed (data not shown). To confirm these data, an independent verification of the results for Lhcb5 and Lhca4, the two proteins that on protein level accumulated most, was made using reverse tran-

![Figure 2: Sucrose density gradient analysis of thylakoids from wt and asLhcb2 plants.](image)

**TABLE 1**

| Sample            | Neoxanthin | Violaxanthin | Antheraxanthin | Lutein | β-Carotene | Xanthophylls cycle carotenoids | Car/chl | Chlorophyll a/b ratio |
|-------------------|------------|--------------|----------------|--------|------------|-------------------------------|---------|-----------------------|
| Thylakoids, wild type | 4.1        | 3.1          | 0.3            | 13.0   | 9.0        | 3.4                           | 0.31    | 3.30                  |
| Thylakoids, asLhcb2 | 3.4        | 4.8          | 0.4            | 14.0   | 15.0       | 5.2                           | 0.40    | 4.20                  |
| Thylakoids, asLhcb2 (S) | 7.1        | 2.4          | 0.0            | 15.0   | 0.5        | 2.4                           | 0.25    | 1.60                  |
| LHCII, wild type   | 5.9        | 5.2          | 0.7            | 16.0   | 3.2        | 5.9                           | 0.31    | 2.00                  |
| LHCII, asLhcb2     | 7.8        | 0.0          | 0.0            | 16.2   | 0.0        | 0.0                           | 0.24    | 1.31                  |
| LHCII, asLhcb2     | 5.7        | 7.3          | 0.0            | 15.0   | 0.0        | 0.0                           | 0.28    | 1.68                  |
| LHCII, asLhcb2     | 5.9        | 8.0          | 0.0            | 15.8   | <0.3       | 8.0                           | 0.30    | 1.95                  |
| LHCII, asLhcb2     | 4.1        | 8.1          | 0.0            | 16.8   | 0.0        | 8.1                           | 0.29    | 2.60                  |

*IEF + FPLC, samples obtained by FPLC separation of IEF fractionated thylakoids as in Fig. 5.*
scription-PCR. Again, the steady-state RNA levels in wt and asLhcb2 were not different (data not shown), indicating that the accumulation of these polypeptides in asLhcb2 may be regulated at the translational or post-translational level.

Analysis of the Oligomeric States of the LHCII Antennae—Gel filtration was used to determine the oligomerization states of the new type of LHCII antenna in the asLhcb2 plants. Unstacked thylakoids were solubilized with α-DM and loaded on a gel filtration column. Fig. 3A shows typical chromatograms. For the wild type, bands of PSII membrane fragments, PSII supercomplexes, PSII core complexes, LHCII trimers, and minor LHCII monomers were resolved and assigned as described previously (15, 16, 33). A similar picture was observed for the asLhcb2 thylakoids (Fig. 3A, trace as), although the ratios of the component fractions clearly differed. In part this reflected the different contents of PSII and PSI, discussed above. However, examining the PSII fractions, there was a decrease in the relative proportions of supercomplexes relative to cores, suggesting that the former are less stable under the detergent solubilization procedures used. Similarly, there were more monomeric LHCII relative to trimeric LHCII in the asLhcb2 thylakoids. In fact, upon solubilization with a slightly increased concentration of α-DM, which has no effect on LHCIIb trimers in the wt, there was almost complete disintegration of trimers in the asLhcb2 thylakoids (trace as'). It should be noted that this disintegration at higher α-DM was not complete, and some complexes with a slightly increased retention time remained. The position of this band matched that for dimeric LHCII prepared from isolated PSI complexes, and indeed polypeptide analysis showed that this fraction contains polypeptides of 20–23 kDa (Fig. 3B) that were positively identified by Western blots as Lhca1–4 (not shown). It is important to note that only Lhca polypeptides are present, not those from the PSI core complex. Consistent with this result, these Lhca polypeptides were found in the trimeric LHCII fraction from asLhcb2 plants, whereas they were absent from the wt. The occurrence of the Lhca components in the LHCII gel filtration profile is unexpected, similar to the finding of LHCII in the sucrose gradient LHCII band (see above). In fact LHCII polypeptides were even found in the PSII supercomplex fraction of the asLhcb2 plants but not at all in that of the wt (Fig. 3, B and C). However, most significantly, PSI membrane fragments did not contain any LHCII, indicating that the presence of the Lhca1–4 proteins in the LHCII preparations is only contamination by a population of loosely bound LHCII found only in asLhcb2 plants; it does not represent Lhca proteins bound to PSII.

Purification of LHCII from asLhcb2 Plants—Isoelectric focusing was used to prepare LHCII fractions from the asLhcb2 plants. Fig. 4 shows the results of IEF of wt and asLhcb2 thylakoids. The sample from the asLhcb2 thylakoids was enriched in monomeric CP26 (identified by its pI position, absorption spectrum, and Western blotting) compared with the wt sample (Fig. 4A). In the wt most of the chlorophyll is found in the area that contains LHCIIb trimers. This area is depleted in the asLhcb2 sample, but one band is very prominent. This band is slightly up-shifted in comparison with that of the wt thylakoids, with pIs ~4.3 and 4.15, respectively. Two distinct bands were collected from the LHCII region and labeled as ief1 and ief2. Polypeptide analysis indicated differences between these two fractions of the asLhcb2 sample (Fig. 4B). The first band contained both Lhcb5 and Lhcb3 polypeptides. The Lhcb3 polypeptide was present almost exclusively in this band and in amounts much higher than in the wt LHCII (Fig. 4C). The second band contained Lhcb5 but very little Lhcb3 and in addition a number of smaller polypeptides from 20 to 23 kDa.

The IEF fractions were applied to a gel filtration column. Here two distinct bands are found, corresponding to trimers and monomers (Fig. 4A). The ief1 fraction from the asLhcb2 plants had almost the same ratio of trimers to monomers (~5:1) as the wt fractions. In contrast, the proportion of trimers relative to monomers for fraction ief2 was reduced to ~0.6:1. Because monomeric CP26 is not found in this region...
of IEF gel, this suggests that all of the Lhcb5 in these IEF fractions was trimeric but that the trimer built of only Lhcb5 dissociated during gel filtration, i.e. the homotrimer is less stable than the heterotrimer of Lhcb5 and Lhcb3. By increasing the detergent concentration used for the solubilization of thylakoids and in the gel filtration, it was possible to further reduce the yield of CP26 homotrimers. In the case of as_ief2, a band with a longer retention time remained, the same as found for a purified dimeric LHCI fraction. With lower DM concentration, as_ief2 and asLhcb2 plants.

FIGURE 4. Isoelectric focusing separation of solubilized unstacked thylakloid membranes from wt and asLhcb2 plants. A, IEF gel of solubilized wt and asLhcb2 (as) thylakoids. The brackets indicate the LHCII fractions 1 and 2 as ief1 and ief2, respectively. B, polypeptide analysis of ief1 and ief2 from wt and asLhcb2 taken from the IEF gel. Labeled are Lhcb3 (arrows) and Lhcb5 and Lhca (brackets) polypeptides. C, Western blot of LHCII fractions 1 and 2 with Lhcb3 antibody.

Pigment Composition of LHCII Trimers from asLhcb2 Plants—The pigment analysis revealed that the homotrimers that contained only CP26 complexes have a chl a/b ratio of ~1.95 (Table 1). This is lower than that found for monomeric CP26, ~2.5 (35). The CP26 monomers prepared here had a ratio of 2.6 (Table 1). In addition, it is known that in comparison with LHCIIb, CP26 is missing three chl b molecules, leaving only three (36). A ratio of 2.67 would then arise if eight chl a were bound. A value of 1.95 would arise if the trimeric complex bound one extra chl b. Fig. 6A supports this conclusion, showing that in the low temperature absorption spectrum of the Lhcb5 homotrimer, the chl b band is more pronounced than in a spectrum of the Lhcb5 monomer. Calculations based on the area under the absorption spectrum also suggest one extra chl b in the Lhcb5 homotrimer. The chl a/b ratio of the Lhcb3/5 heterotrimer was 1.68. Because Lhcb3 binds six chl b molecules (37), it is suggested that the heterotrimer is built from two Lhcb5 and one Lhcb3, giving a predicted chlorophyll a/b ratio of 1.77. The low temperature absorption spectrum of this heterotrimer indeed displays an even more pronounced chl b peak at 650 nm compared with the homotrimer and monomer.

Treatment with phospholipase is well known to cause monomerization of LHCIIb trimers (38), because of the phospholipid bound to the complex (3). The same treatment was found to cause monomerization of the Lhcb5 homotrimer (Fig. 6B). In this experiment the chl a/b ratio of the trimer was 1.91. The monomer derived from this trimer had a ratio of 2.49, consistent with the data in Table 1 and confirming that trimerization causes acquisition of extra chl. Indeed, upon monomerization a free pigment band containing only chl b and no chl a was found; the proportion of chl b found in this band was ~25%, consistent with the loss of one chl b of a population of four. Treatment of wt LHCIIb trimers with phospholipase does not lead to any change in chl a/b ratio or loss of chl b (data not shown) as found previously (35, 39).
content of neoxanthin is reduced. There is little detectable \( \beta \)-carotene in the trimers, consistent with the strong reduction in LHCI contamination. The lutein content is virtually unchanged, suggesting that both the lutein 1 and lutein 2 molecules found in trimERIC LHCIIb (3, 11) are present in the Lhcb5 trimers. The carotenoid composition of the Lhcb5 trimers is similar to CP26 monomers, although not identical, with a smaller neoxanthin content. The composition of the homotrimers differed slightly from that of the heterotrimer; there was more violaxanthin bound to the former.

The Soret band absorption spectra of \( wt \) and Lhcb5 trimers are very similar, and in particular their second derivatives display the presence of the 510-nm absorption band of the same magnitude (Fig. 6C). This band, which arises from lutein 2, is a characteristic for the trimERIC LHCIIb state (31) and is clearly absent from the CP26 monomer, which displayed only the lutein band at the 495-nm band (Fig. 6C).

**Photosystem I Antenna in the asLhcb2 Plants**—The presence of LHCI contamination in various LHCII preparations suggests that there is a new population of this complex that is easily detached from PSI. Analysis of the relative amounts of Lhca protein by quantitative immunoblotting showed large increases in the contents of Lhca1–4 proteins, particularly a 2–3-fold increase in Lhca1 and Lhca4 compared with the wild type (Fig. 7A). It was estimated that in thylakoids, per PSI reaction center (from the PsAD protein), the levels of Lhca1–4 proteins in asLhcb2 plants increased by 140 \( \pm \) 4%, 20 \( \pm \) 6%, 44 \( \pm \) 4%, and 280 \( \pm \) 8%, respectively (Fig. 7A). The same trends, but less pronounced, were also found if PSI preparations rather than thylakoids were analyzed (data not shown), indicating that some of the extra LHCI is retained by the isolated PSI fraction. Moreover, gel filtration of the PSI preparation treated with 1% \( \beta \)-DM showed that a fraction of LHCI of the asLhcb2 plants can be more easily removed from the PSI core than in the \( wt \) (Fig. 7B). A small band of LHCI was found in the profile of the asLhcb2 sample, but not in \( wt \). Comparison of the low temperature absorption spectra of the \( wt \) and asLhcb2 PSI indicated that the latter is more enriched in chl \( b \) and that the major chl \( a \) maximum is blue-shifted (Fig. 7C). These are good indicators that the PSI from the asLhcb2 plants has a larger LHCI antenna than the \( wt \), corroborating the results from the immunoblot analysis.

To assess whether the extra LHCI functions as a part of the PSI antenna, fluorescence excitation spectroscopy was carried out on thylakoid preparations. Fluorescence was detected in the 735-nm region, the maximum of PSI fluorescence associated with the long wavelength energy traps. These final acceptors of excitation at 77 K can be used to estimate the relative size of the PSI antenna (40, 41). Fluorescence excitation spectra of both the \( wt \) and asLhcb2 were normalized at \( \sim 710 \)-nm region, the absorption band giving the F735 emission. It is clear that the amplitudes of the chl \( b \) region and short wavelength chl \( a \) are larger in the asLhcb2 spectrum than in the \( wt \) spectrum. This indicates that there is an increased functional antenna size of PSI in asLhcb2 plants, because of the presence of more LHCI. The estimated average increase in antenna size was found to be \( \sim 25\% \).

**DISCUSSION**

**Features of the New Type of LHCII Antenna**—The experiments described here provide direct proof that in antisense plants in which the main LHCII polypeptides are missing, trimers are assembled composed of the Lhcb5 protein that is normally only found in the monomERIC CP26 complex. Two types of trimers were found: homotrimers of Lhcb5 and heterotrimers of Lhcb5 and Lhcb3. It was estimated that the percentage of homotrimers was much higher than that of the heterotrimers (65\% versus 35\%). Spectroscopic analysis showed that these trimers...
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**Figure 7.** Analysis of the antenna of photosystem I. **A**, Western blot of thylakoids from wt and asLhcb2 (as) plants. The relative amount of each Lhca protein in asLhcb2 compared with wt (100%) is given (as/wt). The results shown are the means ± S.E. (n = 4). **B**, FPLC gel filtration runs of PSI sucrose gradient fractions from wt as compared with the buffer medium containing 0.4% B, FPLC gel filtration runs of PSI sucrose gradient fractions from asLhcb2 (asLhcb2). **C**, low temperature absorption (solid lines) of PSI sucrose gradient preparations and 735-nm fluorescence excitation spectra of thylakoids (dashed lines) from the wt and asLhcb2 (as) plants. The vertical arrow indicates the chl b band.

were functionally coupled to PSII in intact thylakoid membranes, transferring energy to the reaction center core complexes. These observations are consistent with the observation by electron microscopy and image analysis of trimers in the PSII membrane fragments prepared from the asLhcb2 plants (17).

The existence of trimers in the antisense plants suggests a very special requirement for the LHClI trimeric structure. This could be both functional (in light harvesting) and structural (in assembly of the PSII macrostructure). The trimerization of LHClI appears to create features that are important for the avoidance of energy loss by nonradiative dissipation (39) and the promotion of efficient energy transfer (5). The cluster consisting of chl a610, chl a611, and chl a612 may be the conduit for energy transfer to neighboring antenna and core complexes, and the presence of three identical clusters on each trimer could enable efficient energy transfer within the PSII macrostructure, bearing some similarity to the pigment rings found in bacterial antenna complexes. Furthermore, the cluster of chl b601, chl b606, and chl b609 involves pigments in adjacent monomers. The phospholipid molecule that has a key role in the stabilization of the trimer is tightly associated with chl b601, which is not H-bonded at either formyl C-7 or carbonyl C-13 as are other chl b molecules (3). It appears that the inclusion of the phospholipid create the binding pocket for the chl b601. It is suggested that trimerization of CP26 recreates some features of this pocket and causes an extra chl b to be bound, enabling it to function efficiently in energy transfer.

Another feature found exclusively in the trimeric state of LHClI is the red-shifted lutein 2 band at 510 nm (31, 42, 43). This is also present in the CP26 homotrimers, suggesting a similarity between the lutein 2 environment in this complex and that in LHClI. Neither the origin of the red shift of lutein nor its function is well understood. It may arise from new interactions between pigments bound to neighboring monomers in the trimeric structure. For example, trimerization would create an intermonomer lutein 2-chl b603 associate that could red shift the lutein absorption band.

One difference between the CP26 containing trimers and the LHClI trimers is the higher violaxanthin content of the former. Previously, it has been shown that the amount of violaxanthin is ~1/LHClI in monomers but only ~0.3 in the trimer (35). In the case of CP26, the higher amount of violaxanthin is found in both the monomer and trimer. In the known binding site for violaxanthin on LHClI, there are two major interaction regions on the protein, the D-helix (the lumenal site) and the N-terminal loops (the stromal site). These coordinate the alignment of the xanthophyll by interacting with its head groups. Secondary structure prediction indicates that CP26 has a small extra helix on the N-terminal site (between 60th and 70th residues) centering at NH2-DELAK-COOH. This can act as a counterpart to the C-terminal helix D, exerting a more coordinated and stabilizing effect on violaxanthin in comparison with the flexible loop structure in LHClI.

The M-LHClI and S-LHClI trimers, which are part of the LHClI-PSII supercomplex, form an intrinsic part of the macro-organization of PSII in the granal membranes (10), whereas the remaining trimers appear to be located in PSII-free membrane regions. The M trimer consists of Lhcb1 and Lhcb2, whereas the S trimer is composed of Lhcb1 and Lhcb3. We suggested previously, that only the M and S trimers are replaced by CP26 (17), because supercomplexes were observed, but the ratio of LHClI/PSII was much lower (Fig. 2A). On the basis of the results presented here, it is suggested that the Lhcb5 homotrimer replaces the position of LHCl dimers.

The position of LHCl dimers. C, low temperature absorption (solid lines) of PSI sucrose gradient preparations and 735-nm fluorescence excitation spectra of thylakoids (dashed lines) from the wt and asLhcb2 (as) plants. The vertical arrow indicates the chl b band.
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In the wild type plant, the LHCII-PSII supercomplex appears to be more sensitive to the absence of state transitions, in which a portion of LHCIIb trimers, phosphorylated by the redox-sensitive protein kinase, can associate with PSI and contribute to an increase in its absorption cross section. In the asLhcb2 plants the absence of LHCIIb trimers is compensated for by accumulation of CP26 trimers and by an increase in content of PSI. The mobile phospho-LHCII pool is absent, and they are unable to perform state transitions; photosystem I gains extra loosely bound LHCI that may compensate for excitation energy imbalance between photosystems. LHCI (red) and LHCIIb (blue), light harvesting antenna of photosystem I and II, respectively; RCI and II, reaction centers; electrons; PQ and PQH₂, intermediate electron carrier plastoquinone in oxidized and reduced forms. Shown in green are the minor LHCI antennae, totally monomeric in the wt, but including CP26 trimers in asLhcb2.

M-trimer, whereas the Lhcb5/Lhcb3 heterotrimer replaces the S-trimer. These Lhcb5 trimers were less stable in the presence of detergents than the heterotrimers of Lhcb5 and 3 or wt LHCIIb trimers. Because Lhcb3 is involved in trimer formation in the wild type LHCIIb, it stabilizes the Lhcb3/5 trimers.

The LHCII-PSII supercomplex also appears to be more sensitive to detergent attack in the asLhcb2 membranes, and this may arise because of the differences in the S-trimer. The S-trimer is the most tightly bound trimer and is therefore probably more important for supercomplex structure. Despite a lower stability of the supercomplex and trimers, we have not detected any direct physiological consequence of this; for example, the heat stability of PSII in vivo or of the trimers in vitro are the same for wt and asLhcb2. However, the less stable trimers and supercomplexes and/or the decreased proportion of trimers could have effects on the three-dimensional organization; although asLhcb2 plants do have grana, it is possible that the stability of these is reduced. Further analysis of the membrane structure in these plants is needed.

Antenna Function in the Thylakoid Membrane in asLhcb2 Plants—Fig. 8 shows a schematic presentation of the LHC antenna function of both PSI and PSII. Normally the PSI antenna is larger than that of PSI. The taking average number of LHCCI trimers/PSII as 4 with 42 chlorophylls in each and ~35 chlorophylls in the minor antenna, this makes a total chlorophyll cross-section of ~200. For PSI, a recently published structure suggests that the LHC size is equivalent to ~60 chlorophylls (44). Even taking into account chl of the core complexes, PSI would still have a larger antenna size than PSI. Under limiting low light, the efficiency of the linear electron transport would be compromised, because the antennae sizes of PSI and PSII differ. To compensate for this the state transitions allow rapid modulation of the PSI and PSII cross-sections. A part of the larger PSI antenna, the peripheral LHCCIb trimer, works as a shuttle between the two photosystems dependent upon the phosphorylation state of particular residues near the N terminus of the Lhcb1/2 polypeptides. When PSI is overexcited, the protein kinase is activated, and phosphorylated LHCCI serves as an antenna for PSI; when PSI is overexcited by far red-enriched light, the kinase is inactive, and nonphosphorylated LHCCI transfers energy only to PSI. In the asLhcb2 plants, state transitions cannot occur because only Lhcb1 and Lhcb2 polypeptides have the phosphorylation site. As a compensatory response to the absence of state transitions, there is an increase in LHCII, mostly the heterodimer of Lhca1-Lhca4. It is not clear yet whether this extra LHCI is permanently or transiently bound to PSI, reflecting a new mechanism reversibly controlling the PSI cross-section in the asLhcb2 plants.

A Concerted Compensatory Response of the Photosynthetic Light Harvesting System—All of the described alterations in PSII and PSI in asLhcb2 plants reveal a complex and coordinated response. First, extra Lhcb polypeptides accumulate to compensate for the decreased PSI antenna size. Second, extra PSII is synthesized to balance the rate of electron delivery from PSI with the capacity of PSI. Third, trimers are assembled from normally monomeric complexes. Fourth, the pigment properties of the new trimers are adjusted to mimic the wild type LHCCIb trimer. Fifth, extra LHCI is synthesized to compensate for the absence of state transitions. This is the first finding of such a complex and multilevel response of the photosynthetic membrane to a genetic alteration in polypeptide biosynthesis and indicates a high level of plasticity, in which structure and function of PSI-LHCCI may be preserved despite different protein compositions.

To achieve this plasticity there has to be control over the expression of photosynthetic genes. A decrease in LHCII content in the as plants would decrease the reduction state of the electron transfer chain, eliciting a response equivalent to underexcitation of PSII; according to the theory of Pfannschmidt et al. (45), this would increase the transcription of plastocyanin genes encoding PSI reaction center proteins. In addition there would be a decrease in overall PSII electron transport, a decreased excitation pressure, as seen under limiting irradiance (46), which is known modulate the levels of all of the thylakoid membrane complexes, including LHCCI and LHCI (47, 48). An additional factor may be the coordination of chlorophyll and protein synthesis; the absence of the main population of chlorophyll-binding proteins may in some way signal the accumulation of alternative binding proteins. There is currently little understanding of how these latter factors control expression of nuclear encoded genes and which of the numerous putative plastid signals are involved in coordinating chloroplast and nuclear gene expression (49). Which of the numerous possible levels of control is used is also not clear: transcription, translation, or modulation by protein degradation, in which protein folding, pigment binding, assembly of subunits into complexes, and construction of supramolecular organizations may all be regulated. The observation of CP26 accumulation in the asLhcb2 plants provides new insights into these processes. The compensatory accumulation of LHC proteins in the absence of LHCCII seems not to be reflected by an increase in their mRNA levels, suggesting that the protein content is determined at least in part by proteolysis of those not

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**FIGURE 8.** Photosynthetic electron transfer and antenna function in wt and asLhcb2 plants. In the wt, imbalance between the electron transfer rates of PSII and PSI is corrected by means of state transitions, in which a portion of LHCIIb trimers, phosphorylated by the redox-sensitive protein kinase, can associate with PSI and contribute to an increase in its absorption cross section. In the asLhcb2 plants the absence of LHCIIb trimers is compensated for by accumulation of CP26 trimers and by an increase in content of PSI. The mobile phospho-LHCII pool is absent, and they are unable to perform state transitions; photosystem I gains extra loosely bound LHCI that may compensate for excitation energy imbalance between photosystems. LHCI (red) and LHCIIb (blue), light harvesting antenna of photosystem I and II, respectively; RCI and II, reaction centers; electrons; PQ and PQH₂, intermediate electron carrier plastoquinone in oxidized and reduced forms. Shown in green are the minor LHCI antennae, totally monomeric in the wt, but including CP26 trimers in asLhcb2.

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3 M. Wentworth, unpublished data.
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properly assembled into the thylakoid membrane protein complexes. The gene coding for the protease (AtFtsH6) responsible for the degradation of LHCII subunits seems to be constitutively expressed (50), the proteolytic activity appearing to be regulated by the availability of substrate, not the level of the protease. Therefore, trimers or monomers properly assembled into the LHCII-PSII supercomplex may be resistant to the protease, whereas free trimers or monomers can be degraded. Trimerization may be an integral part of the process of supercomplex assembly, in which vacant sites in the growing complex are templates for the trimerization of available proteins, or the availability of chl b and phospholipids may drive the trimerization. In either case, it is assumed that in the wt, the dominant levels of Lhcb1 and Lhcb2 simply out-compete the smaller amounts of Lhcb5 for pigment binding, trimerization, and macroassembly. Therefore, excess Lhcb5 would be degraded.

In the absence of LHCIIb, however, CP26 trimers are stabilized by binding to PSII, resulting in an accumulation of CP26 in the thylakoids. Clearly, the study of plants in which the levels of specific subunits have been genetically manipulated is a promising approach to gaining an understanding the complex events involved in regulating the composition and assembly of thylakoid membrane.

REFERENCES

1. Jansson, S. (1999) Trends Plant Sci. 4, 236–240
2. Kuhlbrandt, W., Wang, D. N., and Fujii, Y. (1994) Nature 367, 614–621
3. Liu, Z., Yan, M., Wang, K., Kuang, T., Zhang, J., Gui, L., An, X., and Chang, W. (2004) Nature 428, 287–292
4. van Amerongen, H., and van Grondelle, R. (2001) J. Phys. Chem. B 105, 604–617
5. Novoderezhkin, V. I., Palacios, M. A., van Amerongen, H., and van Grondelle, R. (2005) J. Phys. Chem. B 109, 10493–10504
6. Haldrup, A., Jensen, P. E., Lunde, C., and Scheller, H. V. (2001) Plant Mol. Biol. 47, 655–684
7. Moya, I., Silvestri, M., Vallon, O., Cignetti, G., and Bassi, R. (2001) Biochemistry 40, 12552–12561
8. Pascal, A. A., Liu, Z., Broess, K., van Oort, B., van Amerongen, H., Wang, C., Horton, P., Robert, B., Chang, W., and Ruban, A. V. (2005) Nature 436, 134–137
9. Dekker, J. P., and Boekema, E. J. (2005) Plant Physiol. 137, 693–703
10. Dekker, J. P., and Jansson, S. (2003) Plant Mol. Biol. 50, 157–169
11. Anderson, J. M., Chow, W. S., and Park, Y.-I. (1995) Trends Plant Sci. 4, 471–478
12. Bailey, S., Walters, R. G., and Horton, P. (2001) Biochemistry 40, 3065–3073
13. Ruban, A. V., and Horton, P. (1991) Biochim. Biophys. Acta 1092, 30–38
14. Ruban A. V., and Trach, V. V. (1991) Biochim. Biophys. Acta 1102, 12–39
15. Yakushevska, A. E., Keegstra, W., Boekema, E. J., Dekker, J. P., Jansson, S., and Horton, P. (2005) Nature 436, 2176–2189
16. Andersson, J., Wentworth, M., Walters, R. G., Howard, C., Ruban, A. V., Dekker, J. P., and Jansson, S. (2005) Biochemistry 44, 10493–10504
17. Porra, R. J., Thompson, W. A., and Kriedemann, P. E. (1989) Biochim. Biophys. Acta 99, 7554–7559
18. Smith, G. K., and Speed, T. (2003) Methods 31, 265–273
19. Porra, R. J., Thompson, W. A., and Kriedemann, P. E. (1989) Biochim. Biophys. Acta 975, 134–137
20. Dainese, P., Hoyer-Hansen, G., and Bassi, R. (1990) Biochim. Biophys. Acta 10493–10504
21. Jansson, S., Andersen, B., and Scheller, H. V. (1996) Annu. Rev. Plant Physiol. Plant Mol. Biol. 47, 655–684
22. Dainese, P., Hoyer-Hansen, G., and Bassi, R. (1990) Biochim. Biophys. Acta 105, 409–420
23. Allemeersch, J., Van Roon, H., Jansson, S., and Horton, P. (2003) Biochim. Biophys. Acta 1501–1510
24. Anderson, J. M., Chow, W. S., and Park, Y.-I. (1995) Trends Plant Sci. 4, 471–478
25. Bailey, S., Walters, R. G., and Horton, P. (2001) Biochemistry 40, 3065–3073
26. Ruban, A. V., Pascal, A., and Robert, B. (2000) FEBS Lett. 477, 181–185
27. Haldrup, A., Jensen, P. E., Lunde, C., and Scheller, H. V. (2001) Trends Plant Sci. 6, 301–305
28. Jansson, S., Andersson, B., and Scheller, H. V. (1996) Plant Physiol. 112, 409–420
29. Porra, R. J., Thompson, W. A., and Kriedemann, P. E. (1989) Biochim. Biophys. Acta 975, 134–137
30. Dainese, P., Hoyer-Hansen, G., and Bassi, R. (1990) Biochim. Biophys. Acta 10493–10504
31. Jansson, S., Chang, W., Dekker, J. P., and Jansson, S. (2005) Biochemistry 44, 10493–10504