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Composition of Photosystem II Antenna in Light-Harvesting Complex II Antisense Tobacco Plants at Varying Irradiances[1]

Ralf Flachmann*

European Molecular Biology Laboratory, Meyerhofstrasse 1, D 69117 Heidelberg, Germany

Plants with genes coding for chlorophyll *a/b*-binding proteins of light-harvesting complex II (LHCII) in antisense orientation (*Lhcb*) that are characterized by severely reduced *Lhcb* transcript levels (below 10% of wild type) do not show a bleached phenotype due to a specific loss of the polypeptide. To produce such a phenotype, a conceptually different antisense approach was tested with a dual-functional transcript encoding the gene for hygromycin phosphotransferase and the transit sequence of *Lhcb1-2* in the antisense orientation. Using increasing concentrations of hygromycin, transformants with *Lhcb* steady-state levels as low as 9% of wild type were regenerated and grown in a growth chamber. Together with *Lhcb* antisense plants obtained in an earlier study, these antisense plants were analyzed biochemically for their photosystem II (PSII) antenna composition under varying light conditions. All antisense plants showed a characteristic low-irradiance-induced increase of their PSII antenna size as determined by higher chlorophyll concentrations, an increased content of LHCII, and a constant chlorophyll *b*-to-lutein ratio in comparison with control plants. One to 5% of the total *Lhcb* transcript amount was sufficient to allow unrestricted formation of the PSII antenna at low irradiance, suggesting that LHCII biogenesis is not controlled primarily by transcription.

The initial steps in the conversion of light energy into chemical energy are performed by multisubunit protein-pigment complexes of the thylakoid membrane. In PSII most of the chlorophylls are attached to LHCII, which alone accounts for roughly 50% of total chlorophyll in the biosphere and for almost all of the Chl *b* in green plants. The atomic model of LHCII indicates that each polypeptide binds a minimum of 12 chlorophylls, 7 Chl *a* and 5 Chl *b* (Kühlbrandt et al., 1994). In addition to two structurally important lutein molecules that form an internal cross-brace (Kühlbrandt et al., 1994), two other carotenoids, neoxanthin and violaxanthin, are bound to the protein, probably at the periphery of the trimer.

In most photosynthetic organisms, the biogenesis of Cab proteins is light-dependent; etiolated plants do not accumulate Cab proteins at all, and plants grown in intermittent light contain only traces of Cab proteins. Upon insertion into the thylakoid membrane the yet-unfolded, pigment-free LHCII apoprotein folds into a protein-pigment complex by accommodating chlorophylls and carotenoids. It has been hypothesized that chlorophylls especially might be essential to induce correct folding of the apoprotein (Apel and Kloppstech, 1980; Mullet et al., 1980; Bennett, 1981; Bellemare et al., 1982). Since chlorophyll synthesis is light-dependent in higher plants, specifically at the step of chlorophyllide formation, the availability of chlorophylls might be one controlling factor in the biogenesis of LHCII at the posttranslational level.

In this study a conceptually different approach was used to create an expression system for highly expressed antisense RNA in transgenic tobacco (*Nicotiana tabacum*) plants. In general, a dual-functional transcript is produced that encodes in antisense orientation the gene of interest, which is physically linked to an antibiotic resistance gene to select for transformants with high-level expression of antisense RNA via increased concentrations of antibiotics. This approach was tested here with the strong, inducible plastocyanin promoter of pea, which transcribed a DNA sequence encoding both the *hph* and the *Lhcb* antisense RNA in a dual-functional transcript. Transformants were selected based on resistance toward high concentrations of hygromycin and analyzed for their content of endogenous *Lhcb* transcripts. Among 21 plants tested, a few showed greatly reduced *Lhcb* mRNA concentrations.

Several *Lhcb* antisense plants with severely reduced *Lhcb* transcript levels had been obtained earlier by the use of the 35S CaMV promoter and *Lhcb* sequences of varying lengths. According to the *Lhcb* sequence used, those plants were designated anticab and antiTP plants (Flachmann and Kühlbrandt, 1995). From the three different antisense RNA approaches, antisense plants with significantly lower amounts of *Lhcb* transcripts were selected for further biochemical and physiological analysis. The plants were tested for their contents of individual antenna proteins of PSII, namely the gene products of *Lhcb1*, *Lhcb2*, and *Lhcb4*, *Lhcb5*, and *Lhcb6*, known as LHCII, and the three minor complexes CP24, CP26, and CP29. Additionally, the question was raised whether low amounts of *Lhcb* mRNA might be limiting for the low-irradiance-induced increase in PSII antenna size. Therefore, antisense and control plants were also analyzed for their LHCII content and pigment composition after adaptation to low irradiance. All *Lhcb* antisense plants appeared to be unchanged from wild type in their composition of PSII antenna complexes under both high

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* E-mail flachmann@embl-heidelberg.de; fax 49-6221-387-306.

Abbreviations: antiTP, anti-transit peptide; Cab, chlorophyll *a*/*b*-binding; CaMV, cauliflower mosaic virus; Chl *a*, chlorophyll *a*; Chl *b*, chlorophyll *b*; F_m, maximal fluorescence; F_o, initial fluorescence; *hph*, gene for hygromycin phosphotransferase; LHCII, light-harvesting complex II; *Lhcb*, genes coding for chlorophyll *a*/*b*-binding proteins of LHCII.

and low irradiances. These results indicated that 1 to 5% of all *Lhcb* transcripts were sufficient to allow adaptation of the PSII antenna size to low irradiance by a 2-fold increase in LHCII concentration.

MATERIALS AND METHODS

Construction of the Binary Vector

The binary vector antiTP containing the 105-bp-long transit sequence of the Cab protein as *Hin*dIII/*Sal*I fragment (Flachmann and Kühlbrandt, 1995) was redesigned in such a way that the coding sequence of *hph* was cloned immediately 5′ of the transit sequence as *Bam*HI/*Hin*dIII fragment. The *hph* coding sequence was amplified by PCR from plasmid pBIB-HYG (Becker, 1990) using the primers 5′Bam-Hyg (5′-TTTGGATCCATGAAAAAGCCTGAA-3′) and 3′HIII-Hyg (5′-CCCCAAGCTTCTATTCCT-TTGCCCT-3′). The construct was driven by the plastocyanin promoter of pea, which was cloned as *Bam*HI fragment. The promoter was amplified by PCR from plasmid pKHn2 (Pwee and Gray, 1993) using the primers 5′Bam-Pet (5′-TTTTGGATCCTATGCAACTTACAACG-3′) and 3′Bam-Pet (5′-CCATGGATCCTTTTTCTCAAGAGTAAT-3′). Using those primers, plasmids were screened for correct fragment orientation by PCR. Recombinant DNA techniques were performed according to standard procedures (Sambrook et al., 1989).

Agrobacterium-Mediated Transformation and Growth of Plant Material

The antisense DNA construct (Fig. 1) was introduced into *Agrobacterium tumefaciens* strain LBA4404 (Hoekema et al., 1983) by electroporation according to the manufacturer's instructions (Bio-Rad). Transformants were used to infect leaf discs of tobacco (*Nicotiana tabacum* cv Petit Havanna SR1) (Maliga et al., 1973). The infected discs were placed on tissue culture medium containing various hygromycin concentrations (see "Results") and regenerated essentially as described by Horsch et al. (1985). After a few weeks putative transformants were transferred to soil and grown under growth chamber conditions (300 μE m^{-2} s^{-1}, 16 h of light/8 h of dark, 25°C). Low-light conditions were 60 to 80 μE m^{-2} s^{-1} (16 h of light/8 h of dark, 25°C).

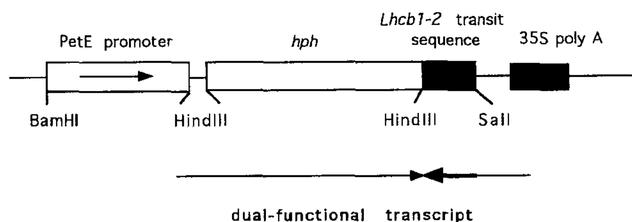

Figure 1. Structure of the dual-functional transcript. The dual-functional transcript encodes in the 5′ to 3′ direction the reading frame for *hph* and in the 3′ to 5′ direction (antisense orientation) the transit sequence of *Lhcb1-2*. The sequences are transcribed from the plastocyanin promoter PetE and terminated by the poly(A) site of the CaMV 35S RNA.

Experiments were repeated independently using in vitro-propagated clones of transformants and plants from the T_1 generation.

Preparation of RNA, Hybridization, and Quantitation

RNA was isolated (Logemann et al., 1987) from the second mature leaf of soil-grown transgenic and wild-type plants. For comparative RNA gel-blot analysis, leaf material was harvested after 4 h of illumination within a time interval of 5 to 10 min. For hybridizations, 15 μg of total RNA was electrophoresed through a 1% agarose gel containing 2.2 M formaldehyde (Sambrook et al., 1989) and blotted to GeneScreen Plus membranes (DuPont). Double-stranded probes were generated by isolation and radioactive labeling (Feinberg and Vogelstein, 1983). Filters were hybridized at 65°C according to Church and Gilbert (1984) with the N-terminal half of the tobacco gene *Lhcb1-2* (305 bp in length, designated Lhcb) and rehybridized with a soybean 18S gene (Eckenrode et al., 1985) to ensure that equal amounts of RNA were present. The hybridization signals were quantitated in cpm directly from the blots using a scanner (PhosphorImager, Molecular Dynamics, Sunnyvale, CA).

Thylakoid Isolation, Chlorophyll Determinations, and Protein Gel Blotting

Chloroplasts were prepared from leaves of wild-type and antisense plants by homogenization in 0.33 M sorbitol/10 mM NaCl, pH 7.8, with a blender (Waring) and passed through cotton wool and Miracloth (Calbiochem). Chloroplasts were collected by centrifugation at 1500*g* for 5 min. Thylakoid membranes were isolated after osmotic shock in 10 mM NaCl/1 mM *N*-tris(hydroxymethyl)methylglycine, pH 7.8, for 15 min at room temperature, centrifuged for 15 min at 6000*g*, and subsequently washed in 10 mM NaCl/1 mM *N*-tris(hydroxymethyl)methylglycine/5 mM EDTA, pH 7.8. Thylakoid membranes were solubilized in 0.8% Triton X-100. Chl *a* and Chl *b* values were determined in 80% acetone according to the method of Porra et al. (1989). The concentration of carotenoids was determined in 80% acetone by the method of Lichtenthaler (1987). For protein gel blots, membranes were resuspended in 10% Suc, 20 mM sodium carbonate, and 100 mM DTT and sonicated twice for 15 s with a sonifier (Branson, Danbury, CT). Protein gel electrophoresis was performed according to Laemmli (1970). Separated proteins were electrophoretically transferred to Immobilon-P (Millipore). Immunodetection by chemiluminescence using polyclonal and monoclonal antibodies was performed according to the manufacturer's instructions (Amersham). Monoclonal antibodies against LHCII and CP29 were produced from the hybridoma cell lines American Type Culture Collection (ATCC) no. CRL1766 and ATCC no. CRL1779.

Reversed-Phase HPLC of Thylakoid Pigments

The HPLC was a chromatographic system (626 pump and 600S controller, Waters) equipped with a photodiode array detector 996 (Waters). All solvents were HPLC grade

and obtained from Merck (Darmstadt, Germany). A Spherisorb ODS-1 column (5 μm particle size, 250 mm $\times$ 4.6 mm i.d.) was purchased from Alltech (Munich, Germany). The flow rate for all separations was 2 mL/min. Pigments were eluted with acetonitrile/methanol/0.1 M Tris-HCl (pH 8.0) (75:12:4) and run isocratically for 0 to 4 min followed by a 2.5-min linear gradient to 100% methanol/hexane (4:1), according to the work of Gilmore and Yamamoto (1991).

In Vivo Chlorophyll Measurements

In vivo chlorophyll fluorescence was measured with leaf discs that had been predarkened overnight at room temperature. Using the pulse-amplitude modulated fluorometer (Walz, Effeltrich, Germany), the determination of F_o and F_m using the saturating flash (3200 μmol m^{-2} s^{-1} for 700 ms) were performed according to the method of Schreiber et al. (1986). Each measurement was performed independently at least twice on three T_1 plants.

RESULTS

Transformants Expressing a Dual-Functional Transcript of "Sense" and "Antisense" Sequences

The 105-bp transit sequence of tobacco *Lhcb1-2* was transcribed in antisense orientation by the strong plastocyanin promoter of pea to create a selection system for highly expressed antisense RNA. This promoter is active in tobacco and initiates transcription five to seven times more frequently than the 35S promoter when analyzed in a transcriptional fusion with the GUS reporter gene (Pwee and Gray, 1993). Simultaneously, the plastocyanin promoter transcribed *hph*, the gene for hygromycin phosphotransferase, producing a dual-functional transcript (Fig. 1). *A. tumefaciens* containing the binary vector was used for transformation via the leaf disc technique. Silencing the expression of *Lhcb* genes could result in transformants with reduced chlorophyll content. Instead of selecting for pale-looking plantlets, the initial hygromycin concentration was elevated from 25 to 50 mg/L to produce calli. For rooting, developing shoots were transferred to rooting medium with a hygromycin concentration of 100 mg/L. Only those plants that grew under the selective pressure of this high hygromycin concentration were kept in tissue culture and transferred to the growth room.

Severely Reduced Steady-State Levels of *Lhcb* mRNA by a Dual-Functional Transcript

Can a strong antisense effect be achieved when a 105-bp short transit sequence is attached to the 3′ end of a much longer transcript? Is the strong plastocyanin promoter comparable to the 35S CaMV promoter in repressing *Lhcb* gene expression? The amount of *Lhcb* transcripts was determined in leaves of transgenic plants after transfer of primary transformants to the growth chamber. Twenty-one plants were analyzed by RNA gel-blot analysis with the radioactively labeled N-terminal half of *Lhcb1-2* (Fig. 2, Lhcb). Hybridization signals were quantitated directly from filters by phosphor imager scanning. From the intensities three classes of antisense plants could be distinguished: the steady-state level of *Lhcb* transcripts was reduced by 30, 60, and more than 60%. The reduction in *Lhcb* steady-state levels was calculated from the ratio of 18S to *Lhcb* mRNA. The most interesting plants of the latter groups were Pla-3, Pla-12, and Pla-20, with 23.1, 22.5, and 9.7% of wild-type *Lhcb* message remaining, respectively (Figs. 2 and 3). These plants were analyzed biochemically in more detail.

Pigment Accumulation in Antisense and Control Plants

Five distinct LHCII pigment-protein complexes have been characterized. LHCII (Chl *a*/Chl *b* ratio of 1.3–1.4; Kühlbrandt et al., 1994) is encoded by the *Lhcb1* and *Lhcb2* genes and binds 42% of the total chlorophyll of the thylakoid membrane; the three minor complexes CP24 (Chl *a*/Chl *b* ratio of 0.9), CP26, and CP29 (Chl *a*/Chl *b* ratio of 2.2) are encoded by the genes *Lhcb6*, *Lhcb5*, and *Lhcb4*, respectively, and each contributes only 3 to 4% of the total chlorophyll in the thylakoid membrane (Dreyfuss and Thornber, 1994). A fourth component, LHCIIe, has only recently been isolated, and the corresponding gene has not yet been sequenced (Jansson, 1994).

A dramatic depletion of LHCII from the thylakoid membrane should have resulted in a phenotypic change of leaf coloration. These phenotypic changes were not observed in the antisense plants when compared with control plants. The total amount of Chl *a* varied between 85 and 107% when compared with the control value (with an average value of 100 $\pm$ 7%; Table I). The content of Chl *b*, which is mainly bound by LHCII, remained between 90 and 103% of wild type in all antisense plants (Table I), with the exception of Pla-6 (81%). More importantly, the ratios of Chl *a* to Chl *b* (with an average of 3.77 $\pm$ 0.2) and chlorophylls to carotenoids (with an average of 5.50 $\pm$ 0.42) were virtually identical in antisense and control plants.

In vivo measurements of chlorophyll fluorescence supported the biochemical data. Photochemical and nonphotochemical quenching parameters were unchanged between antisense and control plants (data not shown). F_o values, thought to demonstrate emission by excited antenna Chl *a* molecules of LHCII before they have migrated to the reaction centers, were unaltered in antisense plants and controls. Additionally, F_m/F_o ratios were almost iden-

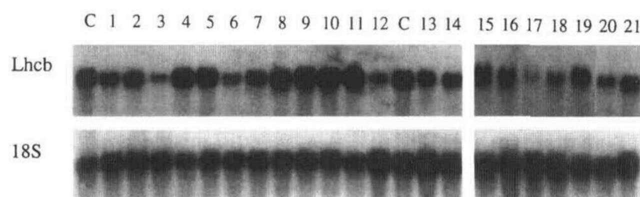

Figure 2. Relative abundance of *Lhcb* transcripts in transgenic tobacco. Fifteen micrograms of total RNA isolated from control and antisense plants Pla-1 to Pla-20 was transferred onto nitrocellulose filters. The filters were successively hybridized with radioactively labeled probes encoding the reading frame for the mature LHCII protein (Lhcb) and a soybean 18S probe to check for equal RNA loading. C, Control plant.

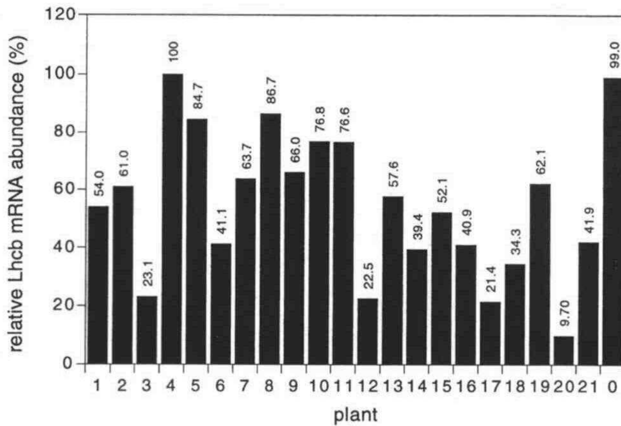

Figure 3. Quantitation of Lhcb mRNA steady-state levels in transgenic tobacco. Hybridization signals obtained with the *Lhcb* and 18S probe (see Fig. 2) were quantitated directly by phosphor imager scanning. Relative transcript levels were calculated from the ratio of 18S to Lhcb in comparison with wild type. Actual numbers (in percentages) are indicated above each column. 0, Control plant SR1.

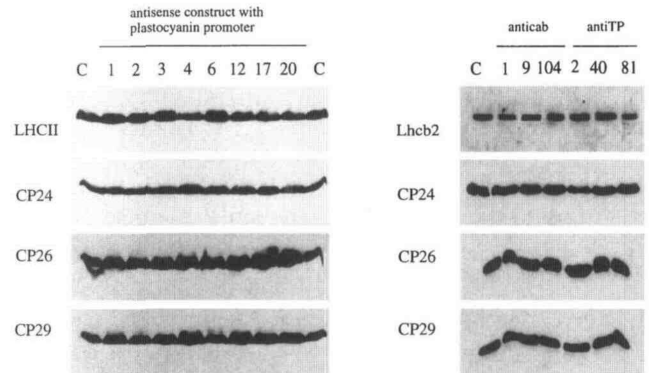

Figure 4. Protein gel analysis of protein-pigment complexes from leaves of transgenic and control plants. Selected Pla plants (left) and various anticab and antiTP plants (right) with reduced *Lhcb* transcript levels were analyzed. Thylakoid membrane proteins were solubilized in Triton X-100, separated by PAGE, and blotted onto nitrocellulose filters. The filter-bound proteins were identified using monoclonal antibodies for LHCII, CP24, CP26, and CP29. LHCB2 was detected by a polyclonal antibody.

tical among the transgenic and control plants (Table I, average of 4.46 ± 0.62).

Accumulation of Cab Proteins in Antisense Plants

Protein gel-blot analysis and immunodetection provided a direct way to compare the amount of LHCII pigment-protein complexes. By far the most abundant antenna protein is LHCII, which accounts for 67% of the antenna chlorophyll in PSII (Peter and Thornber, 1991), whereas the less abundant pigment-containing antenna complexes contribute 15% of the antenna chlorophyll and the PSII core contributes 14% of the chlorophyll. Both of the antisense plants that were produced in this study, and anticab and antiTP plants obtained in a previous study (Flachmann and Kühlbrandt, 1995) were analyzed for their accumulation of antenna proteins. As expected from the unchanged phenotype in antisense plants Pla-3, Pla-6, Pla-12, Pla-17, and Pla-20, no significant changes in the concentration of LHCII (Fig. 4, left) could be observed in comparison with wild type. The amount of the three minor LHCII pigment-protein complexes CP24, CP26, and CP29 is too low in thylakoid membranes so that changes in their concentration were phenotypically invisible. Thylakoids were isolated and solubilized, and 8 μg of protein was separated on PAGE and blotted onto nitrocellulose membranes. As observed for LHCII, the concentration of the minor complexes was reduced neither in absolute terms nor in relation to LHCII when analyzed by immunodetection (Fig. 4, left). When the N-terminal half or the transit sequence of *Lhcb* was transcribed as antisense RNA by the 35S CaMV promoter (anticab and antiTP plants), the endogenous amounts of *Lhcb* transcripts were dramatically reduced (Flachmann and Kühlbrandt, 1995). However, no reduction in the accumulation of antenna proteins was observed (Fig.

Table I. *Amount of pigment and pigment ratios in thylakoid membranes*

Thylakoid membranes were isolated from chloroplasts after osmotic shock. Concentrations of Chl *a* and Chl *b* were measured in 80% acetone (Porra et al., 1989); carotenoid determinations were performed according to the method of Lichtenthaler (1987). All measurements were repeated at least four times. Mean and SD values are indicated.

Plant	*Lhcb* mRNA	Chl *b*	Chl *b*	Chl *a*	Chl *a*	Carotenoid	Chl *a*/Chl *b*	Chl *a* + *b*/ carotenoids	F_m/F_o
	% of control	*μg g⁻¹ fresh wt*	*% of control*	*μg g⁻¹ fresh wt*	*% of control*	*μg g⁻¹ fresh wt*			
Pla-1	54	363 ± 6.6	93	1437 ± 39	103	328 ± 1.2	3.96	5.49	4.48 ± 0.08
Pla-2	61	352 ± 9.0	90	1424 ± 32	102	360 ± 8.7	4.05	4.93	3.80 ± 0.35
Pla-3	23	367 ± 8.7	94	1332 ± 58	95	286 ± 7.2	3.62	5.94	4.06 ± 0.18
Pla-4	100	404 ± 27	103	1471 ± 81	105	328 ± 5.2	3.47	5.72	3.01 ± 0.40
Pla-5	85	351 ± 21	90	1196 ± 70	85	281 ± 3.4	3.41	5.51	4.69 ± 0.06
Pla-6	41	315 ± 16	81	1263 ± 76	90	349 ± 1.1	4.01	4.52	4.66 ± 0.11
Pla-12	23	393 ± 14	101	1495 ± 88	107	337 ± 4.8	3.80	5.60	4.94 ± 0.08
Pla-17	21	389 ± 16	99	1499 ± 75	107	319 ± 2.9	3.85	5.92	4.86 ± 0.23
Pla-20	9	379 ± 27	97	1503 ± 61	107	328 ± 8.5	3.97	5.74	5.09 ± 0.23
Control	100	391 ± 25	100	1401 ± 72	100	317 ± 6.2	3.58	5.65	4.99 ± 0.24
Averaged		371 ± 25	95 ± 6	1402 ± 101	100 ± 7	323 ± 23	3.77 ± 0.2	5.50 ± 0.42	4.46 ± 0.62

Table II. *Pigment content of low-light-grown tobacco plants*

Chloroplasts were isolated from the third and fourth mature leaves of antisense and control plants in two independent experiments. Pigment concentrations for chlorophylls and carotenoids were determined from purified thylakoids in 80% acetone. Lutein concentrations were measured three times by HPLC. Values were measured with three plants in at least six individual measurements. Mean and SD values are indicated.

Plant	Chl *a*	Chl *b*	Carotenoid	Lutein	Chl *b*	Chl *a*	Chl *b*	Carotenoid	Lutein
	μg g⁻¹ fresh wt	*μg g⁻¹ fresh wt*	*μg g⁻¹ fresh wt*	*μg g⁻¹ fresh wt*	*% of control*	*mg m⁻²*	*mg m⁻²*	*mg m⁻²*	*mg m⁻²*
anticab-1	2114 ± 359	835 ± 154	509 ± 56	313 ± 32	76	96 ± 15	38 ± 5	23 ± 5	14 ± 2
antiTP-2	2487 ± 264	991 ± 98	618 ± 58	380 ± 25	90	100 ± 6	40 ± 2	25 ± 2	15 ± 2
Pla-20	2497 ± 494	1006 ± 194	642 ± 122	395 ± 30	92	112 ± 10	45 ± 4	29 ± 2	18 ± 2
Control	2728 ± 453	1099 ± 214	708 ± 105	435 ± 37	100	119 ± 2	48 ± 1	31 ± 1	19 ± 1
Averaged	2457 ± 220	983 ± 95	620 ± 72	381 ± 44	90 ± 8.6	107 ± 9	43 ± 4	27 ± 3	17 ± 2

4, right). It was shown previously that *Lhcb1* and *Lhcb2* genes were equally well repressed in anticab-104 by *Lhcb* antisense RNA (Flachmann and Kühlbrandt, 1995). Although only 1 to 5% of *Lhcb* steady-state RNA remained in anticab-104 when compared with wild type (Flachmann and Kühlbrandt, 1995), the content of LHCII, LHCB2, and the minor antenna complexes CP24, CP26, and CP29 did not decrease.

Can a Phenotype Be Induced by Varying the Growth Condition?

Under normal growth conditions the amount of chlorophyll is not a limiting factor for photosynthesis. Plants adapt to the changing light conditions by adjustments in the thylakoid membrane, in particular via increases or decreases in their PSII antenna size (Anderson, 1986). The three T_1 antisense lines Pla-20, anticab-1, and antiTP-2 (Flachmann and Kühlbrandt, 1995), together with control plants, were transferred to low irradiance (60–80 μE m^{-2} s^{-1}). After 3 weeks of growth all leaves were dark green without visible lack in pigmentation. Quantitation of chlorophylls and carotenoids were performed (Lichtenthaler, 1987; Porra et al., 1989). Generally, the amount of both Chl *a* and Chl *b* as well as carotenoids increased 2-fold (3.4 mg of total chlorophyll/g fresh weight, Table II) in comparison with high irradiance (1.7 mg of total chlorophyll/g fresh weight, Table I). At low irradiance the concentrations of total chlorophyll in the antisense lines were not decreased dramatically in comparison with wild type (2457 ± 220 μg of chlorophyll/g fresh weight); however, the content of Chl *b* in lines anticab-1 (76%) and antiTP-2 (90%) were reduced in relation to wild type (Table II). More predictive, however, were the pigment ratios of chlorophyll and carotenoids (Table III). Although absolute amounts of chlorophyll seemed to be reduced in some antisense plants, the Chl *a*/Chl *b* ratio and in particular the Chl *b*/lutein ratio remained unaltered at low irradiance.

In addition to the spectrophotometrical analysis, pigments were extracted from dark-adapted thylakoids and separated by reversed-phase HPLC. Figure 5 shows a typical chromatogram of 1.2 μg of chlorophyll extract of the antisense plant Pla-20 (left) and the control plant (right). Lutein was quantitated by its extinction coefficients (Gilmore and Yamamoto, 1991) and the ratio of Chl *b* to lutein was calculated. Both pigments are essential ligands of LHCII for functional light-harvesting and protein stability. It is interesting that this ratio remained constant among antisense and control plants (2.59 ± 0.05, Table III), indicating equal amounts of LHCII in thylakoids of antisense and control plants. When judged by a dilution series and immunodetection, altered accumulation of LHCII in antisense and control plants was not observed (Fig. 6). The low irradiance did not restrict the antisense plants in their ability to accumulate PSII antenna proteins and in particular LHCII at normal levels when compared with control plants.

Did de-etiolated plants exhibit a phenotype? The apex and two small, developing leaves of the antisense plants Pla-20, anticab-9, and antiTP-2 were propagated vegetatively on Murashige-Skoog medium and incubated in the dark at 25°C. After 4 weeks plants were grown in soil at low irradiance for 3 weeks. Sections from the base and top of individual leaves were analyzed spectroscopically for their pigment concentration and immunologically (not

Table III. *Pigment ratios in thylakoid membranes of low-light-grown tobacco plants*

Pigments were determined from the third and fourth mature leaves in two independent experiments; ratios were calculated from values given in Table II. Mean and SD values are indicated.

Plant	Chl *b*	Chl *a* + *b*	Chl *b*	Chl *b*	Chl *b*	Chl *a* + *b*	Chl *b*
	μg μg⁻¹ carotenoid g⁻¹ fresh wt	*μg μg⁻¹ carotenoid g⁻¹ fresh wt*	*μg μg⁻¹ lutein g⁻¹ fresh wt*	*μg μg⁻¹ Chl a g⁻¹ fresh wt*	*μg μg⁻¹ carotenoid m⁻²*	*μg μg⁻¹ carotenoid m⁻²*	*μg μg⁻¹ lutein m⁻²*
anticab-1	1.64	5.79	2.67	2.53	1.65	5.83	2.68
antiTP-2	1.60	5.62	2.61	2.51	1.60	5.60	2.60
Pla-20	1.57	5.46	2.55	2.48	1.55	5.41	2.52
Control	1.55	5.41	2.53	2.48	1.55	5.39	2.52
Averaged	1.59 ± 0.03	5.57 ± 0.15	2.59 ± 0.05	2.50 ± 0.02	1.59 ± 0.04	5.56 ± 0.18	2.58 ± 0.07

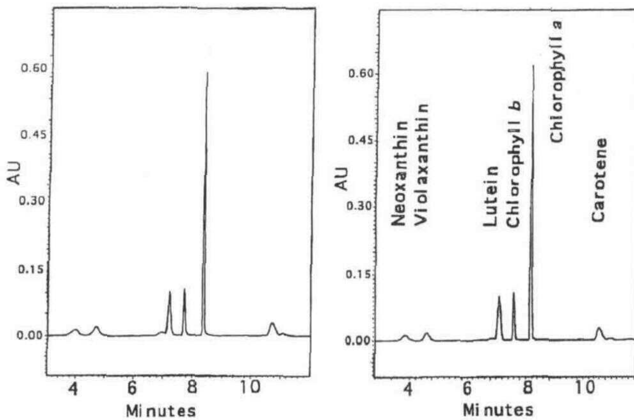

Figure 5. HPLC profile of pigments extracted from thylakoids. Leaf discs of antisense plant Pla-20 (left) and control plant SR1 (right) were dark-adapted overnight. Pigments were extracted, separated by reverse-phase HPLC, and identified by their retention times and absorbance spectra.

shown) for the accumulation of chlorophyll- and carotenoid-binding proteins of the Cab family. The second leaf from the top was analyzed. Control plants accumulated 111 ± 2 mg chlorophyll m^{-2} at their leaf bases and 88 ± 7 mg chlorophyll m^{-2} at their leaf tips. The antisense plants anticab-1, antiTP-2, and Pla-20 accumulated 95 to 120 mg chlorophyll m^{-2} at their leaf bases and between 76 and 125 mg chlorophyll m^{-2} at their leaf tips. These values, however, were statistically not significantly altered in comparison with control plants.

DISCUSSION

High-Level Expression of Antisense RNA

In plants antisense-mediated gene repression has been used almost exclusively in a combination of full-length cDNAs in reverse orientation and the CaMV 35S promoter. Often, antisense RNA was used for "reverse genetics" to analyze the function of unidentified proteins for which cDNAs have been cloned. In these experiments it would be desirable to start with plants that are characterized by frequently transcribed antisense DNA. Generally, the presented antisense approach with a dual-functional transcript allows, on the basis of increasing concentrations of antibiotics, selection for transformants that are characterized by strong expression of the antisense gene. The gene encoding *hph* was cloned 3′ of the plastocyanin promoter, since translation of the second reading frame has been shown to be initiated 500 times less frequently in a dicistronic transcript than translation of the first reading frame (Angenon et al., 1989). However, efficient translation of the *hph* gene was required to screen for transformants that were resistant to high concentrations of hygromycin. Resistance to hygromycin was about four times higher in Pla plants (100 mg/L) than in anticab and antiTP plants (25 mg/L) (Flachmann and Kühlbrandt, 1995).

No Direct Correlation between Amount of *Lhcb* Antisense RNA and *Lhcb* Gene Suppression

Variability in the efficiency of transgenes is seen in almost all antisense approaches and is believed to depend on the site of insertion in the nuclear genome of the different transformants. The use of the plastocyanin promoter in combination with a positive selection for high-level expression of RNA did not lead to a further reduction of endogenous *Lhcb* levels in comparison with the 35S CaMV promoter (Flachmann and Kühlbrandt, 1995). The amounts of antisense transcripts produced did not show a positive correlation with the suppression of the endogenous *Lhcb* genes. With two different promoters similar reductions of the endogenous mRNA levels were achieved, indicating that promoter-controlled parameters such as amount of antisense RNA transcribed, time of antisense RNA expression, and organ specificity were not critical for the suppression of *Lhcb* genes.

Environmental Adaptation of PSII Antenna Is Not Restricted in *Lhcb* Antisense Plants

Neither the amount of chlorophyll (Table I) and LHCII nor the composition of the PSII antenna was altered in most *Lhcb* antisense plants at regular growth conditions. Low irradiance was applied to study the adaptation of LHCII at reduced mRNA levels. It is interesting that even suboptimal light conditions led to increased accumulation of both chlorophylls and xanthophylls (Table II) and to a decrease of the Chl *a*/Chl *b* ratio (from around 3.8 at high light intensity to about 2.5 at low irradiance; Tables I and III) in *Lhcb* antisense plants. This is in agreement with other reports (Anderson et al., 1988) and indicated that the PSII unit size, the ratio of LHCII to PSII core, increased. The

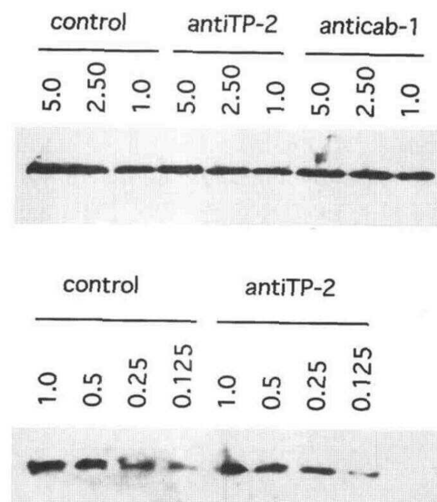

Figure 6. Quantitation of LHCII in plants grown at low irradiance. Thylakoid membrane proteins were solubilized with Triton X-100 and separated by PAGE, and various amounts were blotted onto nitrocellulose filters. Proteins loaded (in μg) are indicated above each lane. Differences in LHCII content were not observed among antisense and control plants when analyzed by immunodetection with monoclonal antibody MLH1.

chlorophyll/carotenoid ratios of 5.50 (at high irradiance) and 5.56 (at low irradiance) were very similar to the ratio of 5.40 in maize thylakoids (Bassi et al., 1993). Additionally, an identical ratio of 5.60 has been determined for isolated LHCII (Bassi et al., 1993). The simultaneous increase of Chl *b* and lutein, which are essential ligands of LHCII (Kühlbrandt et al., 1994), was an additional indication that LHCII accumulated at higher concentration in both antisense and control plants at low irradiance. The Chl *b*/carotenoid ratio of 1.59 ± 0.04 for all tobacco plants tested correlated well with the value of 1.32 determined for maize thylakoids (Bassi et al., 1993). Most interestingly, the Chl *b*/lutein ratio of 2.59 ± 0.04 did not vary significantly among antisense and control plants at low irradiance. Similar values have been determined for maize (2.68 as reported by Bassi et al., 1993) and spinach thylakoids (2.57 as reported by Juhler et al., 1993). The data indicated that in *Lhcb* antisense plants Chl *b* and lutein accumulated at a constant ratio under varying light conditions. The Chl *b*/lutein ratio suggested that five molecules of Chl *b* were needed per two molecules of lutein for proper light-harvesting function at varying light conditions. This relationship is actually reflected by the atomic model of LHCII (Kühlbrandt et al., 1994), in which five molecules of Chl *b* and two molecules of lutein per monomer have been identified. To prevent photooxidative damage, all Chl *b* molecules are in close proximity to the lutein molecules. The minor PSII antenna proteins CP24, CP26, and CP29 also bind carotenoids and lutein in particular. However, their amounts in the thylakoid membrane are too low to affect significantly the Chl *b*/lutein ratio, especially since lutein was shown to associate preferentially with LHCII (Lichtenthaler et al., 1982; Eskins et al., 1983; Siefermann-Harms, 1985; Knoetzel and Simpson, 1991). Immunological quantitation also demonstrated that LHCII accumulated at equal amounts in antisense and control plants under suboptimal light conditions. The low-irradiance-induced increase of LHCII in *Lhcb* antisense plants is a convincing demonstration that, first, about 1 to 5% of *Lhcb* transcripts were sufficient to guarantee normal LHCII biogenesis, and second, posttranscriptional control acts as a key regulatory step in the biogenesis of LHCII in tobacco.

Rieske FeS Antisense Experiments as Model for LHCII-Depleted Plants?

Lhcb transcript levels that were as low as 5% of wild type demonstrated the capacity of antisense plants to adapt to environmental changes by efficiently translating *Lhcb* mRNA. In this respect two results of antisense-mediated suppression of the Rieske FeS subunit are notable. Palomares et al. (1993) reduced the level of Rieske FeS mRNA by 90% without affecting the protein level, whereas a mRNA reduction of 94% induced distinctive phenotypes (Price et al., 1995). In the latter case the antisense phenotypes were recovered at extremely low frequency from 378 independent transformants and were stable at low irradiances only. A further reduction of the *Lhcb* transcript concentration would be needed to achieve a reduction in LHCII content and to induce a phenotype. Astonishingly, the amount of 1 to 5% *Lhcb* mRNA is sufficient to permit the low-irradiance-induced increase in PSII antenna size due to an increased accumulation of LHCII. Additional experiments have to show whether short-term effects during regreening of etiolated antisense plants can be detected. The extreme stability of LHCII, with a turnover of less than 1% per day (Riesselmann and Piechulla, 1992), is probably one of the reasons for the observed differences in mRNA and protein accumulation. This finding prompts the puzzling question: Why are *Lhcb* promoters that strong in transcriptional activities?

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