ABSENCE OF THE PIGMENTS LUTEIN, VIOLAXANTHIN AND NEOXANTHIN AFFECTS THE FUNCTIONAL CHLOROPHYLL ANTENNA SIZE OF PHOTOSYSTEM-II BUT NOT THAT OF PHOTOSYSTEM-I IN THE GREEN ALGA *CHLAMYDOMONAS REINHARDTII*

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Abstract

Chlamydomonas reinhardtii double mutant *npq2 lor1* lacks the β,ε-carotenoids lutein and loroxanthin as well as all β,β-epoxycarotenoids derived from zeaxanthin (e.g. violaxanthin and neoxanthin). Thus, the only carotenoids present in the thylakoid membranes of the *npq2 lor1* cells are β-carotene and zeaxanthin. The effect of these mutations on the photochemical apparatus assembly and function was investigated. In cells of the mutant strain, the content of photosystem-II (PSII) and PSI was similar to that of the wild type, but *npq2 lor1* had a significantly smaller PSII light-harvesting Chl antenna size. In contrast, the Chl antenna size of PSI was not truncated in the mutant. SDS-PAGE and Western blot analysis qualitatively revealed the presence of all LHCII and LHCI apoproteins in the thylakoid membrane of the mutant. The results showed that some of the LHCII and most of the LHCI were assembled and functionally connected with PSII and PSI, respectively. Photon conversion efficiency measurements, based on the initial slope of the light-saturation curve of photosynthesis and on the yield of Chl *a* fluorescence *in vivo,* showed similar efficiencies. However, a significantly greater light-intensity was required for the saturation of photosynthesis in the mutant than in the wild type. It is concluded that zeaxanthin can successfully replace lutein and violaxanthin in the functional light-harvesting antenna of the *npq2 lor1* mutant.

Introduction

Higher plants and green algae collect light energy for photosynthesis by an antenna system consisting of pigment-protein complexes. These complexes contain Chl and a variety of carotenoids (Car) with different cyclic end-groups (Cunningham and Gantt 1998). The various Car are distributed among the photosynthetic complexes in the thylakoid membrane and play multiple roles in photosynthesis. They contribute to light harvesting, maintain structure and function of the photosynthetic complexes, quench Chl triplet states, scavenge reactive oxygen species, and dissipate excess energy (Demmig-Adams et al. 1996, Niyogi 1999).

The most abundant carotenoids in higher plants and green algae are the β,ε-carotenoid lutein and the β,β-carotenoid violaxanthin. They are associated with the light-harvesting complexes of PSI (LHCI) and PSII (LHCII). LHCII comprises several different Chl *a/b*-binding proteins, which are distinguished according to their abundance in the thylakoid membrane into major LHCII and minor LHCII. The minor LHCII are also termed CP29, CP26, and CP24 (Jansson 1994). It is generally accepted that assembly of the major LHCII requires approximately 7 Chl *a* molecules, 6 Chl *b* molecules, two lutein molecules and one neoxanthin molecule (Kuhlbrandt et al. 1994, Croce et al. 1999a, Hobe et al. 2000). In contrast to the major LHCII, the minor LHCII bind fewer Chl molecules, have a higher Chl *a/*Chl *b* ratio and are enriched in violaxanthin (Bassi et al. 1993, Ruban et al. 1994, Green and Durnford 1996, Sandona et al. 1998). Although less is known about the carotenoid composition of light-harvesting proteins associated with PSI (LHCI), all LHC proteins are believed to contain at least 3 carotenoid binding sites that show preferential binding for the xanthophylls lutein, violaxanthin and neoxanthin (Lee and Thornber 1995, Croce et al. 1999b).

Under physiological conditions *in vivo*, the carotenoid zeaxanthin occurs only in trace amounts within the LHC (Ruban et al. 1994, Lee and Thornber 1995, Verhoeven et al. 1999). However, during high-light exposure of photosynthetic tissue, zeaxanthin is formed upon de-epoxidation of violaxanthin through operation of the reversible xanthophyll cycle (Yamamoto 1979, 1985). Although zeaxanthin binds to the LHC during irradiance stress, such association is usually only transient. Upon recovery under low-light or in darkness, zeaxanthin is replaced by violaxanthin (Yamamoto 1985). Because zeaxanthin is present only under irradiance stress conditions, and even then in relatively small amounts, its specific association with the LHC proteins is difficult to establish. Nevertheless, it was concluded from recent *in vitro* studies (Croce et al. 1999b, Hobe et al. 2000), upon analysis of zeaxanthin accumulating *Arabidopsis thaliana* mutants (Pogson et al. 1996, Pogson et al. 1998, Tardy and Havaux 1996), and from the green alga *Scenedesmus obliquus* (Heinze et al. 1997, Bishop et al. 1998), that zeaxanthin could replace lutein and violaxanthin in the LHC of the photosynthetic apparatus.

Although the carotenoid composition of the LHC in lutein and violaxanthin lacking mutants has been investigated before, the photosynthetic apparatus organization of such carotenoid mutants has not been determined. It is still unclear whether zeaxanthin can functionally replace lutein and violaxanthin *in vivo*. In this work, we characterized the photosynthetic apparatus organization and function of a mutant *(npq2 lor1)* of the green alga *Chlamydomonas reinhardtii* with specific deletions in carotenoid biosynthesis. The size of the light-harvesting Chl antenna, the stoichiometry of the photosystems and the functionality of the photosynthetic apparatus in the

absence of lutein, loroxanthin, violaxanthin and neoxanthin are questions that have been addressed in this work. We present evidence that when zeaxanthin is the sole xanthophyll present, LHC complexes are assembled and functionally connected with the reaction center complexes of PSII and PSI. Contrary to results reported for *Scenedesmus obliquus* (Bishop et al. 1998), absence of lutein and violaxanthin did not appear to compromise the photon conversion efficiency of photosynthesis in *Chlamydomonas reinhardtii*. However, lack of these xanthophylls specifically resulted in a truncated light-harvesting Chl antenna size for PSII in the *npq2 lor1* mutant.

Materials and Methods

Organisms, Maintenance and Growth

Cells of *Chlamydomonas reinhardtii* wild type strain CC125 and of the *npq2 lor1* mutant were cultivated photoautotrophically on HS (Harris 1989) agar plates either under 5 μmol photons m^{-2} s^{-1} or 400 μmol photons m^{-2} s^{-1} continuous light (cool-white fluorescent). For measurements cells were grown in liquid culture at 200 μmol photons m^{-2} s^{-1} at 22°C in flat 1 L Roux bottles in bicarbonate enriched medium (Polle et al. 2000). Stirring of the cultures prevented cell settling and ensured uniform illumination.

Cell count and Pigment Analysis

Cell density was monitored by counting the number of cells per milliliter of culture using a Neubauer ultraplane hemacytometer. The absorbance of light $\lambda > 640$ nm of the cell culture was measured using a Klett-Summerson photoelectric colorimeter (Sumner and Somers 1944). For growth curves the logarithm of the reading (Ln *Growth*) was plotted as a function of time.

For pigment determination cells or thylakoid membranes were extracted in 80% acetone and debris was removed by centrifugation at 10,000g for 5 min. The absorbance of the supernatant was measured with a Shimadzu UV-160U spectrophotometer. The Chl (*a* and *b*) concentration of the samples was determined according to Arnon (1949), with equations corrected as in Melis et al. (1987).

For HPLC analysis the HPLC model Hewlett Packard Series 1100 equipped with a Waters Spherisorb S5 ODS1 4.6 X 250 mm cartridge column was used. 1 ml algal suspension was centrifuged at 14,000 RPM for 2 min. The pigments were extracted from algal cells by adding 200 μl 100% acetone to the pellet and vortexing at maximum speed for 1 min. The extract was centrifuged in an Eppendorf centrifuge at 14,000 RPM and 15 μl of the filtered supernatant (0.2 μm nylon filter) subjected to HPLC. HPLC analysis was performed using a modification of the method of Garcia-Plazaola and Becerril (1999). Pigments were eluted with a linear gradient from 100% solvent A (acetonitrile:methanol:[0.1 M Tris-HCl pH 8.0]; 84:2:14) to 100% solvent B (methanol:ethyl acetate; 68:32) for 15 min, followed by 3 min of solvent B. The solvent flow rate was 1.2 mL/min. Pigments were detected by absorbance at 445 nm with a reference at 550 nm, and concentrations of individual pigments were determined using standard curves of purified pigments (VKI, Hørsholm, Denmark) at known concentrations.

Isolation and Separation of Thylakoid Membrane Proteins

Cells were harvested by centrifugation at 1,000g for 4 min at 4°C. The resulting pellets were resuspended in 1-2 ml buffer containing 50 mM Tricine (pH 7.8), 10 mM NaCl, 5 mM $MgCl_2$ and stored frozen at –80°C. Samples were thawed on ice and diluted with ice-cold sonication buffer containing 50 mM Tricine (pH 7.8), 10 mM NaCl, 5 mM $MgCl_2$, 0.2% polyvinylpyrrolidone 40, 0.2% sodium ascorbate, 1 mM aminocaproic acid, 1 mM aminobenzamidine and 100 μM phenylmethylsulfonylfluoride (PMSF). Cells were broken by sonication with a Branson 250 Cell Disrupter operated at 4°C for 30 s (pulse mode, 50% duty cycle, output power 5). This process was repeated three times with 30 s interval. Unbroken cells and starch grains were removed by centrifugation at 3,000 g for 4 min at 4°C. These crude thylakoid membranes were collected by centrifugation of the supernatant at 100,000 g for 25 min at 4°C.

For protein analysis of crude thylakoid membranes the pellet was solubilized in 250 mM Tris-HCl (pH 6.8), 20% glycerol, 7% SDS and 2 M urea. Solubilization of thylakoid membrane proteins was carried out for 30 min at room temperature (a procedure designed to prevent the formation of protein aggregates during denaturation) and was followed by centrifugation of the samples in a microfuge for 4 min to remove unsolubilized material. Aliquots for Chl determination were taken and samples diluted accordingly to yield equal Chl concentrations before the samples were stored at -80°C.

SDS-PAGE and Immunoblot Analysis

Prior to loading for electrophoresis, samples were brought to room temperature. Gel lanes were loaded with an equal amount of Chl (4 nmol Chl per lane). SDS-PAGE analysis was carried out according to Laemmli (1970), with resolving gels containing 12.5% acrylamide. Gels were first run for 30 min at a constant current of 16 mA until the samples migrated into the gels and then for 16 h at a constant current of 9 mA. For protein visualization, gels were stained with 1% Coomassie brilliant blue R. Electrophoretic transfer of the SDS-PAGE resolved proteins onto nitrocellulose was carried out for 3 h at a constant current of 800 mA, in transfer buffer containing 50 mM Tris, 380 mM glycine (pH 8.5), 20% methanol and 1% SDS. Identification of LHC proteins of the thylakoid membrane was accomplished with polyclonal antibodies raised against the LHC complex of *Zea mays,* kindly provided by Dr. R. Bassi (Di Paolo et al. 1990, Bassi et al. 1992) and polyclonal antibodies raised against a polypeptide of CP26. For determination of the level of reaction center core complex proteins polyclonal antibodies against the D1 protein (Kim et al. 1993) or the *psaA/psaB* geneproducts (Kashino et al. 1990) were used. Cross-reaction with the antibodies was visualized by a chromogenic reaction with anti Ig-G secondary antibodies conjugated with alkaline phosphatase (BioRad, Hercules, CA). Immunoblots were scanned with a HP Scanjet 5300C optical scanner connected to a MacIntosh/G3 computer and the NIH Image version 1.6 program was used for deconvolution of the bands.

Chlorophyll Fluorescence

The initial (F_o), variable (F_v) and maximum (F_{max}) Chl fluorescence yield of intact cells was measured upon excitation of the cultures with green light (CS 4-96 and CS 3-69 Corning Filters, actinic light intensity of 75 μmol photons m^{-2} s^{-1}). An aliquot from the culture was incubated in the dark for 10 min prior to the measurement and the Chl fluorescence was recorded in the absence or presence of DCMU (2.5 μM final concentration).

A fluorescence video imaging apparatus (Niyogi et al. 1997a, Niyogi et al. 1998) was utilized to visualize the Chl fluorescence of algal cultures grown on agar plates. Prior to the measurement the plates were dark incubated for at least 5 min. Actinic illumination of ~ 2,000 μmol photons m^{-2} s^{-1}, sufficient to induce the F_{max} emission from the algal colonies, was employed in this fluorescence video imaging analysis. The actinic illumination was administered for a period of 1 s and the resulting fluorescence images were captured by the digital video camera of the apparatus.

Spectrophotometric Analyses

For spectrophotometric measurements the thylakoid membrane pellet was resuspended in a buffer containing 50 mM Tricine (pH 7.8), 10 mM NaCl, 5 mM $MgCl_2$. The concentration of the photosystems in the thylakoid membranes was estimated spectrophotometrically from the amplitude of the light *minus* dark absorbance difference signal at 700 nm (P700) for PSI, and 320 nm (Q_A) for PSII (Melis and Brown 1980). The functional light-harvesting Chl antenna size of PSI and PSII was measured from the kinetics of P700 photo-oxidation and Q_A photoreduction, respectively (Melis 1989).

Oxygen Evolution Measurements

Oxygen evolution activity of the cultures was measured at 22°C with a Clark-type oxygen electrode illuminated with a slide projector lamp. Yellow actinic excitation light was provided by a CS 3-69 Corning cut-off filter in combination with an Ealing 35-5453 VIQ5-8 filter. An aliquot of 5 ml cell suspension (2 μM Chl) was transferred to the oxygen electrode chamber. To ensure that oxygen evolution was not limited by the carbon source available to the cells, 100 μl of 0.5 M sodium bicarbonate solution (pH 7.4) was added to the suspension prior to the oxygen evolution measurements. The measurement of the light-saturation curve of photosynthesis was implemented with the oxygen electrode, beginning with the registration of dark respiration in the cell suspension, and followed by measurements of the rate of oxygen evolution at 30, 70, 125, 240, 375, 525, 700, 1150, 1500, 2250, and 3200 μmol photons m^{-2} s^{-1}. Registration of the rate (slope) of oxygen evolution at each light intensity step was recorded for about 2.0 min. The photosynthetic efficiency of the cells was calculated from the initial slope of the light-saturated curve of photosynthesis.

Results

Isolation and Pigment Analysis of a Secondary Pigment Mutant

From the background of the β,ε-carotenoid-less strain *lor1* of *Chlamydomonas reinhardtii* (Chunaev et al. 1991, Niyogi et al. 1997a, b), we isolated a spontaneous *npq2* mutation, resulting in the absence of the β,β-epoxycarotenoids antheraxanthin, violaxanthin and neoxanthin (Cunningham and Gantt 1998). Lack of xanthophylls derived from zeaxanthin was due to a defect in the activity of the zeaxanthin epoxidase enzyme (Niyogi et al. 1997a). Based on the results of the HPLC analysis (Figure 1), showing that zeaxanthin was the only xanthophyll present, we termed this new carotenoid mutant as *npq2 lor1*.

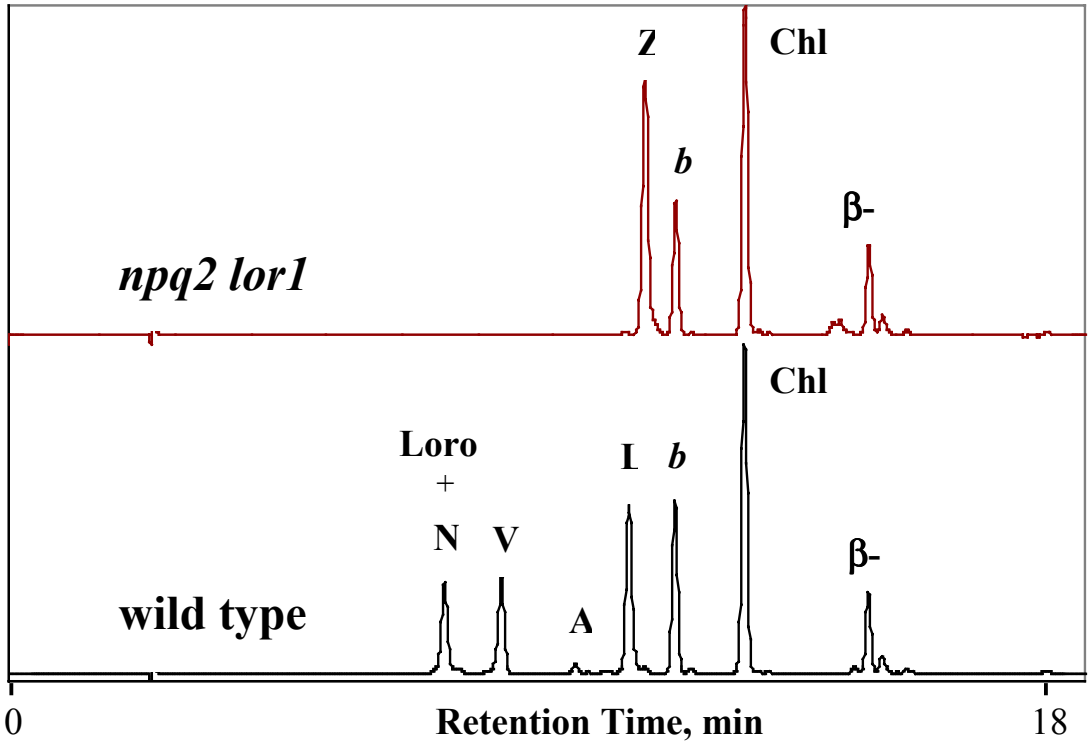

Figure 1 HPLC elution profile of total pigment extract from *Chlamydomonas reinhardtii* wild type and *npq2 lor1* mutant. Traces were normalized to the peak of Chl *a*. Identification of lettered peaks is as follows: Loro, Loroxanthin; N, neoxanthin; V, violaxanthin; A, antheraxanthin; L, lutein; Z, zeaxanthin; *b*, Chl *b*; Chl *a*, Chl a; β-Car, β-carotene.

Table 1 Pigment content of wild type and a mutant of *Chlamydomonas reinhardtii* (*npq2 lor1*) lacking lutein, loroxanthin, violaxanthin and neoxanthin. Values represent means ±SD.

Parameter	wild type	*mutant*
Chl *a* / Chl *b*	2.9 ±0.2	4.1 ±0.4
mol x10^{-15} Chl/cell	4.4 ±0.7	3.1 ±0.33
mol x10^{-15} Carotenoids/cell	1.7 ±0.2	1.2 ±0.2
Car/Chl (mol:mol)	0.39	0.39

Table 1 shows that the Chl *a*/Chl *b* ratio (=4.1) of the *npq2 lor1* mutant, like its *lor1* parent (Niyogi et al. 1997b), was significantly higher than that of the wild type (=2.9). This result suggested a different photosynthetic apparatus organization for the mutant than for the wild type (Anderson 1986, Melis 1991). Table 1 also shows that the total amounts of Chl and Car per cell in the mutant were slightly lower than that of the wild type. However, the total Car to Chl mol:mol ratio was the same in the two strains. Taken together, these results (Fig. 1 and Tab. 1) suggest that, in the *npq2 lor1* mutant, zeaxanthin has replaced and least lutein and violaxanthin in the two of the three xanthophyll-binding sites of the LHCII. The third, neoxanthin-binding site in the LHCII is reported to be highly specific for this xanthophyll species (Croce et al. 1999a,b; Connelly et al. 1997) and, therefore, it is possible that this binding site remains empty in the absence of neoxanthin in the *npq2 lor1* mutant.

Photosynthetic Apparatus Organization

Figure 2 shows growth curves of wild type and *npq2 lor1* mutant in a liquid Tris-Bicarbonate medium (see Materials and Methods) under a light intensity of 200 μmol photons m^{-2} s^{-1}. Wild type and *npq2 lor1* mutant cells had similar growth rates indicating that lack of lutein, loroxanthin, violaxanthin and neoxanthin did not compromise the photosynthetic and growth abilities of the *npq2 lor1* mutant.

In order to investigate the photosynthetic apparatus organization of the *npq2 lor1* mutant, we measured, spectrophotometrically, the amounts of photochemically active primary quinone acceptor Q_A of PSII and the reaction center P700 of PSI.

Table 2 shows that, on a Chl basis, the *npq2 lor1* mutant (4.5 mmol Q_A per mol Chl) had about 50% more photochemically competent PSII centers than the wild type (2.9 mmol Q_A per mol Chl). The amount of P700 per Chl in the mutant (1.74 mmol:mol) was only slightly higher than that of the wild type (1.4 mmol:mol). When compared on a per cell basis, mutant and wild type had similar amounts of Q_A (Table 2). In contrast, the amount of P700 per cell in the mutant was slightly lower than that in the wild type (Table 2). In consequence, the PSII/PSI molar ratio in the mutant (2.6:1) was slightly higher than that in the wild type (2.1:1).

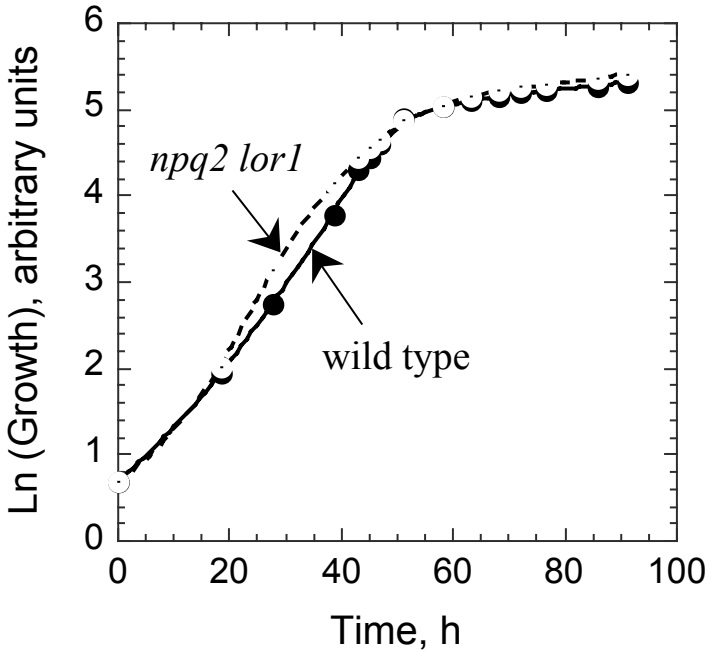

Figure 2 Growth curve of wild type (●) and *npq2 lor1* (○) mutant of *Chlamydomonas reinhardtii*. The logarithm of a photoelectric calorimeter reading of the cultures is plotted as a function of time during cell growth. Cells were cultivated photoautotrophically on a bicarbonate enriched medium under 200 μmol photons m^{-2} s^{-1}.

Table 2 Photochemical apparatus characteristics of wild type and *npq2 lor1* mutant of *Chlamydomonas reinhardtii*.

Parameters	**wild type**	***mutant***
Q_A / total Chl (mmol:mol)	2.9 ±0.18	4.5 ±0.4
P700/ total Chl (mmol:mol)	1.4 ±0.1	1.74 ±0.05
$*10^{-18}$ mol Q_A / cell	13.0	13.9
$*10^{-18}$ mol P700 / cell	6.3	5.4
PSII / PSI	2.1	2.6

The lower Chl per cell content and the elevated Chl *a*/Chl *b* ratio in *npq2 lor1* indicated a truncated Chl antenna size of the photosystems in the mutant relative to that in the wild type. In order to address this point in detail, we performed measurements of the functional Chl antenna size of the photosystems with the "kinetic/spectrophotometric" method (Melis and Anderson 1983, Melis 1989). In this approach, functional Chl molecules are assigned to each photosystem in direct proportion to the rate of light absorption/utilization by PSI and PSII. The rates for PSI and PSII were measured in isolated and DCMU poisoned thylakoids from the kinetics of P700 photooxidation and Q_A photoreduction, respectively (Melis 1989).

Results, summarized in Table 3, showed that thylakoid membranes of wild type contain approximately equal amounts of the two types of PSII, PSIIα and PSIIβ (Melis and Duysens 1979). This distribution is similar to that reported for the *Chlamydomonas reinhardtii* strain *cw15* (Polle et al. 2000). However, in the *npq2 lor1* mutant, PSIIα accounted for only about 23% and PSIIβ for 77% of the total PSII. Further, Table 3 provides values for the functional Chl antenna size (N) of PSIIα, PSIIβ and PSI in the thylakoid membrane of wild type and *npq2 lor1* mutant. In the wild type, PSIIα contained about 300 Chl *a* and Chl *b* molecules and PSIIβ contained 135 Chl molecules. The Chl antenna size of PSI contained about 240 Chl *a* and Chl *b* molecules.

Table 3 Chl antenna sizes of PSII and PSI in wild type and *npq2 lor1* mutant. The concentration of the various forms of PSII is given as a % of total PSII in the thylakoid membrane. The antenna size of PSII and PSI is given as the number of Chl molecules functionally associated with a photosystem. Values represent the average of two experiments.

Parameter	**wild type**	***mutant***	**Minimum Chl antenna size**
PSIIα	55 %	23 %	
PSIIβ	45 %	77 %	
$N_{PSII\alpha}$	301	171	
$N_{PSII\beta}$	135	90	37
N_{PSI}	242	294	95

In the *npq2 lor1* mutant, the functional Chl antenna size of PSIIα (170 Chl molecules) and PSIIβ (90 Chl molecules) were substantially smaller than those of the wild type (Table 3). Taking into consideration the smaller functional Chl antenna in both PSIIα and PSIIβ and the lowered PSIIα/PSIIβ ratio in the mutant, we estimated that PSII in the mutant contained only about 60% of the Chl compared to that in the wild type (not shown). It is evident that absence of lutein, loroxanthin, violaxanthin and neoxanthin resulted in a significantly truncated PSII Chl antenna size. Nevertheless, this functional Chl antenna size was still larger than that of the PSII-core ($N_{PSIIcore}$ = 37 Chl molecules, Manodori et al. 1984, Glick and Melis 1988), suggesting that zeaxanthin alone is sufficient to facilitate the assembly of a significant portion of the auxiliary LHCII. Moreover, the occurrence of PSIIα centers with a relative large Chl antenna size ($N_{PSII\alpha}$ = 171 Chl molecules) suggested that both major and minor components of the LHCII are assembled and functionally associated with PSII in the *npq2 lor1* mutant. Surprisingly, and in contrast to PSII, the functional Chl antenna size of PSI in the *npq2 lor1* (N_I = 294 Chl molecules) was slightly larger than that of the wild type (N_I = 242 Chl molecules). This result suggests that absence of lutein, loroxanthin, violaxanthin and neoxanthin does not bring about an adverse effect on the functional Chl antenna size of PSI.

SDS-PAGE and Western Blot Analysis of Thylakoid Membrane Proteins

The results presented above provide evidence for an altered photosynthetic apparatus organization of the *npq2 lor1* mutant. To probe further the effect of the mutation on the Chl antenna size, we qualitatively and quantitatively compared profiles of thylakoid membrane proteins from wild type and *npq2 lor1* mutant.

Figure 3 shows SDS-PAGE analysis of isolated thylakoid membrane proteins of wild type and *npq2 lor1*. Comparison of wild type (wt) and mutant (mt) lanes in Figure 3 shows quantitative, but not qualitative changes in the polypeptide composition between wild type and mutant, mainly occurring in the 31 kDa electrophoretic migration region. It is suggested that, on a per Chl basis, some of the LHCII proteins are depleted in the *npq2 lor1* mutant whereas LHCI proteins are more abundant when compared to that of the wild type level.

The assessment was further tested by Western blot analysis. Figure 4 shows an immunoblot of thylakoid membrane proteins isolated from wild type and *npq2 lor1* mutant, probed with antibodies against the PsaA/PsaB proteins of PSI (Kashino et al. 1990) and the D1 protein of PSII (Kim et al. 1993). On a per Chl basis, the mutant contained slightly more D1 than the wild type. This is consistent with the results of the spectrophotometric analysis (Table 2), in which the Q_A content was about 50% greater in the *npq2 lor1* than that in the wild type. Figure 4 also shows that the level of PsaA/PsaB proteins is very similar in wild type and *npq2 lor1* mutant indicating that, on a per Chl basis, both strains have approximately the same amount of PSI (Table 2).

Figure 5 shows a Western blot analysis of thylakoid membrane proteins probed with antibodies that recognize most LHC proteins of *Chlamydomonas reinhardtii* (Bassi et al. 1992, Polle et al. 2000). It is evident from the results in Fig. 5 that, on a per Chl basis, wild type and *npq2 lor1* mutant qualitatively contain the same LHC proteins in their thylakoid membranes. However, absence of lutein, loroxanthin, violaxanthin and neoxanthin brought about changes in the quantity of the various LHC proteins in the thylakoid membrane of the mutant. Specifically, loss of these xanthophylls resulted in significantly lower levels of CP26 whereas levels of CP29 and CP24 were higher in the *npq2 lor1* than in the wild type. The level of the major LHCII at 29 kDa was lower in the mutant than in the wild type. However, levels of the LHCII subunit migrating at ~33kDa did not seem to be affected by the mutation. In contrast to this differential effect on LHCII, levels of the various LHCI were uniformly higher in the mutant than in the wild type.

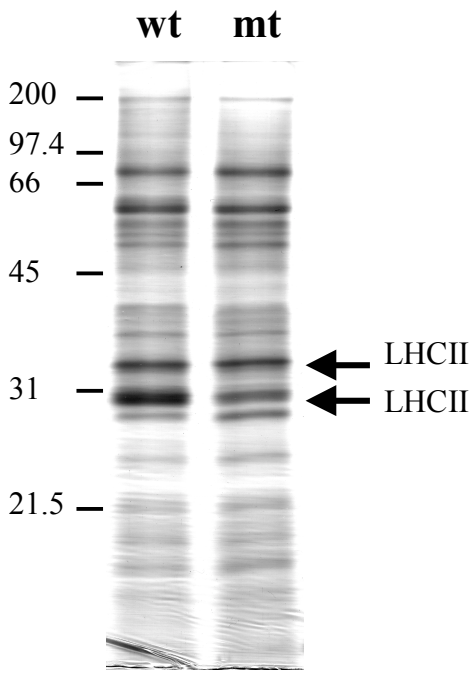

Figure 3 Thylakoid membrane protein profile from wild type (wt) and *npq2 lor1* mutant (mt) of *Chlamydomonas reinhardtii.* Proteins were separated by SDS-PAGE and visualized by Coomassie staining. The lanes were loaded on an equal Chl basis (4 nmol Chl/lane). The electrophoretic mobility of molecular-weight markers is indicated in kDa.

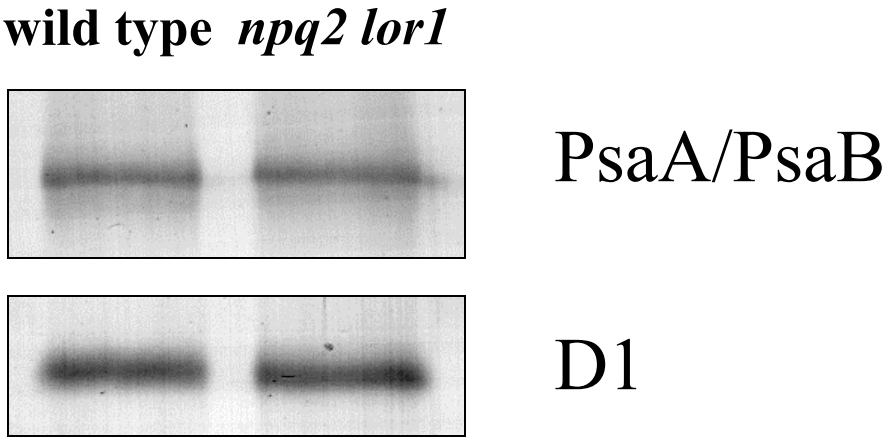

Figure 4 Western blot analysis of thylakoid membrane proteins of wild type and *npq2 lor1,* probed with specific polyclonal antibodies against the PsaA/PsaB (PSI) orD1 (PSII) reaction center proteins.

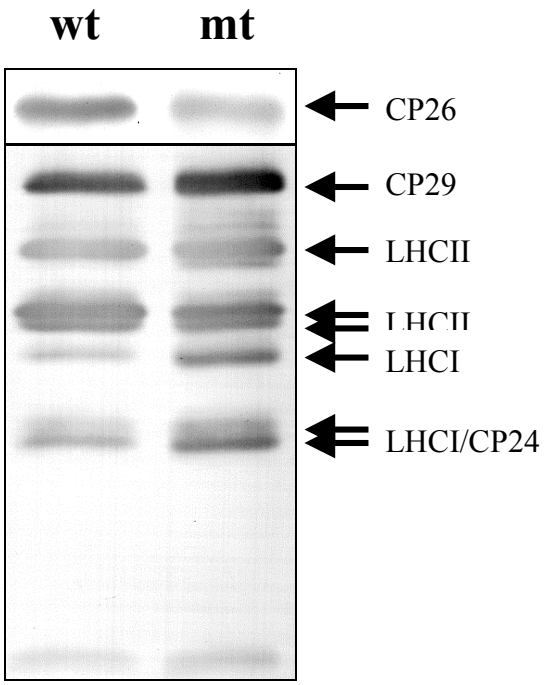

Figure 5 Western blot analysis of thylakoid membrane proteins of wild type and *npq2 lor1,* probed with specific polyclonal antibodies against the LHC proteins of PSII and PSI. Proteins were loaded on a Chl basis. The position of various LHC proteins is indicated. Asterisk denotes a possible degradation product of the LHC.

Since we determined the amount of Q_A, P700 and the LHC proteins on a per Chl basis, it was possible to normalize the level of the LHC proteins to that of PSII or PSI in the cells. The result of such analysis, shown in Figure 6, allows quantitative comparison of the LHC proteins content in the thylakoid membrane of wild type and mutant on a per PSII or PSI basis.

Figure 6A shows a comparison on the basis of Q_A. It is evident from Fig. 6A that the amounts of CP29 and LHCII per PSII in the *npq2 lor1* mutant were lowered to about 60% of that seen in the wild type. The amount of CP26 per PSII was drastically reduced to about 25% of that in the wild type. Figure 6B shows a comparison on the basis of P700. In this case, it is evident that the level of LHCI per PSI in the *npq2 lor1* mutant was significantly higher than that of the wild type.

It is noteworthy that all LHC proteins detected in association with the *npq2 lor1* mutant were fully integrated into the lipid bilayer of the corresponding thylakoid membrane. Treatment of thylakoids with 1 M Tris-HCl pH 9.0, a procedure designed to remove extrinsic proteins from the thylakoid membrane, did not result in loss of any LHC polypeptides associated with this mutant strain (data not shown).

Efficiency and Rate of Photosynthesis

Recently, Bishop et al. (1998) reported that a carotenoid double mutant of the green alga *Scenedesmus obliquus* with a phenotype similar to that of the *npq2 lor1* mutant in *Chlamydomonas reinhardtii* had a slightly increased photosynthetic capacity, but significantly reduced photosynthetic efficiency. To assess the photosynthetic capacity and efficiency of the

npq2 lor1 mutant, the rate of O_2 evolution was measured and plotted as a function of light intensity, thus obtaining the *photosynthesis* versus *irradiance* curve (light-saturation curve). From the slope of the initial linear portion of the light-saturation curve of photosynthesis, information was obtained about the relative efficiency of photosynthesis (Φ) in wild type and mutant (Björkman and Demmig 1987, Neale et al. 1993). The rate of oxygen evolution under light-saturation conditions provided information about P_{max}, the capacity of photosynthesis in the two strains (Powles and Critchley 1980).

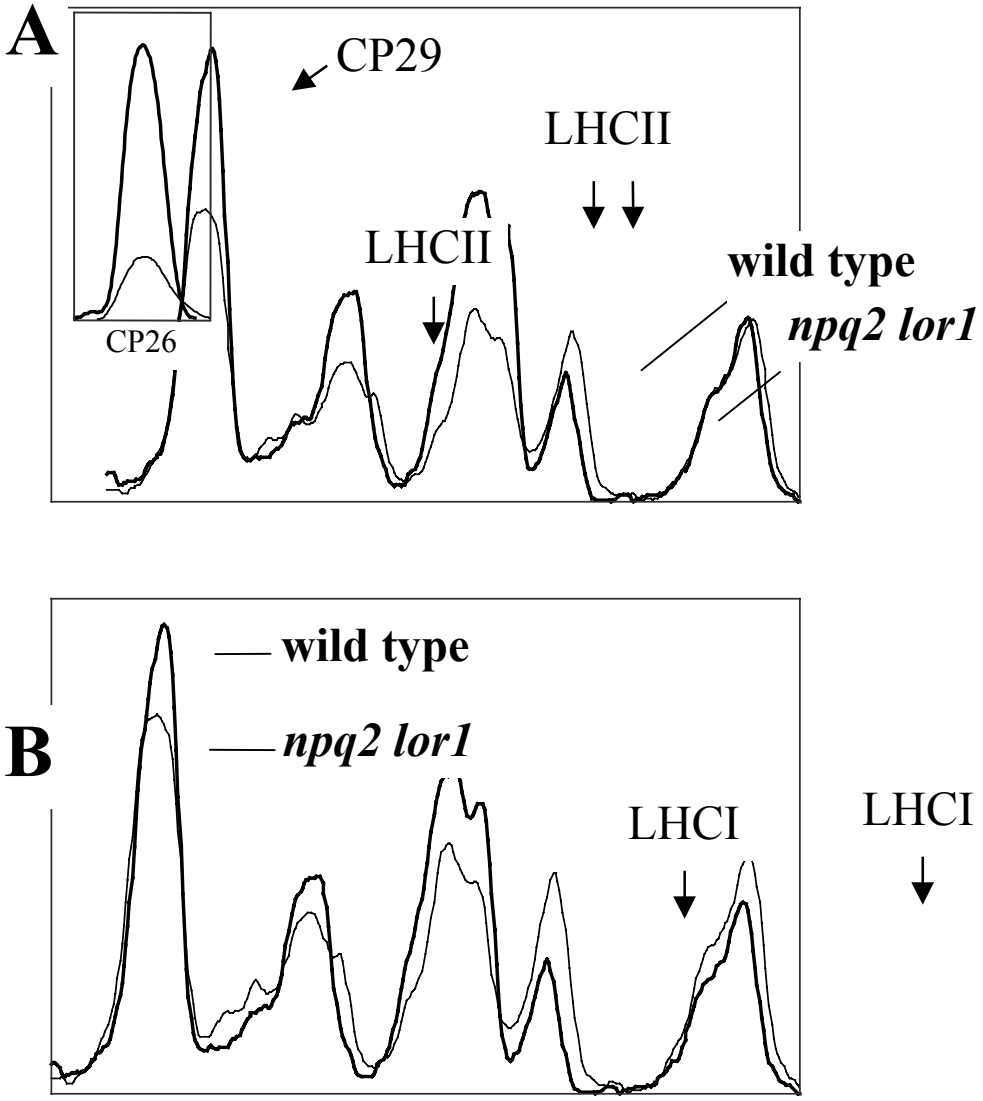

Figure 6 Densitometric scan of Western-blot lanes from Fig. 5, normalized on the basis of PSII/Q_A (A) or PSI-P700 (B). Superimposed scans were normalized on the basis of equal Q_A loading. Arrows indicate the position of the various LHC forms. Note that amounts of CP26, CP29 and LHCII per PSII/Q_A are lower in the *npq2 lor1* mutant compared to wild type (A). Conversely, levels of the LHCI per P700 are greater in the mutant when compared to wild type (B).

Figure 7 shows the light-saturation curve of photosynthesis for wild type and *npq2 lor1* mutant.

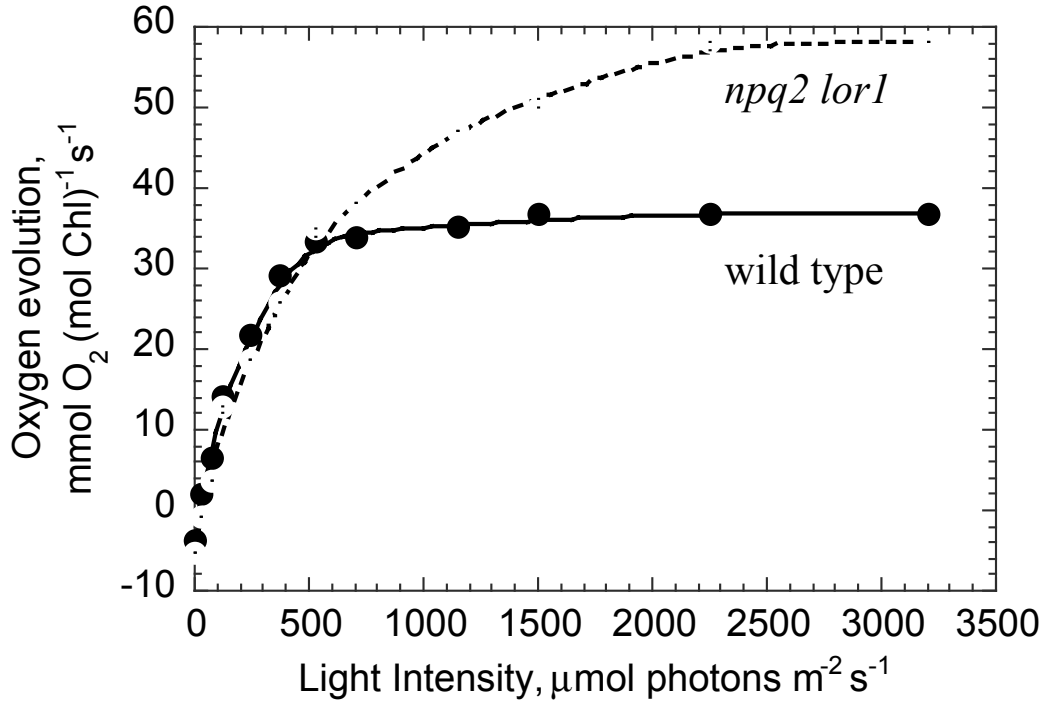

Figure 7 The light-saturation curve of photosynthesis in wild type and *npq2 lor1* mutant.

As summarized in Table 4, wild type (Φ=0.14±0.02) and mutant (Φ=0.125±0.03) had similar photosynthetic efficiencies. The photosynthetic capacity of the *npq2 lor1* mutant (P_{max}=59 mmol O_2 [mol Chl]$^{-1}$ s^{-1}) was about 50% greater than that of the wild type (P_{max}=36 mmol O_2 [mol Chl]$^{-1}$ s^{-1}).

Table 4 Gas exchange and PSII efficiency of wild type and a zeaxanthin accumulating *npq2 lor1* mutant of *Chlamydomonas reinhardtii* expressed on a per Chl basis. Values are means ±SD.

Parameter	**wild type**	***mutant***
Respiration (mmol O_2 (mol Chl)$^{-1}$ s^{-1})	4.0 ±0.04	5.1 ±1.2
Photosynthesis (mmol O_2 (mol Chl)$^{-1}$ s^{-1})	36.5 ±1.8	59.0 ±12.7
Photosynthetic efficiency (relative values)	0.14 ±0.02	0.125 ±0.03
PSII efficiency (F_v/F_{max})	0.69 ±0.04	0.60 ±0.03

Table 4 also compares the photochemical conversion efficiency at PSII, measured from the F_v/F_{max} fluorescence ratio in wild type (F_v/F_{max}=0.69) and *npq2 lor1* mutant (F_v/F_{max}=0.60). The similar F_v/F_{max} ratios in wild type and *npq2 lor1* validate the similar photosynthetic efficiencies as measured from the light-saturation curve of photosynthesis. Taken together, these results suggested that replacement of lutein, loroxanthin and violaxanthin by zeaxanthin, as it

occurred in the *npq2 lor1* mutant, did not bring about a significantly adverse effect on light absorption and utilization in the photochemical apparatus of the mutant. This is clearly evidenced by the similar photon conversion efficiencies observed in the photochemical apparatus of the two strains and ultimately by their similar growth rates.

Discussion

We isolated a carotenoid double mutant of *Chlamydomonas reinhardtii* that lacks not only all α-carotenoids (*lor1*) but is also unable to synthesize violaxanthin and neoxanthin from zeaxanthin (*npq2*). These lesions in carotenoid biosynthesis did not impair photoautotrophic growth of the *npq2 lor1* double mutant. Such observation is consistent with findings in *Scenedesmus obliquus* (Bishop 1996) and *Arabidopsis thaliana* (Pogson et al. 1998). Interestingly, in contrast to reduced virescence and seedling lethality of the analogous *aba1 lut2* mutant of *Arabidopsis* (Pogson et al. 1998), the growth rate of the *npq2 lor1 Chlamydomonas reinhardtii* mutant was not compromised. Accumulation of zeaxanthin in the *npq2 lor1* occurred concomitant with a lowering in the cellular Chl and Car content, by about one-third as compared to that of the wild type. However, levels of PSII per cell were similar and levels of PSI per cell were only slightly lower in the *npq2 lor1* when compared to that of the wild type. Therefore, it is concluded that a reduction in the level of Chl and Car per cell in the *npq2 lor1* mutant is mainly due to a reduction in the amount of LHC proteins.

Previous work with carotenoid mutants of green algae (Chunaev et al. 1991, Bishop et al. 1995, Bishop et al. 1998, Niyogi et al. 1997a,b) and higher plants (Pogson et al. 1996, Tardy and Havaux 1996) showed that loss of lutein and/or violaxanthin was accompanied by alterations in the profile of the LHC proteins in the photosynthetic apparatus. In agreement with these reports, we demonstrated in this work that lack of xanthophylls that are derived from α-carotene, as well as lack of xanthophylls derived from zeaxanthin, resulted in an altered LHC composition in the *npq2 lor1* mutant (Fig. 5). Since all LHC polypeptides are present in the thylakoid membranes of *npq2 lor1* (Fig. 5) and washing of thylakoid membranes did not remove any LHC (data not shown), we conclude that zeaxanthin can successfully substitute for other xanthophylls in the LHC in *Chlamydomonas reinhardtii*. The successful substitution of other xanthophylls by zeaxanthin in the LHCII was also demonstrated *in vitro* when zeaxanthin was used as the only xanthophyll in reconstitution experiments (Croce et al. 1999b, Hobe et al. 2000). Interestingly, the *in vitro* studies revealed that LHCII containing only zeaxanthin were not as efficient in energy transfer as control LHCII (Croce et al. 1999b). Our *in vivo* results, however, showed that *npq2 lor1* had a PSII and overall photon conversion efficiency similar to that of the wild type. These findings are in agreement with results reported for the higher plant *Arabidopsis thaliana* (Hurry et al. 1997). In contrast, stable assembly of the major LHCII was not observed in lutein-lacking mutants of the green alga *Scenedesmus obliquu*s (Bishop 1996, Bishop et al. 1998, Heinze et al. 1997). Obviously, absence of specific xanthophylls may have a variable effect on the assembly of the major LHCII in different organisms.

Quantitative LHC polypeptide analysis revealed that PSII units in the *npq2 lor1* mutant contained lower levels of CP26 (25% of the wt) and CP29 (60% of the wt). They also contained a lower level of the major LHCII (60% of the wt). Consistent with the above-described biochemical analysis, the spectrophotometric/kinetic analysis showed that the functional light-

harvesting Chl antenna size of the *npq2 lor1* is only about 60% of that in the wild type. Taken together, this rigorous analysis suggests that many PSII units in the *npq2 lor1* mutant do not contain a copy of CP26 and/or CP29. In addition, they may lack some of the peripheral components of the trimeric LHCII. In contrast, the level of CP24 per PSII was not affected in the *npq2 lor1,* indicating that every PSII unit in the mutant has a copy of CP24.

Figure 8 shows, as a working hypothesis, the organization and size of the Chl antenna of PSIIα and PSIIβ units in wild type and *npq2 lor1* mutant. This model is consistent with the results presented in this work and also consistent with the analyses presented by other authors (Hankamer et al. 1997, Melis 1998, Nield et al. 2000). The Chl antenna model of PSII, shown in Fig. 8, is based on the assumption of three levels of organization for the Chl-proteins in PSII:

- The PSII-core complex is the first level consisting of D1, D2, CP43, and CP47. This is the minimum PSII configuration that can be stably assembled *in vivo.* The PSII-core contains about 37 Chl *a* molecules (Manodori et al. 1984, Glick and Melis 1988).
- The second level of organization contains, in addition to the PSII-core, one copy of CP29, CP26, CP24, two LHCII monomers and one LHCII trimer. This PSII complex is defined as PSIIβ and contains about 130 Chl *a* and Chl *b* molecules (Melis 1991).
- Further step-wise addition of trimeric LHCII to PSIIβ results in the formation of the PSIIα units (Melis 1998).

On the basis of the quantitative data presented in this work, we estimated that only about 25% of PSII units in the *npq2 lor1* mutant contain CP26. It is thought that CP26 serves as an intermediary in the supramolecular structure of the LHCII by serving to connect peripheral LHCII trimers to the PSII-core. In the absence of CP26 from 75% of the PSII units in the *npq2 lor1* mutant, it follows that an equivalent number of PSII might not be able to bind trimeric LHCII, a prerequisite for the development of the mature PSIIα type of Chl antenna. Therefore, PSII units without a copy of CP26 should have a Chl antenna size that is slightly smaller than that of PSIIβ, owing to the lack of the trimeric LHCII and lack of CP26. This is actually observed in the Chl antenna size of PSIIβ in the *npq2 lor1,* which contains 90 Chl molecules instead of the 135 Chl molecules detected in PSIIβ of the wild type. The difference in Chl antenna size between PSIIβ in wild type and *npq2 lor1* mutant (about 45 Chl molecules) is consistent with the number of Chl molecules predicted to be in association with one LHCII trimer *plus* one copy of CP26. Therefore, we conclude that only monomeric LHCII are structurally associated with PSII in the antenna of the mutant, having an antenna size of 90 Chl molecules (Fig. 8). Since the PSII-core complex contains about 37 Chl *a* molecules, and a LHCII monomer is believed to contain 8-12 Chl molecules (Sandona et al. 1998), it follows that the remaining ~50 Chl *a* and Chl *b* molecules in PSIIβ of the mutant would occupy binding sites in about 4 LHCII monomers.

It is noteworthy that a permanently truncated PSII Chl antenna size has taken place due to alterations in the carotenoid biosynthetic pathway. This truncated Chl antenna mutant exhibited essentially the same photon conversion efficiency as the wild type and had a 50% greater photosynthetic capacity. In contrast to these results with the *npq2 lor1* mutant of *Chlamydomonas reinhardtii*, Bishop et al. (1998) showed that an analogous double mutant of the green alga *Scenedesmus obliquus* had a significantly lower photosynthetic efficiency and a photosynthetic capacity of only about 20% greater than that of the wild type. The difference

between these results could be explained by the fact that *Scenedesmus obliquus* was cultivated under heterotrophic growth conditions and only shifted to photoautotrophy prior to the measurements, whereas *Chlamydomonas* in this study was grown entirely under photoautotrophic conditions. Heterotrophy is known to affect the efficiency and capacity of the photosynthetic apparatus (Polle et al. 2000) and this consideration may be sufficient to explain differences between the *Scenedesmus* and *Chlamydomonas* results.

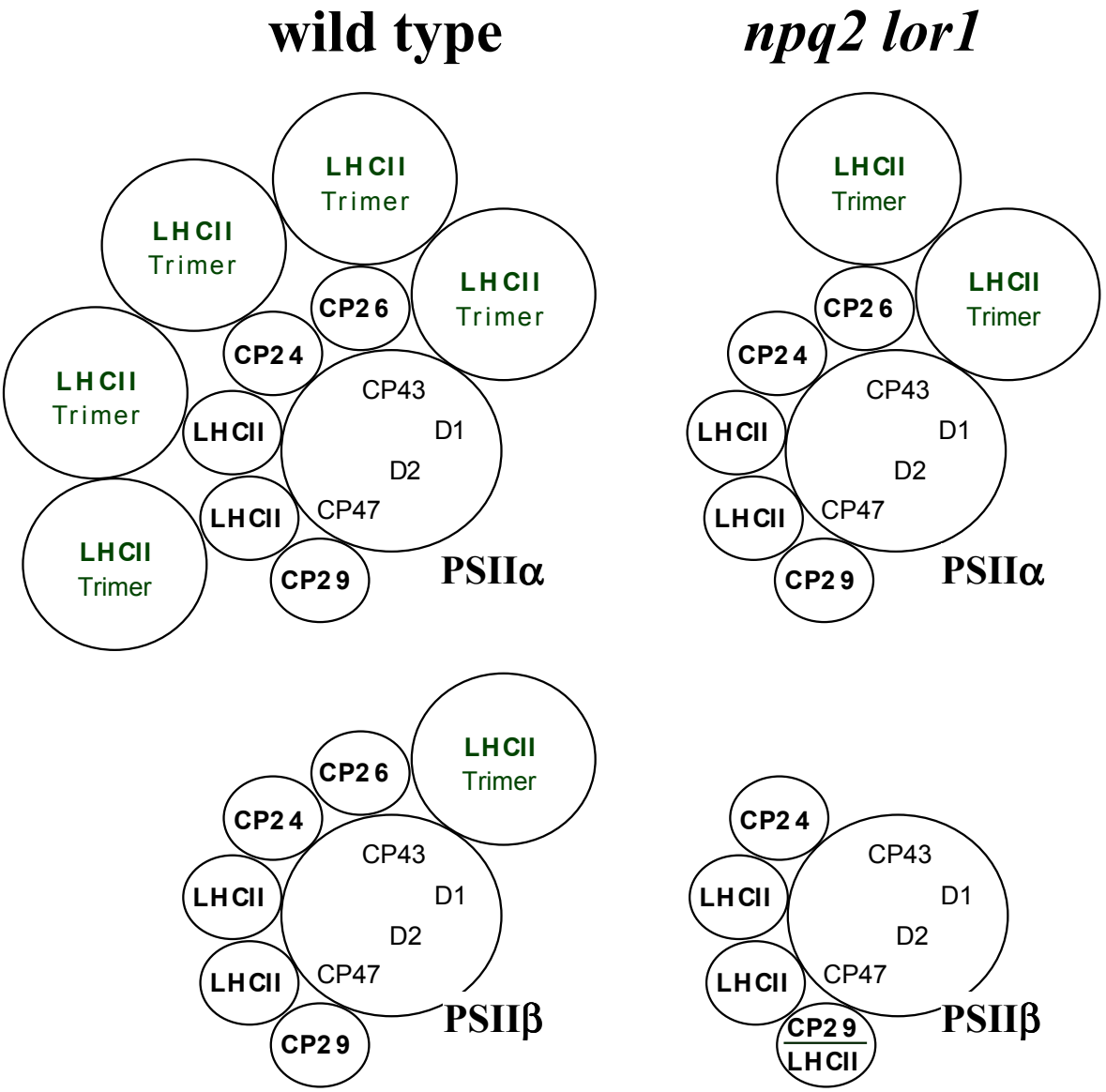

Figure 8 Model of the *Chlamydomonas reinhardtii* PSII light-harvesting antenna organization in wild type and *npq2 lor1* mutant .

In summary, the present work establishes that lutein, loroxanthin, violaxanthin and neoxanthin are not essential for the assembly and function of the LHCII and LHCI in the photosynthetic apparatus. With the possible exception of neoxanthin, these xanthophylls can be successfully substituted by zeaxanthin, resulting in the stable assembly of most, but all, Chl *a/b* LHCs. In consequence, the *npq2 lor1* mutant displayed a truncated PSII Chl antenna size but had a normal PSI Chl antenna size.

Acknowledgements

The work was produced under a DOE-UCB Cooperative Agreement Number DE-FC36-00GO10536 to A.M. Also supported (partially) by NIH grant number GM5879901 to K.K.N. We thank Ms. S.A. Chou for assistance with the preparation of the CP26 antibody, Dr. R. Bassi for providing the LHC antibodies and Dr. I. Enami for the PSI antibody, respectively.

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