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Dynamics of Photosystem II during short and long term response to light intensity: a biochemical and biophysical study

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A Marianna, sempre e comunque grazie…

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SUMMARY

Summary

Photosynthesis is the process by which plants absorb solar energy and convert it to chemical energy and finally biomass. During this process, several key functions are carried out by Photosystems. PhotoSystems I and II are multiproteic complexes responsible for light harvesting, charge separation and play essential part in electron transport from water to NADPH. These events lead to the formation of a transmembrane ΔpH that is used by the ATP-ase enzyme to produce ATP. PSI and PSII represent extraordinary machines for solar energy use, combining high quantum efficiency and the presence of inducible mechanisms in order to avoid photoinhibition which unavoidably derives from performing photosynthesis in oxygenic environment.

The peculiar organization of Photosystems is determinant for function. Both Photosystems are composed by two moieties: a core complex, made up by several plastid-encoded proteins and an antenna system constituted by conserved number of members of the Light Harvesting Complex (LHC) protein family. LHC proteins play a key role in photosynthesis and are involved in both light harvesting and photoprotection. Among other photoprotecting mechanisms, antenna proteins of PSII, so-called Lhcb subunits, are responsible for the mechanism of thermal dissipation of excitation energy in excess (NPQ, non-photochemical quenching). Elucidating the molecular details of NPQ induction in higher plants has proven to be a major challenge. In my phD work, I investigated the reorganization of the protein domains inside grana membranes upon high light treatment and verified its importance for full functioning of NPQ. Below the main results obtained are summarized.

Section A. Zeaxanthin modulates energy quenching properties of monomeric Lhcb antenna proteins.

Among photosynthetic pigments, a special role is played by zeaxanthin (Zea), which is only accumulated under excess light conditions. Zea is formed from Violaxanthin (Viola) under high light and returns to Viola in low light thus forming the so-called “xanthophylls cycle”. Zea binds to antenna complexes thus increasing resistance to light stress and this effect is obtained by up-regulating the capacity for quenching both triplet and singlet chlorophyll excited states energy into heat and by scavenging ROS (Dall'Osto, L. et al. 2007, Noctor, G. et al. 1991).

We studied the dynamics of xanthophylls binding to Lhcb proteins upon exposure of leaves to excess light. We found that Lhcb6 undergoes faster Zea accumulation than any other thylakoid protein so far described. We then studied *in vitro* modulation of Lhcb6 (CP24) functional properties by studying the effects of binding different xanthophyll species by using several spectroscopic techniques. Results show that Zea binding to Lhcb6 not only quenches chlorophyll singlet excited states, but also facilitates chlorophyll a triplet quenching efficiency. These results suggest for Lhcb6 a special role in binding Zea and enhancing photoprotection under excess light. The Lhcb6 subunit is a recent addition to the photosynthetic apparatus of viridiplantae, being absent in algae and first appearing in mosses, together with the adaptation to the highly stressful conditions typical of land environment. Consistently, it is involved in several regulation mechanisms, as evidenced by genetic (de Bianchi, S. et al. 2008, Kovacs, L. et al. 2006) and biochemical analysis (Ballottari, M. et al. 2007). Previously it was reported to be involved in Non-Photochemical Quenching (NPQ), through carotenoid radical cation formation (Ahn, T. K. et al. 2008, Avenson, T. J. et al. 2008).

In the second part of this section we focuses on fluorescence quenching and compared the effect of aggregation, which induces fluorescence quenching in many chlorophyll-binding proteins, on the different members of the Lhcb family

binding or not Zea. Aggregation quenching has been proposed to occur in vivo during NPQ due to a conformational change allowing energy transfer from Chl *a* excited states to the short lived carotenoid S1 excited state (Ruban, A. V. et al. 2007). This aggregation-dependent quenching (ADQ) has been proposed as an alternative to charge transfer quenching (CTQ) (Ahn, T. K. et al. 2008) mechanism proposed by other groups, including our laboratory. We studied the properties, particularly dependence on zeaxanthin binding, of ADQ using time-resolved and steady state spectroscopy. We obtained evidence that monomeric Lhcb proteins undergo ADQ even better than trimeric LHCII for which this mechanism was originally proposed. In these proteins the amplitude of the process is enhanced by zeaxanthin, while this is not the case for LHCII. Nevertheless, when LHCII is mixed with Lhcb6, this provides zeaxanthin-deppendent enhancement. The site of quenching within the Lhcb6 protein was studied by targeted mutagenesis of residues binding Chl ligands in different domains of the protein (Ballottari, M. et al. 2009). ADQ was prevented by deleting Chl molecules proximal to the Lut-binding site L1. This result complements previous studies of CTQ, which localized the quenching site to Chl 603, Chl 609, and Zea in carotenoid-binding site L2 (Ahn, T. K. et al. 2008) and suggests that two different types of quenching may occur in Lhcb proteins.

Section B. Membrane dynamics during NPQ: PsbS and zeaxanthin-dependent reorganization of Photosystem II is controlled by dissociation of a pentameric supercomplex.

Antenna subunits heve been shown to host the site of energy quenching, while the trigger of the mechanism is mediated by PsbS (Bonente, G. et al. 2008), a PSII subunit involved in transducing the signal of over-excitation consisting into lumen acidification (Li, X. P. et al. 2002, Li, X. P. et al. 2004). Recently, it has been shown that PsbS has influence in the overall organization of grana membranes (Kiss, A. Z. et al. 2008).

In this section we investigate the molecular mechanism by which PsbS regulates light harvesting efficiency. We showed that PsbS controls the association/dissociation of a five-subunit membrane complex, composed of two monomeric Lhcb proteins, Lhcb4 and Lhcb6 and the trimeric LHCII-M (namely Band 4 Complex - B4C). We demonstrated that the dissociation of this supercomplex is indispensable for the onset of non-photochemical fluorescence quenching in high light. Consistently, we found that knock-out mutants lacking the two subunits participating to the B4C, namely Lhcb6 and Lhcb4, are strongly affected in heat dissipation. Full NPQ activation also requires zeaxanthin synthesis and we showed that this antenna-bound xanthophylls is a factor regulating the extent of B4C dissociation. Direct observation of grana membranes upon treatment with excess light for different timelengths by electron microscopy and image analysis showed that B4C dissociation leads to the redistribution of PSII within grana membranes, reducing average distances between PSII core complexes. This phenomenon was reversible upon dark relaxation. We interpret these results proposing that the dissociation of B4C makes quenching sites, possibly Lhcb4 and Lhcb6, available for the switch to an energy-quenching conformation. These changes are reversible and do not require protein synthesis/degradation, thus allowing for changes in PSII antenna size and adaptation to rapidly changing environmental conditions.

Section C. New insights on the role of the monomeric Lhcb4 antenna subunit.

PSII is surrounded by a external antenna system composed by trimeric LHCII and monomeric minor antenna complexes. Several evidences suggests that Lhcb4, in particular, is a key factor in both light harvesting and photoprotection: Lhcb4 is a) maintained under long-term HL stresses condition (Ballottari, M. et al. 2007) or under

chronic excitation of PSII (Morosinotto, T. et al. 2006), b) is able to perform charge transfer quenching (Ahn, T. K. et al. 2008) and c) phosphorylation (Bergantino, E. et al. 1995) affects its spectral properties (Croce, R. et al. 1996).
In the first part of this section we characterized the function of Lhcb4 subunits in *Arabidopsis thaliana*, extending the analysis to the different Lhcb4 isoforms. Lhcb4 phosphorylation process in *A. thaliana* is still unclear and not detectable upon HL plus cold treatment as in monocots (Bergantino, E. et al. 1998). In order to determine the function of Lhcb4 in *A. thaliana*, we have constructed knock-out mutants lacking one or more Lhcb4 isoforms and analyzed their performance in photosynthesis and photoprotection. The absence of Lhcb4 also caused a destabilization of PSII supercomplexes, modifying antenna system organization. The distribution of PSII complexes within grana membranes is affected in *koLhcb4* and LHCII enriched domains are formed. While no significant alteration in linear/cyclic electron transport rate and maximal extent of state transition were unchanged, PSII quantum efficiency and NPQ activity were affected. Photoprotection efficiency under high light conditions was impaired in *koLhcb4* plants with respect to either WT or mutants depleted of any other Lhcb subunit. Electron microscopy analysis reveal that PSII supercomplex from koCP29 plants bears a hole in its structure. We conclude that Lhcb4 is a fundamental component of PSII which is essential for maintenance of both the function and structural organization of this photosystem.

Section D. Chlorophyll b reductase affected the regulation of antenna complexes during light stress.

Light-harvesting chlorophyll *a*/*b*-protein complexes are the most abundant membrane proteins in green plants, and its degradation is a crucial process for the acclimation to high light conditions and for the recovery of nitrogen (N) and carbon (C) during senescence. However, the molecular mechanism of antenna breakdown is largely unknown and it is still unclear whether chlorophyll degradation precedes the degradation of the protein moiety or whether protein degradation is the first event.
Recently chlorophyll b reductase mutant has been isolated (Kusaba, M. et al. 2007). This enzyme is responsible for the first step of chlorophyll degradation pathway, the conversion of Chlorophyll (Chl) b in Chl a and the mutant is called "*stay-green*", because of PSII antenna retention upon leaf senescence induction (Horie, Y. et al. 2009). We characterized the response of Chl b reductase ko mutant to acclimation in high light. The mutant showed a slower antenna size reduction with respect to WT. This enzyme is upregulated during HL acclimation. I*n vitro* assay with recombinant Chl b reductase demonstrated that its activity is higher when zeaxanthin, which accumulate during stress, is bound to PSII antenna complexes.

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INTRODUCTION

1. OXYGENIC PHOTOSYNTHESIS

Most organisms on Earth depend directly or indirectly from the solar energy. In particular autotrophic organisms as plants, algae and some kind of bacteria, harvest light and convert it into chemical energy in order to produce bio-mass, which constitutes food for heterotrophic organisms.

Photosynthesis is the process that enables the absorption of light energy and its conversion into chemical energy.

In oxygenic photosynthesis, performed by green plants, algae, and cyanobacteria, water is used as electron donor to reduce CO_2 to carbohydrates, generating molecular oxygen as a secondary product of the reaction. The overall equation of the photosynthetic reaction is the following:

$$nH_2O + nCO_2 + \text{light} \rightarrow (CH_2O)_n + nO_2$$

The whole photosynthetic process can be divided into a light and a dark phase. The light phase is strictly dependent from the presence of solar energy, while dark phase occurs also in the dark, provided that ATP and NADPH are available.

Light phase starts with sunlight absorption by photosynthetic pigments (chlorophylls and carotenoids); followed by transfer of the energy to the reaction centre where charge separation occurs, thus converting light energy into chemical energy. This event induces a set of electron transfer reactions across thylakoid membrane (see next sections), leading to the formation of a trans-membrane proton gradient. Water is the primary electron donor during this process and oxygen is formed as a consequence. Finally, free energy and reducing power, in the form of ATP and NADPH + H^+, are generated.

Light reaction can be summarized by the following equations:

$$2\ NADP^+ + 2\ H_2O + \text{light} \rightarrow 2\ NADPH + O_2 + 2H^+$$

$$ADP + P_i + \text{energy} \rightarrow ATP$$

In dark reactions, ATP and NADPH produced during the light reactions are used to reduce CO_2 to the carbohydrate GlycerAldehyde-3-Phosphate. GAP is then used for the synthesis of various cellular compounds. The process of the dark reactions can be summarised in the following equation:

$$3\ CO_2 + 9\ ATP + 6\ NADPH \rightarrow GAP + 9\ ADP + 8\ P_i + 6\ NADP^+$$

I. The chloroplast

Chloroplast is a specialised organelle in photosynthetic eukaryotes, in which both light and dark reactions take place (Figure 1). This organelle is limited by two membranes (called together *envelope*): the first one is highly permeable, while the second one contains specific transporters which mediate the flux of metabolites with the cytoplasm. The soluble phase delimited by the *envelope* membranes is called *stroma* and it contains all enzymes catalysing the dark reactions as well as the plastidial DNA, RNA and ribosomes. A third membrane system, the *thylakoids*, is found in the stroma and it confines a second compartment, the *lumen*. Thylakoids are organized into two membrane domains: 1) cylindrical stacked structures called *grana*, and 2) interconnecting regions, the *stroma lamellae*. The thylakoid membranes carry a negative charge, but the presence of cations, especially divalent cations like Mg^{2+}, keeps the thylakoid membranes stacked (Barber, J. 1980). In vascular plants chloroplast are found especially in leaves mesophyll

cells and their number is variable between a few to hundreds per cell, depending on species, growth conditions and developmental stage.

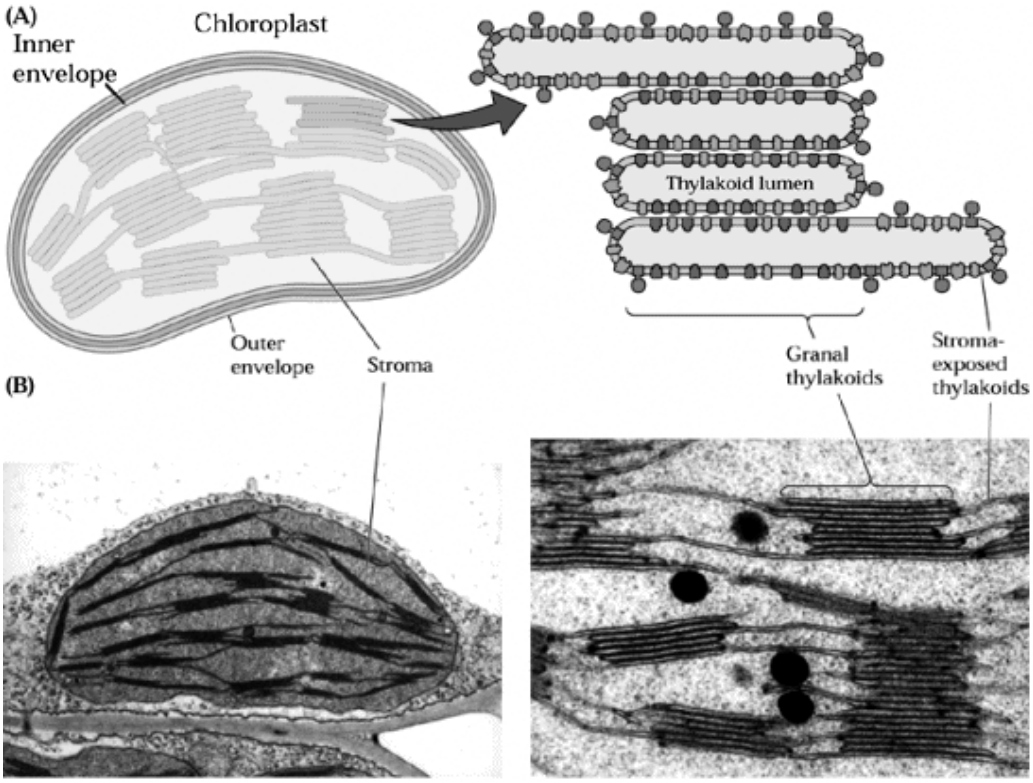

Figure 1. ***A.*** *Schematic diagram of a plant chloroplast. Thylakoids are distinguished in grana and stromal thylakoids (or stroma lamellae).* ***B.*** *Electronic micrographs of a chloroplast. Images from (Malkin, R. and Niyogi, K. K. 2000).*

II. The light phase

The proteins involved in the photosynthetic light reactions are located in the thylakoids and are grouped into four major protein complexes: Photosystem I, cytochrome b_6f complex, Photosystem II and ATP synthase (see Figure 2). These four complexes catalyse the processes of light harvesting, electron transport and photophosphorylation, leading to the conversion of light energy to chemical free energy (ATP and NADPH). Light reactions are catalyzed by two separate photosystems (PSI and PSII), while ATP synthase produces ATP at the expense of the proton-motive force (*pmf*) that is formed by the light reaction. The cytochrome-b_6f complex mediates electron transport between PSII and PSI (Hill, R. and Bendall, F. 1960) and has a role in converting the redox energy into a high-energy intermediate (*pmf*) for ATP formation.

PSI and PSII bind the pigments responsible for light absorption and harvest the solar energy. Energy harvested is transferred to the reaction centre and excite two specialized reaction-centre chlorophylls (primary electron donor, a special chlorophyll pair) which undergo charge transfer, with consequent translocation of an electron across the membrane through a series of cofactors.

Taking account of the redox potential of reagents and products, the energy required for the generation of NADPH cannot be provided by only one photon in the visible range of light (Figure 2): this is the reason why two photosystems act in series, as described in the so called Z-scheme (Hill, R. and Bendall, F. 1960).

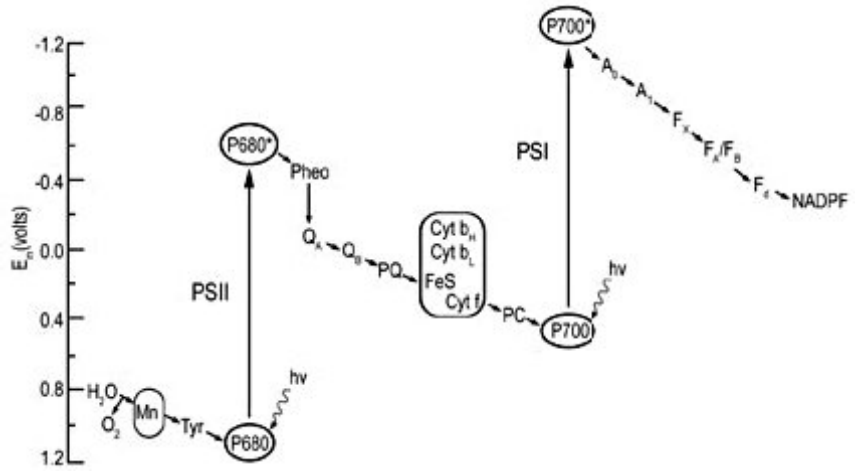

Figure 2. The Z-scheme of Bendall and Hill. *Cofactors involved in electron translocation between H_2O and $NADP^+$, and theirredox potential, are indicated.*

Water, the electron donor for this process, is oxidized to O_2 and 4 protons by PSII. The electrons that have been extracted from water are translocated, through a quinone pool and the cytochrome-b_6f complex, to plastocyanin, a small, copper-containing protein.

In the cytochrome-b_6f complex, at the PQH_2-binding site, two protons are released on the lumenal side of the membrane. An iron–sulphur cluster (Fe_2S_2) attached to the Rieske protein {Kurisu, 2003 8 /id} takes one electron from PQH_2 (Q_p site) and passes it to heme *f* in cytochrome *f*, where it is picked up by plastocyanin. The second electron passes via heme b_L and b_H (b_p and b_n) to a PQ bound at a site near the stromal membrane surface (Q_n site). PQ binds a proton, adding to the pH gradient across the membrane. It is the so-called Q-cycle (Figure 3). Overall, the Q-cycle oxidises two plastoquinols, reduces one plastoquinone, and translocates 4 H^+ for every 2 electrons transported to PSI. Therefore, an electro-chemical gradient is formed across the membrane.

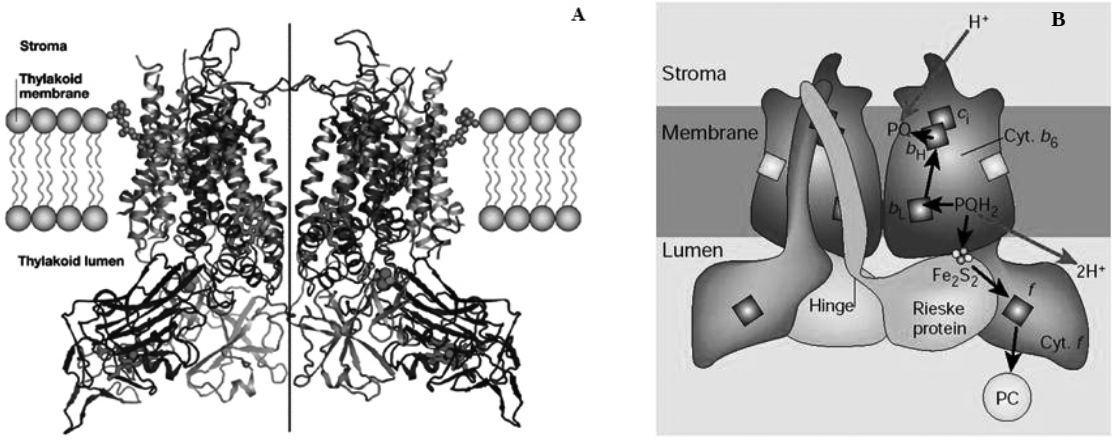

Figure 3. The cytochrome-*b6fcomplex*. *A.* *3D structure of cytochrome-b_6f from Chlamydomonas reinhardtii {Stroebel, 2003 211 /id}* ***B.*** *Schematic view of Q-cycle (Kühlbrandt 2003)*

Light energy absorbed by PSI induces the translocation of an electron from plastocyanin at the inner face of the membrane (thylakoid lumen) to ferredoxin on the opposite side (stroma). Reduced ferredoxin is subsequently used in numerous regulatory cycles and reactions, like nitrate and CO_2 assimilation, fatty-acid desaturation and NADPH production.

Both photosystems operate with a very high quantum yield: PSI works with an almost perfect quantum yield of 1.0, while PSII operates with a lower efficiency (about 0.85).

The charge separation in PSI and PSII, together with the electron transfer through the cytochrome-*b_6f* complex, leads to the formation of an electro-chemical potential gradient, between the stromal and the lumenal side of the membrane, which powers ATP synthesis by the fourth protein complex, the ATPase (Figure 4).

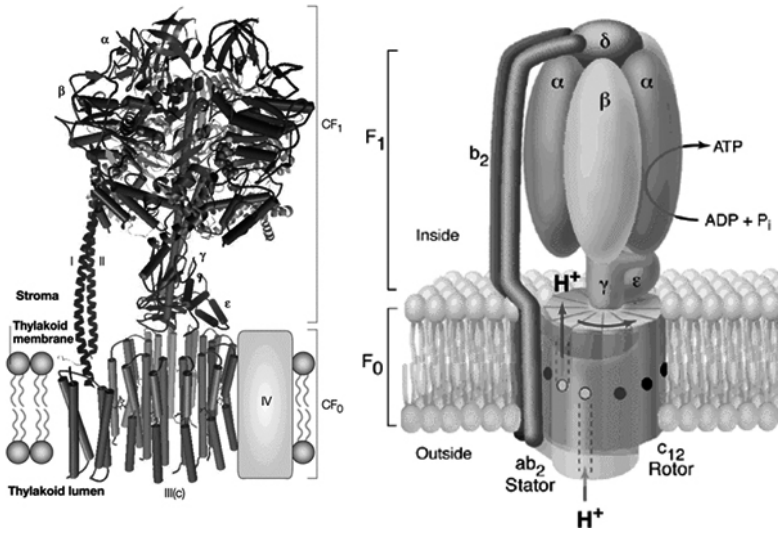

Figure 4. 3D structure of the multimeric complex ATP-ase (Abrahams, J. P. et al. 1994). *Resolution of the complex draws a representation of rotary and stationary subunits acting in ATP synthesis.*

The ATP-ase enzyme is a multimeric complex with both stromal and transmembrane regions that are known as CF_1 and CF_0, respectively, and represents a molecular motor that is driven by a *pmf*. Proton movement through CF_0 is coupled to ATP synthesis/hydrolysis at sites in the β-subunits of CF_1. In chloroplasts, CF_0 contains four subunits I, II, III and IV in a probable stoichiometry of 1:1:14:1; the 14 III subunits form a ring-like structure. The whole CF_0–CF_1 complex is thought to function as a rotary proton-driven motor, in which the stationary subunits are I, II, IV, δ, α and β, and the rotary subunits are III (c), γ and ε {McCarty, 2000 24668 /id}.

When the NADPH/$NADP^+$ ratio is high, the complex can run in a manner that generates a proton gradient (and thus ATP) without producing NADPH or oxygen (PSII is not involved). This process is referred to as cyclic photophosphorylation or cyclic electron flow, and it involves the transfer of electrons from ferredoxin to the cytochrome bf complex, probably through the extra haem c_i (heme x) (Stroebel, D. et al. 2003), producing a proton gradient while transferring the electron to plastocyanin to regenerate the P_{700} reaction centre (Harbinson, J. and Foyer, C. H. 1991).

III. The dark phase

During the dark phase of photosynthesis, atmospheric CO_2 is reduced to carbohydrates, using the chemical free energy (ATP and NADPH) produced during the light reactions (Figure 5). The enzyme RUBISCO is the enzyme responsible for CO_2 fixation to a molecule of Ribulose-bisphospate producing two molecules of 3-phosphoglycerate. A series of reactions, indicated as "Calvin cycle", (Benson, A. A. and Calvin, M. 1950) allow the synthesis of one glyceraldehydes-phosphate (GAP) from three CO_2 molecules and the regeneration of Ribulose-5-phosphate (Ru5P) to preserve the cyclic character of the process. It can be summarised with the following reactions:

$$3\ CO_2 + 3\ Ru5P + 9\ ATP + 6\ NADPH \rightarrow 6\ GAP + 9\ ADP + 6\ NADP^+ + 8\ P_i$$

$$5\ GAP \rightarrow 3\ Ru5P$$

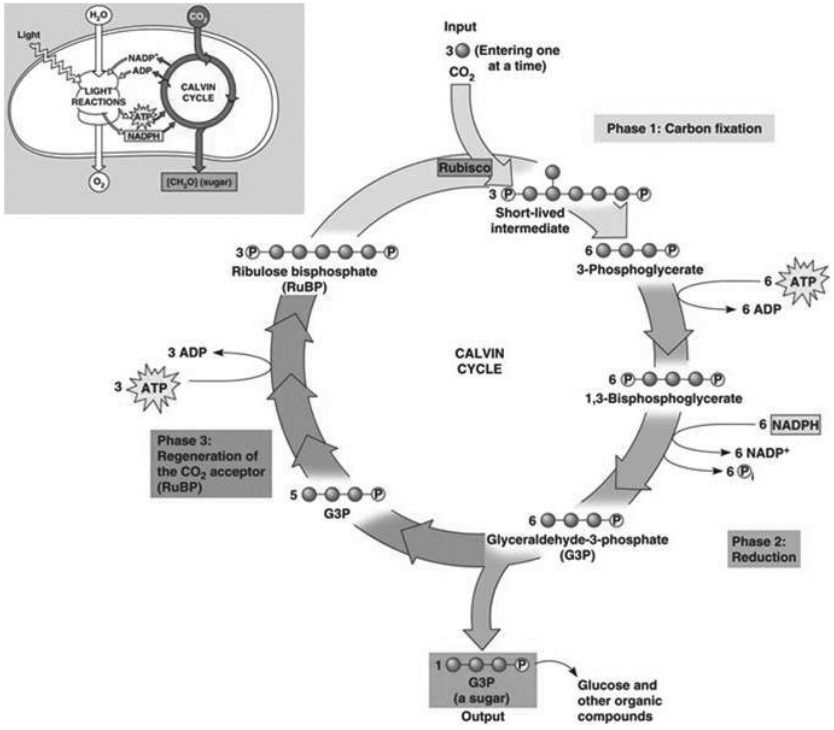

Figure 5. Enzymatic steps involved in Calvin cycle

2. PHOTOSYNTHETIC PIGMENTS IN HIGHER PLANT

Chlorophylls and carotenoids are the two main classes of pigments responsible for light absorption, charge separation and energy transfer toward the reaction centre in both photosystems.

I. Chlorophylls

Chlorophylls (Chls) are synthesized in a pathway starting from glutamic acid (Malkin, R. and Niyogi, K. K. 2000) and are characterized by the presence of a cyclic tetrapyrrole (porphyrin) in which the four nitrogen atoms of the pyrroles coordinate a magnesium atom. A fifth ring and a chain of 20 carbon atoms, called phytol, responsible for their

hydrophobicity, are also present. In photosynthetic organism 5 different types of chlorophylls are present (chlorophyll *a*, *b*, *c1*, *c2*, *d*) differing in their substitutions. In higher plants, two types of molecules are present, differing in a substituent in the second pyrrhole ring: a methyl for the Chl *a*, an aldehyde for the Chl *b* (Figure 6A). The characteristic ability of Chls to absorb light in the visible region is due to the high number of conjugated double bonds present in these molecules.

Light absorption by Chls lead to a transition of an electron to excited states. The absorption spectra in the visible of the Chls are characterized by two main bands with a high extinction coefficients (around 10^5 cm^{-1} M^{-1}): the Qy transition in the red region of the spectrum and the Soret transition in the blue region (Figure 6B). The transition of an electron from S0 to S1 (the first excited state) corresponds to the Qy transition and it is evident in the red region of the absorption spectrum. Another absorption band, called Qx is visible in the red region of the spectrum, even if it is partly masked by the Qy vibronic transitions: it corresponds to the transition of a ground state (S0) electron to the second excited state (S2). The transition to higher states corresponds to the Soret band, visible in the blue region of the absorption spectrum.

Chlorophylls are mainly present in the chloroplast bound by proteins. A key interaction for the stabilization of chlorophyll binding to proteins, is the coordination of the central Mg: in most cases it is bound by nucleophilic amino acids residues, like histidine (Jordan, P. et al. 2001, Liu, Z. et al. 2004); however, chlorophylls bound to proteins by water or lipid molecules was also shown (Liu, Z. et al. 2004). Chlorophyll a and b absorption properties are well known to be modulated by the protein environment in which they are located. Moreover chlorophylls are indispensable for the proper folding of some photosynthetic proteins, as the Lhc proteins (Paulsen, H. et al. 1993).

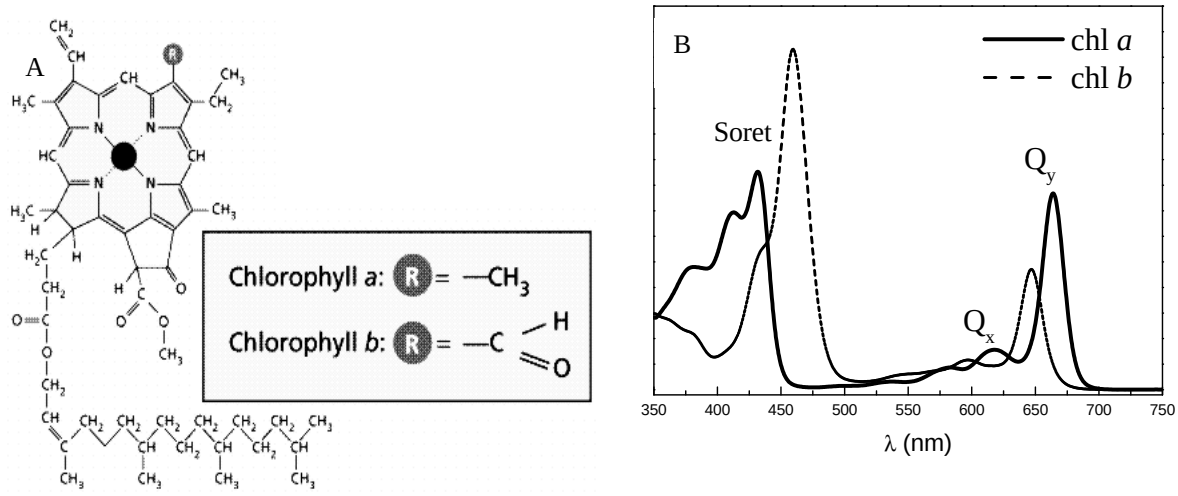

Figure 6. (A) Structure and (B) absorption spectra in acetone 80% of chlorophyll *a* and *b*

II. Carotenoids

Carotenoids are synthesized by bacteria, algae, fungi and plants: they play many essential roles in organisms performing oxygenic photosynthesis and they are especially involved in photoprotection (Moore, A. L. et al. 1982).

Carotenoids are poly-isoprenoid compounds containing 40 carbon atoms. The atomic structure of these pigments is constituted by a long chain of conjugated double bonds in the central part of the molecule and cyclic groups and the extremities. Different level of hydrogenation and introduction of oxygen-containing functional groups create a large family of carotenoids, composed by over 600 natural compounds. In higher plants, two different classes are found into

thylakoid: (i) carotenes (as for example β-carotene), which are hydrocarbons with linear structure and with cyclic groups in one or both extremities, and (ii) xanthophylls (as for example lutein) which are oxygenated derivatives of carotenes.

In higher plants the most abundant carotenoids associated with thylakoid membranes are the α and β carotene and the xanthophylls lutein, violaxanthin, neoxanthin and zeaxanthin. Carotenoids are non-covalently bound to the protein complexes, probably involving hydrophobic interactions (Gastaldelli, M. et al. 2003). Carotenes are bound especially to the core complex of both photosystems, while xanthophylls are mainly located in the antenna complexes (see next sections for details) (Bassi, R. et al. 1993, Caffarri, S. et al. 2001, Ruban, A. V. et al. 1999).

Carotenoids are all synthesised from the terpenoid pathway and they derive from the condensation of 8 IPP (isopentenyl diphosphate) molecules, thus producing phytoene (Figure 7). Phytoene undergoes several reduction steps, introducing four more double bonds and generating lycopene. From lycopene the biosynthesis of carotenoids splits into two branches: one leads to the formation of β- carotene, zeaxanthin, violaxanthin and neoxanthin, while the other to α-carotene and lutein (Pogson, B. et al. 1996).

The conjugated double bond system of carotenoid molecules determines their photochemical properties. The π-electrons delocalisation in the conjugated double bonds system leads to the light absorption in the visible range 400-500 nm. When carotenoids absorb light, electrons are transferred from ground state (S0) to the second excited singlet state (S2); this strongly dipole-dipole allowed transition is responsible for the characteristic absorption spectrum. The first excited singlet state S1 cannot be populated from the ground state by photon absorption due to symmetry reasons.

The absorption spectra of carotenoids are strongly red-shifted *in vivo*, compared to their spectra in organic solvents. This shift represents a lowering of the S2 energy level, which has been ascribed to the mutual polarisability of the carotenoid and protein environment (Andersson, P. O. et al. 1991, Cogdell, R. J. et al. 1992). In contrast to S2, the S1 level is little affected by the surrounding environment (Andersson, P. O. et al. 1991).

Carotenoids have several role in photosynthesis: a) structure stabilisation and assembly of protein complexes in the thylakoid membrane (Paulsen, H. et al. 1993, Plumley, F. G. and Schmidt, G. W. 1987); b) light absorption and excited state energy transfer to the chlorophylls (Gradinaru, C. C. et al. 2000, Mimuro, M. and Katoh, T. 1991) c) protection against photo-oxidative damages (Havaux, M. and Niyogi, K. K. 1999). Caratenoids are important antioxidant in the thylakoid membrane and they are involved in dissipation of excited states of chlorophylls in case of conditions of over-excitation.

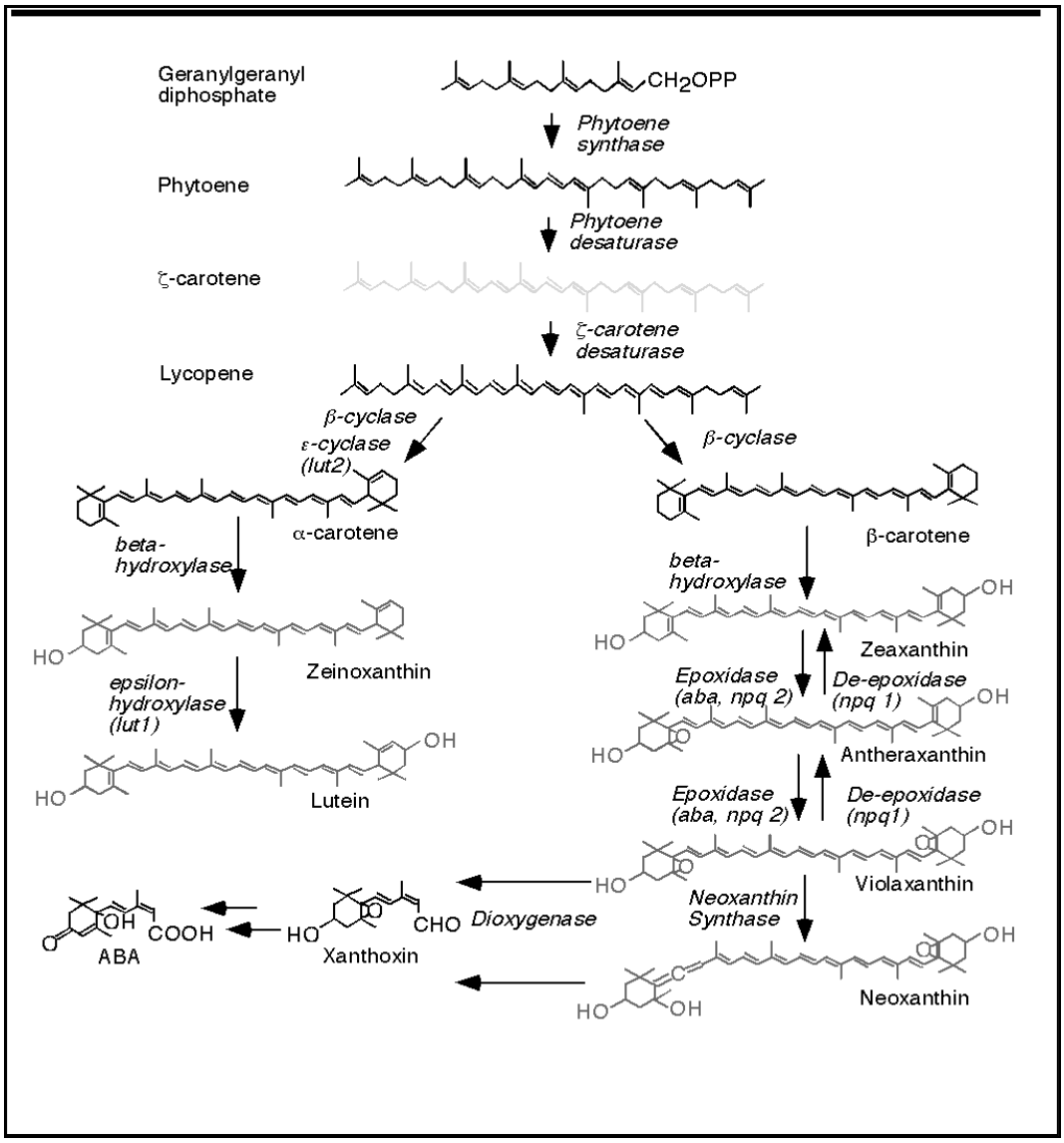

Figure 7. Biosynthetic pathway of carotenoids in higher plants, with enzymes involved.

III. Xanthophyll cycle

During long-term acclimation of plants to stressing condition, as well as rapidly changes following fluctuations of solar light intensity (Demmig-Adams, B. 1990), the carotenoid composition in the thylakoids can undergo modifications. This observation is well explained since different carotenoids, even if sharing a similar structure, are characterized by specific functions (see next sections). An example of carotenoids composition changing in response to a variation of the environmental conditions is the xanthophylls cycle.

The xanthophyll cycle involves the three xanthophylls violaxanthin, antheraxanthin and zeaxanthin, and consists in a light-dependent, reversible de-epoxidation of violaxanthin to zeaxanthin via the intermediate antheraxanthin. Zeaxanthin lowers the light use efficiency by photosynthesis and, if present constitutively, it decreases plant growth (Dall'Osto, L. et al. 2005). Thus, the regulation of the xanthophyll cycle has an important physiological influence. The de-epoxidation reactions converting violaxanthin to zeaxanthin are catalysed by Violaxanthin De-Epoxidase (VDE) (Yamamoto, H. Y. and Kamite, L. 1972), a lumenal enzyme activated by acidification of the lumenal compartment

(Gilmore, A. M. and Yamamoto, H. Y. 1992): this event occurs when high illumination of leaf induce the build-up of a high transmembrane proton gradient (Figure 8).

Xanthophyll cycle is a key component in the activation of several photoprotective mechanisms as thermal dissipation of excitation energy in excess (Holt, N. E. et al. 2004, Niyogi, K. K. 1999) (see next sections for details) or chlorophylls triplets excited state quenching (see next section for details). Carotenoids involved in the xanthophyll cycle are localized in the peripheral antenna proteins of PSII and PSI (Bassi, R. et al. 1993, Bassi, R. and Caffarri, S. 2000, Ruban, A. V. et al. 1999), even if a certain photoprotective role has been assigned also to zeaxanthin in the free lipid phase {Yokthongwattana, 2005 1 /id}.

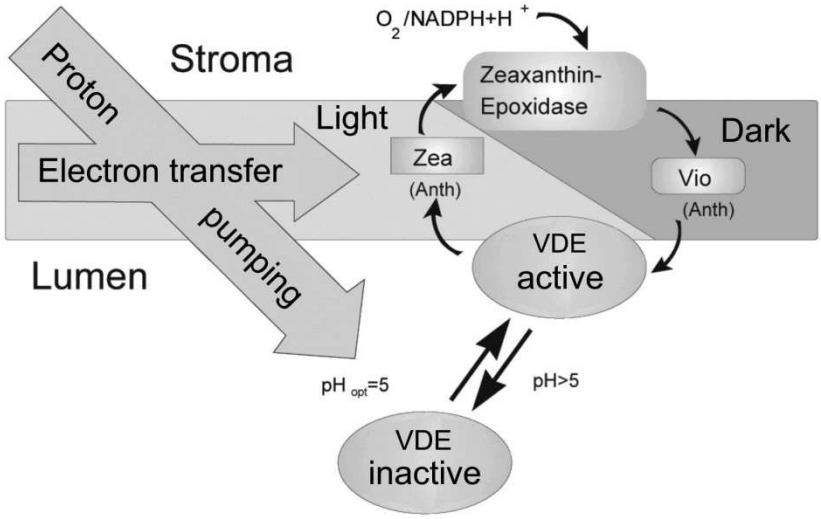

Figure 8. The xanthophyll cycle. *Schematic representation of electron transfer events leading to of ΔpH and activation of enzymes involved in the cycle.*

3. THE LIGHT ABSORBING COMPLEXES: PHOTOSYSTEM I AND II.

Photosystems I and II (PSI, PSII) are two multi-proteic complexes binding the pigments responsible for light harvesting and charge separation. Moreover in PSI and PSII are present some co-factors constituting the electron acceptor and donor in the electron transfer reactions from water to NADPH.

PSI and PSII are located in the thylakoids membranes but their distribution is not homogenous: PSII is mainly located in the stacked *grana* membranes, while PSI is present in the unstacked *stroma lamellae* membranes.

PSI and PSII are structurally different, however in both complexes can be identified two moieties: a) a core complex, in which are located the chlorophyll special pair and the cofactors involved in electron transport; and b) a peripheral antenna system, composed by chlorophylls binding proteins responsible for light harvesting and energy transfer to the reaction center. Chlorophylls and carotenoids are bound both by core complex and antenna system, however core complexes bind only Chl a and carotenes, while antenna proteins bind Chl a, Chl b and xanthophylls.

Core complexes are composed by the polypeptides denominated Psa and Psb respectively for PSI and PSII. Among Psa and Psb proteins there are gene products encoded from both nuclear or plastidic genes. Their sequences are generally well conserved during evolution and similar polypeptides are found in bacteria and eukaryotic organisms.

The antenna system is instead more variable in different organisms: in higher plants, subject of this thesis, it is composed by polypeptides belonging to the multigenic family of *light harvesting complexes* (*Lhc*). All these polypeptides share the same evolutionary origin and a common structural organization (Green and Dunford 1999).

All these gene products are encoded by the nuclear genome and they are denominated Lhca and Lhcb, respectively for the antenna proteins of PSI and PSII (Jansson, S. 1999). In vascular plants 10 different isoforms have been identified to be associated to Photosystem I and II, respectively Lhca1-4 to PSI and Lhcb1-6 to PSII (Jansson, S. 1999). Lhcb1-3 proteins form LHCII trimers, the major PSII antenna complex, with different combinations between the three isoforms. Lhcb 4- 6 instead are present in PSII supercomplexes as monomers and they are also referred to as "minor complexes".

Depending on environmental conditions, a subpopulation of LHCII trimers can be phosphorylated and migrate from PSII, docking to PSI. This process is called State Transition (Allen, J. F. 1992, Andersson, B. and Anderson, J. M. 1980): when electron transport between the two photosystems is inhibited because of an insufficient light absorption of PSI, state transitions provide a mechanism for the equilibration of the excitation energy between photosystems, thus increasing the efficiency of the whole process. In higher plants this mechanism was demonstrated to depend from the presence of the specific kinase Stn7 (Bellafiore, S. et al. 2005).

Four additional isoforms (Lhca5, Lhca6, Lhcb7 and Lhcb8), have been identified from gene sequences but their functional significance is still uncertain (Jansson, S. 1999, Klimmek, F. et al. 2006). Each group can be composed by one or more genes and their number depend on the species, suggesting that they are the result of gene duplication events that occurred quite late in evolution. Genes coding for Lhcb1 gene product comprise the largest class: in fact, 5 different Lhcb1 genes have been identified in *Arabidopsis thaliana*, but they are at least 14 in barley (Caffarri, S. et al. 2004, Jansson, S. 1999).

The cladogram of *Lhc* genes sequence form Arabidopsis thaliana (figure 9A) shows that Lhca and Lhcb cluster in two different groups, with Lhcb6 and Lhca1 located in the middle (figure 9A). In fact, evolutionary studies suggested that antenna proteins of PSI and PSII diverged soon, before the separation of individual classes (Durnford, D. G. et al. 1999). This separated evolution leaded to different characteristics of PSI and PSII antenna proteins, as specificity in carotenoids binding or the presence/absence of long-wavelength absorption forms. In between the two Lhca and Lhcb proteins group some proteins appeared at different evolutionary steps: Lhca4, Lhcb3 and Lhcb6 are not present in green algae, while are present in Bryophytes and Vascular plants, indicating a possible role of this proteins in water to land transition of photosynthetic organisms (Alboresi, A. et al. 2008).

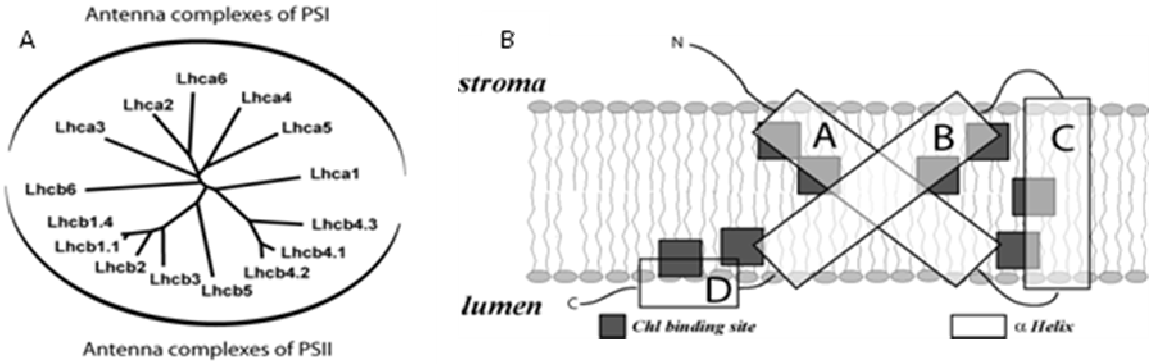

Figure 9. ***A.*** *Cladogram of Lhc sequences from Arabidopsis thaliana. 2 out of the 5 Lhcb1 and one out of 4 Lhcb2 sequences are reported. The association to Photosystem I or II is indicated.* ***B.*** *Schematic representation of the structure of Lhc complexes. α- helices and putative conserved Chl binding residues are indicated*

I. Photosystem I

Photosystem I of higher plants is a light-dependent plastocyanin-ferredoxin oxidoreductase. Higher plants PSI consists of at least 18 polypeptides (Scheller, H. V. et al. 2001) and it binds about 180 chlorophylls and 35 carotenoid molecules (Amunts, A. et al. 2007, Ben Shem, A. et al. 2003, Boekema, E. J. et al. 2001). It is composed of a core complex (PSI core) and the antenna system responsible for light-harvesting (LHCI). In the core complex is bound the P_{700} special chlorophyll pair which undergoes charge separation occurs; core complex includes also the primary electron acceptors A_0 (chlorophyll-*a*), A_1 (phylloquinone) and F_X (a Fe_4–S_4 cluster).

The reaction centre comprises 12 subunits that are denoted PsaA–PsaL, PsaN and PsaO (Scheller, H. V. et al. 2001). PsaG, PsaH, PsaN and PsaO subunits are present only in plants and green algae, not in cyanobacteria. The PsaA–PsaB heterodimer forms the inner core of PSI, binding the P_{700} special chlorophyll pair: here the light-driven charge separation occurs, and it includes the primary electron acceptors A_0 (chlorophyll-*a*), A_1 (phylloquinone) and F_X (a Fe_4–S_4 cluster). This heterodimer binds ~80 chlorophylls that function as inner light-harvesting antenna (Figure 10).

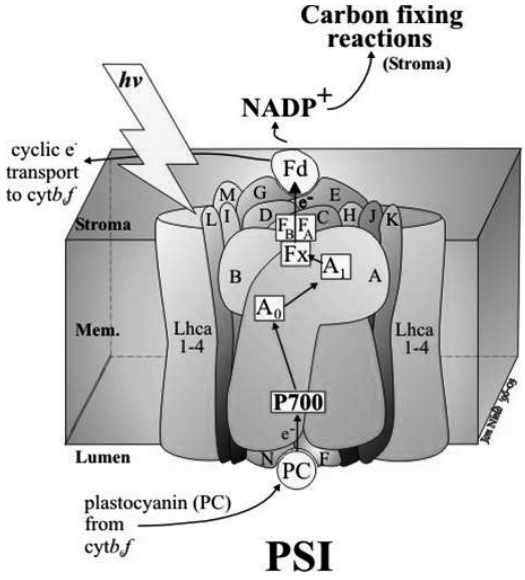

Figure 10. Schematic model of photosystem I. *Subunits organization and cofactors involved in electron transfer are indicated.*

II. Photosystem I antenna system

Lhca 1-4 are the 4 polypeptides constituting LHCI complex, the major component of PSI antenna system (Ben Shem, A. et al. 2003, Croce, R. et al. 2002b, Jansson, S. 1999). These polypeptide are assembled into two dimers, Lhca1-Lhca4 and Lhca2-Lhca3, arranged in half-moon shaped belt (Ben Shem, A. et al. 2003), and they are bound to the core complex mainly through interactions with PsaG subnunit. The characterisation of the individual LHCI components has been difficult, due to the inability to separate different polypeptides in LHCI preparations. A recent study allowed

characterisation of individual Lhca1-4 complexes following a different method, expressing the apoprotein in *E.coli* and reconstituting the protein *in vitro* (Croce, R. et al. 2002b).

Very recently, Lhca5 has been identified at the polypeptide level, with both mass spectrometry and specific antibodies (Ganeteg, U. et al. 2004, Storf, S. et al. 2004). However, it was detected only in very low amounts and not tightly associated to PSI and its physiological relevance is still under debate (Ganeteg, U. et al. 2004): recent results showed a peripherally interaction between Lhca5 and Lhca2-3 dimer and possibly a presence of Lhca5 homodimer as substitute of Lhca1-4 eterodimer (Lucinski, R. et al. 2006). The *lhca6* gene, instead, is expressed in very low levels and the polypeptide has never been detected. Since its sequence is very similar to Lhca2, *lhca6* could result from a recent duplication (Jansson, S. 1999), being a pseudogene, without any physiological function.

Photosystem I from both eukaryotic and prokaryotic organisms has a peculiar spectroscopic feature, the presence of "red forms", chlorophylls adsorbing at lower energies with respect to the reaction centre P_{700} (Gobets, B. and van Grondelle, R. 2001). Red forms are generally found in the PSI core complex, but higher plants are peculiar in this respect: in fact, while they still have red forms in the core complex, the red-most chlorophylls are found in the antenna complex LHCI (Mullet, J. E. et al. 1980). All Lhca complexes have low energy absorption forms, although with different energies (Morosinotto, T. et al. 2003): Lhca3 and Lhca4 are the Lhca proteins with lowest-energy absorption forms. The peculiar red forms located in Lhca3 and Lhca4 proteins were demonstrated to be related to the nature of the ligand of the chlorophyll present in A5 site (Kühlbrandt, W. et al. 1994), as a mutational approach revealed (Morosinotto, T. et al. 2003). Lhca3 and Lhca4 subunits present an asparagine (N), while Lhca1 and Lhca2 present a hystidine residue in A5 binding site: the presence of asparagine induces reinforcement in A5-B5 chlorophylls interactions the lower the energy required for excitation, thus originating the "red forms". In Lhca2 and Lhca1 the histamine presence in A5 chlorophyll binding site sites induce an excitonic interaction between A5 and B5 chlorophyll characterized by absorption at higher energy than Lhca3 and Lhca4, resulting from an higher distance between the two chlorophylls due to histamine respect to asparagine presence (Croce, R. et al. 2004).

In chapter I a time-resolved fluorescence analysis of PSI-LHCI supercomplex is described: our results allowed to propose a kinetic model for energy transfer from antenna to reaction centre based on a trap-limited model, revealing a slowing down effect of "red forms" in energy transfer from antenna to reaction centre. This leads to suggest a possible photoprotective role of "red forms".

III. Supramolecular organization of Photosystem I

The structure of PSI-LHCI complex from higher plants was resolved at 4.4 Å (Ben Shem, A. et al. 2003) and recently at 3.4 Å resolution (Amunts, A. et al. 2007) by 3D crystallography giving details on the supramolecular organisation of the complex (Figure 11).

It shows that only one copy of each Lhca1-4 polypeptides is bound to one core complex and it has been discovered that Lhca/PSI stoichiometry remains unchanged upon adaptation to different light intensities (Ballottari et al. 2007).

3D structure of PSI-LHCI supercomplex revealed also the presence of some pigments at the interface between PSI core and LHCI (*gap* pigments) and between the Lhca dimers (*linker* pigments). This was again unexpected, explaining the reason why the chlorophyll-based estimates were inaccurate.

PsaH is another PSI core subunit present only in green algae and higher plants: it is located on the opposite side with respect to LHCI. Since this protein was shown to be necessary for state transition (Lunde, C. et al. 2000), its localisation suggests that LHCII should dock to PSI in this part of the core complex, on the opposite side of LHCI.

Finally, PSI of higher plants are present as monomers, while in cyanobacterial PSI trimers are found (Jordan, P. et al. 2001).

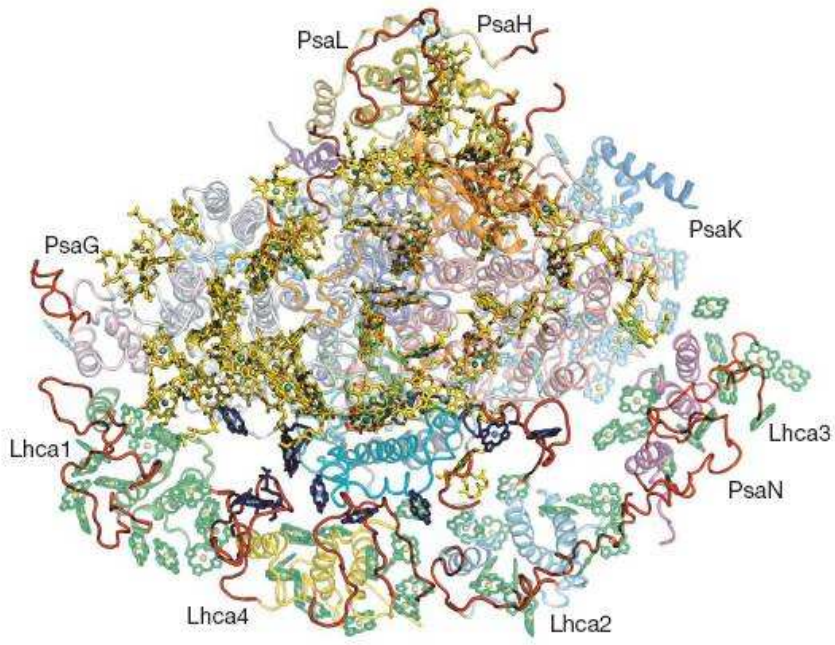

Figure 11. **Supramolecular organization of Photosystem I from *Pisum sativum*** (Ben Shem, A. et al. 2003)

IV. Photosystem II

The PSII complex is a multiproteic complex which catalyses the electron transfer from water to plastoquinone: PSII is thus defined as a water–plastoquinone oxidoreductase. PSII catalyses water oxidation producing oxygen: PSII activity appearance is thus a key feature for the evolution of aerobic organisms. During PSII photosynthetic reaction one molecule of oxygen is produced after 4 light quanta absorption.

The PSII core complex contains the reaction centre where a chlorophyll special pair, named P_{680}, undergoes charge transfer triggered by light energy absorbed. After absorption of the first of the light quanta, an electron is translocated from P_{680} to a pheophytin and then to the quinone Q_A. Quinone Q_A is stably bound to PSII and reduces a mobile quinone Q_B (Figure 12A-B). Upon an additional photochemical cycle, the doubly reduced Q_B (Q_B^{2-}) takes up two protons from the stroma to form Q_BH_2 and is released into the lipid bilayer; it will be then replaced by an oxidized plastoquinone from the lipid-freemembrane pool. After two more photochemical cycles, the manganese cluster is provided with four oxidizing equivalents, which are used to oxidize two water molecules to produce O_2. {Trumpower, 1990 4 /id}.

Three subunits, D1, D2 and Cyt b_{559} coordinate the electron transport cofactors P_{680} (the reaction centre), pheophytin, Q_A and Q_B. The special pair is bound by D1 and D2 subunits, which are arranged in a symmetrical geometry. The P_{680}^+ oxidizes a nearby tyrosine (Tyr_Z) on D1 polypeptide; the homologous tyrosine D (Tyr_D) on the D2 polypeptide forms a dark stable radical ($Tyr_D^{\bullet}$) influencing the oxidation of the nearby P_{680} {Diner, 2001 17 /id}. On the appendix section of this thesis is reported a study of the properties of $TyrD^{\bullet}$ and the influence of the protein environment on its stability based on FTIR spectroscopy.

Y_Z^+ species extracts an electron from a cluster of four manganese ions (OEC, oxygen-evolving complex), which binds two substrate water molecules. On the lumenal side of the complex, three extrinsic proteins of 33, 23 and 17 KDa (OEC1-3) compose the oxygen evolving complex (Zouni, A. et al. 2001), and have a calcium ion, a chloride ion and a bicarbonate ion as necessary cofactors.

CP43 and CP47 form the inner antenna of PSII, and bind respectively 14 and 16 Chl *a* molecules (Ferreira, K. N. et al. 2004). Up to 12 other small subunits are associated with the PSII core; some are involved in the dimerisation or in Chl and carotenoids binding stabilisation, but they do not all have a well clarified function.

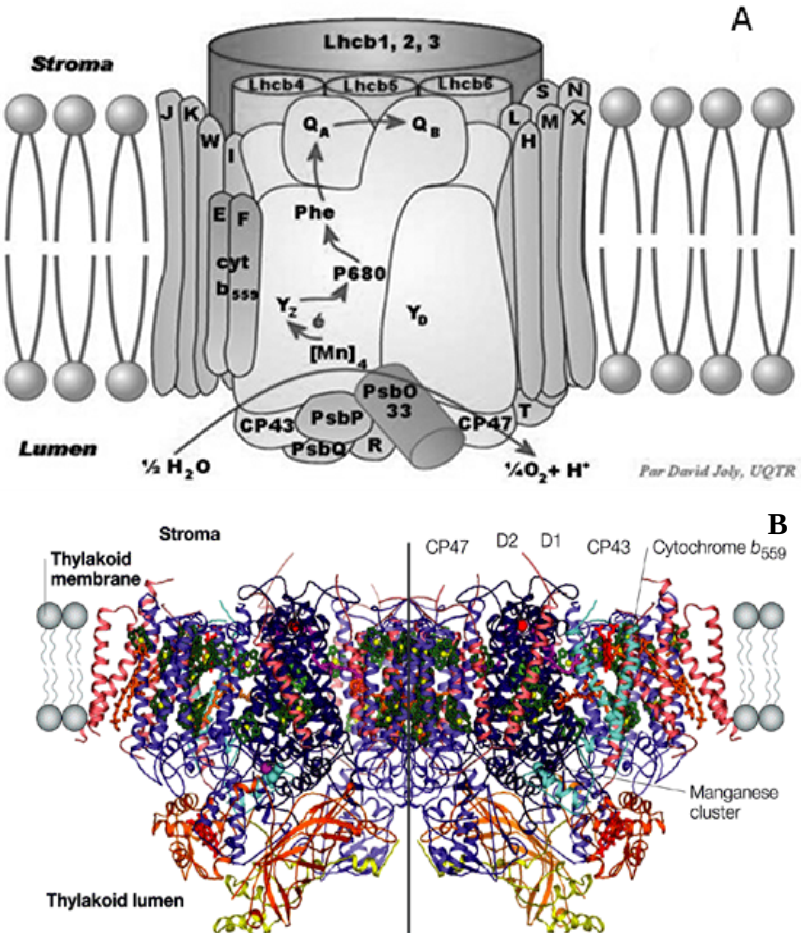

Figure 12. (A) Schematic model for PSII and (B) 3D crystal structure of PSII core complex. *Crystal structure of photosystem II from cyanobacterium T. elongatus were resolved by Ferreira et al. 2004.*

V. Photosystem II antenna system

The peripheral antenna of photosystem II is composed of two classes of light-harvesting complexes: a) the trimeric major light-harvesting complexes of PSII, called LHCII (Thornber et al. 1967) and b) the monomeric minor antennae (Bassi, R. et al. 1996).

PSII Antenna proteins bind Chl a, Chl b and xanthophylls and their function is to harvest light energy and transfer it to the P_{680}, but they are also involved in photoprotection (see next section).

Crystal structure of LHCII has been recently resolved (Liu, Z. et al. 2004) at 2.72Å being a model for the other antenna protein encoded by the Lhc multigene family (Jansson, S. 1999). LHCII trimers are heterotrimers constituted by the subunits Lhcb1, Lhcb2 and Lhcb3, with the different isoforms present with different ratio depending from numerous factor as environmental conditions and the plant species (Caffarri, S. et al. 2001). In Arabidopsis, the products of these genes have an average length of 232, 228 and 223 aminoacids respectively (after removal of the chloroplast import signal).

In the trimeric complex of LHCII, each monomer is constituted of 3 transmembrane domains with α-helix conformation (helices A, B and C) (Kühlbrandt, W. et al. 1994, Liu, Z. et al. 2004). The N-terminal and the C-terminal peptides are exposed respectively on the stromal and lumenal spaces; N-terminal peptide is fully hydrophilic, thus protruding into the stroma space. Two amphipathic helix, named D and E, were found respectively on the C-terminal peptide and in the BC loop region; both helices lies on the lumenal surface (Liu, Z. et al. 2004) (Figure 13).

The trimerization domain covers the amino-terminal domain, the carboxy terminus, the stromal end of helix B, several hydrophobic residues from helix C and also pigments and lipid PG (phosphatidylglycerol) bound to these parts of the polypeptide chain. Six chl *a* (two from each monomer) constitute the core of the trimer (Liu, Z. et al. 2004).

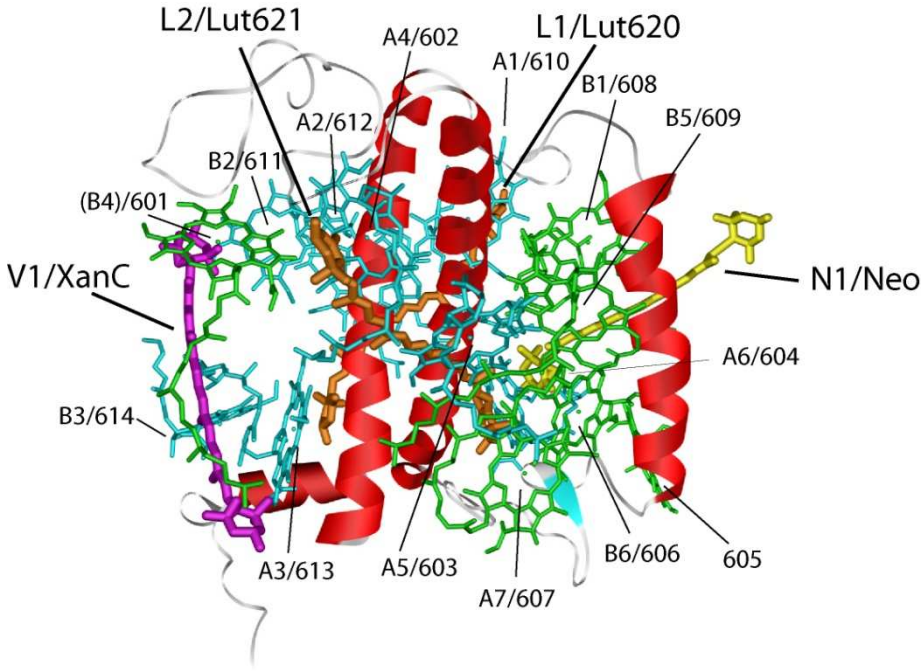

Figure 13. 3D Structure of the light-harvesting complex II (LHCII). *Structure of LHCII from spinach were resolved by Liu et al. Cofactors bound (chlorophylls and carotenoids) are indicated with nomenclatures from (Kühlbrandt et al 1994) and (Liu et al 2004)*

Each monomer binds 14 chlorophylls and 4 xanthophylls. All ligands of the 14 chlorophylls have been identified as side chains of seven amino-acid residues, two backbone carbonyls, four water molecules and the phosphodiester group of a PG. Part of the chlorophylls binding sites are selective for chlorophyll a or chlorophyll b, while in other cases chlorophyll binding sites with mixed occupancy are present. The spectroscopic properties of each chlorophyll bound by Lhcb1 monomer has been investigated through a mutational approach in the chlorophyll binding sites and reconstitution *in vitro* of recombinant Lhcb1 mutants (Remelli, R. et al. 1999).

Two central lutein molecules are bound in the grooves on both sides of the helices A and B cross-brace, forming the internal L1 and L2 carotenoid binding sites; the polyene chains are firmly fixed in two hydrophobic cavities, providing strong linkage between helices A and B. The third xanthophyll, 9'-cis neoxanthin is located in the Chl *b*-rich region around helix C in the carotenoid binding site N1; side chains from helices B and C as well as phytyl chains form a hydrophobic space that accommodates the hook-shaped polyene chain of neoxanthin, while ring on the other ends stretches into the exterior solvent region. Recently a Tyr residue in the lumenal loop has been found to form hydrogen bound with neoxanthin, stabilizing N1 site {Hobe, 2006 1 /id;Caffarri, 2007 24838 /id}. The fourth carotenoid is located in a peripheral site named V1 (Caffarri, S. et al. 2001, Liu, Z. et al. 2004). V1 site is constituted by a hydrophobic pocket at the interface monomer-monomer formed by several chlorophylls, hydrophobic residues and the PG; part of the xanthophyll is located inside this pocket, while the opposite end group is located outside the binding pocket, toward the stromal surface.

Monomeric minor antennae, named also minor complexes are constituted by three Chl *a/b*- and xanthophyll-binding proteins: Lhcb4, Lhcb5and Lhcb6, named from their apparent mass in non-denaturing SDS-PAGE gels (Bassi, R. et al. 1987, Peter, G. F. and Thornber, J. P. 1991).

The minor complexes are encoded respectively by the nuclear genes *Lhcb4*, *Lhcb5* and *Lhcb6*, which are highly homologous to each other and to the other members of the *Lhc* multigene family. The three subunits are probably monomeric proteins that are present in one copy per PSII unit, and bind about 10% of the total chlorophylls and 20% of the violaxanthin of the total complex (Nield, J. et al. 2000). Single genes encode Lhcb5 (CP26) and Lhcb6 (CP24), while three Lhcb4 genes are present in Arabidopsis. The expression levels of minor antennae are similar and the proteins are found in equimolar amounts in the thylakoid (Jansson, S. 1999). However recently the expression profile of the third gene coding for Lhcb4, *Lhcb4.3*, was shown to be different than *Lhcb4.1* and *Lhcb4.2*. *Lhcb4.3* was then re-named into *Lhcb8* (Klimmek, F. et al. 2006). Another gene similar to the genes coding for minor complexes is *Lhcb7*: however Lhcb8 and Lhcb7 proteins are not present in substantial amount into thylakoids membranes and their role is still unclear.

Lhcb4 is composed by 256-258 amino acids in its mature form in Arabidopsis, and it is the largest among Lhc encoded proteins. The overall sequence identity between Lhcb4 and LHCII is 34%, but most of the substitutions are conservative, especially in the helix regions. Lhcb4 binds 6 chl a, 2 chl b: as for Lhcb1, Lhcb4 chlorophylls spectroscopic properties has been analyzed through site-specific mutagenesis on residues responsible for chlorophyll binding and in vitro reconstitution of recombinant mutants (Bassi, R. et al. 1999). The results obtained demonstrated the presence of 4 chlorophyll binding sites specific for Chl a, and 4 chlorophyll binding site with mixed occupancy. About carotenoids Lhcb4 binds one molecule of lutein and substoichiometric amounts of violaxanthin and neoxanthin (for a total of 2 xanthophylls) (Dainese, P. and Bassi, R. 1991). Recently a third carotenoid binding sites specific for neoxanthin, equivalent to the N1 site in LHCII, has been proposed also for Lhcb4, since the Tyr residue responsible for N1 stabilization is present. However native and recombinant Lhcb4 was found with only 2 carotenoids bound, revealing a probable low stability of N1 due to the absence of most of the chlorophyll binding sites nearby helix C involved in N1 stabilization (Caffarri, S. et al. 2007). Lhcb4 can be phosphorylated, especially when plants are exposed to low temperature and high light stress (Croce, R. et al. 1996), and it has been shown that the ability to accumulate cold-induced phosphorylated Lhcb4 is correlated to cold tolerance(Mauro, S. et al. 1997). Recently it has been shown that Lhcb4 phosphorylation is linked also to exposure to several other environmental stresses, that however affected the photosynthetic apparatus (Liu, W. J. et al. 2009). The N-terminal portion of the protein protrudes into the stroma space and can be reversibly phosphorylated on Thr83 (Testi, M. G. et al. 1996). CP29 phosphorylation is supposed to induce

conformational rearrangement or modification in the PSII supercomplex that could facilitate thermal energy dissipation (Croce, R. et al. 1996).

Lhcb5 is 243 amino acids long in Arabidopsis, shows 48% identity with respect to LHCII and coordinates 7 Chl a, 3 Chl b and 2-3 xanthophylls (lutein, violaxanthin and neoxanthin) (Bassi, R. et al. 1996, Croce, R. et al. 2002a). Lhcb5, as Lhcb1 and Lhcb4, presents a Tyr residue which is suggested to stabilize the third carotenoid binding site N1 (Caffarri, S. et al. 2007).

Lhcb6 is the smallest of Lhc proteins (211 amino acids in Arabidopsis), due to the lack of the major part of the C-terminal region of the protein. Sequence homology and absorption spectra suggest that 5 Chl a, 5 Chl b and 2 xanthophylls are bound (Bassi, R. et al. 1996, Pagano, A. et al. 1998). Lhcb6, differently from other Lhcb proteins doesn't bind neoxanthin, indeed the Tyr residue involved in N1 stabilization is absent in this protein (Caffarri, S. et al. 2007).

VI. Supramolecular organisation of Photosystem II

Photosystem II forms a supramolecular complex together with its antenna (Figure 14). Electron microscopy measurements provided 2D and 3D structures of PSII supercomplexes at around 2 nm resolution (Boekema, E. J. et al. 1999b, Nield, J. et al. 2000); from these measurements we have the better description of the PSII structure, due to absence till now of a crystallographic structure. Electron micrographs show that organisation of PSII is very different from PSI: PSII is dimeric, while PSI in higher plants is a monomer.

Lhc subunits are organized into two layers around the PSII core: the inner one is composed of Lhcb4, Lhcb5 and a LHCII trimer (S-type LHCII), forming the so-called C_2S_2 particle together with PSII core (Boekema, E. J. et al. 1999a, Morosinotto, T. et al. 2006). The outer layer is made of LHCII trimer (M-type LHCII) and Lhcb6, to build up the large $C_2S_2M_2$ complex in which the number of LHCII-L trimers depends on the light intensity during growth (Bailey, S. et al. 2001). In particular Lhcb4, Lhcb6 and a LHCII-M trimer form a stable supercomplex bound to PSII core (Bassi, R. and Dainese, P. 1992). Moreover part of LHCII-L trimers can be phosphorylated by Stn7 kinase and move to PSI, during the state transitions, in order to balance the excitation energy receipt by PSI and PSII.

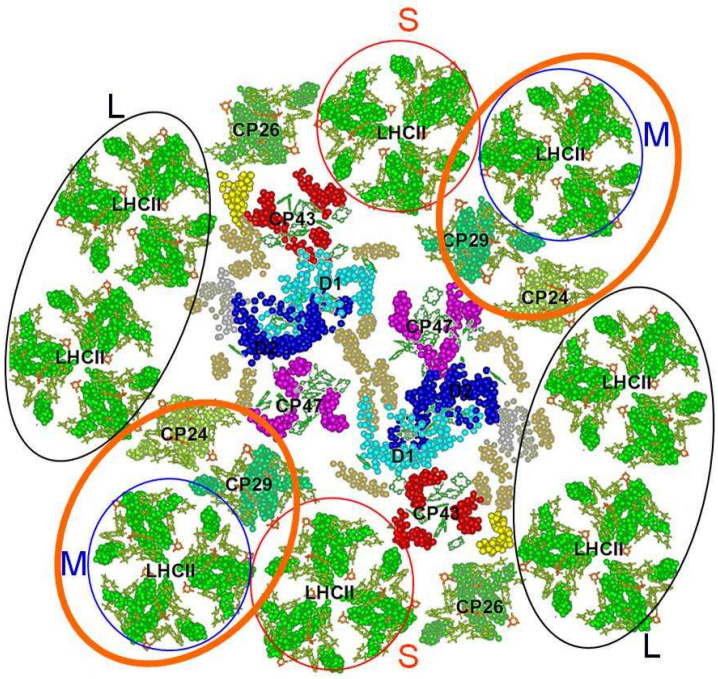

Figure 14. Schematic rappresentation of higher plant PSII supercomplex. *Structural model shows the core dimmer, trimeric outer antennae (LHCII) and the monomeric minor complexes (Lhcb4, Lhcb5 and Lhcb6) (Dekker and Boekema, 2005). Different trimers L, S and M are indicated. Orange circles indicates the supramolecular complexes Lhcb6-Lhcb4-LHCII-M .*

Recently, it has been shown that it is possible to purify omogeneous preparations of LHCII-PSII supercomplexes with increasing antenna size (Figure 15), which are suitable for biochemical, structural and spectroscopic analysis (Caffarri, S. et al. 2009). This procedure is based on mild solubilisation of grana membranes, thus preserving the association of LHCII-PSII supercomplexes.

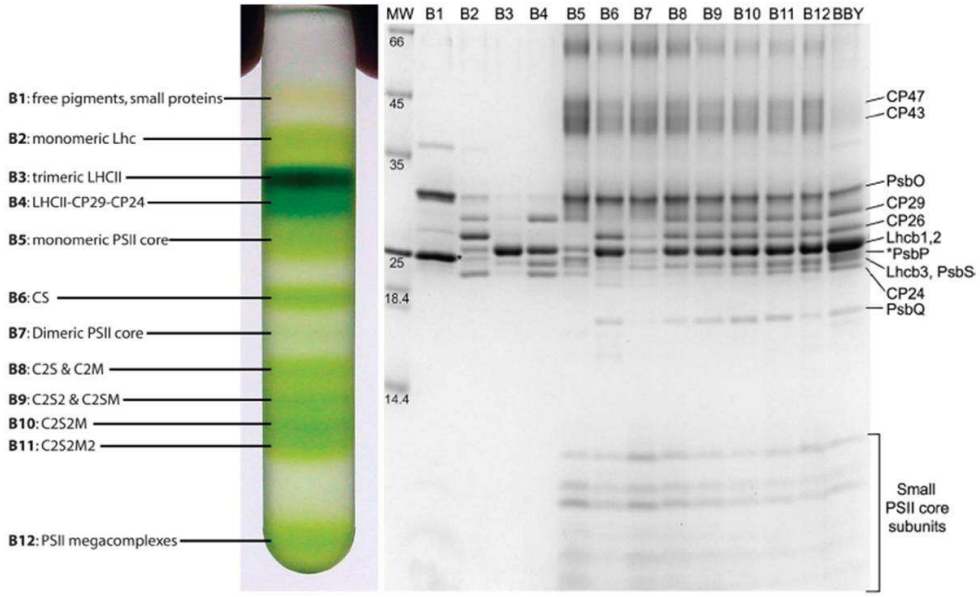

Figure 15. Isolation and characterization of PSII supercomplexes. *Sucrose gradient of solubilized membranes, showing 12 green bands, and following SDS-Page of sucrose gradient fraction (Caffarri, S. et al. 2009)*

4. PHOTOINHIBITION AND PHOTOPROTECTION

Light is needed by plants in order to trigger the photosynthetic reactions. However light can also be dangerous for the photosynthetic apparatus, since its absorption can lead to the formation of reactive intermediates or by-products of the photosynthetic process which can damage the thylakoids membranes. The whole process in which light damage some photosynthetic structures is called “photoinhibition”, since these photo-oxidative damages can decrease efficiency the rate of photosynthesis (Aro, E.-M. et al. 1993, Hideg, E. et al. 1998, Powles, S. B. and Bjorkman, O. 1982).

Plants growth conditions are often dynamic, involving diurnal and seasonal fluctuations in light intensities, temperatures and availability of nutrients. All these situations can lead to the absorption of higher amount of light energy than needed. When absorbed light energy exceeds the capacity for light energy utilization for photochemistry, the excitation energy in excess can induce the formation of dangerous molecules as reactive species of oxygen (Prasil, O. et al. 1992, Tjus, S. E. et al. 1998, Tjus, S. E. et al. 2001).

Light is absorbed by chlorophyll and carotenoid molecules, bound to the light-harvesting complexes: excited pigments rapidly (<ps time scale) transfer the excitation energy to the reaction centres, in order to drive electron transport. Absorption of excess photons can cause accumulation of excitation energy in the LHCs and thereby increase the

lifetime of singlet Chl (^{1}Chl, lifetime ~ns time scale): ^{1}Chl can thus convert into triplet chlorophyll (^{3}Chl), through intersystem crossing. Triplet Chl excited state is a long-lived state (~ms time scale) and thus can react with triplet oxygen, converting it to singlet oxygen ($^1O_2$), a highly reactive oxygen specie (Knox, J. P. and Dodge, A. D. 1985, Krieger-Liszkay, A. 2005). Production of $^1O_2$ within the Lhcs causes oxidation of lipids (Tardy, F. and Havaux, M. 1996), proteins and pigments (Formaggio, E. et al. 2001). Thylakoid membrane lipids, with their abundance in unsaturated fatty acid side chains, are susceptible of singlet oxygen attack: products are hydroperoxides and peroxyl radical chain reactions in the thylakoid membrane (Havaux, M. and Niyogi, K. K. 1999).

Another site for ROS production is the PSII reaction centre: after primary charge transfer P_{680}^+ and Ph^- species are formed. Ph^- is reconverted to Ph after electron transfer to Q_A, while P_{680}^+ is reconverted to P_{680} through Y_Z oxidation. However if Q_A is already reduced and electron transport is blocked, which is the case of excess light absorption, a charge recombination can occur between P_{680}^+ and Ph^-, producing a triplet P_{680} ($^3P_{680}$). $^3P_{680}$ can generate singlet oxygen (Melis, A. 1999) inducing photoinhibition, and in particular photo-damages on the D1 subnunit of PSII (Aro, E.-M. et al. 1993). The following equations summarize the electron transfer and dissipation events that occurs in PSII when Q_A is reduced at the time of primary charge separation.

$$P680^*\ Ph\ Q_A^- \rightarrow P680^+\ Ph^-\ Q_A^- \rightarrow {}^3P680\ Ph\ Q_A^-$$

$$^3P680\ \ ^3O_2 \rightarrow P680\ \ ^1O_2$$

In the case of PSI, the oxidized reaction centre P_{700}^+ is more stable than P_{680}^+ and behave as a quencher of excitation energy (Dau, 1994). Nevertheless, at the acceptor side of PSI, ferrodoxin can reduce molecular oxygen to the superoxide anion (O_2^-). This short-living specie can be metabolised to hydrogen peroxide (H_2O_2) or hydroxyl radical ($OH^\bullet$), the latter one being an extremely aggressive ROS.

Reactive oxygen species produced on the acceptor side of PSI are able to damage key enzymes of photosynthetic carbon metabolism such as phosphoribulokinase and NADP-glyceraldehyde-3-phosphate dehydrogenase, as well as subunits of PSI reaction centre. There are evidences that also PSII can be photoinhibited by PSI-produced ROS in vivo (Tjus, S. E. et al. 2001).

I. Photoprotection mechanisms

Plants evolved some mechanisms in order to use light avoiding as much as possible ROS formation, in order to prevent photoinihibition.

These photoprotective mechanisms can be divided into two different classes, depending on the time-scale of action: a) short-term photoprotective mechanisms and b) long-term photoprotective mechanisms.

In short-term photoprotective mechanisms the absorbed energy is dissipated as heat, in order to prevent ROS formation, while in long-term photoprotective mechanisms plants adjust their photosynthetic machineries to the disposable light. In particular short-term photoprotective mechanisms don't need synthesis of proteins *de novo*, while in long-term photoprotective mechanisms proteins are modulated in order to adapt their amount and composition to the energy requirements for photochemistry reactions.

- ***Short term responses:***

Non-Photochemical Quenching (NPQ)

Light fluctuations happen often in natural conditions: leaves can be suddenly exposed to very high light as a consequence of clouds transit or wind induced leaves movement. In order to avoid photoinhibition events, plants evolved a rapidly light-inducible mechanism, termed NPQ (Non-Photochemical Quenching), that allows the harmless thermal dissipation of excess absorbed photons in PSII (Demmig-Adams, B. and Adams, W. W. 1992). NPQ is a real feed-back regulation mechanism for excitation energy in excess (Figure 16), since it's activation and modulation depends from the extent of thylakoid transmembrane ΔpH deriving from photosynthetic electron transport (Horton, P. 1996). Triggering of NPQ is accompanied by a change in Chl fluorescence lifetime distribution, that leads PSII into a quenched state.

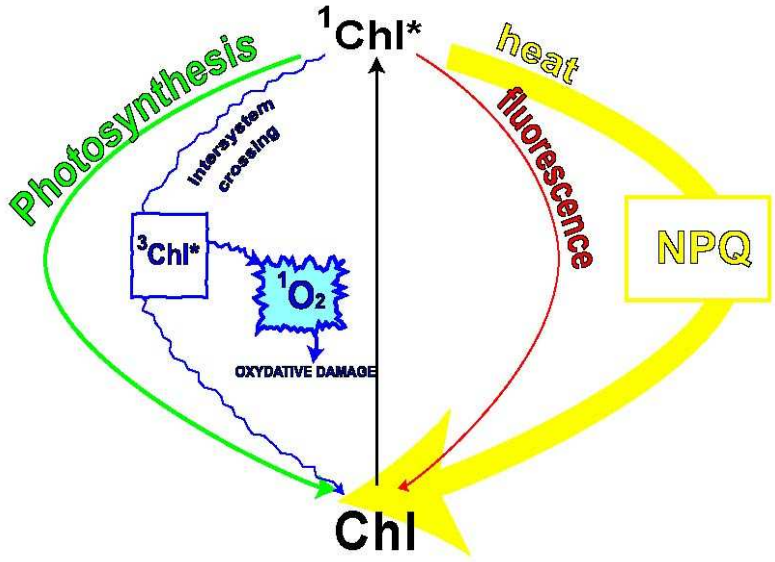

Figure 16. Scheme of de-excitation pathway for ^{1}Chl*. *Photochemistry, fluorescence emission, intersystem crossing and thermal energy emission compete for de-excitation of excited singlet state of Chls.*

NPQ is a very complex phenomenon and it involves several processes. Its fastest component is called qE (energy dependent quenching) and it is activated very rapidly upon illumination (Muller, P. et al. 2001). NPQ and in particular its fastest component qE were demonstrated to require both xanthophyll cycle activation (Demmig-Adams, B. 1990, Niyogi, K. K. et al. 1998) and the presence of the protein PsbS (Li, X. P. et al. 2000). The decrease in lumenal pH induces the activation of the xanthophyll cycle, triggering the violaxanthin de-epoxidase enzyme (VDE). Zeaxanthin production and accumulation into the thylakoids have been correlated to the NPQ extent for a wide variety of plants (Demmig-Adams, B. and Adams, W. W. 1994, Niyogi, K. K. et al. 1998).

PsbS instead is an integral membrane protein component of PSII and member of the Lhc-protein superfamily. This protein has a key role in qE induction, since the *npq4* mutant, missing PsbS, is impaired in NPQ (Li, X. P. et al. 2000). PsbS is able to sense the transmembrane ΔpH induced by electron transport, through protonation of two conserved glutamic acid exposed to the lumen (Li, X. P. et al. 2002). Substitution of these glutamic residues results in PsbS

inactivation. Recently PsbS was also suggested to modulate the interaction between PSII and LHCII, controlling PSII organization (Kiss, A. Z. et al. 2008).

The molecular mechanism of NPQ is still unclear. The question about how is the "quencher" which should be able to dissipate the excess energy absorbed still doesn't have a clear and convincing answer. PsbS has been proposed to be the "quencher", but this hypothesis seems not consistent since recently PsbS was shown not to bind any pigments (Bonente, G. et al. 2007), and thus it's not clear how PsbS could receive and dissipate the excitonic energy. Recently PsbS was demonstrate to interact with Lhc proteins (Teardo, E. et al. 2007).

Low lumenal pH also results in the protonation of several Lhc subunits associated with PSII. VDE enzyme is also activated by low lumenal pH activating the xanthophyll cycle. Upon violaxanthin de-epoxidation, the newly synthesized zeaxanthin is distributed among LHCII and minor complexes: zeaxanthin is known to cause a conformational change able to switch LHCs into a state with efficient thermal dissipation (Dall'Osto, L. et al. 2005, Gilmore, A. M. et al. 1995). However zeaxanthin binding to LHCII is performed by a peripheral, low affinity site and does not induce conformational changes nor decrease of fluorescence quantum yield of the complex (Caffarri, S. et al. 2001). In the minor complexes, instead binding is operated by an intrinsic site carotenoid binding site (L2) (Morosinotto, T. et al. 2002, Ruban, A. V. et al. 1999) and induces a conformational change of the protein, leading to decrease of fluorescence lifetime and quantum yield (Dall'Osto, L. et al. 2005, Moya, I. et al. 2001). However zeaxanthin accumulation in thylakoids membranes by itself is not sufficient for NPQ induction, since in absence of PsbS NPQ is not active, even in presence of zeaxanthin (Niyogi, K. K. et al. 1998). Moreover isolated Lhc proteins enriched in zeaxanthin are not able to dissipate enough absorbed energy as leaves during NPQ induction.

The more consistent mechanism proposed for NPQ induction involve binding of zeaxanthin to LHCs, activation of PsbS by low lumenal pH, eventually protonation of antenna subunits: all these factors, combined with some reorganization of the PSII supercomplexes may cause a conformational change able to switch Lhcs into a state and ^{1}Chl* de-excitation. Recently the formation of a carotenoid radical cation in thylakoids in a "quenched state" (Holt, N. E. et al. 2005) was strictly correlated with qE, since it requires PsbS and zeaxanthin. This dissipation mechanism, called charge transfer quenching (CT quenching), involve minor antenna complexes activity, since there is no trace of zeaxanthin radical cation in isolated LHCII trimer (Ahn et al. 2008; Avenson et al. 2008). In particular it has been identified that the excitonic interaction between chlorophylls A5 and B5 in minor complexes, modulated by zeaxanthin, is responsible for zeaxanthin radical cation formation and consequent energy dissipation.

State Transitions

As previously described, short-term changes in the antenna size of both photosystems can occur, and they are provided by the state-transition mechanism, involving migration of Lhcb proteins from PSII to PSI, in order to balance the PSI and PSII excitation. Here LHCII can transfer the energy absorbed to PSI, rather than PSII. The state transition takes several minutes to be activated, since it involves also the migration of LHCII from grana to stroma lamellae.

The importance of state-transition mechanism in photoprotection is still a subject of debates (Wollman, F. A. 2001).

Non-Assimilatory Electron Transport Mechanisms

Especially under conditions of CO_2 limitation, non-assimilatory electron transport to oxygen acts as important photoprotection mechanism. In C3 plants, photorespiratory metabolism can sustain linear electron transport, and thus allowing utilization of excitation energy; this feature has been verified showing that mutants blocked in photorespiration undergo inhibition of photosynthesis and photoxidative damages {Wallsgrove, 1987 24849 /id}.

The so called pseudocyclic electron transport, or water-water cycle, consists in an alternative electron transport pathway starting with direct reduction of O_2 by PSI, that act as another important photoprotective mechanism (Ruuska, S. A. et al. 2000). On the acceptor side of PSI, reduction of O_2 leads to superoxide (O_2^-) production; this aggressive ROS is readily metabolised by thylakoid-bound superoxide dismutase (SOD) and ascorbate peroxidase (APX) to generate H_2O and monodehydroascorbate; the latter product can be directly reduced by PSI to yield ascorbate. This pseudocyclic pathway, in which electrons produced from water oxidation into PSII are used to reduce O_2 to H_2O, allows generation of a ΔpH for ATP synthesis without production of O_2 or NADPH (Schreiber, U. and Neubauer, C. 1990); the mechanism allows dissipation of excitation energy in excess through electron transport (Asada, K. 1999).

Antioxidant species

ROS production is often unavoidable for plants. Reactive species produced during photooxidative stress can be deactivated by several antioxidant species present in the chloroplast as ascorbate, glutathione, tocopherols and especially carotenoids (Figure 17);

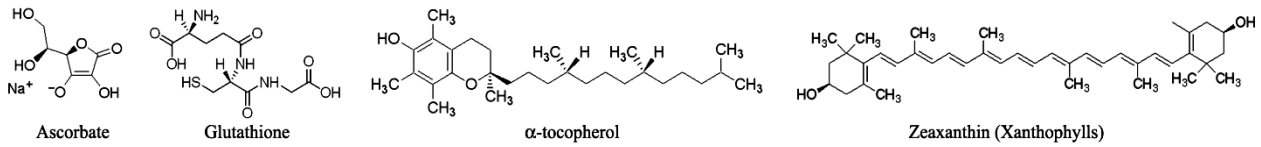

Figure 17. Molecular structures of the most common chloroplastic antioxidants.

Carotenoids play a key role as photoprotective agents: they are membrane-bound antioxidant able to quench both ^{3}Chl and $^1O_2$, thus stabilizing membrane by inhibition of lipid peroxidation. Carotenoids bound to Lhcs and PSII core complex are involved in efficient quenching of respectively ^{3}Chl of antenna proteins (Formaggio, E. et al. 2001) and singlet oxygen produced by $^3P_{680}$ (Telfer et al., 1994). Lutein, Neoxanthin and Zeaxanthin in particular were shown to be strongly involved in photoprotection (Baroli, I. et al. 2004, Dall'Osto, L. et al. 2006, Dall'Osto, L. et al. 2007, Muller-Moule, P. et al. 2003).

- ***Long-term photoprotective mechanisms***

When plants are exposed for a long time to stress conditions, long-term photoprotective mechanisms are activated. These adaptations involve several phenomena, for instance the expression or repression of specific proteins, but also chloroplast movements and modification of plant morphology. One of the mechanisms involves adjustment of changes in Lhc gene expression or Lhc protein degradation (Escoubas, J. M. et al. 1995), yielding a reduction of light-harvesting antenna size.

Another important long-term mechanism is the accumulation of antioxidant molecules during acclimation to excess light (Foyer, C. H. et al. 1994). Carotenoids and in particular zeaxanthin are known to increase their level in high light stressed plants, especially in overwintering evergreen plants, inducing a constitutive not reversible absorbed light quenching (Gilmore, A. M. and Ball, M. C. 2000).

The PSI and PSII antenna systems are characterized by different behaviour upon acclimation in high light condition. The number of antenna complexes bound to PSI is not modified during growth of *Arabidopsis*, instead the PSII antenna

size is largely regulated following environmental conditions: in particular growth in high light cause a reduction of the amount of antenna complexes bound to PSII, instead PSI antenna system is more stable upon long-term exposure (Ballottari, M. et al. 2007). Thus Lhc complexes amount requires a very efficient regulation, but up to now little is known about proteases involved in Lhc apoprotein degradation during long term exposure to high light: one metalloprotease, Ftsh6, has been recently described involved in LHCII degradation upon high light acclimation (Zelisko, A. et al. 2005). Other LHCII protease candidates have also been reported {Yang, 2000 33 /id;Georgakopoulos, 2002 1 /id} .

Instead there are more informations about pigments degradation pathway. Many mutant has been identified that are unable to degrade Chl during leaf senescence. Recently, the genetic defect of some of these mutants was shown to be due to mutations in a gene called *Stay Green* (*Sgr*) mutants has been discovered, which retain intact Lhc complexes upon senescence induction (Park, S. Y. et al. 2007). Originally, absence of Sgr was considered to inhibit Chl breakdown, but these recent findings indicate that SGR is not directly in a Chl catabolic step; instead, it is required for the dismantling of photosynthetic chl-protein complexes, thus allowing chl-breeakdown enzymes to access their substrate (Hortensteiner, S. 2009). The first step of Chl catabolic pathway is the conversion of antenna complexes bound Chlorophyll b (Chlb) to Chlorophyll a (Chla) through the intermediate 7-hydroxymethyl chlorophyll a (HMChla) (Kusaba, M. et al. 2007). Mutants deleted in Chlb reductase codifying genes are not able to degrade Chlb and LHCII is selectively retained during senescence (Yamaji, M. et al. 2009), suggesting that this enzyme participate in the initiale step of LHCII degradation. Chl breakdown continues with the successive removal of phytol and Mg by chlorophyllase and metal chelating substances {Suzuki, 2005 5 /id}, finally obtaining non fluorescent Chl catabolite.

5. ELECTRON MICROSCOPY

Microscopy techniques are among the most frequently and useful investigation methods used in biology. In plants research the level of detail of the microscopy observation increased with the development of imaging techniques, from anatomical details with early optical microscopy, to near-atomic resolution of single particle observation with modern electron microscopes.

The first electron microscope (EM) was built in the early 1930's and in a decade the technique resolution passed from that of the optical microscope to 10Å, exploiting the development of better optical systems and electron sources. Today instruments are commonly able of 10 Å resolution, while 1Å resolution is occasionally still attainable. In particular for protein observation, even if electron microscopy can not compete with X-ray crystallography or NMR in terms of resolution, it is still an invaluable method to investigate structure, function and dynamic of macromolecules and their supramolecular assemblies.

Infact obtaining clear pure crystallization of biological proteins for X-ray imaging is a complex and uncertain task and this is particularly true for membrane proteins and even more for membrane protein supercomplexes, which are also usually too large to be resolved using NMR spectroscopy. Hence electron microscopy have been extensively used in the study of thylakoids membranes and even in the identification of singular antenna moieties.

The available models of supramolecular organization of PSI-LHCI complex are based on electron microscopy data (Boekema, E. J. et al. 2001, Germano, M. et al. 2002), and this technique has been very useful also for the analysis of PSII-LHCII supramolecular organization: PSII is more dynamic than PSI, forming large aggregates of complexes characterized by diverse PSII core-antenna complexes interactions. Electron microscopy was successfully used in

revealing supramolecular geometric and stoichiometric details of these arrangements in isolated membranes (Boekema et al. 1999b; Boekema et al. 1999a; Boekema et al. 1995).

Singular detectable masses of similar proteins are usually not distinguishable with EM resolution, but it is still possible to identify them in some cases. It is noteworthy the PSII antenna proteins moieties identification, despite their very high secondary structure omology (Barros and Kuhlbrandt 2009): a result attained analyzing with EM specific mutants, lacking one or more PSII antennae (Morosinotto et al. 2006; Caffarri et al. 2009) or using the cross-linking (Bassi and Dainese 1992; Harrer et al. 1998; Jansson et al. 1996) technique, where fluorescence signals are detectable on selectively marked interacting proteins. The localization of singular proteins helped to define their structural role and in many cases also their functional contribution to photosynthetic phenomena (de Bianchi et al. 2008a). For an general review on the microscopy applied to plant research see (Vacha et al. 2005).

Part of this work is focused on the study of the organization of PSII complexes on grana membranes in response to light treatment. To this purpose transmission electron microscopy (TEM) on negatively stained samples were used, and will be thus outlined.

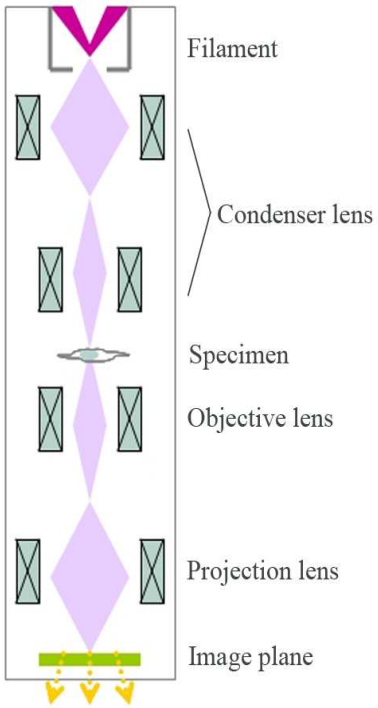

Figure 1. Schematic structure of TEM. *Schematic diagram showing the principle of image formation in Trasmission Electron Microscopy (TEM). The rhomboid shapes indicate the scattered electrons. See text for the description.*

In a transmission electron microscope, electrons are speeded up in a vacuum and then projected onto the specimen via a set of condenser lens (see Figure 1), that render the beam parallel. Electrons scattered by the specimen interact with the magnetic field of the objective lens for a first magnification up to 30 times. The successive magnification is obtained by the passage through the projection lenses that produce the final image on the image plane.

If compared to other EM techniques, where specimen surface is scanned by the electrons beam, in TEM the sample -a few hundreds of nanometers thick- interacts readily with the beam and thus a specific preparation phase is needed.

Specimen preparation

Biological specimen that undergo an electron microscopy measure are exposed to radiation, that can cause ionization as a result of the inelastic interaction of the electrons with those in the orbitals of organic material. The collision leads to the rearrangement of the molecule chemical configuration, inducing the formation of free radical. Other damages to the specimen can come from the dehydration, that is greatly enhanced by the strong vacuum necessary in the electron microscope.

The technique of negative staining (Horne, R. W. 1991), that is used in the present research work for the detection of PSII inside grana membranes of thylakoids, aims to overcome the aforementioned problems by enhancing the visibility of the biological specimen -due to their low weight atoms- by surrounding the sample with a heavy-metal salt, that is able to strongly scatter electrons. The salt is added to the specimen on a support film in carbon, which is magnetically-treated to become hydrophilic, thus increasing the mixture homogeneity and absorption (see Figure). The salts mainly used are uranyl acetate, uranyl formate, but also ammonium molybdate and others.

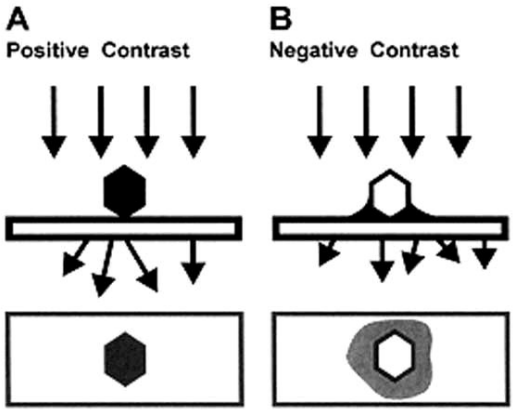

Figure19. A. *Without negative staining.* ***B.*** *Negative staining. The electron-dense stain surrounds the specimen and embeds the particle in a matrix of stain. Due to density differences between the stain and weakly scattering biological components of the sample, it appears as a transparent and detailed reverse (negative) image.*

The negative staining technique is highly reproducible and relatively simple. The typical resolution achievable is 10-20 Å, as the salt introduction produces a grainy noise in the image and coating the sample enhances particularly its quaternary structure, visually rendering protruding structure as brighter in a darker, more salt-rich, landscape.

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SECTION A

New evidences of zeaxanthin modulation of monomeric Lhc quenching properties.

- Section A.1: Dynamics of zeaxanthin binding to the photosystem II monomeric antenna proteinLhcb6 (CP24) and modulation of its photoprotection properties

- Section A.2: Identification of the chromophores involved in aggregation-dependent energy quenching of the monomeric Photosystem II antenna subunits.

Section A.1: Dynamics of zeaxanthin binding to the photosystem II monomeric antenna protein Lhcb6 (CP24) and modulation of its photoprotection properties

Lhcb6 (CP24) is the less known complex among monomeric antenna protein of Photosystem II, due to difficulty for native complex purification. In this section we characterize Lhcb6 functional properties *in vivo* and *in vitro* We show that this protein, upon activation of the xanthophyll cycle, accumulates zeaxanthin into inner binding sites faster and to a larger extent than any other pigment-protein complex. By comparative analysis of violaxanthin or zeaxanthin binding Lhcb6 complexes, we demonstrate that zeaxanthin not only down-regulates chlorophyll singlet excited states, but also increases the efficiency of chlorophyll triplet quenching, with consequent reduction of singlet oxygen production and significant enhancement of photo-stability. On these bases we propose that Lhcb6, the most recent addition to the Lhcb protein family which evolved concomitantly to the adaptation of photosynthesis to land environment, has a crucial role in zeaxanthin-dependent photoprotection.

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1. INTRODUCTION

Higher plants absorb light and convert it into chemical energy during photosynthesis. Two multi-subunit protein complexes, Photosystems I and II (PSI, PSII), bind pigments and cofactors responsible for light harvesting, charge separation and electron transport during the light phase of photosynthesis. Photosystems are composed by two moieties, a reaction centre and a peripheral antenna system, responsible respectively for the conversion of light into chemical energy, and for extending light harvesting cross-section and ensuring photoprotection. The antenna systems of PSI and PSII are composed by Light Harvesting Complexes (LHC), a multi-genic protein family comprising 14 different members in *Arabidopsis thaliana*, sharing high sequence similarity. Thus, these proteins have a common structural model revealed by the resolution of LHCII complex (Kühlbrandt, W. et al. 1994, Liu, Z. et al. 2004), characterized by three transmembrane and two amphipatic helices, named, respectively, A-C and D-E (Liu, Z. et al. 2004). Their light harvesting and photoprotection functions are catalyzed by up to 18 chromophores: 4-8 chlorophylls (Chl) a, 4-6 Chl b and 2-4 xanthophylls. Two carotenoid binding sites, named L1 and L2 (Kühlbrandt, W. et al. 1994), are conserved in all LHC proteins, while a third site, specific for neoxanthin (Neo), has been detected in Lhcb1-3, Lhcb5, Lhcb4 but not in Lhcb6 (Caffarri, S. et al. 2007a, Croce, R. et al. 1999a). A fourth peripheral carotenoid binding site, named V1, has been detected in Lhcb1-3 only (Caffarri, S. et al. 2001, Liu, Z. et al. 2004). Besides light harvesting, LHC proteins catalyze multiple reactions, namely (i) Chl triplet quenching; (ii) Chl singlet quenching and (iii) Reactive Oxygen Species (ROS) scavenging, in order to prevent/reduce photo-oxidation and photoinhibition, two unavoidable effects of oxygenic photosynthesis (Niyogi, K. K. et al. 1997, Niyogi, K. K. 1999). These functions are catalyzed by chromophores bound: Chl a and b are involved in light harvesting; lutein (Lut) is specialized in Chl

a triplet quenching (Dall'Osto, L. et al. 2006); Neo and violaxanthin (Vio) are involved in scavenging superoxide and singlet oxygen, respectively (Dall'Osto, L. et al. 2007).

Among photosynthetic pigments, a special role is played by zeaxanthin (Zea), only accumulated under excess light conditions. Zea is formed from Vio in the so-called xanthophyll cycle (Yamamoto, H. Y. et al. 1967), and Zea binding leads to an increased level of resistance to light stress. This effect is obtained by up-regulating the capacity for quenching singlet Chl excited states energy into heat. (Demmig-Adams, B. and Adams, W. W. 1992, Noctor, G. et al. 1991, Wentworth, M. et al. 2000). Molecular details of the xanthophyll cycle have been elucidated recently: Vio, initially bound to a low affinity binding site named V1 of LHCII trimers (Caffarri, S. et al. 2001, Liu, Z. et al. 2004) is released to the lipid phase and converted to Zea by VDE. In this state it may act as ROS scavenger (Havaux, M. and Niyogi, K. K. 1999). In addition, Lhcb4-6 and Lhca1-4 subunits bind Zea to the high affinity inner site L2 (Jahns, P. et al. 2001, Morosinotto, T. et al. 2002, Wehner, A. et al. 2004, Wehner, A. et al. 2006) which has an allosteric nature (Formaggio, E. et al. 2001, Moya, I. et al. 2001). Recent work has elucidated the mechanistic basis of the activation of singlet Chl excited state dissipation into heat (Ahn, T. K. et al. 2008, Dall'Osto, L. et al. 2005, Ruban, A. V. and Horton, P. 1999) thus revealing the effect of Zea binding to LHC proteins *in vitro* (Ahn, T. K. et al. 2008, Dall'Osto, L. et al. 2005, Phillip, D. et al. 1996) and *in vivo* with respect to photosynthetic efficiency, Non-Photochemical quenching (NPQ) induction, ROS production and scavenging (Fiore, A. et al. 2006) (Dall'Osto, L. et al. 2007) (Havaux, M. et al. 2004, Niyogi, K. K. et al. 1997) (Dall'Osto, L. et al. 2006, Mozzo, M. et al. 2008). In contrast, little is known about quenching of chlorophyll triplet excited states. Previous work has reported a similar (Passarini, F. et al. 2009) or reduced (Mozzo, M. et al. 2008) chlorophyll triplet quenching efficiency in the presence of zeaxanthin in Lhcb6 and LHCII trimers respectively, despite increased quenching of singlet excited states.

In this work we have focused on one of the monomeric antenna complex Lhcb6 (CP24). This subunit is a recent addition to the photosynthetic apparatus of the green lineage, being absent in algae and first appearing in mosses and hepaticas, together with the adaptation to the highly stressful land environment. Consistently, it is involved in several regulation mechanisms, as evidenced by genetic (de Bianchi, S. et al. 2008, Kovacs, L. et al. 2006) and biochemical analysis (Ballottari, M. et al. 2007) (Betterle, N. et al. 2009). Lhcb6, together with Lhcb4 and a LHCII (S) trimer, forms a stable supercomplex (Bassi, R. and Dainese, P. 1992) whose dissociation, upon treatment with high intensity light, is necessary for triggering NPQ (Betterle, N. et al. 2009). The abundance of Lhcb6 was shown to be regulated by light intensity during growth (Ballottari, M. et al. 2007) through the reduction state of the plastoquinone pool (Morosinotto, T. et al. 2006). This subunit was recently reported to be involved in the photoprotective mechanism called Non-Photochemical Quenching (NPQ), responsible for singlet chlorophyll excited state quenching through carotenoid radical cation formation upon Zea binding {Ahn, 2008 24858 /id;Amarie, 2009 24896 /id;Holt, 2005 24620 /id}. Finally, time resolved spectroscopic analysis demonstrated that, in detergent solution, 50% of Lhcb6 complexes are quenched, with the quenching site located near the carotenoid binding site L2 (Passarini, F. et al. 2009).

We studied the *in vivo* xanthophyll composition of Lhcb proteins upon exposure of leaves to excess light and found that upon xanthophyll cycle activation, Lhcb6 undergoes faster Zea accumulation than any other thylakoid protein so far described. We then studied *in vitro* modulation of Lhcb6 functional properties by the binding of different xanthophyll species and we found that Zea binding not only quenches chlorophyll singlet excited states, but also facilitates chlorophyll *a* triplet quenching efficiency. This induces a reduction of singlet

oxygen production and a significant enhancement of photo-stability**.** These results suggest that Lhcb6 is specialized in binding Zea and enhancing photoprotection under excess light.

2. MATERIAL AND METHODS

2.1 DNA Cloning, Mutations and Isolation of Overexpressed Lhcb6 Apoprotein.

cDNA from *Arabidopsis thaliana* encoding Lhcb6 (GenBank accession number AF134130) was supplied by the Arabidopsis Biological Resource Centre (ABRC) at The Ohio State University. Mature *lhcb6* sequence was amplified and cloned in the pQE-50 expression vector as described in (Dall'Osto, L. et al. 2005). Lhcb6 WT apoprotein was overexpressed in SG13009 strain of *Escherichia coli* and purified following a protocol described previously (Paulsen, H. et al. 1993).

2.2 Reconstitution in vitro of recombinant Lhcb6.

Reconstitution and purification of recombinant Lhcb6 pigment-protein complexes (from *Arabidopsis thaliana*) were performed as in (Giuffra, E. et al. 1996) with the following modifications: a total of 60 μg of carotenoids and 250 μg of chlorophylls with a Chl a/b ratio of 3 were added to 420 μg of apoprotein. In the case of complexes with a modified carotenoid composition, pigment mixes were prepared from pure pigments and, when more than one carotenoid was included, all species were added in equal amounts, unless specified in the text. Chl a, b were purchased from Sigma-Aldrich©, while individual carotenoids were purified by HPLC from leaf acetone extracts.

2.3 Pigment analysis.

HPLC analysis was performed according to (Gilmore, A. M. and Yamamoto, H. Y. 1991). Chlorophyll to carotenoid and Chl a/b ratios were independently measured by fitting the spectrum of acetone extracts to the spectra of individual purified pigments (Croce, R. et al. 2002). De-epoxidation indexes (D.I.) were calculated as the ratio between the sum of Zea and half antheraxanthin and the sum of Zea, Vio and antheraxanthin.

2.4 Plant material treatment and fractionation of LHC proteins. Arabidopsis thaliana plants were grown for 3 weeks at 23 °C at 100 μmol $m^{-2}s^{-1}$ (white light, 8 h light/16 h dark). Plants were then kept 1h in the dark and then exposed to light stress at 1500 μmol $m^{-2}s^{-1}$ for different periods at 24 °C. After treatment chloroplast membranes were isolated from leaves as previously reported (Betterle, N. et al. 2009). Thylakoids were solubilised with 0,6% α-DM or β-DM and fractionated by ultracentrifugation in sucrose gradient as previously described (Caffarri, S. et al. 2001). Fractions 2 and 4 were then mixed and monomeric Lhcb subunits were further fractionated through preparative isoelectric focusing (IEF) on acrylamide gel as previously described (Jackowski, G. and Jansson, S. 1998), but using a pH range of 3.5-5.5. Fractions from IEF were purified from co-migrating free pigments by non-denaturing electrophoresis as previously described (Morosinotto, T. et al. 2005, Peter, G. F. and Thornber, J. P. 1991).

2.5 Gel electrophoresis.

SDS-PAGE was performed with Tris-Tricine buffer system (Schägger, H. and von Jagow, G. 1987).

2.6 De-epoxidation Reaction in Vitro. LHC proteins (3 μg of chlorophyll) were incubated with recombinant VDE enzyme in order to perform Vio de-epoxidation *in vitro* assay, as described in (Morosinotto, T. et al. 2002).

2.7 Spectroscopy.

Room temperature absorption spectra were recorded using a SLM-Aminco DK2000 spectrophotometer, in 20 mM Hepes pH 7.5, 0.2 M sucrose and 0.03 % *n*-dodecyl-α-D-maltopyranoside. The wavelength sampling step was 0.4 nm. Fluorescence emission spectra were measured using a Jasco FP-777 spectrofluorimeter and corrected for the instrumental response. Fluorescence quantum yields were calculated as the ratio between the emission spectra area (650-800 nm) with excitation at 625 nm and the absorption at the same wavelength, using in both cases the same 3 nm bandwidth. CD spectra were measured at 10 °C on a Jasco 600 spectropolarimeter: samples were in the same solution as described for the absorption with an OD of 1 at the peak in the Q*y* transition. The measurements were performed in a 1 cm cuvette. Denaturation temperature measurements were performed by following the decay of the CD signal at 480 nm when increasing the temperature from 25 to 80 °C with a time slope of 1 °C/min and a resolution of 0.2 °C. The thermal stability of the samples was determined by finding the $t_{1/2}$ of the signal decay. Carotenoid and chlorophyll triplet formation have been measured through TmS kinetic decay measurements in anaerobic conditions as described in (Mozzo, M. et al. 2008). Kinetics of photobleaching in Lhcb6 have been measured as described in (Croce, R. et al. 1999b) using a light intensity of 750 μmol $m^{-2}s^{-1}$.

2.8 Deconvolution of Spectra into Absorption Forms.

Absorption spectra were analyzed in terms of the contribution of individual pigments by using the absorption spectra of pigments in LHC proteins as previously reported (Croce, R. et al. 2000, Croce, R. et al. 2001). In order to increase the significance of our analysis and choose between multiple fitting solutions, we selected only solutions consistent with biochemical pigment composition such as Chl a/b, Chl/Car ratios and carotenoid content. Among the possible multiple solutions we chose those with the lowest discrepancy with the original absorption spectra.

2.9 Singlet oxygen production analysis.

Lhcb6 samples were diluted at 2 μg/ml concentration in a solution containing 0.03% α-DM, 20 mM Hepes pH 7.5 and 2 μM Single Oxygen Sensor Green™ (Invitrogen)(Flors, C. et al. 2006). Samples were illuminated with red light at 750 μmol $m^{-2}s^{-1}$ for different times. The increase of the concentration of singlet oxygen in solution was detected by measuring the Sensor Green specific fluorescence at 530 nm, with a 480 nm excitation.

3. RESULTS

3.1 Kinetics of violaxanthin de-epoxidation and zeaxanthin binding to LHC proteins.

Dark adapted *Arabidopsis thaliana* plants, grown for 3 weeks in control conditions, were treated with high light (1500 μmol $m^{-2}s^{-1}$) for different periods (5'-10'-30') in order to trigger xanthophyll cycle activity. Thylakoids purified from light treated plants were solubilised with 0.6% detergent, either α-DM or β-DM. The different pigment-binding complexes were separated by sucrose gradient ultra-centrifugation, thus obtaining 5 distinct green bands (Figure 1A): free pigments (fraction B1), monomeric Lhcb proteins (B2), LHCII trimers (B3), a supercomplex composed by Lhcb4, Lhcb6 and a LHCII trimer (B4), PSII core (B5) and PSI-LHCI supercomplex (B6) respectively, as previously described (Caffarri, S. et al. 2001).

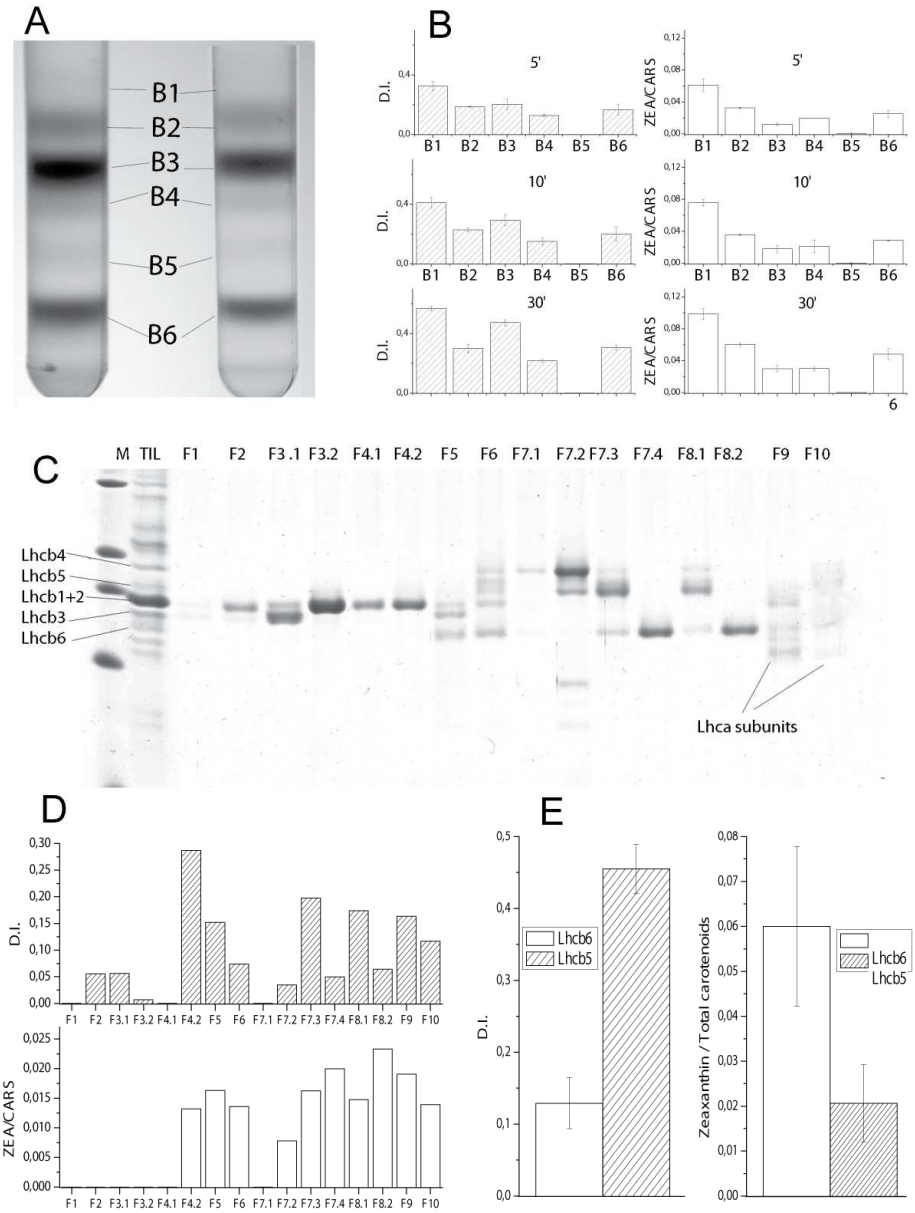

Figure 1: Violaxanthin de-epoxidation analysis *in vivo* and *in vitro*. *A.* *Thylakoid membranes from dark adapted and 5' light-treated leaves were solubilised in α-DM detergent and loaded on sucrose gradients. Similar fraction pattern were obtained at different times of illumination (5'-10'-30'). As previously reported (Ballottari, M. et al. 2004) fraction 1(B1) is composed of free pigments, fraction 2 (B2) by monomeric Lhcb protein, fraction 3 (B3) by LHCII trimers, fraction 4 (B4) by a LHCII trimer-Lhcb4-Lhcb6 supercomplex, fraction 5 (B5) by photosystem II core and fraction 6 (B6) by PSI-LHCI supercomplex.* ***B.*** *Each fraction recovered from sucrose gradients was analysed by HPLC; for each fraction, de-epoxidation indexes (D.I.) and the ratio between zeaxanthin content and total carotenoid content (Zea/ Cars) are reported. In each panel the time of illumination is indicated (5'-10'-30').* ***C.*** *SDS-PAGE analysis of fractions recovered from isoelectric focusing and non-denaturing electrophoresis separation; in each lanes, names of the fractions recovered are indicated; in some cases (F3, F4, F7, F8), IEF fractions were characterized by multiple bands in non-denaturing electrophoresis: in those cases, each band (F3.1-F3.2, F4.1-F4.2, F7.1-F7.4, F8.1-F8.2) was loaded separately in SDS-PAGE. M: Molecular weight marker, Til: Thylakoids.* ***D.*** *de-epoxidation indexes, and ratios between zeaxanthin content and total carotenoid content of fractions recovered by IEF separation (F1-10), after HPLC analysis:* ***E.*** *de-epoxidation indexes (D.I.) and ratios between zeaxanthin and total carotenoids (Zea/Cars) of Lhcb5 and Lhcb6 after in vitro de-epoxidation assay, performed adding recombinant VDE enzyme to recombinant Lhcb5 and Lhcb6 complexes, as described in (Morosinotto, T. et al. 2002). Errors bars are indicated.*

It is worth noting that the B4 supercomplex progressively dissociates during white light treatment, being almost absent after 30' of illumination with strong light, as recently described (Betterle, N. et al. 2009) and is fully dissociated in the β-DM solubilised sample. Pigment composition of fractions from α-DM sucrose gradients was analysed by HPLC: their de-epoxidation index (D.I.) and the amount of Zea per total carotenoids are reported on figure 1B. Already after 5' of illumination Zea was accumulated on B1, B2, B3, B4 and B6, while B5 didn't contain Zea even after 30'. The Zea/Car ratio was higher on the B1 fraction (free pigments) compared to the Lhc containing fractions. At longer illumination times the D.I. and Zea/Car ratio increased in all fractions, particularly B2-B6. When the experiment was repeated using β-DM, the figure was similar but for the case of B3 fraction, whose Zea content was close to zero at 5 and 10 minutes, reaching low values (Figure S1, Supplemental data) after 30 minutes of illumination. Since β-DM was shown to selectively extract xanthophylls from the site V1 with respect to site L2 {Matsubara, 2008 24891 /id} this result implies that Zea binds to different sites in trimeric LHCII (B3) compared to monomeric LHC proteins contained in fractions B2, B4 and B6 (Figure S2, Supplemental data). Zea binding to site L2 was shown to affect the functional properties of LHC proteins while binding to site V1 did not (Caffarri, S. et al. 2001) we therefore focused to fractions containing β-DM-resistant binding proteins in order to determine the sites of early binding of Zea to active sites.

In order to study their function, Lhcb proteins usually are isolated by isoelectrofocusing (Dainese, P. et al. 1990), the mildest and most efficient method described so far. To this aim, we pooled fractions B2 and B4 from the 5' illumination experiment and fractionated this sample using non-denaturing IEF. During IEF free xanthophylls distribute in the matrix in a wide pH range (Caffarri, S. et al. 2001). In order to avoid contamination of the Lhcb proteins by free xanthophylls, fractions from IEF were submitted to a further fractionation by non-denaturing Deriphat-PAGE where free pigments run with the front, well separated from LHC proteins (not shown). Fractions obtained were finally identified by denaturing SDS-PAGE (Figure 1C) and immunoblotting (not shown), and their pigment composition was analyzed by HPLC. The ratio of Zea vs total carotenoids and de-epoxidation index is reported in Figure 1D. Fractions enriched in Lhcb5 (F6-F7.3-F7-F8.1-F10) and Lhcb6 (F5-F7.3-F7.4-F8.1-F8.2) were characterized by the highest de-epoxidation index and higher Zea/total carotenoids ratios. A high level of Zea was also found in fraction F9, containing LHCI complexes while the fraction enriched in Lhcb4 (F7.1 and F7.2), had a lower Zea content. It is important to note that the fractionation procedure applied to samples from leaves illuminated for 10' and 30' yielded a figure almost identical to that obtained after 5' illumination, implying that the binding of Zea to β-DM-resistant sites was virtually saturated upon 5' illumination. A second interesting point was related to fraction, F4.2, which contained a LHCII isoform and yet bound significant amounts of Zea. When compared to other LHCII isoforms (F1, F2, F3.1, F3.2, F4.1), this fraction had a higher number of xanthophylls per polypeptide including two luteins, one Neo (into site N1) and sub-stoichiometric amounts of Vio and Zea per 14 chlorophylls (Liu, Z. et al. 2004) (Table S1).

Since Lut and Neo bind to the L1/ L2 and N1 sites of LHCII respectively (Kühlbrandt, W. et al. 1994, Liu, Z. et al. 2004), we conclude that in F4.2 fraction a small number of the Vio/Zea in V1 sites resisted extraction by β-DM. Fractionation of samples from dark adapted leaves yielded complexes with no Zea but correspondingly higher Vio content (data not shown). We conclude that, upon exposure to excess light, Lhcb5 and Lhcb6 are the subunits of the PSII antenna that more rapidly loose Vio and accumulate Zea in inner (L2) sites.

3.2 Violaxanthin-zeaxanthin exchange in vitro: a comparison between Lhcb6 and Lhcb5.

Native Lhcb5 and Lhcb6 proteins are difficult to separate from each-other and from others LHC proteins, due to their similar molecular weights and isoelectric points. In order to obtain a more complete comparative analysis of the Vio to Zea exchange capacity of these two antenna proteins, we proceeded to the use of recombinant proteins, which have been widely used in order to investigate Lhc protein function (Formaggio, E. et al. 2001, Giuffra, E. et al. 1997, Paulsen, H. et al. 1993), and Vio de-epoxidation assay *in vitro* according to a previously reported procedure (Jahns, P. et al. 2001, Morosinotto, T. et al. 2002). The *Arabidopsis thaliana* genes *lhcb5* and *lhcb6* were cloned in an expression vector, and the apoproteins were overexpressed in *E.coli*. Lhcb5 and Lhcb6 complexes were then reconstituted *in vitro*, (Dall'Osto, L. et al. 2005, Giuffra, E. et al. 1996). The complexes obtained were stable and well folded as judged by the efficient energy transfer from Chl b and from xanthophylls to Chl, determined by spectroscopic analysis (data not shown). Table 1 reports pigment analysis of Lhcb6 recombinant complex (Lhcb6-LV), based on 10 chlorophylls per holo-protein, as previously reported (Bassi, R. et al. 1993, Pagano, A. et al. 1998).

	Den. T (°C)	Chl	Chl a/b	Chl/Car	Chl a	Chl b	Cars	N	V	L	Z
Lhcb6-LV	59,1	10	1,48	4,72	5,97	4,03	2,12	0	0,88	1,24	0
SD	*1,35*	-	*0,08*	*0,13*	*0,12*	*0,12*	*0,06*	-	*0,10*	*0,04*	-
Lhcb6-LL	53,8	10	1,42	4,90	5,87	4,13	2,04	0	0	2,04	0
SD	*2,31*	-	*0,09*	*0,17*	*0,15*	*0,15*	*0,07*	-	-	*0,04*	-
Lhcb6-VV	57,6	10	1,60	7,11	6,16	3,84	1,41	0	1,41	0	0
SD	*1,67*	-	*0,19*	*1,28*	*0,32*	*0,32*	*0,34*	-	*0,10*	-	-
Lhcb6-ZZ	54,6	10	1,34	6,20	5,73	4,27	1,42	0	0	0	1,42
SD	*1,98*	-	*0,04*	*0,41*	*0,07*	*0,07*	*0,39*	-	-	-	*0,39*
Lhcb6-LZ	56,7	10	1,49	4,65	5,98	4,02	2,15	0	0	0,84	1,31
SD	*1,85*	-	*0,10*	*0,12*	*0,16*	*0,16*	*0,06*	-	-	*0,03*	*0,09*

Table 1: Denaturation temperatures and pigments content analysis of Lhcb6 samples having differing carotenoid compositions. *Pigment content of the different complexes was normalized to 10 chlorophylls. Denaturation temperatures (Den. T (°C)) were determined by measuring the decrease of the CD signal at 480 nm upon temperature increase. Abbreviations: Chl, number of chlorophylls per molecule; Chl a/b, Chl a /b ratio; Cars, number of carotenoids per molecule; Chl a: Chlorophyll a; Chl b: Chlorophyll b; Chl /Car: ratio between total chlorophylls and total cars; N: neoxanthin; V: violaxanthin; L: lutein; Z: zeaxanthin. Standard deviations (SD) are indicated.*

It is worth to note that both pigment binding properties and absorption spectra of recombinant proteins were similar to the native ones isolated from thylakoids (Dainese, P. and Bassi, R. 1991). When the Vio de-epoxidation assay was applied to reconstituted proteins *in vitro* by adding recombinant VDE, ascorbate and incubating at low pH some Chl *a* was lost, as previously described for other LHC proteins (Morosinotto, T. et al. 2002). Moreover, a decrease in Vio content and concomitant increase in Zea was observed in both Lhcb5 and Lhcb6 (Figure 1E). The de-epoxidation index was higher in Lhcb5 as compared to Lhcb6. Nevertheless, the ratio between Zea and total carotenoids was higher in the case of Lhcb6, consistent with an higher amount of Vio available for de-epoxidation in Lhcb6-LV, compared to Lhcb5-LNV.

3.3 Reconstitution of Lhcb6 complexes with different xanthophyll composition

The biochemical and spectroscopic properties of monomeric LHC proteins have been reported to be modulated by the xanthophyll species bound, in particular by Zea (Ahn, T. K. et al. 2008, Avenson, T. J. et al. 2008, Avenson, T. J. et al. 2009, Ballottari, M. et al. 2009, Croce, R. et al. 2002, Frank, H. A. et al. 2001, Passarini, F. et al. 2009, Ruban, A. V. et al. 1996, Ruban, A. V. and Horton, P. 1999). In order to investigate the effect of changing xanthophyll composition to Lhcb6, we produced Lhcb6 complexes with differing xanthophyll contents as previously reported for Lhcb1 (Formaggio, E. et al. 2001). In particular, the following samples were refolded *in vitro*: (i) Lhcb6 with only Lut (Lhcb6-LL), (ii) only Vio (Lhcb6-VV), (iii) only Zea (Lhcb6-ZZ), (iv) Lut + Zea (Lhcb6-LZ), (v) Lut + Vio (Lhcb6-LV). Neo did not bind to Lhcb6, as previously described (Caffarri, S. et al. 2007b, Pagano, A. et al. 1998). Pigment composition of complexes is reported in Table 1. They showed similar thermal stability (Table 1) implying the different xanthophyll species could occupy binding sites with similar effects on folding. Chl a/b ratio (~1.45) and the number of carotenoids bound (~ 2) are similar in all different Lhcb6-samples, except for the case of Lut-less samples Lhcb6-VV and Lhcb6-ZZ complexes, characterized by a Chl a/b ratio of 1.6 and 1.3 respectively. Interestingly, under our conditions, adding equimolar amounts of Lut and Zea during reconstitution resulted into production of a complex (Lhcb6-LZ) with an excess of Zea over Lut (1.35 Zea vs 0.76 luteins per polypeptide), suggesting that Zea, besides binding to site L2, can compete with Lut for site L1.

3.4 Spectroscopic analysis of Lhcb6 complexes with different xanthophylls

The function of LHC proteins consists of the absorption of light energy by chromophores and energy transfer to nearby photosystem subunits and finally to reaction centers; moreover, carotenoids bound to LHC proteins are active in photoprotection by singlet and triplet Chl excited states quenching and ROS scavenging. Thus, optical properties of these proteins are closely related to their function *in vivo*. We first proceeded to analyze the absorption spectra in the Soret region of the different Lhcb6-samples in order to obtain information on the properties of multiple binding sites for pigments. In fact, the spectroscopic properties of Chls and carotenoids are tuned by their protein environment (Croce, R. et al. 2000). Absorption spectra of recombinant complexes were fitted with a sum of chlorophyll and carotenoid spectral forms in protein environment as previously described (Croce, R. et al. 2000). The result of such deconvolution in the case of Lhcb6-LV is shown in Figure 2: two lutein spectral forms , with $S_0 \rightarrow S_{2o,o}$ transition at 491 and 497 nm, and two spectral forms of Vio, peaking at 487 and 498 nm, were needed in order to properly fit the absorption spectrum, together with multiple Chl a and Chl b forms. Since two xanthophyll binding sites are available for Lhcb6 complex, the presence of 4 xanthophyll spectral forms clearly implies that Vio and Lut can both bind into L1 and L2 site, as previously suggested (Wehner, A. et al. 2006). In other LHC proteins, xanthophylls binding to site L2 induces a stronger absorption red shift than binding to L1 (Mozzo, M. et al. 2008). According to this assumption, Lut (497) and Vio (498) are proposed to be located in binding site L2 while Lut (491) and Vio (487) in binding site L1. This attribution is supported by the high amplitude (4X) of Vio (498) and of the most blue-shifted form of Lut (491), which is consistent with the preferential binding of Lut and Vio to sites L1 and L2 respectively, as described for all LHC proteins (Croce, R. et al. 1999b, Peterman, E. J. et al. 1997). Similarly red-shifted Lut and Vio spectral forms were detected in Lhcb6-LL and Lhcb6-VV (Figure S3, Supplemental data). Spectra of Lhcb6-ZZ and Lhcb6-LZ were fitted with two Lut (491 and 498 nm) and two Zea spectral forms (497 and 502 nm). Nevertheless, the blue-most Zea spectral form (497 nm) has always a smaller amplitude than the red-most one

(502 nm, Figure S3 Supplemental data) implying that Zea preferentially binds to site L2. The presence of two Zea spectral forms in Lhcb6-ZZ and Lhcb6-LZ implies that Zea can bind to both L1 and L2 sites, but the relative abundance of Lut and Zea spectral forms indicates that in Lhcb6-LZ ~95% of L2 is occupied by Zea, while 70% of L1 is occupied by Lut.

Spectral deconvolution was used in order to investigate if one or both xanthophyll binding sites in Lhcb6 are involved in xanthophyll cycle. Lhcb6 spectrum was analyzed before and after the de-epoxidation assay (Figure 2).

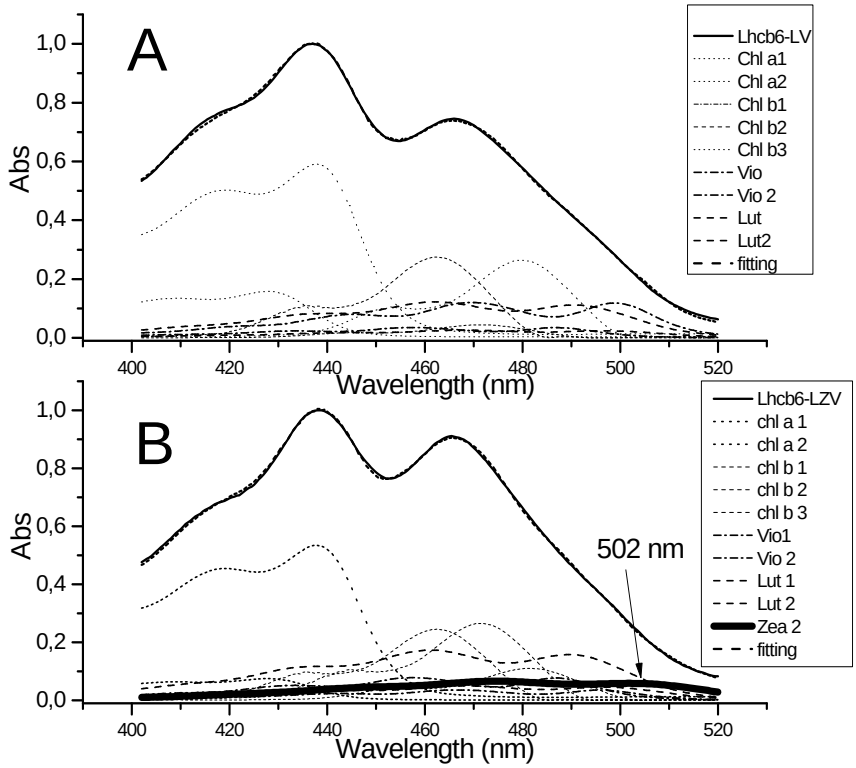

Figure 2: Deconvolution of absorption spectra of Lhcb6 before and after *in vitro* de-epoxidation assay. *Spectral deconvolution of Lhcb6 before (Lhcb6-LV, Panel A) and after (Lhcb6-LZV, Panel B) de-epoxidation in vitro is shown. Fitting procedure was performed in both cases using 2 Chl a (Chl a 1, Chl a 2, dash), 3 Chl b(Chl b 1, Chl b 2, Chl b 3, dot), 2 lutein (Lut 1, Lut 2, dash) and 2 violaxanthin (Vio 1, Vio 2, dash dot) spectral forms protein environment properly shifted in the Soret region. One additional zeaxanthin spectral form (Zea 2, solid) is detected after de-epoxidation in vitro.*

Following the de-epoxidation assay, five carotenoids spectral forms were needed to achieve a best fit of the absorption spectra in the Soret region: two lutein (491 nm and 497), two Vio (487 and 498 nm) and one Zea (502 nm). Thus, only the red-most form of Vio, was decreased as compared to the control sample (Lhcb6-LV), and only the red-most Zea (502 nm) observed. Thus, in Lhcb6, irrespective of the location of xanthophyll species, the ligands of site L2 undergo more rapid exchange during xanthophyll cycle.

In the following, we will concentrate on the analysis of the Lhcb6-LZ complex which appears to be the most representative of the *in-vivo* situation of the complex upon activation of the xanthophyll cycle. In fact, site L2 is occupied by Zea and site L1 is mostly occupied by Lut.

The $S_0 \rightarrow S_1$ transition of carotenoids is forbidden and consequently it does not appear in the Qy region of the Lhcb6 absorption spectra. The changes in the 600–700 nm region of the complex thus represent modifications in the energy levels of the Chl a and Chl b transitions induced by the protein and xanthophyll environment (Croce, R. et al. 1999b), or by changes in Chl a/b ratio. The Lhcb6-L *minus* Lhcb6-LV difference spectrum (Figure 3A) has negative/positive terms, respectively, at 673 nm and 654 nm, which represent partial substitution of Chl a with Chl b in this complex and is consistent with a small reduction in Chl a/b ratio in Lhcb6-LL as determined by chemical analysis.

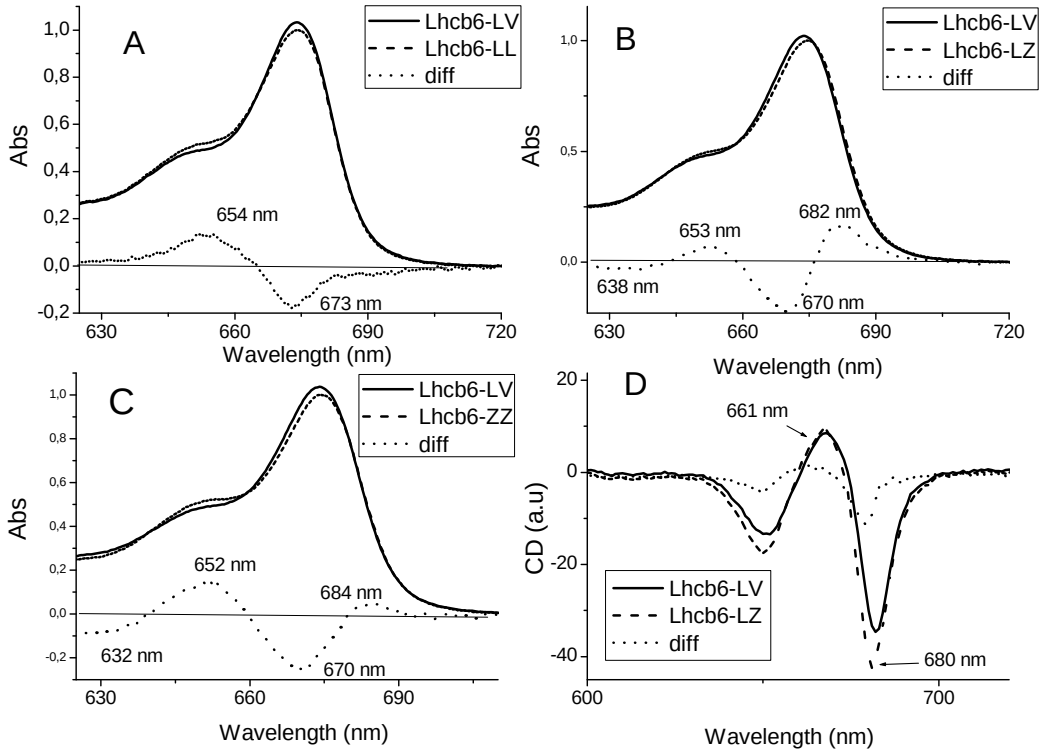

Figure 3: Absorption and CD spectra in the visible region of Lhcb6 samples reconstituted with different carotenoids. *Absorption spectra of Lhcb6 recombinant complexes Lhcb6-L (A), Lhcb6-LZ (B), Lhcb6-ZZ (C) and Lhcb6-LV (A,B,C,D) and difference absorption spectra (diff) in the Qy region obtained subtracting the Lhcb6-LV spectrum from Lhcb6-L (A), Lhcb6-LZ (B), Lhcb6-ZZ (C) absorption spectra. Difference absorption spectra were calculated after normalization to the Chl a/b ratio and multiplied 5 times. D) CD spectra in the Qy region of Lhcb6-LV and Lhcb6-LZ samples and difference spectrum (multiplied for 5 times), calculated subtracting Lhcb6-LV from Lhcb6-LZ. Difference spectrum is characterized by a conservative signal at 661 (+) and 680 (-) nm.*

When comparing Lhcb6-ZZ and -LZ to Lhcb6-LV no Chl a/b ratio change was detected by chemical analysis, while difference spectra showed negative/positive terms with similar amplitude within Chl b forms (638/653nm) and Chl a (670/682 nm) (Figure 3B-C) implying they originated by the modulation of the Chl transition energy level by Zea binding. The red-shift of Chl a and b spectral forms imply an enhancement of the chlorophyll low-energy transitions, possibly due to increased excitonic interactions between adjacent chlorophylls (Ahn, T. K. et al. 2008, Morosinotto, T. et al. 2003). This interpretation is supported by the conservative shape of signals in difference CD spectra between LZ-Lhcb6 and LV-Lhcb6 (Figure 3D). Similar results were obtained in the case of Lhcb6-ZZ complexes (data not shown).

3.5 Light harvesting

Light harvesting in LHC proteins is mainly due to the absorption of the chlorophylls (350-550 and 600-700nm); xanthophylls, however, absorb in the blue (440-550 nm) and can transfer absorbed energy to chlorophylls. The chlorophyll *b* / carotenoid to chlorophyll *a* energy transfer efficiency can be calculated by comparing the relative amplitude of each spectral form in 1-T spectra vs fluorescence excitation spectra (Croce, R. et al. 2000). The results, in terms of energy transfer efficiency for the various forms of Lhcb6, Chl b>Chl a and Car>Chl a, are reported in Table 2.

Sample	Chlb >Chla ET (%)	Car >Chla ET (%)
Lhcb6-LV	83.3	91.0
SD	*2.1*	*2.3*
Lhcb6-LL	82.7	65.4
SD	*2.1*	*1.9*
Lhcb6-VV	82.5	85.0
SD	*2.4*	*1.7*
Lhcb6-ZZ	84.1	56.2
SD	*2.9*	*3.8*
Lhcb6-LZ	83.8	67.6
SD	*3.0*	*3.4*

Table 2: Energy transfer in Lhcb6 samples binding different carotenoid species. Energy transfer (ET) efficiency, from chlorophyll b (Chlb>Chla ET (%)) or carotenoids (Car>Chla ET (%)) to chlorophyll a, were calculated comparing the 1-T absorption and fluorescence excitation spectra in the Soret region; both absorption and fluorescence spectra were deconvoluted using the same chlorophylls and carotenoids spectral forms. Chla > Chla energy transfer efficiencies were considered 100%. Standard deviations (SD) are indicated.

The relative efficiencies of energy transfer Chl b>Chl a are similar in Lhcb6 with differing xanthophyll complements. Car>Chl a energy transfer is maximal in Vio-containing Lhcb6, either Lhcb6-VV or Lhcb6-LV, while it is reduced in complexes containing Lut and/or Zea (Lhcb6-L, Lhcb6-LZ and Lhcb6-ZZ).

3.6 Chlorophyll singlet excited states quenching

LHC proteins ensure photoprotection under intense irradiation by multiple mechanisms: quenching of (i) singlet and (ii) triplet Chl excited states, and (iii) ROS scavenging (Demmig-Adams, B. and Adams, W. W. 1992, Havaux, M. 1993, Niyogi, K. K. 1999, Walters, R. G. and Horton, P. 1991). In order to investigate the activity and the xanthophyll-dependence of these mechanisms in Lhcb6, we measured the fluorescence quantum yield, time-resolved triplet minus singlet (TmS) spectra and the production of singlet oxygen upon illumination of the different Lhcb6 complexes.

The relative fluorescence quantum yield is proportional to chlorophyll singlet excited state average lifetime. Fluorescence quantum yield of our Lhcb6 complexes were clearly different and dependant on the identity of the xanthophylls bound (Figure 4): Zea binding complexes (Lhcb6-LZ, -Z) had a fluorescence yield reduced by ~50% as compared to the control (Lhcb6-LV), implying that Zea induced the activation of thermal dissipation pathway, as previously reported (Passarini, F. et al. 2009).

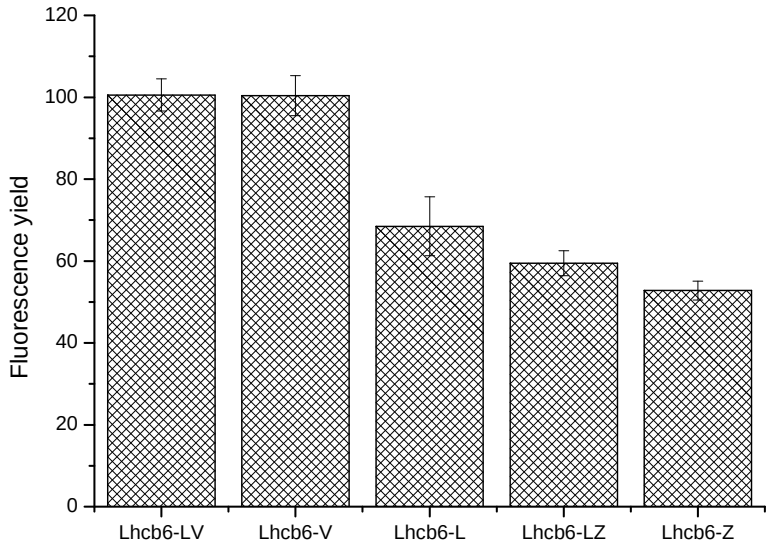

Figure 4: Relative fluorescence quantum yield in Lhcb6 having differing xanthophyll compositions. *Fluorescence quantum yield was measured as the ratio between the absorption at 625 nm and area of the fluorescence emission spectra between 650 nm and 800 nm upon 625 nm excitation. Data are shown as percentage of fluorescence quantum yield with respect to Lhcb6-LV sample. Error bars are indicated.*

No major difference was observed for the presence of one versus two Zea in the complex consistent with Zea activating quenching in site L2, not in L1. A similar effect was also observed for Lut, since in Lhcb6-LL a 30% reduction of fluorescence yield is observed as compared to Lhcb6-LV; yet LV and VV complexes had similar fluorescence yield. Thus, xanthophyll composition of site L2 regulates average singlet excited states lifetime in Lhcb6.

3.7 Chlorophylls triplet excited states quenching

When energy is absorbed in excess, chlorophyll triplet states formation may occur through intersystem crossing. Carotenoids quench chlorophyll triplets by energy transfer to the xanthophylls, whose triplet states are short-lived and vibrationally decay to the ground state, preventing singlet oxygen formation by reaction with ^{3}Chl. Carotenoids bound to LHC proteins have been shown to quench 75%-95% of chlorophyll triplets, with different efficiency on monomeric and trimeric Lhc subunits (Mozzo, M. et al. 2008). In order to verify if xanthophyll composition affects the Chl triplet quenching properties of Lhcb6, we measured flash-induced triplet formation through TmS measurements, under anaerobic conditions, in order to avoid the competing reaction of chlorophyll triplets with oxygen (Knox, J. P. and Dodge, A. D. 1985), as described in (Mozzo, M. et al. 2008) (Figure 5).

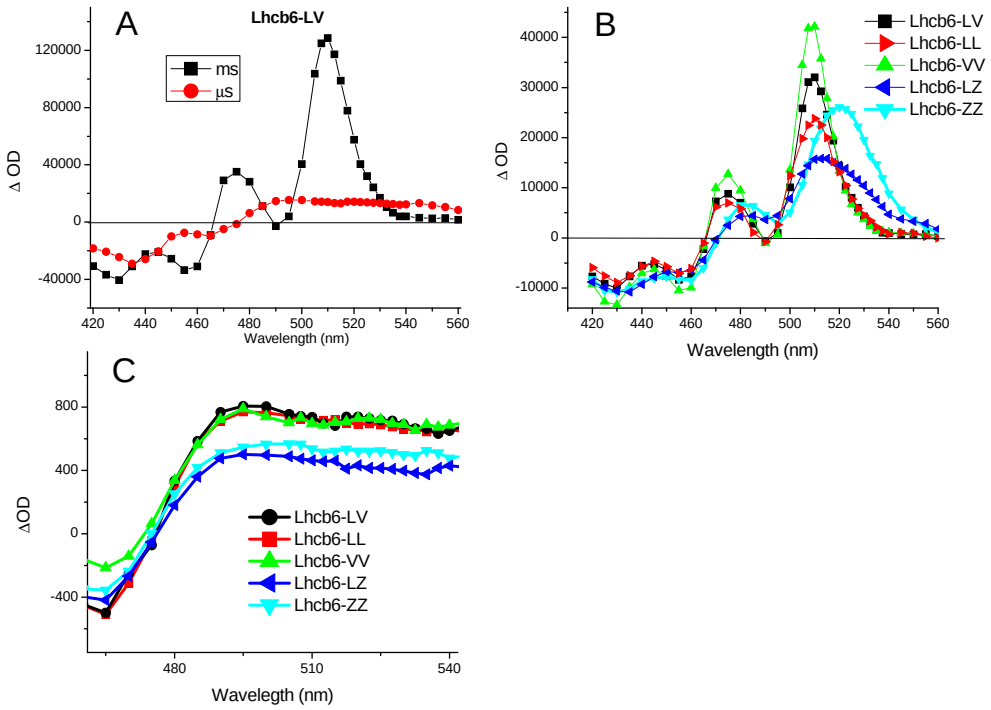

Figure 5: Triplets minus Singlet spectra of Lhcb6 complexes with differing xanthophyll compositions. *The spectral components were obtained by global fitting of the kinetics in anaerobic condition. A) Lhcb6-LV Triplet minus Singlet spectrum, black line: component in the µs representing carotenoid triplets; red line: component in the ms, representing chlorophyll triplet formation. B) µs (7,5–8,8 µs) components of Triplet minus Singlet spectra (ΔOD) in the carotenoid triplets absorption region (490-570 nm) of Lhcb6 complexes with different xanthophyll composition; spectra were normalized to the total chlorophyll content in measured samples. C) ms components of Triplet minus Singlet spectra of Lhcb6 complexes with different xanthophyll composition; spectra were normalized to total chlorophyll content in measured samples.*

Briefly, difference absorption (ΔA) decay kinetics in the ns to ms time range of illuminated and dark samples were measured in a pump-probe laser system: the differential absorption (ΔA) signal in dark samples is due to formation of singlet excited states by chlorophylls and carotenoids, while the signal from the pre-illuminated sample exhibits an additional transient component due to triplet formation, a consequence of high intensity illumination (pumping). Decay of ΔA, thus, accounts for triplets decay after pumping of the sample. In absence of oxygen this ΔA decay can be fitted by a bi-exponential function, characterized by a fast component (µs) and a slower component (ms): the former is associated with carotenoid triplets, appearing upon Chls triplet quenching, while the latter is associated with residual unquenched Chl triplets (Mozzo, M. et al. 2008). The decay-associated spectra of carotenoids (µs) and chlorophyll (ms) triplets in Lhcb6-LV complexes are shown in Figure 5. Carotenoid triplet lifetimes are similar for all samples (7,5 - 8,8 µs, Table S2 Supplemental data) and consistent with a previous report on monomeric LHC proteins (Mozzo, M. et al. 2008). No significant differences can be resolved in the decay-associated spectra of Lhcb6-LV, -LL and -VV: the reconstructed spectra peak at 510 nm (Figure 5B). In the presence of Zea, best evidenced by the spectrum of Lhc6-ZZ in which Lut and Vio are not present, a ~ 20 nm red-shifted component appeared relative to complexes lacking Zea. In all samples, a small fraction of unquenched chlorophyll triplets excited states was evident (Figure 5C). In the case of Lhcb6-LV we calculated the amount of unquenched chlorophyll triplets states corresponded to ~20% of the total chlorophylls present in the complex. The fraction of unquenched chlorophyll triplet states was

calculated as previously described (Mozzo, M. et al. 2008), by normalizing the proportion of residual triplet Chls to the chlorophyll concentration in the sample rather than normalizing to the total triplet absorption (Car+Chl), as previously reported (Passarini, F. et al. 2009). The result obtained in the case Lhcb6-LV is similar in Lhcb6-VV, and –LL complexes. However, in the case of Lhcb6-LZ and Lhcb6-ZZ, unquenched chlorophyll triplets were reduced from 20% to ~12% and ~13% respectively. Since this reduction was observed in both Lhcb6-ZZ and Lhcb6-LZ, with similar L2 composition, but different L1 composition. Therefore the quenching of Chl triplet effect by Zea effect on Chl triplet quenching is catalyzed by the occupation of the L2 site but not the L1 site (see above for the localization of Lut and Zea in these complexes).

3.8 Singlet oxygen production

In sections 3. 6 and 3.7 we demonstrated the role of carotenoids on both singlet and triplet Chl excited states quenching. However, unquenched chlorophyll triplets can react with molecular oxygen, producing singlet oxygen (Knox, J. P. and Dodge, A. D. 1985). The production of singlet oxygen in Lhcb6 upon illumination, was measured using a chemical probe named " Singlet Oxygen Sensor Green™ "(Flors, C. et al. 2006), added to samples illuminated at 750 µmol $m^{-2}s^{-1}$. Increase of Singlet Oxygen Sensor Green™ fluorescence at 530 nm is correlated to production of singlet oxygen in the illuminated samples: results are reported in Figure 6A.

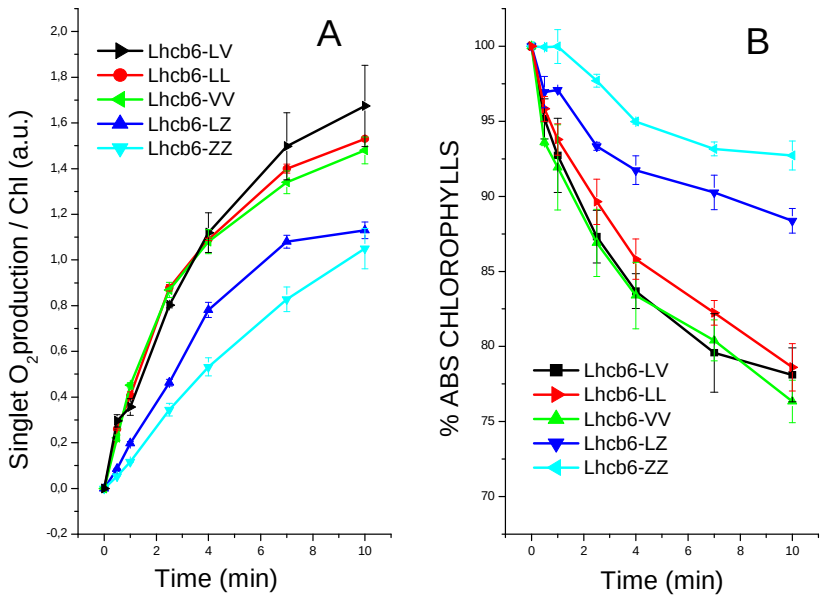

Figure 6: Singlet oxygen production and chlorophyll photobleaching in Lhcb6 having differing carotenoid compositions at different light intensities. ***A.*** *Singlet oxygen production at different time points in Lhcb6 samples: singlet oxygen accumulation was detected measuring the increase of the 530 nm fluorescence emission of the fluorescent probe Singlet Oxygen Sensor Green, normalized to the chlorophylls content of each sample.* ***B.*** *Photobleaching of chlorophylls bound by Lhcb6 complexes, measured as the decrease of the chlorophyll absorption area in Qy region caused by high light illumination. For both measurements samples were treated with high intensity light at 750 µmol $m^{-2}s^{-1}$ for 10 minutes.*

Lhcb6-LV, -V, and -L are characterized by similar singlet oxygen production. However, in the case of Lhcb6-LZ and Lhcb6-ZZ, we monitored a lower levels of singlet oxygen production upon illumination.

3.9 Photobleaching

Chlorophylls are very sensitive to singlet oxygen produced upon illumination, since ROS quickly oxidises them leading to photobleaching. This effect can be monitored *in vitro* following decay of chlorophylls absorption during illumination of Lhc complexes (Croce, R. et al. 1999b). In Figure 6B photobleaching measurements of Lhcb6 are reported: Lhcb6-LV, -VV, and -LL have similar photobleaching kinetics, with a final loss of ~25% of the chlorophyll absorption. However, Lhcb6-LZ and Lhcb6-ZZ are characterized by slower kinetics, with final loss of only 7% and 12% of the chlorophylls absorption respectively.

4. DISCUSSION

Lhcb6 is an antenna protein involved in photoprotection. K.o. mutants in the *lhcb6* gene have a slower kinetic of thermal dissipation of energy absorbed in excess (NPQ) (de Bianchi, S. et al. 2008, Kovacs, L. et al. 2006). This subunit is part of a supramolecular complex including also Lhcb4 and LHCII-M (Bassi, R. and Dainese, P. 1992) whose dissociation is needed for the establishment of the quenching process (Betterle, N. et al. 2009) together with the synthesis of Zea. In this work we analyzed *in vivo* and *in vitro* the carotenoid binding properties of Lhcb6 and in particular the tuning of its physiological properties upon Zea binding. Lhcb6 has 2 carotenoid binding sites with high affinity for both Lut and Vio whereas Neo is not bound, consistent with the non-conservation of a specific tyrosine residue critical for Neo binding, conserved in Lhcb1, Lhcb4 and Lhcb5 (Caffarri, S. et al. 2007b, Pagano, A. et al. 1998, Passarini, F. et al. 2009).

4.1 Zeaxanthin accumulation in Lhcb6

Analysis *in vivo* upon induction of Zea synthesis by high light treatment (Figure 1) show that PSI-LHCI and Lhcb complexes have already accumulated Zea already after only 5’ of illumination. Lhcb6, Lhcb5 and LHCII are the fastest Zea-accumulating subunits in PSII: we show that this corresponds to Zea binding to the internal site L2 in the case of Lhcb5 and Lhcb6. LHCII binds Zea to the peripheral site V1 without major effects on protein functional properties (Caffarri, S. et al. 2001). Instead, Zea bindig induces a destabilization of the LHCII complex (Croce, R. et al. 1999b). Further *in vitro* analysis using a de-epoxidation assay shows that Lhcb6 has a higher capacity for Zea binding as compared to Lhcb5 due to higher amount of exchangeable Vio bound to the complex prior to de-epoxidation. However, the de-epoxidation index is higher in Lhcb5 due to the presence in Lhcb6 of a fraction of Vio bound to site L1 which less accessible for exchange, in agreement with a previous report (Wehner, A. et al. 2006) (Jahns, P. et al. 2001). Due to the lower fraction of exchangeable Vio in Lhcb5 (Morosinotto, T. et al. 2002, Wehner, A. et al. 2006), the number of Lhcb6 molecules statistically capable of accumulating Zea is higher than in the case of Lhcb5. We conclude that Lhcb6 undergoes binding of Zea to a larger extent than any other PSII antenna complex (Figure 1E), upon xanthophyll cycle induction.

The localization of Vio (Zea) in both L1 and L2 binding sites has been clearly established by the presence of a less red-shifted Zea spectral form (497 nm) in addition to the 502 nm Zea typical of site L2 (Morosinotto, T. et al. 2002) for Zea binding complexes (Lhcb6-LZ, -ZZ). The capacity of both the L1 and the L2 sites to bind Zea, even when Lut is available, is a unique feature of Lhcb6, implying a lower selectivity of site L1 in contrast to other LHC proteins where this site is completely selective for Lut (Croce, R. et al. 2002) (Ruban, A. V. et al. 1999) (Gastaldelli, M. et al. 2003) (Caffarri, S. et al. 2004). At present, we cannot exclude that Zea can bind to site L1 of Lhcb6 *in vivo* although we do not have clear evidence for this event. This difference in L1 selectivity, as compared to other LHC proteins, reflects a specificity in the properties of this carotenoid binding site in

Lhcb6. This is in agreement with the recent finding that occupancy of this site is not indispensable for folding in Lhcb6 as is the case for all others LHC proteins (Passarini, F. et al. 2009).

4.2 Zeaxanthin binding induces switching from light harvesting to energy dissipation states.

Zea binding to Lhcb6 decreases its Chl fluorescence quantum yield (Figure 4). The replacement of Vio with Lut in site L2 has also a similar effect, while the occupancy of site L1 does not affect fluorescence quantum yield of Lhcb6 (Figure 4). This evidence implies that, whatever the mechanism which modulates energy dissipation pathways in Lhcb6, either carotenoid radical cation formation {Holt, 2005 24620 /id;Amarie, 2009 24896 /id;Ahn, 2008 24858 /id}, or direct energy transfer from Chl a Qy transition to the xanthophyll S1 state (Ruban, A. V. et al. 2007) it takes place in site L2, as recently suggested by mutational analysis (Passarini, F. et al. 2009). Others events are controlled by the occupancy of site L2: Vio induces maximal Car→Chl a energy transfer efficiency, while the presence of Lut and/or Zea in this site yields into a lower efficiency. These findings are consistent with an allosteric role for Zea binding at site L2, as previously proposed for Lhcb1, Lhcb5 and Lhcb4 (Crimi, M. et al. 2001, Croce, R. et al. 2002) (Ballottari, M. et al. 2009) (Formaggio, E. et al. 2001). Thus, a switch from light harvesting to energy dissipating state results when Zea or Lut are bound to site L2. It appears that the xanthophyll cycle acts by switching LHC proteins between two states with contrasting energy conservation/dissipation properties. Biochemical proof of this conformational change has been provided by pI shift (Dall'Osto, L. et al. 2005), while functional characterization of the two states has shown that Zea/Vio binding modulates the excitonic interaction between adjacent chlorophylls (Ahn, T. K. et al. 2008), increasing low energy excitons. Moreover, in zeaxanthin-binding Lhcb6 a closest interaction between carotenoid in L2 and Chls as compared to violaxanthin binding complex was recently shown (Passarini, F. et al. 2009). Here, we demonstrate a significant tuning in chlorophyll spectroscopic properties (Figure 3B) with enhancement of low energy chlorophyll spectral forms (Figure 3D) upon Zea binding. This is consistent with the demonstration that Lhcb6 is directly involved in the transient formation of carotenoid radical cations dissipating excitation energy through charge recombination *in vitro* {Holt, 2005 24620 /id;Amarie, 2009 24896 /id;Ahn, 2008 24858 /id;Avenson, 2008 24852 /id} and *in vivo* (Li, Z. R. et al. 2009). The fractions of Lhcb6 molecules undergoing formation of radical cation in detergent solution is small with respect to that *in vivo* (Avenson, T. J. et al. 2008) while shift of the equilibrium towards energy-dissipation state has been proposed to be induced by PsbS upon dissociation of the Lhcb4/Lhcb6/LHCII-M supercomplex (Betterle, N. et al. 2009). Our finding that Lhcb6 is the most effective complex of Zea binding supports the model proposed and is consistent with the strong phenotype of koLhcb6 plants (de Bianchi, S. et al. 2008, Kovacs, L. et al. 2006).

4.3 Zeaxanthin binding up-regulates chlorophyll triplet quenching in Lhcb6

Although dissipative mechanisms reduces excess singlet Chl excited states (Demmig-Adams, B. and Adams, W. W. 1992), this might not be enough to prevent ROS formation and additional photoprotection is provided by ^{3}Chl quenching and up-regulation of ROS scavenging (Havaux, M. et al. 2004, Havaux, M. et al. 2007, Havaux, M. and Niyogi, K. K. 1999, Johnson, M. P. et al. 2007). Singlet oxygen is produced by the reaction between O_2 of ^{3}Chl* (Knox, J. P. and Dodge, A. D. 1985), produced by intersystem crossing from ^{1}Chl* (Chauvet, J.-P. et al. 1985). This reaction can be prevented by quenching of the ^{3}Chl* by xanthophylls, mainly Lut (Dall'Osto, L. et al. 2006). Recently, it has been demonstrated that, although carotenoid are very efficient at chlorophyll triplet quenching within LHC proteins, fraction of the chlorophyll triplets (5% - 30%) cannot be quenched, due to the molecular organization of Lhcb proteins (Mozzo, M. et al. 2008) while unquenched Chl triplets have been

suggested to be a major source of photoinhibition (Santabarbara, S. et al. 2002). Most of the unquenched chlorophyll triplets have been located in monomeric Lhcb proteins (Santabarbara, S. et al. 2001). Our data show that in Lhcb6-LV ~20% of the excited chlorophyll triplets states cannot be quenched by xanthophylls (Figure 5C), and thus can react with oxygen producing singlet oxygen (Figure 6A). Moreover, in Lhcb6-LV, Lhcb6-LL and Lhcb6-VV about 20% of excited chlorophyll triplets states are not quenched by xanthophylls (Figure 5C) while in Lhcb6-LZ and Lhcb6-ZZ this figure is reduced by 40%, resulting in a decreased singlet oxygen production (Figure 6A). Unchanged triplet transfer efficiency in Lhcb6-LV and Lhcb6-LZ was recently reported by Passarini and co-authors (Passarini, F. et al. 2009). In that work, the fraction of unquenched triplet chlorophylls was calculated from the TmS spectra of triplet carotenoids and chlorophylls, using the molar extinction coefficients of the singlet absorptions of each pigment in ethanol. Assuming the extinction coefficients of carotenoid singlet and triplet state to be proportional, identical triplet transfer efficiencies were obtained for Lhcb6-LZ and Lhcb6-LV. However, the extinction coefficient of different xanthophyll species differ significantly: (Vio, 6*10-5 at 490 nm; Lut, 2*10-5 at 500 nm; Zea, 1.1*10-5) (Jhutti, C. S. et al. 1998) (Naqvi, R. and Javorfi, T., personal communication). By normalizing the triplet yield to the total chlorophyll content of the sample (Figure 5B) the actual efficiency of quenching can be estimated and an increased triplet transfer efficiency for Lhcb6-LZ and Lhcb6-ZZ as compared to Lhcb6-LV and Lhcb6-LL is evidenced. This is consistent with the observed acceleration of triplet transfer in Lhcb6-LZ (Passarini, F. et al. 2009).

The decrease in the chlorophyll triplet level observed in Zea-containing Lhcb6 samples (Figure 5C) could be, in part, ascribed to the decreased fluorescence yield (Figure 4); nevertheless, since this effect is not detected in Lhcb6-LL, where Lut binding in L2 induces similar fluorescence quenching without a concomitant effect on the chlorophyll triplet yield, we conclude that Zea has a specific effect in enhancing Chl a triplet quenching with respect to Lut and Viol.

4.4 Regulation of ROS scavenging by Zeaxanthin.

The last photoprotective effect of Zea consists of the scavenging of reactive oxygen species (Havaux, M. et al. 2004, Havaux, M. et al. 2007, Havaux, M. and Niyogi, K. K. 1999, Muller-Moule, P. et al. 2003) (Havaux, M. et al. 2007). Indeed, we measured a lower production of singlet oxygen upon illumination in the case of Lhcb6-LZ, further reduced when both L1 and L2 sites are occupied by Zea (Figure 6, Lhcb6-ZZ complex). Since Lhcb6-LZ and Lhcb6-ZZ do not differ in fluorescence yield nor in the level of unquenched ^{3}Chl triplets (Fig. 4, 5), and yet the latter has slower kinetic in $^1O_2$ production, we conclude that Zea has also a scavenging effect with respect to the ROS produced within Lhcb6, proportional to the amount of Zea bound. Zea is indeed effective in scavenging irrespectively of its binding to site L1 or L2.

5. CONCLUSIONS

Lhcb6 carotenoid binding properties have been investigated in details; in particular this protein is the Lhcb antenna subunit that accumulates Zea most rapidly in internal sites, activating photoprotection. Upon Zea binding to site L2 we detected an increase in chlorophyll-chlorophyll excitonic interaction(s), likely as a consequence of protein conformational change, switching the protein to a dissipative state for singlet excited states (Ahn, T. K. et al. 2008). This conformational change might also be involved in the recognition of Lhcb6 by PsbS during triggering of NPQ (Betterle, N. et al. 2009). An up-regulation of Chl a triplet quenching is also associated to Zea binding in site L2. A third effect of Zea binding to Lhcb6 is an increase of ROS scavenging. This is proportional to the amount of Zea bound to Lhcb6 complex, irrespective from its binding site. These

combined effects explain the Zea-mediated increased resistance to high light stress *in vivo* (Havaux, M. et al. 2007, Havaux, M. and Niyogi, K. K. 1999) and are consistent with the first appearance of Lhcb6 in mosses (Alboresi, A. et al. 2008) the first organisms to colonize terrestrial environment.

SUPPLEMENTAL DATA

Fraction	Chl	Chl a/b	Chl/Car	Chl a	Chl b	Cars	N	V	L	Z	A	β-car	D.I.	Z/Cars
F1	100	1.17	5.32	53.92	46.08	18.80	3.68	0.63	14.49	0.00	0.00	0.00	0.000	0.000
F2	100	1.00	4.85	49.88	50.12	20.63	5.54	0.44	14.55	0.00	0.05	0.02	0.055	0.000
F3.1	100	1.29	4.71	56.42	43.58	21.22	5.67	0.26	15.18	0.00	0.03	0.00	0.056	0.000
F3.2	100	1.03	4.78	50.73	49.27	20.91	5.96	0.62	14.32	0.00	0.01	0.00	0.007	0.000
F4.1	100	1.29	5.11	56.41	43.59	19.57	5.92	0.21	13.43	0.00	0.00	0.00	0.000	0.000
F4.2	100	1.12	4.20	52.78	47.22	23.80	7.62	0.89	14.88	0.31	0.09	0.00	0.287	0.013
F5	100	1.21	3.88	54.67	45.33	25.78	8.30	2.66	14.23	0.42	0.11	0.01	0.152	0.016
F6	100	1.37	4.07	57.74	42.26	24.55	6.22	6.05	11.62	0.33	0.30	0.00	0.074	0.014
F7.1	100	2.07	4.82	67.43	32.57	20.77	3.86	7.91	8.81	0.00	0.00	0.00	0.000	0.000
F7.2	100	2.34	4.08	70.08	29.92	24.53	6.76	7.89	9.45	0.19	0.18	0.01	0.035	0.008
F7.3	100	1.75	4.05	63.68	36.32	24.67	7.40	3.23	12.73	0.40	0.79	0.02	0.198	0.016
F7.4	100	0.89	4.89	47.16	52.84	20.45	0.96	9.33	9.48	0.41	0.16	0.01	0.050	0.020
F8.1	100	1.99	4.10	66.57	33.43	24.39	7.23	3.42	12.61	0.36	0.72	0.00	0.174	0.015
F8.2	100	0.99	4.81	49.75	50.25	20.80	0.81	8.96	10.16	0.48	0.26	0.01	0.064	0.023
F9	100	1.58	4.09	61.19	38.81	24.42	5.74	4.88	12.28	0.47	0.98	0.01	0.164	0.019
F10	100	1.97	3.85	66.31	33.69	25.94	7.69	4.26	12.99	0.36	0.40	0.02	0.117	0.014

Table S1: Pigment analysis of LHC proteins fractions separated by IEF and non-denaturing electrophoresis. *HPLC pigments analysis was performed on fractions F1-F10, obtained through non-denaturing PAGE separation of LHC complexes from leaves light treated for 5' at at 1500 μmol $m^{-2}s^{-1}$ (Figure 1 and Figure S2). Pigments were normalized to 100 chlorophylls content. Composition of each fraction is described in the text and in Figure 1. Abbreviations: Chl, number of chlorophylls per molecule; Chl a/b: Chl a /b ratio; Cars: number of carotenoids; Chl a : Chlorophyll a; Chl b: Chlorophyll b; Chl /Car: ratio between total chlorophylls and total cars; N: neoxanthin; V: violaxanthin; L: lutein; Z: zeaxanthin; A: antheraxanthin, β-car: β-carotene, D.I.: de-epoxidation index, Z/Cars: ration between zeaxanthin and total carotenoids per molecule. Errors are less than 10% in each case.*

Sample	Max T-S	τ
	nm	*μs*
Lhcb6-LV	510,0	8,7
Lhcb6-L	510,0	8,8
Lhcb6-V	510,0	8,3
Lhcb6-LZ	512,5	8,6
Lhcb6-Z	520,0	7,5

Table S2: Triplet minus Singlet spectral features and carotenoid triplet lifetimes (τ) of Lhcb6 complexes reconstituted with different carotenoids.
Absorption peaks of the Triplet minus Singlet spectra (Max T-S) and carotenoid triplet lifetimes were obtained by global analysis of a data set consisting of time traces measured at different wavelengths in anaerobic conditions, as described in (Mozzo, M. et al. 2008). Absorption maxima of carotenoid triplets are similar for Lhcb6-LV, Lhcb6-L and Lhcb6-V complexes, while in presence of zeaxanthin the peaks are shifted. Triplet carotenoid lifetimes (τ) are indicated: all Lhcb6 complexes show similar triplet carotenoid lifetimes.

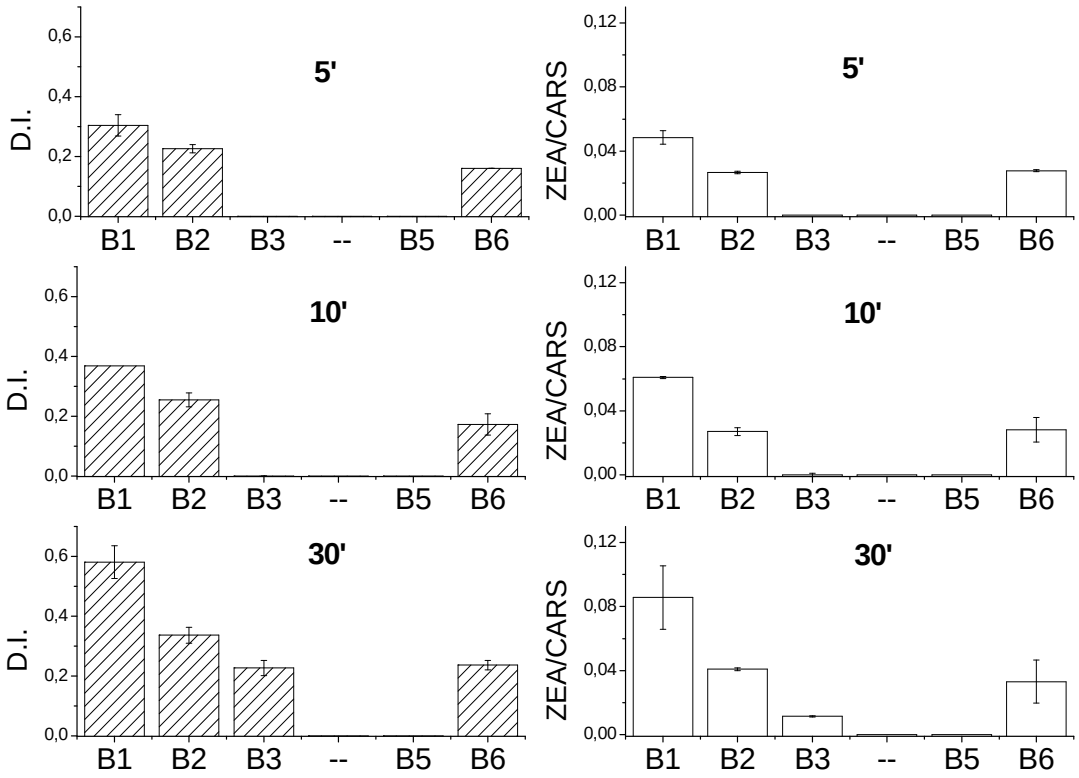

Figure S1: Violaxanthin de-epoxidation in chlorophyll binding fractions obtained after solubilization of thylakoids with β-DM *Thylakoid membranes from dark adapted and leaves light treated for 5' at at 1500 µmol* $m^{-2}s^{-1}$ *were solubilised in β-DM detergent and loaded on sucrose gradients. Similar fraction pattern was obtained at different times of illumination (5'-10'-30'). Fraction composition is the same as described in Figure 1A (main text) with the exception that B4 is not present upon β-DM treatment (-- in the figure). Each fraction recovered from sucrose gradients was analysed by HPLC: de-epoxidation indexes (D.I.) and the ratios between zeaxanthin content and total carotenoid content (Zea / Cars) are reported. In each panel time (minutes) of illumination is indicated (5'-10'-30').*

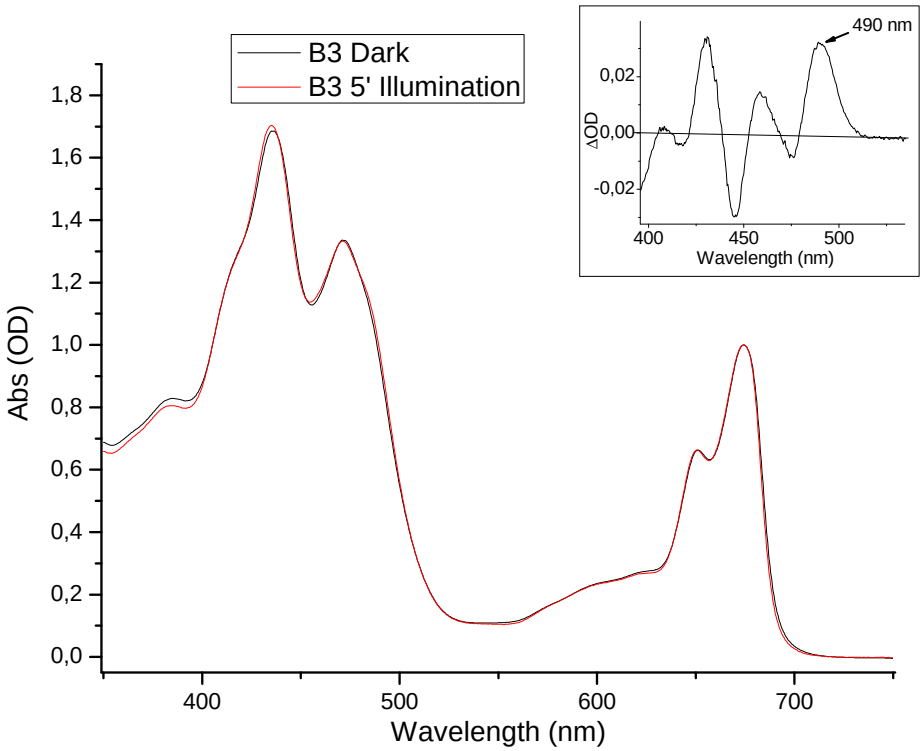

Figure S2: Absorption spectra of LHCII complexes isolated from dark adapted and 5' light treated leaves. *LHCII trimers composing B3 from sucrose gradients is reported in Figure 1A. Black trace: LHCII trimers (B3) from dark adapted leaves; Red trace: LHCII trimers (B3) from 5' light treated leaves. LHCII trimers from light treated leaves bind zeaxanthin in V1: the presence of zeaxanthin in V1 is suggested by the difference spectrum obtained subtracting dark adapted LHCII spectrum to light treated LHCII spectrum (inset). The difference spectrum in the 400 – 520 nm is characterized by a positive peak at 490 nm: this peak is related to the presence of a zeaxanthin with a small red shift induced by protein environment: this is a clear indication of zeaxanthin binding in V1 in light treated sample, as previously reported (Caffarri, S. et al. 2001)*

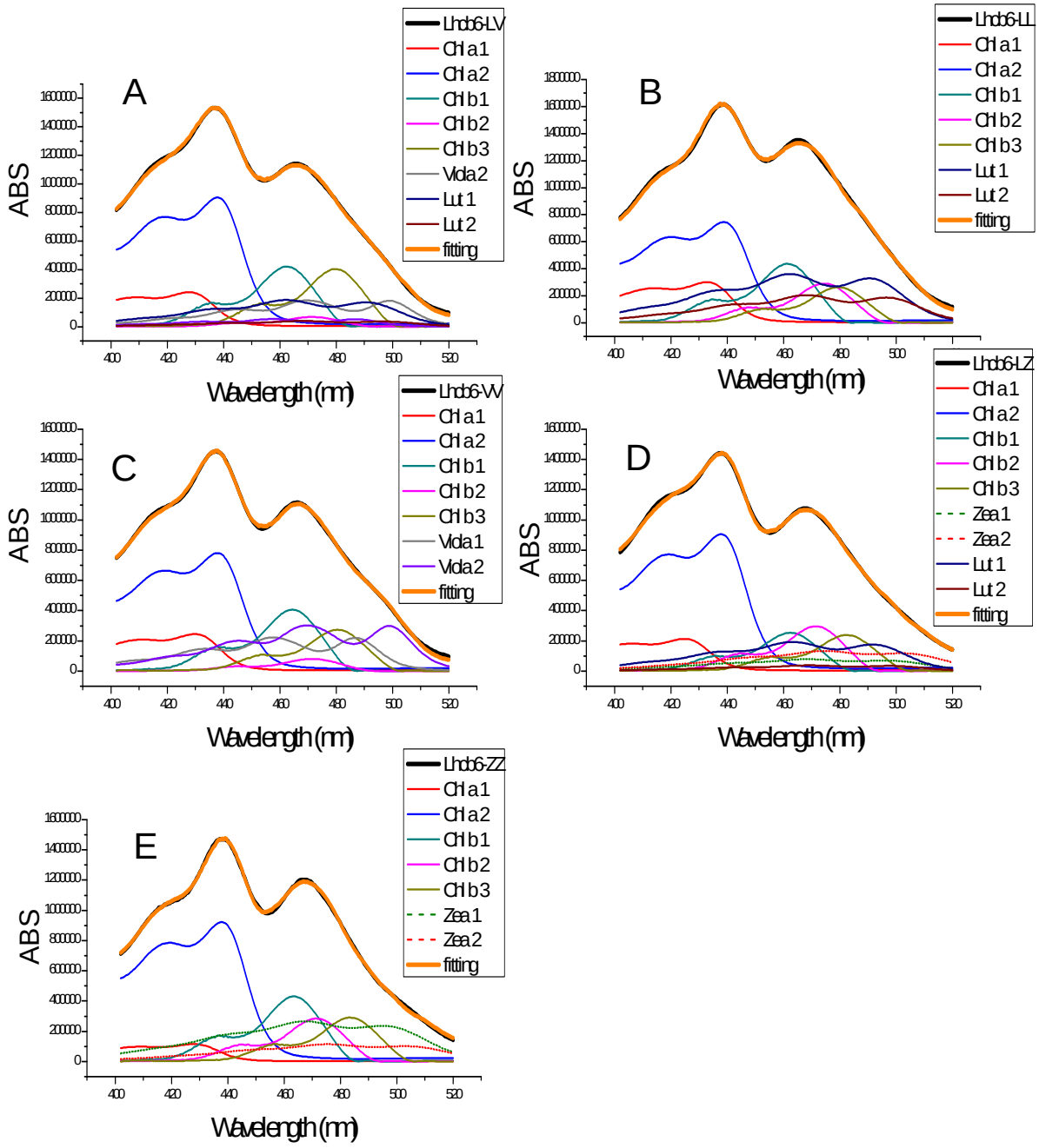

Figure S3: Deconvolution of absorption spectra of recombinant Lhcb6 complexes. *Deconvolution of absorption spectra of recombinant Lhcb6 complexes reconstituted with chlorophyll a, b and i) lutein and violaxanthin (Lhcb6-LV, panel A); ii) only lutein (Lhcb6-L, panel B); iii) only violaxanthin (Lhcb6-V, panel C); iv) lutein and zeaxanthin (Lhcb6-LZ panels D); v) only zeaxanthin (Lhcb6-Z, panel E), are reported. Fitting procedure was performed using properly Chl a, Chl b and carotenoids spectral forms as described in (Croce, R. et al. 2000). Lutein, violaxanthin and zeaxanthin spectral forms were used for different spectra fitting, basing on carotenoid content of the different complexes. Chlorophyll a (Chl a 1, Chl a 2, Chla 3) , Chlorophyll b (Chl b 1, Chl b 2, Chl b 3), Lutein (Lut 1, Lut 2), Violaxanthin (Viola 1 , Viola 2) and Zeaxanthin (Zea 1, Zea 2) spectral forms used are indicated.*

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Section A.2: Identification of the chromophores involved in aggregation-dependent energy quenching of the monomeric Photosystem II antenna subunits

Non-photochemical quenching (NPQ) of excess absorbed light energy is a fundamental process that regulates photosynthetic light harvesting in higher plants. Among several proposed NPQ mechanisms, aggregation-dependent quenching (ADQ) and charge transfer quenching (CTQ) have received the most attention. *In vitro* spectroscopic features of both mechanisms correlate with very similar signals detected in more intact systems and *in vivo*, where full NPQ can be observed. A major difference between the models is the proposed quenching site, which is predominantly the major trimeric light harvesting complex II (LHCII) in ADQ, and exclusively monomeric Lhcb proteins in CTQ. Here, we studied ADQ in both monomeric and trimeric Lhcb proteins, investigating the activities of each antenna subunit and their dependence on zeaxanthin (Zea), a major modulator of NPQ *in vivo*. We found that monomeric Lhcb proteins undergo stronger quenching than LHCII during aggregation, and that this is enhanced by binding to Zea, as occurs during NPQ *in vivo*. Finally, the analysis of Lhcb5 mutants showed that chlorophyll (Chl) 612-613, in close contact with lutein bound at site L1, are important facilitators of ADQ.

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For the experiment done in this article I reconstituted PSII antenna complexes *in vitro*, I performed the preliminary experiments for aggregation quenching analysis and I contributed with the other authors with suggestions in the discussion of the experimental design and results.

1. INTRODUCTION

Plants fix CO_2 and synthesize sugars by absorbing light energy using two multiprotein complexes named photosystems I and II (PSI and PSII). Each complex has a core where charge transfer and electron transport occur, and a light-harvesting antenna system composed of Lhc (light-harvesting complex) proteins. Lhc proteins belong to a superfamily with a highly conserved amino acid sequence, suggesting a common structure (Amunts, A. et al. 2007b, Jansson, S. 1999, Kühlbrandt, W. et al. 1994, Liu, Z. et al. 2004). Different members are associated with PSI (Lhca proteins) and PSII (Lhcb proteins), and their potential for aggregation also varies, such that they exist as trimers (LHCII is a trimer of Lhcb1-3), dimers (LHCI exists as Lhca1/4 and Lhca2/3 dimers) or monomers (Lhcb4-6) (Bassi, R. et al. 1987, Ben Shem, A. et al. 2003).

Structural analysis of LHCII has shown that each subunit comprises three transmembrane helices (designated A, B and C) and two amphipatic helices exposed on the thylakoid lumen surface, designated D and E (Liu, Z. et al. 2004). Each monomer coordinates four xanthophylls and 14 porphyrins, either Chl *a* or Chl *b*. Two carotenoid-binding sites, defined in ref. (Kühlbrandt, W. et al. 1994) as L1 and L2, lie close to helices A and B and usually bind lutein (Lut), whereas a third site named N1, which specifically binds neoxanthin (Neo), lies near helix C (Croce, R. et al. 1999). Finally, a peripheral and less stable binding site named V1 has been shown to accommodate violaxanthin (Vio) and/or zeaxanthin (Zea) (Caffarri, S. et al. 2001, Liu, Z. et al. 2004, Ruban, A. V. et al. 1999), depending on the metabolic state of the chloroplast.

There are no structural data on monomeric Lhc proteins, so the structures are modeled on studies of LHCII trimers (Kühlbrandt, W. et al. 1994, Liu, Z. et al. 2004) and PSI-LHCI (Amunts, A. et al. 2007c, Amunts, A. et al. 2010b, Ben Shem, A. et al. 2003). However, biochemical data indicate that Lhcb4 and Lhcb5 lack carotenoid binding site V1 and

have a different selectivity at site L2 (which binds Vio rather than Lut), and that Lhcb6 lacks site N1 (Caffarri, S. et al. 2007b, Passarini, F. et al. 2009i, Ruban, A. V. et al. 1999, Schmid, V. H. R. 2008). These three proteins bind 8, 9 and 10 Chl molecules, respectively (Caffarri, S. et al. 2007a, Dainese, P. and Bassi, R. 1991, Giuffra, E. et al. 1996, Pagano, A. et al. 1998, Ruban, A. V. et al. 1996). One important feature of monomeric Lhcb proteins is their ability to exchange the Vio ligand at site L2 with newly-formed Zea, which is produced following lumen acidification under excess light conditions (Caffarri, S. et al. 2001, Jahns, P. et al. 2001, Morosinotto, T. et al. 2002a, Wehner, A. et al. 2006), a process strictly related to photoprotection (Alboresi, A. et al. 2009, Horton, P. and Ruban, A. V. 1992). Excess light energy induces the accumulation of Chl singlet excited states, increasing the probability of Chl triplet formation. Such triplets can react with O_2 to form reactive oxygen species (ROS) resulting in photoinhibition (Niyogi, K. K. 1999). Lhcb proteins prevent photoinhibition by quenching Chl triplets (Dall'Osto, L. et al. 2006, Mozzo, M. et al. 2008), by scavenging ROS (Dall'Osto, L. et al. 2007, Dall'Osto, L. et al. 2010, Niyogi, K. K. 1999) and by preventing their formation by feedback de-excitation of singlet excited states (Niyogi, K. K. 1999), a mechanism known as non-photochemical quenching (NPQ). Lhcb subunits are known to play a key role in this process, as shown by mutants with a low Lhc protein content (Havaux, M. et al. 2007). Mechanisms proposed for ^{1}Chl* quenching include charge transfer quenching (CTQ) and aggregation-dependent quenching (ADQ).

CTQ involves the formation of a carotenoid radical cation and a Chl radical anion upon excitation, which recombine at the ground state to dissipate the excitation energy (Ahn, T. K. et al. 2008, Avenson, T. J. et al. 2008, Holt, N. E. et al. 2005). ADQ may occur in the trimeric LHCII, which is thought to undergo a conformational change to transfer energy from Chl *a* excited states to the short-lived carotenoid S1 excited state, a conformational change that can be reproduced accurately *in vitro* during LHCII aggregation (Berera, R. et al. 2006, Ruban, A. V. et al. 2007). Recently, quenching has also been associated with the formation of Chl-Chl charge transfer states during the aggregation of LHCII trimers *in vitro* (Muller, M. G. et al. 2010b).

It is possible that ADQ occurs both in LHCII and in monomeric Lhcb proteins, possibly by different mechanisms. Indeed, two quenching sites have been identified by time-resolved fluorescence analysis *in vivo*, one located in the PSII core and the other in the peripheral antenna system (Holzwarth, A. R. et al. 2009e). Electron microscopy coupled with reverse genetics has shown that the outer antenna, comprising LHCII trimers and Lhcb6, detaches from PSII super-complexes, segregating into LHCII + Lhcb6 enriched domains (Betterle, N. et al. 2009, de Bianchi, S. et al. 2008). Spectroscopic changes induced by the aggregation of LHCII trimers *in vitro*, notably changes in Raman resonance and low-temperature fluorescence spectra, were also detected in leaves and chloroplasts under quenching conditions (Johnson, M. P. et al. 2009, Miloslavina, Y. et al. 2008, Ruban, A. V. et al. 2007), suggesting that similar conformational changes can be induced by *in vitro* aggregation and NPQ activation *in vivo*. Zea was also shown to increase fluorescence quenching *in vitro* in aggregated LHCII, Lhcb5 and Lhcb4 (Wentworth, M. et al. 2000), although its role in LHCII trimers is currently unclear (Caffarri, S. et al. 2001, Miloslavina, Y. et al. 2008, Wentworth, M. et al. 2000).

Although ADQ has been studied widely, there are no experimental data to show which chromophores are involved. Previous reports have suggested that ADQ occurs within the protein domain encompassing carotenoid-binding site L1 and Chl 610-611-612 through strong carotenoid/Chl coupling and energy transfer to the carotenoid excited state S1 (Miloslavina, Y. et al. 2008, Pascal, A. A. et al. 2005, Rogl, H. and Kuhlbrandt, W. 1999, Ruban, A. V. et al. 2007) or through Chl-Chl charge transfer (Muller, M. G. et al. 2010a). This putative quenching site is highly conserved in trimeric and monomeric Lhcb proteins (Mozzo, M. et al. 2008).

Here, we report the localization and Zea-dependence of ADQ using time-resolved and steady-state spectroscopy on the different Lhcb proteins. In addition, targeted mutagenesis of Lhcb5 (Ballottari, M. et al. 2009t) showed that ADQ is strongly influenced by Chl molecules proximal to the Lut-binding site L1. This result complements previous studies of CTQ, which localized the quenching site to Chl 603, Chl 609 and Zea in carotenoid-binding site L2 (Ahn, T. K. et al. 2008), and suggests that different types of quenching may occur in different Lhc protein domains.

2. MATERIAL AND METHODS

2.1 DNA cloning, mutations and isolation of overexpressed Lhcb4-6 apoproteins. The *Arabidopsis thaliana lhcb5*, *lhcb4.1* and *lhcb6* genes were cloned as previously reported (Ballottari, M. et al. 2009s, Mozzo, M. et al. 2008, Passarini, F. et al. 2009h). Mutations in *lhcb5* were generated as previously described (Ballottari, M. et al. 2009r) using the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene). Wild-type Lhcb4, Lhcb6 and Lhcb5, and mutant Lhcb5 apoproteins were overexpressed in *Escherichia coli* strain SG13009, and reconstituted *in vitro* as previously described (Giuffra, E. et al. 1996) with modifications (Ballottari, M. et al. 2009q, Bassi, R. et al. 1999). Pure pigments were purchased from Sigma-Aldrich© (Chl *a* and Chl *b*) or purified by HPLC (xanthophylls). When more than one carotenoid was present in the pigment mix, all species were added in equal amounts.

2.2 LHCII isolation procedures. In order to induce Zea accumulation in LHCII trimers, leaves were illuminated for 30 min at 1200 μmol m^2 s^{-1}. LHCII trimers were isolated from dark-adapted or illuminated *A. thaliana* leaves as previously described (Caffarri, S. et al. 2001).

2.3 Pigment analysis. HPLC analysis was performed as described (Gilmore, A. M. and Yamamoto, H. Y. 1991). The Chl/carotenoid and Chl *a*/*b* ratios were determined independently by fitting the spectrum of acetone extracts to the spectra representing individual purified pigments (Croce, R. et al. 2002).

2.4 Steady-state spectroscopy. Samples were diluted in 20 mM HEPES (pH 7.5), 0.2 M sucrose, 0.03% *n*-dodecyl-β-D-maltopyranoside (β-DM) to maintain proteins in a non-aggregated state, or in 20 mM citrate (pH 5.5), 0.2 M sucrose, 0.003% β-DM for the induction of aggregation. Room temperature absorption spectra were recorded using a SLM-Aminco DK2000 spectrophotometer, with a sampling step wavelength of 0.4 nm. Fluorescence emission spectra were measured using a Horiba Jobin-Yvon Fluoromax-3 spectrofluorometer and corrected for the instrumental response. Low-temperature (77 K) fluorescence was measured in a cryostat with 80% glycerol added to each mixture. Circular dichroism (CD) spectra were measured at 10°C on a Jasco 600 spectropolarimeter using a R7400U-20 photomultiplier tube and samples dissolved in the same solvents used for absorption, with an OD of 1 at the maximum in the Q*y* transition. The measurements were performed in a 1-ml cuvette.

2.5 Time-resolved fluorescence analysis. LHCII trimers, Lhcb4-6 wild type proteins and Lhcb5 mutants with or without bound Zea were diluted to the same chlorophyll concentration (80 ng/ml) in buffers promoting non-aggregation or aggregation, as described above. Mixtures of LHCII trimers and Lhcb6 monomers (3:1 molar ratio), each binding either Vio or Zea, were prepared in the same buffers. The level of aggregation in different samples was assessed by measuring the amplitude of light scattering at 750 nm. Time-resolved fluorescence spectroscopy was carried out at room temperature using the single-photon-timing method with a PicoQuant Fluotime 200. Kinetic analysis was performed with a PicoQuant FluoFit, at an excitation wavelength of 435 nm and detection wavelengths of 685 and 705 nm. Each sample was measured ten times in two independent experiments. The fluorescence quantum yield (ϕ) was calculated from fluorescence decay lifetimes as τ_f / τ^f_0, where τ_f is the average fluorescence lifetime and τ^f_0 is the time constant of

Chl spontaneous emission in a constant protein environment (Moya, I. et al. 2001), extrapolated from the LHCII fluorescence quantum yield (Formaggio, E. et al. 2001).

2.6 Dynamic light scattering. The size of aggregates induced by detergent dilution was determined by dynamic light scattering using a ZETASIZER NANO S instrumentation as described in ref. (Cellini, B. et al. 2006).

3. RESULTS

3.1 Time-resolved fluorescence analysis of monomeric Lhcb4-6 subunits and LHCII trimers.

In order to investigate ADQ and its dependence on Zea, monomeric and trimeric Lhcb proteins were refolded *in vitro* or isolated from thylakoids. Monomeric Lhcb proteins were reconstituted *in vitro* after adding pigment mixtures, as previously described (Ballottari, M. et al. 2009p, Bassi, R. et al. 1999, Giuffra, E. et al. 1996, Mozzo, M. et al. 2008, Passarini, F. et al. 2009g, Plumley, F. G. and Schmidt, G. W. 1987). All mixtures contained the same amounts of Chl *a*, Chl *b*, Neo and Lut, but each mixture contained either Vio or Zea but never both, so that two batches of each Lhcb protein were prepared, consistently binding Lut and Neo but differing according to whether Vio or Zea was bound at site L2 (Ahn, T. K. et al. 2008, Connelly, J. P. et al. 1997, Morosinotto, T. et al. 2002a, Wehner, A. et al. 2006). Table S1 (Supplemental Data) shows the pigment composition of each refolded or native complex, and depending on the subunits, each complex contained different relative amounts of Lut, Neo, Vio and Zea as previously reported and consistent with the composition of complexes purified from thylakoid membranes (Bassi, R. et al. 1993, Dainese, P. et al. 1992a, Giuffra, E. et al. 1996). All the complexes were stable and properly folded resulting in efficient energy transfer from Chl *b* and the xanthophylls to Chl *a* as determined by fluorescence spectroscopy (not shown). Vio-binding LHCII trimers were purified from the thylakoid membranes of dark-adapted leaves, whereas Zea-binding LHCII trimers were obtained by illuminating the leaves (1200 µmol m^{-2} s^{-1}) to induce partial substitution at the V1 site prior to isolation, with a final de-epoxidation index of 0.33 (Caffarri, S. et al. 2001) (Supplemental Data, Table S1).

All complexes were analyzed in both concentrated detergent (0.03% β-DM) and in diluted detergent (0.003% β-DM), the latter falling below the critical micelle concentration of 0.01% for β-DM and thus promoting aggregation (Horton, P. et al. 1991). Furthermore, the pH of the solution was adjusted to either 7.8 and or 5.5, generating samples in unquenched and quenched states, respectively (Ruban, A. V. and Horton, P. 1994). Quenching was quantified by time-resolved fluorescence (Miloslavina, Y. et al. 2008, Mullineaux, C. W. et al. 1993, van Oort, B. et al. 2010) with an emission wavelength of 685 nm.

Figure 1 shows the fluorescence decay traces and fitted curves for Lhcb5 and LHCII as examples, although the results for all complexes are provided in Table 1.

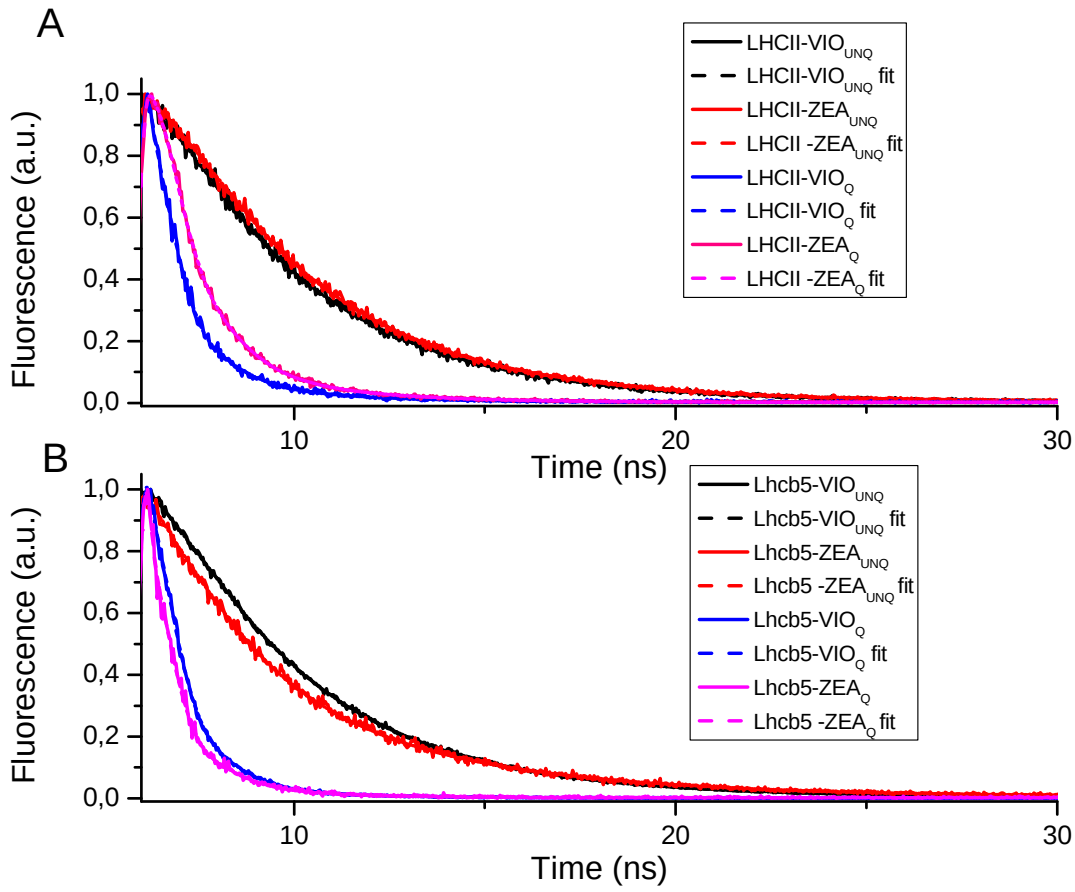

Figure 1: Fluorescence decay of Lhb5 and LHCII under "quenching" and "unquenching" conditions *Fluorescence decay traces (solid lines) of LHCII (A) and Lhcb5 (B) proteins recorded at 685 nm, under either "quenching" (Q) or "unquenching" (UNQ) conditions (see text for details). Decay traces were then fitted using either two or three exponential functions for "unquenching" or "quenching" samples, respectively: resulting fitting curves (fit) are shown as dashed lines.*

Two exponential components were sufficient to fit all the decay traces for unquenched samples, with decay components at 3.70–4.53 and 1.37–2.72 ns, respectively. The average fluorescence lifetime for Lhcb4-6 proteins was reduced when Zea rather than Vio was bound at site L2, in agreement with previous reports (Crimi, M. et al. 1998, Moya, I. et al. 2001, Passarini, F. et al. 2009f). In contrast, Zea binding at site V1 in trimeric LHCII had no effect on the fluorescence lifetime.

For the quenched samples, at least three exponential components were needed to fit the decay traces, including a short component at 40–130 ps, an intermediate component at 380–700 ps and a long-lasting component (in the ns range) with a small amplitude. Average fluorescence lifetimes were significantly reduced in all aggregated samples compared to the corresponding non-aggregated complexes, ranging from 3–4 ns to 0.13–0.55 ns depending on the sample. The strongest quenching induced by aggregation was observed for Lhcb6 in the presence of both Vio and Zea (Figure 2). The effect of Zea differed according to which protein was analyzed. LHCII + Zea had longer-lasting fluorescence (0.55 ns) than LHCII + Vio (0.28 ns), whereas the opposite trend was observed for monomeric Lhcb4 and Lhcb5. For Lhcb5, the average fluorescence lifetime was 240 ps in the presence of Vio and 130 ps in the presence of Zea due to the reduction of time constants associated with all three exponential components (Table 1).

	Amp_1	τ_1	Amp_2	τ_2	Amp_3	τ_3	τ_{AV}	χ^2	$\phi_{(x\ 10^{-2})}$
LHCII V_{UNQ}	21.41%	1.42	78.59%	4.01	-	-	3.46	1.10	8.90
LHCII Z_{UNQ}	17,31%	1,14	82,69%	3,92	-	-	3.44	1.06	8.95
Lhcb4 V_{UNQ}	40.72%	2.42	59.28%	4.56	-	-	3.69	1.05	9.49
Lhcb4 Z_{UNQ}	57.94%	1.76	42.06%	4.18	-	-	2.78	1.14	7.15
Lhcb5 V_{UNQ}	33.10%	2.50	66.90%	4.15	-	-	3.60	1.07	9.26
Lhcb5 Z_{UNQ}	56.13%	1.44	43.87%	3.70	-	-	2.43	1.15	6.25
Lhcb6 V_{UNQ}	49.58%	1.82	50.42%	4.22	-	-	3.030	1.19	7.79
Lhcb6 Z_{UNQ}	69.78%	1.52	30.22%	4.33	-	-	2.369	1.13	6.09
LHCII V_{UNQ} + Lhcb6 V_{UNQ} (3:1)	25.96%	1.352	74.04%	3.969	-	-	3.28978	1.197	8.47
LHCII Z_{UNQ} + Lhcb6 Z_{UNQ} (3:1)	38.04%	1.227	61.96%	3.822	-	-	2.8349	1.135	7.30
LHCII V_Q	66.85%	0.11	31.46%	0.50	1.69%	3.09	0.28	1.03	0.72
LHCII Z_Q	45.23%	0.13	50.10%	0.70	4.66%	2.92	0.55	1.12	1.41
Lhcb4 V_Q	67.18%	0.09	31.40%	0.49	1.42%	2.56	0.25	1.22	0.64
Lhcb4 Z_Q	70.42%	0.08	28.38%	0.48	1.20%	2.33	0.22	1.09	0.57
Lhcb5 V_Q	65.30%	0.08	33.93%	0.50	0.77%	2.73	0.24	1.22	0.62
Lhcb5 Z_Q	84.40%	0.06	14.99%	0.43	0.61%	2.39	0.13	1.08	0.33
Lhcb6 V_Q	87.05%	0.07	12.05%	0.39	0.90%	3.52	0.14	0.98	0.36
Lhcb6 Z_Q	84.31%	0.05	15.03%	0.37	0.66%	2.51	0.11	1.01	0.26
LHCII V_Q + Lhcb6 V_Q (3:1)	87.36%	0.11	12.04%	0.50	0.60%	2.95	0.17	1.32	0.44
LHCII Z_Q + Lhcb6 Z_Q (3:1)	83.55%	0.12	16.00%	0.45	0.44%	3.70	0.19	1.31	0.49

Table 1: Time resolved fluorescence analysis on Lhcb proteins in detergent or under conditions favoring aggregation. *Fitting results of fluorescence decay traces (emission detected at 685 nm) measured on recombinant Lhcb4-6 proteins, native LHCII trimers and on a mixture of Lhcb6 and LHCII trimers in a 3:1 molar ratio. Samples were reconstituted (Lhcb4-6) or purified (LHCII trimers) with violaxanthin (V) or zeaxanthin (Z). Different complexes were diluted in presence of 0.03% β-DM and 20 mM HEPES (pH 7.8) or 0.003% β-DM and 20 mM citrate (pH 5.5), in order to induce unquenching (UNQ) or quenching (Q) conditions, respectively.. Amp_{1-3}: amplitude of the exponential components 1-3; τ_{1-3}: decay time constants (ns) of the exponential curves 1-3 used to fit the fluorescence decay curves; τ_{AV}: average fluorescence decay lifetime (ns); ϕ: fluorescence quantum yield. Errors are less than 12% in each case.*

By plotting the ratio of the average fluorescence lifetimes in the unquenched and quenched samples, the effect of Zea and Vio on ADQ could be compared among the different Lhcb proteins (Figure 2B). Lhcb6 showed the highest ratio in the presence of either Zea or Vio, indicating that fluorescence lifetime was highly dependent on aggregation, whereas trimeric LHCII showed the lowest ratio, indicating that fluorescence lifetime was only minimally dependent on aggregation, and in this context Zea appears to prevent quenching rather than stimulating it.

Recently, NPQ was shown to be triggered by the dissociation of the LHCII-M/Lhcb6/Lhcb4 super-complex catalyzed by protonation of PsbS (Betterle, N. et al. 2009) and enhanced by Zea synthesis. This event segregates two domains in grana membranes, one of which contains Lhcb6 together with LHCII-M and LHCII-L trimers. Two different quenching sites have been detected, one connected to the PSII core and the other unconnected (Holzwarth, A. R. et al. 2009d, van Oort, B. et al. 2010).

In order to mimic the formation of the LHCII-Lhcb6 domain, we analyzed a 3:1 molar ratio mixture of LHCII trimers and Lhcb6 monomers in the non-aggregated and aggregated states. Fluorescence lifetime data are shown in Table 1.

Two exponential components were sufficient to fit the unquenched traces but at least three were required to fit the quenched traces, with decay components similar to those observed in samples containing a single protein species. Figure 2 compares the average fluorescence decay lifetimes of the mixtures containing Vio and Zea with the corresponding data from samples containing single subunits.

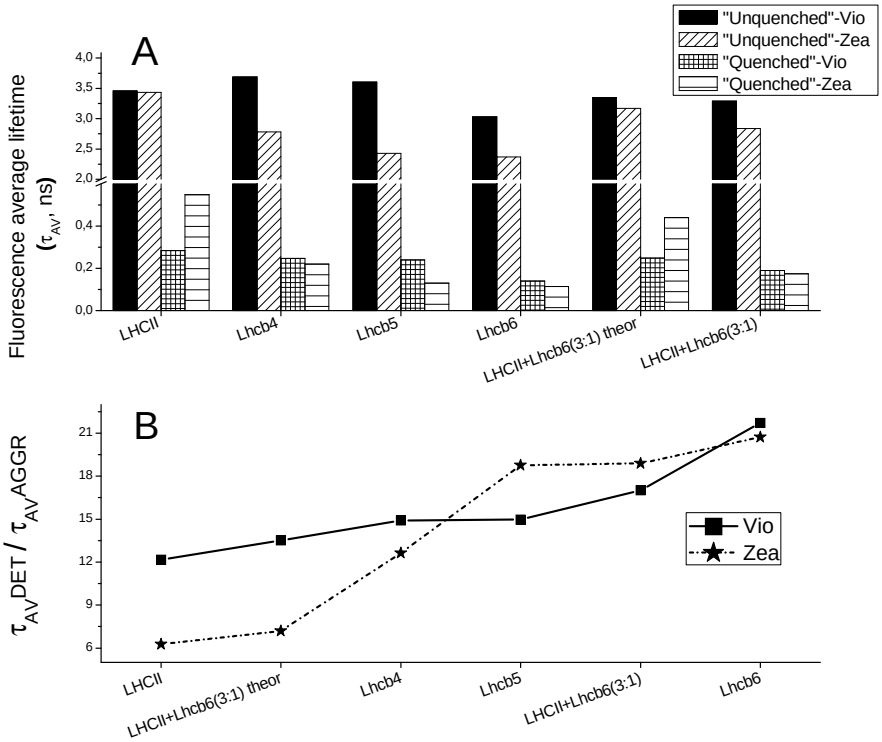

Figure 2: Average fluorescence lifetime ratios between samples in detergent and under conditions favoring aggregation. ***A.*** *fluorescence average lifetimes of monomeric Lhcb4-6, trimeric LHCII proteins and of a mixture of Lhcb6 and LHCII trimers (3:1 molar ratio) in detergent (τ_{AV} UNQ) or under aggregation conditions (τ_{AV} Q). Time resolved fluorescence analysis is reported in Table 1. Lhcb4-6 and LHCII trimers average fluorescence lifetimes were measured either in violaxanthin binding (Vio) or zeaxanthin binding (Zea) samples; calculated fluorescence average lifetimes of the LHCII and Lhcb6 mixture is reported ("theor").* ***B.*** *average fluorescence lifetime ratios among samples measured in detergent (τ_{AV} UNQ) or in aggregation conditions (τ_{AV} Q), showing actual and calculated fluorescence average lifetimes of the LHCII and Lhc6 mixture under aggregation conditions.*

Lifetimes predicted by computing linear combinations of individual samples are also shown to represent the absence of interactions between LHCII and Lhcb6. In the non-aggregated state, the observed and predicted lifetimes are the same, whereas in the aggregated state the observed lifetimes are shorter than the predicted values (Figure 2), indicating that the quenching of LHCII (the predominant species in the sample) is enhanced in the presence of Lhcb6. This effect is exacerbated in the presence of Zea. Similar results were observed when fluorescence emission was measured at 705 nm (Supplemental Data, Table S2), with a higher amplitude for the shortest component (<170 ps), implying the quenching species has a greater far-red emission, in agreement with previous *in vivo* data (Miloslavina, Y. et al. 2008).

3.2 77K steady state fluorescence analysis of Lhcb4-6 and LHCII trimers

The formation of excitation traps by aggregation in LHCII is associated with the appearance of long-wavelength emitting forms, reflecting the mixed excitation/charge transfer state. These spectral forms have recently been associated with similar red-shifted forms detected *in vivo* in quenched leaves or chloroplasts (Johnson, M. P. et al. 2009, Miloslavina, Y. et al. 2008). In order to verify the appearance of these far-red emitting forms in Lhcb proteins and their dependence on Zea, we measured the 77K emission fluorescence spectra of non-aggregated and aggregated Lhcb proteins (Figure 3).

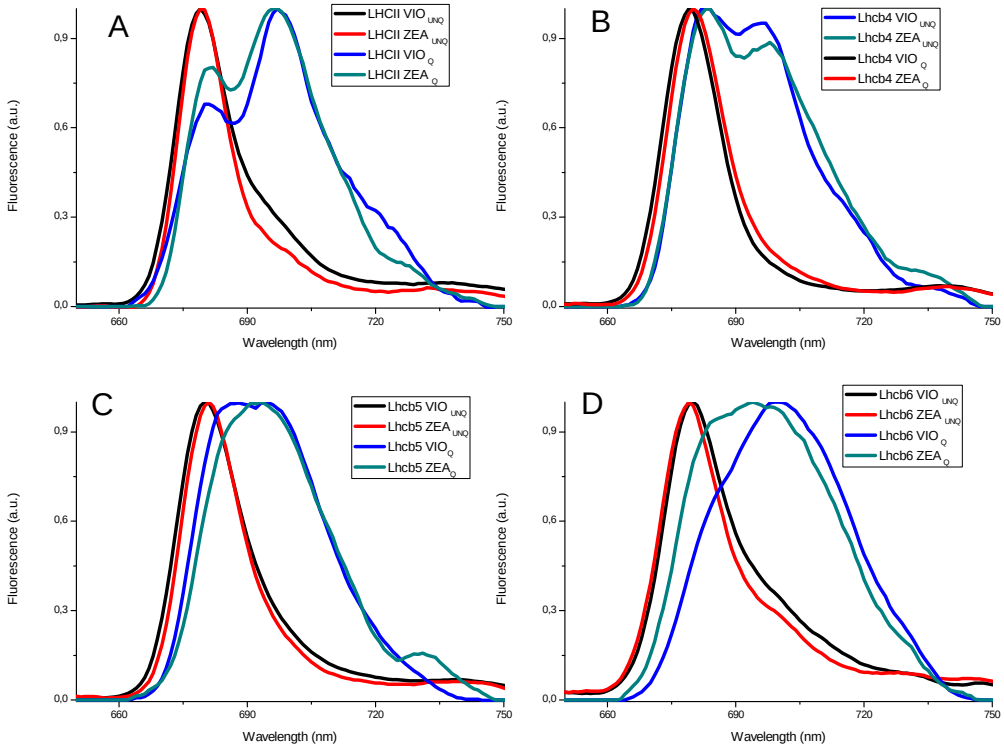

Figure 3: 77K emission spectra of Lhcb proteins in detergent or under conditions favoring aggregation *Fluorescence emission spectra (recorded at 77K) of Lhcb proteins binding either violaxanthin (Vio) or zeaxanthin (Zea). Different samples were diluted with detergent (UNQ) or under aggregation conditions (Q) as described in the text.* ***A.*** *LHCII trimers,* ***B.*** *Lhcb4 complexes,* ***C.*** *Lhcb5 complexes;* ***D.*** *Lhcb6 complexes. Black traces: violaxanthin binding complexes in detergent; red traces: zeaxanthin binding complexes in detergent; blue traces: violaxanthin binding complexes under aggregation conditions; cyan traces: zeaxanthin binding complexes under aggregation conditions.*

The emission spectra of all unquenched samples had a characteristic single peak at ~680 nm, whereas an additional signal at 697 nm appeared in the quenched samples, irrespective of which carotenoid was bound at the V1 site (Miloslavina, Y. et al. 2008). A broadening of the high-energy peaks was also observed. In the case of aggregated Lhcb4, emission spectra were characterized by two peaks at 681 and 698 nm, regardless of which carotenoid was present. Lhcb6 and Lhcb5 also showed significant spectral changes, although in both cases aggregation produced a single broad peak encompassing both the 685/695 and 684/699 nm ranges rather than the two discrete peaks observed for LHCII and Lhcb4. Nevertheless, the broad emission spectrum represents the convolution of two bands, as more evident in the case of Lhcb5. Carotenoid selectivity at the L2 site therefore contributed only small differences to the spectra, and it is interesting to note that Lhcb6 showed the most significant red-sift following aggregation, regardless of whether Zea or Vio was present.

3.3 In vitro refolding of Lhcb5 mutants lacking chlorophyll-binding residues

The sites of energy dissipation in Lhc proteins are thought to be the Chl molecules with the lowest transition energies, namely Chl 612-610-611 {Liu, 2004 24308 /id;Novoderezhkin, 2005 24860 /id;Calhoun, 2009 24900 /id;van Grondelle, 2006 24936 /id}, and Lut bound at the L1 site (Johnson, M. P. et al. 2009, Miloslavina, Y. et al. 2008, Ruban, A. V. et al. 2007). To verify this model and/or identify other chromophores involved in quenching, we analyzed a series of

Lhcb5 mutants in which the Chl-binding residues were disrupted by targeted mutagenesis (Ballottari, M. et al. 2009o). Lhcb5 was chosen for a series of reasons: (i) it undergoes ADQ even more efficiently than LHCII (Figure 1, Figure 2, Table 1) {Wentworth, 2001 24909 /id;Wentworth, 2004 24518 /id}, (ii) most Chl-binding sites can be specifically targeted in Lhcb5, whereas LHCII and Lhcb6 have sites without a ligand that could be experimentally modified, (iii) quenching in Lhcb5 is enhanced by Zea just like NPQ *in vivo* whereas LHCII and Lhcb4 show little dependence on this xanthophyll (Figure 2), and (iv) the occupancy of each Chl-binding site in Lhcb5 has been well characterized (Ballottari, M. et al. 2009n). On this basis we prepared two samples for each Lhcb5 mutant (containing either Vio or Zea in addition to the standard Chl *a*, Chl *b*, Lut and Neo). Our nomenclature for the mutants reflects the targeted Chl-binding site as previously described (Kühlbrandt, W. et al. 1994) with a suffix indicating whether Vio or Zea are present. For example mutant $A2_{VIO}$ is mutant N179F described in ref. (Ballottari, M. et al. 2009m), which has a mutated Chl-binding site A2 (Kühlbrandt, W. et al. 1994) coordinating Chl 612 (Liu, Z. et al. 2004) (Table 2).

SAMPLE	Mutated residues	Chl coordinated	Amp_1	τ_1	Amp_2	τ_2	Amp_3	τ_3	τ_{AV}	χ^2	ϕ (x 10^{-2})
WT Vio_{UNQ}	-	-	33.10%	2.50	66.90%	4.15	-	-	3.60	1.07	9.26
A2 Vio_{UNQ}	N179F	Chl 612	29.99%	2.18	70.01%	4.17	-	-	3.57	1.01	9.18
A3 Vio_{UNQ}	Q193L	Chl 613	65.06%	2.77	34.94%	4.87	-	-	3.50	1.03	9.00
A4 Vio_{UNQ}	E65V/R181L	Chl 602	73.98%	3.16	26.02%	5.25	-	-	3.71	1.16	9.54
A5 Vio_{UNQ}	H68F	Chl 603	61.08%	3.07	38.92%	4.98	-	-	3.81	1.14	9.80
B3 Vio_{UNQ}	H208L	Chl 614	37.20%	2.52	62.80%	4.19	-	-	3.57	1.09	9.18
B5 Vio_{UNQ}	E137V	Chl 609	41.29%	2.54	58.71%	4.07	-	-	3.44	1.10	8.85
B6 Vio_{UNQ}	E129V	Chl 606	47.70%	2.38	52.30%	4.74	-	-	3.62	1.09	9.31
WT Zea_{UNQ}	-	-	56.13%	1.44	43.87%	3.70	-	-	2.43	1.15	6.25
A2 Zea_{UNQ}	N179F	Chl 612	49.29%	1.75	50.70%	4.07	-	-	2.93	1.08	7.54
A3 Zea_{UNQ}	Q193L	Chl 613	52.05%	1.71	47.95%	4.69	-	-	3.14	1.06	8.08
A4 Zea_{UNQ}	E65V/R181L	Chl 602	59.20%	1.94	40.80%	4.57	-	-	3.01	1.13	7.74
A5 Zea_{UNQ}	H68F	Chl 603	59.94%	2.37	40.06%	4.70	-	-	3.30	1.08	8.49
B3 Zea_{UNQ}	H208L	Chl 614	64.29%	1.80	35.71%	4.51	-	-	2.77	1.19	7.13
B5 Zea_{UNQ}	E137V	Chl 609	50.68%	1.69	49.32%	3.88	-	-	2.77	1.04	7.13
B6 Zea_{UNQ}	E129V	Chl 606	60.94%	1.79	39.06%	4.73	-	-	2.94	1.06	7.56
WT Vio_Q	-	-	65.30%	0.08	33.93%	0.50	0.77%	2.73	0.24	1.22	0.62
A2 Vio_Q	N179F	Chl 612	50.26%	0.11	49.74%	0.73	3.13%	3.08	0.52	1.20	1.34
A3 Vio_Q	Q193L	Chl 613	60.82%	0.08	34.74%	0.71	4.44%	2.98	0.43	1.17	1.11
A4 Vio_Q	E65V/R181L	Chl 602	74.91%	0.06	23.17%	0.57	1.92%	2.93	0.23	1.07	0.59
A5 Vio_Q	H68F	Chl 603	81.74%	0.19	16.53%	0.60	1.73%	3.17	0.31	1.20	0.80
B3 Vio_Q	H208L	Chl 614	80.22%	0.03	18.15%	0.69	1.63%	3.11	0.20	1.12	0.51
B5 Vio_Q	E137V	Chl 609	73.14%	0.05	25.24%	0.58	1.62%	2.78	0.22	1.28	0.57
B6 Vio_Q	E129V	Chl 606	80.83%	0.04	18.18%	0.47	1.00%	3.22	0.15	1.26	0.39
WT Zea_Q	-	-	84.40%	0.06	14.99%	0.43	0.61%	2.39	0.13	1.08	0.33
A2 Zea_Q	N179F	Chl 612	73.45%	0.09	23.16%	0.49	3.40%	2.13	0.25	1.00	0.64
A3 Zea_Q	Q193L	Chl 613	70.54%	0.24	22.47%	1.12	6.99%	3.06	0.63	1.16	1.62
A4 Zea_Q	E65V/R181L	Chl 602	78.94%	0.06	18.60%	0.46	2.46%	2.60	0.20	0.96	0.51
A5 Zea_Q	H68F	Chl 603	83.23%	0.07	16.15%	0.41	0.62%	2.71	0.14	1.05	0.36

B3 Zea$_Q$	H208L	Chl 614	79.11%	0.05	20.42%	0.35	0.48%	2.34	0.12	0.99	0.31
B5 Zea$_Q$	E137V	Chl 609	65.05%	0.09	32.86%	0.47	2.09%	2.33	0.26	1.02	0.67
B6 Zea$_Q$	E129V	Chl 606	87.93%	0.04	11.69%	0.29	0.39%	2.18	0.08	0.95	0.21

Table 2: Time resolved fluorescence analysis on Lhcb5 chlorophyll binding site mutants in detergent or under conditions favoring aggregation. *Fitting results of fluorescence decay traces (emission detected at 685 nm) measured on recombinant Lhcb5 complexes mutated at different chlorophyll binding sites, reconstituted in presence of chlorophyll a, b, lutein, neoxanthin and violaxanthin (Vio) or zeaxanthin (Zea). Samples were diluted in presence of 0.03% β-DM and 20 mM HEPES (pH 7.8) or 0.003% β-DM and 20 mM citrate (pH 5.5), in order to induce unquenching (UNQ) or quenching (Q) conditions, respectively. Fluorescence emission was recorded at 685 nm. Amp_{1-3}: amplitude of the exponential components 1-3; τ_{1-3}: decay time constants (ns) of the exponential curves 1-3 used to fit the fluorescence decay curves; τ_{AV}: average fluorescence decay lifetime (ns). ϕ: Fluorescence quantum yield. Mutations and chlorophylls coordinated by mutated residues are indicated. Errors are less than 12% in each case.*

All the pigment-protein complexes folded correctly *in vitro* in the presence of either Zea or Vio, resulting in efficient energy transfer from Chl *b* and xanthophylls to Chl *a* as previously reported (Ballottari, M. et al. 2009l). Table S3 (Supplementary Material) summarizes the results of pigment analysis for each of the mutants as compared to wild-type Lhcb5. In samples containing Vio, our results are consistent with previous studies, with most mutants losing one Chl, but mutants $A4_{VIO}$ and $A5_{VIO}$ each losing two: Chl 611 and 609, respectively, in addition to Chl 602 and 603 (Ballottari, M. et al. 2009k). The carotenoid content was conserved in all mutants, although a marginal loss of Lut and Neo was observed in $A4_{VIO}$, $A5_{VIO}$, $B5_{VIO}$ and $B6_{VIO}$ (Ballottari, M. et al. 2009j). Samples containing Zea and Vio had similar pigment contents, although $A3_{ZEA}$ and $B5_{ZEA}$ presumably lose more than one Chl, since the loss of a single chlorophyll would imply that only Chl *a* is bound at these sites, a situation not found in Lhcb5-Vio or any other Lhc protein analyzed thus far (Ballottari, M. et al. 2009i, Morosinotto, T. et al. 2002b, Morosinotto, T. et al. 2005, Mozzo, M. et al. 2008, Passarini, F. et al. 2009e, Remelli, R. et al. 1999). We suggest that $A3_{ZEA}$ and $B5_{ZEA}$ lose Chl 614 and 603 respectively, similar to Lhca1 (Morosinotto, T. et al. 2002b). It is worth noting that, as previously reported (Ballottari, M. et al. 2009h), it was not possible to produce a Lhcb5 complex with a mutation in the A1 Chl-binding site, as the refolded complex was extremely unstable in the presence of both Vio and Zea.

3.4 Time-resolved fluorescence analysis of Lhcb5 mutants

Each mutant Lhcb5 protein was prepared in the detergent solutions described above to yield non-aggregated and aggregated samples, and each was prepared in the presence of either Vio or Zea. Time-resolved fluorescence analysis (Table 2) revealed that the fluorescence decay traces of unquenched samples could be fitted to curves using two exponential functions, with time constants in the ns range similar to the wild-type Lhcb5 protein. All complexes containing Vio had average fluorescence lifetimes similar to the wild type protein (~3.6 ns), except mutant A5 which showed a slight delay in both decay components and thus an average fluorescence lifetime of 3.8 ns. In samples containing Zea, the average fluorescence lifetimes were reduced to 2.2–2.8 ns, with $A3_{ZEA}$ and $A5_{ZEA}$ mutants having the longest lifetimes (Table 2). Interestingly, all the mutants had longer fluorescence lifetimes than the wild-type protein. This suggests that delocalized quenching promoted by Zea is not specifically dependent on one particular Chl and instead is likely to reflect multiple weak interactions.

The fluorescence decay traces of mutants in the aggregated state in the presence of either Vio or Zea required three exponential curves, characterized by two short components (< 100 ps and 300–730 ps) and a longer component in the ns range with small amplitude, similar to the wild type. All samples showed at least five-fold more quenching after aggregation, irrespective of which Chl site was lost. The decay kinetics and average fluorescence lifetimes of Vio-binding mutants A4-$A5_{VIO}$, $B3_{VIO}$ and $B5_{VIO}$ were similar to the wild type in the presence of Vio (0.24–0.30 ns),

whereas $B6_{VIO}$ showed even faster decay (0.15 ns). In contrast, $A2_{VIO}$ and $A3_{VIO}$ mutants had a longer fluorescence lifetime (0.52 and 0.43 ns, respectively) due to the prevalence of the intermediate component from 500 to 700 ps. A similar, but smaller, effect was observed for $B3_{VIO}$, which lacks Chl 614 (the closest chlorophyll to Chl 613). Almost all the quenched Zea-binding mutant complexes were characterized by decay components similar to wild type complexes in the presence of Zea, with minor differences reflecting small changes in the amplitude of the longest component. Clear differences in the decay time constants were detected in $A3_{ZEA}$, with the two short components delayed to 240 ps and 1.12 ns, implying that the loss of Chl 613 reduces the efficiency of ADQ. The florescence decay was more rapid in $B6_{ZEA}$ compared to the wild type under quenching conditions, and similar results were obtained when fluorescence emission was detected at 705 nm, although the amplitude of the faster component (<100 ps) was higher.

3.5 77K steady state fluorescence analysis of Lhcb5 mutants

We recorded 77K emission fluorescence spectra for Lhcb5 mutants in the aggregated state in order to identify the Chl molecules responsible for the red-shifted fluorescence emission, i.e. those involved in the formation of mixed excitation/charge transfer states. Figure 4 compares the emission spectra for Lhcb5 mutants in the unquenched and quenched states, showing that all unquenched mutants have fluorescence emission spectra similar to the wild type, although $A2_{VIO}$ and $A3_{ZEA}$ have 77K fluorescence emission peaks that are blue-shifted by 2 nm compared to the wild type.

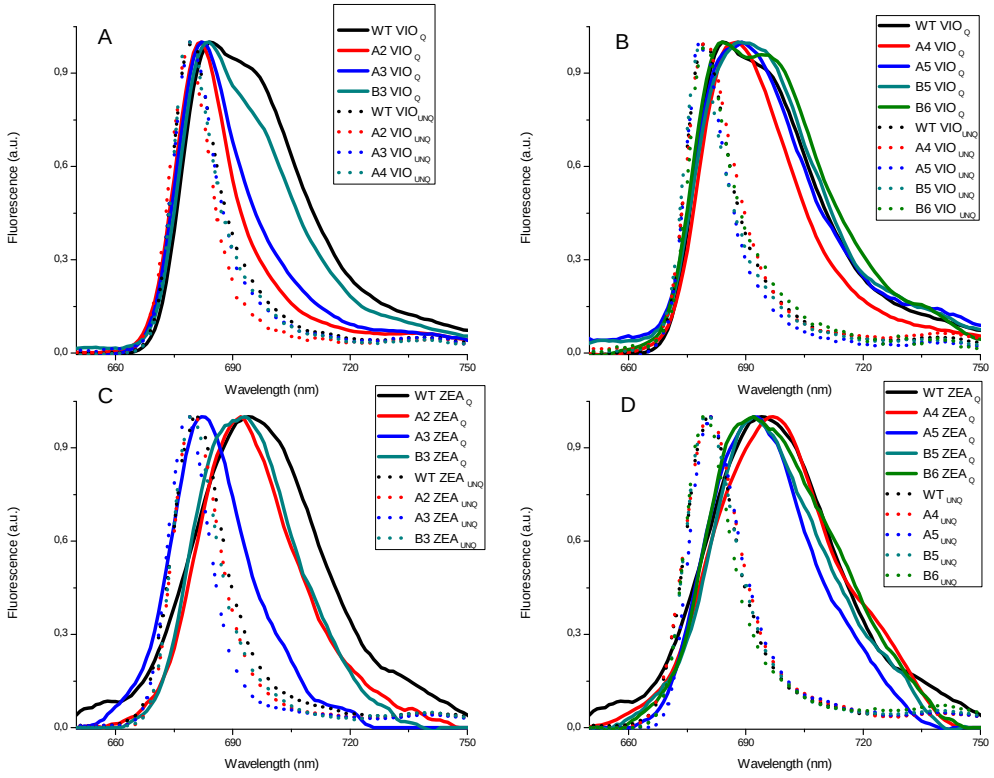

Figure 4: 77K emission spectra of Lhcb5 WT and mutants in detergent or under conditions favoring aggregation
77K fluorescence emission spectra for Lhcb5 wild type and chlorophyll binding mutants. Different samples, binding either violaxanthin (Vio) or zeaxanthin (Zea), were diluted with detergent (UNQ, dashed traces) or under aggregation conditions (Q, solid lines) as described in the text. ***A.*** *wild type (black traces), A2 (red traces), A3 (blue traces), B3 (cyan traces) violaxanthin binding complexes;* ***B.*** *wild type (black traces), A4 (red traces), A5 (blue traces), B5 (cyan*

traces), B6 (green traces) violaxanthin binding complexes; ***C.*** *wild type (black traces), A2 (red traces), A3 (blue traces), B3 (cyan traces) zeaxanthin binding complexes;* ***D.*** *wild type (black traces), A4 (red traces), A5 (blue traces), B5 (cyan traces), B6 (green traces) zeaxanthin binding complexes.*

Under conditions promoting aggregation, mutants A4-5_{VIO}, $B5_{VIO}$ and $B6_{VIO}$ still present emission spectra similar to the wild type protein in the presence of Vio (Figure 4B), with two main peaks at 685 and 700 nm. In contrast, the amplitude of the 700 nm broad component is strongly reduced in the $A2_{VIO}$ and $A3_{VIO}$ mutants, and the 685 nm peak is blue-shifted to 680 nm, possibly due to the missing low-energy band (Figure 4A). Mutant $B3_{VIO}$ is intermediate, showing a slight reduction in the far-red fluorescence emission tail. With Zea bound to the L2 binding site, only $B6_{ZEA}$ and $A4_{ZEA}$ show emission spectra similar to the wild type in the presence of Zea, with a broad peak at 698 nm, representing the sum of the red and far-red components (Figure 4C-D). The amplitude of the far-red tail was reduced in the spectra from mutants $A2_{ZEA}$, $B3_{ZEA}$, $B5_{ZEA}$ and $A5_{ZEA}$, particularly in the case of $A3_{ZEA}$, where the single peak showed a significant blue shift and the far-red component was completely absent.

3.6 *Conformational changes induced by aggregation in Lhcb5.*

Conformational changes induced by aggregation in LHCII correlate with specific changes in circular dichroism (CD) spectra (Ruban, A. V. et al. 1997a). In order to confirm the involvement of specific Chl binding sites in ADQ, we used CD spectroscopy to investigate the occurrence of conformational changes induced by aggregation in wild type Lhcb5 and mutants, with Vio or Zea bound at site L2. As shown in Figure 5, when the unquenched spectrum of Lbcb5 + Vio is subtracted from the equivalent quenched spectrum, characteristic components were observed at 680 (+), 669 (-), 500 (-), 491 (+), 475 (+), 459 (-) and 434 (-) nm.

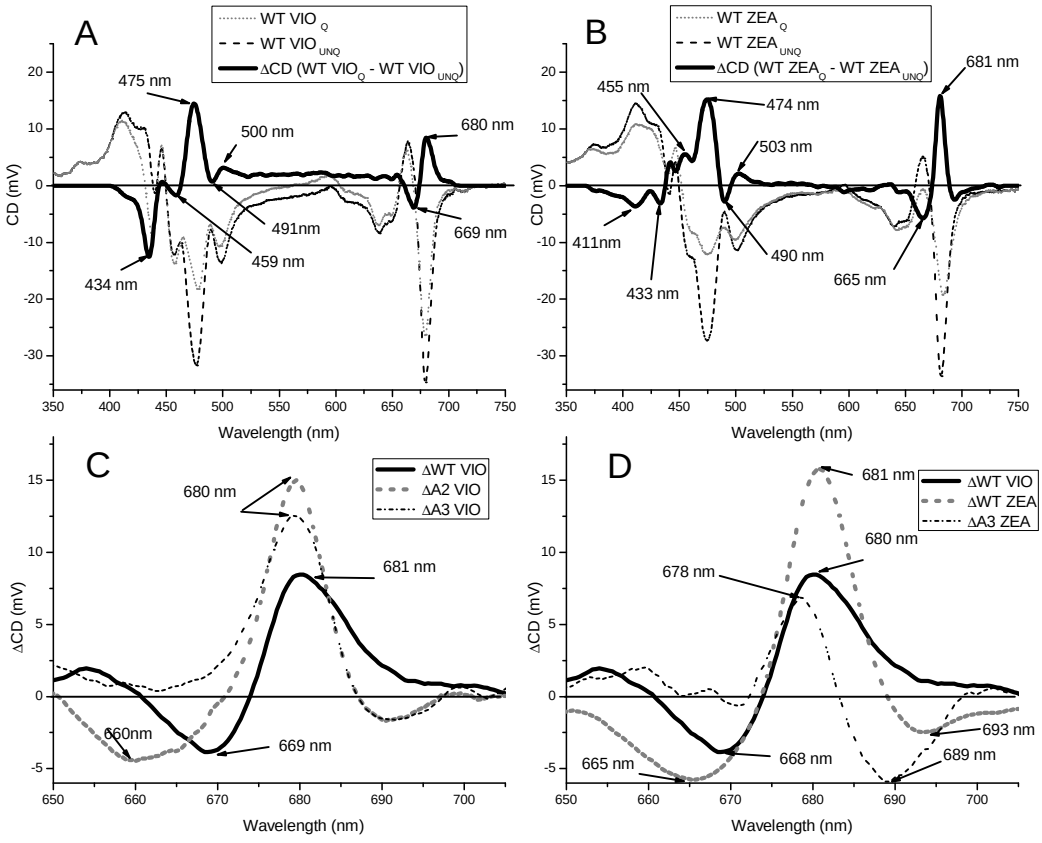

Figure 5: Circular dichroism spectra of Lhcb5 WT and mutants *A.* *circular dichroism spectra of violaxanthin (Vio) binding Lhcb5 wild type in detergent (UNQ, dashed black line), under aggregation conditions (Q, solid black line); and the difference between them (gray dashed traces). Difference spectrum peaks are indicated.* ***B.*** *circular dicroism spectra of zeaxanthin (Zea) binding Lhcb5 wild type in detergent (UNQ, dashed black line), under aggregation conditions (Q, solid black line); and the difference between them (gray dashed traces). Difference spectrum peaks are indicated.* ***C.*** *circular dicroism difference spectra in the 650-750 region of violaxanthin (Vio) binding Lhcb5 wild type (solid black line), A2 (dashed black line) and A3 (dashed gray line) mutants, calculated by subtracting the spectrum measured in detergent (UNQ) from the spectrum measured under aggregation conditions (Q).* ***D.*** *circular dicroism difference spectra in the 650-750 region of violaxanthin (Vio) binding Lhcb5 wild type (solid black line), zeaxanthin binding Lhcb5 wild type (dashed black line) and the zeaxanthin binding mutant A3 (dashed gray line), calculated by subtracting the spectrum measured in detergent (UNQ) from the spectrum measured under aggregation conditions (Q).*

In Lbcb5 + Zea, these components were shifted to 681(+), 665(-), 503(+), 490(-), 474 (+), 455 (+) and 433 (-) nm and two additional signals appeared at a 411nm (-) and 693 (-) nm. These results demonstrate that conformational changes induced by the aggregation of Lhcb5 are partially dependent on which carotenoid binds at the L2 site. The CD spectra of Lhcb5 mutants + Vio showed similar modulations upon aggregation (data not shown), with the exception of $A2_{VIO}$ and $A3_{VIO}$. In these cases, the differences resulted in a sharper signal at 680 nm (+), with the red-most contribution eliminated. The CD spectra of Lhcb5 mutants + Zea were also similar to the wild type, with the exception of $A3_{ZEA}$ where differences in the red region of the spectrum were blue-shifted, with negative components at 689 and 665 nm, and a positive peak at 678 nm (Figure 5).

3.7 Aggregation states of different Lhcb proteins

The results presented above show that different Lhcb proteins undergo similar conformational changes upon aggregation, and these conformational changes are associated with fluorescence quenching. To determine whether the efficiency of quenching relates quantitatively to the degree of aggregation, we measured the distribution of particle sizes by dynamic light scattering, under the same conditions used for fluorescence lifetime measurements. We observed particles of two different sizes, 100 nm and 1 μm, under conditions promoting aggregation (Figure S1). Lhcb6 and LHCII formed 1 μm particles. It should be noticed that by aggregating LHCII monomers or trimers the same aggregated size is obtained, implying a determinant role of the protein monomers in this process. Lhcb4 formed 100 nm particles, and Lhcb5 switched from 100 nm to 1 μm particles depending on whether it bound Viola or Zea (Figure S1). Single point mutations in Lhcb5 did not influence particle size distribution (Figure S1).

4. DISCUSSION

We have analyzed the behavior of different Lhcb proteins undergoing ADQ, in the light of multiple reports over the last 20 years suggesting a correlation between fluorescence quenching induced by *in vitro* aggregation of LHCII trimers and the induction of NPQ *in vivo* {Horton, 1991 15577 /id;Mullineaux, 1993 16425 /id;Barzda, 1994 24910 /id;van Oort, 2007 24907 /id;Wentworth, 2001 24909 /id;Ruban, 1996 19114 /id;Miloslavina, 2008 24898 /id;Johnson, 2009 24902 /id;Ruban, 1997 20952 /id;Wentworth, 2000 23534 /id;Ruban, 2007 24842 /id}. In particular, changes in the Raman resonance spectrum of LHCII trimers during aggregation correlate with similar signals detected in leaves when NPQ is induced, suggesting that Lhc proteins undergo the same conformational change during aggregation *in vitro* and NPQ activation *in vivo* (Ruban, A. V. et al. 2007). Similarly, far-red fluorescence in quenched leaves is associated with red-shifted emission forms in LHCII oligomers *in vitro* (Johnson, M. P. et al. 2009, Miloslavina, Y. et al. 2008). However, these reports focused primarily on LHCII, in either its monomeric or trimeric aggregation state, whereas earlier reports suggested that Lhcb4 and Lhcb5 were even more efficient facilitators of ADQ than LHCII {Ruban, 1996 19114 /id;Wentworth, 2004 24518 /id;Wentworth, 2001 24909 /id}. Moreover, although Zea is a strong inducer of NPQ *in*

vivo (Demmig-Adams, B. 1990), its role in ADQ has never been clarified. Finally, the precise location of the quenching sites, i.e. the identity of the chromophores involved in NPQ, has yet to be determined.

4.1 ADQ in Lhcb proteins.

Comparing the average fluorescence lifetime of different Lhcb proteins before and after ADQ (Table 1) revealed that monomeric Lhcb4-6 and trimeric LHCII are strongly quenched following aggregation (Figure 2), with a 10–30-fold reduction in fluorescence quantum yield (Table 1). The effect appears to be greater in monomeric antennas, with Lhcb6 showing the shortest fluorescence lifetime and the lowest quantum yield (Figure 2B). The amplitude of quenching appears unrelated to particle size in solutions containing 0.003% β-DM because Lhcb proteins that differ in the amplitude of quenching and its dependence on Zea (e.g. Lhcb6 and LHCII) have the same particle size. Instead, the size of the aggregates appears to depend on the properties of individual Lhcb monomers, since both monomeric and trimeric LHCII have similar-sized aggregates (Supplemetal Data, Figure S1). Furthermore, wild-type Lhcb5 and Lhcb5 Chl-binding mutants with different fluorescence lifetimes exhibit the same aggregation behavior, and undergo a switch between small and large aggregates when Zea binds at the L2 site, consistent with a previously reported change in conformation (Dall'Osto, L. et al. 2005). We conclude that the observed changes in aggregate particle size are unrelated either to changes in the fluorescence properties of the complexes under aggregating conditions or to the conformational changes detected by CD spectroscopy. This is consistent with previous reports showing that strong CD spectra and fluorescence lifetime changes are observed when comparing proteins dissolved in buffers containing detergent concentration above and below the critical micellar concentration, whereas a further reduction in detergent concentration, although increasing particle size, does not significantly affect either the CD spectrum or the fluorescence lifetime (Bassi, R. and Wollman, F.-A. 1991). We cannot completely exclude the possibility that changes in the fluorescence lifetime of Lhcb5 induced by Zea might in part reflect changes in aggregation size.

The formation of a quenched state in aggregated LHCII was previously correlated with the presence of red-shifted forms due to the formation of mixed excitation/charge transfer states (Miloslavina, Y. et al. 2008, Ruban, A. V. et al. 1991). Here, we observed a similar red-shift and broadening of emission spectra in all Lhcb complexes upon aggregation. LHCII and Lhcb4 show two peaks with amplitudes at 680 and 697-698 nm, whereas red-shifting of the emission peaks is even more pronounced in Lhcb5 and Lhcb6. Another spectroscopic signature of LHCII in the quenched state induced by aggregation is a conformational change that can be monitored by CD spectroscopy (Ruban, A. V. et al. 1997a). We observed similar changes in the CD spectra for Lhcb5 (Figure 5), which suggests that all Lhcb proteins have the ability to undergo similar conformational changes in response to aggregation. In addition to these common features, we also observed unique properties such as the red-most emission forms and the lowest aggregation-induced fluorescence yield in Lhcb6. These results imply that, whenever processes similar to ADQ occur in plants during the induction of NPQ, monomeric Lhcb proteins can be involved along with LHCII and show higher efficiency excitation energy quenching. In this context, it is interesting to note that the addition of Lhcb6 monomers to LHCII trimers enhances the intensity of quenching compared to LHCII alone. Moreover, Zea binding enhances this effect even further (Table1, Figure 2). The simplest explanation is that, upon aggregation, excitation energy is delocalized among the LHCII/Lhcb6 aggregates and is quenched in the most efficient manner, namely by Lhcb6 (Table 1 and Table S2). The formation of specific domains within grana membranes enriched in LHCII and Lhcb6 oligomers has been recently demonstrated *in vivo* following NPQ (Betterle, N. et al. 2009, de Bianchi, S. et al. 2008, Holzwarth, A. R. et al. 2009c, van Oort, B. et al. 2010). Although it is rather unlikely that membrane proteins can aggregate in the lipid phase of the thylakoid membrane due to the inaccessibility of the hydrophobic membrane surfaces, multiple protein-protein

interactions between antenna proteins have been shown to induce quenching in liposomes likely by inducing conformational change(s) by their cooperative effect (Moya, I. et al. 2001).

4.2 Effect of zeaxanthin on ADQ in Lhcb proteins

All Lhcb4-6 complexes undergo a reduction in fluorescence yield in solution when Zea binds to the L2 site, as previously reported (Crimi, M. et al. 2001, Dall'Osto, L. et al. 2005, Wentworth, M. et al. 2000). Interestingly, Zea present in the LHCII purified from plants exposed to excess light does not affect the complex fluorescence yield in solution nor upon aggregation (Caffarri, S. et al. 2001). Previously, Zea has been shown to have a positive effect on ADQ in both monomeric Lhcb proteins (Ruban, A. V. et al. 1997b) and LHCII trimers isolated from *A. thaliana npq2* mutants (Miloslavina, Y. et al. 2008). This appears to conflict with our results but the apparent discrepancy can be explained because of the different pigment composition of LHCII trimers from *npq2* mutants and wild-type plants exposed to intense light. The *npq2* mutant lacks the enzyme zeaxanthin epoxidase and is therefore unable to synthesize Neo or Vio. LHCII proteins must therefore fold in the presence of Lut and Zea as the only xanthophylls, and these are incorporated into the inner L1 and L2 sites and the external V1 site, respectively, leaving the N1 site empty (Miloslavina, Y. et al. 2008, Mozzo, M. et al. 2008). In wild-type plants, LHCII is synthesized in the presence of Vio and Neo, whereas Zea is only produced under excess light stress and binds to the only accessible external V1 site (Caffarri, S. et al. 2001). The absence of Neo, in particular, may have a strong influence on ADQ, as discussed below.

We used LHCII isolated from plants exposed to excess light and the N1 site therefore correctly occupied by Neo. Hence we found that Zea, solely occupying the V1 site, has no effect in ADQ in agreement with previous results (Caffarri, S. et al. 2001). The LHCII-Zea preparation used in our investigation has a de-epoxiation index of 0.33, i.e. it is not highly enriched in Zea, suggesting this might be the reason for the poor quenching effect. However, the absence of a Zea-quenching effect in LHCII has been reported before (Caffarri, S. et al. 2001, Wentworth, M. et al. 2000) and we observed an increase in the average fluorescence lifetime compared to LHCII-Vio (Table 1) supporting the view that Zea accumulation is ineffective in promoting quenching in LHCII.

In contrast to LHCII, the monomeric Lhcb4-6 proteins all bind Zea at the L2 site *in vivo* (Jahns, P. et al. 2001, Morosinotto, T. et al. 2002a, Ruban, A. V. et al. 1999, Wehner, A. et al. 2006). The same site is occupied in our recombinant proteins, making them representative of the native proteins when plants are exposed to excess light *in vivo*(Ballottari, M. et al. 2009g, Gastaldelli, M. et al. 2003, Passarini, F. et al. 2009d). Zea-Lhcb5 undergoes ADQ much more efficiently than Vio-Lhcb5, whereas the differential for Lhcb6 is smaller, possibly due to the fact that this protein is already quenched in the presence of Vio (Figure 2). These results imply that the capacity of individual Lhcb proteins to undergo enhanced ADQ upon Zea binding is specifically dependent on the properties of the individual gene product.

4.3 ADQ is regulated by chlorophylls located near Lut bound at the L1 site.

Site-specific mutation allows the functions of different Chl-binding sites to be determined (Ahn, T. K. et al. 2008, Ballottari, M. et al. 2009f, Formaggio, E. et al. 2001, Passarini, F. et al. 2009c), and this has been used to investigate the architecture of the CT quenching site (Ahn, T. K. et al. 2008) and the origin of the red-shifted fluorescence emission forms in LHCI (Morosinotto, T. et al. 2003). We used this approach with Lhcb5 to identify the chromophores involved in ADQ. First, we showed that mutations do not significantly affect the fluorescence lifetimes of Lhcb5-Vio in solution, implying that quenching is only triggered upon aggregation. The only exception was mutant A5, whose fluorescence lifetime exceeded that of wild-type Lhcb5-Vio reflecting its involvement in a different type of quenching mechanism localized in the L2 domain (Ahn, T. K. et al. 2008, Ballottari, M. et al. 2009e, Passarini, F. et al. 2009b). The strongest effects on fluorescence decay were observed in the $A2_{VIO}$ and $A3_{VIO}$ mutants, and 77K fluorescence emission analysis

showed the same mutants were unable to produce a red-shifted emission band, a signature representing the formation of quenching sites (Ahn, T. K. et al. 2008, Johnson, M. P. et al. 2009, Miloslavina, Y. et al. 2008, Morosinotto, T. et al. 2003). This result is consistent with the absence of features induced by aggregation in the CD spectra of the same mutants (Figure 5). It is important to note that this effect is specific, since other mutants with intact Chl 612 and 613 show similar spectral properties to the wild type protein. In mutant $B3_{VIO}$, small differences in fluorescence and CD spectra were observed, consistent with the proximity of the mutation to Chl 613 (Liu, Z. et al. 2004). On this basis, we conclude that Chls 612 and 613 are both involved in the conformational changes induced by ADQ. Chl 614, even if not indispensable, might be involved in modulating the energy level of the Chl 613 S1 transition through its ability to establish excitonic interactions (Ballottari, M. et al. 2009d, Liu, Z. et al. 2004).

When Zea is bound to Lhcb5, multiple Chl binding sites regulate the singlet chlorophyll excited state in the unquenched and non-aggregated protein, since average fluorescence lifetimes are in all cases longer than those of the wild type protein + Zea. However, the $A5_{ZEA}$ mutant has a longer fluorescence lifetime than any other mutant (Table 1), suggesting a preferential role for Chl 603. Upon aggregation, the florescence lifetime of the $A3_{ZEA}$ mutant is far longer than the wild type or any other mutant, the most red-shifted 77K fluorescence emission is absent (Figure 4), and aggregation-specific CD signals are lost (Figure 5). On this basis we conclude that Chl 613 becomes, or participates in the formation of, the major quenching site in Zea-binding Lhcb5. It has been suggested that efficient energy quenching by aggregation requires the S1 transition energy level obtained through the establishment Chl-Chl excitonic interactions to be repressed, probably creating a mix of charge transfer state and exciton delocalization, responsible for 77K fluorescence emission red-shift (Figures 3 and 4) (Johnson, M. P. et al. 2009, Miloslavina, Y. et al. 2008). Excitonic interactions are therefore essential for efficient ADQ whereas a lone Chl cannot achieve the quenching effect. Indeed, Chl 613 is known to share a strong excitonic interaction with Chl 614, whereas Chl 612 forms excitonic interactions with Chl 611 and Chl 610. All these chlorophylls are located close to Lut in L1 site and our results therefore support the hypothesis that the domain containing L1 is preferentially involved in ADQ (Kühlbrandt, W. and Wang, D. N. 1991, Liu, Z. et al. 2004). Chl 612 and 613 play a prominent role, especially the latter when Zea is bound to the L1 site. It is worth noting that, although some mutants are affected more than others, the average fluorescence lifetime of all of them is reduced at least five-fold following aggregation, suggesting that Lhcb proteins in this state undergo conformational changes to multiple dissipative states with the contribution of all Chl molecules bound to the protein. Interestingly, mutant B6 in both its Vio and Zea-binding forms shows increased fluorescence quenching compared to the wild type. Since this mutant has lost Chl 606 and, partially, the Neo bound at site N1, we conclude that, although Chl 606 and Neo are not needed for ADQ, Neo somehow prevents the complete transition to the dissipative conformation. This, in turn, is consistent with the fact that a larger ADQ effect is observed in Lhcb6 (Figure 2), the only PSII antenna protein that cannot bind Neo (Caffarri, S. et al. 2007c). We hypothesize that the conformational change induced by aggregation involves helix C twisting with respect to helices A and B, and this is prevented when Neo binds in the groove in between these two domains (Croce, R. et al. 1999, Liu, Z. et al. 2004).

The identification of the chlorophylls and carotenoids primarily responsible for ADQ does not provide a complete explanation of the molecular mechanisms facilitating energy dissipation following aggregation, which has previously been attributed to energy transfer to carotenoid excited state S1 or the formation of a Chl-Chl charge transfer state (Miloslavina, Y. et al. 2008, Ruban, A. V. et al. 2007). Our results cannot exclude either of these mechanisms. Strong Chl-Chl excitonic interactions are detectable in our quenched samples by examining CD and 77K fluorescence spectra, as previously reported (Miloslavina, Y. et al. 2008). Although our data suggest that Chl-Chl excitonic interactions may be involved in ADQ, possibly through their ability to reduce the energy level of S1 transition(s), the final quenching site

is likely to be the Lut molecule bound to L1. Furthermore, the induction of NPQ *in vivo* has been positively correlated with the S1 carotenoid excited state (Bode, S. et al. 2009).

4.4 ADQ versus CTQ in vitro.

The physico-chemical nature of the NPQ mechanism has long been debated, owing to the importance of this process for photosynthesis and plant life (DemmigAdams, B. and Adams, W. W. 1996, Horton, P. and Hague, A. 1988, Noctor, G. et al. 1991). Full induction of NPQ requires a trans-thylakoid pH differential, the protonation of PsbS, the accumulation of Zea or Lut and the presence of Lhcb proteins (Demmig-Adams, B. 1990, Havaux, M. et al. 2004, Li, X. P. et al. 2000, Li, X. P. et al. 2002, Li, Z. R. et al. 2009b, Niyogi, K. K. et al. 1998, Niyogi, K. K. et al. 2001, Pogson, B. J. et al. 1998). Evidences supporting both ADQ and CTQ can be generated *in vitro* using native or recombinant Lhcb proteins (Ahn, T. K. et al. 2008, Avenson, T. J. et al. 2008, Horton, P. et al. 1991, Ruban, A. V. et al. 2007). We have previously shown that CTQ is localized in monomeric Lhcb4-6 subunits, and does not occur in LHCII (Ahn, T. K. et al. 2008, Avenson, T. J. et al. 2008, Avenson, T. J. et al. 2009b). Zea bound at site L2 has a dual role in CTQ, directly participating in the reaction, and also playing an allosteric role, allowing the formation of a Lut radical cation in the L1 site of Lhcb5 (Ahn, T. K. et al. 2008, Avenson, T. J. et al. 2008, Avenson, T. J. et al. 2009a). Here, we have shown that all Lhcb proteins can undergo ADQ, but the process is much more efficient in Lhcb5-6 and much less efficient in LHCII (Figure 2). Zea is not indispensable for ADQ, but enhances the process in Lhcb5 and Lhcb6. Although CTQ involves Chl 603 and 609, and strongly depends on carotenoids occupying site L2 (Ahn, T. K. et al. 2008), ADQ mainly involves chlorophylls proximal to Lut occupying site L1.

4.5 Relevance of CTQ and ADQ for NPQ in vivo.

The mechanism responsible for NPQ *in vivo* is difficult to determine since it is impossible to perform detailed spectroscopic analysis in optically challenging materials such as leaves and unicellular algae. Nevertheless, CTQ has been demonstrated in isolated thylakoids (Holt, N. E. et al. 2005, Li, Z. R. et al. 2009a) and, although the aggregation of proteins within a lipid membrane might appear unlikely, the induction of NPQ in leaves gives rise to spectral signatures similar those of Lhc proteins aggregating *in vitro* (Miloslavina, Y. et al. 2008, Ruban, A. V. et al. 2007, Ruban, A. V. and Johnson, M. P. 2009). It has also been difficult to establish whether NPQ occurs in the monomeric Lhcb proteins (Ahn, T. K. et al. 2008, Avenson, T. J. et al. 2008, de Bianchi, S. et al. 2008, Kovacs, L. et al. 2006) or in the major LHCII (Ruban, A. V. et al. 2007, van Oort, B. et al. 2010). The recent discovery that the PSII super-complex dissociates during NPQ and its components segregate into different membrane domains (Betterle, N. et al. 2009), while two distinct quenching sites are activated (Holzwarth, A. R. et al. 2009b), suggests that NPQ may involve multiple mechanisms.

Our results indicate that wherever ADQ occurs in plants during the induction of NPQ, both monomeric Lhcb proteins and trimeric LHCII can be involved, but the former contribute more efficiently to excitation energy quenching.

5.SUPPLEMENTAL DATA

SAMPLE	Chl a/b	Chl/ Car	Chls, n°	Tot Cars	Neo	Vio	Lut	Zea
LHCII VIO	1.31	3.66	14	3.82	0.91	0.65	2.26	-
LHCII ZEA	1.28	3.70	14	3.78	0.90	0.49	2.15	0.24
Lhcb4 VIO	2,39	3,64	8	2,20	0,80	0,27	1,13	-
Lhcb4 ZEA	2.21	3.68	8	2.17	0.77	-	0.81	0.59
Lhcb5 VIO	2.09	3.32	9	2.71	0.84	0.39	1.48	-
Lhcb5 ZEA	2.17	3.43	9	2.62	1.04	-	0.98	0.61
Lhcb6 VIO	1,48	4,72	10	2,12	-	0,88	1,24	-
Lhcb6 ZEA	1,48	5,03	10	1,99	0,00	0,00	0,94	1,05

Table S1: Pigment binding properties of different Lhcb subunit violaxanthin or zeaxanthin binding. *Monomeric Lhcb4, Lhcb5 and Lhcb6 subunits were reconstituted in vitro adding chlorophylls and carotenoids as described in ref. (Giuffra, E. et al. 1996), with modifications reported in ref. (Ballottari, M. et al. 2009c, Bassi, R. et al. 1999, Passarini, F. et al. 2009a). Two batches were produced for each subunit, one in presence of violaxanthin, the other in presence of zeaxanthin. LHCII trimers were instead purified native from dark adapted or high light treated Arabidopsis thaliana leaves, in order to induce violaxanthin or zeaxanthin binding into V1 site, as described in (Caffarri, S. et al. 2001). Pigments binding properties of each sample were analyzed through a combined approach of fitting analysis of pigment acetone extract absorption spectra and HPLC. Data are normalized to the number of chlorophylls present in each samples according to (Dainese, P. et al. 1992b) for monomeric LHcb4-6 and (Liu, Z. et al. 2004) for LHCII trimers. Chl a/b: molar ratio between Chl a and Chl b; Chl/car: molar ratio between chlorophylls and carotenoid; Chls, n°: number of chlorophylls bound by each complex; Tot Cars: number of carotenoids bound by each complex; Neo: neoxanthin, Vio: violaxanthin, Lut: lutein; Zea: zeaxanthin. Relative standard deviations should be considered less than 15% in each case.*

	Amp_1	τ_1	Amp_2	τ_2	Amp_3	τ_3	τ_{AV}	χ^2	$\phi_{(x\ 10^{-2})}$
LHCII V_{UNQ}	33.45%	0.58	66.55%	3.86	-	-	2.76	1.14	7.11
LHCII Z_{UNQ}	33.84%	0.29	66.16%	4.02	-	-	2.76	1.08	7.09
Lhcb4 V_{UNQ}	33.75%	1.26	66.25%	4.21	-	-	3.22	1.20	8.27
Lhcb4 Z_{UNQ}	51.13%	1.09	48.87%	3.83	-	-	2.43	1.29	6.25
Lhcb5 V_{UNQ}	26.93%	0.76	73.07%	3.81	-	-	2.99	1.19	7.69
Lhcb5 Z_{UNQ}	53.23%	0.99	46.77%	3.53	-	-	2.18	1.26	5.60
Lhcb6 V_{UNQ}	43.07%	1.32	56.93%	4.25	-	-	2.99	1.11	7.68
Lhcb6 Z_{UNQ}	62.44%	1.58	37.56%	4.76	-	-	2.78	1.36	7.15
LHCII V_{UNQ} + Lhcb6 V_{UNQ} (3:1)	38.00%	0.73	63.00%	3.95	-	-	2.77	1.10	7.12
LHCII Z_{UNQ} + Lhcb6 Z_{UNQ} (3:1)	41.00%	0.66	58.00%	4.11	-	-	2.65	1.20	6.83
LHCII V_Q	82.21%	0.09	16.83%	0.57	0.97%	2.98	0.20	1.00	0.52
LHCII Z_Q	64.83%	0.17	31.58%	0.74	3.59%	2.85	0.44	1.05	1.14

Lhcb4 V_Q	83.28%	0.08	15.90%	0.48	0.82%	2.41	0.16	1.02	0.41
Lhcb4 Z_Q	81.87%	0.07	17.27%	0.45	0.86%	2.14	0.16	1.04	0.40
Lhcb5 V_Q	78.55%	0.07	21.00%	0.44	0.46%	2.61	0.16	1.08	0.41
Lhcb5 Z_Q	93.49%	0.04	6.32%	0.41	0.18%	2.66	0.07	1.01	0.17
Lhcb6 V_Q	89.13%	0.07	10.33%	0.40	0.54%	1.69	0.12	0.93	0.30
Lhcb6 Z_Q	96.48%	0.04	3.20%	0.43	0.32%	3.12	0.06	0.95	0.16
LHCII V_Q + Lhcb6 V_Q (3:1)	90.77%	0.10	9.05%	0.38	0.18%	3.57	0.13	1.23	0.33
LHCII Z_Q + Lhcb6 Z_Q (3:1)	88.99%	0.10	10.80%	0.51	0.21%	3.00	0.15	1.22	0.38

Table S2: Time resolved fluorescence analysis on Lhcb proteins in detergent or in aggregation. *Fitting results of fluorescence decay traces (emission detected at 705 nm) measured on recombinant Lhcb4-6 proteins, native LHCII trimers and on a mixture of Lhcb6 and LHCII trimers in a 3:1 molar ratio. Samples were reconstituted (Lhcb4-6) or purified (LHCII trimers) with violaxanthin (V) or zeaxanthin (Z). Different complexes were diluted in presence of 0.03% β-DM and 20 mM HEPES (pH 7.8) or 0.003% β-DM and 20 mM citrate (pH 5.5), in order to induce unquenching (UNQ) or quenching (Q) conditions, respectively.. Amp1-3: amplitude of the exponential components 1-3; τ1-3: decay time constants (ns) of the exponential curves 1-3 used to fit the fluorescence decay curves; τAV: average fluorescence decay lifetime (ns); ϕ: fluorescence quantum yield. Errors are less than 12% in each case.*

	Mutated residues	**Chl coordinated**	**Chl a/b**	**Chl/ Car**	**Chls, n°**	**Tot Cars**	**Neo**	**Vio**	**Lut**	**Zea**	**Chl a**	**Chl b**	**Δ Chl a**	**Δ Chl b**
WT_{VIO}	-	-	2.09	3.32	9	2.71	0.84	0.39	1.48	-	6.09	2.91	-	-
$A2_{VIO}$	N179F	Chl 612	1.83	2.88	8	2.78	0.86	0.34	1.58	-	5.18	2.82	0.91	0.09
$A3_{VIO}$	Q193L	Chl 613	2.02	3.18	8	2.52	0.67	0.35	1.5	-	5.35	2.65	0.74	0.26
$A4_{VIO}$	E65V/ R181L	Chl 602	1.94	3.11	7	2.25	0.44	0.16	1.65	-	4.61	2.38	1.47	0.52
$A5_{VIO}$	H68F	Chl 603	1.97	3.17	7	2.2	0.52	0.1	1.53	-	4.64	2.36	1.45	0.55
$B3_{VIO}$	H208L	Chl 614	2.13	3.01	8	2.66	0.75	0.48	1.67	-	5.45	2.56	0.64	0.36
$B5_{VIO}$	E137V	Chl 609	2.08	3.13	8	2.56	0.85	0.24	1.44	-	5.40	2.60	0.69	0.31
$B6_{VIO}$	E129V	Chl 606	3.19	3.65	8	2.19	0.22	0.35	1.64	-	6.09	1.91	0.00	1.00
WT_{ZEA}	-	-	2.17	3.43	9	2.62	1.04	-	0.98	0.61	6.16	2.84	-	-
$A2_{ZEA}$	N179F	Chl 612	1.83	3.17	8	2.52	1.02	-	1.01	0.49	5.17	2.83	0.99	0.01
$A3_{ZEA}$	Q193L	Chl 613	1.84	2.63	7	2.66	1.06	-	1.08	0.51	4.53	2.47	1.63	0.37
$A4_{ZEA}$	E65V/ R181L	Chl 602	2.33	3.46	7	2.02	0.79	-	0.78	0.46	4.89	2.11	1.27	0.73
$A5_{ZEA}$	H68F	Chl 603	1.94	3.98	7	2.35	0.95	-	0.90	0.50	4.62	2.38	1.55	0.45
$B3_{ZEA}$	H208L	Chl 614	1.88	2.97	8	2.70	1.03	-	1.06	0.61	5.22	2.78	0.94	0.06
$B5_{ZEA}$	E137V	Chl 609	1.87	3.1	7	2.26	0.97	-	0.79	0.49	4.56	2.44	1.61	0.39
$B6_{ZEA}$	E129V	Chl 606	3.01	3.35	8	2.09	0.65	-	0.91	0.53	6.00	1.99	0.16	0.85

Table S3: Pigment binding properties of mutants in individual Chl binding residues. *Lhcb5 mutants in specific chlorophyll binding sites were obtained as described in (Ballottari, M. et al. 2009b), including violaxanthin or*

zeaxanthin in the pigment mixture added to holoproteins for protein refolding. Two batches were thus produced for each mutant, one in presence of violaxanthin, the other in presence of zeaxanthin. Pigment binding properties of WT and mutant-Lhcb5 were analyzed through a combined approach of fitting analysis of acetonic pigment extracts absorption spectra and HPLC. Data are normalized to the number of chlorophylls present in each mutant. Chl a/b: molar ratio between Chl a and Chl b; Chl/car: molar ratio between chlorophylls and carotenoid; Chls, n°: number of chlorophylls bound by each Lhcb5 complex; Tot Cars: number of carotenoids bound by each Lhcb5 complex; Neo: neoxanthin, Vio: violaxanthin, Lut: lutein; Zea: zeaxanthin; Chl a,b: number of Chl a and Chl b bound by each Lhcb5 complex. Variations of Chl a and Chl b (Δ Chla; Δ Chlb) with respect to WT are also shown: these values were calculated subtracting the number of Chl a(b) bound by each mutant to the number of Chl a(b) bound by WT. Relative standard deviations should be considered less than 15% in each case. Mutated residues and chlorophyll coordinated by amino acids subject to mutations are indicated for each mutant.

SAMPLE	Mutated residues	Chl coordinated	Amp_1	τ_1	Amp_2	τ_2	Amp_3	τ_3	τ_{AV}	χ^2	ϕ (x 10^{-2})
WT Vio_{UNQ}	-	-	26.93%	0.76	73.07%	3.81	-	-	2.99	1.19	7.69
A2 Vio_{UNQ}	N179F	Chl 612	66.66%	4.04	33.34%	0.49	-	-	2.85	1.21	7.35
A3 Vio_{UNQ}	Q193L	Chl 613	60.47%	4.11	39.53%	1.55	-	-	3.10	1.06	7.97
A4 Vio_{UNQ}	E65V/R181L	Chl 602	31.85%	0.79	68.15%	4.16	-	-	3.08	1.02	7.94
A5 Vio_{UNQ}	H68F	Chl 603	24.43%	0.96	75.57%	4.06	-	-	3.30	1.03	8.50
B3 Vio_{UNQ}	H208L	Chl 614	30.30%	0.46	69.70%	3.87	-	-	2.84	1.14	7.30
B5 Vio_{UNQ}	E137V	Chl 609	70.36%	3.73	29.64%	0.63	-	-	2.81	1.15	7.24
B6 Vio_{UNQ}	E129V	Chl 606	59.70%	4.34	40.30%	1.53	-	-	3.21	**1.14**	8.26
WT Zea_{UNQ}	-	-	53.23%	0.99	46.77%	3.53	-	-	2.18	1.26	5.60
A2 Zea_{UNQ}	N179F	Chl 612	43.83%	1.03	56.17%	3.60	-	-	2.47	1.17	6.36
A3 Zea_{UNQ}	Q193L	Chl 613	55.52%	1.63	44.48%	4.64	-	-	2.97	1.20	7.64
A4 Zea_{UNQ}	E65V/R181L	Chl 602	49.86%	3.96	50.14%	1.18	-	-	2.57	1.16	6.61
A5 Zea_{UNQ}	H68F	Chl 603	59.47%	4.03	40.53%	1.36	-	-	2.95	1.15	7.59
B3 Zea_{UNQ}	H208L	Chl 614	47.04%	3.75	52.96%	1.12	-	-	2.36	1.17	6.07
B5 Zea_{UNQ}	E137V	Chl 609	53.49%	3.68	46.51%	1.12	-	-	2.48	1.14	6.40
B6 Zea_{UNQ}	E129V	Chl 606	45.22%	4.04	54.78%	1.04	-	-	2.40	1.21	6.17
WT Vio_Q	-	-	78.55%	0.07	21.00%	0.44	0.46%	2.61	0.16	1.08	0.41
A2 Vio_Q	N179F	Chl 612	59.05%	0.11	36.71%	0.57	4.24%	2.19	0.36	1.09	0.94
A3 Vio_Q	Q193L	Chl 613	73.10%	0.11	25.50%	0.68	1.40%	3.27	0.30	1.20	0.78
A4 Vio_Q	E65V/R181L	Chl 602	83.07%	0.08	15.12%	0.57	1.81%	2.56	0.20	1.11	0.51
A5 Vio_Q	H68F	Chl 603	91.21%	0.15	7.88%	0.72	0.91%	3.26	0.22	1.11	0.57
B3 Vio_Q	H208L	Chl 614	78.27%	0.07	19.90%	0.68	1.84%	3.01	0.24	1.04	0.62
B5 Vio_Q	E137V	Chl 609	78.39%	0.06	19.78%	0.68	1.83%	3.01	0.24	1.05	0.62
B6 Vio_Q	E129V	Chl 606	86.95%	0.06	12.39%	0.48	0.66%	3.38	0.14	1.12	0.35
WT Zea_Q	-	-	93.49%	0.04	6.32%	0.41	0.18%	2.66	0.07	1.01	0.17
A2 Zea_Q	N179F	Chl 612	88.19%	0.06	9.83%	0.47	1.99%	1.87	0.13	0.95	0.35
A3 Zea_Q	Q193L	Chl 613	80.05%	0.11	14.34%	0.79	5.62%	2.68	0.35	0.93	0.90
A4 Zea_Q	E65V/R181L	Chl 602	88.78%	0.11	9.30%	0.72	1.92%	2.91	0.22	0.94	0.57
A5 Zea_Q	H68F	Chl 603	90.60%	0.08	8.87%	0.43	0.54%	2.39	0.13	0.97	0.32

B3 Zea$_Q$	H208L	Chl 614	91.74%	0.07	8.02%	0.42	0.24%	2.46	0.10	0.98	0.26
B5 Zea$_Q$	E137V	Chl 609	88.54%	0.12	10.72%	0.65	0.75%	2.60	0.19	0.97	0.50
B6 Zea$_Q$	E129V	Chl 606	44.49%	0.10	54.85%	0.11	0.66%	1.70	0.11	0.92	0.29

Table S4: Time resolved fluorescence analysis on Lhcb5 chlorophyll binding site mutants in detergent or in aggregation. *Fitting results of fluorescence decay traces (emission detected at 705 nm) measured on recombinant Lhcb5 complexes mutated on different chlorophyll binding sites, reconstituted in presence of chlorophyll a, b, lutein, neoxanthin and violaxanthin (Vio) or zeaxanthin (Zea). Samples were diluted in presence of 0.03% β-DM and 20mM HEPES pH 7.8 or 0.003% β-DM and 20mM Citrate pH 5.5, in order to induce unquenching (UNQ) or quenching (Q) condition respectively. Fluorescence emission was recorded at 705 nm. Amp$_{1-3}$: amplitude of the exponential components 1-3; τ_{1-3}: decay time constants (ns) of the exponential curves 1-3 used to fit the fluorescence decay curves; τ_{AV}: average fluorescence decay lifetime (ns). ϕ: Fluorescence quantum yield. Mutations and chlorophylls coordinated by mutated residues are indicated.*

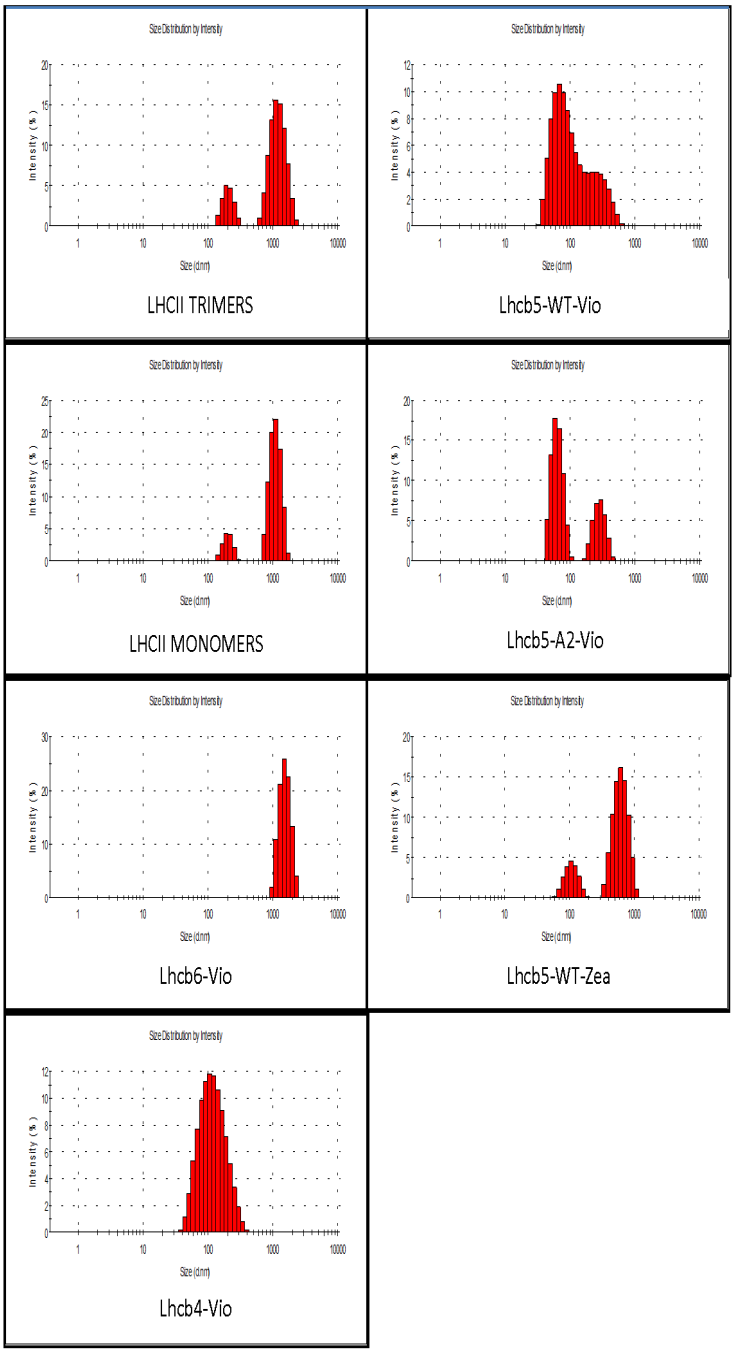

Figure S1: Size distribution from Dynamic light scattering measurements of Lhc proteins in aggregation condition. *The size of aggregates induced by detergent dilution was determined by dynamic light scattering using a ZETASIZER NANO S instrumentation as described in ref. (Cellini, B. et al. 2006). LHCII monomers were prepared from violaxanthin binding LHCII trimers as described in (Ruban, A. V. et al. 1999).*

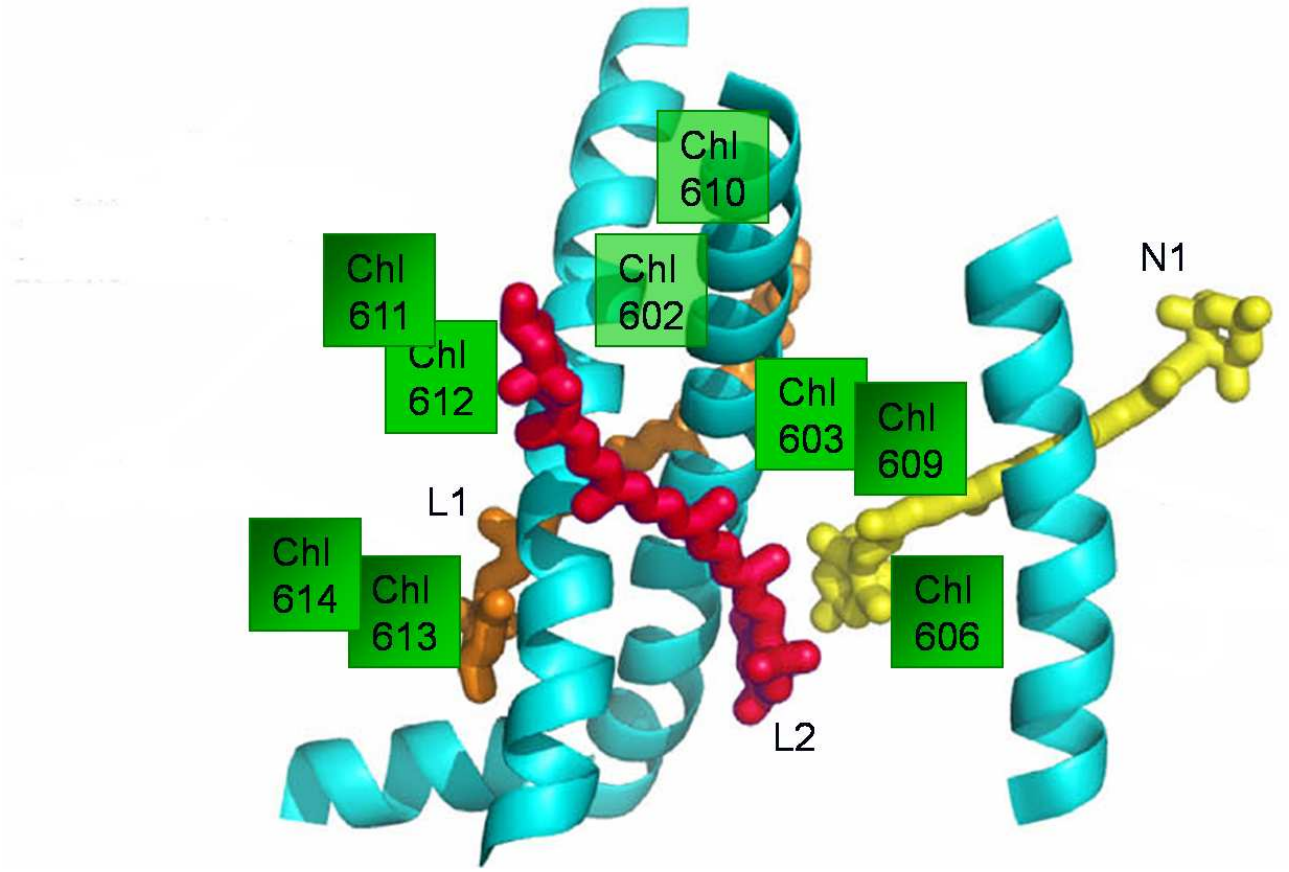

Figure S2: Model structure of Lhcb5 with chromophores bound. *Model structure of Lhb5 complex is reported: chlorophyll (Chl), carotenoids and alpha helices positions are from LHCII (Liu et al. 2004, pdb 1rwt). Different chlorophylls are shown in green: chlorophyll number is indicated according to (Liu, Z. et al. 2004). Carotenoid binding sites are indicated: according to (Ballottari, M. et al. 2009a) and (Caffarri, S. et al. 2007d), carotenoid site L1 (orange) is occupied by lutein, site L2 is occupied by lutein, neoxanthin and violaxanthin or zeaxanthin, site N1 is occupied by neoxanthin.*

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SECTION B

Membrane dynamics and reorganization for the quenching events: PsbS and zeaxanthin-dependent dissociation of a PSII pentameric supercomplex.

PsbS plays a major role in activating the photoprotection mechanism known as “Non Photochemical Quenching” which dissipates chlorophyll excited states exceeding the capacity for photosynthetic electron transport. PsbS activity is known to be triggered by low lumenal pH. However, the molecular mechanism by which this subunit regulates light harvesting efficiency is still unknown. Here we show that PsbS controls the association/dissociation of a five-subunit membrane complex, composed of two monomeric Lhcb proteins (Lhcb4 (CP29) and Lhcb6 (CP24)) and the trimeric LHCII-M. Dissociation of this supercomplex is indispensable for the onset of non-photochemical fluorescence quenching in high light, strongly suggesting that protein subunits catalyzing the reaction of heat dissipation are buried into the complex, thus not available for interaction with PsbS. Consistently, we show that knock out mutants on two subunits participating to the B4C complex are strongly affected in heat dissipation. Direct observation by electron microscopy and image analysis shows that B4C dissociation leads into re-organization of PSII distribution within grana membranes.
We interpret these results as the dissociation of B4C making available quenching sites, possibly Lhcb4 and Lhcb6, to the interaction with structures inducing switch to an energy quenching conformation, possibly PsbS. These changes are reversible and do not require protein synthesis/degradation, thus allowing for changes in PSII antenna size and adaptation to rapidly changing environmental conditions.

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This article has been selected by Alexey Amunts and Nathan Nelson for “Faculty of 1000" as new finding and interested hypothesis.

1. INTRODUCTION

Photosynthetic reaction centers (RC) exploit solar energy for driving electrons from water to $NADP^+$. This transport is coupled to H^+ transfer from chloroplast stroma to thylakoids lumen, building a proton gradient for ATP synthesis (Nelson, N. and Ben Shem, A. 2004). The capacity for light absorption is increased by pigment binding proteins composing the antenna system. In higher plants, antenna is composed of the nuclear-encoded Chl *a*/*b* binding Light Harvesting Complexes (*Lhc*). The major constituent of the Photosystem II (PSII) outer antenna is LHCII, a heterotrimer composed by different combinations of *Lhcb1*, *Lhcb2* and *Lhcb3* gene products (Jansson, S. 1999). Three additional monomeric antenna complexes (Lhcb4, Lhcb5, and Lhcb6) encoded by *lhcb4, lhcb5* and *lhcb6* genes respectively, are localized in between the core complex and LHCII (Boekema, E. J. et al. 1999a). Similarly, PSI has four Lhca antenna proteins, yielding a total of 10 distinct Lhc isoforms (Jansson, S. 1999). Differences between the mentioned isoforms have been largely conserved in all higher plants during at the last 350 million years of evolution, strongly indicating that each pigment–protein complex has a specific function (Alboresi, A. et al. 2008), although the specific role of each gene product in light harvesting and/or photoprotection is still under debate (Horton, P. et al. 2005). Their topological organization into the supercomplex has been analyzed by electron microscopy and biochemical methods (Bassi, R. and Dainese, P. 1992, Boekema, E. J. et al. 1995, Boekema, E. J. et al. 1999a, Nield, J. et al. 2000) showing that *Lhcb* subunits are organized into two layers around the PSII core: the inner one is composed of CP29, CP26 and the S-type LHCII trimer, forming, together with PSII core, the so-called C_2S_2 particle (Boekema, E. J. et al. 1999b, Morosinotto, T. et al. 2006). The outer layer is made of LHCII trimers and Lhcb6, to build up the larger $C_2S_2M_2L_x$ complexes (Boekema, E. J. et al. 1999b) in which the number of LHCII-L trimers depends on the light intensity during growth (Bailey, S. et al. 2001, Ballottari, M. et al. 2007).

This structural organization responds to requirements of light harvesting regulation: in high light, when absorbed energy is not limiting growth, PSII antenna loses the components of the external antenna layer, namely Lhcb6, LHCII-M and LHCII-L (Ballottari, M. et al. 2007) while the internal antenna components, Lhcb5, Lhcb4 and LHCII-S, are always retained in 1:1 stoichiometry with PSII core complex. This is consistent with the composition of a mutant exhibiting chronic plastoquinone reduction, mimicking over-excitation (Morosinotto, T. et al. 2006). Such an acclimation to contrasting light conditions, however, requires days to weeks (Ballottari, M. et al. 2007, Lindahl, M. et al. 1995), while plants are often exposed to rapid changes in light intensity, temperature and water availability. In these conditions, incomplete photochemical quenching leads to increased Chl excited state (^{1}Chl*) lifetime and increased probability of Chl *a* triplet formation (^{3}Chl*) by intersystem crossing. Chl triplets react with oxygen (^{3}O2) and form harmful reactive oxygen species (ROS), responsible for photoinhibition and oxidative stress (Barber, J. and Andersson, B. 1992). These harmful events are counteracted by photoprotection mechanisms consisting either in scavenging of generated ROS (Asada, K. 1999) or in prevention of their production through dissipation of the ^{1}Chl* in excess (Kulheim, C. et al. 2002, Niyogi, K. K. 2000). This latter process is known as Non Photochemical Quenching (NPQ) and is observed as light-dependent quenching of Chl fluorescence. NPQ was shown to be composed by at least two components with different activation timescale: one, feedback de-excitation quenching (qE), is rapidly activated upon increasing light intensity while a second component (qI) is slower. Full NPQ activation requires zeaxanthin synthesis (Demmig-Adams, B. 1990, Niyogi, K. K. et al. 1997) and the PsbS protein (Li, X. P. et al. 2000) which senses low luminal pH through two lumen-exposed protonable residues (Li, X. P. et al. 2002, Li, X. P. et al. 2004). Mutants lacking Chl b, thus lacking *Lhc* proteins, or exhibiting alterations in the topological organization of PSII antenna also undergo a strong reduction in NPQ, demonstrating the involvement of antenna proteins in its activation (Briantais, J.-M. 1994, de Bianchi, S. et al. 2008, Kovacs, L. et al. 2006).

In this work we have analyzed the changes in the organization of PSII antenna during exposure to strong light and NPQ development. We found that a supramolecular complex, named B4C, composed of Lhcb4, Lhcb6 and LHCII-M, connecting the inner and the outer antenna moieties, dissociates during light exposure and re-associates during subsequent dark recovery. Dissociation of the B4C complex appears to be necessary for the establishment of non-photochemical fluorescence quenching. Upon illumination, PSII distribution within grana membranes was also affected and we observed a shorter distance between PSII reaction centers, implying enrichment in C_2S_2 complexes and depletion in the outer LHCII components. These results suggest that the NPQ process includes a rapid and reversible change in the organization of grana membranes with disconnection of a subset of Lhcb proteins from the PSII reaction Centre.

2. MATERIAL AND METHODS

2.1 Plants growth, light treatment and thylakoids isolation. WT plants of *Arabidopsis thaliana* ecotype Columbia and mutants *npq1, npq2, npq4, koLhcb3, koLhcb6 and koLhcb5*, were obtained from the *Arabidopsis* Stock center; the complemented *npq4* mutant with PsbS WT and E122Q, E226Q and double E122Q/E226Q mutants were obtained as described in (Li, X. P. et al. 2002); the *npq2/npq4* double mutant was obtained crossing *npq2* and *npq4* mutants; the triple mutant *koLhcb4* was obtained crossing the single mutants *Lhcb4.1, Lhcb4.2, Lhcb4.3* obtained from *Arabidopsis thaliana* Stock center. Plants WT and mutants were grown for 4-6 weeks at 100 μmol photons m^{-2} s^{-1}, 21 °C, 90% humidity, and 8 h of daylight. Detached leaves from 3 weeks grown plants were adapted for 1 hour in the dark and eventually treated for 30' at 1500 μmol photons m^{-2} s^{-1}, or for different times as indicated in the text. Unstacked

thylakoids were isolated from leaves dark adapted or light treated as previously described (Bassi, R. et al. 1988). Membranes corresponding to 150 mg of chlorophylls were washed with 5mM EDTA and then solubilized with 0.6% α-DM. Solubilized samples were then fractionated by ultracentrifugation in a 0.1 to 1 M sucrose gradient containing 0.03% α-DM and 20 mM HEPES, pH 7.5 (rotor SW60ti, 5 h 30 min at 60,000rpm, 4°C).

2.2 Membranes isolation. Grana membranes have been isolated from dark and light adapted samples using α-DM solubilization of stacked thylakoids, as described in (Morosinotto, T. et al. 2006).

2.3 Quantification of B4C dissociation. The image of each sucrose gradient fractionation has been analyzed by using Gel-Pro Analyzer© software to determine the integrated optical density (IOD) of the different bands. B4C was quantified as the ratio between the IOD of the corresponding band and the sum of all bands corresponding to antenna proteins (band 2, 3 and 4).

2.4 Pigment analyses. Pigments were extracted from leaf discs or grana membranes (see below) with 80% acetone (v/v) and then separated and quantified by HPLC (Gilmore, A. M. and Yamamoto, H. Y. 1991).

2.5 In vivo fluorescence analysis. Non-photochemical quenching of Chl fluorescence was measured on whole leaves at RT with a PAM 101 fluorimeter (Walz, Effeltrich, Germany). Minimum fluorescence (F_0) was measured with a 0.15 µmol m^{-2} s^{-1} beam, maximum fluorescence (F_m) was determined with a 0.6 sec light pulse (4500 µmol m^{-2} s^{-1}), white continuous light (100-2000 µmol m^{-2} s^{-1}) was supplied by a KL1500 halogen lamp (Schott, Mainz, Germany). NPQ was calculated according to the following equation (Van Kooten, O. and Snel, J. F. H. 1990): $NPQ = (F_m-F'_m)/F'_m$, where F_m is the maximum Chl fluorescence from dark-adapted leaves, F'_m the maximum Chl fluorescence under actinic light exposition. For photoinhibition analyses, Fv/Fm was determined after 5 or 15 dark incubation following light treatment, with equivalent results.

2.6 Electron microscopy and image analysis. Electron microscopy (EM) on isolated grana membranes was conducted using an FEI Tecnai T12 electron microscope operating at 100 kV accelerating voltage. Samples were applied to glow-discharged carbon coated grids and stained with 2% uranyl acetate. Images were recorded using a CCD camera. Best stained grana patches were identified and PSII core position identified using Boxer software, manually edited in case of uncertain attributions. Distribution of PSII cores within the image distribution have been analyzed by a homemade procedure written in Matlab© (available upon request), determining the distance between each core and the closest (or *n* closest) neighbor. In total around 1000 points, resulting from at least 3 independent biological replications, have been considered for the analysis for each sample.

3. RESULTS

3.1 A pentameric Lhcb complex (B4C) is dissociated upon light treatment.

PSII antenna organization has been shown to be fundamental for a full establishment of NPQ, which, in fact, is significantly impaired in plants depleted of antenna proteins or where the antenna organization is affected (Briantais, J.-M. 1994, de Bianchi, S. et al. 2008, Kovacs, L. et al. 2006). In order to test whether a re-organization of grana membranes is involved in NPQ, we have analyzed the interactions between the PSII-LHCII supercomplex subunits using sucrose gradient ultracentrifugation upon mild solubilization of thylakoid membranes. To this aim, *Arabidopsis* plants were either dark adapted or illuminated with saturating light for 30'. Upon illumination, leaves were cooled in iced-water slurry and thylakoids were isolated, solubilized with α-DM and fractionated by ultracentrifugation. Different pigment-protein complexes migrated as green bands depending on their sizes, as shown in Figure 1A.

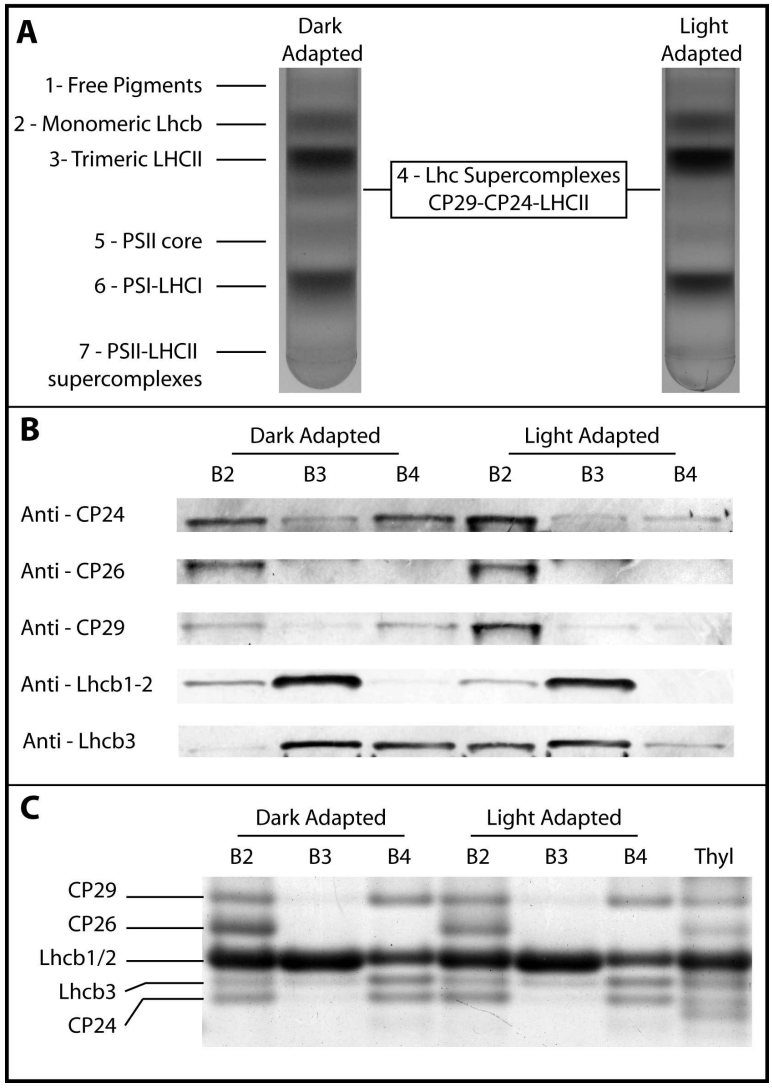


Figure 1. Light dependent dissociation of B4C protein complex in *Arabidopsis thaliana*. A. *Sucrose gradient fractionations of mildly solubilized thylakoids membranes purified from dark and light adapted (30' at 1500 μE) leaves. In dark thylakoids pigment binding complexes separates into seven distinct bands, the fourth one (B4C) being depleted in light treated sample.* ***B.*** *Distribution of monomeric antenna proteins in sucrose gradients between bands no. 2, 3 and 4 from dark and light adapted samples. Western blotting analysis was carried on using specific antibodies against Lhcb6 (CP24), Lhcb5 (CP26) and Lhcb4 (CP29), Lhcb1-2 and Lhcb3 respectively. Samples from different bands were loaded in amounts proportional to their abundance in the sucrose gradient. In Lhcb1-2 blotting each band was loaded with five time less protein to avoid antibody signal saturation.* ***C.*** *Coomassie stained SDS PAGE loading equal Chl amounts (2 μg) of sucrose gradient bands no 2, 3 and 4 from dark adapted samples. Bands corresponding to Lhcb4, Lhcb5, Lhcb1/2, Lhcb3 and Lhcb6, as identified by western blotting, are indicated. Only the gel region were antenna polypeptides are migrating is shown.*

Grana							
Sample	**Chl a/b**	**neo**	**viola**	**antera**	**lut**	**zea**	**β-car**
WT Dark	**2.19**	**4.9**	**3.1**	**0.0**	**12.1**	**0.0**	**6.5**
SD	0.04	0.4	0.2	0.0	0.3	0.0	0.4
WT Light (30')	**2.16**	**5.3**	**1.6**	**0.5**	**12.0**	**0.9**	**6.0**
SD	0.03	0.5	0.2	0.1	0.3	0.1	0.6
WT Light (90')	**2.33**	**4.8**	**1.4**	**0.4**	**11.2**	**1.1**	**5.9**
SD	0.03	0.3	0.1	0.1	0.2	0.1	0.2
WT Recovery (90')	**2.42**	**4.8**	**1.9**	**0.8**	**10.9**	**0.7**	**6.2**
SD	0.07	0.4	0.1	0.1	0.5	0.0	0.1
N4 Dark	**2.37**	**5.2**	**2.4**	**0.0**	**12.0**	**0.0**	**6.6**
SD	0.02	0.7	0.1	0.0	0.0	0.0	0.1
N4 light (30')	**2.31**	**5.7**	**1.5**	**0.3**	**12.2**	**0.6**	**6.1**
SD	0.04	1.1	0.2	0.1	0.1	0.1	0.3
N4 light (90')	**2.22**	**6.2**	**1.5**	**0.3**	**12.5**	**0.8**	**5.5**
SD	0.04	1.3	0.2	0.1	0.4	0.1	0.3

Leaves							
Sample	**Chl a/b**	**neo**	**viola**	**antera**	**lut**	**zea**	**β-car**
WT Dark	**3.12**	**4.5**	**4.3**	**0.2**	**12.7**	**0.0**	**7.1**
SD	0.26	0.4	0.3	0.0	1.1	0.0	0.0
WT Light (30')	**3.31**	**4.9**	**1.5**	**0.9**	**11.5**	**2.3**	**7.6**
SD	0.02	0.4	0.1	0.1	0.1	0.1	0.3
WT Light (90')	**3.20**	**4.3**	**1.2**	**0.6**	**12.3**	**2.4**	**6.5**
SD	0.07	0.3	0.0	0.0	1.0	0.2	1.5
WT Recovery (90')	**3.20**	**4.3**	**2.9**	**1.4**	**12.5**	**1.1**	**6.7**
SD	0.03	0.1	0.4	0.3	1.1	0.1	0.3

Table 1. Pigment binding properties of light treated leaves and isolated grana membranes. *Pigment composition of leaves during the light treatment was analyzed by HPLC (neo, neoxanthin; vio, violaxanthin; antera, anteraxanthin; zea, zeaxanthin, β-car, β-carotene). Values are reported as normalized to 100 total Chl molecules.*

Light treatment affected the band pattern and the sucrose band n° 4 (B4) disappeared. This band is made of a Lhc supercomplex composed by monomeric Lhcb6 (CP24), Lhcb4 (CP29) and a LHCII trimer (Bassi, R. and Dainese, P. 1992). From now on we will refer to this pentameric Lhc complex as B4C. The distribution of individual *Lhcb* proteins among different green bands was analyzed in detail by using specific antibodies. As shown in figure 1B, a large fraction of Lhcb6 and Lhcb4 subunits are present in the B4C from the dark adapted sample, while the rest, in monomeric form, is found in fraction n°2 (B2). After light treatment, Lhcb6 and Lhcb4 are barely detectable in the gradient fraction corresponding to B4C, while they are now increased in B2 fraction. These results show that the reduction of B4C in the gradients is indeed due to the dissociation of this oligomeric antenna complex. Among the other polypeptides analyzed, it is very interesting to observe that Lhcb3 is an enriched component of B4C in the dark. Upon light treatment, Lhcb3 is instead found in the monomeric fraction, confirming this subunit is participating to light dependent dissociation of B4C supercomplex. Lhcb1-2, the major components of LHCII trimers are mostly found in B3, as expected, and in monomeric, B2, fraction. A fraction of Lhcb1-2 is also detected in B4C: their relative abundance appears to be rather

low. However, this is only due to their high enrichment in B2 and B3 fractions. In fact, by loading equal Chl amounts of B2, 3 and 4 in a Coomassie stained gel, it is clear that Lhcb1-2 are indeed present in significant amounts in B4C according to a previous report (Bassi, R. and Dainese, P. 1992). As a further confirmation that Lhcb1-2 are genuine B4C components we can observe that their content in B4C is reduced upon light treatment as all the other previously mentioned components of this complex (figure 1B). Lhcb5, instead, is not involved in B4C formation, as shown by the fact that it is found in B2, in a monomeric state, irrespective from the treatment.

We thereafter analyzed the dependence of B4C dissociation on light intensities: dissociation increased with the level of illumination, reaching saturation above 1500 μE (figure 2A).

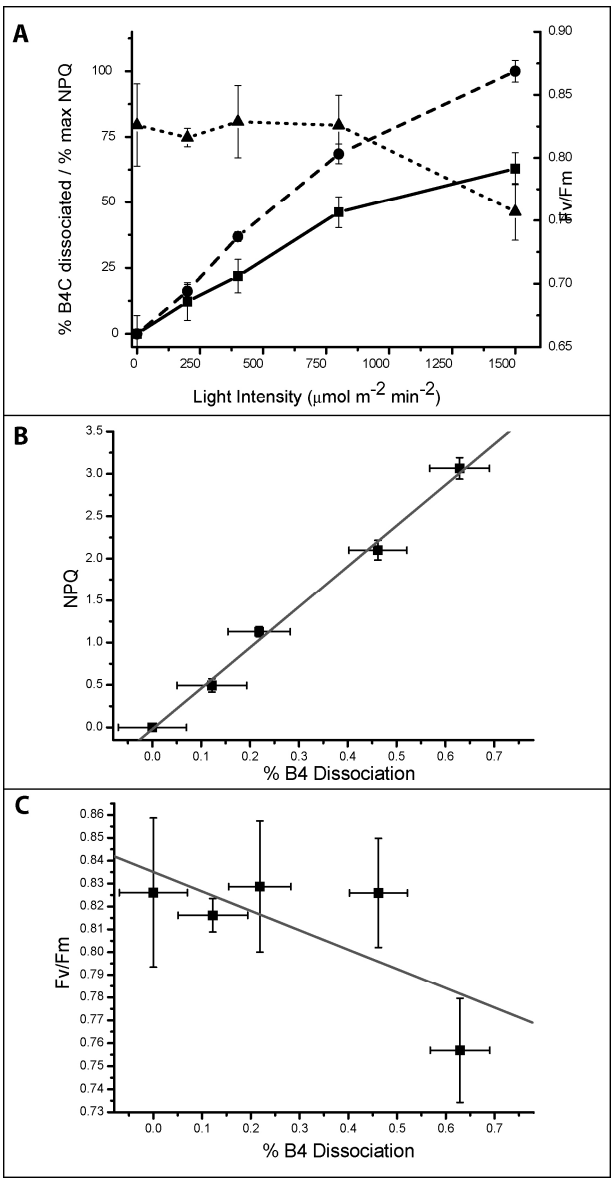


Figure 2. ***A.*** *Comparison of B4C dissociation (solid line, squares), NPQ (dashed line, circles) and PSII yield (Fv/Fm, dotted line, triangles). All parameters were evaluated after 30 minutes of light treatment with the indicated intensity. B4C is quantified as the ratio of the band with respect to the sum of all bands containing antenna upon separation of pigment binding complexes in sucrose gradient or non denaturing gel. NPQ values in order to have comparable scale with B4C dissociation is expressed as fraction of maximal value, 3.1. Fv/Fm values where measured after 15 minutes of dark incubation following light treatment to allow for PSII reduction and NPQ relaxation but not recovery from photo-inhibition.* ***B.*** *Correlation between B4C dissociation and NPQ measured with different light intensities is analyzed in more detail. Linear fitting of reported points is shown in red (R value is 0.997).* ***C.*** *Correlation analysis of Fv/Fm and*

B4C dissociation determined using different illumination intensities. Best linear fitting of reported points is shown in red (R value is 0.71).

State transitions are known to involve dissociation of LHCII subunits from PSIIRC (Wollman, F. A. 2001) and cannot be excluded as the cause of B4C complex dissociation. However, state transition are saturated at low light (200 μE) and are inhibited by high light, in agreement with their role in balancing PSI vs. PSII photon trapping under limiting light conditions (Bellafiore, S. et al. 2005, Tikkanen, M. et al. 2006) and thus are unlikely to be correlated with B4C dissociation.

Also, we did not observe any increase in phosphorylated Lhcb4 in light treated samples, consistently with previous results (Tikkanen, M. et al. 2006), an indication that this phenomenon is also unrelated to B4C dissociation.

At least two processes can be active in high light conditions leading to B4C dissociation: one is photoinhibition, meaning light induced damages in PSII, and the other is Non Photochemical Quenching. In order to test whether one of these processes (or both) are involved in B4C dissociation, we have measured the recovery of variable fluorescence, a marker of photoinhibition, upon illumination with different intensities (1500 μE, Figure 2A). It clearly appears that Fv/Fm is significantly affected only with the highest light intensity, while B4C dissociation, instead, occurs with weaker illuminations, suggesting the two phenomena are not correlated. Instead, the level of B4C dissociation at different light intensity closely matches the amplitude of NPQ obtained in the same light conditions (Figure 2A); in fact, both NPQ and B4C shows activation with increasing illumination. This correlation is evidenced in figure 2B, where it can appreciated how B4C dissociation and NPQ have a similar dependence on light intensity, differently from Fv/Fm (figure 2C). We thus tentatively conclude that B4C dissociation correlates with NPQ rather than with photoinhibition.

We analyzed the time dependence to light of B4C dissociation: as shown in figure 3A dissociation increases with time.

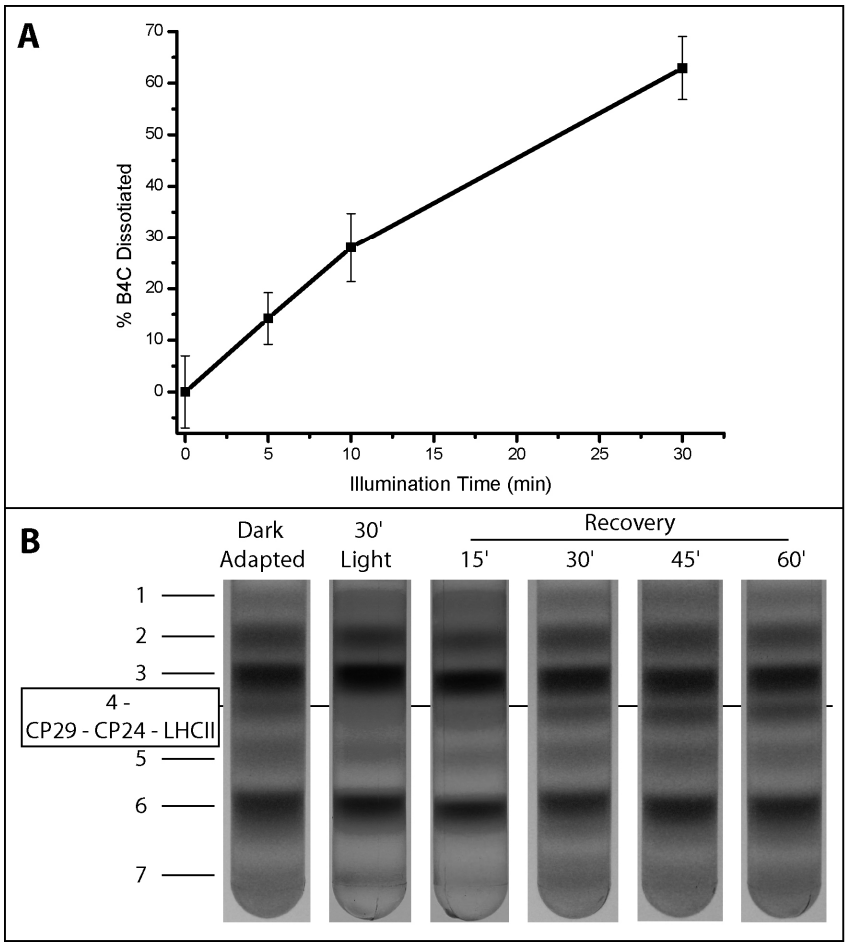


Figure 3. Time dependence of B4C dissociation and re-association in *Arabidopsis thaliana*. *A.* *B4C dissociation was determined from sucrose gradients after illumination with different duration of 1500 μE illumination. B4C is quantified as above.* ***B.*** *Re-association of B4C complex in samples left in the dark dissociation for 15, 30, 45 and 60' after a 30 minutes long illumination at 1500 μE.*

This process is reversible and, following dark incubation, B4C slowly re-associates: upon a lag phase of 15 min, B4C can be again detected at 30' and the dark control level is reached upon 45 minutes of dark adaptation (Fig 3B). We also verified that these results were not due to the thylakoid fractionation technique we used, by analyzing the kinetic of B4C dissociation by non-denaturing Deriphat-PAGE: we obtained results fully consistent with sucrose gradient analysis (data not shown).

3.2 Pharmacologic and genetic analysis of B4-complex dissociation.

To investigate in more detail the correlation between NPQ and B4C dissociation, light treatment was performed on leaves treated with nigericin, a NPQ inhibitor through its uncoupler effect for the trans-membrane pH gradient (Figure S1A). As shown in figure 4A, in nigericin-treated leaves, the B4C complex is stable, implying its light dependent dissociation requires the presence of a trans-membrane pH gradient.

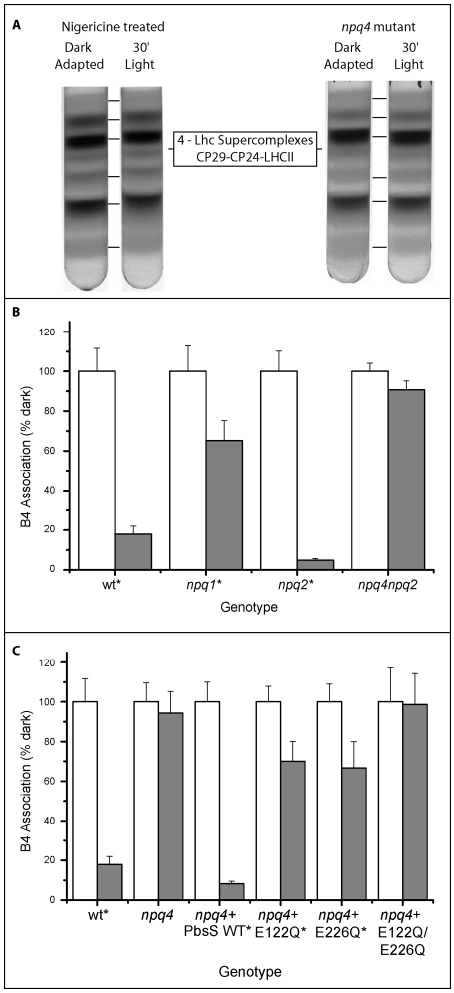


Figure 4. Dependence of B4C dissociation on ΔpH, PsbS and Zeaxanthin *A.* *light dependent B4C dissociation in nigericin treated leaves (left) and PsbS depleted mutants (npq4, right);* ***B.*** *Quantification of B4C in leaves either dark adapted (white) or treated with 1500 μE for 30 minutes (grey). WT is compared with mutants affected in zeaxanthin biosynthesis (npq1) or accumulating constitutively zeaxanthin (npq2) and in npq2npq4 double mutant in dark adapted (white).* ***C.*** *Quantification of B4C in leaves either dark adapted (white) or treated with 1500 μE for 30 minutes (grey).*

*WT is compared to npq4, also complemented with PsbS WT or mutated E122Q and/or E226Q. Quantifications in B-C are done as in figure 1. All genotypes where dark and light treated samples are significantly different (p>0.05, n=3) are indicated with *. NPQ measurements for all genotypes are reported in supplemental figure S1.*

To dissect the components of the relation between B4C and NPQ, we analyzed a series of mutants affected in NPQ at different levels, namely: *npq4* impaired in qE, the NPQ fastest component, due to the absence of PsbS (Li, X. P. et al. 2000); *npq1* (no zeaxanthin and reduced NPQ (Niyogi, K. K. et al. 1998)) and *npq2*, with constitutive zeaxanthin and accelerated NPQ (Niyogi, K. K. et al. 1998) as well as the double *npq2/npq4* mutant. NPQ kinetics of these genotypes have been reported in earlier work and are here shown in Figure S1 (Dall'Osto, L. et al. 2005, Li, X. P. et al. 2000, Niyogi, K. K. et al. 1998). Results in figure 4B and C show that the *npq4* mutation completely abolishes the light dependent B4C dissociation, even in the presence of constitutive zeaxanthin, implying a key role of PsbS protein in the process. Zeaxanthin is also a factor for B4C dissociation: in fact, in its absence, the *npq1* mutant undergoes only 30% dissociation of B4C upon illumination, while, when present constitutively, as in the *npq2* mutant, B4C is dissociated faster than in WT (not shown).

PsbS activity in NPQ has been reported to be dependent on protonation of two lumen-exposed glutamate residues (Li, X. P. et al. 2002, Li, X. P. et al. 2004). The analysis of B4C dissociation on mutants at these Glu residues, thus, allows verification of the hypothesis that the PsbS activity in B4C dissociation and in NPQ is mediated by the same mechanism, i.e. the protonation of E122 and E226 residues. To this aim, we analyzed *npq4* mutants complemented with PsbS-encoding sequences carrying either WT, E122Q, E226Q or double E122Q/E226Q mutant sequence (Li, X. P. et al. 2002, Li, X. P. et al. 2004). Data in figure 4C show that the double mutant is unable to dissociate B4C, while each of the single glutamate mutants undergoes approximately 40% B4C dissociation. The complementation with WT PsbS sequence, instead, fully restored the capacity for light dependent B4C dissociation. Thus, the extent of B4C dissociation was completely correlated with NPQ activity of each genotype (all kinetics are shown in additional figure S1). It is worth mentioning that PsbS protein was detected in sucrose gradients in several of the pigmented fractions, as previously reported (Dominici, P. et al. 2002, Teardo, E. et al. 2007) and that this distribution did not change upon light treatment.

3.3 Specific Lhcb mutants show constitutive dissociation of B4C

PsbS crucial role in NPQ is known since several years (Li, X. P. et al. 2000); upon an intense debate, it is now widely recognized that its role is to sense the low lumenal pH thus inducing a quenching conformation of specific antenna complexes where the quenching reaction(s) occur (Ahn, T. K. et al. 2008, Bonente, G. et al. 2008a, Ruban, A. V. et al. 2007). Since antenna polypeptides are involved both in structurally building the B4C and in catalyzing the quenching reactions, we have investigated the effect of knocking out individual components of the PSII antenna system on the levels and kinetics of NPQ and on the structural stability of the B4C. In figure 5A sucrose gradients fractionation patterns of thylakoids purified from dark adapted plants depleted in Lhcb6, Lhcb5 (de Bianchi, S. et al. 2008), Lhcb1+2 (Andersson, J. et al. 2003), Lhcb4 and Lhcb3 (this work) are shown.

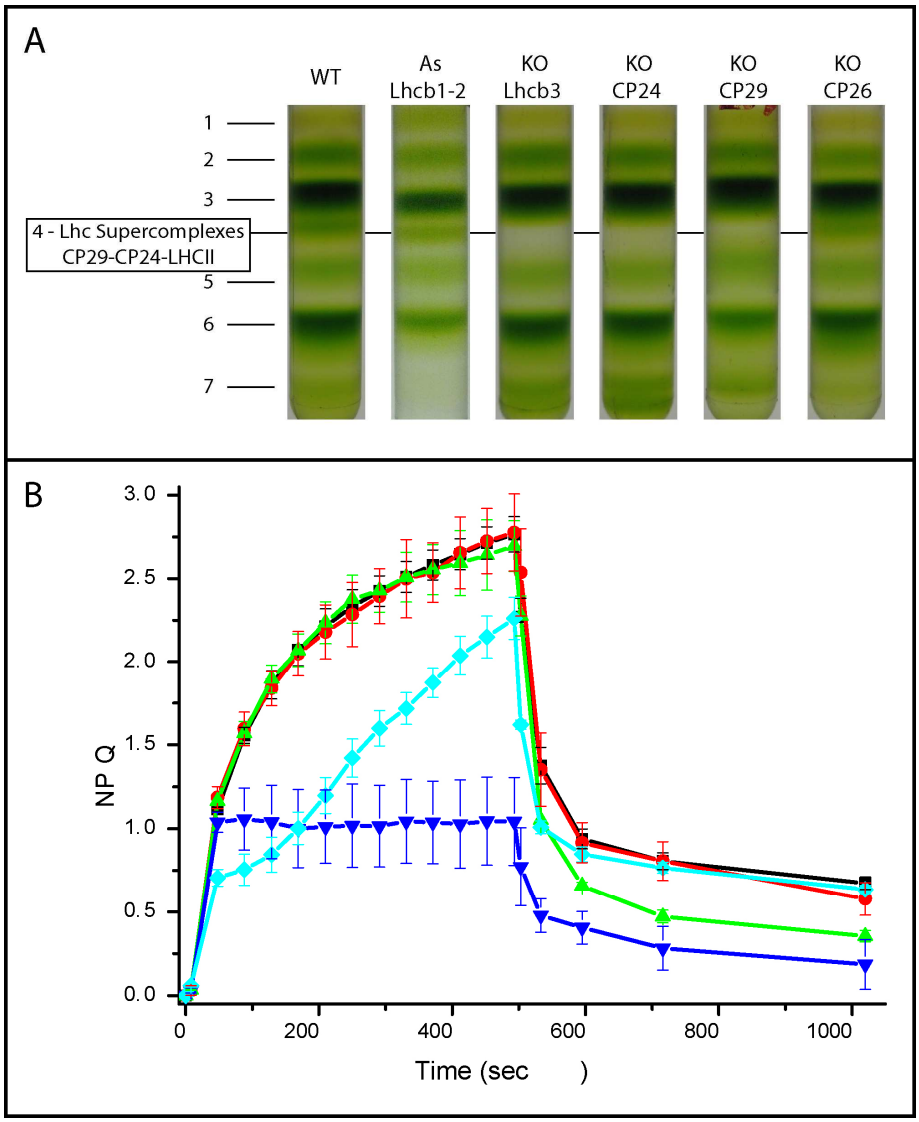


Figure 5. Dependence of B4C and NPQ on the presence of individual Lhcs. ***A.*** *The capacity of B4C formation was analyzed, in plants depleted in individual Lhcb proteins, by separating different pigment binding complexes from dark-adapted thylakoid membranes. Genotypes analyzed were WT, koLhcb3, koCP24 (Lhcb6), koCP29 (Lhcb4) (which is a triple KO in all Lhcb4 Arabidopsis isoforms, Lhcb4.1, Lhcb4.2, Lhcb4.3) and koCP26 (Lhcb5) .* ***B.*** *NPQ dependence on antenna composition, analyzed by comparing WT (black) with plants depleted in CP26 (green), CP24 (blue), CP29 (KO in Lhcb4.1, Lhcb4.2 and Lhcb4.3, cyan), Lhcb3 (red).*

We observe that KO mutants for some B4C components, namely Lhcb4, Lhcb6 and Lhcb3, do actually miss B4C. Instead, depletion of Lhcb1+2 does not affect B4C abundance in the sucrose gradient, implying that Lhcb3 is not equivalent to the other LHCII components as for its contribution for the building of B4C. On this basis we propose that B4C complex formation requires the direct interaction of Lhcb3, Lhcb4 and Lhcb6. On the contrary, Lhcb1 and Lhcb2, although participating to the trimeric LHCII M component, are not indispensable for the B4C formation. Consistently, B4 is present in *koLhcb5* plants.

Analysis of the above genotypes by pulse fluorometry during light treatment showed that the mutants on B4C components were not equivalent as for their NPQ behavior: ko-Lhcb3 did not show any effect while Lhcb4 and Lhcb6 were deeply affected, showing significant decrease in the NPQ amplitude and slower induction kinetic. While the effect of Lhcb6 depletion on NPQ process was recently well described (de Bianchi, S. et al. 2008, Kovacs, L. et al. 2006), the NPQ kinetics of *koLhcb4* reveals that also this subunit has a relevant role on NPQ induction, differently from previous suggestions coming from antisense plants (Andersson, J. et al. 2001). Lhcb1+2 depleted plants showed very similar

kinetic but slightly lower amplitude of NPQ with respect to WT (not shown), in agreement with previous reports (Andersson, J. et al. 2003). CP26 is the only Lhcb complex not directly involved in B4C assembly, in agreement with its location in a different domain of the PSII supercomplex (Boekema, E. J. et al. 1999a). The NPQ kinetic of *koLhcb5* plants is unaffected in the onset during light treatment but has a faster recovery of fluorescence in the dark implying an effect in the qI component (figure 5B). We conclude that the presence of a stable B4C complex is not a pre-requisite for NPQ and that the NPQ properties of the mutants depend on the residual composition in Lhcb complexes of the different genotypes. However, when B4C complex is present, its dissociation is needed for NPQ expression.

3.4 NPQ upon prolonged illumination

NPQ and B4C dissociation have in common a dependence on light intensity and presence of PsbS. These phenomena, however, are activated with different timescales: literature data show that onset of NPQ requires only few minutes, while B4C dissociation needs a longer illumination. In order to compare the two processes over the same time span, we measured NPQ kinetics using longer exposure to actinic light with respect to what is usually reported in the literature. In figure 6 we show the NPQ kinetic in WT *Arabidopsis* plants upon illumination for 8, 30 and 90 minutes.

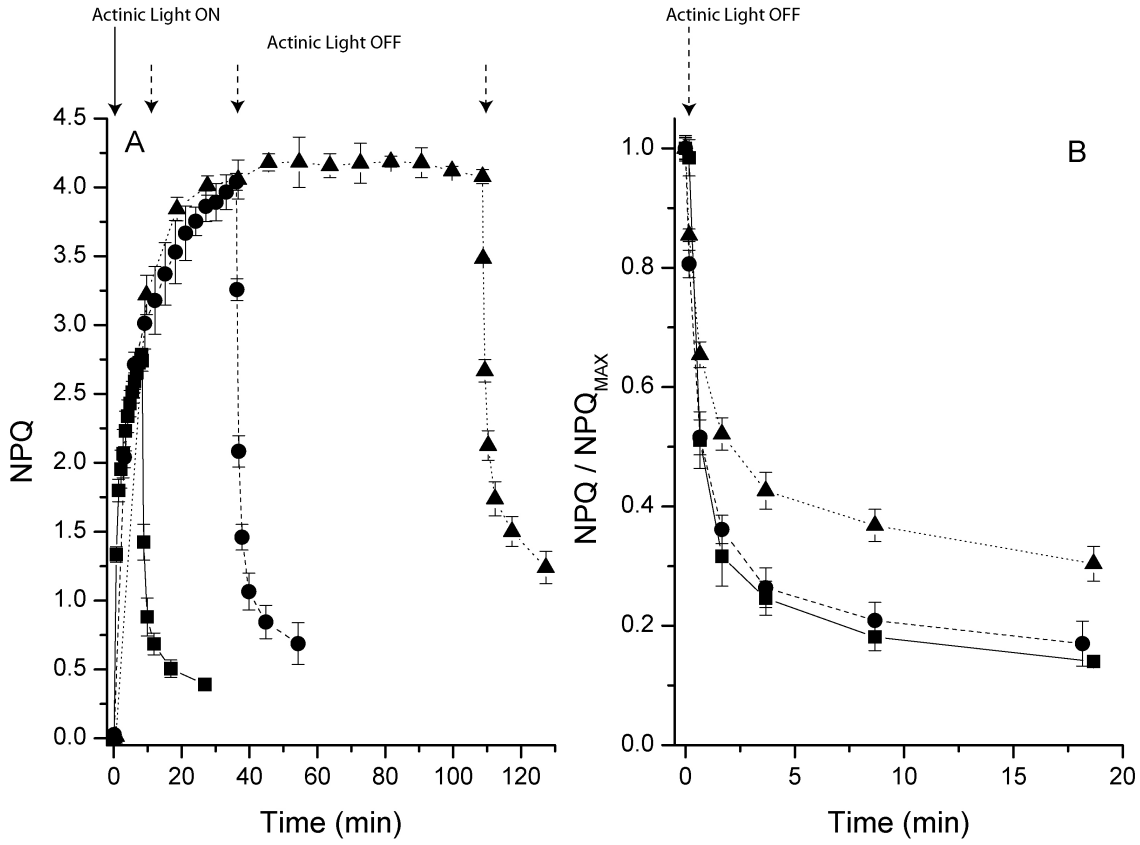


Figure 6. Long term NPQ induction and recovery. ***A.*** *NPQ kinetics in WT leaves was recorded using different durations of actinic light. For all samples NPQ was recorded in 12 points during illumination. Curves with 8, 36 and 108 minutes of actinic light are shown respectively in solid line and squares, dashed and circles, dotted and triangles. In all samples six points were recorded during the dark recovery with the same time intervals.* ***B.*** *Enlargement of recovery kinetics shown in A, after normalization to maximal NPQ.*

These kinetics show that the largest part of NPQ is activated within a few minutes from illumination ($t_{1/2}$ is 3 minutes) but continues to rise for up to 30 minutes. After 90 minutes of illumination NPQ amplitude is stable at its saturated

level. However, the kinetic of dark relaxation was slower upon 90' illumination with respect to what observed after 8 or 30 minutes light. This is evidenced in figure 6B, where NPQ decay curves are normalized to the maximal NPQ value: while relaxation kinetics are similar upon 8 or 30 minutes of illumination, they are slower upon 90 minutes treatment. We can exclude that this effect is due to the normalization procedure, since the level of quenching is very similar following 30 and 90 minutes of illumination.

The possibility that photoinhibition could be a reason for the slowdown of fluoresce recovery was assayed by measuring dark recovery of PSII quantum yield after light treatment. Since this was essentially the same in plants light treated for 30 or 90 minutes, we conclude that differential photoinhibition is not the cause of the different kinetics of fluorescence recovery after NPQ (supplementary figure S2). Alternatively, sustained quenching could be due to zeaxanthin accumulation (Dall'Osto, L. et al. 2005); however, no difference was observed in Zea content in leaves treated with 30 or 90 minutes of actinic light and the recovery of violaxanthin content was very similar (supplementary figure S2).

We conclude that, besides the two well known phases of NPQ consisting into a rapidly developing step dominated by qE and by a slower phase depending on zeaxanthin synthesis (Demmig-Adams, B. 1990) we can distinguish a later phase with sustained quenching during which the properties of the photosynthetic apparatus undergo changes that cannot be explained in terms of photoinhibition or zeaxanthin quenching and yet significantly affect the recovery of fluorescence.

3.5The organization of grana membranes is modified upon PsbS-dependent dissociation of B4C complex.

In order to test the hypothesis that the slowly developing changes of the NPQ slowest phase described above involve a reorganization of the thylakoid membrane, we have studied the organization of the PSII components in grana membranes through direct observation by electron microscopy. In grana partitions, the PSII complex form C_2S_2 particles (Boekema, E. J. et al. 1999a) constitute the inner antenna system which is maintained even in HL adapted plants (Ballottari, M. et al. 2007) and include a subset of the B4C complex, namely CP29. The rest of B4C components are part of the outer antenna which is assembled in low light conditions and leads to the formation of larger $C_2S_2M_2L_X$ particles (Boekema, E. J. et al. 1999a). This suggests that integrity of B4C is required for the formation of large supercomplexes and its dissociation could lead to a reorganization of the antenna system with a consequent reduction of antenna size. In order to test this hypothesis we have analyzed the organization of grana membranes in WT and selected mutants upon light treatment. To this aim we isolated grana membranes from plants illuminated for different periods, using a mild solubilization with α-DM, which preserves the interaction between PSII components and the overall mutual organization of PSII units (Morosinotto, T. et al. 2006). Photosystem II forms tightly packed domains in grana partition membranes with very little lipids interspersed between protein complexes (Tremolieres, A. et al. 1994). When observed by TEM upon negative staining, PSII membranes are characterized by stain-excluding particles with a tetrameric structure which can be readily identified as PSII cores (Figure 7). Lhcs protrude less from the membrane plane with respect to PSII RC and are thus covered by electron-dense stain. Observation of grana membranes isolated from leaves treated with different light intensities did not show any eye catching difference (Figure 7).

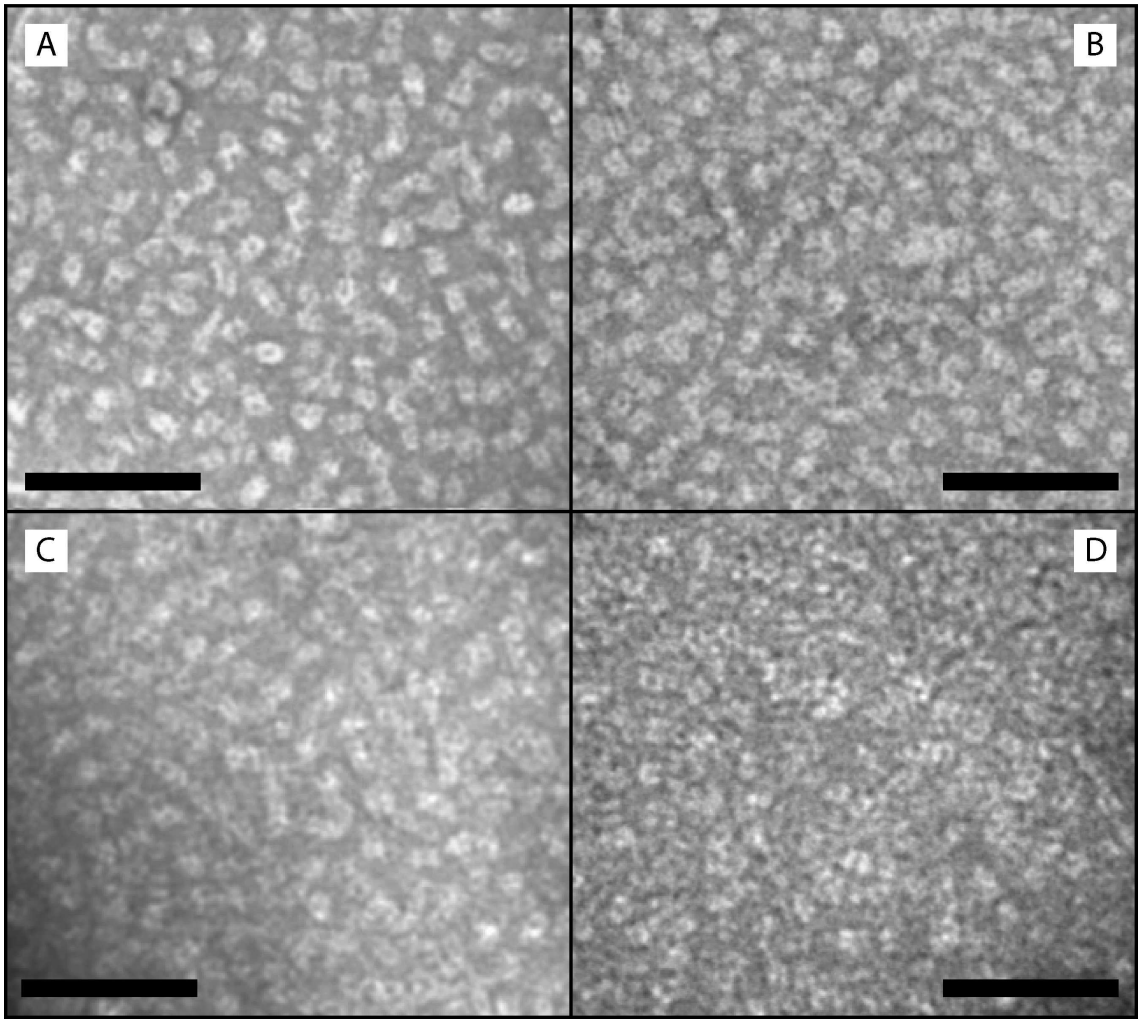


Figure 7. EM of grana membranes isolated upon light treatment. *EM micrograph of grana membrane isolated from WT leaves either dark adapted (A), treated with 30 (B), 90 light (C) or with 90 minutes of light and 90 of dark (D). Space bar represents 100 nm.*

Searching for more subtle differences, we identified in each image the position of the PSII cores and calculated the distance of the closest neighbor. The distribution of the distances is reported in figure S3: in most cases, the closest PSII core is located at 16-17 nm. Two shoulders in the distribution are also visible, one at 14 nm and a second one at 20-21 nm. This distribution with one main peak and two symmetric shoulders, is close to that obtained previously using different microscopic methods: AFM and freeze fracture (Kirchhoff, H. 2008a, Kirchoff, B. K. et al. 2008). This allows concluding that, despite the use of a different approach, results we obtained for dark adapted sample are well consistent with published data, strongly supporting the reliability of grana isolation and image analysis procedures.

In order to reduce the dependence of the results on possible imprecise assignment of PSII cores, we repeated the analysis by calculating for each core the average distance from the four closest PSII cores, rather than from the closest neighbor only. As shown in figure 8, data deviation in this case is smaller, since the error in PSII core location is partially compensated by the larger sampling. This method was thus employed to analyze grana membranes isolated from light treated leaves (data considering only the closest neighbor are shown in supplementary material).

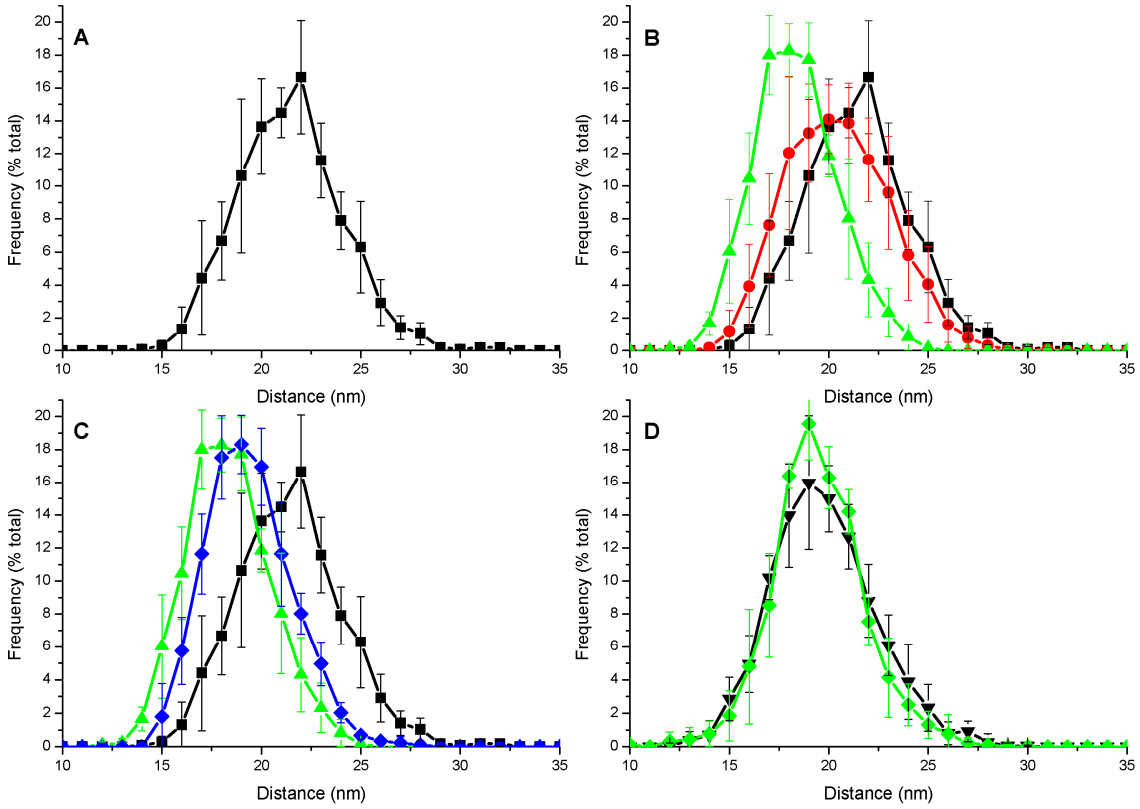


Figure 8. Analysis of PSII distribution in isolated grana isolated from dark/light treated leaves. ***A.*** *Distribution of PSII complexes in grana membranes from dark adapted leaves, expressed as the average distance from the four nearest PSII core complexes.* ***B.*** *Comparison of PSII distribution in samples dark adapted (black squares) or treated with 30 (red circles) and 90 minutes (green triangles) of light before grana isolation.* ***C.*** *Comparison of light adapted sample (green triangles) with sample treated successively with 90 minutes of dark following light treatment (blue diamonds).* ***D.*** *Effect of light treatment on PSII distribution in npq4 grana membranes. Dark adapted sample is in black triangles, 90 minutes light treated in green diamonds. SD in the case of X axes is maximum 1.5 nm*

As shown in figure 8B, upon 30 minutes of illumination the PSII distribution shows a decrease in the population with average distance of 22 nm and a compensating increase of the 18-19 nm population. The difference is significantly larger in the sample illuminated for 90 minutes: in this case, there is a far lower abundance of PSII cores with 22 nm of average distance, while the highest frequency is found at 18 nm.

We also verified the reversibility of the phenomenon by analyzing samples that, after 90 minutes of illumination, were further incubated at dark for 90 minutes before grana isolation. In this case (figure 8C), we observed that the average distance between PSII increased, showing a decrease in content of PSII cores <17 nm and an increase in abundance for all points >19 nm. Thus, although the recovery is partial and likely requires longer time to be completed, these data strongly suggest the phenomenon is reversible. As a control, it is interesting to observe the phenotype of PsbS less mutants *npq4*. Data from mutant grana isolated before and after 90 minutes of light treatment are shown in figure 8D: remarkably, the distribution is substantially unaffected by light treatment. These results, thus, allow concluding that light induces a redistribution of PSII cores within grana membranes but only in the presence of PsbS protein.

4. DISCUSSION

4.1 B4C complex has a key role in building the PSII supercomplex.

Lhcb4 and Lhcb5 are located in direct contact with the PSII core complex (Boekema, E. J. et al. 1999b, Dekker, J. P. and Boekema, E. J. 2005, Yakushevska, A. E. et al. 2003) and interact with the LHCII-S trimer to form the so-called

C_2S_2 particles (Boekema, E. J. et al. 1999b). This PSII complex organization is found in plants grown in any conditions, even very intense light and in mutants with constitutively reduced plastoquinone where PSII is constantly over-excited (Morosinotto, T. et al. 2006). C_2S_2 particles thus represents the minimal PSII supercomplex.

Growth in low or moderate light leads to the accumulation of additional antenna complexes, Lhcb6, LHCII-M and LHCII-L trimers (Ballottari, M. et al. 2007, Frigerio, S. et al. 2007) and formation of an outer antenna system in $C_2S_2M_2$ particles or higher mass supercomplexes (Boekema, E. J. et al. 1999b). PSII-LHCII supramolecular complexes thus appear to include two layers of Lhc proteins: the inner, composed by Lhcb5, Lhcb4 and LHCII-S trimer and the outer, including Lhcb6 and at least two LHCII trimers (M and L).

The B4C is a Lhc supercomplex composed by Lhcb4 (inner layer), Lhcb6 and LHCII-M (outer) (Yakushevska, A. E. et al. 2003). B4C was described (Bassi, R. and Dainese, P. 1992) as a product of incomplete solubilization of PSII membranes with mild detergents. It was later shown to represents a structural brick of PSII-LHCII supercomplexes as shown by electron microscopy and cross-linking studies showing the interactions of these subunits (Boekema, E. J. et al. 1999a, Boekema, E. J. et al. 1999b, Harrer, R. et al. 1998). Its presence is fundamental for the structure of PSII supercomplexes with larger antenna sizes. A further confirmation of this view comes from the observation that green algae, which do not have Lhcb6 and Lhcb3 subunits, are not able to form B4C complex and showed the present of C_2S_2 particles only (Dekker, J. P. and Boekema, E. J. 2005). It can be asked if all Lhcb4 and Lhcb6 subunits are organized into a B4 supercomplex with a LHCII trimer. Figure 1 shows that a non negligible amount Lhcb4 and Lhcb6 is found as monomers. However, in a previous report from *Zea mays* 100% of Lhcb4 and Lhcb6 were found in the B4C (Bassi, R. and Dainese, P. 1992). These consideration suggest that monomerization of Lhcb6 and Lhcb4 is most likely due to a partial destabilization of B4C supercomplex due to detergent treatment during thylakoids solubilization.

4.2 Dissociation of the B4C complex is correlated to Non Photochemical Quenching.

In this work we report on a light-dependent dissociation of the supramolecular pentameric complex composed of Lhcb4, Lhcb6 and the LHCII-M trimer (Bassi, R. and Dainese, P. 1992) which is connecting the inner and outer moieties of the light harvesting system. We noticed that the fractionation pattern of PSII subunits in sucrose gradients or non-denaturing PAGE was modified upon strong illumination: an oligomeric green band, B4C, present in dark-adapted leaves, dissociated in its monomeric and trimeric components (Figure 1).

Pharmacologic and genetic analysis showed that B4C dissociation fully correlates with NPQ. In fact, we observed that this event is sensitive to the uncoupler nigericin and is up-modulated by zeaxanthin, as shown by the *npq1* mutant, which cannot synthesize Zea and is inefficient in both B4C dissociation and NPQ. The most striking evidence of this correlation, however, comes from the phenotype of PsbS-depleted plants, which are unable to activate NPQ and do not show the light dependent dissociation of B4C (Figure 4). Furthermore, the mutation of each of two lumen-exposed glutamate residues in PsbS, essential for NPQ activation (Li, X. P. et al. 2000, Li, X. P. et al. 2002) leaded to a NPQ decrease of approximately 50% (Li, X. P. et al. 2002). Here, we show that the same mutations impair dissociation of B4C by approximately the same extent (Figure 4), making very unlikely the possibility that these two phenomena are independent but occurring simultaneously upon illumination.

4.3 Functional role of B4C dissociation in Non Photochemical Quenching.

The above results suggest a tight relationship between B4C dissociation and NPQ. It is, however, unclear if dissociation of B4C is sufficient for feed-back de-excitation or if additional operations are involved in the process that might well be fulfilled by PsbS. One way to address this question is to compare WT with mutants in which B4C is constitutively dissociated such as *koLhcb6* (Figure 5). In this mutant dark adapted plants have an higher fluorescence level with respect to WT, implying that the disconnected LHCII was not efficiently quenched (de Bianchi, S. et al. 2008, Kovacs,

L. et al. 2006). Furthermore, koCP24 plants are able, despite the complete absence of B4C, to activate NPQ, although to a reduced level with respect to WT. By analyzing different mutants depleted in antenna proteins, *koLhcb3* and *koLhcb4* in figure 4, we noticed that the absence of B4C in a number of mutants does not abolish completely NPQ, although kinetics are differently affected depending on the residual complement of Lhcb proteins in each mutant.

B4C dissociation by itself is not thus sufficient for NPQ; however, in all cases where the B4C is present in the dark, we never observed NPQ without B4C dissociation: this suggests that B4C is stabilizing an unquenched state and that its dissociation is required for quenching activation. On these bases, we hypothesize that the quenching activity is performed by one or more B4C components. Integrity of B4C prevents quenching, which can be triggered only when this is dissociated. It is not clear at present whether the role of PsbS is limited to the dissociation of B4C or if it extends to establishing an interaction with the resulting dissociated Lhcb subunits. We favor the latter hypothesis since in koCP24 the disconnected LHCII-M+L are clearly detectable, however, they are not quenched (Kovacs, L. et al. 2006). Indeed the koCP24 genotype mimics the constitutive dissociation of B4C and the EM analysis of its grana membranes clearly shows that in the absence of B4C PSII core is non-homogeneously distributed in grana membrane disks with PSII rich areas and LHCII–enriched stain depleted domains (Supplementary figure S4).

It might be asked which of the different subunits might be the “quenching site”. Considering the similarity between antenna proteins it is unlikely that quenching occurs in only one subunit and they may well all contribute. Nevertheless, recent reports are helpful in the identification of some preferential quenching sites based on the *in vivo* effect of knocking out individual gene products on NPQ amplitude/kinetics and on the capacity of catalyzing the transient formation of carotenoid radical cation, a chemical species which has been proposed to be responsible for excess energy quenching (Holt, N. E. et al. 2005), by isolated pigment-protein complexes. Thus, depletion of Lhcb1+2 (Andersson, J. et al. 2003) or Lhcb3 (Supplementary Figure S1) has a small effect on NPQ *in vivo* and these antenna subunits cannot produce *in vitro* any carotenoid radical cation (Avenson, T. J. et al. 2008).

Monomeric Lhcb4, 5 and 6 do indeed perform charge transfer quenching *in vitro* (Ahn, T. K. et al. 2008, Avenson, T. J. et al. 2008) while depletion *in vivo* strongly affects NPQ components: qE in the case of Lhcb6 and Lhcb4 (de Bianchi, S. et al. 2008)(figure S1) and qI in the case of Lhcb5. The involvement of two components of B4C complex in qE, together with the NPQ depletion in all mutants/conditions where B4C complex cannot be dissociated is a strong evidence for a direct interaction of a PsbS-minor Lhc complexes in triggering NPQ.

4.4 A NPQ model based on the rearrangement of PSII supercomplex antenna.

A possible discrepancy between B4C dissociation and NPQ can be found in their activation timescales. In fact, a large fraction of NPQ is activated within few minutes after dark to light transition ($t_{1/2}$ is around 3 minutes, figure 6), while we observed that B4C dissociation is developed with a $t_{1/2}$ of 12-15 minutes (figure 3). This phenomenon, thus, appears to have the same timescale of the slow phase of NPQ activation. However, the method we have used for biochemical detection, of B4C dissociation i.e. sucrose gradient ultracentrifugation, is all but rapid and sensitive. It is likely that dissociated B4C need to be accumulated in order to be detected by biochemical fractionation and/or that unstable B4C dissociation is involved in the fastest phase of NPQ. In fact, after light treatment thylakoids isolation and solubilization requires about 60 minutes and thus, despite all operations were performed in ice, we cannot exclude early changes to revert before thylakoids solubilization and gradient loading. On the contrary, the physiological effects eliciting NPQ might be activated in a few seconds by a partial and unstable B4C dissociation which, if the high light conditions are maintained, is increased by a complete B4C dissociation and stabilized by the structural redistribution of complexes within the grana membrane.

Our results provide experimental support to previous suggestion that i) PsbS functions by interacting with other antenna proteins; ii) PsbS controls the grana membrane organization and iii) structural modification in Photosystems are implied in NPQ (Bonente, G. et al. 2008b, Johnson, M. P. et al. 2008, Kiss, A. Z. et al. 2008, Teardo, E. et al. 2007). Such an extensive PSII re-organization might appears surprising; nevertheless recently reported data are consistent with such a model: FRAP measurements showed that the largest part of complexes (75%) within grana membranes are substantially immobile. However, the remaining complexes, likely identified as antenna complexes, have instead a high diffusion coefficient. According to this hypothesis, an antenna complex might travel from the centre to the limit of the grana membrane in about 2 seconds (Kirchhoff, H. 2008b). These results thus suggest that a rearrangement of antenna complexes, also implying protein displacement, would be compatible with NPQ kinetics. Furthermore, the latter study suggests this movement is not only diffusion driven but it is likely to be "oriented": in this respect a role could be played by PsbS activity, which might help in creating the so-defined diffusion channels (Kirchhoff, H. et al. 2008a). Concerning the role of PsbS, it interesting to mention that we observed a small but significant difference between WT and *npq4* in PSII core distribution in the dark. This is consistent with the observation that *npq4* mutants grows better in low light conditions with respect to WT (Dall'Osto, L. et al. 2005), suggesting PsbS activity is not fully restricted to excess light conditions.

4.5 B4C dissociation induces a fast decrease in the antenna size of PSII supercomplex.

Structural analysis of grana membranes clearly shows that light treatment induces a decrease in the distance between PSII reaction centers. This effect is reversible upon dark recovery in parallel to the dissociation and re-association of B4C. Grana membranes are densely packed with PSII components and contain little free lipids (Tremmel, I. G. et al. 2003, Tremolieres, A. et al. 1994); thus the distance between PSII core complex is mainly determined by the Lhcb protein complement of Photosystems (Dekker, J. P. and Boekema, E. J. 2005, Kirchhoff, H. et al. 2008b, Kovacs, L. et al. 2006, Morosinotto, T. et al. 2006). We conclude that light treatment, with the involvement of PsbS activity, leads into a reversible dissociation of the outer layer of PSII antenna system from the reaction centre complex, thus reducing the size of the antenna system feeding excitation energy to PSII reaction centers.

We observed that such a PSII redistribution requires more than 30 minutes (figure 8) and thus it correlates with the stabilization of NPQ occurring in the 30 to 90 minutes interval (figure 6), suggesting that NPQ stabilization occurs by rearranging PSII complexes and their antenna.

The advantage of such a regulatory mechanism is clear if we consider that over-excitation of PSII is the major source of photoinhibition (Barber, J. and Andersson, B. 1992). Long term acclimation to high light is well known to lead to reduction of peripheral antenna size (Bailey, S. et al. 2001, Ballottari, M. et al. 2007). However, the acclimatory process requires protein degradation and takes several days and is not useful nor energetically affordable when light intensity varies within minutes. NPQ, which is instead the major regulatory mechanism acting within these timescales (Kulheim, C. et al. 2002), could, thus, work by functionally dissociating part of the antenna from the core complex while avoiding degradation and re-synthesis.

4.6 The fate of Lhcbs upon disconnection from PSII reaction centers.

It might be asked where do antenna complexes travel to upon disconnection from the PSII core particles. Possible destinations are the grana periphery and the stroma lamellae. The former hypothesis is likely for at least a fraction of the antenna, since the PSII distribution was shown to be reversible. In the case of *koLhcb6* it was also observed that regions with lower PSII core density were generally found in the grana periphery (Supplementary figure S4). On a longer timescale (hours or even days), it is also possible that antenna complexes might migrate even further. Early work in fact showed that in light treated leaves LHCII isoforms disappeared from grana membranes are found in stroma membranes

(Kyle, D. J. et al. 1983). Since proteolysis is particularly active in stroma membranes, antenna migrating here might be degraded, leading to a stable down-sizing of PSII antenna size. This hypothesis suggests the idea that domain segregation process described here under prolonged illumination contributes to the acclimatory modulation of the PSII antenna size.

4.7 *Evolution of NPQ mechanisms in Viridiplantae*

It is interesting pointing out that CP24 and Lhcb3, two of the three components of B4C complex, are late addition to the PSII antenna system, being only present in land plants (Alboresi, A. et al. 2008) and never detected in algae. *Arabidopsis* mutants missing any of these polypeptides miss B4C (Figure 5), demonstrating they are fundamental for the formation of the supercomplexes. Algae and plants also differ for their PsbS content: in fact, although the gene is present in several algal genomes, the corresponding polypeptide is not accumulated in any algal species analyzed so far (Bonente, G. et al. 2008b, Morosinotto, T. et al. 2006). Furthermore, in algae, only C_2S_2 supercomplexes have been found, while larger supercomplexes, such as those of land plants have never been observed (Dekker, J. P. and Boekema, E. J. 2005). This suggests the hypothesis that the regulatory mechanism we described in this work might have evolved upon land colonization in order to defend plants from the highly variable conditions found in the land environment (Kulheim, C. et al. 2002). On the contrary, algal water environment is more stable for temperature, light and CO_2 supply thus making a fast quenching mechanism less important in algae with respect to plants (Wollman, F. A. 2001).

4.8 Conclusion

We have here described new experimental evidences on PsbS function, consisting into the reorganization of protein domains in grana membranes as recently suggested (Kiss, A. Z. et al. 2008). This is obtained by controlling the assembly of a pentameric complex, called B4C, which includes components of both the inner (Lhcb4) and outer (Lhcb6, LHCII-M) moieties of PSII antenna system. In low light the complex is assembled and PSII antenna size is maximal; in high light the complex dissociates and we proposed that C_2S_2 particles dissociates from CP24/LHCII-M/LHCII-L complexes which migrates to the periphery or the grana. Upon dissociation of PSII supercomplexes the formation of quenching sites in PSII antenna, which we propose to be Lhcb6 and Lhcb4, is allowed by dissociation of B4C and accessibility of these minor Lhcs to the interaction with PsbS.

We also suggest that this regulation of PSII supercomplexes assembly is part of a control cycle connecting short term energy dissipation to long term regulation of PSII antenna size upon acclimation.

5. SUPPLEMENTAL DATA

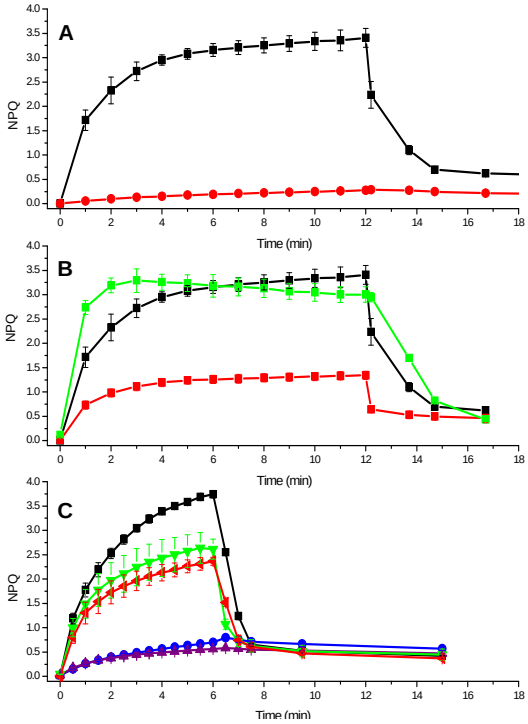
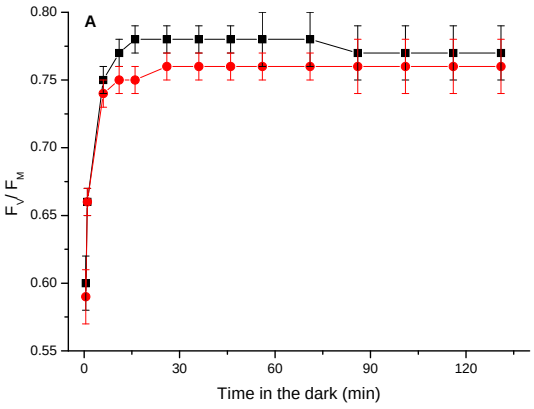


Figure S1. NPQ kinetics dependence from nigericine and genotype. ***A.*** *NPQ measured in leaves infiltrated with nigericine (red), an uncoupler dissipating ΔpH across the thylakoids membranes, as compared with control leaves infiltrated with buffer only (black).* ***B.*** *NPQ Dependence on carotenoid composition, analyzed by comparing plants depleted (npq1, red) or accumulating constitutively zeaxanthin (npq2, green).* ***C.*** *NPQ dependence on PsbS is analyzed by comparing PsbS-less plants (npq4, blue) with npq4 plants complemented with PsbS in the WT form (npq4+PsbS, black) and PsbS where either one or both lumen exposed glutamate residues binding DCCD were mutated into glutamine (E122Q, E226Q and E122Q/E226Q, respectively green and red and purple).*



	30 minutes light	90 minutes light
Dark	n.d	n.d.
End light treatment	2.29± 0.10	2.43±0.23
15' recovery	1.50±0.13	1.72±0.30
90' recovery	1.03±0.04	1.06±0.05

Figure S2. PSII recovery and zeaxanthin content in leaves after prolonged NPQ measurements. ***A.*** *PSII yield (Fv/Fm) in dark adapted conditions was measured in leaves treated with 30 (black squares) and 90 minutes (red circles) of high light following light treatment leaves were incubated in the dark for 60 minutes before measurement.* ***B.*** *Zeaxanthin content in leaves was also analyzed during the prolonged NPQ measurements. Values for samples treated for 30 and 90 minutes of actinic light are shown.*

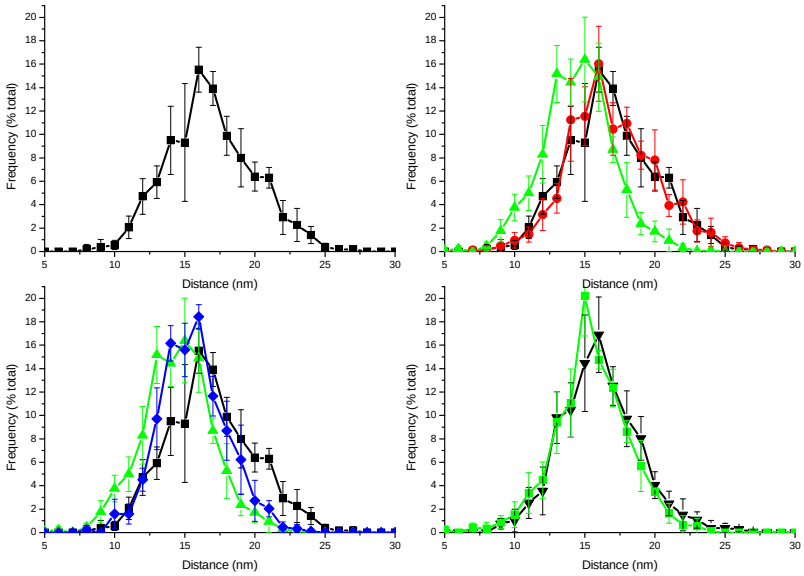


Figure S3. Analysis of PSII core distribution, considering only the closest neighbor. *Distribution of PSII complexes in grana membranes was analyzed as shown in figure 4 of the main text, but considering only the distance of the closest PSII core rather than the average of the closest four.* ***A.*** *Distribution in dark adapted sample.* ***B.*** *Comparison of PSII distribution in samples dark adapted (black squares) or treated with 30 (red circles) and 90 minutes (green triangles) of light before grana isolation.* ***C.*** *Comparison of sample either light adapted (green triangles) or incubated in the dark for 90 minutes following light treatment (blue diamonds). D) effect of light treatment in PSII distribution in npq4 grana. Dark adapted sample is shown in black triangles, sample light treated for 90 minutes in green diamonds.*

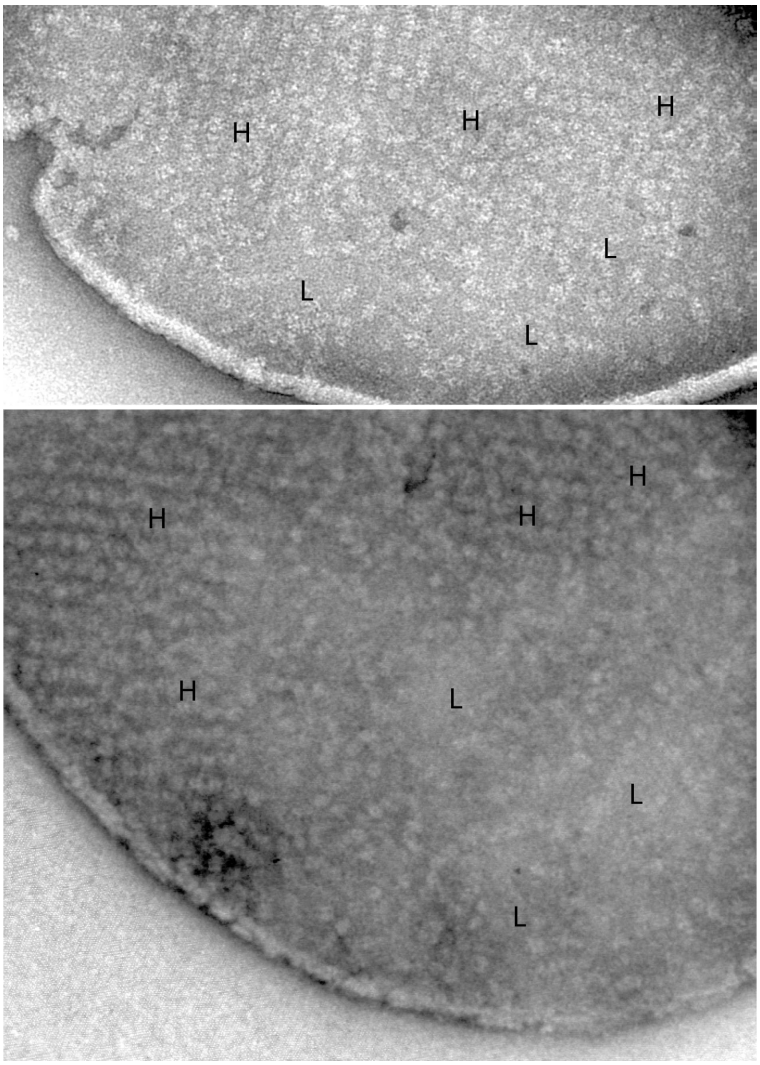


Figure S4. EM images from *KOLhcb6*. *EM pictures of negatively stained grana membranes purified from KOLhcb6 plants. Regions with high or low density of PSII core complexes are indicated with H and L respectively.* {Bonente, 2008 2 /id}{Kirchoff, 2008 1 /id}{Kirchoff, 2008 1 /id}{Kirchhoff, 2008 21 /id}{Kiss, 2008 6 /id}

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SECTION C

New insights on the role of minor subunit antenna Lhcb4 in photoprotection.

The role of the light-harvesting complex Lhcb4 (CP29) in photosynthesis and photoprotection was investigated in *Arabidopsis thaliana* by characterizing knockout lines for each of the three Lhcb4 isoforms (Lhcb4.1/4.2/4.3). Plants lacking all isoforms (*koLhcb4*) showed a compensatory increase of Lhcb1 and a reduced Photosystem II/Photosystem I ratio with respect to wild-type plants. The absence of Lhcb4 results in a lower Non-Photochemical Quenching activity with respect to either WT or mutants retaining a single Lhcb4 isoform. *KoLhcb4* plants were more sensitive to photoinhibition under stress, while this effect was not observed in knockout lines for any other Photosystem II antenna subunit. Interestingly, deletion of either Lhcb4.1 or Lhcb4.2 was compensated by over-accumulation of the remaining subunit, while the double mutant *koLhcb4.1/4.2* did not accumulate Lhcb4.3. Ultrastructural analysis of thylakoid grana membranes showed a lower density of PSII complexes in *koLhcb4*. Moreover, analysis of isolated supercomplexes showed a different overall shape of the C_2S_2 particles due to a different binding mode of the S-trimer to the core complex. Also, an empty space was observed within the PSII supercomplex at Lhcb4 position, implying the missing Lhcb4 was not replaced by other Lhc subunits. This suggests that Lhcb4 is unique among subunits of the PSII antenna and determinant for PSII macro-organization and photoprotection.

This section is based on the submitted paper: de Bianchi*, S., Betterle*, N., Kouril, R., Cazzaniga, S., Boekema, E., Bassi, R., and Dall'Osto,. L. (2011); (*,these authors equally contributed to the work).

For the experiments done in this chapter I contributed by analyzing and characterizing PSII supercomplexes of *koLhcb4* mutant, by observing PSII distribution inside *koLhcb4* grana membranes with EM microscope and by contributing with the other authors with suggestions in the discussion of the experimental design and results.

1. INTRODUCTION

Oxygenic photosynthesis is performed in the chloroplast by a series of reactions that exploits light as an energy source to fuel ATP and NADPH production for CO_2 fixation and synthesis of organic compounds. Light harvesting is the primary process in photosynthesis and consists of absorption of photons by an array of hundreds of chlorophylls (Chls), which are structurally organized into a light-harvesting system. Excitons are transferred among Chls and to the photosynthetic reaction centre (RC), where charge separation occurs. In the case of Photosystem II (PS II), electrons are transferred to the quinonic acceptor plastoquinone (PQ) leading to charge accumulation in the oxygen evolving complex and water splitting (Nelson, N. and Ben Shem, A. 2004). RC of PSII consists of the D1/D2/cyt b559 complex carrying the co-factors for electron transport, which forms, together with the nearest subunits CP43 and CP47, two homologous plastid-encoded proteins binding Chl *a* and β-carotene, to form a "core complex" (Ferreira, K. N. et al. 2004). Core complexes form dimers (C_2), which bind an extended system of nuclear-encoded light-harvesting proteins (Lhc), each binding Chl *a*, Chl *b* and xanthophylls. Near to the core Lhcb4 (CP29) and Lhcb5 (CP26) are located, which mediate a binding of a trimeric antenna complex, made by Lhcb1-3 gene-products called LHCII-S (strongly bound). The above components form the basic PSII supercomplex structure, called C_2S_2 (Boekema, E. J. et al. 1999), which is the major PSII form found in plants grown under high light (HL) conditions (Frigerio, S. et al. 2007, Morosinotto, T. et al. 2006). In plants cultivated under low/moderate light, one additional monomeric subunit, Lhcb6 (CP24) and two LHCII trimers (LHCII-M and LHCII-L, moderately and loosely bound, respectively) are accumulated to extend the light harvesting capacity of PSII (Ballottari, M. et al. 2007). Under these conditions, larger supercomplexes are formed called $C_2S_2M_2$ (Caffarri, S. et al. 2009) with LHCII-L trimers loosely associated to the supercomplex (Boekema, E. J. et al. 1999). In *Arabidopsis*, single genes encode Lhcb5 (CP26) and Lhcb6 (CP24) subunits, while Lhcb4 (CP29) is encoded by three,

highly conserved genes. Namely, *Lhcb4.1* and *Lhcb4.2* are similarly expressed, while the *Lhcb4.3* messenger level is 20 times lower under control condition (Jansson, S. 1999). The polypeptide encoded by *Lhcb4.3* is predicted to lack a large part of the C-terminal domain, a peculiar feature of Lhcb4.1 and Lhcb4.2 isoforms, but it has not yet been detected in thylakoids. Because of these differences, the proposal was made to rename it as Lhcb8 (Klimmek, F. et al. 2006).

A remarkable property of the antenna system is the ability to actively regulate PSII quantum efficiency in order to avoid the damaging effects of excess light. Indeed, under constant moderate light conditions, the efficiency of energy conversion is high, due to photochemical reactions, which efficiently quench Chl singlet excited states (^{1}Chl*). On the other hand, fluctuations of light intensity/temperature/water availability may yield into the excitation of PSII over the capacity for photochemical quenching of ^{1}Chl*. The consequent lifetime increase enhances the probability of Chl triplet (^{3}Chl*) formation by intersystem crossing and yields into single oxygen ($^1O_2$) production (Melis, A. 1999). Since formation of ^{3}Chl* is a constitutive property of Chls (Mozzo, M. et al. 2008), photoprotection mechanisms are activated in order to prevent damage and improve fitness in the ever-changing environment experienced by plants. Safety systems have evolved to either detoxify the reactive oxygen species (ROS) that are produced (Asada, K. 1999), or to prevent their formation (Niyogi, K. K. 2000) by (i) down-regulating ^{1}Chl* lifetime through a process called non-photochemical quenching (NPQ) that dissipates excess excited states into heat (Horton, P. 1996); (ii) by quenching ^{3}Chl*; (iii) by scavenging ROS. Sustained over-excitation is counteracted by the long-term reduction of PSII antenna size (Ballottari, M. et al. 2007). Additional regulation is activated in limiting light conditions by transferring LHCII complexes between photosystems, which balances excitation delivery to PSI and PSII (Allen, J. F. 1992, Haldrup, A. et al. 2001).

The conservation of the different Lhcb gene products through evolution suggests each has a specific functional role which, however, is not yet fully clarified. Two major approaches have been used to this aim: namely, the analysis of individual gene products either purified from thylakoids (Bassi, R. et al. 1987, Caffarri, S. et al. 2001), or recombinant (Caffarri, S. et al. 2004, Formaggio, E. et al. 2001) and the reverse genetics, by which plants lacking one or more Lhcbs have been produced and characterized (Andersson, J. et al. 2001, Damkjaer, J. et al. 2009, de Bianchi, S. et al. 2008, Kovacs, L. et al. 2006). These studies have provided evidence for differential roles of LHCII components (Lhcb1-3) with respect to monomeric subunits (Lhcb4-6). Antisense lines of *Arabidopsis* devoid of Lhcb1+2 (Ruban, A. V. et al. 2003) showed a reduced fitness for plants grown in field, while only minor differences were observed for growth rates, PSII quantum yield, photosynthetic rate at saturating irradiances and capacity for NPQ in controlled environment (Andersson, J. et al. 2003). Similar considerations apply to Lhcb3: the major effect of its depletion consisting of a faster kinetic of state I-state II transitions (Damkjaer, J. et al. 2009). More specific effects have been reported for monomeric Lhcb4 and Lhcb5, which have been shown to (i) carry protonatable (DCCD-binding) sites (Pesaresi, P. et al. 1997, Walters, R. G. et al. 1996); (ii) undergo conformation changes upon exchange of violaxanthin (Viola) to zeaxanthin (Zea) in their L2 xanthophyll binding site (Morosinotto, T. et al. 2002), an important feature since Zea is only synthesized in excess light conditions and correlates with NPQ (Demmig-Adams, B. et al. 1989). The Zea-dependent allosteric change was shown to produce profound effects on the properties of these pigment-proteins, including decrease in fluorescence lifetime (Crimi, M. et al. 2001, Moya, I. et al. 2001) and formation of carotenoid radical cations (Ahn, T. K. et al. 2008, Avenson, T. J. et al. 2008), suggesting a major role in NPQ. The strong decrease in NPQ observed in *koLhcb6* plants (de Bianchi, S. et al. 2008, Kovacs, L. et al. 2006)is consistent with the above suggestion, while *koLhcb5* plants were affected in a different, slowly relaxing component of NPQ called qI. Nevertheless, many properties of Lhcb proteins, which are essential for photoprotection, appear to be redundant. In fact, while the total depletion of Lhcb proteins clearly induces photosensitivity in *ch1* mutants (Dall'Osto, L. et al. 2010), no individual

knockout (KO) lines have showed major impairment in their capacity to resist HL treatment so far (de Bianchi, S. et al. 2008, Ruban, A. V. et al. 2003).

Arabidopsis KO lines that completely lack either Lhcb5, Lhcb6 or both, have recently been described. As for Lhcb4, its role in light harvesting and energy dissipation was investigated using antisense RNAi lines depleted in the target protein: NPQ kinetic and amplitude, photosynthetic electron transport rate (ETR) and light sensitivity were similar to the wild type (Andersson, J. et al. 2001). However, since this work only employed mild stress conditions, the question is open for the role of Lhcb4 in photoprotection. Indeed, several evidence suggests that Lhcb4 might be a key factor in both light harvesting and photoprotection: Lhcb4 is maintained in HL stress conditions leading to the reduction of Lhcb6 and LHCII (Ballottari, M. et al. 2007), while Lhcb4 phosphorylation in both monocots (Testi, M. G. et al. 1996) and dicots (Hansson, M. and Vener, A. V. 2003, Tikkanen, M. et al. 2006) affects the spectral properties of the protein (Croce, R. et al. 1996) and correlates with higher resistance to HL + cold (Bergantino, E. et al. 1995, Mauro, S. et al. 1997) and water stress (Liu, W. J. et al. 2009). Although the mechanism of this protective effect is unknown, there is evidence for a link between Lhcb4 and NPQ, since the protein has been identified as an interaction partner of PsbS (Teardo, E. et al. 2007), the pH-dependent trigger of qE (Li, X. P. et al. 2000) and it is part of a pentameric complex whose dissociation is indispensable for the establishment of NPQ (Betterle, N. et al. 2009). Finally, two Chl ligands in Lhcb4 have been identified as components of the quenching site, catalyzing the formation of xanthophyll radical cation (Avenson, T. J. et al. 2008, Holt, N. E. et al. 2005).

In the present work, we have addressed the function of Lhcb4 isoforms in *Arabidopsis thaliana*. To this aim, we have constructed KO mutants for Lhcb4 isoforms and analyzed their performance in photosynthesis and photoprotection. We found that PSII quantum efficiency and capacity for NPQ were affected by lack of Lhcb4 and that depletion of Lhcb4, unlike that of any other Lhcb subunit, does affect the capacity of resisting excess light conditions. Although depletion of Lhcb subunits is usually complemented by the over-accumulation of other members of the subfamily (de Bianchi, S. et al. 2008, Ruban, A. V. et al. 2003), this is not the case for Lhcb4. In fact, PSII supercomplexes isolated from *koLhcb4*, although retaining their C_2S_2 organization, showed a low density region at Lhcb4 position, consistent with a missing subunit, which was confirmed by protein analysis. This caused a different mode of binding of LHCII-S trimer within the PSII super-complex and changed the overall shape of the C_2S_2 particle. The three isoforms were not equivalent, since, while deletion of Lhcb4.1 isoforms yielded into a compensatory accumulation of Lhcb4.2 and vice versa, the double mutant *koLhcb4.1/4.2* was unable to accumulate Lhcb4.3. We conclude that Lhcb4 is a fundamental component of PSII, which is essential for maintenance of both the function and structural organization of this photosystem.

2. MATERIAL AND METHODS

2.1 Plant material and growth conditions Arabidopsis thaliana T-DNA insertion mutants (*Columbia* ecotype) GK Line ID282A07 (insertion into the *Lhcb4.1* gene), SAIL_910_D12 (insertion into the *Lhcb4.2* gene) and SALK_032779 (insertion into the *Lhcb4.3* gene) were obtained from NASC collections (Alonso, J. M. et al. 2003). Homozygous plants were identified by PCR analysis using the following primers: forward 5'-TCACCAGATAACGCAGAGTTTAATAG-3' and reverse 5'-CACATGATAATGATTTTAAGATGAGGAG-3' for *Lhcb4.1* sequence, 5'-CATCATACTCATTGCTGATCC ATG -3' for the insertion; forward 5'-GCGTTTGTGTTTAGCGTTTCGACATCTGTCTG-3' and reverse 5'-GGTACCCGGGTGGTTTCCGACATTAGC-3' for *Lhcb4.2* sequence, 5'-GCCTTTTCAGAAATGG ATAAATAGCCTTGCTTCC-3' for the insertion; forward 5'-

GTGAGCTGATCCATGGAAGGTGG-3' and reverse 5'-GGCCGGTTTTGAACGATTGATGTGAC-3' for *Lhcb4.3* sequence, and 5'-GCGTGGACCG CTTGCTGCAACT-3' for the insertion. Genotypes *koLhcb4*, *koLhcb4.1/4.2*, *koLhcb4.2/4.3* and *koLhcb4.1/4.3* were obtained by crossing single mutant plants and selecting progeny by PCR analysis. For reverse transcriptase-mediated-PCR, total RNA was isolated from 4-weeks old plants with Trizol protocol for RNA extraction. Reverse transcription was performed using M-MLV reverse transcriptase with oligo-dT primer and 1,5 μg of total RNA from wild type and mutant plants. For normalization purposes, actin2 (At3g18780) was chosen as an endogenous control. The primers used were as follows: 5'-GTGGCTCCCGGTATCCATCC-3' and 5'-TTGAACCGCGAATCCCAAGAAGG-3' for *Lhcb4.1* cDNA; 5'-GGTTTCCGACATTAGCTCCAATTC-3' and 5'-CTGAACCGCAAAACCCAAGAATC-3' for *Lhcb4.2* cDNA; 5'-CCGGTTCGGGTTCAGTTTCGG-3' and 5'-GGCAAGGAAGCTGACAGGGC-3' for *Lhcb4.3* cDNA; 5'-CCTCATGCCATCCTCCGTCTTG-3' and 5'-GAGCACAATGTTACCGTACAGATCC-3' for actin2 cDNA.

Arabidopsis T-DNA insertion mutant *koLhcb3* (SALK_020342) from NASC was obtained by selecting progeny by western-blotting using specific antibody against Lhcb3 subunit; asLHCII (Andersson, J. et al. 2003) was obtained by NASC. Double mutant *koLhcb4 npq1* was obtained by crossing single mutants and selecting progeny by western-blotting (α-Lhcb4) and HPLC. Insertion mutants *koLhcb6* and *koLhcb5/Lhcb6* were isolated as previously described (de Bianchi, S. et al. 2008). Mutants were grown for 5 weeks at 100 μmol photons m^{-2} s^{-1}, 23°C, 70% humidity, and 8 h of daylight.

2.2 Stress conditions. Short-term high-light (HL) treatment was performed at 1200 μmol photons $m^{-2}s^{-1}$, 45', RT (24° C) in order to measure kinetic of zeaxanthin accumulation, on detached leaves floating on water. Samples were rapidly frozen in liquid nitrogen prior to pigment extraction. Longer photo-oxidative stress was induced by exposing whole plants to either 550 μmol photons m^{-2} s^{-1} at 4°C for 2 days, 900 μmol photons $m^{-2}s^{-1}$ at 4°C, for 10 days or 1600 μmol photons $m^{-2}s^{-1}$ at 24°C, for 10 days. Light was provided by halogen lamps (Focus 3, Prisma, Italy) and filtered through a 2-cm recirculation water layer to remove infrared radiation.

2.*3 Pigment analysis* - Pigments were extracted from leaf discs, either dark-adapted or HL-treated, with 85% acetone buffered with Na_2CO_3, then separated and quantified by HPLC (Gilmore, A. M. and Yamamoto, H. Y. 1991).

2.4 Membrane isolation Unstacked thylakoids were isolated from dark-adapted or HL-treated leaves as previously described (Bassi, R. et al. 1988).

2.5 Gel Electrophoresis and Immunoblotting. SDS-PAGE analysis was performed with the Tris-Tricine buffer system (Schägger, H. and von Jagow, G. 1987), with the addition of 7M urea to the running gel in order to separate Lhcb4 isoforms. Non-denaturing Deriphat-PAGE was performed following the method developed by (Peter, G. F. et al. 1991) with modification described in (Havaux, M. et al. 2004). Thylakoids concentrated at 1 mg/ml chlorophylls were solubilised with a final 1% α/β-DM, and 25 mg of chlorophyll were loaded in each lane. For immunotitration, thylakoid samples corresponding to 0.25, 0.5, 0.75, and 1 μg of chlorophyll were loaded for each sample and electroblotted on nitrocellulose membranes. Filters were incubated with antibodies raised against Lhcb1, Lhcb2, Lhcb3, Lhcb4, Lhcb5, Lhcb6, or CP47 (PsbB) and were detected with alkaline phosphatase-conjugated antibody, according to (Towbin, H. et al. 1979). Signal amplitude was quantified (n = 4) using GelPro 3.2 software (Bio-Rad). In order to avoid any deviation between different immunoblots, samples were compared only when loaded in the same slab gel.

2.6 In Vivo Fluorescence and NPQ Measurements. PSII function during photosynthesis was measured through chlorophyll fluorescence on whole leaves at room temperature with a PAM 101 fluorimeter (Heinz-Walz) (Andersson,

J. et al. 2001); saturating light pulse: 4500 µmol photons $m^{-2}s^{-1}$, 0.6 s; white actinic light: 100-1100 µmol photons $m^{-2}s^{-1}$, supplied by a KL1500 halogen lamp (Schott). NPQ, ϕ_{PSII}, and relative ETR were calculated according to the following equation (Van Kooten, O. and Snel, J. F. H. 1990): NPQ=(Fm-Fm')/Fm', ϕ_{PSII}=(Fm'-Fs)/Fm', relETR=ϕ_{PSII} · PAR, qP=(Fm'-Fs)/(Fm'-F_0) where F_0 is the fluorescence determined in darkness by a weak measuring beam, Fm is the maximum chlorophyll fluorescence from dark-adapted leaves measured after the application of a saturating flash, Fm' the maximum chlorophyll fluorescence under actinic light exposure, Fs the stationary fluorescence during illumination, and PAR the photosynthetic active radiations.

State transition experiments were performed using whole plants according to established protocols (Jensen, P. E. et al. 2000). Preferential PSII excitation was provided by illumination with blue light (40 µmol photons m^{-2} s^{-1}), excitation of PSI was achieved using far-red light from a LED light source (Heinz-Walz; 102-FR) applied for 15 min simultaneously with blue light. Fm level in state I (Fm') and state II (Fm'') was determined at the end of each cycle by the application of a saturating light pulse. The parameter qT (PSII cross section changes) was calculated as (Fm_1-Fm_2)/Fm_1 (where $Fm_{1/2}$ is the maximal fluorescence yield in state I/ II).

Fluorescence induction kinetics was recorded with a home-built apparatus in order to measure functional antenna size on leaves. S_m/t_{Fmax} was calculated from variable fluorescence curves induced with green light (1100 µmol m^{-2} s^{-1}) according to (Strasser, R. J. et al. 1995). For measurements of PSII functional antenna size, leaves were infiltrated with 30 µM DCMU and 150 mM sorbitol, and variable fluorescence was induced with a green light of 7 µmol m^{-2} s^{-1}. The reciprocal of time corresponding to two-thirds of the fluorescence rise ($T_{2/3}$) was taken as a measure of the PSII functional antenna size (Malkin, S. et al. 1981). For measurements of the PSII repair process, whole plants were illuminated at 900 µmol m^{-2} s^{-1}, 4° C for 4 h to induce photoinhibition of PSII, and restoration of the F_v/F_m ratios was subsequently followed at irradiances of 20 µmol m^{-2} s^{-1}, 4° C (Aro, E. M. et al. 1994).

2.7 Analysis of P700 redox state. Spectrophotometric measurements were performed using a LED spectrophotometer (JTS10, Biologic Science Instruments, France) in which absorption changes are sampled by weak monochromatic flashes (10-nm bandwidth) provided by light emitting diodes (LED). $P700^+$ reduction following a flash was assayed as detailed by (Golding, A. J. et al. 2004). Leaves were infiltrated with 50 µM DCMU in 150 mM sorbitol. A 400-ms saturating flash of red light (λ_{max}=630 nm; 3,000 µmol m^{-2} s^{-1}) was delivered to the leaf, and changes in absorbance at 705 nm were used to measure the kinetic of $P700^+$ reduction**.** Oxidized P700 (ΔA_{max}) was recorded during far-red light illumination (1500 µmol m^{-2} s^{-1}, λ_{max}=720 nm). The level of oxidized P700 in the leaf (ΔA) was determined during illumination with red light (from 100 to 980 µmol m^{-2} s^{-1}, λ_{max}=720 nm) as previously described (Zygadlo, A. et al. 2005). Antenna size of PSI was estimated according to (Kim, E. H. et al. 2009).

2.8 Determination of the sensitivity to photooxidative stress Photooxidative stress was induced in detached leaves by a strong light treatment at low temperature. Detached leaves floating on water were exposed to HL (1500 µmol m^{-2} s^{-1} for 8 hours) in a growth chamber at low temperature (4° C), then immediately frozen in liquid nitrogen. Photoxidative stress was assessed by measuring malondialdehyde (MDA) formation, as indirect quantification of lipid peroxidation (Havaux, M. et al. 2005). Lipid peroxidation was measured on whole plants by thermoluminometry with custom-made apparatus (Ducruet, J. M. 2003). The amplitude of the TL peak at 135°C was used as an index of lipid peroxidation (Havaux, M. 2003). Measurements of singlet oxygen production from leaves were performed with SOSG (Molecula Probe, Eugene), a $^1O_2$-specific fluorogenic probes, as described in (Dall'Osto, L. et al. 2010).

2.9 Electron microscopy Samples were negatively stained with 2% uranyl acetate on glow discharged carbon-coated copper grids. Electron microscopy was performed on a Philips CM120 electron microscope equipped with a LaB6 tip

operating at 120 kV. Images were recorded with a Gatan 4000 SP 4 K slow-scan CCD camera at 80,000 magnification at a pixel size (after binning the images) of 0.375 nm at the specimen level with GRACE software for semi-automated specimen selection and data acquisition (Oostergetel, G. T. et al. 1998). Single particle analysis of a data set of 1350 PSII particles was performed using GRIP (Groningen Image Processing) software, including multireference and no-reference alignments, multivariate statistical analysis, classification, and averaging of homogeneous classes (van Heel, M. et al. 2000).

2.10 Accession Number Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under accession numbers At5g01530 (Lhcb4.1), At3g08940 (Lhcb4.2), At2g40100 (Lhcb4.3), At5g54270 (Lhcb3), At4g10340 (Lhcb5), At1g15820 (Lhcb6), At1g08550 (npq1). The KO lines mentioned in the article can be obtained from the NASC under the stock numbers N376476 (*koLhcb4.1*), N877954 (*koLhcb4.2*), N532779 (*koLhcb4.3*), N520342 (*koLhcb3*), N514869 (*koLhcb5*), N577953 (*koLhcb6*), N6363 (asLHCII).

3. RESULTS

3.1 Isolation of koLhcb4 mutant

The construction of a plant without CP29 complex (hereafter named *koLhcb4*) requires the isolation of KO mutants at three distinct loci, namely *Lhcb4.1*, *Lhcb4.2* and *Lhcb4.3* (Jansson, S. 1999). We identified *koLhcb4.1*, *koLhcb4.2*, and *koLhcb4.3* homozygous plants in T-DNA F_5 seed pools, obtained from NASC, by PCR analysis of genomic DNA using isoform-specific primers (Figure 1A).

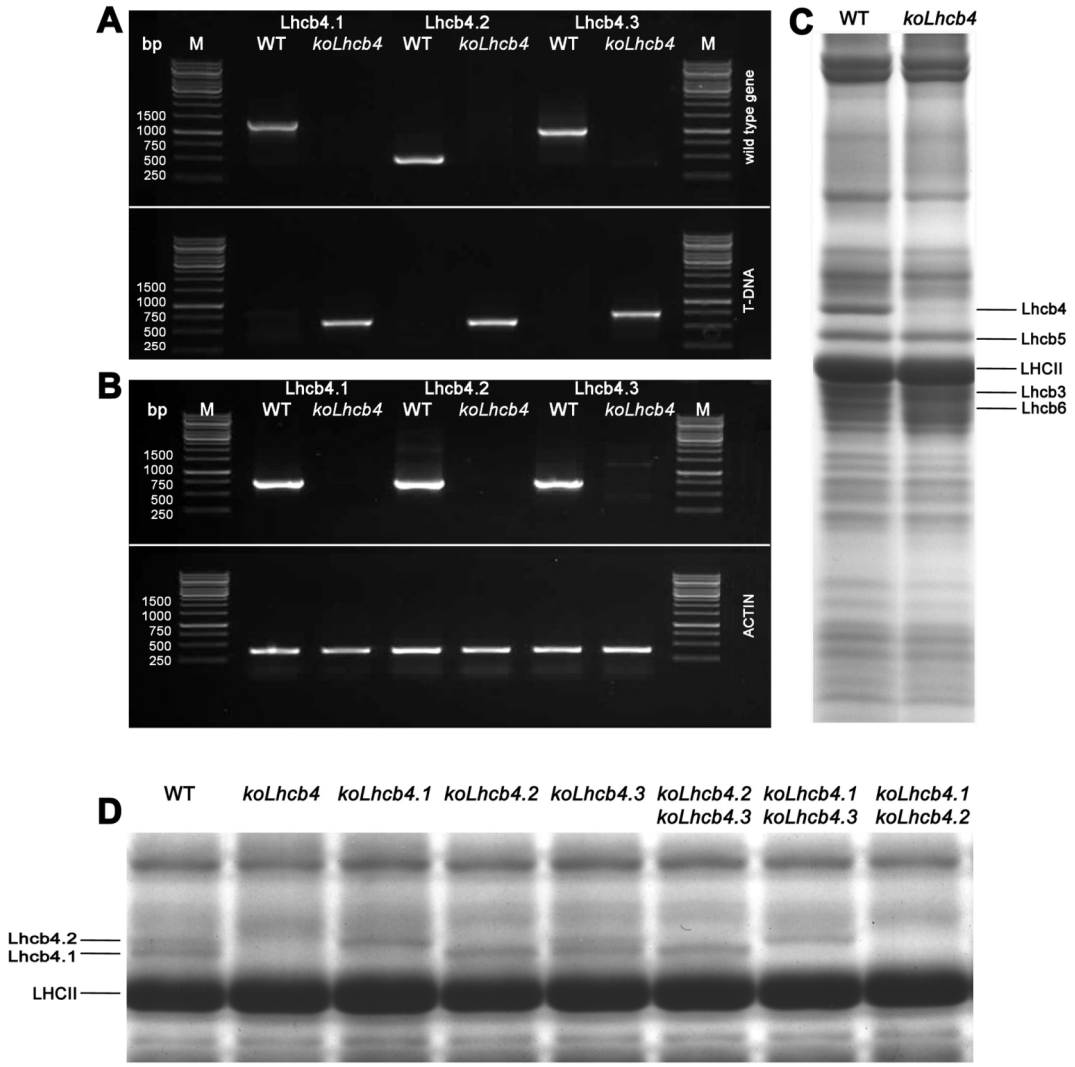


Figure 1. Genetic and biochemical characterization of *koLhcb4* mutant (triple mutant for the three isoforms of Lhcb4). ***A.*** *Amplification of Lhcb4.1, Lhcb4.2, Lhcb4.3 loci with allele-specific PCR primer. Top panel: amplification using gene specific primers: 1378 bp, 520 bp, 1046 bp for the amplification respectively of Lhcb4.1, Lhcb4.2, Lhcb4.3 locus. Bottom panel: amplification using a T-DNA specific primer: 685 bp, 661 bp, 773 bp for the amplification respectively of Lhcb4.1, Lhcb4.2, Lhcb4.3 knockout loci. Details of primer sequences are reported in Material and Methods.* ***B.*** *RT-PCR measurement of gene-specific transcripts. Sequences of the oligonucleotides used are reported in Material and Methods. Top panel: for each gene, RNA extracted from WT and the corresponding mutant was subjected to reverse transcription, followed by 30 cycles of PCR amplification. Bottom panel: amplification of the housekeeping gene actin2 transcript from the same RNAs used as loading control. M: molecular weight marker. The expected sizes of the PCR products are: Lhcb4.1: 724 bp; Lhcb4.2: 715 bp; Lhcb4.3: 730 bp; actin: 384 bp.* ***C.*** *SDS-PAGE analysis of WT and koLhcb4 mutant thylakoid proteins performed with the Tris-Tricine buffer system (Schägger, H. and von Jagow, G. 1987). Selected apoprotein bands are marked. Purified thylakoid sample, corresponding to 15 µg of chlorophylls, was loaded in each lane.* ***D.*** *SDS-PAGE analysis performed with the Tris-Tricine buffer system with the addition of 7M urea to the running gel in order to separate Lhcb4 isoforms in the Lhcb4 KO mutants. Selected apoprotein bands are marked. Fifteen micrograms of chlorophylls were loaded in each lane.*

The triple KO mutant *koLhcb4* was obtained by selection of the progeny from crossing single mutants. PCR analysis confirmed that all Lhcb4 coding regions carried T-DNA insertion in both alleles (Figure 1A), while reverse-transcriptase – mediated PCR showed that mRNAs encoding Lhcb4 isoforms were absent in mutant (Figure 1B).

During the course of this work, two additional lines (N124926 and SK32480) with a T-DNA insertion mapped respectively to the *Lhcb4.1* and *Lhcb4.2* genes became available. Both lacked the corresponding protein. The lines

showed very similar phenotypic characteristics to the N376476 (*koLhcb4.1*) and N877954 (*koLhcb4.2*), respectively. Below, we present data from the N376476 and N877954 lines, unless otherwise stated.

By screening F_2 generation, it was possible to isolate double mutants expressing single Lhcb4 isoforms, namely *koLhcb4.2/4.3* (retaining Lhcb4.1), *koLhcb4.1/4.3* (retaining Lhcb4.2) and *koLhcb4.1/4.2* (retaining Lhcb4.3). All the isolated genotypes did not show significant reduction in growth with respect to the wild type under control light condition (100 µmol photons m^{-2} s^{-1}, 24°C, 8/16 day/night) (not shown). Electron microscopy analysis of plastids from mesophyll cells of wild-type and mutants was performed to verify if the thylakoid structure was changed as an effect of the missing Lhcb (Supplemental Figure 1). All the mutants showed a membrane organization very similar to that of wild-type chloroplasts. All genotypes formed well defined grana, containing about 6 discs per granum, as well as showed similar amounts of grana, stroma lamellae and end membranes per plastid (Supplemental Table I).

Thylakoid membranes isolated from *koLhcb4* mutant lacked the corresponding gene product (Figure 1C). A better resolution in the 22-35 kDa MW range was obtained by using a gel incorporating 7M urea, by which Lhcb4 was split into a doublet in WT thylakoids, the upper band corresponding to Lhcb4.2 as shown by comparison with the pattern from double KO genotypes (Figure 1D). The gel without urea (Figure 1C), however revealed that an additional band with lower MW was also missing, which corresponded to Lhcb6, as revealed by immunoblotting (Figure 2A, B).

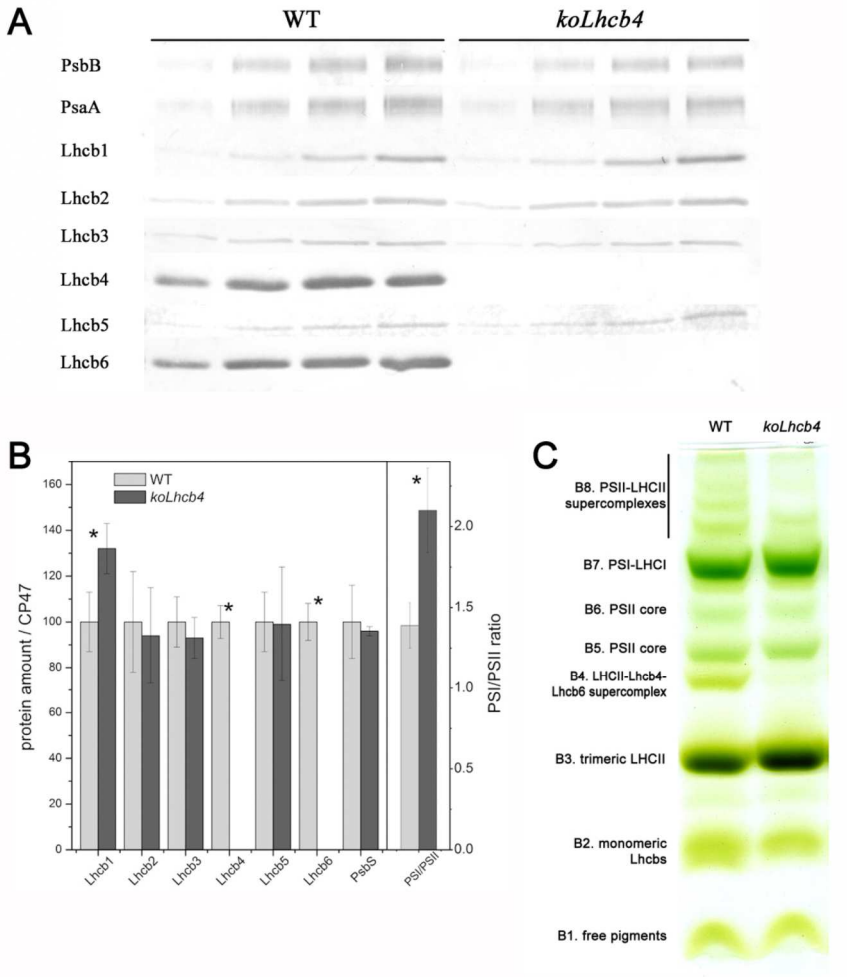


Figure 2. Polypeptide composition of thylakoid membranes from wild-type and *koLhcb4* mutant. ***A.*** *Western blotting used for the quantification of photosynthetic subunits in WT and koLhcb4 thylakoids. Immunoblot analyses were performed with antibodies directed against individual gene products: minor antenna proteins, LHCII subunit, PSII core subunit PsbB (CP47) and PSI core subunit (PsaA). Thylakoid samples corresponding to 0.25, 0.5, 0.75, and 1 µg of chlorophyll were loaded for each sample. All samples were loaded on the same SDS-PAGE slab gel.* ***B.*** *Results*

of the immuno-titration of thylakoid proteins. Data of PSII antenna subunits were normalized to the core amount, PsbB content (Ballottari, M. et al. 2007) and expressed as a percentage of the corresponding wild-type content. Significantly different values from wild type membranes are marked with an asterisk. ***C.*** *Thylakoid pigment-protein complexes were separated by non-denaturing Deriphat-PAGE upon solubilisation with α-DM. Thylakoids corresponding to 25 μg of chlorophylls were loaded in each lane.*

As for the pigment content, the *koLhcb4* mutant did not differ from WT in either chlorophyll content per leaf area or Chl/Car ratio. Instead, *koLhcb4* plants showed a small but significant decrease in the Chl *a*/Chl *b* ratio with respect to WT (Table I).

	Chl a/b	Chl/Car	μg Chl/cm2	Fo	Fv/Fm
WT	3.06 ± 0.07	3.63 ± 0.08	20.7 ± 3.0	0.195 ± 0. 002	0.790 ± 0.007
koLhcb4	2.83 ± 0.06 *	3.58 ± 0.03	19.0 ± 1.1	0.253 ± 0. 021 *	0.747 ± 0.021 *
koLhcb4.2/4.3	2,97 ± 0,03	3,70 ± 0,13	22,5 ± 1,2	0.213 ± 0. 012	0.796 ± 0.004
koLhcb4.1/4.3	2,97 ± 0,03	3,61 ± 0,09	22,6 ± 4,5	0.231 ± 0. 012 *	0.777 ± 0.007 *
koLhcb4.1/4.2	2,83 ± 0,09 *	3,63 ± 0,08	22,9 ± 2,5	0.266 ± 0. 004 *	0.745 ± 0.007 *

Table I. *Chlorophyll content and fluorescence induction parameters determined for leaves of Arabidopsis wild-type, koLhcb4 plants and mutants retaining a single Lhcb4 isoform. Data are expressed as mean ± SD (n>5), significantly different values (Student's t test, p=0.05) with respect to WT are marked with an asterisk.*

To determine whether the capacity of the antenna system and its ability to transfer absorbed energy to reaction centers was affected in the mutant, the functional antenna size of PSII was measured on leaves by estimating the rise time of chlorophyll florescence in the presence of DCMU. No significant differences were observed in $t_{2/3}$ F_{max} (see material and methods for details) between *koLhcb4* and WT (Table I), suggesting that Lhcb4 depletion did not impair the overall light-harvesting capacity. Analysis of the fluorescence induction in dark-adapted leaves (Butler, W. L. and Strasser, R. J. 1978) revealed a higher F_0 value and a significant decrease of maximum quantum efficiency of PSII (F_v/F_m) in *koLhcb4* with respect to WT. Thus, a larger fraction of absorbed energy is lost as fluorescence in the mutant, implying that the connection between the major LHCII complex and PSII RC is less efficient in the absence of Lhcb4 (Table I). Nevertheless, parameter S_m/t_{Fmax}, which is used for quantifying PSII electron transport (ET) activity, was essentially the same in *koLhcb4* and WT, thus implying that ET was not limited downstream from Q_A^- in mutant leaves.

3.2 Organization and stoichiometry of pigment-protein complexes

The organization of pigment-binding complexes was analyzed by non-denaturing Deriphat-PAGE upon solubilisation of WT and *koLhcb4* thylakoid membranes with 0.7% dodecyl-α-D-maltoside (α-DM). Seven major green bands were resolved (Figure 2C). In WT, the PSI-LHCI complex was found as a major band (B7) in the upper part of the gel, while the components of the PSII-LHCII migrated as multiple green bands with different apparent masses; namely the PSII core dimer and monomer (B6 and B5, respectively) and the antenna moieties, including the Lhcb4-Lhcb6-LHCII-Msupercomplex (B4) (Bassi, R. and Dainese, P. 1992), LHCII trimer (B3) and monomeric Lhcbs (B2). Four faint green bands with high apparent molecular mass were detected in the upper part of the gel (B8) containing un-dissociated PSII

supercomplexes with different LHCII composition. The major differences detected in *koLhcb4* with respect to WT were in the lack of B4 and a reduced level of PSII supercomplexes. Secondly, band B2 appears to be only composed of one sub-band in *koLhcb4*, which indicates that the lower sub-band of B2 observed in WT, contains Lhcb4 and/or Lhcb6. Finally, densitometric analysis of the Deriphat-PAGE showed a higher PSI/PSII ratio in *koLhcb4* mutant (1.38 ± 0.11) with respect to WT (1.04 ± 0.03).

In order to detect possible alterations in the relative amount of pigment-protein components, we determined the stoichiometry of photosynthetic subunits by immunoblotting titration using CP47 (PsbB) as internal standard (Ballottari, M. et al. 2007). *KoLhcb4* plants lacked both Lhcb4 and Lhcb6 proteins (Figure 2A), while there was a 30% increase in Lhcb1 (Figure 2B) with respect to the WT level. The other examined proteins were present in wild-type amounts. A quantitative immunoblotting using PsaA and PsbB specific antibodies also confirmed a higher PSI/PSII ratio in *koLhcb4* compared to WT (Figure 2B) detected by Deriphat PAGE (Figure 2C).

We also analyzed the Lhc protein composition and PSI/PSII ratio in double mutants expressing individual Lhcb4 isoforms (Supplemental Table II, Figure 1D and Supplemental Figure 2). A high resolution gel incorporating 7M urea allowed separating Lhcb4.1 from Lhcb4.2 (Figure 1D). The *koLhcb4.2/4.3* mutant which only has Lhcb4.1, retained 87% of WT Lhcb4 while *koLhcb4.1/4.3*, retaining isoform Lhcb4.2, had a significantly lower amount of Lhcb4 (59%) with respect to WT (Supplemental Table II). The *koLhcb4.1/4.2* mutant retaining the *Lhcb4.3* gene did not show any band with mobility similar to Lhcb4.1 and Lhcb4.2 (Figure 1D). Nevertheless, the *Lhcb4.3* gene product is expected to have a MW significantly lower than the .1 and .2 isoforms. We cannot exclude, therefore, that it migrates together with the bulky LHCII band. Immunoblotting analysis also revealed that the level of Lhcb6 polypeptide underwent changes in agreement with the amounts of Lhcb4.1 + Lhcb4.2 protein. In the *koLhcb4.1/4.2* mutant, Lhcb6 was not detectable (Supplemental Table I).

3.3 Photosynthesis related functions: electron transport rate, state transition and P700 redox state

Since pigment-protein complexes participate in modulating electron transport between photosystems, PSII and PSI function during photosynthesis was further analyzed by chlorophyll fluorometry. *KoLhcb4* showed no significant differences with respect to WT plants neither in the photochemical quenching activity (qP) nor in the ETR through PSII, as measured at different light intensities in the presence of saturating CO_2 on leaves (Figures 3A, B).

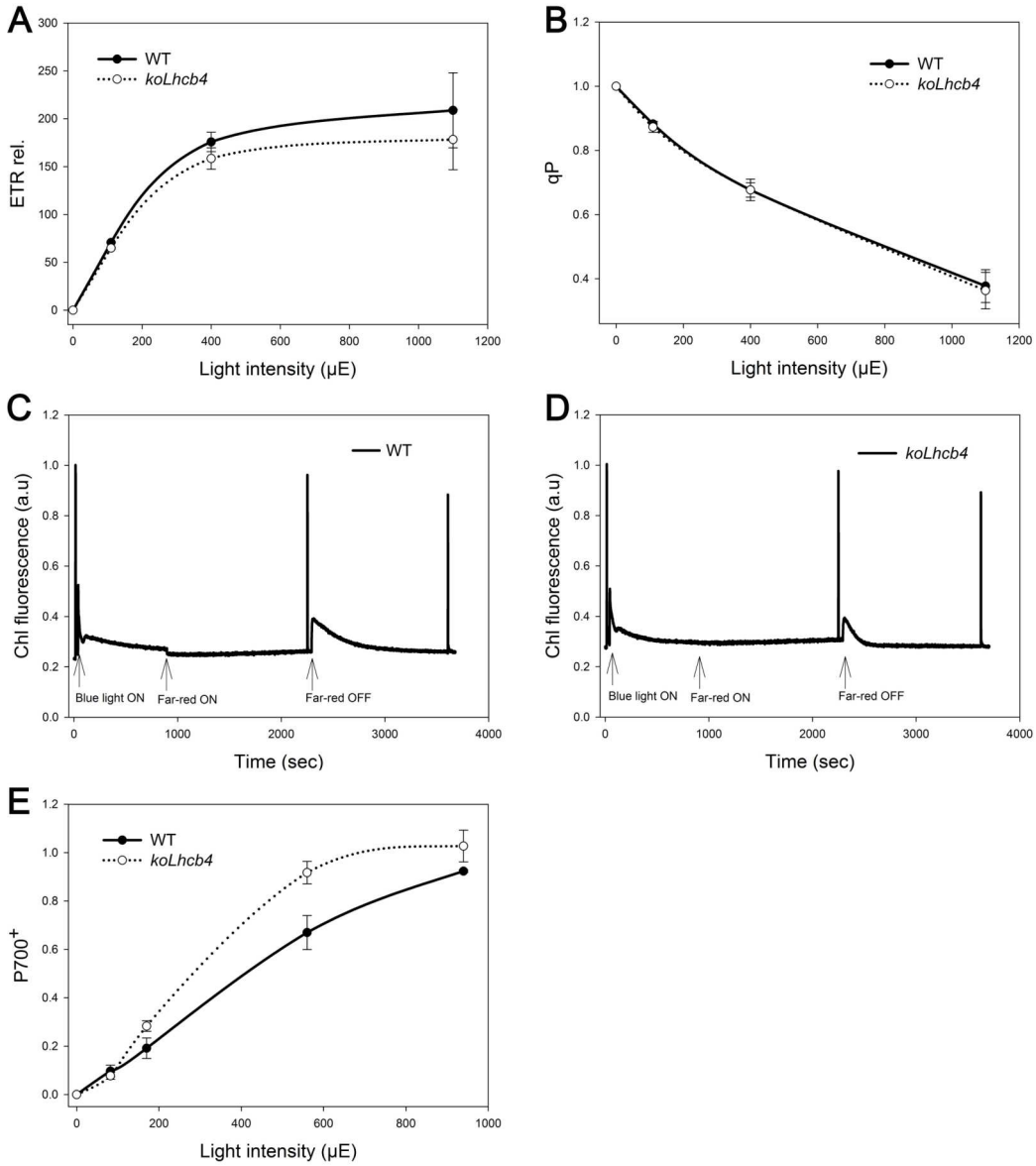


Figure 3. Characterization of photosynthetic electron flow on WT and *koLhcb4* plants. *Dependence of the relative electron transport rate (rel. ETR) on light intensity on wild-type and koLhcb4 leaves. Relative ETR is calculated as rel. ETR=$\phi_{PSII} \cdot PAR$, where ϕ_{PSII} is the quantum yield of electron transfer at PSII, a measure of the overall efficiency of PSII reaction centers in the light; PAR is the photosynthetic active radiation* ***B.*** *Amplitude of photochemical quenching (qP) at different light intensities on wild-type and koLhcb4 leaves. qP reflects the redox state of the primary electron acceptor of PSII, Q_A.* ***C-D.*** *Measurement of State 1–State 2 transition on WT and koLhcb4. Upon 1 hour dark adaptation, plants were illuminated with blue light (40 µmol photons m^{-2} s^{-1}, wavelength <500 nm) for 15 min to reach State II. Far-red light source was then superimposed to blue light in order to induce transition to State I. Values of F_m, F_m', and F_m'' were determined by light saturation pulses (4500 µmol photons m^{-2} s^{-1}, 0.6 s).* ***E.*** *Dependence of the P700 oxidation ratio ($\Delta A/\Delta A_{max}$) on light intensity.*

The capacity for state transitions (Allen, J. F. 1992) was measured from the changes in chlorophyll fluorescence on leaves (Jensen, P. E. et al. 2000). The final amplitude of the state transitions (qT) after a 15 min illumination was the same in both genotypes (0.091 ± 0.002 and 0.091 ± 0.006, respectively in WT vs. *koLhcb4*) (Figure 3C, D). However, the kinetic of the transition from state I to state II upon switching off far-red light was three times faster in *koLhcb4* ($t_{1/2}$ =71 ± 5 sec) with respect to WT ($t_{1/2}$ =204 ± 17 sec) (Figure 3C, D). Furthermore, it was observed that the switching on of far-red light produced a sudden decrease of fluorescence intensity in WT but not in *koLhcb4*. In fact, fluorescence

level in the latter was already very low, consistent with a faster reoxidation of the PQ pool in *koLhcb4* with respect to WT under low intensity blue light.

Fluorescence induction analysis on intact leaves showed a F_0 value up to 40% higher in *koLhcb4* vs. WT, implying the absence of Lhcb4 caused a lower efficiency in energy transfer between LHCII and PSII reaction centre (Table I). Photosynthetic electron flow through PSI during steady state photosynthesis *in vivo*, was measured from the dependence of the $P700^+/P700$ ratio on light intensity (Figure 3E). In WT, this value approached a saturation level at >1000 μmol photons m^{-2} s^{-1}. In *koLhcb4*, the saturation was observed already at 600 μmol photons m^{-2} s^{-1} in *koLhcb4*, which implies a lower electron supply rate from upstream complexes. In order to investigate the origin of this difference, cyclic electron flow (CEF) was determined by following the re-reduction of $P700^+$ upon far-red saturating flashes, on leaves infiltrated with DCMU using absorbance changes at 705 nm (Supplemental Figure 3A). The fast decay component of $P700^+$ that has been attributed to CEF was the same in both WT and *koLhcb4*. Analysis of the functional antenna size of PSI, measured by the rate coefficient of P700 oxidation in steady far-red light following a saturating flash, did not reveal any difference between both genotypes (Supplemental Figure 3B). These results suggest that the different kinetics of P700 oxidation can be attributed to the higher PSI/PSII ratio of *koLhcb4* with respect to WT, as shown in Figure 2B.

3.4 Non-photochemical quenching of chlorophyll fluorescence

Thermal energy dissipation is a major photoprotection mechanism in plants. Although a quenching component has been localized in PSII core (Finazzi, G. et al. 2004), most quenching activity was associated to the antenna system (Havaux, M. et al. 2007, Horton, P. and Ruban, A. 2005). In order to identify the role of Lhcb4 in NPQ, we measured NPQ activity of *koLhcb4* plants (Figure 4A).

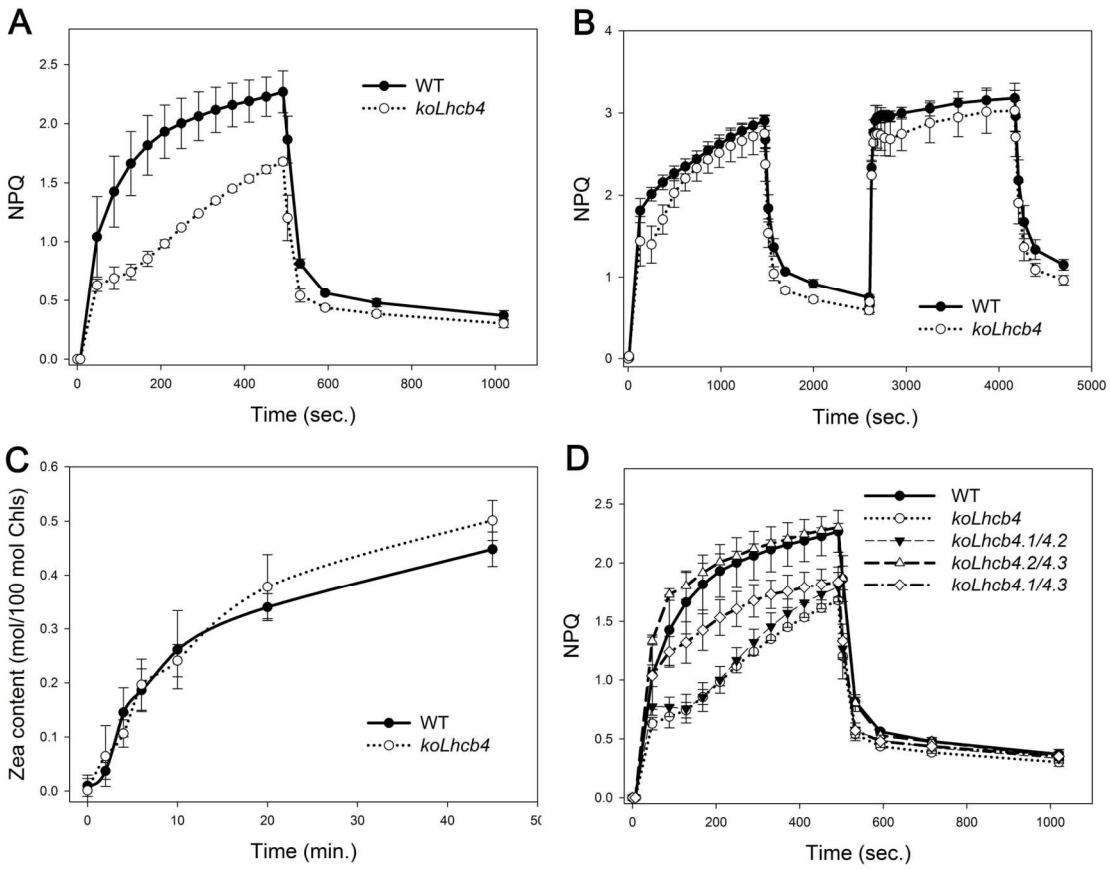


Figure 4. Kinetics of the formation and relaxation of photoprotective energy dissipation. ***A.*** *Measurements of NPQ kinetics on WT and koLhcb4 leaves illuminated with 1200 μmol photons $m^{-2}s^{-1}$, 24°C.* ***B.*** *NPQ kinetics of WT and koLhcb4 plants during two consecutive periods of illumination with white light (1200 μmol photons m^{-2} s^{-1}, 25 min, 24°C) with a 18 min period of darkness in between, as indicated by the white and black bars.* ***C.*** *Time course of violaxanthin de-epoxidation in wild-type and koLhcb4 plants. Leaf discs from dark-adapted leaves were illuminated at 1200 μmol photons m^{-2} s^{-1}, 24° C (white actinic light). At different times, discs were frozen in liquid nitrogen and total pigments extracted before HPLC analysis.* ***D.*** *NPQ kinetics of WT, koLhcb4 and mutants retaining single Lhcb4 isoforms. The expression of the isoform Lhcb4.1 (koLhcb4.2/4.3) leads to complete compensation of the NPQ phenotype of koLhcb4; instead the expression of the Lhcb4.2 isoform partially recovered the quenching ability in the first minutes of induction, nevertheless mutant failed to fully match the WT quenching capacity within 8 min of illumination. The presence of the Lhcb4.3 gene did not contribute to NPQ activity. Symbols and error bars show means ± SD (n > 3).*

In agreement with previous reports (Niyogi, K. K. 1999), upon exposure of WT to saturating light intensity (1200 μmol photons m^{-2} s^{-1}, 24°C) NPQ showed a rapid rise to 1.4 in the first minute followed by a slower raise, reaching a value of 2.3 after 8 minutes. Induction of NPQ in *KoLhcb4* was slower (0.7 at t=1 min) and reached a lower amplitude (1.6 t=8 min). Recovery in the dark was faster and more complete in *koLhcb4* compared to WT (Figure 4A). NPQ activity of *koLhcb4* recovered to WT level upon longer light treatment (25 min). When a second illumination period was applied (Figure 4B) the delay in NPQ rise was less evident, while a recovery in the dark remained still faster in *koLhcb4*. It should be noted that zeaxanthin synthesis had the same kinetic in WT and *koLhcb4* (Figure 4C), implying that the delayed NPQ onset was not due to a delayed zeaxanthin synthesis or slower acidification of the thylakoid lumen.

We also investigated whether the three Lhcb4 isoforms had the same activity in NPQ by analyzing the kinetic of NPQ rise in double mutants retaining one single *Lhcb4* gene. The results reported in Figure 4D show that mutants conserving the *Lhcb4.1* gene had slightly faster onset of NPQ than WT, while those retaining *Lhcb4.2* gene were unable to reach the final NPQ level of WT plants. Mutant plants retaining only *Lhcb4.3* behaved like *koLhcb4*. The faster fluorescence recovery observed in *koLhcb4* compared to WT is present in the mutants retaining only *Lhcb4.2* or *Lhcb4.3* genes, whereas mutant retaining *Lhcb4.1* recovered with the slower WT-type kinetic.

3.5 Photosensitivity under short- and long-term stress conditions

Treatment of plants with strong light produces photoxidative stress, whose severity is enhanced by low temperature. Under these conditions, enhanced release of $^1O_2$ leads to a bleaching of pigments, lipid oxidation and PSII photoinhibition, which is accompanied by a decrease in F_v/F_m (Zhang, S. and Scheller, H. V. 2004). The sensitivity to photoxidative stress of WT and Lhcb4-depleted plants was assessed upon transfer from control conditions to HL + low temperature (500 μmol photons m^{-2} s^{-1}, 4°C). The level of F_v/F_m was monitored for 2 days (Figure 5A). In WT plants, the F_v/F_m parameter gradually decreased from 0.8 to 0.35 during the treatment, while in *koLhcb4* plants the decrease was stronger, reaching a value of 0.15 at the end of the treatment period. As a reference, we compared the photosensitivity of different antenna mutants, namely *koLhcb5/Lhcb6*, *koLhcb3* and LHCII antisense plants (asLHCII). Interestingly, mutants lacking antenna components other than Lhcb4, namely Lhcb6, Lhcb5, LHCII or Lhcb3, showed the same level of photoinhibition as WT plants, implying that Lhcb4 is the only antenna component indispensable for full level of photoprotection under photoxidative stress conditions (Figure 5A). Measurements of F_v/F_m recovery after photoinhibitory treatment (Supplemental Figure 4), clearly showed that WT and *koLhcb4* leaves had the same capacity of PSII quantum efficiency recovery, implying that a higher photosensitivity of mutant plants is due to a less effective photoprotection rather than to impaired PSII repair mechanism (Aro, E. M. et al. 1994). Loss of Lhcb4 caused a decrease in PSII quantum yield (Figure 5A) that could be caused either by damage of the PSII core complex or by an incomplete excitation transfer to PSII RC.

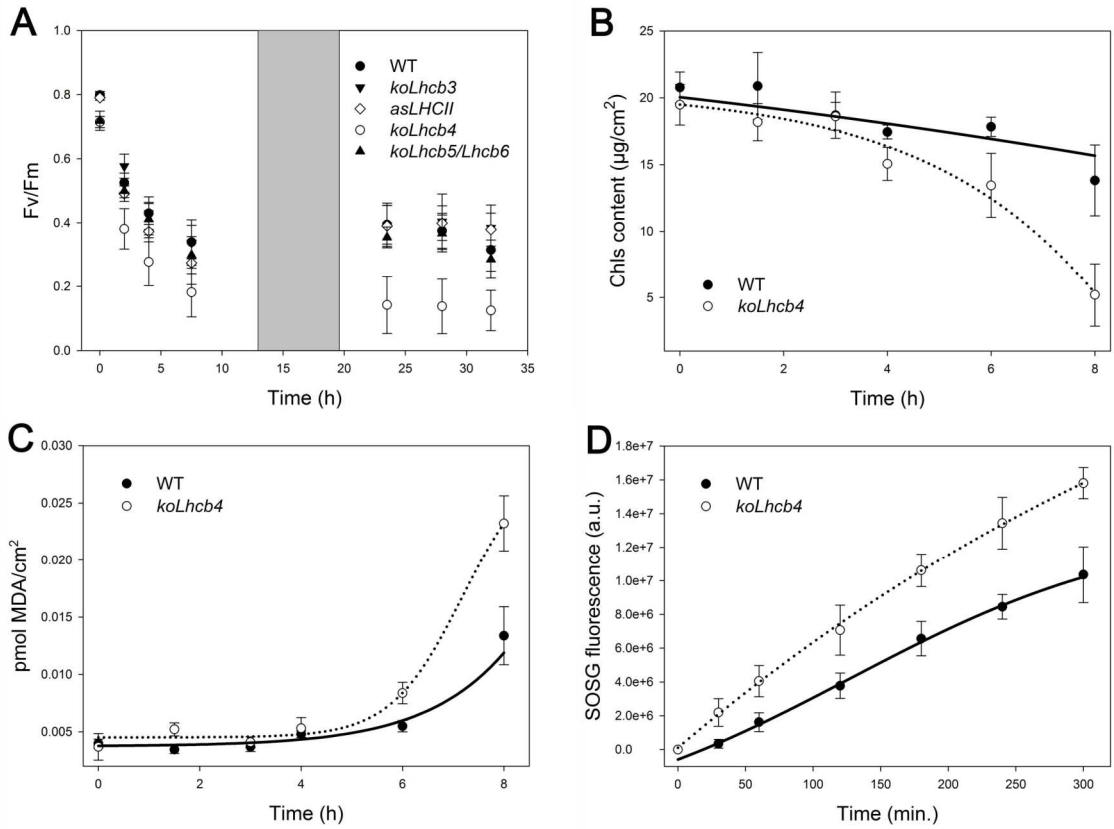


Figure 5. Photo-oxidation of Arabidopsis WT and *koLhcb4* mutant exposed to high-light and low temperature. ***A***. *PSII photoinhibition (F_v/F_m decay) was followed on WT, koLhcb4 and antenna-depleted mutants (koLhcb5/Lhcb6, koLhcb3 and LHCII antisense) plants, treated at 550 µmol photons m^{-2} s^{-1}, 4°C for 30 hours with a 6-hour period of low light (20 µmol photons m^{-2} s^{-1}) between the 12 hours of HL stress; low light interval permitted the PSII efficiency recovery.* ***B, C****. Detached leaves floating on water were treated at 1500 µmol photons m^{-2} s^{-1} at 4° C, and kinetics of chlorophyll bleaching (B) and MDA formation (C) were recorded.* ***D.*** *WT and mutant detached leaves were vacuum-infiltrated with 5 µM SOSG, a $^1O_2$-specific fluorogenic probes. SOSG increases its fluorescence emission upon reaction with singlet oxygen. The increase in the probe emission was followed with a fiber-optic on the leaf surface during illumination with red actinic light (550 µmol photons m^{-2} s^{-1}) at 4°C.*

In order to provide a more complete characterization of the photodamage, leaf discs from WT and *koLhcb4* were submitted to HL + cold stress (1500 µmol photons m^{-2} s^{-1}, 4° C), and the time-course of pigment photobleaching and lipid peroxidation were measured (Figure 5B, C). Analysis indicates that chlorophyll bleaching was faster and malondialdehyde (MDA) production was higher in *koLhcb4* with respect to wild-type leaves, implying a higher level of lipid peroxidation (Havaux, M. et al. 2005). Photodamage can be caused by the production of different ROS species, including singlet oxygen ($^1O_2$) (Triantaphylides, C. et al. 2008), H_2O_2 and superoxide anion (Asada, K. 2000). In order to identify if one or more of these species were involved in the preferential photodamage of *koLhcb4*, we quantified production of different ROS species directly in WT and *koLhcb4* leaves using vacuum-infiltrated ROS-specific chemical probes (see Methods for details). After illumination with strong light at 4°C, *koLhcb4* leaves clearly showed a significantly higher release of singlet oxygen compared to WT (Figure 5D), whereas the production of reduced ROS species was unchanged (data not shown).

An interesting question was whether the different Lhcb4 isoforms had a specific importance in photoprotection. To get the answer, we measured the level of PSII photoinhibition on KO mutants retaining individual *Lhcb4* genes. Plants retaining either *Lhcb4.1* or *Lhcb4.2* showed WT level of resistance to HL+cold stress, while those retaining *Lhcb4.3* gene (*koLhcb4.1/4.2* mutant) were similar to *koLhcb4* (Figure 6).

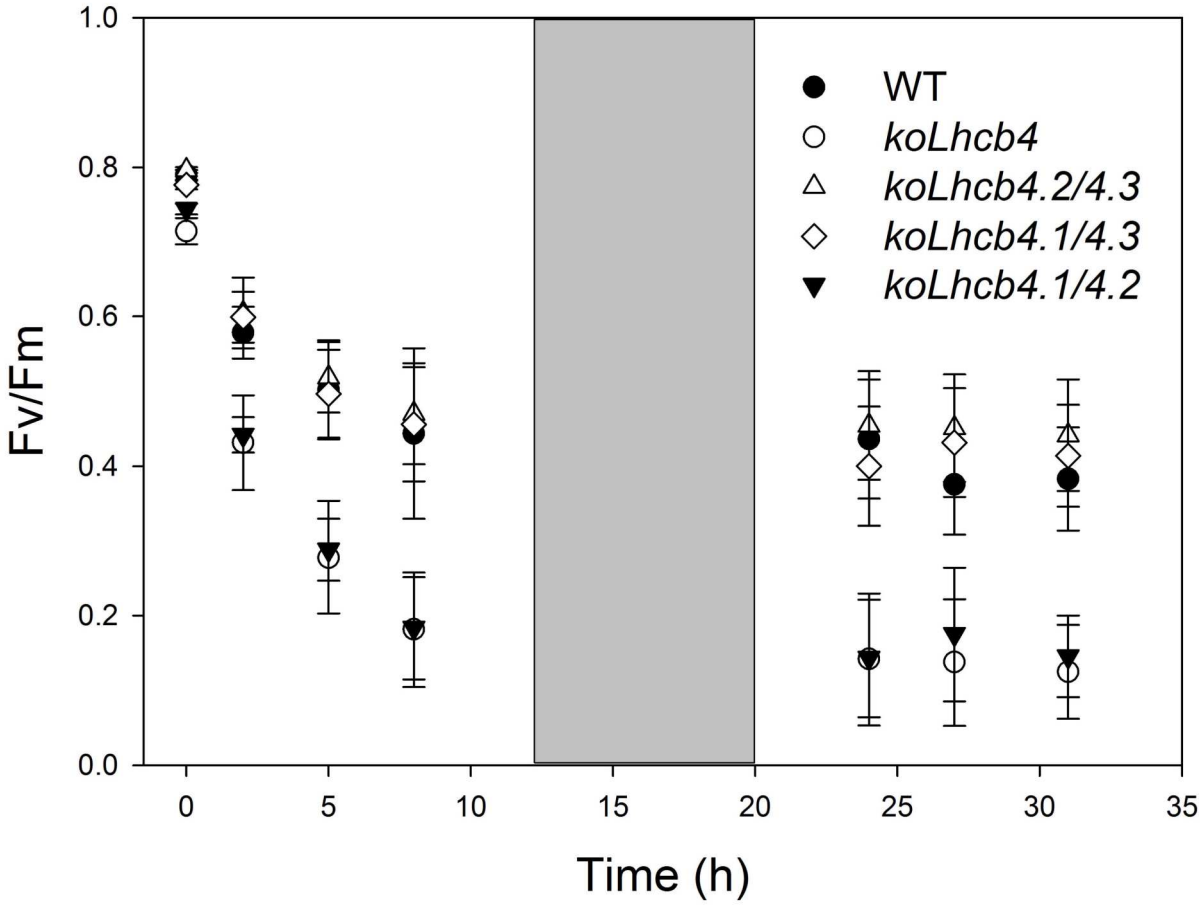


Figure 6. Photoinhibition of WT and mutants retaining a single Lhcb4 isoform exposed to high-light and low temperature. *Kinetics of F_v/F_m decay were measured on WT, koLhcb4, koLhcb4.1/4.2, koLhcb4.1/4.3 and koLhcb4.2/4.3. Whole plants were treated as described for Figure 5A.*

More detailed analysis was performed after 3 and 8 days of treatment by determining the leaf chlorophyll content, a target of photoxidative stress. Mutants *koLhcb4* and *koLhcb4.1/4.2* underwent a significant reduction of leaf chlorophyll content (Table II), while mutants retaining either Lhcb4.1 or 4.2 did not show this effect.

		µg Chl/cm^2				
		WT	***koLhcb4***	***koLhcb4.2/4.3***	***koLhcb4.1/4.3***	***koLhcb4.1/4.2***
Days of stress	**0**	21.7 ± 2.86	19.5 ± 0.16	22.5 ± 1.2	21.5 ± 2.1	22.2 ± 2.6
	3	19.1 ± 0.7	16.5 ± 1.6 *	18.9 ± 2.6	17.7 ± 0.9	17. 2 ± 1.4 *
	8	20.3 ± 2.2	13.7 ± 1.7 *	19.7 ± 2.0	18.1 ± 2.3	15.8 ± 1.8 *

Table II. *Chlorophyll content determined for WT and Lhcb4 mutants after 3 and 8 days of stress (900 µmol photons m^{-2}s^{-1}, 4° C). Data are expressed as mean ± SD (n=5); for each genotype, significantly different values (Student's t test, p=0.05) with respect to t_0 are marked with an asterisk.*

This indicates that a compensatory accumulation of Lhcb4.1 or Lhcb4.2 isoforms can restore photoprotection of the *koLhcb4.1* and *koLhcb4.2* mutants to WT level. Interestingly, the presence of wild type alleles of Lhcb4.3 as the only Lhcb4 isoform did not restore photoprotection. The latter observation prompted us to get further insight into the Lhcb4.3 expression. We investigated accumulation of the Lhcb4.3 isoform upon several stress conditions, namely: (a) 500 μmol photons $m^{-2}s^{-1}$, 4° C for 2 days; (b) 900 μmol photons $m^{-2}s^{-1}$, 4° C for 10 days; (c) 1600 μmol photons $m^{-2}s^{-1}$, 24° C for 10 days. At the end of these treatments, thylakoids were isolated from WT, *koLhcb4* and *koLhcb4.1/4.2* and analyzed by SDS-PAGE / immunoblotting using a polyclonal antibody. Under all experimental conditions no Lhcb4 immune-reactive bands were detected in the *koLhcb4.1/4.2* sample from all the tested conditions. In order to verify that our antibody was indeed able to detect Lhcb4.3, we cloned the three Lhcb4 isoforms and expressed the apoproteins in bacteria. When the three apoproteins were assayed by the anti-Lhcb4 antibody, they were recognized with the same efficiency (Supplemental Figure 5). Based on these experiments we conclude that plants retaining the *Lhcb4.3* gene only were unable to accumulate the encoded protein to a significant level in the conditions assayed in this work.

3.6 Role of the zeaxanthin–Lhcb4 interaction in long-term membrane lipid photoprotection

Earlier work has shown that Lhcb4 exchanges Viola with Zea both *in vitro* (Bassi, R. et al. 1997, Morosinotto, T. et al. 2002) and *in vivo* upon HL treatment (Bassi, R. et al. 1993), which leads to an increased activity of several photoprotection mechanisms, including ROS scavenging, improved Chl triplet quenching (Mozzo, M. et al. 2008) and formation of carotenoid radical cations (Avenson, T. J. et al. 2008, Holt, N. E. et al. 2005). In order to investigate the role of Zea on Lhcb4-dependent photoprotection, we crossed *koLhcb4* lines with *npq1* and selected a genotype lacking both the capacity of producing Zea and Lhcb4. The level of stress caused by HL + cold treatment in WT, *npq1*, *koLhcb4* and *koLhcb4 npq1* was measured from the extent of lipid peroxidation detected by high-temperature thermoluminescence (HTL) at 135 °C (Ducruet, J. M. and Vavilin, D. 1999). Figure 7 shows plots of HTL amplitudes at different time points during exposure of leaf discs to HL stress (800 μmol photons m^{-2} s^{-1}, 4°C). In *koLhcb4* genotypes, HL treatment produced higher levels of lipid peroxidation with respect to WT while *npq1* behavior was intermediate. Experimental points were fitted using a first-order exponential function ($Y = A\ e^{bx}$) and the resulting equations were used to obtain the differential effect of Zea in the presence or absence of Lhcb4 (Figure 7, inset).

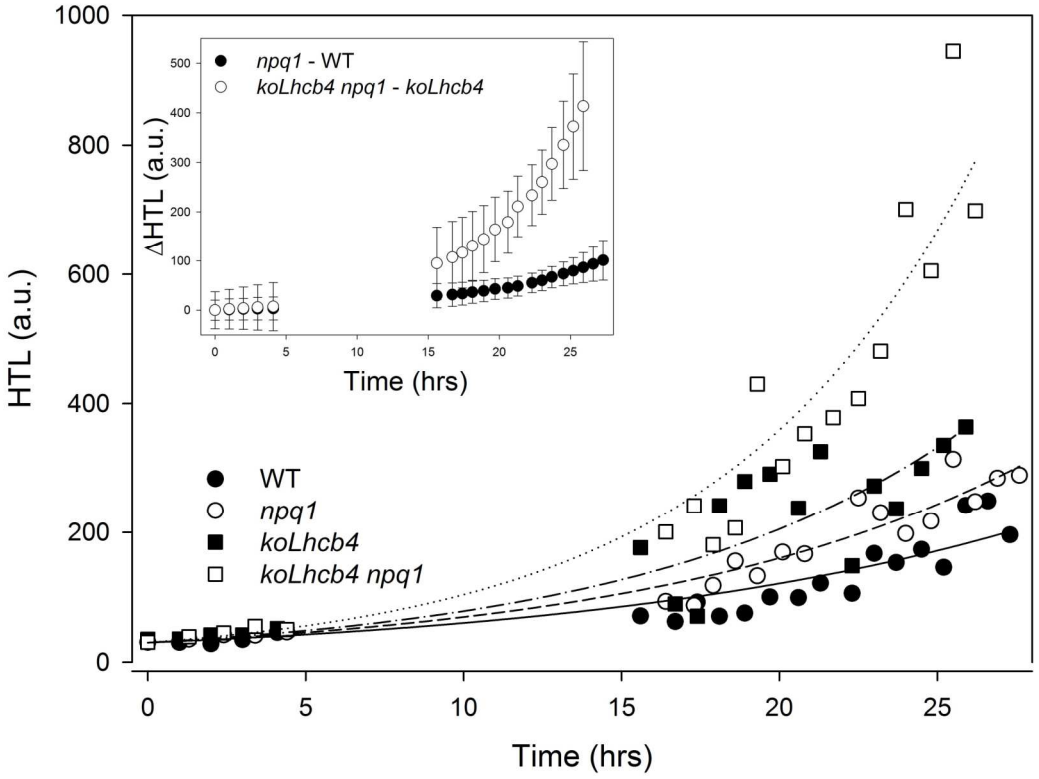


Figure 7. **Kinetics of lipid peroxidation of Arabidopsis detached leaves exposed to high-light stress**. *WT, npq1, koLhcb4 and koLhcb4 npq1 mutant leaves floating on water were exposed to HL (800 µmol photons m^{-2} s^{-1}, 4° C) and photo-oxidation was estimated from the extent of lipid peroxidation measured by high-temperature thermoluminescence (HTL). Each experimental point corresponds to a different sample. The kinetic of oxidized lipid accumulation was described by fitting the dataset with first-order exponential functions: differential kinetics npq1 - WT and koLhcb4npq1 - koLhcb4 were calculated on the basis of the first-order exponential functions obtained by fitting experimental points (error bars, 95% confidence level).*

Clearly, a much stronger differential effect of the *npq1* mutation was observed in *koLhcb4* vs. WT background, implying that photoprotection mediated by Lhcb4 is enhanced in the presence of Zea.

3.7 Structural analysis of isolated grana membranes

The above results suggest that *koLhcb4* mutant was more strongly affected than genotypes lacking other components of the PSII antenna system. Previous work with *koLhcb6* (koCP24) (de Bianchi, S. et al. 2008, Kovacs, L. et al. 2006) and *koLhcb3* (Damkjaer, J. et al. 2009) has shown that mutations in members of the Lhcb sub-family can affect the macro-organization of PSII supercomplex and PSII-associated regulatory functions. In order to verify whether a structural effect was induced by lack of Lhcb4, we analyzed both the organization of PSII supercomplexes in grana membranes and a structure of isolated PSII supercomplexes from *koLhcb4* plants by transmission electron microscopy and single particle analysis. Analysis of negatively stained grana membranes from wild-type and *koLhcb4* plants, isolated as previously described (Morosinotto, T. et al. 2010), exhibit randomly distributed tetrameric stain-excluding particles, corresponding to the PSII-OEC complexes exposed on the luminal surface of dimeric PSII core complexes (Figure 8A,B) (Betterle, N. et al. 2009, Boekema, E. J. et al. 2000).

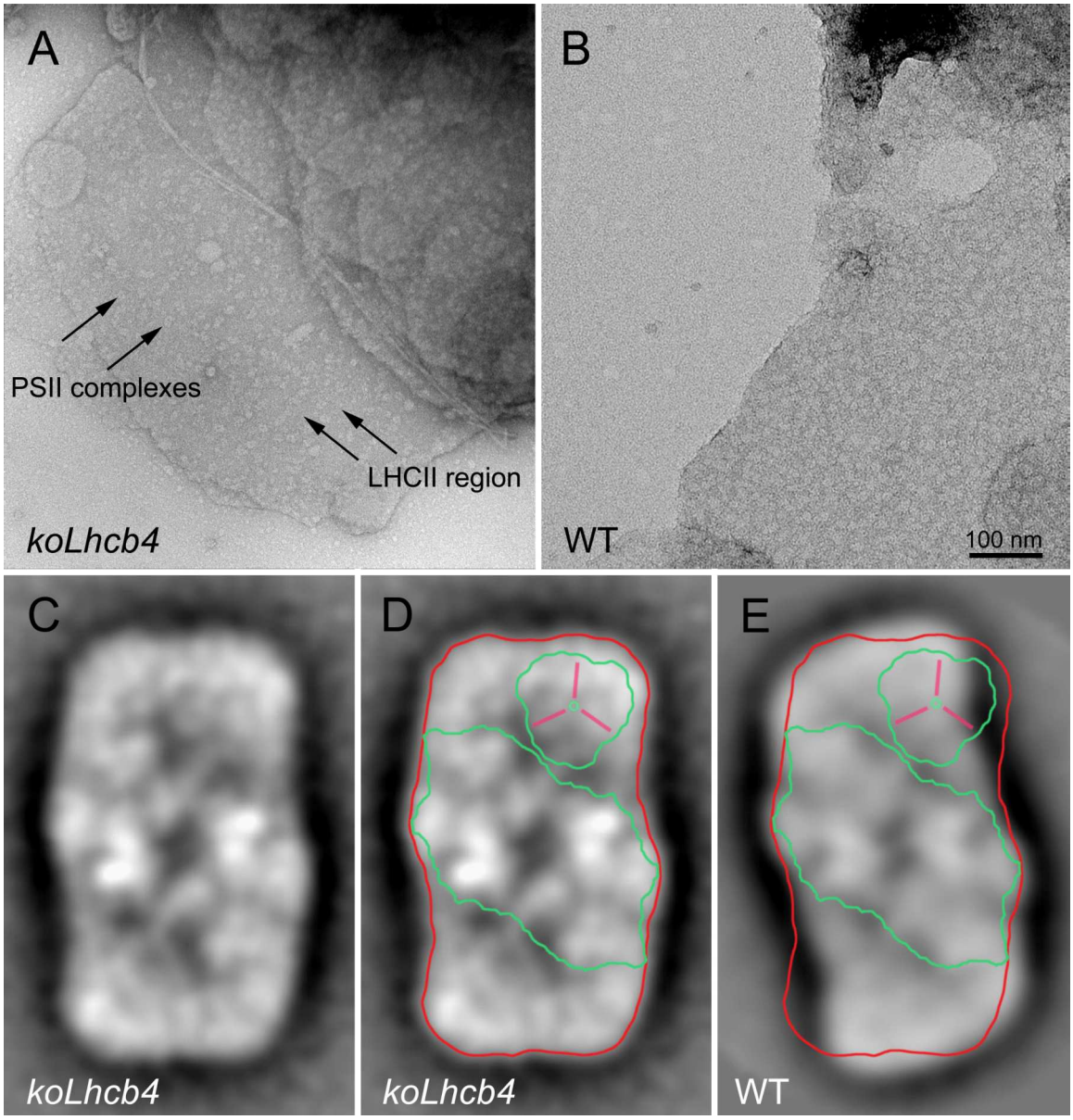


Figure 8. Electron microscopy of grana membranes and PSII particles. ***A,B.*** *EM of negative staining grana partition membranes were obtained by partial solubilisation of thylakoids from koLhcb4 (A) or wild-type plants (B) with α-DM. High-resolution micrographs show the distribution of stain-excluding tetrameric particles (arrows). Grana partitions from koLhcb4 (A) were characterized by the presence of tetrameric particles more widely spaced than wild-type membranes (B). Arrows indicate PSII core complexes (magenta) and LHCII region (violet).* ***C-D****. Average projection map of a set of 1024 projections of C_2S_2 supercomplexes from plants lacking Lhcb4 (C, D) and wild-type (E). Contours representing PSII dimeric core (green), Lhcb4 (yellow) and whole wild-type C_2S_2 supercomplex (red) are superimposed.*

In case of WT, a density of PSII complexes in the grana membrane was about 1.5 PSII per 1000 nm^2 (Figure 8B). Samples from *koLhcb4* were clearly different (Figure 8A), being characterized by a wider spacing of the tetrameric particles (0.9 PSII particles per 1000 nm^2). To investigate a lack of Lhcb4 on a structure of PSII supercomplex in more details, purified PSII supercomplexes were analyzed by single particle analysis. To this aim, freshly isolated thylakoids of *koLhcb4* were mildly solubilized with 0.3% α-DM followed by rapid fractionation by gel filtration (Damkjaer, J. et al. 2009). The first eluted fractions, containing membrane fragments and PSII supercomplexes, were analyzed by EM. Single projections of PSII supercomplexes images, identified on the basis of their shape as determined by earlier work (Caffarri, S. et al. 2009); supercomplexes were collected, and subjected to image analysis, including translational and

rotational alignments, multivariate statistical analysis and averaging of homogeneous classes. Figure 8C shows the average projection map from a set of 1024 projections of C_2S_2 supercomplexes from plants lacking Lhcb4. Clearly, the projection map is rather similar to the projection map of the WT C_2S_2 supercomplex (Figure 8E). In both genotypes, typical features of the core complex were resolved, together with the S-trimer within the peripheral antenna. However, a detailed comparison between the complexes from *koLhcb4* (Figure 8C, D) and from WT (Figure 8E) (adopted from (Boekema, E. J. et al. 2000)) showed a different mode of binding of the S-trimer to the core complex and thus a different overall shape of the C_2S_2 particles. Indeed, the S-trimer in *koLhcb4* has a different position with respect to the core complex than in WT. In addition the electron density at the position where Lhcb4 binds in WT C_2S_2 particles is localized much closer to the core complex in *koLhcb4* and thus space between the core complex and LHCII-S seems to be too small in order to accommodate an Lhcb subunit, suggesting it could represent a lipid-filled area. The simplest way to interpret this result is that PSII supercomplex associates in the absence of Lhcb4 leading to a reorganization of the overall shape of the particle with partial occlusion of the Lhcb4 area and bending of the LHCII-S mass towards the CP47 complex of the core. In order to verify this hypothesis, we determined the polypeptide composition and relative amounts in the PSII particles from WT vs. *koLhcb4*. In particular, we aimed at determining whether any Lhcb subunit could substitute Lhcb4 in the complex from mutant. To this aim, grana membranes from WT and mutant plants were solubilized with a very low concentration of α-DM (0.3%) and fractionated by non-denaturing Deriphat-PAGE (Supplemental Figure 6A), yielding a pattern superimposable to that previously reported (Caffarri, S. et al. 2009). Fractions containing the C_2S_2M (Band 10) and $C_2S_2M_2$ (Band 11) supercomplexes were still detectable in *koLhcb4*, albeit their amounts were reduced compared to WT (14% and 9% of C_2S_2M and $C_2S_2M_2$) (Supplemental Figure 6A). We chose to analyze C_2S_2M supercomplexes (Band 10) since this fraction is homogeneous with respect to other fractions with higher mobility (Caffarri, S. et al. 2009) (see Supplemental Figure 7). Band 10, and other bands as a reference, were eluted from the gel and their protein composition and relative abundance were determined by SDS-PAGE (Supplemental Figure 6B), densitometry and quantitative western blotting analysis (not shown). In the C_2S_2M supercomplex (Band 10) from WT, polypeptides of PSII core were found along with Lhcb4, Lhcb5 and LHCII. In the same fraction from *koLhcb4*, the amounts of Lhcb5 and LHCII with respect to PSII core subunits were the same while Lhcb4 was not found, implying that this C_2S_2M supercomplex, although exhibiting a similar overall organization and mobility in green gels, missed one of its inner subunits. Lhcb4 was not substituted by any other protein component that we could confirm by Coomassie staining or immunoblotting. (see Supplemental Figure 6 for details).

4. DISCUSSION

4.1 The role of Lhcb4/Lhcb6 in the topology of grana membranes and in the assembly of the PSII-LHCII supercomplex

Lhcb4 is one of the six homologous Lhc proteins composing the PSII antenna system. These pigment-protein complexes are expected to share a common 3D organization on the basis of the structural data available for five members of the Lhc family ((Ben Shem, A. et al. 2003, Liu, Z. et al. 2004). Previous work with antisense lines has evidenced a high degree of redundancy among Lhcb subunits; in fact, the PSII supercomplex organization was maintained in the absence of Lhcb1+2 components by over-accumulating Lhcb5 (Ruban, A. V. et al. 2003). Similarly, plants lacking Lhcb5 and/or Lhcb6 over-accumulated Lhcb1 or Lhcb4 gene products, leading to maintenance of the C_2S_2 central architecture of the PSII supercomplex, only the organization of the outer shell of PSII antenna being affected in the *koLhcb6* plants (de Bianchi, S. et al. 2008). In the case of *koLhcb4*, structural redundancy is broken as shown by the de-stabilization of all supercomplex bands in green gels (Figure 2C, Supplemental Figure 2) and by the

differences in structure of C_2S_2 particles with respect to WT (Figure 8). These results are consistent with Lhcb4 being the only Lhc subunit that can occupy the position between CP47 and LHCII-M building blocks within the PSII supercomplex (Figure 8 C-E). This conclusion is strongly supported by the polypeptide composition of C_2S_2M particles isolated from *koLhcb4* that lack this subunit without a compensation by other gene products (Supplemental Figure 6B-C).

Our ability to isolate C_2S_2 supercomplex from *koLhcb4* grana membranes shows that antenna proteins can be associated to the core complex in the absence of Lhcb4 as a docking subunit, which is consistent with a recent report showing that a complex of a monomeric core with CP26 and LHCII-S trimer was stable enough to be isolated (Caffarri, S. et al. 2009). Supercomplexes with a similar shape around the Lhcb4 position were previously found after salt treatment (Boekema, E. J. et al. 2000). We conclude that in the absence of Lhcb4, PSII does assemble in grana membranes. However, because of the missing subunit, the complex is less stable and assumes a different overall structure with a low density area located in the position where Lhcb4 is present in WT, likely occupied by lipids. As for the organization of the outer shell of PSII antenna system, formed by LHCII-M, LHCII-L and Lhcb6, it appears to be strongly affected in *koLhcb4*. Titration of the different Lhcb proteins with respect to PSII RC showed that the Lhcb6 complex was completely missing in *koLhcb4* (Figure 2B). Since the *Lhcb6* messenger level was unchanged with respect to WT, this implies that removal of Lhcb4 decreases Lhcb6 stability. This is consistent with Lhcb4 being the docking site for Lhcb6 (Andersson, J. et al. 2001, Caffarri, S. et al. 2009), both participating to a pentameric complex called B4C (band 4 complex), which connects inner and outer antenna moieties (Bassi, R. and Dainese, P. 1992, Betterle, N. et al. 2009). One component of B4C, interacting with Lhcb4 and/or Lhcb6, is Lhcb3 (Betterle, N. et al. 2009) which, at variance with Lhcb6, is still present in *koLhcb4* membranes; it is consistent with the observation that, besides Lhcb4, Lhcb3 can interact with Lhcb1 and Lhcb2 in complexes containing LHCII-M (Supplemental Figure 6B, lanes B9-B11), while it is absent in complexes containing only LHCII-S such as CS complexes (lanes B6). Less stable $C_2S_2M_2$ supercomplexes can still form in *koLhcb4* mutant lacking both Lhcb4 and Lhcb6, although their molecular interactions are rather weak and the abundance of this complex is only 5% with respect to WT. Previous work with *koLhcb6* has shown that a large part of the outer antenna formed by LHCII-M and LHCII-L is not directly bound to PSII supercomplexes; rather, they form LHCII-only domains segregating from arrays of C_2S_2 particles (de Bianchi, S. et al. 2008). No such arrays were observed in *koLhcb4* membranes (Figure 8A), nor was PQ diffusion restricted (Figure 3B), suggesting that the altered shape of C_2S_2 particles and/or their instability prevents cooperative interactions into arrays. Besides this, the distance between neighbor PSII centers is higher than in WT (Figure 8A-B) implying a higher number of LHCII trimers is interposed between PSII centers, consistent with the densitometric analysis of green gels (Figure 2B) and ultrastructural analysis of thylakoid grana membranes (Figure 8A,B).

4.2 Consequences for photosynthesis: light harvesting and electron transport.

The loss of Lhcb4 in *A. thaliana* did not strongly affect growth rate, pigment compositions and growth of plants under control light conditions (Figure 3, Table I). Indeed, linear and cyclic electron transport rates were similar to wild-type plants as well as the functional antenna size. Alterations in the PSII macrostructure in mutant plants, however, did result in differences in chlorophyll fluorescence parameters (Table I). Increased F_0 (Table I) clearly showed that the efficiency of excitation energy transfer from the antenna to the PSII reaction centre is decreased in mutant plants. In grana membranes from the wild type (Figure 8B), the distribution of PSII particles is homogeneous through the whole surface. This is not the case for *koLhcb4*, whose grana membranes contained discrete patches of LHCII trimers that are interspersed by fewer PSII core complexes, that are randomly distributed (Figure 8A); therefore, in some discrete areas of *koLhcb4* grana membranes the LHCII/PSII core ratio is increased. In these domains, LHCII fluorescence is probably

not efficiently photochemically quenched, thus yielding higher F_0 (Table I). This is consistent with the results obtained in KO mutants for Lhcb5 and Lhcb6 (de Bianchi, S. et al. 2008, Kovacs, L. et al. 2006) while no such an effect was observed in Lhcb1/Lhcb2 (Andersson, J. et al. 2003). According to previous evidence (de Bianchi, S. et al. 2008) this is likely due to LHCII domains that are not coordinated by PSII core complexes. Increased F_0 suggests that vacancy of Lhcb4 site in mutant PSII-supercomplex causes excitation energy transfer from the peripheral antenna to the core to follow restricted pathways; indeed, the migration time of excitations from antenna to PSII reaction centre was significantly reduced in *koLhcb4* with respect to the wild-type (van Oort, B. et al. 2010). Thus, despite fluorescence induction in the presence of DCMU it showed no changes in functional antenna size, yet the number of LHCII per PSII RC in grana membranes was higher (Figure 8A,B). We conclude that in the absence of a well organized PSII-LHCII supercomplex, the efficiency of excitation energy trapping in *koLhcb4* was lower than in WT and that the compensatory increase in peripheral LHCII complexes (Figure 2A-B) contributed with lower efficiency to light harvesting.

Although functional measurements indicate that there was no major perturbation of PSII and PSI function, an increase in the PSI/PSII ratio was observed in *koLhcb4*: both densitometric analysis of Deriphat-PAGE and immunotitration with specific antibodies revealed that, while the mutant has a full complement of LHCII and PSI-LHCI (PsaA), the PSII (PsbB) content is decreased by 25% in Lhcb4-less plants (Figure 2). The consequences of a lower than WT PSII level were not very strong: the parameter S_m/t_{Fmax}, expressing the average fraction of open reaction centers during the time needed to complete their closure, was similar in *koLhcb4* and WT plants, thus making unlikely ET restriction in PSII. However, the lack of fluorescence quenching in far-red light (Figure 3 C-D) and the higher level of $P700^+$ accumulation (Figure 3E) imply that, at both HL and limiting light, PSII activity was lower in *koLhcb4* than in WT.

4.3 Consequences for regulation of light harvesting: State Transitions.

State transition is the mechanism by which the complement of LHCII bound to PSII vs. PSI is balanced depending on the reduction state of the intermediate electron carrier plastoquinone (PQ) through its reversible phosphorylation, which induces its disconnection from PSII and binding to PSI (Allen, J. F. and Nilsson, A. 1997, Jensen, P. E. et al. 2000). Lhcb4 can be phosphorylated in monocots (Bergantino, E. et al. 1995, Bergantino, E. et al. 1998) and in *Arabidopsis* (Hansson, M. and Vener, A. V. 2003). In *C. reinhardtii* P-Lhcb4 was found to be connected to PSI-LHCI supercomplexes in state II conditions only (Takahashi, H. et al. 2006). Thus, changes in state transitions can be expected in Lhcb4-less plants. Results displayed on Figure 3C-D show that *koLhcb4* was not affected in its state transition total activity, in agreement with the similar reduction state of the plastoquinone pool at all light intensities (Figure 3B), (Bellafiore, S. et al. 2005). We observed that in *koLhcb4* the fluorescence changes, upon switching off the far-red light, are faster than the wild-type, implying that the transiently reduced state of the free PQ pool is more promptly relaxed by migration of the LHCII to PSI. The same state transition phenotype has been described for *koLhcb6* and *koLhcb5/Lhcb6* plants (de Bianchi, S. et al. 2008), and this is a clear indication that the connection between PSII core and the bulk trimeric LHCII are weaker in these mutants with respect to WT. In the case of the *koLhcb6* plants, the faster state transition was attributed to the displacement of the M-trimer from the PSII macrostructure, thus enhancing its migration to PSI. We suggest that, in the *koLhcb4* mutant, the weaker binding of S trimer (and, possibly, of the M trimer) and the higher amount of weakly connected LHCII trimers available, increases the probability of migrating towards PSI upon phosphorylation. In *C. reinhardtii*, state transitions do not only fulfill the role of balancing light absorption between photosystems, they also increase PSI electron flow at the expense of PSII, acting as a switch between linear and cyclic electron pathways (Vallon, O. et al. 1991). This was not the case in *Arabidopsis* since, despite a clear effect on state transitions (Figure 3C, D) no changes in linear (Figure 3A) vs. cyclic electron transport rates (Supplemental Figure 3) was observed.

The major effect of the mutation on electron transport activity was faster saturation of the P700 oxidation ratio in *koLhcb4* with respect to WT (Figure 3E). This can be due to (i) a smaller PSI antenna size, (ii) a lower rate of ET from cytochrome b_6/f complex or (iii) a higher PSI/PSII ratio. As the PSI functional antenna size that was measured was the same in both genotypes (Supplemental Figure 3) and cyclic ET was also unaffected, differential kinetics of P700 oxidation ratio can reasonably be attributed to the higher PSI/PSII ratio of *koLhcb4* with respect to WT (Figure 2B).

4.4 Non-photochemical fluorescence quenching

Non-photochemical dissipation of excess energy is affected in *koLhcb4* plants with respect to the wild-type, consisting into a delayed rise and a lower NPQ amplitude after 8 min light: a clear plateau was observed in the NPQ kinetic between 1 and 3 min, after which the curve rose again (Figure 4A). Upon prolonged illumination and/or upon repeated light treatments (Figure 4B) the amplitude of NPQ was similar to WT. A similar effect was previously observed in *koLhcb5/Lhcb6* double mutant (de Bianchi, S. et al. 2008). Since Lhcb6 is de-stabilized in *koLhcb4* (Figure 2), this mutant phenocopies a double *koLhcb4/Lhcb6* mutant as for the lack of Lhcb6. NPQ kinetic can be modified by changes in the trans-thylakoid ΔpH gradient (de Bianchi, S. et al. 2008), by changes in the level of the pH sensor PsbS (Li, X. P. et al. 2002, Li, X. P. et al. 2004) or by changes in the number/relative abundance of protein subunits hosting quenching sites (Bonente, G. et al. 2007). Changes in luminal pH appear unlikely: in fact, the kinetic of zeaxanthin synthesis, catalyzed by the pH-dependent enzyme VDE (Yamamoto, H. Y. and Higashi, R. M. 1978) was the same in WT and mutant leaves (Figure 4C). Moreover, the level of PsbS was unchanged (Figure 2B), leaving modification in the abundance and identity of quenching sites localized in Lhcb proteins as the most likely cause for the observed phenotype. We interpret our results in the framework of the recently proposed model (de Bianchi, S. et al. 2010, Miloslavina, Y. et al. 2008) based on the formation, upon lumen acidification, of quenching sites within each of two distinct protein domains in grana discs: (i) C_2S_2 particles (containing PSII RC, Lhcb4, Lhcb5 and LHCII-S) and (ii) the peripheral antenna including Lhcb6, LHCII-M and LHCII-L (Betterle, N. et al. 2009, Miloslavina, Y. et al. 2008), which segregates because of the action of PsbS. Zea-dependent quenching activity, has been detected within monomeric Lhcb 4, 5 and 6 in detergent solution (Avenson, T. J. et al. 2008) while, in LHCII, quenching was activated by aggregation and was independent from Zea (Ruban, A. V. et al. 2007). The similar NPQ kinetics of *koLhcb4* and of *koLhcb5/Lhcb6* can thus be explained because they both retain a quenching site within the C_2S_2 domain; the faster quenching kinetic upon repeated illumination is due to the enhancement by Zea of the quenching activity (Niyogi, K. K. et al. 1998) associated to Lhcb4 or Lhcb5. This is consistent with the capacity of exchanging Viola with Zea in binding site L2 observed in monomeric Lhcbs (Morosinotto, T. et al. 2002, Wehner, A. et al. 2006) rather than in trimeric LHCII. In *koLhcb5/Lhcb6* the recovery of NPQ upon the initial delay is faster and more complete than in *koLhcb4* implying that Lhcb4 is more active as a quencher than Lhcb6, consistent with the little impact of Lhcb5 deletion on NPQ amplitude and kinetic when Lhcb4 is unaffected (de Bianchi, S. et al. 2008). If there are two distinct quenching domains within PSII antenna (Miloslavina, Y. et al. 2008) and monomeric Lhcb4-6 proteins are the only sites of quenching *in vivo*, the peripheral antenna domain should remain unquenched in both *koLhcb5/Lhcb6* (de Bianchi, S. et al. 2008) and *koLhcb4*, because they both lack Lhcb6. Since both genotypes reach NPQ levels similar to WT, although with a delayed kinetic, it is likely that some kind of quenching does occur in the peripheral antenna domain disconnected from C_2S_2 particles, which, in these genotypes, only contains LHCII isoforms. Previous work with *koLhcb6* has shown that this component has a quenching effect on the major LHCII antenna (van Oort, B. et al. 2010); here, we observe that, even in the absence of Lhcb6, the overall quenching activity is high, consistent with quenchers being activated in both PSII antenna domains, and conclude that LHCII is likely to contribute to NPQ *in vivo*, in spite of the fact that it does not exhibit spectroscopic and structural features of quenching sites (Ahn, T. K. et al. 2008, Avenson,

T. J. et al. 2008). Thus it is likely to activate quenching by a different mechanism (Ruban, A. V. et al. 2007). Consistent with this view is the finding that a red-shifted fluorescence lifetime component was observed *in vivo* under NPQ conditions (Holzwarth et al. , unpublished results) having properties similar to LHCII aggregated *in vitro*. A more detailed fluorescence lifetime analysis is needed in order to distinguish between quenching activities in the two protein domains.

While differences in qE were minimized by prolonged/repeated illumination, we observed that the qI component, responsible for the slowly relaxing component of quenching, is equally decreased upon short/long or repeated light treatments (Figure 4B). Previous work suggested that qI was specifically due to Zea binding to Lhcb5 (Dall'Osto, L. et al. 2005); instead, removal of Lhcb6 has a negligible effect on qI amplitude, since double mutant *koLhcb5/Lhcb6* did not further decrease its qI level (de Bianchi, S. et al. 2008). Here, we show that removal of Lhcb4 reduces the extent of qI, implying that qI is catalyzed by both Lhcb4 and Lhcb5.

4.5 Consequences for photoprotection: resistance to photoxidative stress is decreased in koLhcb4 plants with respect to wild-type

Lhcb4-less plants showed a reduced photoprotection capacity when exposed to high irradiance at low temperature. The highest sensitivity to photoxidative stress of *koLhcb4*, amongst all other Lhcb KO mutants, is consistent with the higher reduction in fitness of plants lacking Lhcb4 (Andersson, J. et al. 2001, Ganeteg, U. et al. 2004), supporting the importance of this gene product for PSII performance and chloroplast photoprotection. Structural integrity of photosynthetic supramolecular complexes is essential for the resistance to photoxidative stress (Horton, P. and Ruban, A. 2005) although the reasons are not completely clear. Depletion of light harvesting antenna has been reported to favor photoinhibition of both PSI (Alboresi, A. et al. 2009) and PSII (Dall'Osto, L. et al. 2010). *KoLhcb4* mutant plants are more sensitive to photoxidative stress than WT (Figure 5) and this effect is associated to increased production of $^1O_2$ rather than other ROS species. This effect cannot be ascribed to a pleiotropic effect on photosynthetic electron transport efficiency, since lack of Lhcb4 does not significantly affect either the rate or the regulation of photosynthetic electron transport (Figure 3 A-D). A putative mechanism for photoinhibition is the reduction of ^{1}Chl* dissipation (qE and qI) (Johnson, M. P. et al. 2007); however, in *koLhcb4*, qE activity is affected at the onset of illumination while it is similar to WT on a longer timescale. Since our photoinhibitory light treatment was performed at constant light intensity, we conclude that photosensitivity of *koLhcb4* plants is not due to differences on qE or qI.

Connection between PSII core complex and outer LHCII was partially impaired, indeed a steady increase in F_0 was measured in *koLhcb4* plants with respect to wild type (Table I); a higher ^{1}Chl* level than WT might lead to ^{3}Chl* formation through intersystem crossing. Although we cannot exclude that the inefficient connection of LHCII to PSII RC could contribute to the higher photosensitivity of *koLhcb4*, we notice that the *koLhcb5/Lhcb6* double mutant was clearly as resistant as wild-type plants (Figure 5A), despite a F_v/F_m reduction comparable to that of Lhcb4-less plants (de Bianchi, S. et al. 2008). Since photoprotection in *koLhcb5/Lhcb6* plants was similar to wild-type (Figure 5A), neither the increase in F_0 per se nor the absence of Lhcb6 can be the cause of higher photosensitivity of *koLhcb4* plants. Instead, this effect appears to be specific for the absence of Lhcb4 implying this gene product is of particular importance in providing PSII photoprotection with respect to all other Lhcb proteins (Figure 5A). Indeed, Lhcb4 appears to be the most conserved among Lhc proteins associated with PSII (Koziol, A. G. et al. 2007), and it maintains its stoichiometry with respect to the reaction centre even under stressing growth conditions leading to depletion of Lhcb1, 2 and 6 (Ballottari, M. et al. 2007) and in xanthophyll mutants that destabilizes other Lhc members, such as Lhcb5 (Havaux, M. et al. 2004). The most obvious peculiarity of Lhcb4 is that it cannot be replaced in PSII supramolecular architecture by other Lhcbs and is thus required for allowing structural integrity of PSII (Boekema, E. J.

et al. 1999) (Figures 8C-E). Thus, the higher photosensitivity in *koLhcb4* could be the effect of a less stable PSII supercomplex: indeed the extreme photosensitivity of *Arabidopsis* mutants such as *ch1* (Dall'Osto, L. et al. 2010) can be ascribed to the absence of light-harvesting complexes surrounding the PSII reaction centre, leaving the PSII core complex exposed to the lipid phase, where radical chain reactions of peroxy-lipids occur during photo-oxidative stress.

4.6 The role of Zeaxanthin-Lhcb4 interaction in PSII photoprotection.

Measurement of lipid peroxidative damage by thermoluminescence in intact leaves of WT vs. *koLhcb4* and *koLhcb4 npq1* plants showed that Zea and Lhcb4 are synergically active in protection from photodamage (Figure 7). Therefore, it appears that a specific effect for Zea in providing efficient photoprotection is preferentially amplified when bound to Lhcb4. This observation is consistent with a previous report of a protective effect of Zea (Dall'Osto, L. et al. 2010) additional to qE enhancement (Niyogi, K. K. et al. 1998) and to ROS scavenging in the lipid phase (Baroli, I. et al. 2003, Baroli, I. et al. 2004, Havaux, M. et al. 2007, Havaux, M. and Niyogi, K. K. 1999). Upon light induced synthesis, Zea enters the protein-bound xanthophyll pool by binding to the L2 site of minor Lhc (Avenson, T. J. et al. 2008) and to the V1 site of LHCII (Caffarri, S. et al. 2001), in addition to the accumulation in the lipid phase. The molecular mechanism of enhanced resistance to photoxidation by Zea is not yet clear. The hypotheses include: (i) draining excitation energy from PS core complexes by xanthophylls bound at the interface with Lhcbs; (ii) quenching ^{3}Chl* energy from RC, possibly by the Dexter exchange mechanism (Nayak, L. et al. 2002) by Zea located in site L2 on specific Lhcs; (iii) preferential scavenging of $^1O_2$ by zeaxanthin into site V1 of LHCII (Johnson, M. P. et al. 2007). Although we cannot exclude any of these hypotheses, we favor the view that it forms a protective shield partly covering the PSII reaction centre, thus subtracting PSII core complexes from radical chain reactions of peroxy-lipids during photo-oxidative stress.

4.7 Effect of the accumulation of the individual isoforms on koLhcb4 phenotype

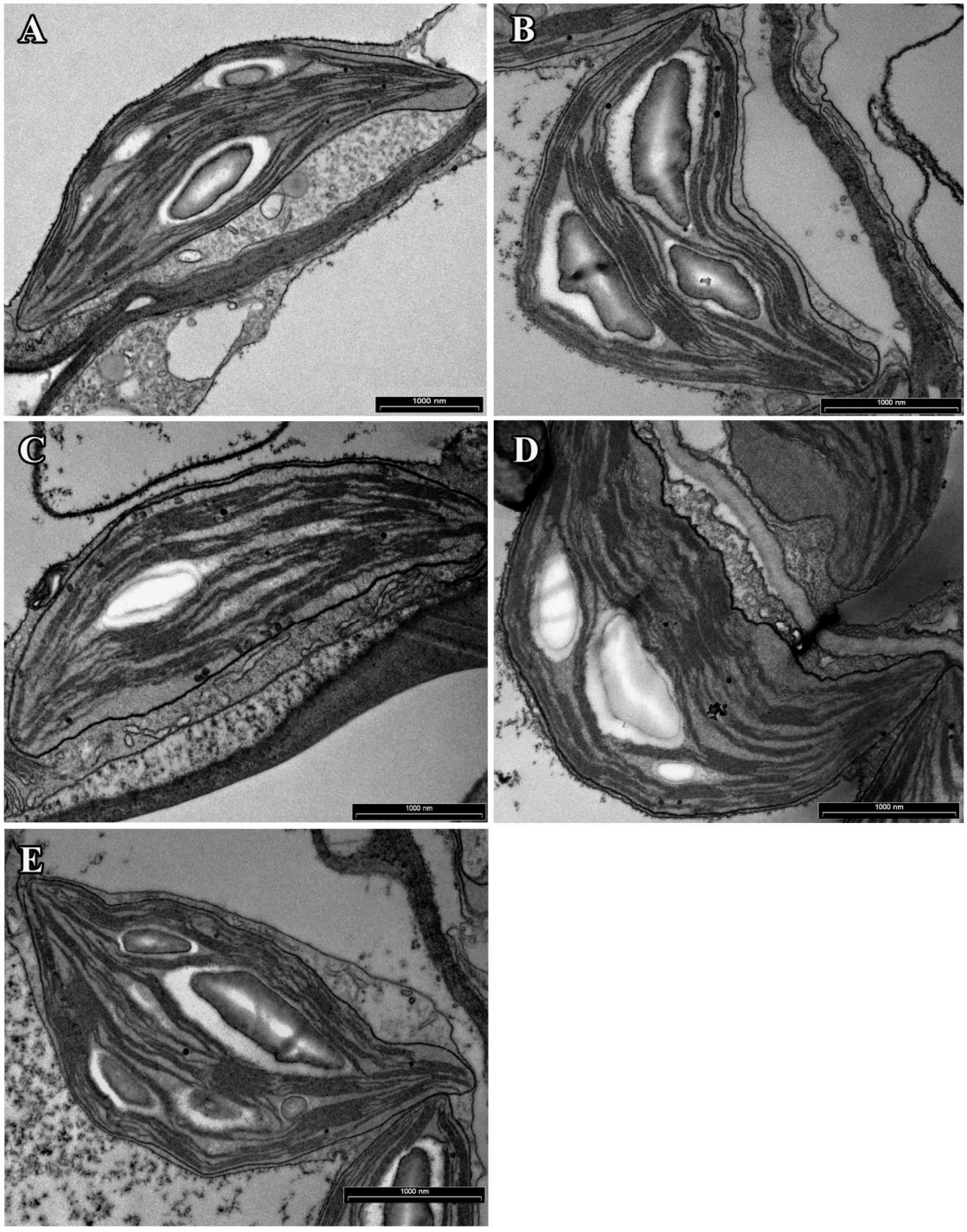
The attenuation of the F_0 and NPQ phenotypes produced by the triple *koLhcb4* mutations is different depending on which *Lhcb4* gene is in double *koLhcb4* mutants. Retention of Lhcb4.1 restores F_v/F_m and NPQ to WT levels, while Lhcb4.2 is only partially effective in this function; Lhcb4.3 is completely inefficient (Figure 4D, Figure 6, Table I). Indeed, plants retaining *Lhcb4.3* only show the same reduction in Chl *a*/*b* ratio (Table I), increased photosensitivity (Figure 6) and delayed NPQ kinetic as measured in triple *koLhcb4*. Although we cannot exclude that Lhcb4.1, 4.2 and 4.3 gene products are intrinsically different, the penetration of the phenotype appears to be essentially due to the level of the gene product: indeed, levels of Lhcb4.1, Lhcb4.2 and Lhcb4.3 in plants retaining only the corresponding genes reacahes the stoichiometry of 1, 0.6 and 0, respectively, per PSII RC (Supplemental Table II). In fact, although *Lhcb4.3* is transcribed under stress conditions (Alboresi et al 2010 unpublished results), we were unable to detect the corresponding protein even by keeping plants under a variety of stress conditions, implying it is either non translated or rapidly turned-over. It should be noted that our antibody is effective in detecting recombinant Lhcb4.3 expressed in *E. coli* (Supplemental Figure 5).

The *Lhcb4.3* gene appeared recently in plant genome. Its sequence is not present in algal genomes and no orthologous were found in the moss *Physcomitrella patens*; therefore, it appeared only at later stages in the evolution of the green lineage (Alboresi, A. et al. 2008). It seems unlikely that a new gene conserved in higher plants has no function or is not translated at all. EST data from *Arabidopsis* show that *Lhcb4.3* is expressed at very low levels (Jansson, S. 1999), and its transcription seems to be confined to dicots (Goff, S. A. et al. 2002, Yu, J. et al. 2002); furthermore, clustering analysis of Lhc superfamily expression data (Klimmek, F. et al. 2006) confirmed that *Lhcb4.3* is regulated in an opposite way with respect to *Lhcb4*.1 and *Lhcb4.2*. The putative Lhcb4.3 protein is shorter than other Lhcb4 isoforms

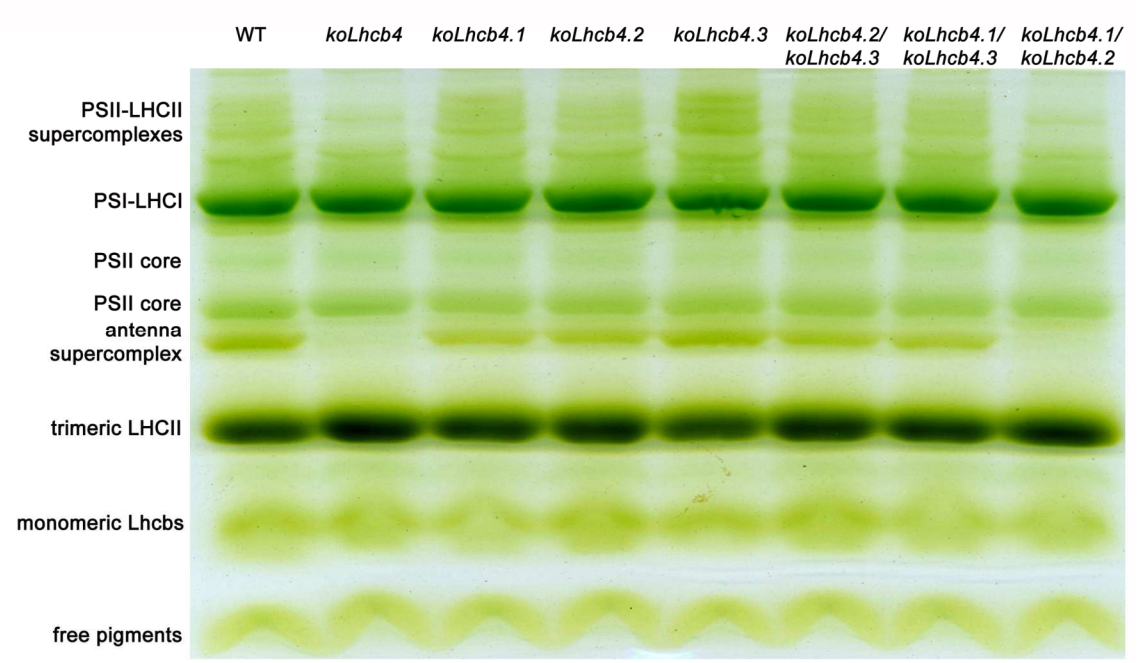
and its sequence is considerably different from both Lhcb4.1 and Lhcb4.2 isoforms, suggesting it might have a different function in the stress conditions in which this gene is actively transcribed. We cannot, however, exclude that Lhcb4.3 isoform might be accumulated in special and still un-investigated environmental/developmental conditions; so far, the role of this isoform remains elusive.

We have shown that Lhcb4 has a specific function in protecting PSII from photoinhibition, which accounts for the *koLhcb4* higher sensitivity to high-light stress. This was possible as this gene product, different from any other PSII antenna component, cannot be replaced by homologous subunits as a building block of the PSII-LHCII supramolecular complex. This leads to the formation of incomplete PSII particles with a gap in the position normally occupied by Lhcb4, they also have a modified shape and a lower stability in favor of the dissociated form of pigment-protein complexes in green gels. This is consistent with evidence that a "core" group of antenna proteins developed prior to green algal diversification (Koziol, A. G. et al. 2007) and included Lhcb4 protein associated with PSII RC. The weaker interaction between PSII-LHCII components leads to a faster migration of LHCII complexes to stroma membrane upon phosphorylation, similar to the case of *koLhcb6* and *koLhcb3*. Although PSII activity is not impaired in moderate light conditions, there is a lower level of PSII RC in grana membranes probably because PSII core complex is less strongly retained within grana discs, because one of the two monomeric Lhcbs that bridges it to LHCII is missing. Under stress conditions *koLhcb4* is photoinhibited and this effect is selectively enhanced in the absence of Zea, which is active in preventing synthesis of ROS species and promoting their scavenging. We propose that Lhcb4, by binding in between LHCII trimers and the CP47 subunit of PSII, is crucial for the protection of PSII RC from ROS produced during photosynthesis either by neighbor damaged PSII complexes (Krieger-Liszkay, A. et al. 2008) or by overexcited light harvesting antennas (Mozzo, M. et al. 2008, Santabarbara, S. et al. 2002). We show that the absence of a specific antenna subunit, although it does not restrict light harvesting and photosynthetic electron transport rate, produces and impairs PSII photoprotection capacity through its effect in the assembly of supercomplexes. This is a clear example of optimization of the building blocks of photosynthetic complexes and the tuning of their interactions with each other towards overcoming the inhibitory effect of increasing oxygen concentration over evolution time. Non-photochemical quenching is affected in *koLhcb4* because two of the quenching sites, namely Lhcb4 itself and Lhcb6 which is destabilized in *koLhcb4* (Avenson, T. J. et al. 2008), are missing. Nevertheless, although a quenching activity is delayed, it is still high suggesting that LHCII, the only component remaining in the outer antenna domain, might have a quenching activity *in vivo*. A detailed fluorescence lifetime analysis is required in order to further clarify this issue.

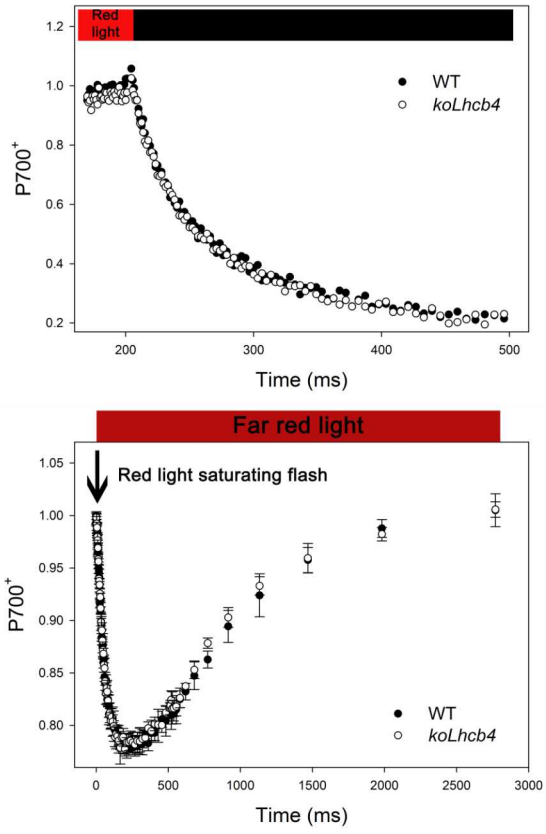
5. SUPPLEMENTAL DATA



Supplemental Figure 1. Transmission electron micrographs of plastid from mesophyll cells of wild type and mutants. *Ultrastructure of chloroplasts from 5 weeks old WT (A), koLhcb4 (B), koLhcb4.2/Lhcb4.3 (C), koLhcb4.1/Lhcb4.3 (D) and koLhcb4.1/Lhcb4.2 (E) plants was analyzed by electron microscopy. Representative thin sections are shown. The scale bar indicates 1 µm. The number of grana, discs per grana stack, stroma lamellae and end membranes was counted (at least 15 chloroplasts per genotype) and reported in Supplemental Table I.*

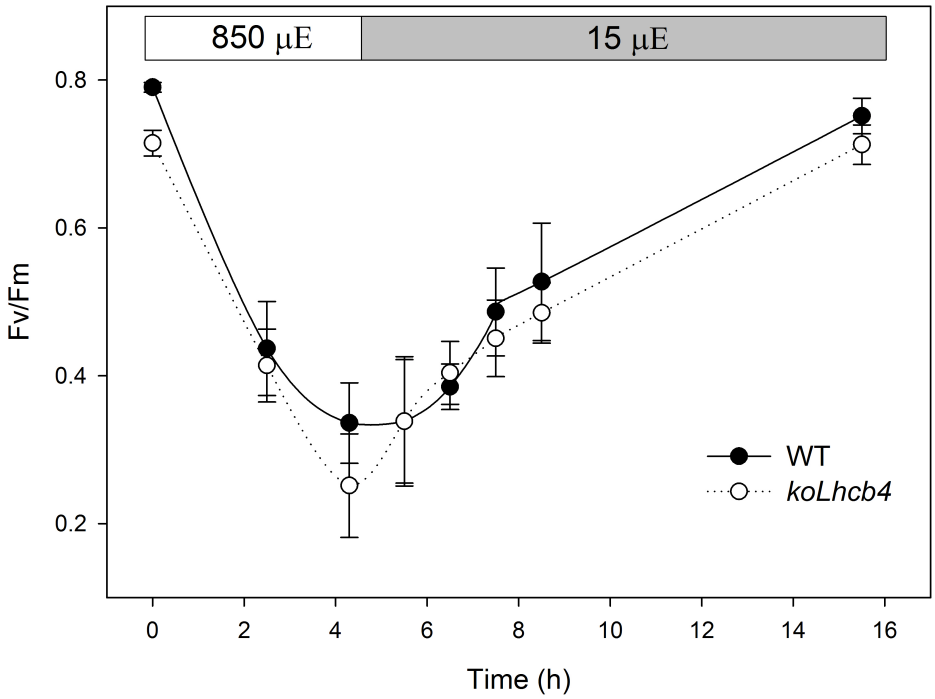


Supplemental Figure 2. Biochemical characterization of *koLhcb4* mutants. *Thylakoid pigmented complexes were separated by non-denaturing Deriphat-PAGE upon solubilization with 0.6% α-DM. 25 mg of chlorophyll were loaded in each lane.*

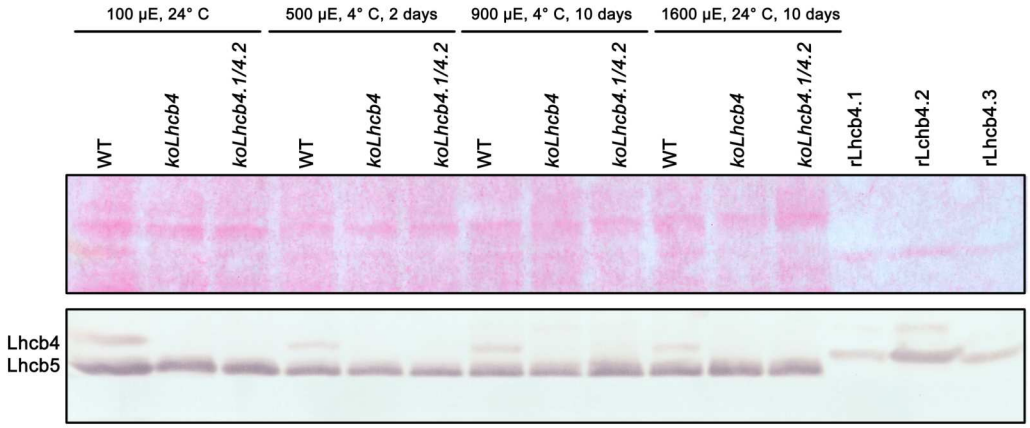


Supplemental Figure 3. P700 measurements: cyclic electron transport (CEF) and PSI functional antenna size. *A.* *The reduction of $P700^+$ was followed upon a 400-ms flash of saturating red light (3000 µmol photons m^{-2} s^{-1}) in leaves of WT and koLhcb4 infiltrated with 50 µM DCMU. Leaves were infiltrated in the presence of 150 mM sorbitol, to avoid*

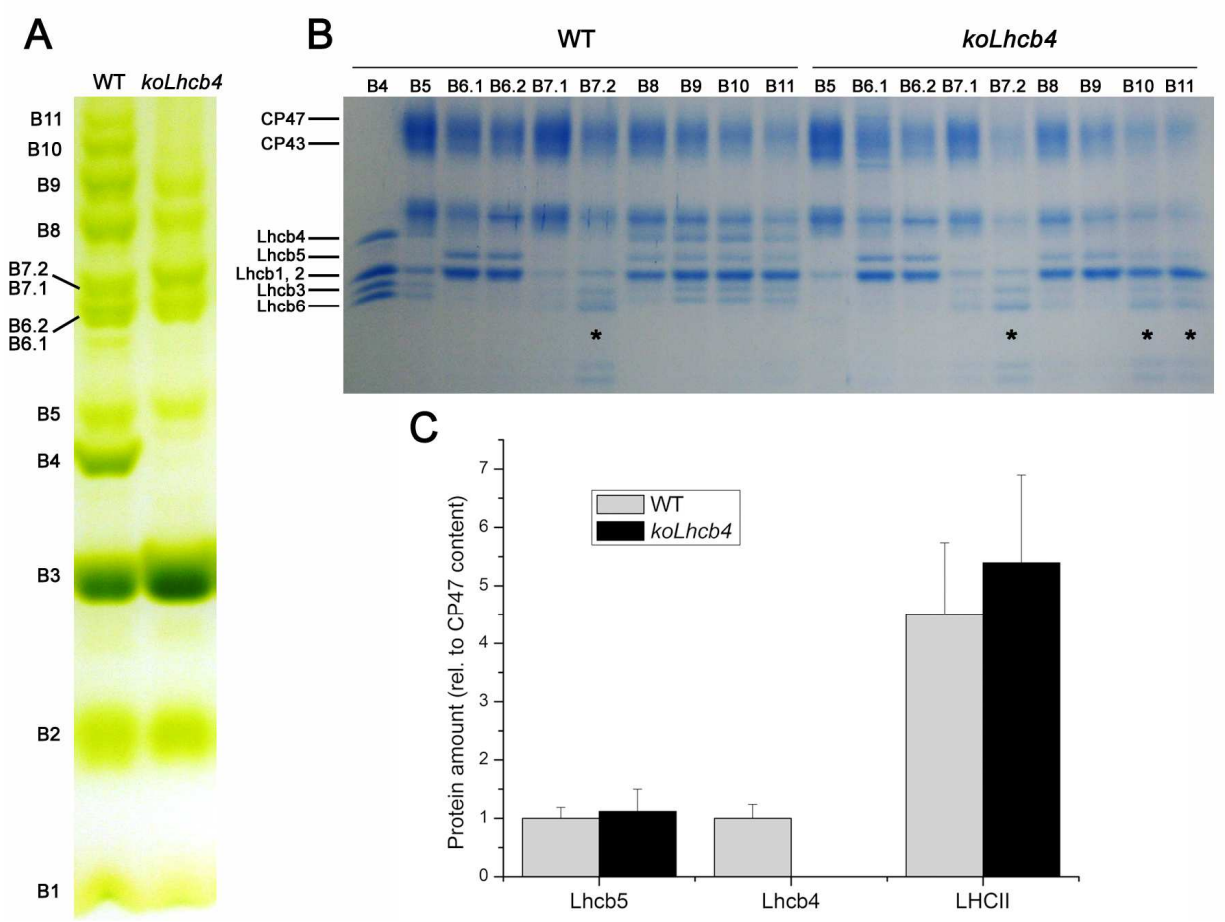
osmotic effects. The decay followed a complex curve, as described previously (Johnson, G. N. 2005). The fast component in this decay has previously been attributed to CEF and the slower reduction phases are thought to indicate the slower processes of redox equilibration occurring in the chloroplast. ***B.*** *P700 oxidation of leaves illuminated with a far-red background light (140 µmol photons m^{-2} s^{-1}) after the application of a saturating flash of red light (3000 µmol photons m^{-2} s^{-1}).*



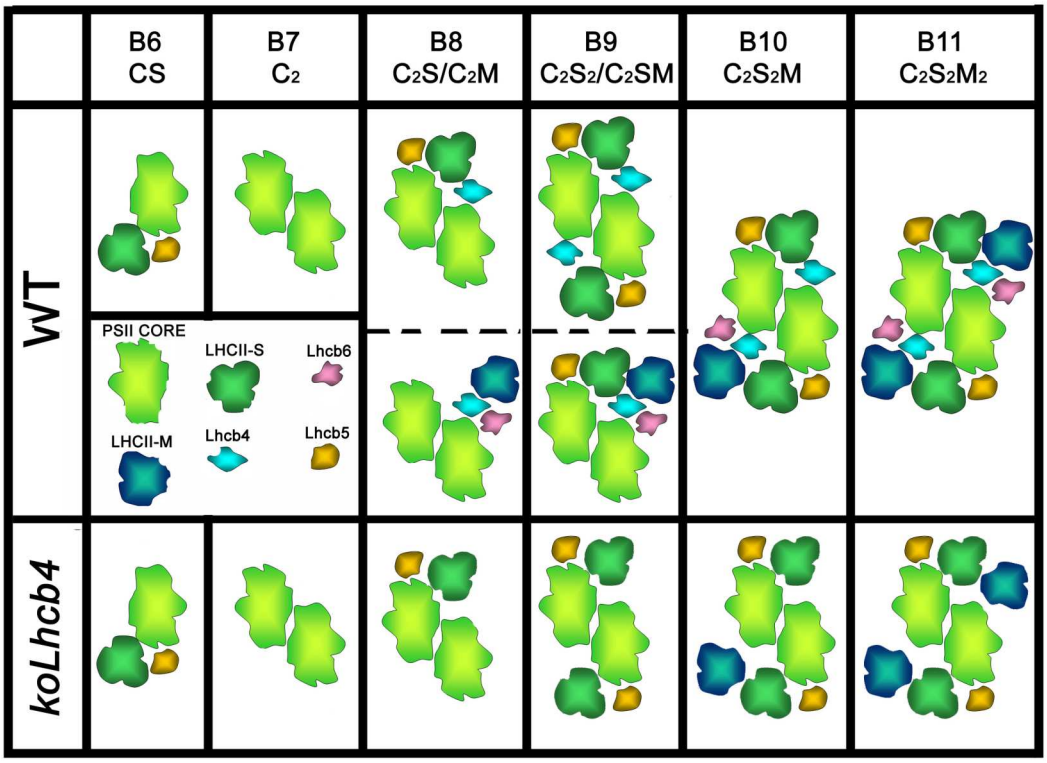
Supplemental Figure 4. PSII repair efficiency under photoxidative stress. *PSII repair efficiency was quantified on WT e koLhcb4 plants by measuring F_v/F_m recovery in low light (15 µmol photons m^{-2} s^{-1}, 4°C) after photoinhibitory treatment (900 µmol photons m^{-2} s^{-1}, 4° C). Data are expressed as means ± S.D. (n = 4).*



Supplemental Figure 5. Insights on the Lhcb4.3 expression and function. *Thylakoids were isolated from WT, koLhcb4 and koLhcb4.1/4.2 plants either grown in control conditions (100 µmol photons m^{-2} s^{-1}, 24° C) or at the end of stress treatments (500 µmol photons m^{-2} s^{-1}, 4° C for 2 days; 900 µmol photons m^{-2} s^{-1}, 4° C for 10 days; 1600 µmol photons m^{-2} s^{-1}, 24° C for 10 days); protein content was analyzed by immunoblotting using an antibody that recognize all Lhcb4 apoproteins. Thylakoid samples corresponding to 1 µg of chlorophyll were loaded for each sample. Top panel: Ponceau-stained nitrocellulose filter for check of equal loading; Lower panel: immunoblot assay. rLhcb4.x, recombinant proteins.*



Supplemental Figure 6. Insights on the PSII supercomplexes composition. *Grana membranes were isolated from WT and koLhcb4 plants as described in methods. In order to determine the protein composition of PSII supercomplexes found in koLhcb4, grana membranes from WT and mutant plants were solubilized with a very low concentration of α-DM (0.3% α-DM) and different complexes were separated by non-denaturing Deriphat-PAGE (Panel A); a pattern of green bands similar to that recently described by (Caffarri, S. et al. 2009) was obtained. Fractions from B6 to B11 contained PSII supercomplexes with increasing Lhc content, as determined by SDS-PAGE (Panel B) and absorption spectra of bands 6-11 (not shown). Lanes containing Lhca complexes are marked (*). According to previous result of single particle image analysis (Caffarri, S. et al. 2009), bands 10 and 11 in wild-type contained homogeneous PSII populations, respectively $C_2S_2M_1$ and $C_2S_2M_2$ supercomplexes; the amount of these complexes were extremely reduced in koLhcb4, showing a 86% decrease in $C_2S_2M_1$ and 91% reduction in $C_2S_2M_2$ amounts. Residual $C_2S_2M_1$ complexes were extracted from acrylamide matrix and protein composition were determined by quantitative western blot (Panel C). In the fraction from koLhcb4, the amounts of CP26 and LHCII are not significantly different than wild-type, suggesting that these subunits are bound in a similar stoichiometry to the PSII core of both genotypes.*



Supplemental Figure 7. Schematic panel of WT and *koLhcb4* PSII supercomplexes composition. *Model based on the results described in (Caffarri, S. et al. 2009) of PSII supercomplexes from WT and koLhcb4 genotypes for bands 6–12. C, core; S, LHCII trimer strongly bound; M, LHCII trimer moderately bound.*

	No. of grana	No. of discs per granum	No. of stroma lamellae	No. of end membranes
WT	36.1 ± 3.7^{a}	6.5 ± 2.7^{b}	42.5 ± 4.9^{c}	26.5 ± 4.2^{d}
koLhcb4	33.5 ± 4.7^{a}	6.3 ± 3.2^{b}	41.6 ± 7.8^{c}	27.6 ± 7.6^{d}
koLhcb4.2/4.3	35.2 ± 4.1^{a}	6.5 ± 3.0^{b}	38.1 ± 7.3^{c}	25.4 ± 4.4^{d}
koLhcb4.1/4.3	35.3 ± 6.0^{a}	6.2 ± 2.5^{b}	39.2 ± 6.5^{c}	24.8 ± 1.9^{d}
koLhcb4.1/4.2	34.1 ± 4.5^{a}	6.4 ± 3.5^{b}	40,2 ± 7.8^{c}	25.5 ± 5.0^{d}

Supplemental Table I. Quantitative analysis of thylakoid morphological traits. *Data were obtained by analysis of transmission electron micrographs of plastid from mesophyll cells of wild type and mutants. Values for 95% confidence interval in a single column with the same superscript letters a, b,c or d do not differ significantly (Student's t test, n>15).*

	Lhcb4	Lhcb5	LHCII	Lhcb6
WT	100.0 ± 10.0	100,0 ± 12,0	100,0 ± 14,4	100,0 ± 4,6
koLhcb4.1/4.3	58,4 ± 3,7 *	111,1 ± 11,4	118,0 ± 19,9	53,3 ± 5,1*
koLhcb4.2/4.3	87,8 ± 8,8	103,7 ± 15,7	112,0 ± 22,8	102,1 ± 11,5
koLhcb4.1/4.2	nd	99,0 ± 25,0	122,0 ± 11,0	nd

Supplemental Table II. Polypeptide composition of thylakoid membranes from wild-type and KO mutant expressing single Lhcb4 isoform. *Western blotting used for the quantification of photosynthetic subunits in WT and*

koLhcb4 thylakoids. Immunoblot analysis were performed with antibodies directed against individual gene products: minor antenna proteins, LHCII subunit, PSII core subunit PsbB (CP47). Thylakoid samples corresponding to 0.25, 0.5, 0.75, and 1 μg of chlorophyll were loaded for each sample. All samples were loaded on a single SDS-PAGE slab gel. Data of PSII antenna subunits were normalized to the core amount, PsbB content. For each sample, linear fit of points were verified. Significantly different values (Student's t-test) with respect to WT are marked (*).

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SECTION D

Chlorophyll b reductase affected the regulation of antenna complexes during light stress.

Light-harvesting chlorophyll *a*/*b*-protein complexes are the most abundant membrane proteins in green plants, and its degradation is a crucial process for the acclimation to high light conditions and for the recovery of nitrogen (N) and carbon (C) during senescence. However, the molecular mechanism of antenna breakdown is largely unknown and it is still unclear whether chlorophyll degradation precedes the degradation of the protein moiety or whether protein degradation is the first event.

Recently chlorophyll b reductase mutant has been isolated (Kusaba, M. et al. 2007). This enzyme is responsible for the first step of chlorophyll degradation pathway, the conversion of Chlorophyll (Chl) b in Chl a and the mutant is called "*stay-green*", because of PSII antenna retention upon leaf senescence induction (Horie, Y. et al. 2009). We characterized the response of Chl b reductase ko mutant to acclimation in high light. The mutant showed a slower antenna size reduction with respect to WT. This enzyme is upregulated during HL acclimation. I*n vitro* assay with recombinant Chl b reductase demonstrated that its activity is higher when zeaxanthin, which accumulate during stress, is bound to PSII antenna complexes.

1. INTRODUCTION

Photosynthesis is a indispensable process for plants to generate chemical energy for biological processes in which chlorophyll plays a central role by harvesting light energy (Green, B. R. and Durnford, D. G. 1996) and driving electron transfer (Fromme, P. et al. 2003). This pigment is bound to proteins, forming complexes that can be divided into two groups (Barber, J. et al. 2000). The first group consists of the core antenna complexes, which include CP43/CP47 of Photosystem II (PSII) and P700-chlorophyll *a*-protein complexes of Photosystem I (PSI). The composition and organization of these complexes is conserved in oxygenic phototrophs. The second group consists of peripheral antenna moieties, which harvest and transfer light energy to the core antenna complexes. These proteins, called LHC (Light Harvesting Complexes) are less conserved and display diversity depending on evolutionary and environmental issues (Alboresi, A. et al. 2008, Jansson, S. 1999). LHC proteins bind chlorophyll *a* and *b* and account for >60% of the total chlorophyll in plants and green algae. Nevertheless, their relative amount with respect to Core protein complexes varies depending on developmental stages and on light intensity (Ballottari, M. et al. 2007). Biogenesis and degradation of antenna proteins appear to be crucial for the acclimation of plants allowing survival in their highly variable environment.

Besides chlorophylls, oxygenated carotenoids, called xanthophylls: Lutein, Lut; Violaxanthin, Vio; Zeaxanthin, Zea are also ligands for LHC proteins, acting in modulating antenna function and favoring energy dissipation by quenching (Dall'Osto, L. et al. 2005, Formaggio, E. et al. 2001, Moya, I. et al. 2001). Zeaxanthin plays a special role in this dynamic behaviour because it accumulates only under excess light conditions, upon synthesis from Vio in the so-called xanthophyll cycle (Yamamoto, H. Y. et al. 1967), and its presence leads to an increased level of resistance to light stress (Ballottari, M. et al. 2007, Niyogi, K. K. et al. 1998).

Antenna proteins biogenesis has been extensively studied by using ko mutants and/or transgenic plants down-regulated in LHC accumulation in various species. LHCs cannot accumulate in chlorophyll *b*-less mutants, because Chl a-only complexes are unstable (Havaux, M. et al. 2004). In contrast, LHCII level of accumulation increases when chlorophyll *b* synthesis is accelerated by the overexpression of chlorophyllide *a* oxygenase (Tanaka, A. et al. 1998).

Conversely, LHC degradation mechanisms are largely unknown. Several evidences indicated that such a degradation occurs when photosystems re-organizes upon changes of light intensity and during leaf senescence. Degradation of

antenna complexes implies two distinct processes: one is the proteolitic degradation of the protein moiety, and the second is chlorophyll degradation. It is still unclear whether chlorophyll degradation precedes the degradation of the protein moiety or whether protein degradation occurs first (Hortensteiner, S. 2009).

Chlorophyll degradation pathway is common in higher plants and all chlorophyll catabolites derive from chlorophyll a (Krautler, B. et al. 1991). The conversion of chlorophyll *b* to 7-hydroxymethyl chlorophyll *a* is the first step of chlorophyll degradation and is catalyzed by Chlorophyll *b* Reductase (CbR) enzyme. The *non-yellow coloring1* (*nyc1*) stay-green mutant was recently isolated (Kusaba, M. et al. 2007a, Takahashi, S. et al. 2009), which retained PSII antenna complexes after induction of leaf senescence. This mutant is defective in the *Nyc1* gene encoding CbR and phylogenetic analysis has revealed the presence of Nyc1-like (Nol) protein as the most closely related protein to Nyc1 in plants.

In this work we show that CbR activity is upregulated in HL acclimated plants of *Arabidopsis thaliana*. In order to analyze the molecular details of the mechanisms, we constructed a *in vitro* system using isolated thylakoid membranes using which, we could determine the effect of zeaxanthin on the activity of CbR. We conclude that, besides it important for photoprotection zeaxanthin has a role in up-regulating the CbR activity and thus increasing the turnover rate of antenna complexes.

2. MATERIAL AND METHODS

2.1 Plant Materials and Growth Conditions Arabidopsis thaliana (Columbia ecotype) was grown at 23 °C under continuous light in a chamber equipped with white fluorescent lamps at a light intensity of 100 µmol/$m^{-2}s^{-1}$. For the dark-induced leaf senescence experiments, 4-week-old *Arabidopsis* plants were kept in darkness at 23 °C for 2, 4, 6, and 8 days. The T-DNA insertion mutants, lacking either AT4G13250 (SALK 091664) (*NYC1*) or AT5G04900 (AL759262) (*NOL*) or both in the case of the double mutants, were kindly provided by prof. Ayumi Tanaka (Institute of Low Temperature Science, Hokkaido University, Japan).

2.2 Expression and Purification of Recombinant Nol The expression plasmid containing the coding region of *Nol* gene, kindly provided by prof. Ayumi Tanaka (Horie et al., 2009) was introduced into *Escherichia coli* Rosetta DE3 (Novagen) cells. Two milliliters of an overnight culture of the transformed *E. coli* was diluted with 300 ml of Luria-Bertani medium containing kanamycin (50 µg/ml) and chloramphenicol (10 µg/ml). The culture was grown at 37 °C until the optical density at 600 nm reached 0.6. The expression of the *Nol* gene was induced with 0.1 mM IPTG for 2 h. After incubation, the culture was harvested by centrifugation at 8000 *g* for 10 min at 4 °C. The collected cells were resuspended in 100 mM phosphate buffer (pH 7.8) containing 300 mM NaCl and disrupted by sonication. Triton X-100 was added at a final concentration of 1%, and the mixture was incubated for 1 h at room temperature. It was then centrifuged at 8000 *g* for 20 min at 4 °C to remove the cell debris. The soluble fraction containing recombinant NOL was loaded onto a nickel column (Novagen) pre-equilibrated with the buffer (20 mM Tris-HCl, pH 7.9, 500 mM NaCl, 5 mM imidazole, and 0.8% Triton X-100). The unbound proteins were washed out with the buffer used for equilibration of the column.

Subsequently, the recombinant proteins were eluted with 20 mM Tris-HCl, pH 7.9, 500 mM NaCl, 300 mM imidazole, and 0.8% Triton X-100. The purified protein was stored at 4 °C and used within 24 h of purification.

2.3 Pigment analysis. HPLC analysis was performed as described (Gilmore, A. M. and Yamamoto, H. Y. 1991). The Chl/carotenoid and Chl *a*/*b* ratios were determined independently by fitting the spectrum of acetone extracts to the spectra representing individual purified pigments (Croce, R. et al. 2002).

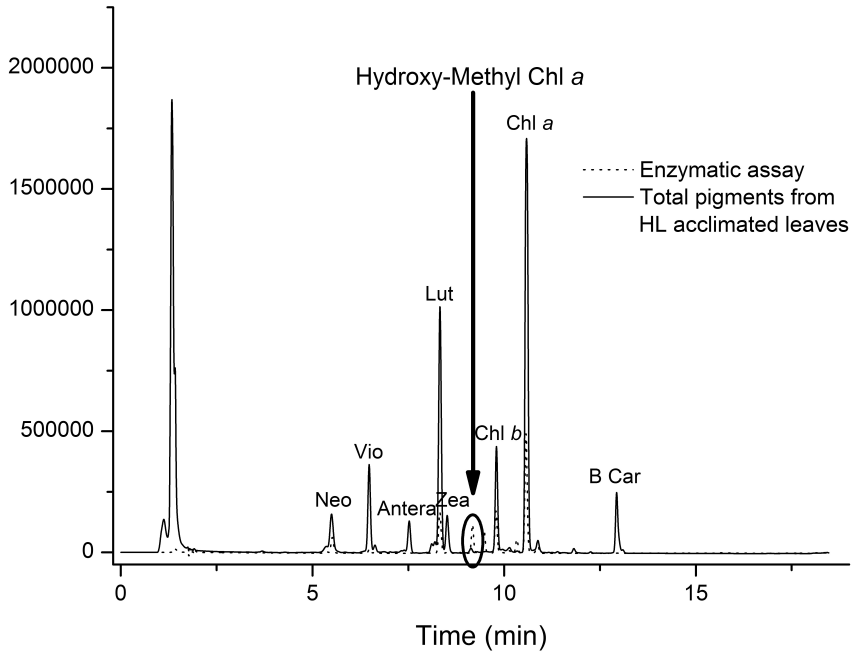
2.4 Membrane isolation Unstacked thylakoids were isolated from dark-adapted or HL-treated leaves as previously described (Bassi, R. et al. 1988).

2.5 Gel Electrophoresis and Immunoblotting SDS-PAGE analysis was performed with the Tris-Tricine buffer system (Schägger, H. and von Jagow, G. 1987). For western-blot analysis, antibodies against Nol and Nyc proteins were kindly provided by prof . Ayumi Tanaka.

3.RESULTS

It has been recently reported (Ballottari, M. et al. 2007) that *Arabidopsis thaliana* modulates the PSII antenna size depending on light exposure: in particular, acclimation to high light (HL) conditions causes a reduction of the abundance of antenna complexes per PSII core. PSI antenna system, instead, is unaffected by growth conditions.

We investigated the relation between HL stress response and degradation of PSII antenna complexes by analyzing the pigment composition of HL acclimated leaves of *Arabidopsis thaliana* WT. In these conditions we observed the emergence of a new peak in the HPLC chromatogram (Figure 1A-B), that is not detectable in plants grown in control conditions. This pigment is hydroxy-methyl chlorophyll *a* (HMCa), an intermediate produced by the first reaction of chlorophyll degradation pathway, that is the conversion of Chlorophyll b (Chlb) to Chlorophyll a (Chla) (Kusaba, M. et al. 2007b, Takahashi, S. et al. 2009): indeed the same pigment is found upon *in vitro* enzymatic assay with the chlorophyll b reductase (CbR) enzyme (Horie et al., 2009), which will be discussed further below.



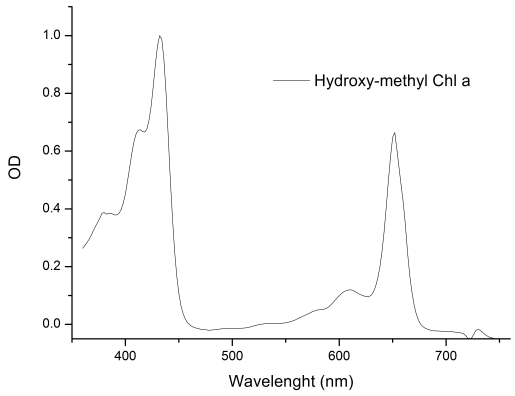
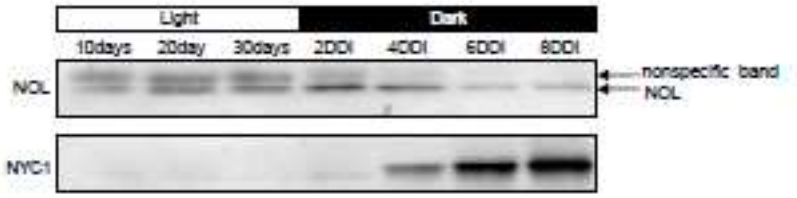


Figure 1. HPLC-chromatogram of thylakoid pigments at 430nm. ***A.*** *Analysis of the pigments extracted from high light acclimated leaves (straight line) leads to the emergence of a new species from the ellipse high-lighted peak. The same pigment is produced after the enzymatic assay with the Chlorophyll b Reductase enzyme, whose products are represented by dotted chromatogram. This corresponds to hydroxy-methyl chlorophyll a (HMCa) pigment (Folly and Engel, 1999).* ***B.*** *Absorption spectra of HMCa between 350 and 750 nm.*

This finding suggests that HMCa is detected in a condition where plants are subjected to prolonged stress and it is linked to antenna complexes degradation. We thus investigated the ability of CbR enzyme mutants to acclimate to different light conditions.

The chlorophyll *b* reductase enzyme is encoded by two homologous, but not redundant, genes in *Arabidopsis thaliana Nyc1* and *Nol* (Takahashi, S. et al. 2009). The corresponding proteins are expressed at different stages of plant growth. Western blot analysis indicates that Nol protein is characterized by a basal level of expression in normal growth conditions, and gradually disappears during senescence (Figure 2A, Tanaka A. personal comunication). Nyc1 enzyme is instead involved in the senescence response (Horie et al., 2009), indeed it is not present during development, but accumulated during leaf senescence, as shown by western-blot analysis, determining senescence induced antenna degradation (Figure 2B-C).



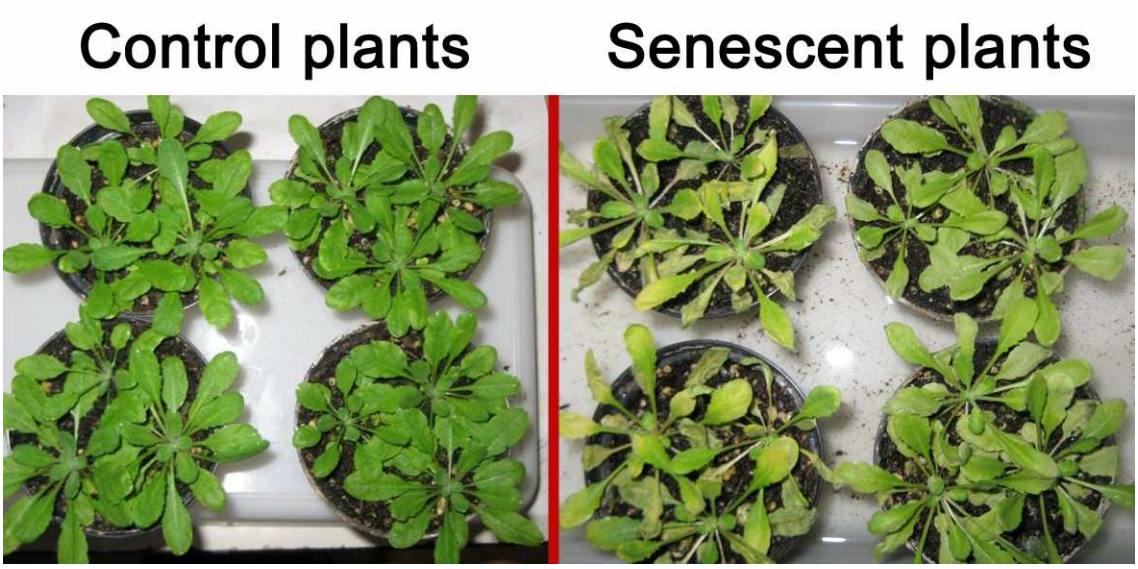


Figure 2. Nyc and Nol functonal role upon senescence induction *A.* *Western blot analysis of leaf extracts at different time of growth in light or dark adaptation using antibodies against Nol (above) and Nyc1 (below) proteins (Tanaka A., personal comunication). The first part (left, light) of the figure shows the signal from plants grown at 100 μE for 10, 20 and 30 days. On the right (dark) the expression of the same proteins in senescent plants, which are placed in the dark for 2, 4, 6, 8 days is shown. DDI = Day Dark After Induction.* ***B.*** *Comparison between control plants (left) and 7 days senescent plants (right). In each panel starting from the top left with anti-clockwise sense: WT, nol, nyc1 and nol/nyc1 double mutant plants.*

Figure 2B clearly shows that mutants deleted in *Nyc1* gene are characterized by pale green leaves without yellowing upon 7 days of dark-induced senescence, due to antenna complex retention. Instead, WT and *nol* mutant leaves looks older because of chlorophyll degradation during natural senescence.

In order to further investigate the role Nol enzyme with respect to light acclimation, we investigated its expression in HL acclimated plants (2 weeks – 1000 μE). Thus we purified thylakoid membranes and then proteins has been analyzed using western-blot technique, with an antibody specific for Nol protein. Nol had a basal expression in control plants grown at 100 μE and it is upregulated during HL treatment, using as a reference CP47 amount, a component of PSII (Figure 3).

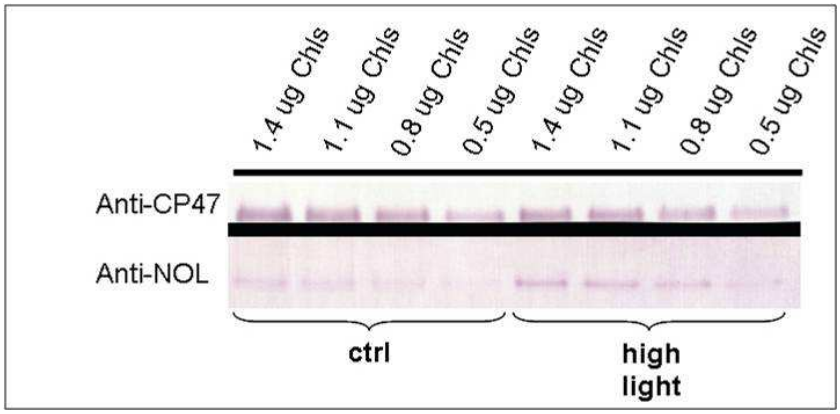
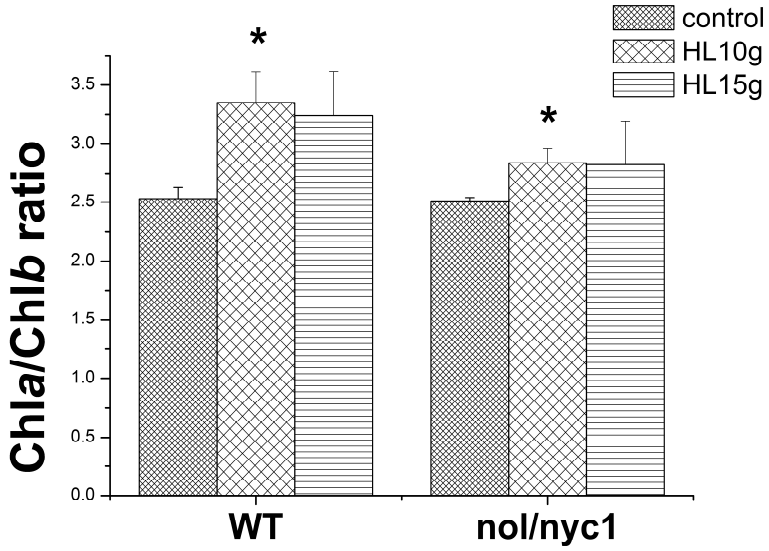


Figure 3. Nol protein expression in HL acclimated plant. *Western-blot analysis on thylakoids extracted from WT plants grown in control conditions (100 μE) and held for 2 weeks in high light (~1000 μE) using anti-Nol and anti-*

CP47 antibody, a component of the PSII core used as a reference. A range of chlorophyll concentrations was loaded in order to have a linear signal (between 1.4 and 0.5 ug of Chls).

Up-regulation of *Nol* upon HL acclimation with respect to control plants suggested that this protein could be important for antenna size regulation under environmental stress conditions. We investigated CbR function during HL acclimation by analyzing the behavior of mutant lacking the two homologous enzymes. **T**he seeds of double knock-out insertional mutants for *Nol* (At5g04900) and *Nyc* (At4g13250) genes (Horie et al. 2009) were kindly provided by the laboratory of Professor Tanaka (Institute of Low Temperature Science, Hokkaido University, Kita-ku, Sapporo) and these mutants were grown in growth-chamber under controlled condition showing no defects in growth or changes of morphology with respect to WT plants (data not shown).

WT and the *nol/nyc* double mutant have been acclimated to different HL intensities as described before and the stoichiometric Chla / Chlb ratio by RP-HPLC system was determined. This parameter is directly related to the stoichiometry between photosystems core complexes and antenna moieties, since Chl b is bound to the latter only (Figure 4).



	control	10 days	15 days
wild type	2.53 ± 0.1	3.35 ± 0.26	3.05 ± 0.38
nol/nyc1	2.51 ± 0.03	2.84 ± 0.12	2.67 ± 0.47

Figura 4. HL acclimation of *nol/nyc* double mutant. Plot and table of Chla/Chlb ratio of wild type (WT) and *nol/nyc* double mutant plants in control condition (100 µE, control) and acclimated to HL for 10 and 15 days (1000 µE), determined by HPLC analysis.

After 10 days of acclimation mutant shows a minor increase in Chla / Chlb ratio as compared to WT. In the long term, after 15 days of acclimation, Chla / Chlb ratio reaches WT level, indicating that the lack of CbR slows the rate of acclimation process but does not prevent it..

Zeaxanthin (Zea) has a fundamental role in plants photoprotection activity (Dall'Osto, L. et al. 2005, Moya, I. et al. 2001, Niyogi, K. K. et al. 1998), by determining Lhc proteins conformational change between energy conserving and energy-dissipative (quenching) conformations. This xanthophylls is strongly accumulated during HL acclimation, thus we decided to investigate if Zea-induced conformational change can also affected Nol activity.

The plasmid containing *Nol* coding sequence was kindly provided by the working group of Professor Tanaka (Institute of Low Temperature Science, Hokkaido University, Kita-ku, Sapporo) and the recombinant Nol protein was obtained by expression in *E. coli*. Because of the inability to conserve the enzyme in its active form (it is denatured by freezing), we had to use it immediately after purification so that it was impossible to carefully quantify the purified protein in order to use the enzyme at a constant concentration for the different essays. We thus decided to standardize synthesis and purification steps in order to obtain approximately the same amount of enzyme in different preparations. In order to make comparison possible, samples to be compared were treated with aliquots from the same expression event. It should be noted that the results were very reproducible in different repetitions of this experiment. In order to study the degradation of pigment-protein moieties, the assay was performed on native complexes, inserted in the membranes or purified by sucrose gradient fractionation of solubilised thylakoid complexes.

We compared complexes binding Vio or Zea in the L2 carotenoid binding site of antenna complexes (Formaggio, E. et al. 2001). The enzymatic reaction occurred at 25° C for times of 15 and 45 minutes, following which we analyzed the aceton extracts by RP-HPLC in order to determine pigments stoichiometry in samples subjected to enzymatic assay. Thylakoids from WT and *npq2* plants were isolated and further quantified by their chlorophyll content using a Unicam spectrophotometer. The assay was performed using NADPH as cofactor (Takahashi, S. et al. 2009).

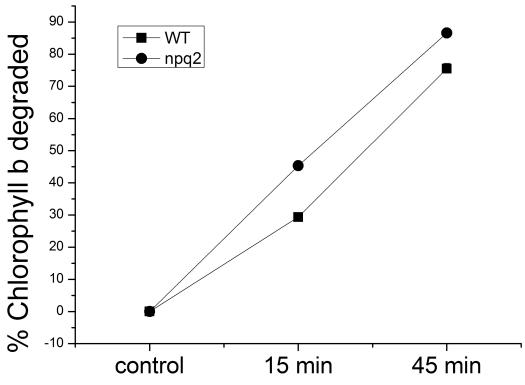


Figura 5. Enzymatic assay on unstacked thylakoids isolated from WT and npq2 plants, *incubated with concentrated Nol for 15 and 45 minutes at 25°C (2ug of Chls). Control samples have been incubated for 45 min at 25°C without enzyme. Data are normalyzed to the amount of chlorophyll b of the untreated sample.*

Plot in Figure 5 shows, that enzyme is able to convert *Chl b* bound to native complex inserted in thylakoid membranes to HMCa. Samples isolated from *npq2* plants are also characterized by a faster conversion of chlorophyll b with respect to WT. This higher activity of CbR is not due to a different level of the endogenous enzyme present within thylakoid

from WT and *npq2* plants. Figure 6, in fact, shows that there is no significant difference in protein accumulation between the two genotypes.

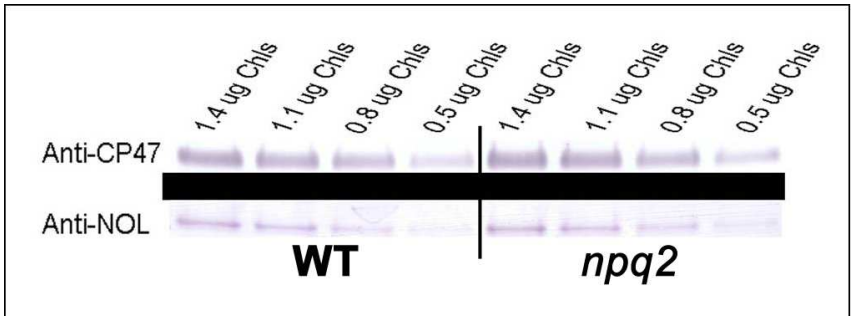


Figure 6. Western blot on thylakoids extracted from wild type and npq2 plants. *The immunodetection was performed with anti-Nol and anti-CP47 antibody (used as reference).*

Thus, the differential activity of Nol is not associated to different level of the endogenous enzymes. Indeed there is no detectable activity of endogenous Nol in the condition used for the enzymatic assay on thylakoids (data not shown). We conclude that the observed conversion of chlorophyll b is associated to the unique activity of the exogenous enzyme that we used for enzymatic assay.

The relation between presence of Zea and Nol activity has been more deeply studied by comparing WT and *npq2* samples treated for different time lengths with HL, in order to analyze Nol activity using samples characterized by different level of bound zeaxanthin (Figure 7).

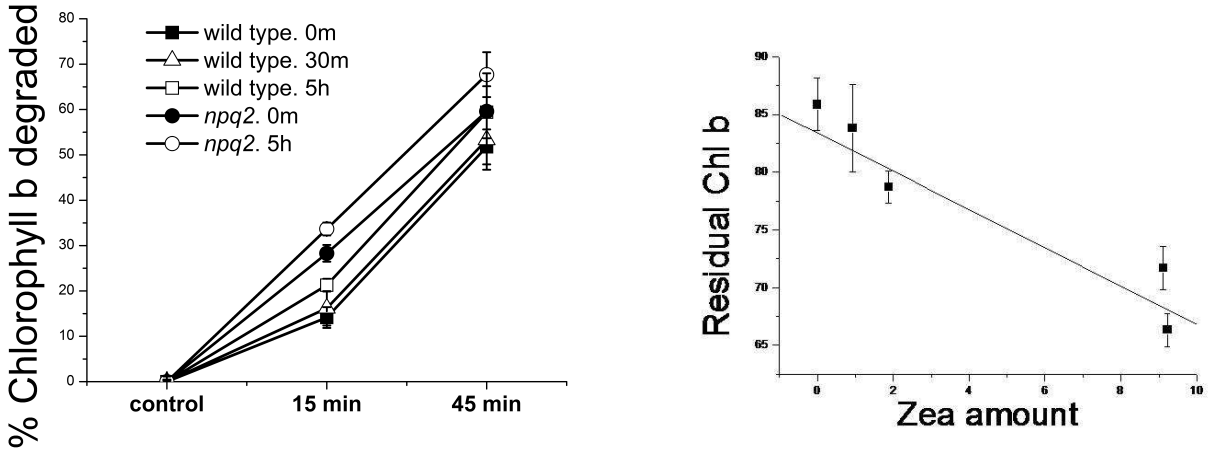


Figure 7. Relation between presence of Zea and Nol activity. *A. Enzymatic assay on unstacked thylakoids (2ug of Chls) isolated from WT and npq2 plants, dark adapted (0m) or treated for 30 minutes (30m) or 5 hours (5h) at 1000 uE, incubated with concentrated Nol for 15 and 45 minutes at 25°C. Control samples have been incubated for 45 min at 25°C without enzyme. Data are normalyzed to the amount of chlorophyll b of the untreated sample. B. Correlation between Nol enzyme activity and amount of zeaxanthin present in samples treated for 15 minutes*.

The activity of Nol enzyme is directly dependent on Zea amount, as evidenced in Figure 7. In particular it was observed a faster Zea-related activity of Nol after 15 minutes of reaction, instead after 45 min the reaction was close saturation and the differences between the analyzed samples were smaller.

We finally analyzed samples containing mixture of monomeric Lhcbs (monLhcbs)or trimeric LHCII, purified upon sucrose gradient centrifugation of WT and *npq2* solubilized thylakoid membranes. Monomeric Lhcbs fractions are composed by monomeric antenna proteins Lhcb4, Lhcb5, Lhcb6 and by components of trimeric LHCII monomeric by the detergent treatment (Bassi and Dainese, 1992). Figure 8 shows that monomeric antenna fraction are more susceptible to CbR activity with respect to trimeric LHCII, confirming that Zea affected the activity of Nol enzyme.

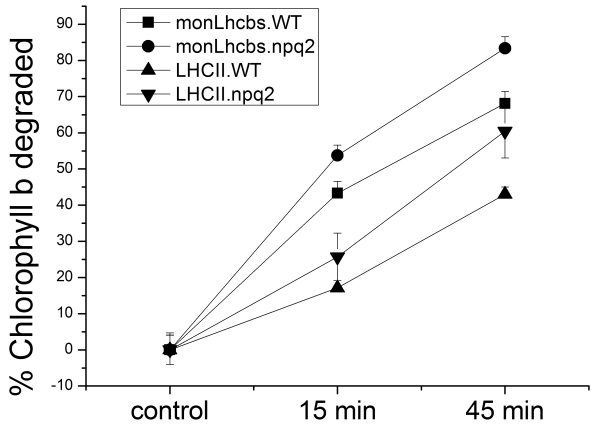


Figura 8. Comparison between monomeric and trimeric Lhcb fractions. *Enzymatic assay on sucrose gradient fractions from solubilized WT and npq2 thylakoid membranes. Fractions containing mixture of monomeric Lhcb complexes (monLhcb) and trimeric LHCII are incubated with concentrated Nol for 15 and 45 minutes at 25°C (2ug of Chls). Control samples have been incubated for 45 min at 25°C without enzyme. Data are normalized to the amount of chlorophyll b of the untreated sample.*

4. DISCUSSION

Nol and *Nyc1* are two closely related genes that encode Chlorophyll b Reductase enzyme (Fromme, P. et al. 2003, Horie, Y. et al. 2009, Hortensteiner, S. 2009, Krautler, B. et al. 1991, Kusaba, M. et al. 2007b, Park, S. Y. et al. 2007, Takahashi, S. et al. 2009); Horie et al. 2009), which is responsible for the first step of Chlorophyll (Chl) degradation pathway, promoting Chlb conversion into Chla. *nol* and *nyc* mutant are called "*stay green*", because of their retention of antenna complexes upon induction of senescence (Hortensteiner et al., 2009).

Preliminary expression data of CbR protein (Figure 2A, Tanaka A. personal communication) lead to hypothesize a specific expression of Nyc1 during leaf senescence, while the enzyme Nol would be expressed in all stages of plant development but senescence. *Nyc1* involvement in senescence is evident in Figure 2B, because plants deleted for this gene retained antenna complexes during senescence, without yellowing of leaves. Based on these results it seemed interesting to further investigate functional properties of *Nol* with respect to light response, in order to study the relation between high light (HL) stress and PSII antenna complexes degradation. We verified by western blotting that *Nol* accumulates under HL acclimation (Figure 3), thus strengthening the idea that this enzyme is involved in HL

acclimation and its HL-related upregulation promotes the mobilization and conversion of Chlb in Hydroxy-methyl Chla (HMCa) (Figure 1).

Thus we tested *nol/nyc1* double mutant for its ability to acclimate to HL and it was grown for 15 days at 1000μE together with WT and we evaluated HL acclimation phenotype, observing no significant differences in terms of plants dimension and anthocyanins synthesis (data not shown) between WT and mutant. We then measured Chla /Chlb ratio, indicative of the ratio between reaction centers of photosystems and antenna complexes: the ratio is usually increased in HL acclimated plants following the reduction of the antenna associated to the photosystems, which bind chlorophyll b (Bailey et al, 2004; Ballottari et al., 2007). In the case of *nol /nyc1* mutant, the relationship grows more slowly than in the wild type, indicating slower antenna reduction (Figure 4). These data support the hypothesis of a key role of Chlb mobilization in promoting antenna proteins degradation, not only during senescence but also during the acclimation (Kusaba et al. 2007).

Despite this, in the continuing adaptation, double mutant reached a Chla / Chlb ratio comparable to WT. This indicates the presence of degradation mechanisms that compensate for the lack of Chlorophyll b Reductase (CbR). In particular, it is known that chlorophyllase enzymes are able to convert, although with lower efficiency, Chlb in chlorophyllide b and phytol, a different substrate for the enzyme than Chla (Hörtensteiner S. 1999). It can be assumed, therefore, that chlorophyllase reacts also with Chlb, although with less efficiency, due to a) the low affinity for Chlb than Chla, b) its stromal localization, which requires the transport of pigment out from thylakoid membranes. Chlorophyll b Reductase (CbR) is instead anchored to thylakoid membranes (Kusaba et al., 2007), allowing a high efficiency in their functional role.

Furthermore, despite much research, very little is known about the proteases involved in antenna moieties degradation. Chlorophyll degradation is a necessary step for antenna degradation, as determined by the analysis of several *stay green* mutants (Hortensteiner, S. 2009, Krautler, B. et al. 1991, Kusaba, M. et al. 2007b, Park, S. Y. et al. 2007, Sener, M. et al. 2003, Yamaji, M. et al. 2009); however we cannot exclude that proteases are able to degrade antenna without pigments destabilization upon prolonged stress, for instance HL acclimation.

Plants exposed to HL convert violaxanthin (Vio) to zeaxanthin (Zea), which increases their photoprotection ability (Demmig-Adams, B. 1990). Zea is also accumulated during HL acclimation, in which antenna complexes are degraded to obtain antenna size reduction (Ballottari, M. et al. 2007). It is well known that Zea binding causes conformational change of antenna complexes into a quenched state (Ahn, T. K. et al. 2008, Dall'Osto, L. et al. 2005, Morosinotto, T. et al. 2003). It could be hypothesized that this Zea-induced conformational reorganization could affect the substrate properties such as the interaction with CbR activity, the first step known for chlorophyll degradation (see above). It is clear, in fact, that in HL the Zea binding to antenna complexes favors their degradation: this has been observed for example by analysis of mutants that constitutively accumulate Zea (npq2, npq2lut2): they have a lower amount of antenna complexes, particularly LHCII trimers (Havaux, M. et al. 2004).

The *in vitro* enzymatic assay by using recombinant Nol protein expressed in *E. coli* and thylakoid membranes from *npq2* and WT plants (Figure 5) was useful to assess whether the binding of Zea to the complexes makes them more sensitive to CbR activity. We could first show the ability of CbR to work with natural complexes inserted in thylakoid membranes implying that CbR activity is not dependent on denaturation on antenna complexes or to their extraction from the membrane. We further observed that the thylakoids from *npq2* were more susceptible to degradation of chlorophyll b bound, as compared to WT, suggesting that Zea-induced conformational change could facilitate CbR activity. A more detailed experiment allowed to determine that *in vitro* CbR activity is really affected by the presence of Zea and it is characterized by a direct relation as shown in Figure 7.

Subsequently, CbR activity was evaluated on complex isolated from sucrose gradient fractions, particularly fractions containing mixtures of monomeric Lhcb proteins (monLhcbs) and LHCII trimers (LHCII) (Caffarri, S. et al. 2001). The enzyme is active on both fractions, demonstrating its ability to degrade chlorophyll b bound to the complex, as reported in Horie Y. et al., 2009. The activity was significantly higher for fraction containing monomeric Lhcbs. Thus, monomers are more susceptible to degradation of chlorophyll b bound than LHCII trimers. Moreover, this experiment confirmed that fractions purified from *npq2* are more sensitive to the activity of CbR, as compared to fractions from WT: thus, Zea binding seems to promote Chlb degradation, in particular in monomeric antenna complexes. The monomerization of LHCII trimers is, in fact, the first step necessary for their proteolytic degradation (Yang, D.-H. et al. 2000), and it is promoted during HL acclimation with accumulation of Zea (Ballottari, M. et al. 2007, Havaux, M. et al. 2004). LHCII are probably made more accessible to the attack of CbR upon monomerization and proteolytically degraded because of Chlb removal. This hypothesis is supported by the results of *in vitro* LHC protein renaturation experiments, which determine that Chlb is fundamental to obtain a stable complex (Giuffra, E. et al. 1996) and the Lhc complexes instability in *Ch1*mutant, that lacks of Chlb (Havaux, M. et al. 2007).

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CONCLUSIONS

CONCLUSIONS

Thylakoid membranes are the site in which light is absorbed and converted into chemical energy. Photosynthetic organisms like higher plants are subjected to a extremely wide range of environmental conditions which extend to stress. Plant need to develop ad-hoc mechanisms in order to cope with these stresses and survive.

One important conclusion of this thesis work is the understanding that plants evolved rapid and dynamic defense systems for reversibly switch from an energy conserving to an energy-dissipating physiological condition of their photosynthetic apparatus. The common issue of many stressing conditions is the production of ROS, particularly singlet oxygen, in all conditions in which photosynthetic electron transport cannot use all the energy associated to the absorbed light. In these conditions the lumenal pH decreases because ATPase is blocked by lack of ADP and Pi substrates. This pH drop is the trigger by at least two signaling pathways starting with:

A) Violaxanthin De-Epoxidase activation
B) PsbS protonation

VDE is responsible for zeaxanthin synthesis when plants are exposed to stress. This xanthophyll plays a central role in the response to high light stress. Mechanisms by which its function is fulfilled are several:

i) Up-regulates qE and qI components of NPQ. This is obtained by binding to site L2 of monomeric Lhcb complexes, particularly Lhcb6 and Lhcb5.
ii) Up-regulates Ros scavenging activity with respect to the molecule (violaxanthin) present in low light.

In section A.1, I investigate the role of zeaxanthin upon binding to the PSII antenna complex Lhcb6 (CP24). Our results showed that, upon activation of the xanthophyll cycle, zeaxanthin rapidly accumulates and Lhcb6 binds it into inner L2 site faster and to a larger extent than any other pigment–protein complex. The higher affinity of Lhcb6 for zeaxanthin and the faster exchange of violaxanthin with respect to the other antenna complexes is important in understanding the functional role of Lhcb6. This minor antenna complex is an evolutionarily recent addition to the photosynthetic apparatus, appearing only in land plants. In particular, Lhcb6 is absent in photosynthetic organisms like algae, which grow in water environments which are not subjected to continuous variations like emerged land. The presence of Lhcb6 into plant photosynthetic apparatus appears to be crucial for the concurrent activation of defensive responses upon exposure to stress. In fact, Lhcb6 is essential for the reorganization of PSII which both triggers qE and decreases the antenna size of PSII (Section B). The mechanism for qE enhancement has been elucidated recently (Ahn et al. 2008): zeaxanthin binding to site L2 is needed for the activation of Charge Transfer quenching process, which is characterized by a zeaxanthin radical cation (Z●+) formation and subsequent charge recombination at the ground state, dissipating excitation energy. The special arrangement of two chlorophyll *a* and one zeaxanthin interacting is found in monomeric Lhcbs while the more abundant LHCII trimers cannot undertake this mechanism since one of the chlorophylls involved is chlorophyll *b* thus making difficult the formation of Charge transfer states.

Recently, other molecular models has been proposed for qE, which are based on different principles with respect to CT quenching. Aggregation Dependent Quenching is a mechanism based on experimental evidences that similar spectral changes has been observed upon *in vitro* aggregation of isolated LHCII trimers and upon NPQ induction *in vivo*. So far this model has been developed focusing mainly for LHCII. One major objection is that while zeaxanthin is a major factor modulating qE, LHCII is unable to bind this xanthophyll on site L2 but only to site V1. It should be noticed that binding to site V1 is not efficient in quenching (Caffarri et al. 2001).

This problem was discussed in section A.2 in which we studied ADQ in both monomeric and trimeric Lhcb proteins, investigating the activities of each antenna subunit. *In vitro* analysis showed that monomeric Lhcb proteins undergo stronger quenching than LHCII. In addition it is enhanced in the presence of zeaxanthin, as it occurs during NPQ *in vivo*. We also showed that ADQ in LHCII becomes zeaxanthin-dependent when Lhcb6 is added to the system. This result is interesting because the process of domain reorganization that we have studied (Section B) leaves LHCII together with Lhcb6, implying that Lhcb6 might be the element conferring quenching properties to this domain.

Findings shown in Section B can help to answer to this question. We observed that PsbS, which is the trigger of NPQ activated by the protonation of two lumenal exposed glutamate residues, is responsible for the light induced dissociation of a specific pentameric PSII supercomplex, composed by the minor antenna complex Lhcb4 and Lhcb6 and by a trimer LHCII. This dissociation, which is also promoted by zeaxanthin binding to Lhc proteins, leads to a reorganization of complexes inside *grana* membranes. Our suggestion, strenghtened by consistent findings by prof. Alfred Holzwarth research group, is that upon dissociation of the pentameric supercomplex two distinct quenching domains are formed: one associated to the antenna complexes that remains strictly bound to PSII core (one LHCII trimer, Lhcb5 and Lhcb4, which are constitutively associated to PSII core, even under chronic PSII over-excitation) and one composed by segregated LHCII trimers associated to Lhcb6, and several mechanisms including CT quenching and ADQ may be involved for fully induction of energy dissipation state.

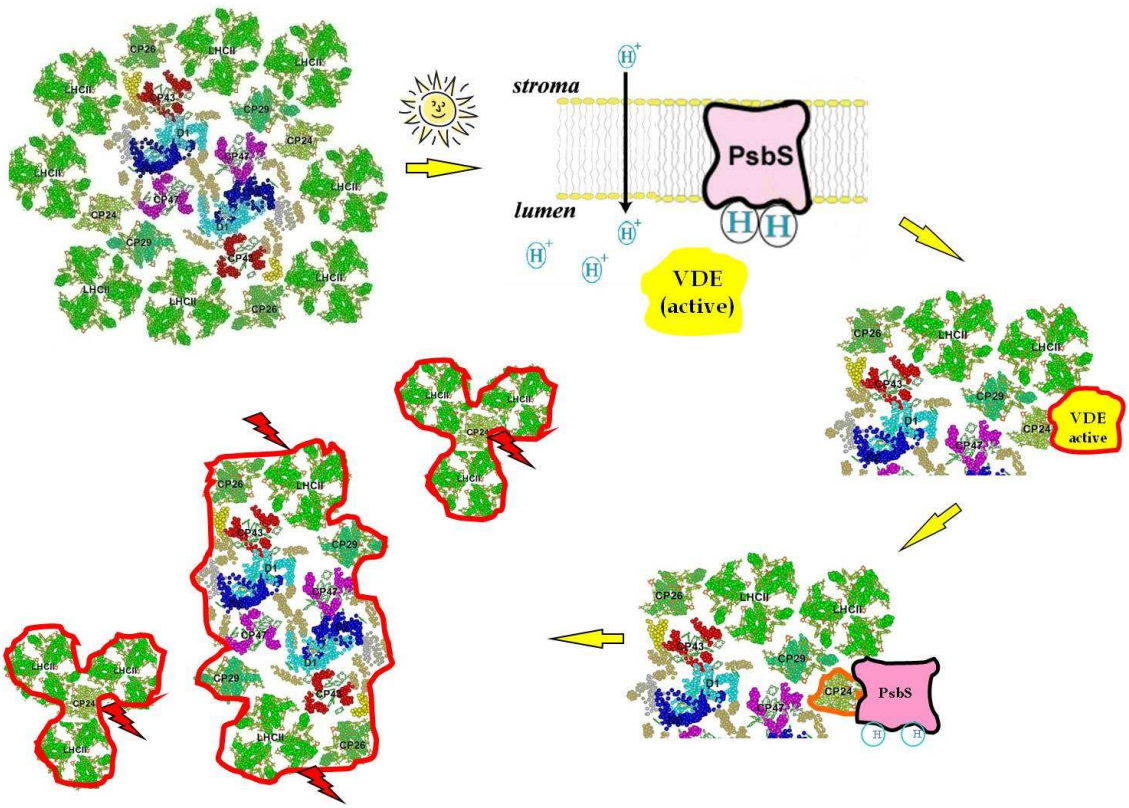

Figure 1 Scheme of NPQ induction based on the results shown in this work of thesis. *Upon light exposure Violaxanthin De-Epoxidase is induced by lumenal acidification and promote zeaxanthin binding to Lhcb6 (CP24), inducing a conformational change that is recognized by PsbS, which interact with PSII, determining a dissociation of a penthameric supercomplex and the formation of two quenching domains, one associated to PSII core and one to detached antenna complexes.*

Minor antenna complexes that remain associated to PSII core during stress exposure are essential for PSII phototoprotection. In section C, we deeply characterized Lhcb4 (CP29) functional properties in *Arabidopsis* thaliana: this antenna protein, which is part of the pentameric supercomplex described in the previous section, has a fundamental role for protecting PSII from photo-inhibition, confirmed by the evidence that lack of Lhcb4, unlike that of any other Lhcb subunit, increases photosensitivity to strong light stress. These data further support the view that monomeric antenna complexes are essential for photoprotection, and Lhcb4, in particular; acts as a defensive shield for PSII core, protecting from ROS diffusing in the lipid membrane. In non stressing conditions, Lhcb4 is important for structurally bridging dimeric PSII core complexes to the major trimeric LHCII antenna. Indeed in its absence, a large fraction of the LHCII becomes badly connected to the reaction centre and PSII supercomplexes are destabilized.

In the last section of this thesis, we studied the relationship between antenna degradation and plant photoprotection. Our data shows that the first enzyme of the chlorophyll degradation pathway, chlorophyll *b* reductase, is important for the regulation of PSII antenna size upon acclimation to high light, indeed *Arabidopsis thaliana* mutants that lack of this protein show a higher retention of antenna proteins in excess light conditions, leading to overexcitation and photodamage. These findings suggest that antenna complexes degradation relies firstly on destabilization of chlorophyll ligands and only subsequently on apoprotein degradation. It should be noticed that this area of research is still very far from being elucidated: in continuous strong light conditions antenna complexes degradation proceeds even in the absence of chlorophyll *b* reductase, although more slowly. Interestingly, we observed that the presence of zeaxanthin determined a higher activity of chlorophyll *b* reductase. Thus this xanthophylls is not only important as a modulator of NPQ, but appears also to be important for the regulation of protein turnover as it was previously suggested based on the strong reduction of antenna size in zeaxanthin-only mutants (Havaux et al. 2004).

In conclusion, in this work of thesis I described mechanisms that are essential during short and long term response to excess light exposure, involving a strict interaction between protein subunits, small molecules and supercomplexes of the photosynthetic apparatus, acting at different levels. Indeed, I showed the importance of the presence of zeaxanthin, which modulates antenna complexes properties and modulates the PsbS-related reorganization of PSII upon light exposure. We also suggest that this regulation of PSII supercomplex assembly is part of a control cycle connecting short term energy dissipation to long term regulation of PSII antenna size upon acclimation. This network must be rigorously regulated in order to be the most effective as possible and to ensure plant survival in unfavorable conditions.

GLOSSARY

Antenna system: component of photosystems responsible for light harvesting and energy transfer to the reaction centre. For both PSI and PSII two antenna systems are present: an inner antenna system located in the core complex and a peripheral antenna system composed by LHC proteins. In particular the peripheral antenna systems are formed by Lhca and Lhcb proteins for PSI and PSII respectively. Antenna systems are responsible for light harvesting and photoprotection.

ATP: Adenosine 5'-triphosphate. It is a multifunctional nucleotide that is most important in intracellular energy transfer. In this role, ATP transports chemical energy within cells for metabolism. It is produced as an energy source during the processes of photosynthesis and cellular respiration and consumed by many enzymes and a multitude of cellular processes including biosynthetic reactions, motility and cell division

ATP-ase: a multimeric complex involved in the light phase of photosynthesis: this complex is responsible for ATP production, coupling ATP synthesis with transmembrane proton movement. During photosynthetic electron transport, protons are pumped in the lumen compartment: the transmembrane ΔpH formed constitutes a proton motive force used by ATP-ase to produce ATP. ATP-ase is characterized by stromal and transmembrane regions that are known as CF_1 and CF_0, respectively, and represents a molecular motor that is driven by proton movement across the membrane. Proton movement through CF_0 is coupled to ATP synthesis/hydrolysis.

PSII-C2S2: the basic unit of PSII supercomplex composed by a PSII dimer, and the subunits CP26, CP29 and a trimeric LHCII bound to each PSII core.

Calvin cycle: the series of reactions in which atmospheric CO_2 is reduced to carbohydrates, using the chemical free energy (ATP and NADPH) produced during the light reactions. These reactions occur in the stroma compartment of the chloroplast.

$Car^{\bullet+}$: carotenoid radical cation. The formation of this species, and in particular zeaxanthin radical cation, has been correlated to qE induction and belongs to a charge separation in a heterodimer composed by a chlorophyll a and a carotenoid. The subsequent charge recombination allows the thermal dissipation of the energy used for charge separation, in a mechanism called Charge Transfer quenching. Carotenoid radical cation is characterized by absorption in the NIR at ~1000nm

Ch1: mutant of Arabidopsis thaliana lacking the chlorophyll a oxygenase, resulting in absence of chlorophyll b production. Absence of chlorophyll b leads to an impairment in the assembly of Chl *b*-containing light-harvesting complexes.

$^1Chl^*$: singlet chlorophyll excited states. This specie is formed upon light absorption or excitonic energy transfer; singlet chlorophyll excited states can decay to the ground states through several pathways: fluorescence emission, heat production, intersystem crossing or photosynthetic reactions

$^3Chl^*$: triplet chlorophyll excited states. This specie is formed through intersystem crossing from singlet chlorophyll excited states. Triplet Chl excited state is a long-lived state (~ms time scale) and thus can react with triplet oxygen, converting it to singlet oxygen ($^1O_2$), a highly reactive oxygen specie. Carotenoid have a determinant role in $^3Chl^*$ quenching

Core complex: the inner part of the photosystems binding the reaction centre and the co-factors involved in electron transport. Core complexes are composed by the products of the genes denominated *Psa* and *Psb* respectively for PSI and PSII. Among them there are

both nuclear and chloroplast encoded polypeptides. At the reaction centre level a chlorophyll special pair undergo charge separation after being excited by the excitonic energy. Core complexes have also an inner antenna system responsible for light harvesting and energy transfer to the reaction centre.

CP24: Lhc antenna protein, named also Lhcb6. This protein is one of the PSII antenna minor complexes and it's found in monomeric form in Photosystem II.

CP26: Lhc antenna protein, named also Lhcb5. This protein is one of the PSII antenna minor complexes and it's found in monomeric form in Photosystem II.

CP29: Lhc antenna protein, named also Lhcb4. This protein is one of the PSII antenna minor complexes and it's found in monomeric form in Photosystem II.

CP43: PSII core complex subunit encoded by the plastidial gene *psbC*. This subunit binds 14 chlorophylls a and forms with CP47 the inner antenna of photosystem II

CP47: PSII subunit encoded by the plastidial gene *psbB*. This subunit binds 14 chlorophylls a and forms with CP47 the inner antenna of photosystem II

CT quenching: mechanism of thermal dissipation of absorbed light energy by chlorophylls trough carotenoid radical formation. When this mechanism is activated the absorbed energy is used for charge separation in a heterodimer composed by a chlorophyll a and carotenoid heterodimer. The subsequent charge recombination allows the thermal dissipation of the energy.

Cyclic electron transport: electrons from ferredoxin are transferred (via plastoquinone) to a proton pump, cytochrome b_6f. They are then returned (via plastocyanin) to P700. This cycle does not produce reducing power, NADPH, but ATP.

Cytochrome-b_6f: a multiproteic complex involved in the light phase of the photosynthesis. Cytochrome-b_6f is a plastoquinol—plastocyanin reductase located in the thylakoids. It transfers electrons between the two reaction center complexes of oxygenic photosynthetic membranes, photosystem I and photosystem II, and participates in formation of the transmembrane electrochemical proton gradient by transferring protons from the stromal to the internal lumen compartment. It is minimally composed of four subunits: cytochrome b_6, carrying a low- and a high-potential heme groups (b_L and b_H); cytochrome *f* with one covalently bound heme *c*; Rieske iron-sulfur protein (ISP) containing a single [Fe_2S_2] cluster; and subunit IV (17 kDa protein).

D1: PSII subunit forming dimers with D2 subunit. D1 and D2 subunits bind the PSII reaction centre P_{680}. D1 subunit is encoded by the plastidial *psbA* gene.

D2: PSII subunit forming dimers with D1 subunit. D1 and D2 subunits bind the PSII reaction centre P_{680}. D1 subunit is encoded by the plastidial *psbD* gene.

Dark phase: the photosynthesis phase in which the ATP and NADPH produced during light phase are used in order to produce biomass trough CO_2 fixation. The reactions constituting the dark phase are indicated as the Calvin cycle.

Ferredoxin: iron-sulfur protein that mediate electron transfer during light phase of photosynthesis. The chloroplast ferredoxin is involved in both cyclic and non-cyclic photophosphorylation reactions of photosynthesis. In non-cyclic photophosphorylation, ferredoxin is the last electron acceptor, being reduced by PSI, and reduces the enzyme $NADP^+$ reductase, which finally produces NADPH. Ferredoxins are small proteins containing iron and sulfur atoms organized as iron-sulfur clusters. These biological "capacitors" can accept or discharge electrons, the effect being change in the oxidation states (+2 or +3) of the iron atoms.

Ferredoxin-NADP$^+$ reductase: enzyme that catalyzes the reduction of NADP$^+$ to NADPH. This enzyme is present in the stroma compartment and it's reduced by ferredoxin.

Gap chlorophylls: chlorophylls found at the interface between LHCI complex and PSI core complex.

Grana: the stacked structures formed by thylakoids

L1: carotenoid binding site, located in the inner part of antenna proteins close to helix A. L1 site is prevalently filled by lutein in all plant species and in all LHC proteins.

L2: carotenoid binding site, located in the inner part of antenna proteins close to helix B. L2 sites is prevalently filled by lutein in LHCII trimers, while in monomeric antenna proteins L2 can be occupied by lutein, violaxanthin, neoxanthin and zeaxanthin, depending by several factors, as environmental condition, plant species, protein properties. L2 in LHCI and Lhcb minor complexes binds the product of the xanthophyll cycle.

LHC proteins: antenna proteins of photosystem I and II. These proteins are responsible for light harvesting and photoprotection; LHC proteins bind chlorophyll a, chlorophyll b and xanthophylls. Lhc proteins are encoded by nuclear genes forming a multigenic family. The structure of Lhc proteins is similar.

Lhcb proteins: members of the Lhc antenna proteins family associated to Photosystem II. Lhcb proteins are divided into two classes: the more abundant LHCII trimers, constituted by Lhcb1, 2, 3 subunits and the monomeric minor complexes constituted by Lhcb4, 5, 6 subunit. The amount of Lhcb protein associated to PSII depends from environmental conditions and the plant species. The subunit Lhcb7 and Lhcb8 are only rarely expressed and their role is still uncertain.

Lhcb1: Lhc antenna protein associated to PSII. It's a subunit of the heterotrimeric antenna complex LHCII

Lhcb2: Lhc antenna protein associated to PSII. It's a subunit of the heterotrimeric antenna complex LHCII

Lhcb3: Lhc antenna protein associated to PSII. It's a subunit of the heterotrimeric antenna complex LHCII

Lhcb4: Lhc antenna protein, named also CP29. This protein is one of the PSII antenna minor complexes and it's found in monomeric form in Photosystem II.

Lhcb5: Lhc antenna protein, named also CP26. This protein is one of the PSII antenna minor complexes and it's found in monomeric form in Photosystem II.

Lhcb6: Lhc antenna protein, named also CP24. This protein is one of the PSII antenna minor complexes and it's found in monomeric form in Photosystem II.

Lhcb7: rarely expressed antenna protein similar to the Lhcb4-6 subunit associated to PSII

Lhcb8: rarely expressed antenna protein similar to the Lhcb4-6 subunit associated to PSII

LHCI: antenna complex associated to PSI. It's composed by Lhca1, 2, 3 and 4 subunits and it's positioned in a half-moon shape at one side of PSI.

LHCII: the trimeric more abundant antenna complex associated to PSII. Each trimmer is a heterotrimer with different levels of Lhcb1, 2, 3 subunits. The structure of each monomer is constituted by three transmembrane and one amphypathic helices, indicated respectively as A-C and D. Each monomer binds 14 chlorophylls and 4 carotenoids.

LHCII-L: LHCII trimers forming the outer layer of antennae protein bound to PSII core. LHCII-L amount per PSII core depend from environmental condition.

LHCII-S: LHCII trimers bound to the basic unit of PSII, the C2S2 particle. LHCII-S amount per PSII core doesn't depend from environmental condition.

Light phase: the photosynthesis phase in which sunlight is absorbed by photosynthetic pigments (chlorophylls and carotenoids); absorbed energy is subsequently transferred to the reaction centre where charge separation occurs, thus converting light energy into chemical energy. Photosystems I and II, Cytochrome b_6f and ATP-ase are the proteic complexes involved in the light phase. During light phase a water molecule is consumed in order to produce O_2, ATP and NAPH

Linker: chlorophylls found at the interface between LHCI subunits.

Lumen: the soluble compartment in the chloroplast delimited by thylakoids: in the lumen protons are pumped during photosynthetic light phase, forming a transmembrane ΔpH which constitutes the force used by ATP-ase to produce ATP.

Minor complexes: monomeric antenna proteins associated to PSII. Minor complexes are composed by CP24, CP26 and CP29 antenna proteins and bind 2-3 carotenoids per molecule. They are located between PSII core and the peripheral LHCII trimers.

N1: carotenoid binding site, located close to helix C of antenna proteins. N1 site is present in LHCII and it's specific for neoxanthin, even if violaxanthin or lutein binding was found therein. N1 site is stabilized by a Tyrosine residue which can form hydrogen bounds with the carotenoid therein. N1 site is also stabilized in LHCII by the chlorophylls located near Helix C and D. The presence of N1 site also in CP26 and CP29 has been suggested recently and it's discussed in this thesis.

NADPH: Nicotinamide adenine dinucleotide phosphate. This specie is used in anabolic reactions, such as lipid and nucleic acid synthesis, as a reducing agent. NADPH is the reduced form of NADP+, and NADP+ is the oxidized form of NADPH. In chloroplasts, $NADP^+$ is reduced by ferredoxin-$NADP^+$ reductase in last step of the electron chain of the light reactions of photosynthesis. The NADPH produced is then used as reducing power for the biosynthetic reactions in the Calvin cycle of photosynthesis.

NPQ: Non Photochemical Quenching. It's a light-induced photoprotective process by which plants are able to rapidly dissipate the excess absorbed energy as heat. When light is absorbed in excess the photosynthetic electron transport establish a low lumenal pH, which activate the mechanisms inducing NPQ. NPQ is detectable monitoring the decrease of leaf fluorescence during illumination. NPQ is characterized by a fast component called qE with relaxation time within minutes, and a slower component called qI, correlated to photoinhibition, which has relaxation time of hours.

Npq1: double mutant of Arabidopsis thaliana lacking the Violaxanthin de-epoxidase enzyme. This mutant is impaired in xanthophyll cycle and cannot convert violaxanthin to zeaxanthin.

Npq2: mutant of Arabidopsis thaliana lacking the Zeaxanthin epoxidase enzyme. This mutant constitutively accumulates zeaxanthin but not violaxanthin and neoxanthin.

Npq4: double mutant of Arabidopsis thaliana lacking the PsbS protein. This mutant is constitutively impaired on NPQ induction.

OEC: Oxygen Evolving Complex. It's a multiproteic complex characterized by the presence of four manganese ions cluster. This complex it's associated to PSII and extracts electrons from water producing O_2

and H^+ , through redox reactions with the manganese cluster. Electrons extracted from water reduce the oxidized P_{680}^+. On the lumenal side of the complex, three extrinsic proteins of 33, 23 and 17 KDa (OEC1-3) compose the oxygen evolving complex and have a calcium ion, a chloride ion and a bicarbonate ion as necessary cofactors.

P_{680}: the chlorophyll special pair constituting the reaction centre of PSII. These chlorophylls are characterized by absorption at 680nm and upon excitation undergo charge transfer, transferring one electron to the electron acceptors located in PSII. The oxidized P_{680}^+ is reduced by a Tyrosine residue named Tyr_Z, which was previously reduced by the Oxygen Evolving Complex (OEC).

P_{700}: the chlorophyll special pair constituting the reaction centre of PSI. These chlorophylls are characterized by absorption at 700nm and upon excitation undergo charge transfer, transferring one electron to the electron acceptors located in PSI. The oxidized P_{700}^+ is reduced by a plastocyanin which was previously reduced at the cytochrome b_6f level.

Photoinhibition: reduction of photosynthetic efficiency in plants due to damages deriving from light energy absorbed in excess. When light is absorbed in excess and photosynthetic pathway become saturated, the energy can diverted to produce ROS, which causes damage through oxidation of lipids, proteins and pigments, reducing the photosynthetic efficiency**.**

Photoprotection: the whole mechanisms developed by photosynthetic organisms in order to avoid photoinhibition. Photoprotective mechanisms can be divided into two different classes, depending on the time-scale of action: a) short-term photoprotective mechanisms and b) long-term photoprotective mechanisms.

Photorespiration: an alternate pathway for production of glyceraldehyde 3-phosphate, an intermediate of the Calvin cycle, by RuBisCO. Although RuBisCO favors carbon dioxide to oxygen, (approximately 3 carboxylations per oxygenation), oxygenation of RuBisCO occurs frequently, producing a glycolate and a glycerate. This usually occurs when oxygen levels are high; for example, when the stomata (tiny pores on the leaf) are closed to prevent water loss on dry days. It involves three cellular organelles: chloroplasts, peroxisomes, and mitochondria. Photorespiration produces no ATP, but consumes ATP and NADPH: it may function as a "safety valve", preventing excess NADPH and ATP from reacting with oxygen and producing free radicals.

Photosystems: multiproteic complexes involved in the light phase of photosynthesis. Photosystems are responsible for light absorption and its conversion into chemical energy trough charge separation at the reaction centre level. In higher plants are present two photosystem : photosystem I (PSI) and photosystem II (PSII). Both photosystem are composed by a core complex where is located the reaction center and an antenna system.

Plastocyanin: a monomeric copper-containing protein involved in photosynthetic electron-transfer. Plastocyanin functions as an electron transfer agent between cytochrome *f* of the cytochrome b_6f complex from photosystem II and P_{700}^+ from photosystem I.

PsbS: an integral membrane protein component and member of the Lhc-protein superfamily, even if it doesn't bind pigments. Its presence is fundamental for NPQ induction, and in particular for the qE component. PsbS has two conserved glutamic acid exposed to the lumen (Li, X. P. et al. 2002) which substitution results is PsbS inactivation.

Pseudocyclic electron transport: called also water-water cycle, consists in an alternative electrons transport pathway with direct reduction of O_2 by PSI. This pseudocyclic pathway, in which electrons produced from water oxidation into PSII are used to reduce O_2 to H_2O, allows generation of a ΔpH for ATP synthesis without production of O_2 or NADPH that act as another important photoprotective mechanism.

PSI: Photosystem I. It's one of the multiproteic complexes responsible for the light phase of the photosynthesis and it is mainly located in the unstacked *stroma lamellae* membranes. PSI is a light-dependent plastocyanin-ferredoxin oxidoreductase. PSI is composed by a core complex and a peripheral antenna system. The core complex is constituted by 12 about subunits, encoded by the *psa* genes and binds the reaction center P_{700} and the primary electron acceptors A_0 (chlorophyll-*a*), A_1 (phylloquinone) and F_X (a Fe_4–S_4 cluster). The oxidized P_{680}^+ is reduced by the plastocyanin at the lumen exposed side, while ferrodoxin is reduced at the stroma exposed side. The inner antenna system of PSI is constituted of 97 chlorophylls a while the outer antenna system, the LHCI complex, is constitutes of 56 Chls bound by Lhca1-4 subunits. 9 "gap" chlorophylls are present at the interface between LHCI ad PSI core. PSI is characterized by the presence of the "red forms".

PSII: Photosystem II. PSII is water–plastoquinone oxidoreductase. It's one of the multiproteic complexes responsible for the light phase of photosynthesis and it is mainly located in the stacked *grana* membranes. PSII is composed by a core complex and a peripheral antenna system. The core complex is constituted by 16 subunits, encoded by the *psb* genes and binds the reaction center P_{680} and the primary electron acceptors: pheophytin and the quinones Q_A and Q_B. The oxidized P_{680}^+ is reduced by a Tyr_Z residue, which is reduced by electrons extracted from water by the Oxygen Evolving Complex. The final electron acceptor of PSII is the plastoquinone pools. Reduced plastoquinones (plastoquinols) migrates to cytochrome b_6f. The inner antenna system of PSII is constituted by CP43 and CP47 subunits, while the outer antenna system is composed by the Lhcb proteins. The amount of Lhcb proteins bound to PSII core depends from environmental conditions and plant species.

PSI-LHCI*: PSI-LHCI complex with a reduced amount of Lhca antenna proteins bound. PSI-LHCI* complex is mainly found in mutants of Arabidopsis thaliana lacking Lhca1, Lhca2, Lhca3 or Lhca5 proteins (Δa1, Δa2, Δa3, Δa5 plants). Lhca4 depleted plants (Δa4) have a PSI supercomplex with almost no Lhca proteins bound.

qE: the faster component of leaf fluorescence decrease due to NPQ induction; it's associated to the reduction of lumenal pH upon extreme electron transport reactions as a consequence of light absorption in excess. qE depends from the presence of the PSII associated protein PsbS and from zeaxanthin accumulation. qE component of NPQ has relaxation time of few minutes.

qI: the slower component of NPQ. When leaves are placed to dark, after being illuminated to activate NPQ, their fluorescence level is lower than the initial level: this difference represents the qI component of NPQ, which has a relaxation time of hours. qI is associated to photoinhibition events and to zeaxanthin accumulation, which takes hour to be re-converted to violaxanthin and activates some long-term photoprotective mechanisms.

Red forms : low-energy absorption forms associated to photosystem I. Photosystem I, respect to Photosystem II, is characterized by absorption forms at long-wavelengths, over 700 nm. This absorption forms are called "red forms" and are mainly located in the

antenna system LHCI. “Red forms” originate from an excitonic interaction between chlorophylls located in A5 and B5 chlorophyll binding sites. In particular Lhca3 and Lhca4 show the red-most shifted absorptions, determined by the modulation of the A5-B5 excitonic interaction given by the presence of an asparagine residue in A5 chlorophyll binding sites.

ROS: Reactive Oxygen Species. These reactive species are byproduct of photosynthesis and their products is increased during abiotic stresses which impair the photosynthetic reactions. The main classes of ROS present in the chloroplast are: singlet oxygen, superoxide anion, hydrogen peroxide or hydroxyl radical. ROS production has mainly three different sites in the thylakoids: LHC proteins of PSII, PSII reaction centre and PSI acceptor side. Singlet oxygen is produced mainly at LHC proteins level when light is absorbed in excess and cannot be transferred to the reaction centre: in this case $^3Chl^*$ is formed, which can convert O_2 to singlet oxygen. Singlet oxygen can be produced also at P_{680}^+ level, since it can become a triplet P_{680} ($^3P_{680}$) due to charge recombination and other back-reactions of PSII. Superoxide anion, hydrogen peroxide or hydroxyl radical are mainly produced at PSI acceptor side. ROS accumulation causes damages to the photosynthetic apparatus though oxidation of lipids, proteins and pigments. Their accumulation induce a situation known as oxidative stress

RuBisCO: Ribulose-1,5-bisphosphate carboxylase/ oxygenase. It is an enzyme that is used in the Calvin cycle to catalyze the first major step of carbon fixation, a process by which the atoms of atmospheric carbon dioxide are made available to organisms in the form of energy-rich molecules such as sucrose. RuBisCO catalyzes either the carboxylation or oxygenation of ribulose-1,5-bisphosphate (also known as RuBP) with carbon dioxide or oxygen.

State transitions: migration of Lhcb proteins from PSII to PSI, in order to balance the PSI and PSII excitation here LHCII can transfer the energy absorbed to PSI, rather than PSII. The state transition takes several minutes to be activated, since it involves also the migration of LHCII from grana to stroma lamellae

Stroma lamellae: the interconnecting regions of thylakoids between grana.

Stroma: the soluble compartment of the chloroplast. In the stroma are present the enzymes involved in the dark phase of photosynthesis, that use the ATP and NADPH produced during light phase in order to produce biomass.

Thylakoids: the inner membranes of the chloroplast. The complexes involved into the light phase of the photosynthesis (PSI, PSII, ATP-ase, Cytochrome b6*f*) are located in the thylakoids membranes. Thylakoids are organized into two membrane domains: 1) cylindrical stacked structures called *grana*, and 2) interconnecting regions, the *stroma lamellae*. Thylakoids confine a compartment called *lumen*: in the lumen protons are pumped during photosynthetic light phase, forming a transmembrane ΔpH which constitutes the force used by ATP-ase to produce ATP.

Transfer-to-Trap limited kinetics: theoretical model in order to explain the energy trapping kinetics of photosystems. Light is absorbed by photosystems and converted into chemical energy through charge separation at the reaction center level (the “energy-trap”). The energy trapping kinetic is “transfer-to-trap-limited” when the diffusion of the absorbed energy to the reaction center is the slowest phase of the whole process from light harvesting to charge separation.

V1: carotenoid binding site, located at the peripheral part of antenna proteins LHCII. V1 site is a specific site for violaxanthin and it’s involved in xanthophyll cycles product binding in LHCII.

VDE: Violaxanthin de-epoxidase enzyme. This enzyme catalyzes the xanthophyll cycle reactions: it de-epoxidates violaxanthin to antheraxanthin and finally to zeaxanthin. VDE has a maximum active at low pH (~5.2 pH): in this way the xanthophyll cycles and the photoprotection mechanisms correlated, are activated only when photosynthesis reaction are saturated, inducing a strong reduction of the lumenal pH, the compartment where VDE enzyme is localized.

Xanthophyll cycle: a series of enzymatic reactions by which the carotenoid violaxanthin is de-epoxidated to zeaxanthin, through the intermediate antheraxanthin. These reactions are catalyzed by the Violaxanthin de-epoxidase enzyme (VDE), which is activated at acid pH around 5.2. When photosynthetic reactions are saturated the lumenal pH is reduced by proton pumping: this event activates VDE enzyme which induces the xanthophyll cycle. Accumulation of zeaxanthin is responsible for activation of several photoprotective mechanism as NPQ, $^{3}Chl^{*}$ quenching and ROS scavenging.

$Z^{\bullet+}$: zeaxanthin radical cation. The formation of this species has been correlated to qE induction and belongs from charge separation in a heterodimer composed by a chlorophyll a and zeaxanthin heterodimer. The subsequent charge recombination allows the thermal dissipation of the energy used for charge separation, in a mechanism called Charge Transfer quenching. Zeaxanthin radical cation is characterized by absorption in the NIR at ~1000nm

ZE: Zeaxanthin epoxidase enzyme. This enzyme catalyzes the zeaxanthin epoxidation to produce violaxanthin through antheraxanthin intermediate.

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