

Antisense Inhibition of the Photosynthetic Antenna Proteins CP29 and CP26: Implications for the Mechanism of Protective Energy Dissipation

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The specific roles of the chlorophyll *a/b* binding proteins CP29 and CP26 in light harvesting and energy dissipation within the photosynthetic apparatus have been investigated. *Arabidopsis* was transformed with antisense constructs against the genes encoding the CP29 or CP26 apoprotein, which gave rise to several transgenic lines with remarkably low amounts of the antisense target proteins. The decrease in the level of CP24 protein in the CP29 antisense lines indicates a physical interaction between these complexes. Analysis of chlorophyll fluorescence showed that removal of the proteins affected photosystem II function, probably as a result of changes in the organization of the light-harvesting antenna. However, whole plant measurements showed that overall photosynthetic rates were similar to those in the wild type. Both antisense lines were capable of the qE type of nonphotochemical fluorescence quenching, although there were minor changes in the capacity for quenching and in its induction kinetics. High-light-induced violaxanthin deepoxidation to zeaxanthin was not affected, although the pool size of these pigments was decreased slightly. We conclude that CP29 and CP26 are unlikely to be sites for nonphotochemical quenching.

INTRODUCTION

Solar energy is efficiently absorbed and transferred to the photosynthetic reaction centers by a complex of pigment binding proteins, the light-harvesting antenna. In higher plants, the antenna is composed of two classes of proteins together with their associated chlorophylls and carotenoids: the plastid-encoded proteins of the inner antenna, which bind chlorophyll *a* and β -carotene, and the chlorophyll *a/b* binding light-harvesting complexes (LHCs) of the outer antenna. The photosystem (PS) II outer antenna consists of the minor antenna complexes CP29, CP26, and CP24 (whose apoproteins are encoded by *Lhcb4*, *Lhcb5*, and *Lhcb6*, respectively) and the major antenna (LHCII), which comprises heterotrimers of Lhcb1 plus Lhcb2 or Lhcb3 and homotrimers of Lhcb1 (Jansson, 1994; Bassi et al., 1996; Jackowski and Jansson, 1998). In addition, four antenna proteins are confined to PSI, giving a total of 10 distinct LHC proteins in higher plants. Angiosperms and gymnosperms diverged ~350 million years ago, yet they share the same set of 10 LHC proteins. This strongly indicates that each pigment-protein complex has a specific function, because no LHC proteins have been lost during 350 million years of evolution.

The common ancestor of the LHC proteins (encoded by members of the *Lhc* gene family) is believed to be a cyanobacterial high-light-inducible protein with one membrane-spanning helix (Dolganov et al., 1995). Some time after endosymbiotic chloroplast formation, the *Lhc* gene progenitor was transferred to the nuclear genome, where subsequent duplication and deletion events took place, eventually leading to the large *Lhc* gene family that exists today (Green and Kühlbrandt, 1995). The extended *Lhc* gene family includes members encoding proteins such as PsbS and early light-inducible proteins (Jansson, 1999). These genes are all nucleus encoded, and the protein precursors contain an N-terminal transit peptide that is cleaved off during chloroplast import, after which the mature proteins are inserted into the thylakoid membrane with one to four membrane-spanning helices.

In the variable conditions of the natural environment, plants experience states of excitation stress, for example, during periods of high light or low temperature, when the capacity for photosynthesis is insufficient to utilize all light quanta that are absorbed. To prevent damage, several safety systems have evolved. Of particular interest is the process known as qE, which is observed as nonphotochemical quenching of chlorophyll fluorescence (NPQ), in which the excess energy absorbed by the antenna is harmlessly dissipated as heat. qE is formed under conditions in

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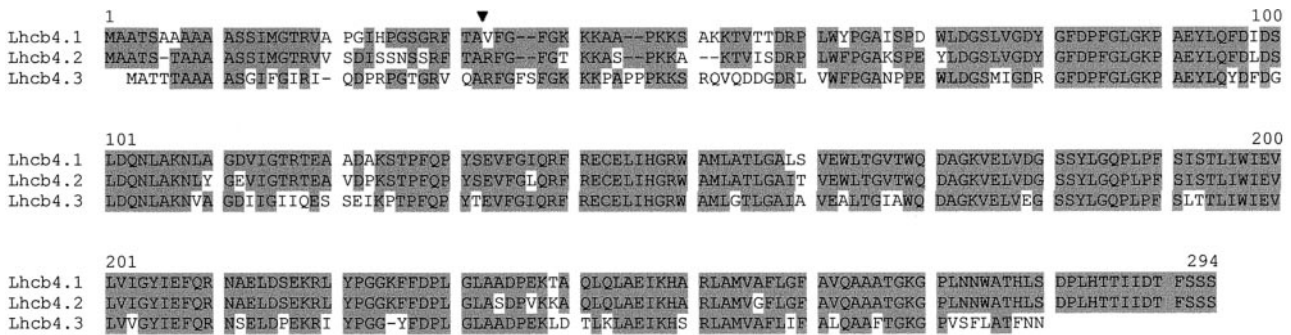


Figure 1. Homology between CP29 Isoforms.

Alignment of the predicted amino acid sequences of the products of the three *Lhcb4* genes of *Arabidopsis* showing their strong sequence similarity. Shading denotes residues conserved between at least two of the sequences. The arrowhead indicates the putative transit peptide cleavage site.

which there is a large proton gradient across the thylakoid membrane, and it is particularly sensitive to the pH of the thylakoid lumen; it is suggested that antenna polypeptides become protonated, inducing structural or organizational changes (Horton et al., 1996). Its Δ pH dependence means that qE forms and relaxes rapidly (within minutes), and it can be distinguished from other protective processes on this basis (Horton et al., 1996). An additional effect of a low lumen pH is that the enzyme violaxanthin deepoxidase is activated, so that in the so-called xanthophyll cycle (XC), violaxanthin is converted via antheraxanthin to zeaxanthin, an allosteric regulator of qE. Because qE depends substantially on zeaxanthin, it frequently is resolved into two phases when leaves are initially exposed to light: a rapidly forming, largely zeaxanthin-independent component, and a slower-forming component that mirrors XC activity (Demmig-Adams and Adams, 1996; Horton et al., 1996; Niyogi, 1999).

Several authors have suggested that CP29 and CP26 are involved in qE. The evidence for this view comes from the location of these proteins between the inner antenna and the major light-harvesting LHCII, the level of XC pigments within these complexes and the kinetics of their conversion (Bassi et al., 1997), and the in vitro quenching characteristics and dicyclohexylcarbodiimide binding of CP29 and CP26 (dicyclohexylcarbodiimide is an inhibitor of qE; Jahns and Schweig, 1995; Walters et al., 1996; Ruban et al., 1998). However, it has been shown that the LHC-related protein PsbS is indispensable for the rapid formation and relaxation of qE; *npq4*, an *Arabidopsis* mutant deficient in this protein, does not perform qE. It is not known if PsbS is itself responsible for qE or if other proteins (e.g., CP29 or CP26) are needed for energy dissipation to take place. PsbS, for example, has not been demonstrated to bind XC pigments (Li et al., 2000).

We are interested in the functions of the different LHC proteins in light harvesting and light dissipation, in particular in the functions of the PSII minor antenna proteins CP29 and CP26. Are they involved in energy dissipation, perhaps

as a link between antenna and quencher? How do they contribute to PSII function and/or antenna organization, perhaps as anchor points for the bulk LHCII? Do the different proteins depend on each other for stability? We are using reverse genetics, in this case antisense inhibition of gene expression, to seek the answers to these questions. Previous attempts to use antisense inhibition to prevent the expression of the LHC protein have not been successful (Flachmann and Kühlbrandt, 1995; Flachmann, 1997). We have shown, however, that antisense inhibition can be used successfully to selectively block the expression of LHC proteins in *Arabidopsis* (Zhang et al., 1997; Ganeteg et al., 2001). A potential problem is that the genes encoding the CP29 and CP26 apoproteins are members of the large LHC supergene family. The mechanism of antisense inhibition is not understood, and other homologous genes might be inhibited by the same antisense construct. Furthermore, the CP29 apoprotein is encoded by three highly homologous genes (Jansson, 1999) that may or may not be affected by the introduced antisense gene.

In this article, we report on the successful production of CP29 and CP26 antisense lines of *Arabidopsis*. Analysis of the photosynthetic performance of these lines shows that both CP29 and CP26 are involved in light harvesting and that they also influence NPQ, although probably in an indirect fashion.

RESULTS

The Three *Lhcb4* Genes of *Arabidopsis* Encode Slightly Different CP29 Proteins

The CP26 apoprotein is encoded by a single *Lhcb5* gene in *Arabidopsis*, but CP29 is encoded by three genes, *Lhcb4.1*, *Lhcb4.2*, and *Lhcb4.3*, which are similar but not identical.

Two of these genes have a high level of expression, whereas the third gene encodes a somewhat smaller protein and has a much lower mRNA level (Jansson, 1999). Because it is not clear how similar an antisense construct needs to be for a complete antisense effect, we compared the decrease in expression from the three *Lhcb4* genes in the antisense plant. The CP29 antisense construct was prepared using a full-length *Lhcb4.2* cDNA. When sequences of the three *Lhcb4* gene products are aligned (Figure 1), it is clear that Lhcb4.1 and Lhcb4.2 are highly similar (91% identity between the mature polypeptides) and that Lhcb4.3 is more divergent (70 and 67% identity, respectively, with the Lhcb4.1 and Lhcb4.2 mature polypeptides). If DNA sequences are considered, there is 82% identity between *Lhcb4.1* and *Lhcb4.2* and 74% identity between *Lhcb4.2* and *Lhcb4.3* in the coding region.

Efficient and Specific Antisense Inhibition of *Lhcb4* and *Lhcb5* Expression

Plants were infiltrated with *Agrobacterium* containing the antisense plasmids, and seed were collected (the T1 generation) and spread on kanamycin selection plates. More than 25 kanamycin-resistant plantlets of each antisense transformation were recovered and screened immunologically for reduced levels of the antisense target proteins (Figure 2). Note that the CP29 antibody cross-reacted with the PSI antenna protein Lhca2 (Knoetzel and Simpson, 1991), providing an internal control for the amount of protein on the protein gel blot. Both antisense constructs were effective at reducing the level of the target protein, and almost all of the kanamycin-resistant T1 plants of CP29 and CP26 antisense

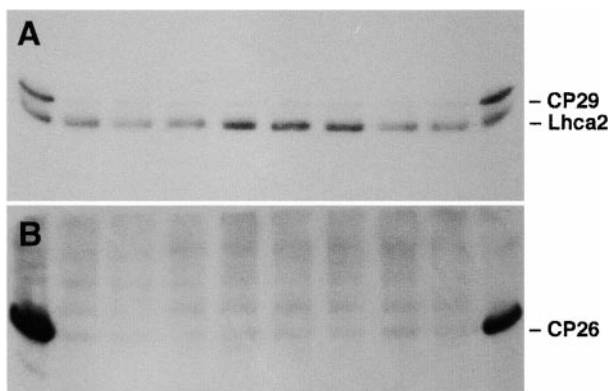


Figure 2. Immunoblot Analysis of Primary Transformants.

Thylakoid protein samples from primary transformants (T1 generation) of CP29 (A) and CP26 (B) were screened using antibodies against CP29 and CP26, respectively. The outer lanes contain samples from wild-type plants, flanking samples from eight arbitrarily selected primary transformants of each antisense construct.

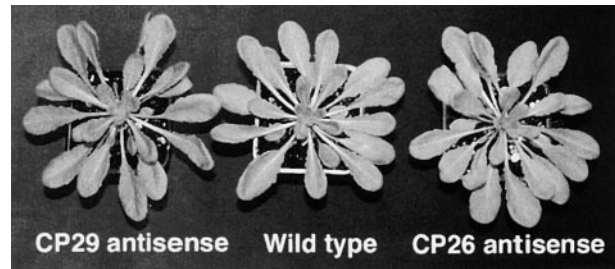


Figure 3. Growth and Morphology of CP29 and CP26 Antisense Plants.

Antisense plants were grown in low light under standard conditions as described in the text. Growth rate, morphology, and pigmentation were indistinguishable between the antisense plants and the wild-type parental line.

transformations were found to have the target proteins reduced to very low levels (Figure 2). In the case of CP26, no trace of the protein was visible even after prolonged exposure of the immunoblots, whereas small amounts (<5%) of CP29 were present. As discussed below, it is possible that the remnant Lhcb4 polypeptides are products of the *Lhcb4.3* gene.

Three individual lines of each antisense transformant were chosen for further analysis and selfed, and T2 or T3 plants were used for the following experiments. DNA gel blots using radiolabeled probes hybridizing with *Lhcb4* or *Lhcb5* revealed different restriction patterns for the different lines, confirming that the lines were the result of independent transformation events (data not shown). Subsequent experiments were performed on all three lines of each antisense construct; in all cases, variation between different independent antisense lines was no greater than batch-to-batch variation for a single line of plants. Because the lines always behaved equally, we show the results either for a single representative of each transformant or as aggregated data for all three lines. The antisense lines, lacking the different target proteins, had under growth chamber conditions no obvious phenotype (Figure 3) in terms of pigmentation or morphology and had growth rates comparable to those of the untransformed control.

Reduced CP24 Levels in CP29 Antisense Lines

Removal of one protein in a multiple protein complex can result in decreased stability of the others; for example, we have shown that the two LHCI proteins Lhca2 and Lhca3 are mutually dependent on each other for maximum stability (Ganeteg et al., 2001), probably due to physical interaction between the polypeptides. Alternatively, there might be compensation for the loss of one pigment-protein complex by increasing the levels of another. To determine the protein

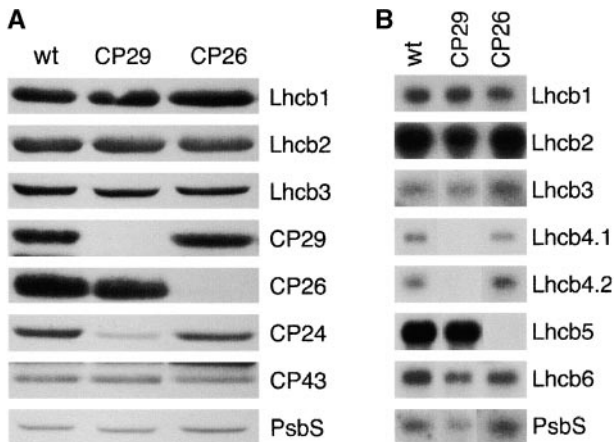


Figure 4. Immunoblot and RNA Gel Blot Analysis of CP29 and CP26 Antisense Lines.

(A) Thylakoid samples were probed with antibodies specific for the indicated proteins.

(B) RNA samples were hybridized with radiolabeled, gene-specific polymerase chain reaction fragments homologous with the genes encoding PSII antenna proteins, as indicated. wt, wild type.

composition of the PSII antenna in the antisense plants, we performed immunoblot analyses by using antibodies against all Lhcb proteins, PsbS, and CP43 (Figure 4A). In the CP26 antisense lines, no pleiotropic effects were found, the other proteins examined being present in wild-type amounts. However, thylakoids from CP29 antisense plants had significantly reduced amounts of CP24; this finding was reproduced in several batches of plants grown under lights of varying quantity and spectral quality. In some experiments, it also appeared that the amount of PsbS might be increased in CP26 antisense plants and decreased in CP29 antisense plants; however, this effect was neither large nor consistent. The other proteins were not affected, to the detection limit of this method, by the reduced levels of CP29 or CP26.

All *Lhcb* genes are homologous, to varying degrees, and it was possible that an antisense construct against one gene could affect the mRNA level of another gene. To determine if this was the cause of the decreased CP24 levels in the CP29 antisense lines, and to confirm the absence of *Lhcb4* and *Lhcb5* transcripts in the respective antisense lines, we hybridized gene-specific probes for *Lhcb1*, *Lhcb2*, *Lhcb3*, *Lhcb4.1*, *Lhcb4.2*, *Lhcb4.3*, *Lhcb5*, *Lhcb6*, and *PsbS* to RNA gel blots of RNA preparations from the different lines (Figure 4B). The antisense effect was highly specific; only the target mRNA was absent. Therefore, the decreased CP24 protein levels observed in the CP29 antisense lines were not caused by degradation of *Lhcb6* mRNA and probably were due to the decreased stability of the CP24 com-

plex in the absence of CP29. Because the levels of *Lhcb4.1* and *Lhcb4.2* transcripts were below the level of detection, the residual CP29 protein might be the product of the *Lhcb4.3* gene. However, we were not able to detect *Lhcb4.3* transcripts in leaves of either wild-type or CP29 antisense plants under our experimental conditions. In any case, it is apparent that in the antisense lines >95% of PSII centers lack CP29.

Altered PSII Function in Antisense Lines

The impact of the loss of CP26 or CP29 on photosynthesis was investigated by measuring rates of O_2 evolution in CO_2 -saturating conditions in leaves from antisense lines grown in either low light (LL) or high light (HL) conditions (Figure 5 and Table 1). There was a clear increase in the maximum photosynthesis rate (P_{max}) in HL-grown CP26 antisense plants and a small but significant reduction in the P_{max} of LL-grown CP29 antisense plants; P_{max} was unchanged in HL-grown CP29 antisense plants and LL-grown CP26 antisense plants. No reduction in the quantum yield of photosynthesis in light-limiting conditions was observed in any of the antisense lines; this was confirmed from measurements of CO_2 consumption using light with a different spectral composition (data not shown), implying that efficient light capture

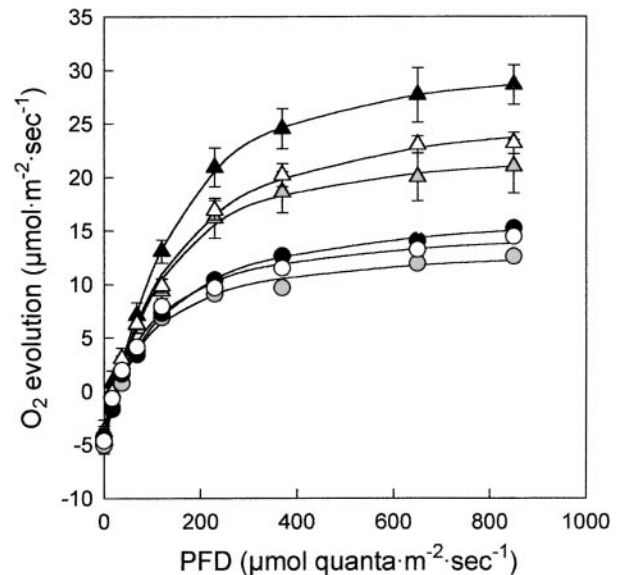


Figure 5. Photosynthetic Oxygen Evolution in Saturating CO_2 .

The rate of oxygen evolution from leaf discs was measured during illumination with varying levels of broadband red light for plants (wild type, white symbols; CP29 antisense, gray symbols; CP26 antisense, black symbols) grown under HL (triangles) or LL (circles). Symbols and error bars show means \pm SE ($n \geq 4$). PFD, photon flux density.

Table 1. Composition and Function of the Photosynthetic Apparatus^a

Lines	P_{\max} ($\mu\text{mol O}_2 \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$)	Functional PSII ($\text{mmol} \cdot \text{mol Chl}^{-1}$)	Chl <i>a/b</i>
Wild type			
HL	23.7 ± 1.0	3.85 ± 0.12	3.82 ± 0.05
LL	14.2 ± 0.5	3.03 ± 0.16	3.33 ± 0.01
CP29			
HL	20.8 ± 2.0	4.42 ± 0.18 ^b	3.88 ± 0.06
LL	12.3 ± 0.5 ^b	2.98 ± 0.19	3.31 ± 0.02
CP26			
HL	28.8 ± 2.0 ^b	5.04 ± 0.44 ^b	3.85 ± 0.10
LL	15.2 ± 0.5	4.03 ± 0.15 ^b	3.28 ± 0.02

^aMaximum photosynthetic rate (P_{\max}) was determined, and functional PSII and chlorophyll were assayed in leaf discs from plants grown under HL or LL. Data are means ± SE ($n \geq 4$). Chl, chlorophyll.

^bSignificant differences from the corresponding wild-type parental line ($P < 0.05$).

was not compromised by the loss of CP29 (plus CP24) or CP26. Measurements of functional PSII indicated that with the exception of LL-grown CP29 antisense plants, the loss was compensated for by increasing the levels of PSII (Table 1).

Although PSII levels increased, there was no marked increase in levels of LHCII, because chlorophyll *a/b* ratios were unchanged (Table 1). This implies that the antenna sizes of individual PSII units were reduced. To estimate the magnitude of the change, we used published pigment binding properties of LHC and reaction center complexes (Bassi et al., 1996) and made the assumption that the proportion of chlorophyll associated with PSII and PSI remained constant (otherwise, quantum yield would be affected). This calculation indicated that under all growth conditions there was a decrease in the PSII antenna in CP26 antisense plants of ~1 LHCII trimer per PSII, in addition to the reduction in light-harvesting capacity due to the loss of CP26. CP29 antisense plants showed only marginal changes in the PSII antenna other than the loss of CP29 and a fraction of CP24. The loss of CP26 or CP29 had no effect on the acclimation of the PSII antenna to light quantity; for all lines, growth light-dependent changes in LHCII content were equivalent to between 1 and 1.5 extra trimers per PSII in LL-grown plants, consistent with previous observations for *Arabidopsis* and other species (Mäenpää and Andersson, 1989; Walters et al., 1994; Walters and Horton, 1999).

Although there were only minor effects on photosynthesis, room temperature chlorophyll fluorescence measurements revealed that the loss of either CP29 or CP26 altered the characteristics of PSII: the variable fluorescence yield/maximum fluorescence yield ratio (F_v/F_m) of dark-adapted plants, a parameter that is regarded as reflecting changes in PSII photochemical efficiency (Maxwell and Johnson, 2000), was reduced significantly in both antisense lines (Table 2).

This effect was observed irrespective of growth conditions but was particularly pronounced in LL-grown anti-CP29 lines.

A careful analysis of fluorescence measurements from intact leaves indicated that the reductions in F_v/F_m in CP26 and CP29 antisense lines were due to differing effects: CP26 antisense and HL-grown CP29 antisense lines appeared to have unchanged dark-level fluorescence (F_o) but decreased F_m ; in contrast, in addition to a small reduction in F_m , LL-grown CP29 antisense lines had a substantially increased F_o . Because the absolute level of leaf fluorescence is affected by numerous factors unrelated to PSII function, fluorescence also was measured from broken chloroplasts suspended at identical chlorophyll concentrations (Figure 6A). As suggested by the observations with intact leaves, the F_m level was decreased in CP26 antisense lines, whereas in CP29 antisense lines there was a smaller decrease in F_m and an increase in F_o . During these experiments, it also was observed that there were significant effects on the apparent stability of PSII in vitro: F_v/F_m was maintained over many hours when wild-type chloroplasts were stored in the dark on ice; however, chloroplasts from antisense plants (particularly those from antisense CP29 plants) exhibited a clear increase in the rate at which F_v/F_m decreased (Figure 6B).

We also investigated the 77K chlorophyll fluorescence emission spectra from leaves (Figure 7). Relative to fluorescence from PSI (with a maximum at ~732 nm), the magnitude of the fluorescence emission in the 680- to 700-nm region (emanating from PSII) was largely unchanged in the CP29 antisense line compared with that in the wild type; in contrast, in the CP26 antisense line it was increased significantly,

Table 2. Altered PSII Fluorescence in CP29 and CP26 Antisense Lines^a

Lines	F_v/F_m		
	Untreated	Nigericin	Water Control
Wild type			
HL	0.824 ± 0.007		
LL	0.821 ± 0.012	0.823 ± 0.007	0.822 ± 0.002
CP29			
HL	0.782 ± 0.012 ^b		
LL	0.767 ± 0.010 ^b	0.779 ± 0.001 ^b	ND ^c
CP26			
HL	0.792 ± 0.013 ^b		
LL	0.790 ± 0.011 ^b	0.806 ± 0.001 ^b	ND

^aPlants were grown under HL or LL and dark adapted for 1 hr, and the F_v/F_m was determined directly (means ± SD, $n \geq 8$) or after infiltration of leaf discs with nigericin or water (means ± SE, $n = 3$).

^bSignificant difference from the corresponding wild-type parental line ($P < 0.001$).

^cND, not determined.

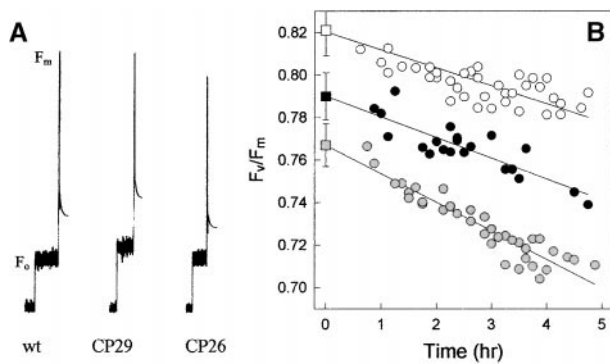


Figure 6. Room Temperature Chlorophyll Fluorescence from Thylakoids.

(A) Fluorescence traces from broken chloroplasts from LL-grown wild-type (wt), CP29 antisense, and CP26 antisense plants showing the dark (F_o) fluorescence level achieved after application of the low-intensity measuring beam and the maximum (F_m) level achieved after application of an intense light pulse.

(B) F_v/F_m measurements from broken chloroplasts from LL-grown plants (wild type, white symbols; CP29 antisense, gray symbols; CP26 antisense, black symbols) after storage on ice. Squares and error bars denote the F_v/F_m of intact leaves (mean \pm SE, see Table 2). Data are from four or five separate chloroplast preparations for each set of plants.

which we believe to be a consequence of the increased numbers of functional PSII centers in the CP26 antisense line. This change was due to an increase in the absolute levels of PSII fluorescence and not to a decrease in PSI fluorescence (data not shown). There also was a change in the shape of the emission spectrum in the 680- to 700-nm region in the CP26 antisense line. Second derivative analysis (data not shown) showed that PSII fluorescence for all three sets of plants was composed of two separate emissions centered at 684 and 694 nm; in CP26 antisense plants, the ratio between these emissions was altered. There also was a small but reproducible change in the relative magnitudes of the two peaks in CP29 antisense plants. Similar experiments with finely ground, diluted thylakoids showed essentially the same results (data not shown). Thus, it appears that the loss of CP29 and CP26 alters the energetics of PSII so that exciton distribution within the antenna is altered. One possible reason for these effects is direct energy transfer by CP26 to the species fluorescing at 694 nm and by CP29 to the species fluorescing at 684 nm.

Both Antisense Lines Were Capable of Performing qE

PSII function during photosynthesis was investigated by further analyzing chlorophyll fluorescence (Figure 8). HL-grown CP26 antisense plants showed clear increases both in the extent to which PSII was in an oxidized state and in PSII

photochemical efficiency compared with untransformed controls (Figures 8A and 8B). These results presumably reflect the higher rate of photosynthetic electron transport in these plants (Figure 5). PSII photochemistry in other antisense lines was indistinguishable from that in the wild type.

Both sets of antisense lines exhibited appreciable levels of rapidly relaxing energy dissipation (qE, measured as reversible NPQ [NPQ_{rev}]), demonstrating that neither CP26 nor CP29 is crucial for its formation (Figures 8C and 8D). Nevertheless, the absence of either CP26 or CP29 gave rise to clear changes in quenching: HL-grown antisense plants showed reduced quenching at intermediate light levels, although maximum quenching was largely unchanged; LL-grown antisense plants had reduced maximum values for NPQ_{rev}, particularly CP29 antisense plants.

CP29, CP26, and CP24 bind significant amounts of the XC pigments—violaxanthin, antheraxanthin, and zeaxanthin—which are implicated in the activation of NPQ. Therefore, we measured carotenoid levels, XC pool size, and HL-induced interconversion of XC carotenoids in wild-type and antisense lines (Table 3). Only small (not statistically significant) changes in carotenoid/chlorophyll ratios were observed. However, XC pigments were significantly depleted in the CP29 antisense lines in both LL and HL conditions, as might be expected in view of the XC pigment binding properties of CP29 and CP24; when the increased levels of PSII in CP26 antisense lines are taken into account, it is clear

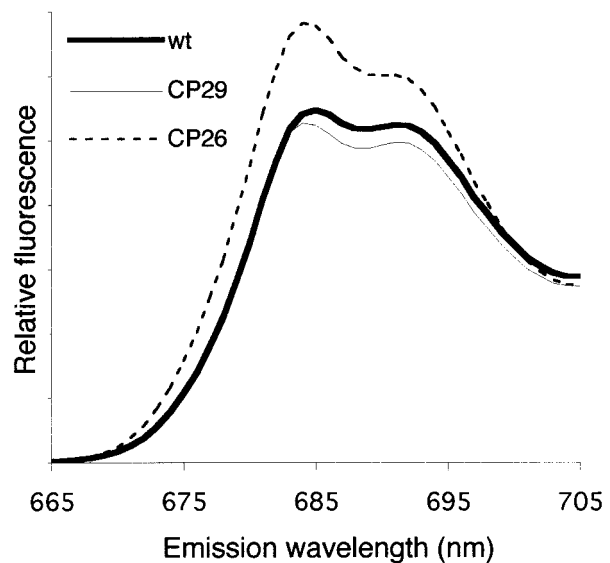


Figure 7. Whole-Leaf Fluorescence Emission Spectra at 77K.

Fluorescence emission spectra were determined for intact leaves from CP29 antisense plants, CP26 antisense plants, and untransformed control plants (wild type [wt]) at 77K. The spectra are normalized to the 732-nm emission peak emanating from PSI. Each curve is the mean of separate measurements on nine to 20 different leaves.

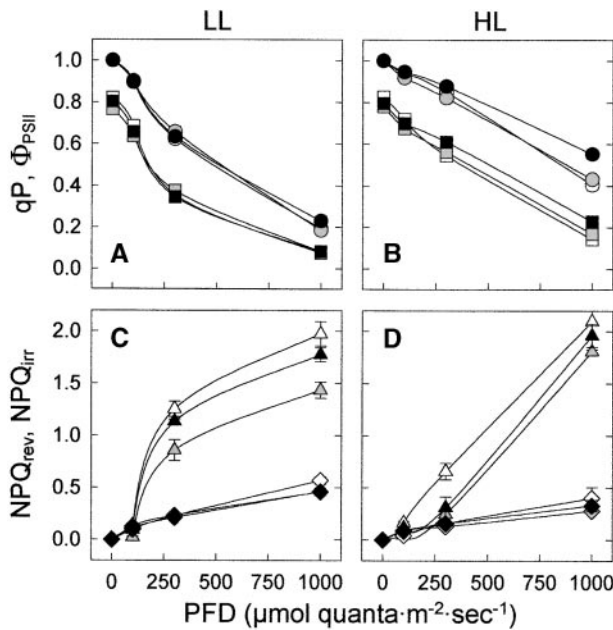


Figure 8. Analysis of Room Temperature Chlorophyll Fluorescence during Photosynthesis.

Fluorescence was monitored in leaf discs from dark-adapted plants (wild type, white symbols; CP29 antisense, gray symbols; CP26 antisense, black symbols) grown in low light (**A**) and **C**) or high light (**B**) and **D**). Leaf discs were given 23 min of illumination in the presence of saturating CO_2 followed by 10 min in the dark.

(A) and **(B)** PSII redox state (qP ; circles) and photosynthetic efficiency (Φ_{PSII} ; squares) during steady state photosynthesis.

(C) and **(D)** Reversible energy dissipation (NPQ_{rev} ; triangles) and irreversible energy dissipation (NPQ_{irr} ; diamonds).

Symbols and error bars show means \pm SE ($n \geq 3$). PFD, photon flux density.

that XC pigments per PSII also were decreased significantly in these plants ($P < 0.01$), again consistent with the pigment binding properties of CP26. Despite this reduction in XC pigment content, the deepoxidation state of the XC pool did not differ from that in wild-type plants, either immediately after commencing a light treatment or at its conclusion. Thus, the reduction in qE did not appear to be due to altered XC activity.

On illumination of dark-adapted samples, NPQ formation takes place in two distinct phases: a rapid phase that occurs within 2 min and that is largely independent of zeaxanthin formation, and a slower phase that is inhibited by treatments that block the XC (Jahns and Schweig, 1995; Härtel et al., 1996). Both components of quenching relax rapidly after darkening and are reinduced quickly upon reillumination, indicating that they are both dependent on the formation of a transthylakoid ΔpH . The kinetics of quenching formation in LL-grown plants (Figure 9) show that for both CP29 and CP26 antisense plants, the reduction in NPQ corresponded almost completely with changes in the rapidly

forming component of quenching. In contrast, the magnitude of the slower phase of qE was indistinguishable from that in control plants. Remarkably, the reduction in reversible quenching was accompanied by a reduction in slowly relaxing or irreversible quenching (NPQ_{irr}); because NPQ_{irr} is a frequently used indicator of photodamage or sustained downregulation of PSII, it seems that such inhibition of PSII activity was, if anything, lower in the antisense plants.

Further analysis suggests that the loss of CP29 and CP26 affects quenching kinetics. Formation of the slow phase of NPQ was somewhat slower in all antisense lines (half-times of ~ 6 min compared with 4.5 min for control plants); indeed, both antisense lines exhibited an increase in NPQ during a second light period, suggesting that formation of the slow phase was incomplete during the first illumination. It was notable that in CP29 antisense plants, reversible quenching relaxed almost completely within 2 min of darkening (half-time = 14 ± 1 sec), significantly faster than control plants (22 ± 1 sec), although it is not clear whether this is simply a consequence of there being less reversible quenching. Quenching relaxed more slowly in CP26 antisense plants (29 ± 1 sec). Similar changes in NPQ kinetics were observed for HL-grown plants (data not shown).

It is difficult to make detailed comparisons of NPQ between different plants with distinct PSII characteristics, because calculated values of NPQ are highly dependent on accurate measurement of the "dark-adapted" reference state. The observed differences in F_v/F_m between control and antisense plants suggest that these states are not necessarily comparable. Infiltration of leaves with nigericin had no effect on the measured F_v/F_m (Table 2), removing the possibility of ΔpH -dependent quenching of F_m in the dark in antisense plants. The reduction in quenching in CP29 antisense plants therefore is likely to be genuine; however, the possibility remains that the small decrease in reversible NPQ observed in CP26 antisense plants is an artifact associated with a reduced F_m .

DISCUSSION

The photosynthetic light-harvesting antenna is highly efficient at capturing light energy for photosynthesis, but equally important is the capacity for a rapid switch to an energy-dissipating mode (Horton et al., 1996). Without delicate tuning between light harvesting and dissipation, the efficiency of the photosynthetic apparatus would be reduced drastically in the fluctuating light conditions of nature, either by suboptimal light absorption or by devastating overexcitation. To study the processes of energy transfer and energy dissipation and the switch between these two states, we have started to systematically create Arabidopsis lines that lack the different antenna proteins.

Here and in previous studies (Zhang et al., 1997; Ganeteg et al., 2001), we have shown that antisense inhibition is a

Table 3. Leaf Carotenoid Content and Xanthophyll Cycle Activity^a

Lines	Carotenoid/Chlorophyll (mol·mol ⁻¹)	XC (% Total Carotenoid)	Postillumination DES (%)	
			2 Min	23 Min
Wild type				
HL	0.30 ± 0.01	17.9 ± 0.5	ND ^b	ND
LL	0.24 ± 0.01	15.9 ± 0.6	12.0 ± 3.3	29.6 ± 4.1
CP29				
HL	0.27 ± 0.01	15.6 ± 0.9 ^c	ND	ND
LL	0.25 ± 0.01	12.9 ± 0.3 ^d	9.4 ± 2.4	30.4 ± 4.4
CP26				
HL	0.28 ± 0.01	17.4 ± 0.8	ND	ND
LL	0.25 ± 0.01	15.1 ± 0.6	10.4 ± 3.1	33.3 ± 4.7

^aCarotenoids and chlorophylls extracted from leaf discs from dark-adapted or light-treated plants (grown under HL or LL) were assayed by reverse-phase HPLC. The deepoxidation state (DES) of the XC pigments zeaxanthin (Z), antheraxanthin (A), and violaxanthin (V) was calculated as $(Z + 1/2 A)/(Z + A + V)$, for leaves exposed to 1000 $\mu\text{mol quanta}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$ in CO₂-saturating conditions for either 2 or 23 min; the preillumination DES was <4% for all lines. Data are means \pm SE, $n \geq 4$.

^bND, not determined.

^cSignificant difference of $P < 0.05$ from the corresponding wild-type parental line.

^dSignificant difference of $P < 0.01$ from the corresponding wild-type parental line.

useful tool for the creation of Arabidopsis lines lacking individual proteins. The method is not only efficient but also specific in the sense that only the mRNA level of the desired protein is affected, leaving the other members of this family unchanged. On the basis of evolutionary arguments (Jansson, 1999), the *Lhcb4.1* and *Lhcb4.2* genes are likely to encode proteins (91% identical) that have the same function. Conveniently enough, we find that transcript levels for both genes are reduced to undetectable levels in antisense lines. The significance of the third *Lhcb4* gene, *Lhcb4.3*, is unclear. The frequency of expressed sequence tag (EST) clones in Arabidopsis is very low (Jansson, 1999), and we have not been able to detect *Lhcb4.3* transcripts in leaves by RNA gel blotting. We hypothesize that residual levels of CP29 in CP29 antisense plants may be due to the expression of this gene.

Antisense inhibition of CP26 expression was both efficient and specific, with no detectable effect on any other LHC. To confirm this, we also have analyzed pigment binding proteins by native isoelectric focusing (data not shown) and found that the only difference between the wild type and antisense plants was the absence of CP26. In contrast, CP29 antisense lines exhibited reductions in the levels of another LHC protein, CP24, despite the presence of normal *Lhcb6* message levels, indicating that CP29 is required for the stable assembly of CP24. One interpretation of this finding is that the two complexes are in physical contact, as suggested previously in biochemical studies (Bassi and Dainese, 1992). We also recently constructed antisense lines that lack CP24, in which the level of CP29 is decreased.

The loss of CP26 or CP29/CP24 did not greatly affect plants' photosynthetic performance or growth rate. Native isoelectric focusing indicated no disturbances in the macro-

molecular organization of PSII (data not shown). Although this indicates that there was no major perturbation of PSII function, distinct changes in PSII characteristics were identified: F_m was decreased to varying degrees in all antisense lines, whereas F_0 levels were increased in LL-grown CP29 antisense plants; PSII levels were increased, especially in the CP26 antisense lines; and PSII appeared less stable in CP29 antisense lines. Apparently, the removal of CP26 and

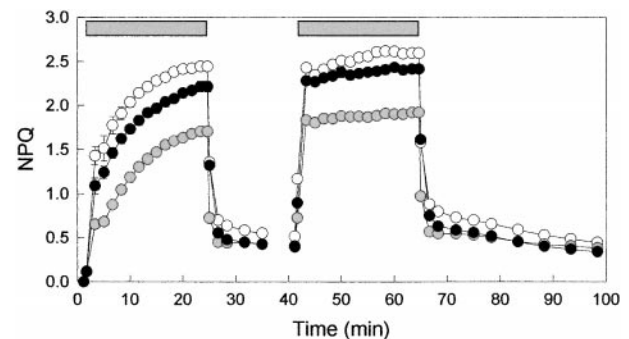


Figure 9. Kinetics of the Formation and Relaxation of Photoprotective Energy Dissipation.

Fluorescence was monitored in leaf discs from LL-grown plants (wild type, white circles; CP29 antisense, gray circles; CP26 antisense, black circles) during two successive periods of illumination with strong light ($1000 \mu\text{mol quanta}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$), as indicated by the gray bars, with a 17-min period of darkness in between. Symbols and error bars show means \pm SE ($n \geq 3$) for the total energy dissipation NPQ.

CP29/CP24 influences PSII activity, but the plant is largely able to compensate for the losses by rearrangements of the photosynthetic apparatus, including increases in the levels of PSII. As a result, the antisense plants performed well under standard laboratory conditions. Indeed, preliminary experiments, using HL and LL in combination with cold or extreme LL ($30 \mu\text{mol quanta}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$) at normal temperature, failed to identify conditions in which the absence of either of the proteins significantly affected the viability or growth rate of the plants. It is interesting that protein levels of CP26 decreased drastically in tobacco plants carrying a deletion of *orf62* (also called *ycf9*), a chloroplast gene suggested to encode a novel structural component of the LHCII (Ruf et al., 2000). This line showed inefficient photosynthesis and very slow growth in LL, which was suggested to be due to the loss of CP26. Our results show that the phenotype of the *orf62* knockout plants is unrelated to the loss of CP26.

One role that has been suggested for CP26 and CP29 is that of organizing the major LHCII light-harvesting antenna of PSII; therefore, it might be expected that the loss of one of these complexes could alter the antenna properties of individual PSII units. The significant reduction in PSII antenna size in CP26 antisense lines, by approximately a full LHCII trimer in both LL and HL growth conditions, is consistent with this hypothesis; we suggest that CP26 may be the primary anchor point for the strongly bound or proximal (nonmobile) LHCII (Mäenpää and Andersson, 1989; Spangfort and Andersson, 1989; Boekema et al., 1998) and that the loss of CP26 prevents the binding of this population of LHCII complexes. Conversely, CP29 and/or CP24 may be the principal site for binding of the distal LHCII (Mäenpää and Andersson, 1989), which is present in significant amounts only during LL growth (Walters and Horton, 1999). Inefficient energy transfer by this LHCII population in the absence of CP29/CP24 can account for the increased F_0 and the marginally reduced P_{max} of LL-grown CP29 antisense plants; CP29 antisense plants do not display this phenotype when grown in HL, under which conditions this LHCII population is largely absent (Walters and Horton, 1999). The observed 77K fluorescence emission spectra are consistent with the occurrence of major changes in the organization of the PSII antenna.

Analysis of the energy-dissipating capacity of the antisense lines showed that they were capable of developing a significant level of the qE type of NPQ. In particular, the maximum values for NPQ determined in CP26 and HL-grown CP29 antisense lines were barely distinguishable from that of the wild type, although there were small changes in the rate of formation and relaxation of quenching. LL-grown CP29 antisense plants had a somewhat different phenotype, with a clear reduction in the rapidly forming, zeaxanthin-independent component of NPQ; the absence of such a phenotype in HL-grown plants again suggests that this is an indirect effect of the loss of CP29, perhaps related to antenna disorganization resulting from the partial disconnection of peripheral LHCII. An alternative explanation might reflect the role of CP29 in proton channel-

ling away from PSII (Walters et al., 1996); because the rapidly forming component is believed to be directly dependent on ΔpH formation, the CP29 antisense phenotype could reflect a change in the pH gradient in these plants.

The simplest interpretation of these data is that CP29 and CP26 are involved in the coordination of the LHCII antenna but are not involved directly in NPQ. The presence of quenching in the antisense lines is clearly consistent with the hypothesis that the PsbS protein forms the quenching species, as suggested by the phenotype of *npq4* mutants (Li et al., 2000). It can be argued that either CP29 or CP26 is sufficient for NPQ formation and that an effect would be seen only if both of these complexes were to be removed. However, the fact that in HL-grown plants the quenching capacity is more or less unaffected suggests that this is unlikely. Attempts to resolve this question by crossing our lines to create plants lacking both CP29 and CP26 have been unsuccessful. Although antisense inhibition in our lines was perfectly stable over several generations, the inhibition partially disappeared when the lines were crossed, perhaps due to cosuppression (identical vectors were used to construct the two sets of lines). This effect was not specific for the CP26 and CP29 antisense lines and has been found in another cross.

In conclusion, we have constructed two antisense lines lacking CP26 or CP29, in which CP29 antisense plants also had decreased amounts of CP24. These pigment-protein complexes have proved to be present, and highly conserved, in all higher plants, indicating that retaining them imparts some evolutionary advantage. The inference is that under some conditions, the different LHC proteins have some nonredundant function; otherwise, there would be species lacking one or more types of LHC. However, when grown under several different (but constant) conditions, none of the antisense plants showed any obvious visible phenotype; they exhibited differences in PSII function but nevertheless were able to show highly efficient photosynthesis. Nor did the data support the idea that these proteins are involved directly in the qE type of NPQ. It appears that a plant, when grown under static conditions for its full life span, has the capacity to adapt the light-harvesting antenna even when it lacks one or more LHC proteins. However, we suggest that under more natural conditions, in which light intensity may change by orders of magnitude within seconds, a full complement of LHC proteins provides increased flexibility to the system.

METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana cv Columbia and antisense lines derived from it were grown in growth chambers in an 8-hr photoperiod with a light intensity of $100 \mu\text{mol quanta}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$ (low light [LL]) or $400 \mu\text{mol}$

quanta \cdot m⁻²·sec⁻¹ (high light [HL]), as described previously (Walters et al., 1999).

Antisense Constructs and Arabidopsis Transformation

Antisense constructs used the plant expression vector pSJ10 (Ganeteg et al., 2001). For the CP29 antisense plasmid, Arabidopsis expressed sequence tag (EST) clone 20D3T7, harboring a full-length *Lhcb4.2* cDNA (Jansson, 1999), was digested with BamHI and SmaI, and the cDNA fragment was inserted into SmaI-digested pSJ10. For the CP26 antisense construct, a full-length Arabidopsis *Lhcb5* cDNA from EST clone 37A1T7 (Jansson, 1999) was amplified by polymerase chain reaction (PCR) with the general primers T7 (5'-TAA-TAGACTCACTATAGGGA-3') and FWE (5'-TCGCGAATTCGCGTACGTAAGCTTGGATCC-3'), digested with EcoRI, and inserted into EcoRI-digested pSJ10. The correct orientations of the inserts were confirmed in both cases by sequencing of the vector/insert junctions.

Arabidopsis cv Columbia was used as the background for antisense transformation. The in planta vacuum infiltration method was performed according to Ganeteg et al., 2001. Transformed seed were selected on Murashige and Skoog (1962) plates containing 50 μ g/mL kanamycin. Kanamycin-resistant plants were collected, screened by immunoblotting to verify the efficiency of the antisense inhibition, and allowed to self-pollinate. Kanamycin-resistant individuals from the T2 and T3 generations were used for the experiments described below.

Immunoblot Analysis

Thylakoids for immunoblot analysis were prepared by homogenizing leaves with a rotating knife in slushy grinding medium (330 mM sorbitol, 5 mM MgCl₂, and 10 mM Na₄P₂O₇, pH 6.5), filtered through cotton wool, and centrifuged for 5 min at 4°C. After resuspension in 330 mM sorbitol and 10 mM Mes, pH 6.5, the extract was centrifuged for 5 min at 4°C and resuspended in 330 mM sorbitol, 0.25 mM EDTA, 3.5 mM MgCl₂, and 50 mM Hepes-KOH, pH 7.6. The chlorophyll concentration of thylakoid suspensions extracted in 80% (v/v) acetone was determined spectrophotometrically according to Porra et al. (1989).

Immunoblotting was performed essentially according to Jansson et al. (1997). Antibodies used to detect Lhcb1, Lhcb2, Lhcb3, CP29, and CP26 were the same as those described by Jansson et al. (1997) for CP24, and new antibodies were prepared using the same antigen (Falbel and Staehelin, 1992). Antibodies specific for PsbS and CP43 were provided by Kris Niyogi (University of California, Berkeley) and Roberto Barbato (University of Padua, Italy), respectively.

RNA Gel Blot Analysis

RNA preparation and RNA gel blotting were according to Ganeteg et al., 2001. RNA was extracted from leaf material sampled 3 hr into the light period (when *Lhcb* gene transcription peaks). Blots were hybridized with ³²P-labeled PCR fragments that were prepared as follows. *Lhcb1* and *Lhcb2* in Arabidopsis are encoded by five and four highly similar genes, respectively, and no attempt was made to measure the transcription of the individual members of these gene families. Full-length cDNA probes were prepared from EST clones 138O13T7 (*Lhcb1.3*) and 31F8T7 (*Lhcb2.1*), amplified with the general PCR primers T7 and SP6 (5'-ATTTAGGTGACACTATAG-3'), and used as

probes for these genes. *Lhcb3*, *Lhcb5*, *Lhcb6*, and *PsbS* genes are singletons in Arabidopsis, and we used specific primers to amplify parts of the coding regions: 98K5T7 (*Lhcb3*) was amplified with 5'-GATGGGCAATGTTGGGAGCTTTTGG-3' and 5'-GGTGCAAC-TTAGTTGCGAAAGCCC-3'; 37A1T7 (*Lhcb5*) was amplified with 5'-GGAGCAGCTGGTTTCATCATTCTG-3' and 5'-GAAATGGCTTAG-AGAGTGGGAGCTC-3'; 23A1T7 (*Lhcb6*) was amplified with 5'-GTACAGAAAGGCTGAGCTGATCCATG-3' and 5'-CGTTTTCTACAAAC-CAAGAGCACC-3'; and 137M5T7 (*PsbS*) was amplified with T7 and 5'-CCTTTTGTCTCAACATGAAGAGAAG-3'. To distinguish between mRNAs derived from the three different *Lhcb4* genes of Arabidopsis, we amplified the divergent 3' untranslated regions: EST clones 103O22T7 (*Lhcb4.1*), 20D3T7 (*Lhcb4.2*), and 149G3T7 (*Lhcb4.3*) were amplified with SP6 in combination with 5'-ATCTTAAAATCA-TTATCATGTGAG-3' (*Lhcb4.1*), 5'-CTGATGTGGTGGCTTTTACG-3' (*Lhcb4.2*), or 5'-GCTTCTTCCACCTTAAAC-3' (*Lhcb4.3*), respectively.

Gas Exchange and Photosystem II Measurements

Measurements of O₂ evolution in saturating CO₂ were performed using an LD2 leaf disc electrode as described previously (Walters et al., 1999). Light response curves were determined using broadband red light, with determination of maximum photosynthetic rate by curve fitting to nonrectangular hyperbolas; assays of active photosystem II (PSII) were performed by measurement of the O₂ flash yield in the presence of background far-red light according to Chow et al. (1991). All data are means \pm SE for four to 15 individual plants.

Chlorophyll Fluorescence Measurements

Measurements of the variable fluorescence yield/maximum fluorescence yield ratio (F_v/F_m) from leaf tissue were performed using a model PAM2000 portable fluorometer (H. Walz, Effeltrich, Germany) either on plants after 1 hr of dark adaptation or on 1.5-cm leaf discs cut from dark-adapted plants and vacuum infiltrated with nigericin/water according to Ruban and Horton (1995). Chloroplasts for fluorescence measurements were prepared as follows. Leaves were homogenized in 450 mM sorbitol, 10 mM EDTA, 10 mM NaHCO₃, 0.1% BSA, and 20 mM Tricine-KOH, pH 8.4, filtered first through two layers of muslin and then through eight layers of muslin and one layer of cotton wool, and centrifuged at 3000g for 20 sec at 4°C. After washing by gently resuspending in 300 mM sorbitol, 5 mM MgCl₂, 2.5 mM EDTA, and 20 mM Tricine-KOH, pH 7.6, centrifugation (1500g for 2 min), and resuspension in wash medium, chloroplasts were stored on ice in the dark until use. Fluorescence measurements were performed according to Noctor and Horton (1990) by using chloroplasts broken in 5 mM MgCl₂ followed by the rapid addition of an equal volume of 660 mM sorbitol, 20 mM KCl, 2 mM EDTA, and 100 mM Hepes-KOH, pH 8.0.

Measurements of room temperature fluorescence during photosynthesis in saturating CO₂ and with illumination from a model KL1500 lamp (Schott, Mainz, Germany), calculation of fluorescence parameters, and deconvolution of nonphotochemical quenching of chlorophyll fluorescence (NPQ) into reversible and nonreversible components were performed as described previously (Walters and Horton, 1999; Walters et al., 1999). Fluorescence emission spectra (77K) were measured on intact leaves as described (Ganeteg et al., 2001). The excitation wavelength was 440 nm.

Pigment Analysis

Reverse-phase HPLC of pigments extracted in ethanol/diethyl ether, dried under N₂, and redissolved in acetone was performed as described previously (Ruban et al., 1994) using modified extinction coefficients derived as follows. The concentrations of purified pigments dissolved in 100% ethanol were determined using published extinction coefficients (Young and Britton, 1993; Britton, 1995); the same samples then were used to determine extinction coefficients when dissolved in ethyl acetate/90% acetonitrile in ratios corresponding to each pigment's retention time. The resulting relative extinction coefficients (at λ_{max} , as eluted during HPLC) were as follows: neoxanthin, 0.91; violaxanthin, 0.94; auroxanthin, 0.68; antheraxanthin, 1.00; zeaxanthin, 1.00; lutein, 1.04; chlorophyll *b*, 1.05; chlorophyll *a*, 0.70; and β -carotene, 1.00. Data for violaxanthin and its isomer auroxanthin were summed.

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Antisense Inhibition of the Photosynthetic Antenna Proteins CP29 and CP26: Implications for the Mechanism of Protective Energy Dissipation

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