The Plant Cell, Vol. 7, 149–160, February 1995 © 1995 American Society of Plant Physiologists

RESEARCH ARTICLE

Accumulation of Plant Antenna Complexes Is Regulated by Post-Transcriptional Mechanisms in Tobacco

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Transgenic tobacco plants expressing antisense RNA directed against the multigene family of the light-harvesting complex of photosystem II (LHCII) were raised and analyzed biochemically and physiologically. A partial 5′ terminal sequence with 509 nucleotides complementary to *cab* (chlorophyll *a/b* binding protein) genes reduced the amount of transcript to almost undectectable levels. We demonstrated for endogenous genes that a 5′ terminal sequence with only 52 to 105 nucleotides complementary to the transit sequence of *cab* can be equally efficient in gene repression. Chlorophyll content and chlorophyll *a*-to-chlorophyll *b* ratios of thylakoid membranes isolated from transgenic plants were unchanged in comparison with the wild type. Photosynthetic oxygen evolution and in vivo–measured chlorophyll fluorescence of the transformants showed that LHCII accumulates to normal levels. The reduced level of *cab* mRNA did not correlate with the amount of LHCII in thylakoids. This indicates that transcriptional regulation is not the rate-limiting step in the biogenesis of the LHCII apoprotein. The antenna size of photosystem II is therefore modulated by yet undiscovered post-transcriptional mechanisms.

INTRODUCTION

In photosynthesis, the initial steps in the conversion of light energy into chemical energy are performed by multisubunit protein–pigment complexes of the thylakoid membranes. Oxygen-evolving photosynthetic organisms contain two separate photosystems, photosystem I (PSI) and photosystem II (PSII), which operate in tandem. The photosystems consist of a reaction center core that performs the charge separation and electron transfer reaction and a set of light-harvesting or antenna complexes that collect solar energy and deliver it to the reaction centers. In PSII, the majority of light-absorbing chlorophylls (~200 to 400 per reaction center) are attached to LHCII, the light-harvesting complex associated with this photosystem.

LHCII is the most abundant member of the family of chlorophyll *a/b* binding (Cab) proteins, which include LHCI of four different types, LHCII of types I to III, CP29, CP26, CP24, and early light-induced proteins (Green et al., 1991). LHCII alone accounts for ~50% of total chlorophyll in the biosphere and for most of the chlorophyll *b* (Chl *b*) in green plants. In addition to chlorophyll, LHCII contains the carotenoids lutein, neoxanthin, and violaxanthin. Currently, LHCII is the most thoroughly investigated and best characterized chlorophyll binding protein in chloroplast thylakoids. Recently, the structure of the complex has been determined at 3.4 Å resolution (Kühlbrandt et al., 1994). The structure indicates that each polypeptide binds a minimum of 12 chlorophylls and two carotenoids.

The biogenesis of LHCII involves a number of different post-translational processes. Analysis of dark-grown plants indicates that accumulation of LHCI, LHCII, and reaction center complexes requires chlorophyll (Anderson, 1986). Chlorophyll synthesis is light dependent in higher plants, specifically at the step of chlorophyllide formation. Darkness results in the complete absence of LHCII complexes. In vitro reconstitution of pea LHCII expressed in *Escherichia coli* suggests that chlorophyll may be required to induce correct folding (Paulsen et al., 1993). In vivo folding probably occurs in a similar way. It is clear that upon insertion into the thylakoid membrane, the conformation of the LHCII apoprotein undergoes a major structural change to accommodate the chlorophyll and carotenoid molecules.

In all plant species examined to date, LHCII is encoded by a multigene family consisting of at least five genes in Arabidopsis, six in *Nicotiana tabacum*, eight in *N. plumbaginifolia*, and up to 15 genes in tomato (Jansson et al., 1992). Transcription of LHCII genes is controlled by several different mechanisms that have been widely investigated. Many previous reports have shown that LHCII expression is stimulated by light and that this regulation pattern involves both the phytochrome and the blue-light regulatory systems. Other reports have demonstrated that the expression of LHCII genes is organ

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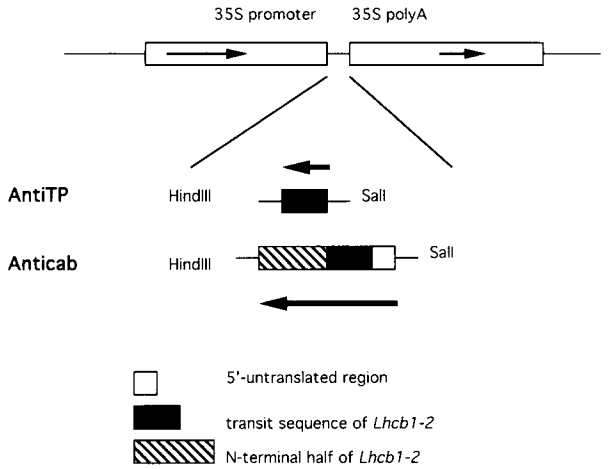


Figure 1. Structure of the Chimeric *cab* Genes.

Partial sequences of the tobacco *Lhcb1-2* cDNA were driven by the promoter of the CaMV 35S RNA and subsequently by the poly(A) site of the CaMV 35S RNA. The 5′ to 3′ direction of the DNA sequences used is indicated by arrows.

and tissue specific and is subject to circadian patterns and developmental control (Kuhlemeier et al., 1987; Piechulla and Gruissem, 1987; Kellmann et al., 1990). These data suggest that high-level expression of *cab* genes coincides with accumulation of large amounts of antenna complexes under appropriate light conditions. We have tested this assumption by using an antisense approach with two different *cab* constructs; our goal was to suppress the accumulation of LHCII in the thylakoid membrane of transgenic tobacco plants. We report here that the LHCII content of transgenic tobacco plants appears to be normal, even when *cab* mRNA is reduced to an almost undetectable level by antisense RNA. Biochemically and physiologically, these antisense plants do not appear to be different from their wild-type parents. These results strongly suggest that there is an as yet undiscovered post-transcriptional process that regulates translation into LHCII from a pool of *cab* RNA molecules.

RESULTS

Construction of the LHCII Antisense Gene and Regeneration of Transgenic Tobacco Plants

Two constructs were used as antisense probes: one containing the *cab* transit peptide (TP) of the tobacco gene *Lhcb1-2* (antiTP) and the other containing the N-terminal half of the same gene together with 62 bp of the 5′ untranslated region (anticab). These two constructs thus differed only in length. One or the other was fused in reverse orientation to the enhanced promoter of the cauliflower mosaic virus (CaMV) 35S promoter (Figure 1). Agrobacterium containing binary vectors with these inserts linked to the hygromycin phosphotransferase gene were used for transformation via the leaf disc technique. Because a deficiency in antenna proteins could result in transformants with a reduced chlorophyll content, slow-growing and pale-looking calli were regenerated preferentially to select for putative LHCII-deficient transformants. Plantlets were selected on the basis of resistance to hygromycin, which was added to the growth medium.

Reduced Levels of *cab* mRNA Are Due to Antisense Inhibition

Six genes encoding five LHCII type I complexes (new nomenclature: *Lhcb1-1* to *Lhcb1-5*; see Jansson et al., 1992) and one LHCII type II complex (*Lhcb2-1*) have been identified in *N. tabacum*. Sequences used in antisense orientation were the 105-bp transit sequence or the 509-bp N-terminal half of *Lhcb1-2* having 65 to 87% and 85 to 88% homology, respectively, with the five other tobacco genes. The antisense genes should therefore reduce the expression of all *cab* genes in the transgenic plants. The level of steady state mRNA was determined in leaves of transgenic plants after transfer of primary transformants to a growth chamber. Twenty (of 36 analyzed) plants revealed a reduction; nine of them showed a very strong reduction of *cab* mRNA. Transcripts were barely detectable even after overexposure. F_1 plants from both transgenic lines all showed a severely reduced level of expression and were selected for more detailed biochemical and physiological analysis.

The Reduction of the *cab* mRNA Level Is Not Proportional to the Expression Level of Antisense RNA

Several transgenic plants displaying differences in the steady state level of *cab* mRNA or antisense plants that did not show the antisense effect were chosen for a more detailed analysis at the RNA level. RNA gel blots were probed with the random primer–labeled tobacco gene *Lhcb1-2* and rehybridized with a soybean 18S probe. The amount of transcript was quantitated by Phosphorimager scanning. The reduction in *cab* steady state levels was calculated from the ratio of 18S to *cab* mRNA. In both transgenic lines, a few plants containing a very low level of LHCII-encoding RNA (5 to 8%) were identified. The data revealed three classes of transgenic plants carrying sequences of anticab or antiTP constructs: no reduction of *cab* mRNA in comparison with the wild type, reduction by ~70 to 80%, and very strong reduction by up to 95% (Figure 2). Because both sense and antisense transcripts are detected by the labeled double-stranded *Lhcb1-2* probe, oligonucleotides were used as primers to produce strand-specific probes. Antisense transcripts were detected in most transgenic plants. However, their amount varied greatly. In anticab plants, reduction of *cab* mRNA levels coincided with comparatively low

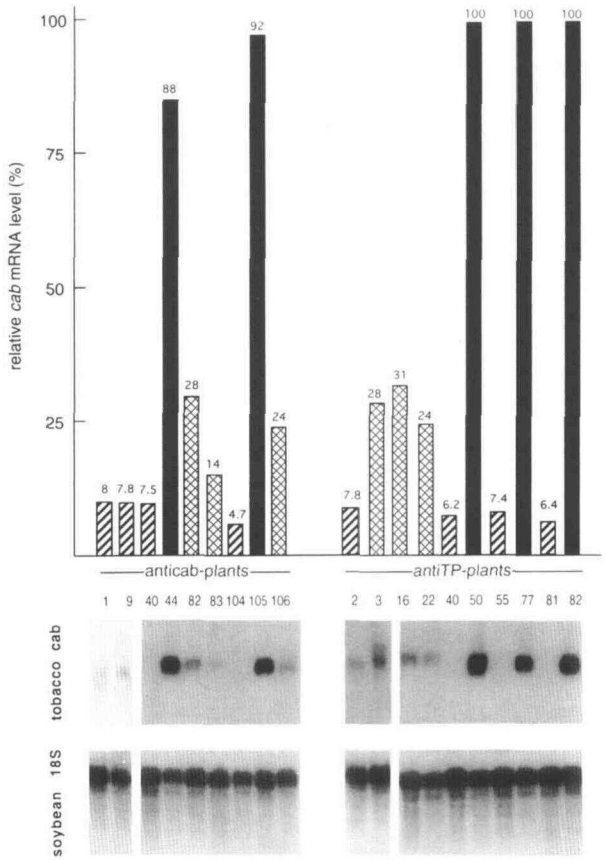


Figure 2. Quantitation of *cab* mRNA Steady State Levels in Transgenic Tobacco.

Total RNA isolated from individual anticab and antiTP plants was transferred onto nitrocellulose filters. The identification code for each plant is given above each lane. Filters were successively hybridized with a radioactively labeled tobacco *Lhcb1-2* probe and a soybean *18S probe* to check for equal RNA loading. Signals were quantified directly by Phosphorimager scanning, and relative transcript levels (in percentages) were calculated from the ratio of 18S to *cab* in comparison with the wild type. The transformants can be divided roughly into three classes: black bars represent plants without significant transcript reduction; crosshatched bars, plants with a distinct reduction of *cab* mRNA (15 to 30% of the wild-type level); striped bars, plants with severely reduced levels of *cab* mRNA (5 to 8% of the wild-type level). Numbers above bars represent the steady state level of *cab* mRNA (in percentages).

antisense RNA concentrations, whereas almost unchanged *cab* transcript levels coincided with higher antisense RNA levels. The amount of antisense transcript in anticab plants thus correlated inversely with the degree of reduction in the *cab* steady state level (Figure 3). This inverse correlation was less pronounced in antiTP plants. Relatively high concentrations of antisense RNA (antiTP-40 and antiTP-81) were observed even in antiTP plants with a strong reduction in *cab* message. The higher steady state level of antiTP antisense RNA might indicate that efficient degradation of RNA duplexes is dependent on the length of the double strands formed.

Growth Rates and Plant Morphology of Transformants

Several independent transformants with severely reduced levels of *cab* mRNA showed normal or retarded growth in the growth chamber after transfer from tissue culture medium to soil. Surprisingly, retarded growth of some plants did not coincide with the amount of *cab* mRNA present (Figure 4). However, the observed phenotypic differences did not manifest themselves in F_1 plants grown in soil under identical conditions. Differences therefore appeared to be due to tissue culture conditions or the preferential selection of pale calli for regeneration; these tend to be slow growing. Flower induction and production of seed occurred simultaneously with control plants. No obvious phenotypic changes were detected either in the morphology or in the coloration of immature and mature leaves.

Lhcb Genes Are Almost Equally Suppressed in Antisense Plants

Antenna complexes of PSII are organized in two subpopulations: an inner compartment that is more tightly bound to PSII and encoded by *Lhcb1* genes and a more peripheral pool that contains a mixture of 25- and 27-kD polypeptides (Bassi et al.,

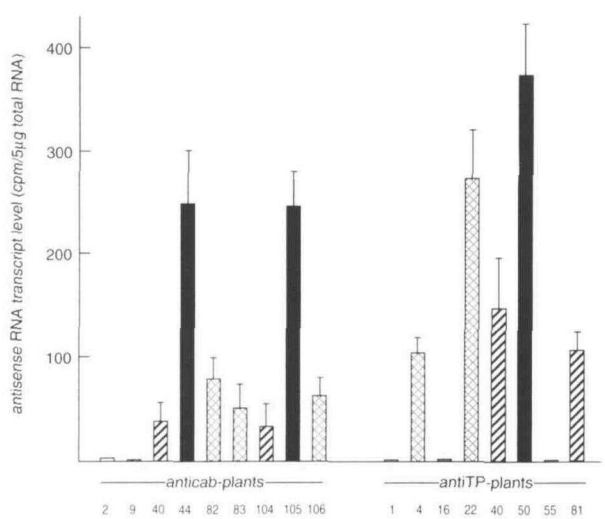


Figure 3. Quantitation of Antisense Transcripts.

Five micrograms of total RNA isolated from individual transgenic plants was blotted onto a nitrocellulose filter and hybridized with a single-stranded probe using the oligonucleotide AS-1 as primer (the plant number is given below each bar). Signal intensities were directly determined from the blots by Phosphorimager scanning. Mean values with standard deviations of two experiments are given in counts per minute per 5 µg of total RNA. Shading for bars is as given in Figure 2.

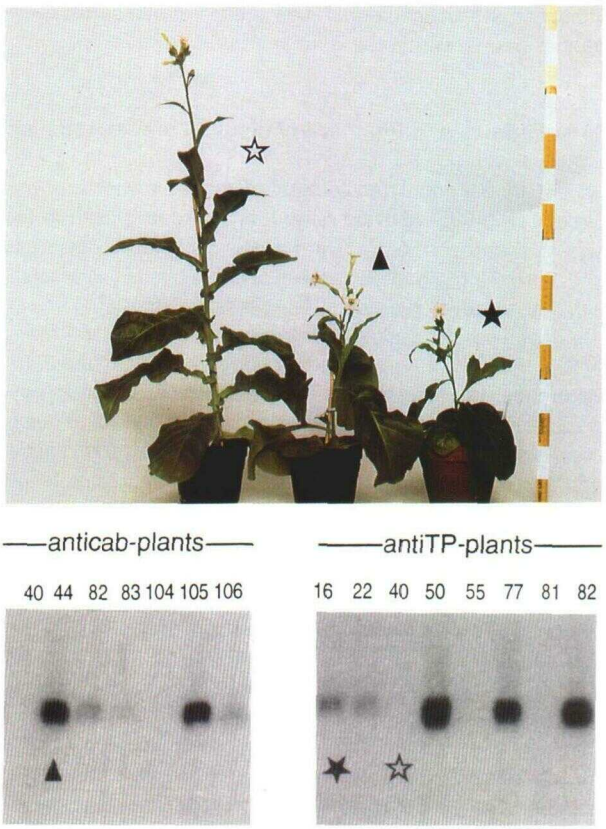


Figure 4. Growth of Primary Transformants.

Primary transformants, which were randomly selected from tissue culture, were potted and raised in a growth chamber. No phenotypic differences were detectable in leaf color and leaf morphology. Although some transformants were characterized by retarded growth, they were not delayed in flower development. RNA gel blot analysis showed that retarded growth of plants was not related to a reduction in *cab* mRNA levels, as indicated by three transgenic tobacco plants (marked individually with a triangle and open and filled stars) and their corresponding *cab* transcript levels (marked with the same symbols).

1988; Spangford and Andersson, 1989) encoded by both the *Lhcb2* and the *Lhcb1* genes (Jansson et al., 1990). In tobacco, the *Lhcb1* genes are highly homologous (85 to 94%) to each other, whereas homology with *Lhcb2-1* is only between 71 and 77%. We therefore addressed the question of whether antisense genes affect the expression of *Lhcb* genes equally or affect primarily *Lhcb1* gene expression. Oligonucleotides specific for individual genes at their 3′ ends (Figure 5) were used to quantitate the transcript concentrations in transgenic and wild-type plants. Plant anticab-104 showing the lowest level of *cab* mRNA was chosen for the analysis. Total RNA was slot blotted, hybridized with gene-specific, end-labeled probes, and quantitated using the Phosphorimager. In wild-type plants, *Lhcb1-2* was the most highly expressed gene among the type I genes and accounted for 42% of *cab* mRNA; *Lhcb1-3* (28%), *Lhcb1-1* (9%), and *Lhcb1-4* (4%) were expressed only weakly (Figures 6 and 7). The type II gene, *Lhcb2-1*, was well expressed (17%). The expression pattern was not significantly different in the transgenic plant anticab-104 even though only 5% of total *cab* mRNA was present. In this plant, *Lhcb1-2* accounted for 62% of the mRNA, with *Lhcb1-3* accounting for 18%, *Lhcb1-4* for 8%, *Lhcb2-1* for 7%, and *Lhcb1-1* for 5%. In anticab-104, a large contribution to the steady state level was due to *Lhcb1-2*; this portion was even larger than that in the wild-type plant SR1. The *Lhcb1-2* gene is the most prominent member of the *cab* gene family and therefore accounted for most of the steady state mRNA present in both wild-type and antisense plants.

Accumulation of LHCII Polypeptides

Protein gel blot analysis and immunodetection indicated that the amount of the PSII antenna complexes was not drastically reduced (Figure 8). In the total protein extract (lanes 1 to 6), only LHCII monomers reacted with the antibody. In the thylakoid membrane preparations, the main band was due to LHCII monomers, the upper band to LHCII trimers, and the lower band to lower molecular weight homologs, probably CP24. Quantitation via immunostaining was difficult. Because LHCII complexes bind most of the Chl *b* in plants, the ratio of Chl *a* to Chl *b* is an excellent indicator of changes in the amount of chlorophyll binding proteins. Isolated thylakoid membranes from 100 regenerated plants were screened for changes in the Chl *a*-to-Chl *b* ratio. The ratios in wild-type, anticab, and antiTP plants were highly reproducible, but not significantly different (Table 1). Moreover, the amount of total Chl *a* and Chl *b* in the

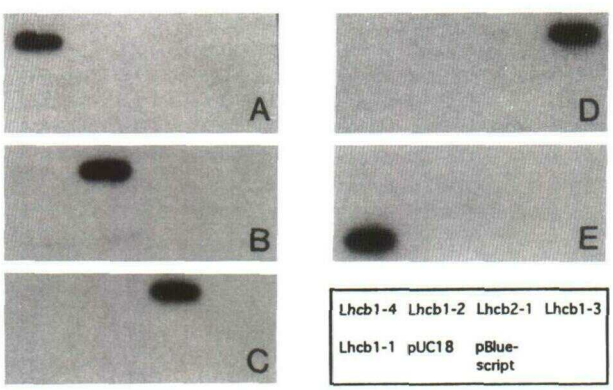


Figure 5. Gene-Specific Hybridization of Oligonucleotides to *Lhcb* Genes.

Equal amounts (0.3 pmol) of plasmid DNA containing five tobacco *Lhcb* genes and vector DNAs pUC18 and pBluescript KS− were blotted onto nitrocellulose filter and hybridized with each 3′ gene-specific oligonucleotide labeled to the same specific radioactivity. The pattern of DNA loading is as indicated in the box. Filter A was probed with b1-4, filter B with b1-2, filter C with b2-1, filter D with b1-3, and filter E with b1-1.

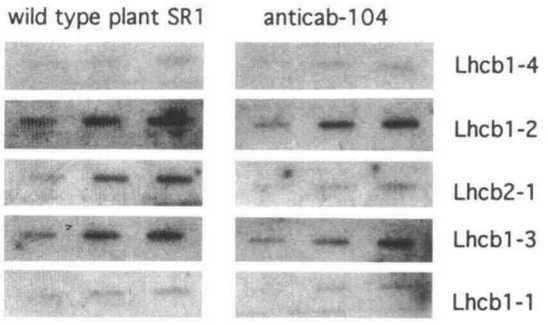


Figure 6. Expression of Tobacco *Lhcb* Genes in Leaves.

Total RNA from wild type and transgenic plant anticab-104 was isolated after 3 to 5 hr of illumination. RNA from the wild type (1, 2, and 3 μg; from left to right) and from anticab-104 (8, 12, and 20 μg; from left to right) was blotted onto nitrocellulose filters. Filters were probed with the same *Lhcb* 3′ gene-specific oligonucleotides as in Figure 5. Hybridization and washing conditions were the same as for filter-bound DNA.

membrane was not significantly altered in all of the plants that we analyzed. Examples can be found in Figure 9 (e.g., anticab-44 and anticab-83).

Functional Analysis of Antenna and PSII Components

The biochemical data previously shown indicate that the amount of PSII antenna proteins was not reduced in the transgenic plants. To determine whether there would be any effect on antenna function, three F_1 plants from different anticab and antiTP lines were analyzed for in vivo chlorophyll fluorescence and photosynthetic rates. Proper functioning of the photosynthetic apparatus requires balanced excitation of PSI and PSII; this is thought to be due to the appropriate association of LHC complexes with the two photosystems. The photochemical quenching coefficient (values between 0.81 and 0.87) and the nonphotochemical quenching parameter (values between 0.52 and 0.62), which was determined in predarkened leaf discs, were virtually unchanged in transgenic plants when compared with the wild type (Table 2). The F_0 level, which is a measure for the initial energy distribution to PSII and for the efficiency of excitation trapping at P680, is slightly lower in the transformants than in wild-type plants. Leaf discs from mature leaves, grown at 430 μE m^{-2} sec^{-1} and 25°C, were predarkened overnight, and photosynthetic rates were followed by the oxygen evolution at increasing light intensity. Substantial reduction of LHCII would result in lower efficiency of light capturing and hence in a lower efficiency of oxygen evolution by PSII. When compared with wild-type plants (27 μmol of O_2 m^{-2} sec^{-1}), some transgenic plants did indeed show lower oxygen evolution, varying from 13 μmol of O_2 m^{-2} sec^{-1} (anticab-1) to the wild-type level (25 μmol of O_2 m^{-2} sec^{-1} in antiTP-40; Figure 10). Clear differences between plants of anticab and antiTP lines were not detectable. Some plants showed significantly reduced rates of oxygen evolution, whereas others were unaffected. When photosynthetic rates were based on the chlorophyll concentration in the leaf discs used in the oxygen electrode, only anticab-1 and antiTP-3 showed significantly lower rates of oxygen evolution in comparison with untransformed control plants; however, there were no apparent differences in Chl *a*-to-Chl *b* ratios and in the photochemical quenching coefficient, qQ, and the nonphotochemical quenching coefficient, qE (Table 3). AntiTP-3 can be considered as an internal control, similar to wild type, because its *cab* mRNA level was reduced only by ~70%. Because all physiological parameters are similar in wild-type and transgenic plants, we are forced to conclude that the amount of antenna proteins of PSII is not altered and, therefore, that protein–pigment complexes accumulate at normal levels in the thylakoid membrane.

DISCUSSION

Expression of *Lhcb* Genes in Tobacco

Extended multigene families encode the polypeptides of LHCI and LHCII, the light-harvesting antennae of PSI and PSII. By far the most abundant complex is LHCII. The genes encoding the LHCII polypeptides can be classified into three types on the basis of gene structure: *Lhcb1* genes do not contain introns

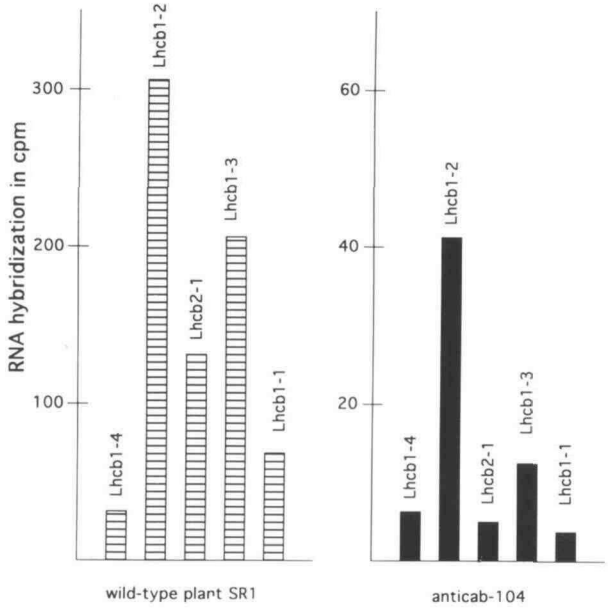


Figure 7. Quantitation of *Lhcb* Transcripts in Leaves.

Hybridization signals from the filters shown in Figure 6 were quantitated directly by Phosphorimager scanning. The amount of transcript is given in counts per minute after subtraction of background activity.

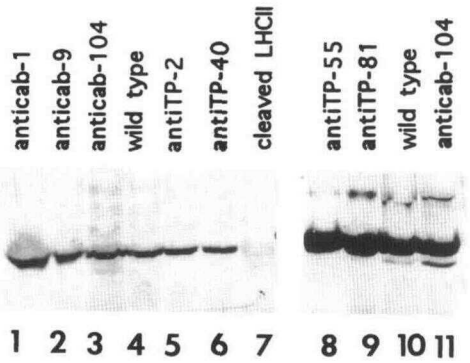


Figure 8. Protein Gel Analysis of Total Protein and Thylakoid Membranes from Leaves of Transgenic and Control Tobacco Plants.

After isolation, total protein (lanes 1 to 6; 20 μg each) and thylakoid membranes (lanes 8 to 11; 30 μg each) were subjected to PAGE under denaturing conditions, transferred to nylon membranes, and analyzed for the presence of LHCII. The polyclonal anti-LHCII antibody used is directed against the first 19 N-terminal residues. Purified LHCII was cleaved with trypsin, which removes the first eight N-terminal residues. Trypsin-treated LHCII does not react with the antibody (lane 7), indicating the specificity of the immunoreaction.

and share a high degree of sequence homology; *Lhcb2* and *Lhcb3* genes are more divergent both from *Lhcb1* genes and from each other, containing introns and encoding more divergent polypeptides (Pichersky et al., 1987; Schwartz et al., 1991). The first *cab* genes were isolated from pea; the largest number of individual gene family members have been identified in tomato and petunia. From tobacco, pea, and other species, only a limited set of *Lhcb* genes has been isolated. Currently, five *Lhcb1* and one of the *Lhcb2* genes are known to be present in tobacco. In pea, two genes, *Lhcb1-1* and *Lhcb1-4*, alone account for 70% of *cab* mRNA in leaves; the *Lhcb3-1* gene contributes 25% to the steady state level of *cab* mRNA in leaves, whereas three other *Lhcb1* genes and one *Lhcb2* gene are weakly expressed (0.4 to 4%; White et al., 1992). In contrast, in tomato only *Lhcb1* genes 1, 2, 5, and 6 are expressed at high level (11, 20, 13, and 28%, respectively), accounting for 72% of *cab* mRNA in leaves. All intron-carrying *Lhcb2* genes are very weakly expressed, with a maximum of 5% (Piechulla et al., 1991). The expression of the five *Lhcb* genes in tobacco leaves has not been determined. Even though these genes are unlikely to represent the complete *Lhcb* family, other members (in particular *Lhcb3*, which so far has not been identified in tobacco) most probably have very similar gene sequences and therefore would be equally subject to the antisense effect. The observed expression pattern resembles that in pea: only two genes, *Lhcb1-2* and *Lhcb1-3*, account for 70% of *cab* steady state mRNA. The third most highly expressed gene is the type II gene *Lhcb2-1*, with 17% of *cab* mRNA. Sequences of *Lhcb1-2* were used to achieve maximal repression by antisense RNA. The fact that all *Lhcb* genes are expressed in leaf tissue suggests that all types of LHCII polypeptides are probably required for efficient light harvesting. This is consistent with the observation that it was possible to identify all products of both *Lhcb* genes in thylakoids of tomato (Green et al., 1992) and *Lhca* genes in pea (Ikeuchi et al., 1991).

Reduced Expression of *Lhcb* Genes by Antisense RNA

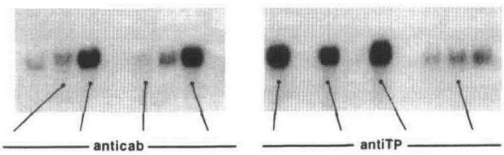
A detailed understanding of the contribution and role of LHCII in plant growth in vivo under various environmental conditions requires appropriate mutants with varying amounts of PSII antenna proteins. We used the antisense RNA approach to try to repress all *Lhcb* genes by fusing sequences of the most highly expressed member of this gene family, *Lhcb1-2*, to the promoter of the 35S RNA of CaMV. This promoter has been chosen for two reasons: it is expressed in a variety of tissues and cells and, unlike the *cab* promoters, is not restricted to photosynthetically active cells. Because data were not available for tobacco on the expression kinetics of *cab* genes, in particular during the early stages of thylakoid formation, or on their developmental regulation, antisense RNA should be accumulating at a very early developmental stage.

So far, full-length cDNA constructs have mainly been used to suppress endogenous genes by the antisense effect. Even partial sequences, when they are used at all, are several hundred base pairs long (see van der Krol et al., 1990). In our experiments, the antiTP construct with a complementarity of only 105 nucleotides was as efficient in reducing the level of *cab* mRNA as the second construct with a five times longer

Table 1. Determination of Chl *a*-to-Chl *b* Ratios

Plant	No. of Experiments	Chl *a*-to-Chl *b* Ratio
Wild-type plant	34	3.590 ± 0.017
Regenerated wild type	18	3.633 ± 0.050
Antisense plants without reduced *cab* mRNA		
Anticab-44	6	3.837 ± 0.020
Anticab-B	14	3.497 ± 0.346
AntiTP-50	6	3.572 ± 0.029
AntiTP-77	12	3.497 ± 0.172
AntiTP-82	6	3.747 ± 0.013
Aantisense plants with reduced *cab* mRNA		
AntiTP-16	6	3.768 ± 0.011
Anticab-40	10	3.764 ± 0.106
AntiTP-55	10	3.008 ± 0.037
AntiTP-81	10	3.754 ± 0.058
Anticab-82	11	3.332 ± 0.006
Anticab-83	6	3.718 ± 0.007
Anticab-104	10	3.556 ± 0.047
Anticab-H	6	3.838 ± 0.020

Thylakoid membranes were obtained from isolated chloroplasts after osmotic shock. Chl *a* and Chl *b* determinations were performed in 80% acetone by the method of Porra et al. (1989).



plant	106	105	83	44	82	77	50	16
freshweight	0.6	0.5	0.7	0.7	0.8	0.8	0.8	0.8
Chla (mg/ml)	0.652	1.471	1.732	1.211	1.279	1.074	1.940	2.190
Chlb (mg/ml)	0.173	0.368	0.467	0.314	0.341	0.288	0.548	0.58
Chla/b-ratio	3.838	3.982	3.718	3.837	3.747	3.729	3.540	3.768

Figure 9. Chlorophyll Content and Steady State Level of *cab* mRNA.

Chloroplasts from the second mature leaf of antisense plants, grown under growth-chamber conditions, were isolated. Chlorophyll content was determined from purified thylakoids in 80% acetone according to Porra et al. (1989), and the Chl *a*-to-Chl *b* (Chla/b) ratio was calculated.

anticab sequence. An even shorter sequence may therefore be as effective. This is suggested by the observation that the antiTP construct is only 65% homologous to *Lhcb2-1* over 52 nucleotides, including a gap of six nucleotides. Yet, *Lhcb2-1* (accounting for 17% of all *Lhcb* transcripts in the wild type) was significantly repressed in antiTP plants because only 5% of the *cab* mRNA remained (see Figure 7). Our results thus show that it might be possible to modulate individually the expression even of highly homologous endogenous genes by short and therefore gene-specific sequences. A stretch of only 41 nucleotides may be sufficient for antisense inhibition, as shown for the artificial marker gene *Gus* which encodes β-glucuronidase in transgenic tobacco (Cannon et al., 1990). However, it is not yet possible to estimate the minimal length of a nucleotide sequence required for the antisense effect. The efficiency of an antisense RNA in gene expression might be determined by the length of RNA–RNA hybrids and its secondary structures that are recognized by double strand–specific RNases. In a few plants with severely reduced *cab* mRNA levels, for example, antiTP-22, antiTP-40, and antiTP-81, antisense transcripts were stably maintained at levels higher than those in anticab plants with low *cab* mRNA levels. Perhaps the formation or degradation of short putative RNA–RNA hybrids is inhibited, or additional mechanisms, such as the unwinding of short RNA double strands, are enhanced in these plants.

In our experiments, the expression of five members of the LHCII family was significantly suppressed in more than half of all anticab and antiTP lines. The level of antisense RNA and of detectable sense transcripts varied considerably among individual anticab and antiTP plants. A similar degree of variation has been observed earlier (Stockhaus et al., 1990; Tieman et al., 1992). The minimal steady state *cab* mRNA concentration is ~1% when calculated after correction for equal loading of RNA or ~5% when calculated from the 18S to *cab* ratio. This result was expected and is in itself not surprising. It had been shown in one of the first antisense RNA experiments in plants (Rodermel et al., 1988) that the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase, also encoded by a multigene family, could be significantly reduced at the RNA (12% of control) and protein (37% of control) level in transgenic tobacco. However, the ratio of sense to antisense RNA does not necessarily correlate with the degree of protein reduction achieved by antisense effects. It was actually found that even

Table 2. Chlorophyll Fluorescence Parameter of Transgenic and Wild-Type Tobacco Plants

Plant	F_m/F_0[a]	n[b]	qQ[c]	n	qE[d]	n
Wild type	4.99 ± 0.24	25	0.857 ± 0.02	3	0.541 ± 0.08	3
Plants expressing anticab[e]						
Anticab-1	3.86 ± 0.39	10	0.807 ± 0.02	3	0.571 ± 0.10	3
Anticab-9	4.74 ± 0.53	18	0.868 ± 0.05	3	0.615 ± 0.04	3
Anticab-104	4.76 ± 0.27	34	0.843 ± 0.02	3	0.601 ± 0.05	3
Plants expressing antiTP[f]						
AntiTP-2	4.31 ± 0.31	21	0.853 ± 0.04	3	0.663 ± 0.09	3
AntiTP-40	3.80 ± 0.61	19	0.867 ± 0.01	3	0.517 ± 0.03	3
Plants expressing anticab and antiTP[g]						
Anticab-4	4.09 ± 0.36	10	0.842 ± 0.02	3	0.642 ± 0.05	3
AntiTP-3	4.77 ± 0.33	14	0.853 ± 0.01	3	0.517 ± 0.03	3

The second mature leaf was measured after dark adaptation. Means and standard deviations are indicated. All selected transformants are characterized by severely reduced *cab* mRNA levels.

[a] F_0, dark-level chlorophyll fluorescence; F_m, maximal chlorophyll fluorescence.

[b] Number of repetitions.

[c] Photochemical quenching coefficient.

[d] Nonphotochemical quenching coefficient.

[e] 95% reduced *cab* mRNA level.

[f] 95% reduced *cab* mRNA level.

[g] 70% reduced *cab* mRNA level.

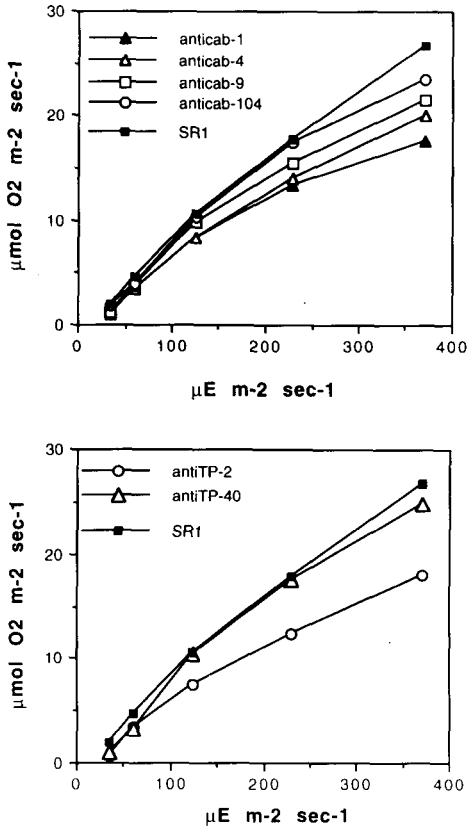


Figure 10. Light Intensity Curves for Oxygen Evolution.

Leaf discs from wild-type and transgenic plants raised in a growth chamber were kept in the dark overnight. Oxygen evolution was determined at 25°C polarographically with an oxygen electrode. The graphs show the rates at increasing light intensities calculated on the basis of equal leaf areas. For mean values and standard deviations, see Table 3.

plants without reduced target RNA had a distinct antisense phenotype but no detectable antisense RNA (Stockhaus et al., 1990). In contrast to this, the data on chlorophyll content, Chl *a*-to-Chl *b* ratio, and photosynthetic oxygen evolution indicate that, despite almost undetectable levels of mRNA, the amount of LHCII was virtually unchanged in all our transgenic anticab and antiTP plants. The strength of the promoter used for the expression of antisense RNA might not be critical. Endogenous promoters for the antisense RNA transcripts can work as efficiently as the CaMV 35S promoter (van der Krol et al., 1990). Instead, the stability of antisense RNA and the formation of heteroduplex RNA might be the more relevant factors. The *Lhc1-2* antisense messages remained stable as they accumulated (Figure 3); the steady state level of several *Lhcb* genes (including perhaps yet unidentified genes in tobacco) was substantially lowered with surprisingly small effects on LHCII concentration. Unlike other successful antisense RNA inhibitions, the message of the various *Lhcb* genes is not the rate-limiting factor for the accumulation of antenna protein. Five percent or less of *Lhcb* message is sufficient to allow for normal protein accumulation, binding of normal amounts of Chl *a* and Chl *b*, and support of photosynthesis at normal rates. This shows that post-transcriptional processes determine the PSII antenna size and the efficiency of light capturing. Perhaps differently processed 5′ and 3′ ends of the *Lhcb* messages or secondary structures determine which mRNA molecules are selected for translation.

Physiological Aspects of LHCII Deficiency

Chlorophyll content and Chl *a*-to-Chl *b* ratios of the transgenic plants indicate that LHCII accumulates to a normal level. These in vitro data were confirmed by various in vivo measurements.

Table 3. Photosynthetic Rates of Transgenic and Wild-Type Tobacco

	Evolution of Oxygen in µmol of O_2 m^{-2} sec^{-1}					Evolution of Oxygen in µmol of O_2 mg^{-1} Chl min^{-1}
	Increasing Light Intensities (µE m^{-2} sec^{-1})					
Plant	33	58	127	230	365	230 µE (m^{-2} sec^{-1})
Wild type	1.9 ± 0.2	4.6 ± 0.6	10.5 ± 1.1	17.8 ± 1.6	26.7 ± 2.6	9.7 ± 0.9
Anticab-1	1.0 ± 0.5	3.4 ± 0.3	8.3 ± 0.8	13.3 ± 1.0	17.6 ± 1.4	6.5 ± 0.5
Anticab-9	1.1 ± 0.1	3.8 ± 0.2	9.8 ± 1.1	15.4 ± 1.9	21.5 ± 1.1	8.3 ± 1.0
Anticab-104	1.8 ± 0.5	4.0 ± 0.9	10.3 ± 1.7	17.4 ± 2.3	23.4 ± 2.8	8.4 ± 1.1
AntiTP-2	0.6 ± 0.2	3.4 ± 1.3	7.4 ± 0.6	12.3 ± 0.5	18.0 ± 0.9	8.4 ± 1.1
AntiTP-3	1.0 ± 0.1	2.6 ± 0.2	6.0 ± 0.8	8.5 ± 1.4	13.2 ± 1.4	8.9 ± 0.4
AntiTP-40	1.0 ± 0.3	3.2 ± 0.6	10.3 ± 1.1	17.5 ± 1.8	24.9 ± 1.8	8.3 ± 0.9

The rates of oxygen evolution at increasing light intensities were calculated per leaf area. The amount of oxygen evolution per milligram of chlorophyll was measured at 230 µE m^{-2} sec^{-1}. Measurements were performed on three to four plants from each transgenic line and on control tobacco plants.

The amount of oxygen evolved at increasing light intensity is a measure of the efficiency of photosynthesis. Based on chlorophyll concentration, transgenic plants showed a trend toward a lower photosynthetic rate. This was significantly lower in anticab-1 and antiTP-3 compared with the wild type. However, because there was no correlation between the level of *cab* mRNA or Chl *b* content and oxygen evolution, we believe that this reduction must be due to other causes, possibly related to culture conditions.

The ratios of maximal chlorophyll fluorescence to dark-level chlorophyll fluorescence (F_m/F_0), which were expected to be higher in LHCII-deficient mutants, were actually lower for these two plants when compared with the wild type; both photochemical (qQ) and nonphotochemical (qE) quenching parameters remained unchanged. Table 2 shows that F_m/F_0 values of all other transgenic plants were also not higher and that the photochemical and nonphotochemical quenching coefficients remained unchanged. That plants having less PSII antenna proteins should indeed have a lower photosynthetic rate was shown by a spontaneous nuclear mutant of tobacco in an 18-year-old seed lot. The mutation *Su/su* is characterized by unchanged photochemical and nonphotochemical quenching coefficients but has an elevated F_m/F_0 ratio of 5.48 (wild type, 4.24; Šantrůček et al., 1992). Photosynthetic oxygen evolution on the chlorophyll basis is three to four times lower in the wild type than in *Su/su* mutants (Specht et al., 1987). This is in agreement with the calculated reduction in LHCII concentration between 2.3- and 5.6-fold (Specht et al., 1987). Whether PSII antenna proteins (and perhaps PSI antenna proteins) can be reduced by antisense RNA is yet to be determined. It is interesting to note that an Arabidopsis mutant, *cue1*, which has been identified by screening for "*cab* underexpression" after γ-ray mutagenesis, displays only 14% of control *cab* message. Similar to the transgenic tobacco plants, this mutant is not an albino plant; it has a reticulate pattern with veins greener than the interveinal regions in leaves (H.-M. Li and J. Chory, personal communication). Albino tobacco plants are known to have spontaneously occurring mutations (e.g., the dominant negative *Su* mutation). In this nuclear mutation, the chlorophyll concentration is reduced seven- to eightfold, the Chl *a*-to-Chl *b* ratio is increased (Schmid and Gaffron, 1967), the rate of oxygen evolution is fivefold higher than that in the wild type on the basis of chlorophyll (Schmid, 1967), and the PSII antenna size in *Su/Su* is reduced sixfold (Specht et al., 1990).

A detailed molecular analysis showed that the *Su* mutation exhibits pleiotropic effects on chloroplast proteins in homozygous plants (Kawata and Cheung, 1990). In heterozygous plants, most transcripts and corresponding proteins accumulate at normal levels. The notable exception is LHCII, which accumulates to only 25% of the wild-type protein level, whereas the level of mRNA is not significantly reduced. This phenomenon is just the opposite to what is observed in our antisense plants and Arabidopsis *cue1*. The homozygous plant *Su/Su* has only 10% of wild-type *cab* mRNA but undetectable levels of LHCII. Although expression of an *Lhcb* gene from the CaMV 35S promoter increased the steady state level of *cab* mRNA, LHCII did not accumulate to a higher level than it did in its untransformed counterpart.

Su is a mutation in an uncharacterized gene whose gene product is involved in the insertion or degradation of LHCII in the thylakoid membrane. Perhaps these mechanisms are related to those processes that regulate LHCII translation from an mRNA pool. *Su* causes primarily depletion of LHCII from the thylakoid membrane and leads to photooxidative damage that results in decreased *cab* mRNA levels. The lowered message level in itself does not cause a depletion of LHCII.

In conclusion, we have shown that antisense RNA does not affect the accumulation of the major PSII antenna protein for two reasons. First, the steady state level of *cab* mRNA does not correlate with the amount of LHCII pigment–protein complexes. This is indicative of post-transcriptional processes that constitute a "bottleneck" in the biogenesis of PSII antenna proteins. Second, the antenna size of PSII is determined through selection among very homologous transcripts by as yet undiscovered post-transcriptional mechanisms.

METHODS

Construction of Binary Vectors

Sequences were cloned from a *Nicotiana tabacum Lhcb1-2* cDNA (kindly provided by L. Bogorad, Harvard University, Cambridge, MA; EMBL accession No. X52743). To construct anticab (Figure 1), a HindII-SmaI fragment extending from 62 bp upstream to 447 bp downstream of the initiation codon of the gene was fused in reverse orientation to the enhanced cauliflower mosaic virus (CaMV) 35S promoter (Kay et al., 1987) followed by the polyadenylation signal of the 35S transcript of CaMV (Guerineau et al., 1988) and inserted as a KpnI fragment into the binary vector pBIB-HYG (Becker, 1990). Plasmid antiTP encodes the 105-bp chlorophyll *a/b* binding protein (*cab*) transit sequence only; it was amplified by polymerase chain reaction with primers AS-1 (5′-TTTTGTCGACAATGGCTGCTTCTACAA-3′) and AS-2 (5′-ATTTTA-AGCTTCATGGTAACTTTCCC-3′) from *Lhcb1-2* and subcloned as a HindIII-SalI fragment. Recombinant DNA techniques were performed according to standard procedures (Maniatis et al., 1982).

Agrobacterium-Mediated Transformation and Growth of Plant Material

The antisense DNA constructs anticab and antiTP were introduced into *Agrobacterium tumefaciens* LBA4404 (Hoekema et al., 1983) by electroporation according to the manufacturer's instruction (Bio-Rad). Transformants were used to infect leaf discs of *N. tabacum* cv Petit Havanna SR1 (Maliga et al., 1973). The infected discs were placed on tissue culture medium (Murashige and Skoog, 1962) containing 25 μg of hygromycin 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 $\mu E\ m^{-2}\ sec^{-1}$; 16-hr-dark/8-hr-light regime; 25°C). Experiments were repeated independently using in vitro–propagated clones of transformants and plants from the F_1 generation.

Preparation of RNA, Hybridization, and Quantitation

For RNA gel blot analysis, RNA was isolated (Logemann et al., 1987) from the second mature leaf of soil-grown transgenic and wild-type plants after 3 to 5 hr of light. For hybridizations, 15 μg of total RNA was electrophoresed through a 1% agarose gel containing 2.2 M formaldehyde (Maniatis et al., 1982) and blotted to GeneScreen Plus membranes (Du Pont). 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 tobacco *Lhcb1-2* and rehybridized with a soybean 18S gene (Eckenrode et al., 1985) to ensure that equal amounts of RNA were present. To quantitate antisense-specific transcripts, the primer AS-1 was used to produce specific single-stranded probes. To analyze the *Lhcb* gene family, we used the following oligonucleotides: b1-4 for *Lhcb1-4* (*cab7*), 5′-CTGATACTAAAAGAGACTCT-3′; b1-2 for *Lhcb1-2* (*cab21*), 5′-CAGAATTTTGAGAATGTTTT-3′; b1-3 for *Lhcb1-3* (*cab40*), 5′-CTG ACACTACTTGAAAATTA-3′; b1-1 for *Lhcb1-1* (*cab50*), 5′-CAGCTAATT GAACAAACTAT-3′; and b2-1 for *Lhcb2-1* (*cab36*), 5′-CAGTAGCCC CACAAATTGTA-3′.

Oligonucleotides were end labeled with T4 polynucleotide kinase. Labeled oligonucleotides were separated from unincorporated label using NucTrap columns (Stratagene). Unlabeled oligonucleotides were added to adjust the specific activities for all probes to ~10^6 cpm per pmol of probe. The relative hybridization efficiencies of the oligonucleotides were determined using 0.3 pmol of plasmid DNA containing the *Lhcb* genes after transfer onto GeneScreen Plus membranes. Filters with bound DNA and RNA were incubated as described by Church and Gilbert (1984) at 42°C for 24 to 36 hr. After hybridization, filters were washed twice at room temperature for 30 min in 6 × SSC (1 × SSC is 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0) and then at 47°C for probes b1-3 and b1-1, at 50°C for b1-2, at 54°C for b1-4, and at 58°C for b2-1. The hybridization signals were quantitated in counts per minute 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 Waring blendor and passed through cotton wool and Miracloth (Petzold, Melsungen, Germany). Chloroplasts were collected by centrifugation at 1500*g* for 5 min. Thylakoid membranes were isolated after osmotic shock in 10 mM NaCl, 1 mM Tricine, pH 7.8, for 15 min at room temperature, centrifugation for 15 min at 6000*g*, and subsequent washing in 10 mM NaCl, 1 mM Tricine, 5 mM EDTA, pH 7.8. Chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*) values were determined in 80% acetone according to Porra et al. (1989). For protein gel blots, membranes were resuspended in 10% sucrose, 20 mM sodium carbonate, 100 mM DTT and sonicated twice for 15 sec with a Branson sonifier (Branson Sonic Power Co., Danbury, CT). PAGE was performed according to Laemmli (1970). Separated proteins were electrophoretically transferred to Immobilon-P (Millipore, Bedford, MA). Immunodetection was with the anti-LHCII antibody, and staining was with alkaline phosphatase as described by Maniatis et al. (1982). The polyclonal anti-LHCII antibody was prepared by using a synthetic peptide with the sequence RKSATTKKVASSGSPWYGP (N-terminal residues of LHCII from pea).

Physiological Measurements

Maximal photosynthesis rates were measured from dark-adapted leaf discs of growth chamber–grown plants (430 μE m^{-2} sec^{-1}; 12-hr-dark/12-hr-light regime; 25°C/20°C) in a Hansatech leaf disc electrode (Bachofer, Reutlingen, Germany) under increasing light intensities. Each measurement was performed independently at least twice on three F_1 plants. In vivo chlorophyll fluorescence was measured with leaf discs predarkened overnight at room temperature. Using the PAM fluorometer (Walz, Effeltrich, Germany), we determined initial fluorescence (F_0) and maximal fluorescence (F_m) using a saturating flash (3200 μmol m^{-2} sec^{-1} for 700 msec) and the photochemical quenching coefficient (qQ) and nonphotochemical quenching coefficient (qE) (at 270 μE m^{-2} sec^{-1}). The photochemical quenching coefficient and the nonphotochemical quenching coefficient were calculated using equations according to Schreiber et al. (1986):

$$qE = [(F_v)m - (F_v)s]/(F_v)m$$
$$qQ = [(F_v)s - Fv]/(F_v)s,$$

where *Fv* is variable fluorescence, $(F_v)m$ is maximal variable fluorescence, and $(F_v)s$ is variable fluorescence after continuous actinic light and pulses of saturating light were applied.

ACKNOWLEDGMENTS

Support by the Deutsche Forschungsgemeinschaft is gratefully acknowledged. We thank Drs. Lawrence Bogorad (Harvard University, Boston) and Tony Kavanagh (University of Dublin) for providing *Lhcb* cDNA clones. We also thank Dr. Mark Stitt, University of Heidelberg, for providing greenhouse space, equipment, and help with the in vivo chlorophyll fluorescence measurements.

Received October 3, 1994; accepted December 1, 1994.

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