Study of factors controlling the photoprotection capacity of the thylakoid membrane

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Abstract

Plants require light for the process of photosynthesis, but excess of light absorption can cause photooxidative damage. To avoid this damage, plants have evolved a photoprotective mechanism to dissipate excess light energy as heat in a process called nonphotochemical quenching (NPQ). This regulatory mechanism of light harvesting involves both pigment and protein constituents of antenna complexes. Two xanthophylls, lutein and zeaxanthin, have been implicated to contribute to the rapidly relaxing qE component of NPQ, acting as quenchers of the chlorophyll excitation energy. To determine the molecular mechanism of NPQ and role of these xanthophylls in it, the kinetics of qE and qE-related conformational changes were measured in *Arabidopsis thaliana* mutant plants with altered xanthophyll contents. The effect of xanthophyll composition on the chlorophyll excited state lifetime was also compared - in leaves and native isolated antenna complexes. The data reveal that the replacement of lutein by either zeaxanthin or violaxanthin in the internal binding sites of the antenna complexes affects the qE kinetics and amplitude as well as the absolute chlorophyll fluorescence lifetime. This demonstrates the role of lutein in maintaining the efficient photoprotective state. The PsbS protein of photosystem II has also been demonstrated to play a significant role in controlling the qE component of NPQ. Thereby, enhancement of PsbS and resultant increase in qE and qE-related conformational changes was achieved in *Arabidopsis* by physiological and genetic means in the absence of zeaxanthin. This helps to dissect the relationship between zeaxanthin and PsbS in NPQ, suggesting both as independent entities. The results support allosteric role of zeaxanthin and not as the direct quencher alone or in combination with the PsbS in the process, whilst the role of PsbS is suggested as kinetic modulator of conformational change which results in NPQ.

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Dedicated to Ammi;

a diligent teacher,

a brave woman

and a loving mother.

Abbreviations

ADP	adenosine diphosphate
ATP	adenosine triphosphate
ATPase	ATP synthase
bp	base pair
BSA	bovine serum albumin
Chl	chlorophyll
Chl*a*	chlorophyll a
Chl*b*	chlorophyll b
Cyt *b559*	cytochrome *b559* complex
Cyt *b6f*	cytochrome *b6f* complex
DCCD	N,N'-dicyclohexylcarbodiimide
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DGDG	digalactosyldiacylglycerol
***α*-DM**	*n*-dodecyl-*α*-D-maltoside
***β*-DM**	*n*-dodecyl-*β*-D-maltoside
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELIP	early light inducible protein
EM	electron microscopy
FAD	flavin adenine dinucleotide
Fd	ferredoxin
FeS	iron-sulphur centre
FNR	ferredoxin NADP reductase
FPLC	fast protein liquid chromatography
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid
HLIP	high light inducible protein
HPLC	high performance liquid chromatography
IEF	iso-electric focusing

IgG	immunoglobulin G
kDA	kilodalton
LED	light emitting diode
LHCI	light harvesting antenna complex of photosystem I
LHCII	light harvesting antenna complex of photosystem II
LHCIIb	major light harvesting antenna complex of PSII
Lut	lutein
MGDG	monogalactosyldiacylglycerol
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
Neo	neoxanthin
NPQ	non-photochemical quenching of chlorophyll fluorescence
OD	optical density
OEC	oxygen evolving complex
OHP	one helix protein
PAGE	polyacrylamide gel electrophoresis
PAM	pulse amplitude modulated
PC	plastocyanin
Pheo	pheophytin
PG	phosphatidylglycerol
PQ	plastoquinone
PQH2	plastoquinol
PSI	photosystem I
PSII	photosystem II
ΔpH	trans-thylakoid pH gradient
RC	reaction centre
ROS	reactive oxygen species
Rubisco	ribulose-1,5-bisphosphate carboxylase/oxygenase
qE	rapidly relaxing, energy dependent non-photochemical quenching
qI	very slowly relaxing, photoinhibitory non-photochemical quenching

qP	photochemical quenching
qT	slowly relaxing non-photochemical quenching related to state transitions
SDS	sodium dodecyl sulphate
SEP	stress enhanced protein
TEMED	tetramethylethylenediamine
Vio	violaxanthin
VDE	violaxanthin de-epoxidase
WT	wild type
XC	xanthophyll cycle
Zea	zeaxanthin
ZE	zeaxanthin epoxidase

Table of contents

List of Tables

List of Figures

Chapter One

Introduction

1.1 Photosynthesis

Solar energy is the ultimate source of all metabolic energy on earth, and capturing this radiant energy and making it available for biochemical reactions is prerequisite for life. Photosynthesis is the metabolic pathway whereby plants use light to convert simple, inorganic and energy-poor compounds, carbon dioxide (CO_2) and water (H_2O), into complex, organic and energy-rich compounds carbohydrates, releasing oxygen (O_2) as a by-product. This process can be represented by this equation:

$$H_2O + CO_2 + \text{solar energy} \rightarrow (CH_2)_xO + O_2$$

1.2 Photosynthetic apparatus

Photosynthesis is a highly efficient process which traps an immense amount of approximately 100 terawatts (10^{12} watts) of energy per annum, a quantity seven times larger than the annual energy consumption by human civilisation (Nealson and Conrad, 1999). Plants perform this energy converting process in the specialised mesophyll cells of leaves (Fig. 1.1. A). These cells are packed with special organelles called chloroplasts. There are ten to hundred chloroplasts in a typical plant cell. A chloroplast is comprised of a system of flattened vesicles called thylakoids embedded in a colourless matrix called stroma, all contained within a double membrane envelope. The thylakoid membrane is a continuous double membrane system, which is differentiated into stacked grana and unstacked stroma lamellae regions (Fig. 1.1. B). This system is organised into a three dimensional network with an interior aqueous phase known as the lumen. There are four major protein complexes embedded in the thylakoid membranes which are important for light harvesting and electron transport functions, photosystem I and II (PSI and PSII), cytochrome *b6/f* complex (Cyt *b6/f*) and adenosine tri-phophosphate (ATP) synthase.

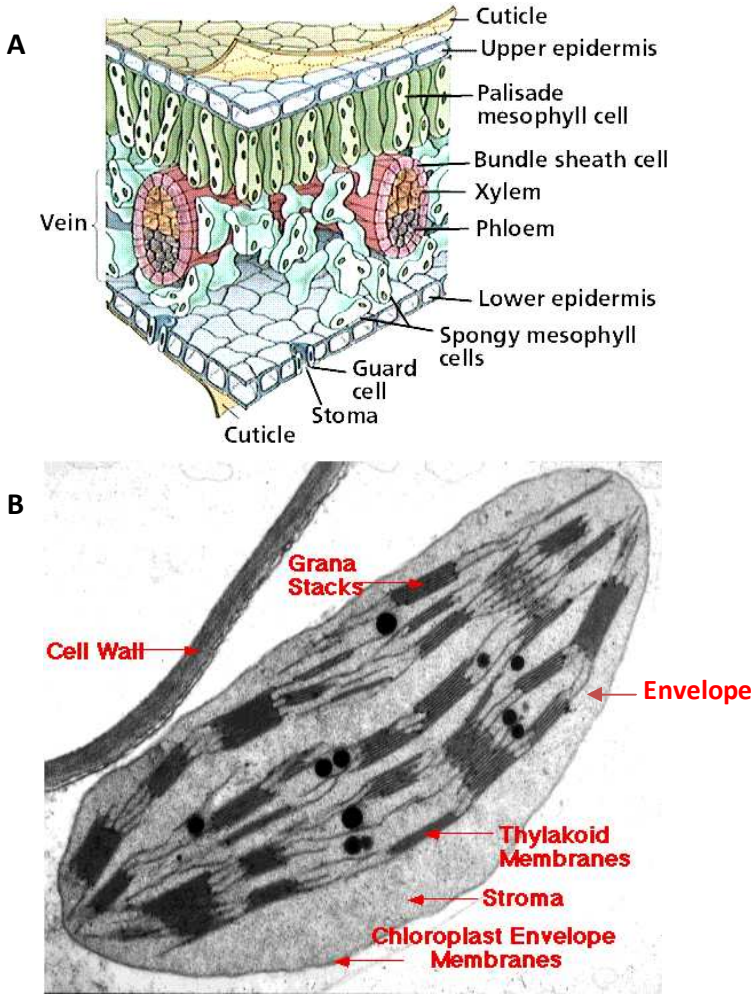


Figure 1.1 The higher plant leaf and chloroplast (A) Structure of the higher plant leaf (B) organisation of the thylakoid membranes within the chloroplast (Allen and Forsberg, 2001).

1.3 Two phases of photosynthesis

Photosynthesis comprises of two phases; light reactions and light-independent or dark reactions. The thylakoid membrane with its pigment-protein complexes is the site of light reactions while the dark reactions occur in stroma, which contains the enzymes responsible for carbon fixation. In the light reactions light energy is captured and used to oxidise water generating the energy-storage molecules ATP and reduced nicotinamide adenine dinucleotide (NADPH). During the dark reactions, the products of light reactions are used to fix and reduce carbon dioxide.

1.3.1 Overview of light reactions and photosynthetic electron transport

Plants harness light energy in the form of photons by the help of pigments, chlorophylls and carotenoids. A photosystem has two physiologically distinct components, light harvesting complex (LHC) and reaction centre (RC), the light energy harvested and funnelled by the former to the latter (Melis and Anderson, 1983). Photon absorption excites a chlorophyll or carotenoid molecule by raising an electron from a ground triplet state to an excited singlet state. This excitation energy brings about oxidation, an exit of electron or exciton from the pigment molecule. The RC utilises the excitation energy delivered by LHC to drive a charge separation reaction between a special chlorophyll molecule, called P680 in PSII and P700 in PSI, and an acceptor molecule. The electron generated during charge separation in PSII is passed along a series of acceptor molecules to reduce plastoquinone (PQ). The PQ is in turn oxidised by the Cyt b6/*f* resulting in a simultaneous translocation of protons across the thylakoid membrane into lumen. This translocation generates chemiosmotic potential to synthesise ATP. Cyt b6/*f* then passes the electron through a mobile electron carrier, plastocyanin (PC), to P700 special pair within the RC of PSI. A second charge separation event liberates an electron, this time from P700 special pair, which is passed along a series of carriers to the terminal electron acceptor ferredoxin (Fdx). Fdx is used by ferredoxin NADP reductase (FNR) to reduce $NADP^+$ to NADPH.

The deficiency of electron in the P680 special pair of PSII is completed by the photolysis of water, yielding protons and O_2 as by-products of the reaction. Translocation of protons across the thylakoid membrane occurs concomitantly with the transport of electrons from water to $NADP^+$. A pH gradient is formed by the release of protons into the thylakoid lumen by the photolysis of water in the oxygen-evolving complex of PSII, by the reduction of Cyt *b6f* complex during the electron transport and by the uptake of protons for the reduction of plastoquinone and $NADP^+$ (Fig. 1.2).

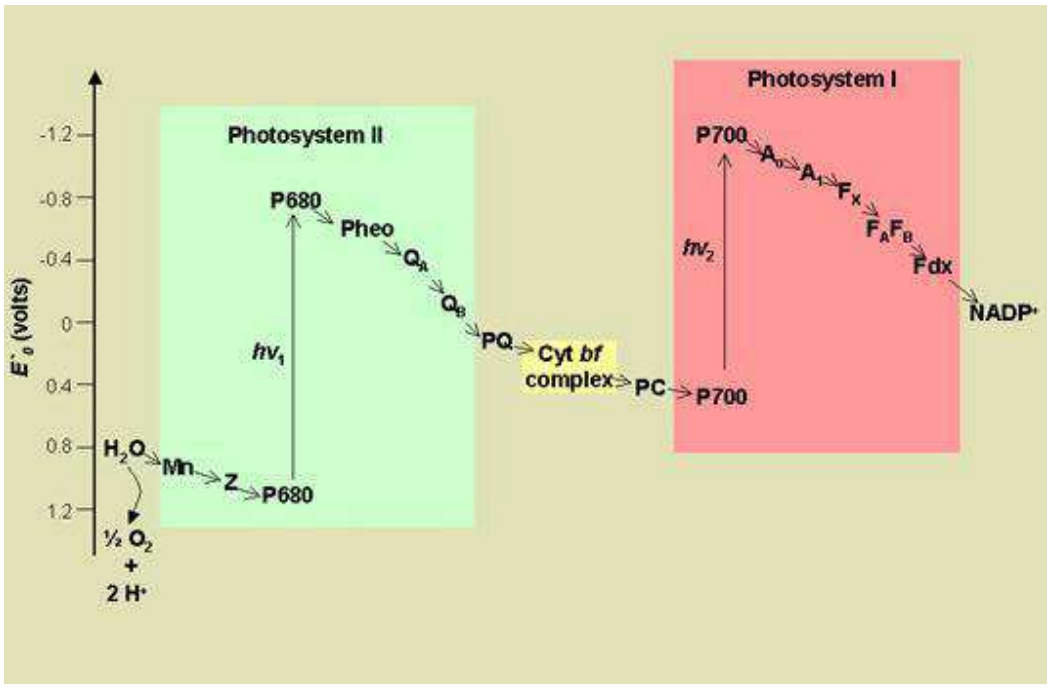
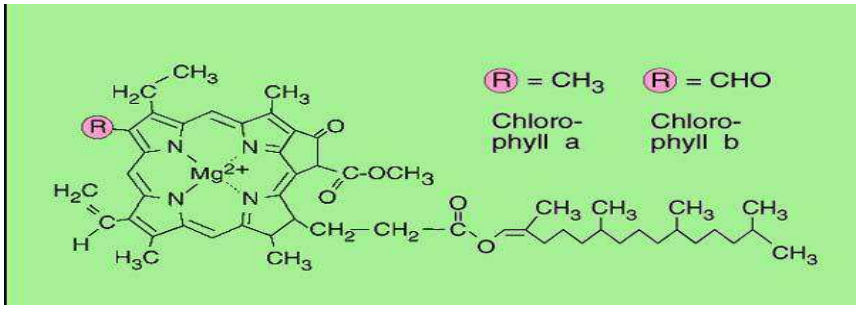


Figure 1.2 The Z-scheme shows linear electron transport from water to NADPH in the thylakoid membrane. Abbreviations: Mn, Manganese cluster; Z, tyrosine z; P680, PSII reaction centre special pair chlorophyll; P680*, excited singlet state of special pair (Primary donor); Pheo, Pheophytin (primary acceptor); QA, Plastoquinone A, QB Plastoquinone B, PQ Plastoquinone pool, Cyt b*f*, Cytochrome b6*f* complex; PC, Plastocyanin; P700, PSI reaction centre special pair chlorophyll; P700*, excited singlet state of special pair (Primary donor); A0, Primary acceptor chlorophyll; A1, secondary acceptor phylloquinone; Fx, iron sulphur cluster; FAFB, iron sulphur cluster; Fdx, ferredoxin (Berg *et al*., 2003).

1.4 Photosynthetic pigments: chlorophylls and carotenoids

The photosynthetic pigments of plants are responsible for capturing the light energy and converting it into the chemical energy. The main pigments are chlorophylls, which absorb in the blue and red visible regions of the electromagnetic spectrum. These are substituted tetra pyrroles, constituting a porphyrin ring system of alternating double and single bonds, also called conjugated double bonds, which plays important role in light capture. The magnesium atom in the centre of porphyrin ring carries the electrons involved in photosynthesis. Attached to the porphyrin ring is an ester of a long-chain phytol, which makes chlorophyll hydrophobic and aids the pigment binding within protein complexes of

A



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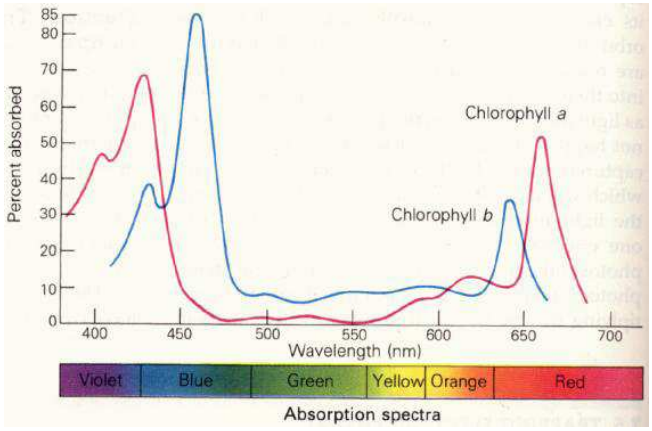


Figure 1.3 The light absorbing chlorophyll pigments. (A) Structure of chlorophyll *a* and chlorophyll *b* the primary photosynthetic light absorbing pigments of the chloroplast. (B) The room temperature absorption spectra of chlorophyll *a* (magenta) and chlorophyll *b* (blue). Berg *et al*., (2002).

the membrane (Fig. 1.3.A). The critical and essential photosynthetic pigment in all the plants is chlorophyll *a*, which also constitutes P680 and P700 special pairs of the two photosystems. There are also accessory pigments in plants, such as chlorophyll *b* and carotenoids, which increase the absorption cross-section by absorbing light energy in different areas of the visible spectrum and pass that on to chlorophyll *a*. Chlorophyll *b* differs from chlorophyll *a* by the presence of a formyl group (-CHO) attached to the porphyrin ring instead of a methyl group (-CH_3) found in the latter. The differences between the absorption spectra of these two chlorophylls (Fig. 1.3.B) are significant: the red-most absorption band in the absorption spectrum (also known as the Qy band) of chlorophyll *a* is located at ~662 nm, whereas the red most absorption band for chlorophyll *b* is located at ~642 nm; and the so-called Soret band (in the blue region of the spectrum)

for chlorophyll *a* is situated at ~430 nm and at 460 nm for chlorophyll *b*. All chlorophylls present in the thylakoid membrane are bound by proteins, which provide a specific local environment, resulting in red shifts of the absorption spectra, further increasing the absorption cross-section.

Carotenoids are yellow pigments which are also comprised of extended conjugated double bond systems and cyclic ring structures, most commonly C40. They are derived from isoprenoid precursors and are of two types, the carotenes (cyclic hydrocarbons) and xanthophylls (oxygenated derivatives of the carotenes). Much of the early work on the biosynthesis of carotenoids in higher plants was achieved through traditional biochemical approaches of purification and characterisation of the enzymes involved. More recently a new approach has been taken in which mutants lacking particular carotenoids have been isolated through screenings and subsequently molecular genetics is used to identify the mutated gene. The branching point of the carotenoid biosynthesis is defined by the action of the homodimeric lycopene β,β-cyclase complex which converts lycopene to β-carotene, or the heterodimeric lycopene β,ε-cyclase complex, which converts lycopene to α-carotene via δ-carotene (Cunningham *et al*., 1996; Cunningham, 2002).

The α-xanthophyll branch involves the hydroxylation of α-carotene by a two step process involving two cytochrome mono-oxygenases, the first one (CYP97a3) catalyses the hydroxylation of the β-ring to form zeinoxanthin, and then the second one (CYP97c1) catalyses the ε-ring hydroxylation to yield lutein, the most common xanthophyll in nature (Pogson *et al*., 1996, Tian *et al*., 2004, Kim and DellaPenna, 2006).

The β-xanthophyll branch involves hydroxylation of β-carotene primarily by the action of two ferredoxin dependent non-haem di-iron mono-oxygenase enzymes encoded by the *chyB1* and *chyB2* genes to form zeaxanthin (Bouvier *et al*., 1998, Tian *et al*., 2003). Zeaxanthin is then converted into violaxanthin, via the intermediate antheraxanthin, by the activity of zeaxanthin epoxidase enzyme which adds two epoxy groups across the β-ring double bonds (Yamamoto *et al*., 1962, Bouvier *et al*., 1996, Niyogi *et al*., 1998). The reverse reaction is also possible to convert violaxanthin back into zeaxanthin, via the intermediate antheraxanthin, by the activity of violaxanthin de-epoxidase which removes

two epoxy groups from both β-rings (Yamamoto *et al*., 1962, Avridsson *et al*., 1996, Niyogi *et al*., 1998). Together these two reactions constitute the xanthophyll cycle (Yamamoto *et al*., 1962). The final major xanthophyll that accumulates in thylakoids is neoxanthin which is formed by all-*trans*-violaxanthin. 9-*cis* neoxanthin is an isomer of violaxanthin and differs from the latter as its epoxy group with the conjugated double bond chain forms a hydroxyl group on the β-ring and an allene (= C =) group, this modification of violaxanthin has recently been ascribed as the function of neoxanthin synthase (Dall'Osto *et al*., 2007).

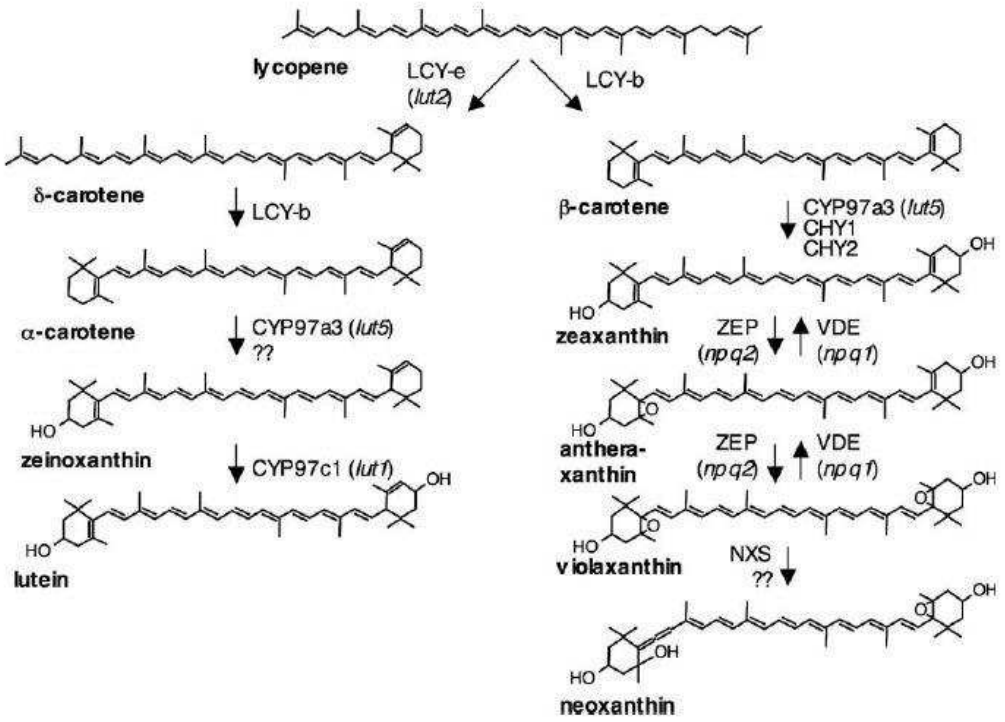


Figure 1.4 The carotenoid biosynthetic pathway in higher plants. Key enzymes are in bold, *Arabidopsis* mutants that led to their identification in italics: LCY-e= lycopene ε-cyclase (*lut2*), LCY-b= lycopene β-cyclase, Chy1/ Chy2 = β-carotene hydroxylases, ZEP= zeaxanthin epoxidase, VDE = violaxanthin de-epoxidase, NXS = neoxanthin synthase, CYP97a3 (*lut5*) = β-ring hydroxylase activity, CYP97c1 (*lut1*) = ε-ring hydroxylase activity (Fiore *et al*., 2006).

Carotenoids are involved in light harvesting, absorbing light of different wavelengths to that of chlorophyll, and passing the energy on to chlorophyll. A major function of carotenoids is in photoprotection, removing chlorophyll triplet states, quenching singlet oxygen species and dissipating chlorophyll singlet states under excess light conditions. The role of carotenoids in photoprotection will be discussed in detail later.

1.5 The pigment-protein complexes of the thylakoid membrane

There are four major pigment-protein complexes, embedded in the thylakoid membrane of the chloroplast, which are responsible for light reaction of photosynthesis. The structural and functional features of these complexes, PSII, PSI, Cyt *b6/f* and ATP Synthase, are discussed in this section:

1.5.1 Photosystem II

Photosystem II (PSII) is a multi-subunit protein-cofactor complex found abundantly in the stacked or granal regions of the thylakoid membrane. It has a water-plastoquinone oxido-reductase function (Hankamer *et al*., 1997; Barber, 1998). It can be separated into three main regions- the oxygen evolving complex, the reaction centre and the core antenna. The structure of the PSII core complex of the cyanobacterium, *Synechococcus elongatus*, has been resolved to a 2.5Å resolution (Zouni *et al*., 2001). Situated at the centre of PSII, the RC incorporates the chloroplast encoded D1 and D2 polypeptides (products of *psbA* and *psbD* genes, respectively), which form a heterodimer. The dimer contains 35 chlorophyll *a*, 11 β-carotene molecules and 14 lipid molecules (Loll *et al*., 2005). Each of the D1 and D2 subunits contains five membrane spanning helices with the n-termini on the stromal side of the membrane. The D1 and D2 subunits are homologues of the L and M subunits of the purple bacterial RC (Deisenhofer *et al*., 1985; Rhee *et al*., 1998). The cofactors involved in charge separation and electron transport bind to the RC's dimeric core, and include the primary electron donor P680 reaction centre chlorophyll (probably PD1), PD2, ChlD1, ChlD2, ChlZD1, ChlZD2 chlorophylls, two pheophytins (PheoD1 and PheoD2), the

secondary electron acceptors plastoquinones QA and QB (situated close to pheophytins), two β-carotenes and finally a non-haem iron molecule situated between the plastoquinones molecules. The RC also contains cytochrome b559 (situated at the periphery), which consists of two subunits (9kDa and 4kDa) encoded by the chloroplast *psbE* and *psbF* genes, respectively. These polypeptides ligate a single haem group. The function of cytochrome b559 is still not proved but it is considered to be important in the protection of the RC against photodamage (Whitmarsh and Pakrasi, 1996; Stewart and Brudvig, 1998).

Several other small subunits are present in the PSII dimer, the roles of most of them also being unclear. It is thought that PsbL, PsbM, and PsbT are involved in dimer formation and that PsbJ, PsbK, PsbN and PsbZ might facilitate the carotenoid binding since they are located close to β-carotene molecules (Ferreira *et al*., 2004). Cross-linking experiments (Tomo *et al*., 1993; Shi *et al*., 1999; Zouni *et al*., 2001) have located the low molecular weight PsbI and PsbX proteins close to the reaction centre D2 and cytochrome b559 proteins, perhaps having a role in stabilising the peripheral ChlZD1 and ChlZD2 chlorophylls (Ferreira *et al*., 2004).

The D1 and D2 proteins bind the core antenna proteins, CP43 and CP47, products of the *psbC* (CP43) and *psbB* (CP47) genes binding 14 and 16 chlorophyll *a* molecules, respectively. These pigments form two layers close to the stromal and lumenal sides of the membrane. This is consistent with the observation by Barry *et al*. (1994) that the majority of conserved histidines, known to coordinate the Mg atoms in the chlorophyll molecules, are located towards the stromal and lumenal parts of the proteins. CP43 and CP47 are also considered to bind β-carotene and lutein (Bassi, 1996), although structural data does not confirm the latter (Zouni *et al*., 2001; Kamiya and Shen, 2003). The 2.5 Å structure of CP47, from Zouni *et al*. (2001) shows six membrane spanning helices as predicted by the topology of both CP43 and CP47 (Rhee *et al*., 1998). CP47 and CP43 function not only in light harvesting, but also in transferring excitation energy from the peripheral antenna to the RC, via the ChlZD1 and ChlZD2 chlorophylls (Fereira *et al*., 2004).

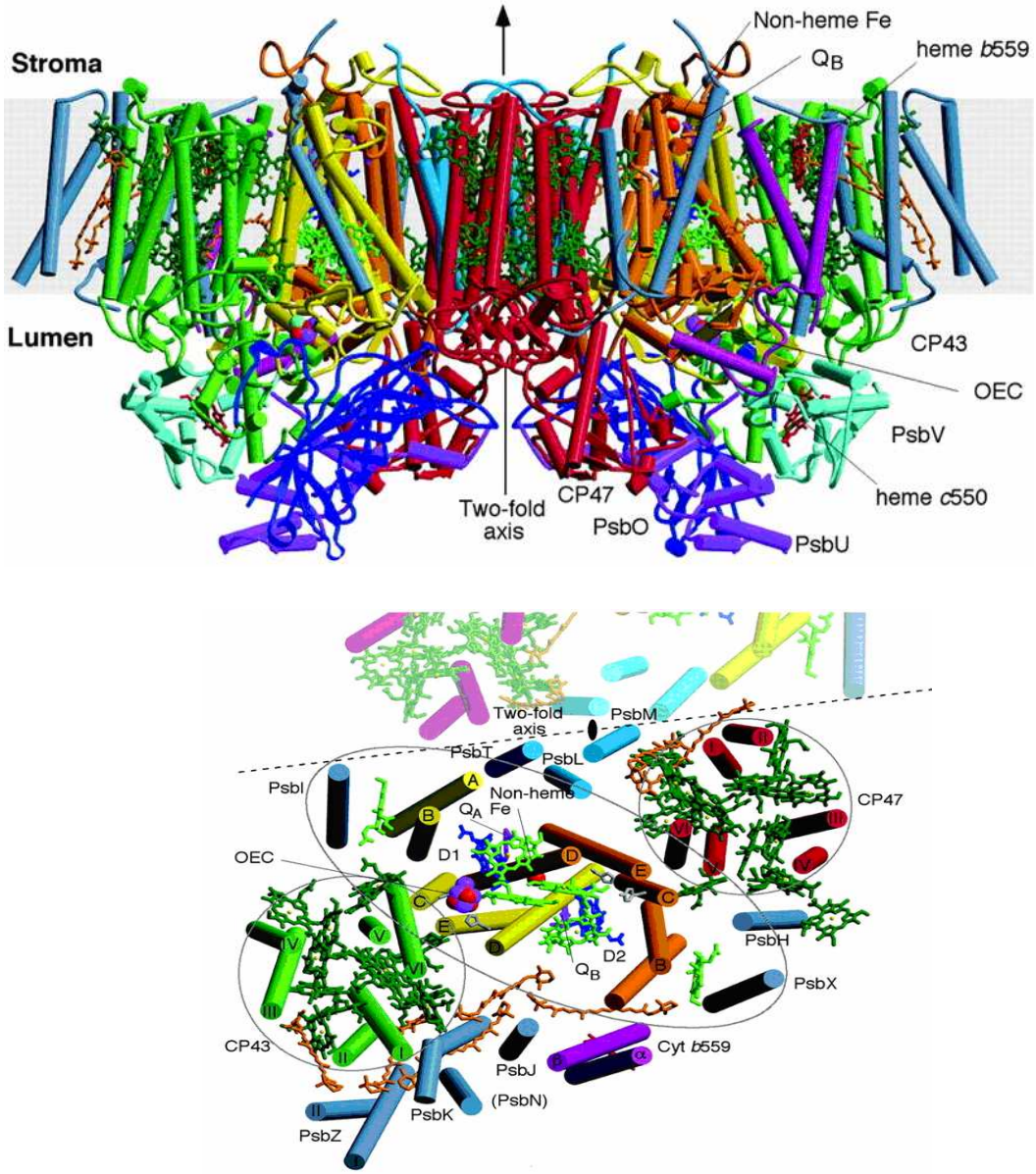


Figure 1.5 Structural organisation of the Photosystem II core complex. View perpendicular to the membrane plane, showing the minor lumenal exposed subunits (top). Helices are represented as cylinders with D1 in yellow; D2 in orange; CP47 in red; CP43 in green; cyt b559 in red; PsbL, PsbM, and PsbT in medium blue; and PsbH, PsbI, PsbJ, PsbK, PsbX, PsbZ, and PsbN in gray. The extrinsic proteins are PsbO in blue, PsbU in magenta, and PsbV in cyan. Chlorophylls of the D1/D2 reaction centre are light green, pheophytins are blue, chlorophylls of the antenna complexes are dark green, β-carotenes are in orange, hemes are in red, nonheme Fe is red, QA and QB are purple. The oxygen-evolving centre (OEC) is shown as the red (oxygen atoms), magenta (Mn ions), and cyan (Ca^{2+}) balls. View vertical from the membrane plane (bottom). Colouring is the same as for the top view (Ferreira *et al.*, 2004).

On the lumenal side of the PSII complex close to the D1 subunit, there are three extrinsic proteins, PsbO, PsbP and PsbQ (Zouni *et al*., 2001), which form a 'cap' over the OEC (De Las Rivas *et al*., 2004). The OEC splits water into molecular oxygen, electrons and protons. The extrinsic proteins play a structural role by keeping the peripheral antenna at an appropriate distance from OEC (Boekema *et al*., 2000b), but their main role is in oxygen evolution. The Mn and Ca ions seen in the cyanobacterial PSII structure form a cuban-like Mn_4CaO_3 cluster that acts like a catalyst in the water splitting (Ferreira *et al*., 2004). The PsbO subunit is critical for the stability of Mn cluster (Ono and Inoue, 1984) and has been suggested to form a hydrophilic "pore" connecting the OEC with the lumenal surface (Ferriera *et al*., 2004). PsbO attaches to the PSII core via the large extrinsic loops of CP43 and CP47 (Bricker and Frankel, 2002). The catalytic cycle of water oxidation involves five intermediate oxidation states, S_0 to S_4, each transition driven by a photon of light (Kok *et al*., 1970; Goussias, 2002). Each of the four incoming photons results in a charge separation event, thus removing four electrons from the Mn cluster in total, with simultaneous de-protonation of two water molecules which ultimately results in liberation of one O_2 for each completed cycle.

1.5.2 Photosystem I

Photosystem I (PSI) is a large pigment-protein complex, which comprises of a reaction centre core and a peripheral antenna. It is located in the unstacked stromal lamellae regions of the thylakoid membrane (Dekker and Boekema, 2005). It mediates the light driven electron transport from plastocyanin to ferredoxin, thus functions at the end of the photosynthetic electron transport chain as a plastocyanin-ferredoxin oxidoreductase (Scheller and Moller, 1990). Reduced ferredoxin is used in numerous regulatory cycles and reactions including nitrate assimilation, fatty acid desaturation and NADPH production. In turn NADPH is used with ATP to reduce CO_2 to carbohydrates in the Calvin Cycle. This complex is composed of a core complex and a peripheral antenna system. The 3-Dimensional crystal structure of PSI from pea, *Pisum sativum*, has been resolved to 4.4 Å showing 12 core subunits and 4 different light harvesting antenna complexes (LHCI),

which are assembled together in a half-moon shape on one side of the core. The complex binds 167 chlorophylls, 3 iron-sulphur clusters and 2 phylloquinones, and comprises of 45 transmembrane helices (Ben-Shem *et al*., 2003). The core complex is made up of 14 subunits and binds about 100 chlorophyll *a* and 22 β-carotene molecules (Jordan *et al*., 2001). The structure of PSI is shown in Figure 1.6.

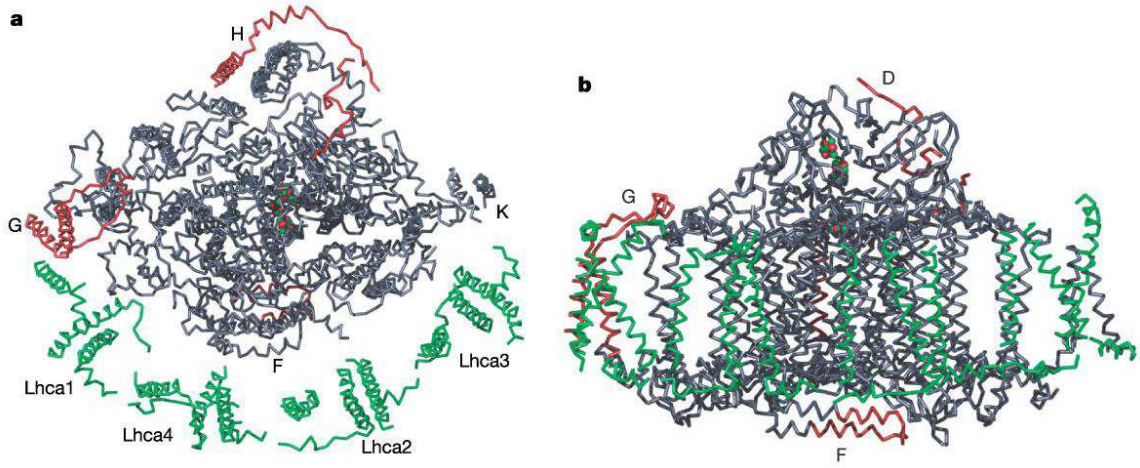


Figure 1.6 Structural model of the plant photosystem I. View from the stromal side of the thylakoid membrane (a) and view from the LHCI side (b). Lhca antenna proteins are shown in green, PSI core proteins (PsaA and PsaB) in grey and the minor subunits in red (Ben-Shem *et al*., 2003).

The large subunits PsaA and PsaB form the catalytic core and bind the majority of the chlorophylls, including the P700 special pair, which forms the primary electron donor. Subunits PsaA and PsaB each contain 11 transmembrane α-helices and share similarities in protein sequence and structure. The structural orientations of the carboxy-terminal regions of PsaA and PsaB have been found to be similar to that of the D1/D2 heterodimer in PSII (Hankamer *et al*., 1999). Six helices from each of the core polypeptides were also found to be arranged in a similar manner to CP43 and CP47 in PSII leading to suggestions that both photosystems may share common evolutionary origin (Hankamer *et al*., 1999). Light absorption causes charge separation in reaction centre, where the P700 transfers an electron to the electron acceptor A0 (a chlorophyll *a* molecule). The electron then passes to A1 (a

phylloquinone) and then through 3 iron-sulphur centres (FX, FA and FB), to ferredoxin on the stromal side of the membrane (Jordan *et al.*, 2001). The electron reduces ferredoxin, which binds in a pocket on the stromal side of PsaA and is surrounded by PsaC, PsaD and PsaE. Oxidised $P700^+$ is reduced by an electron from plastocyanin on the lumenal side of PSI.

1.5.3 Cytochrome *b6/f* complex

The Cytochrome *b6/f* complex (Cyt *b6/f*) links the two photosystems, PSII and PSI, by a linear electron flow. It is located in both granal and stromal regions of the membrane (Dekker and Boekema, 2005). It functions as a plastoquinol-plastocyanin oxidoreductase, simultaneously translocating protons into the lumen (for review, see Cape *et al*., 2006). The complex is dimeric, consisting of 4 large (cytochrome *b6*, cytochrome *f*, Rieske iron-sulfur, and subunit IV) and 4 small (PetG, PetM, PetL and PetN) polypeptide subunits (Widger *et al*., 1984; Kurisu *et al*., 2003). Each of the Cyt *b6/f* monomers is made up of 13 transmembrane helices and binds four haem molecules (Kurisu *et al*., 2003), one chlorophyll *a* (Pierre *et al*., 1997) and one β-carotene (Zhang *et al*., 1999). The ferredoxin NADP reductase (FNR) binds to the Cyt *b6/f* complex, thus enabling the cyclic electron transport around PSI (Joliot *et al*., 2004).

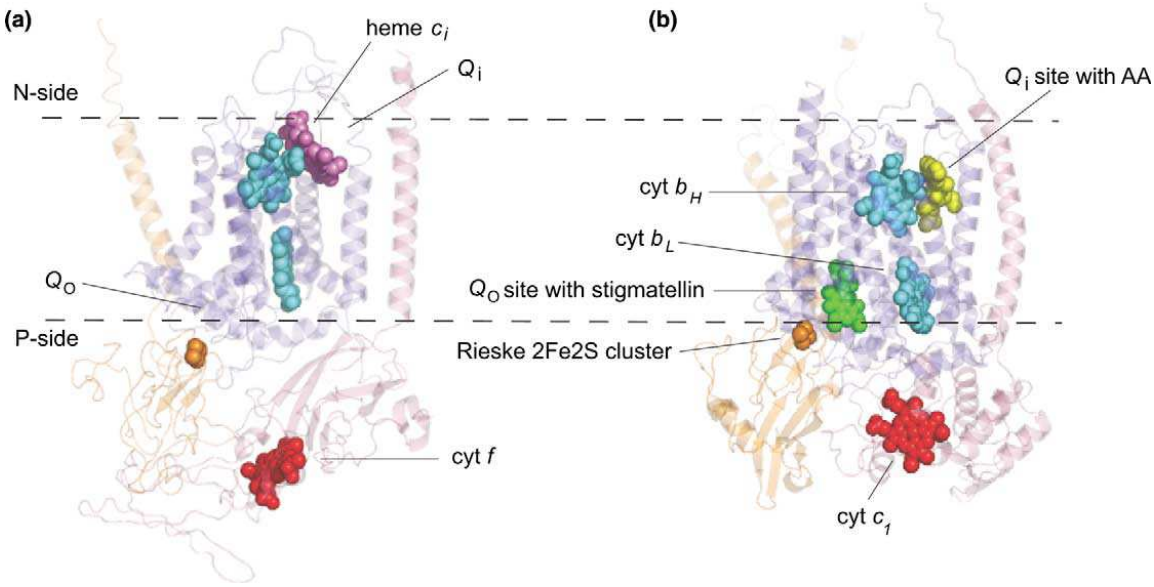


Figure 1.7 Structure of cytochrome *b6/f* (a) and cyt bc_1 (b) from Cape *et al*., (2006). Colour scheme for protein subunits: the Rieske ISP (orange); Cyt *b* (with subunit IV in the b6*f* structure) (slate); Cyt *f* (red).

1.5.4 ATP-synthase complex

The ATP-synthase complex is a large macromolecular multisubunit enzyme of approximately 600 kDa. It is responsible for the generation of ATP by phosphorylation, utilising the chemiosmotic proton gradient created by electron transport. ATP is formed from adenosine diphosphate (ADP) and inorganic phosphate (Pi). The complex is composed of nine polypeptides organised in the CF_1 and CF_o main subunits (for review, see Groth and Pohl, 2001). The CF_1 subunit is found on the stromal surface of the thylakoid membrane, where it is associated with the CF_o intrinsic membrane subunit. CF_1 is water soluble while CF_o is hydrophobic (Mc Carty *et al*., 2000). CF_1 is the large component which contains the active site and is composed of 5 different polypetides in a stoichiometry of $\alpha 3\beta 3\gamma\delta\varepsilon$ (Abrahams *et al*., 1994; Groth and Pohl, 2001). CF_o is made up of 4 subunits (IIV) and its function is to use a proton driving force to rotate the γ subunit of CF_1 (Junge *et al*., 1997), a process that is a key part of the ATP synthase activity (Noji *et al*., 1997). Muller *et al*. (2000) found by atomic force microscopy that the four subunits are present in a 1:1:14:1 stoichiometry.

1.6 The peripheral light harvesting antenna complexes

Both the photosystems, PSII and PSI, are equipped with their own additional membrane-bound peripheral light harvesting antenna complexes. These antennae are meant for efficient light collection by increasing the absorption cross-section by many folds and then delivering the excitation energy to their photosystems. The peripheral antenna is a chlorophyll *a*/*b* binding protein complex which is of two types with reference to its respective photosystem, light harvesting complex I (LHCI) delivers excitation energy to PSI while light harvesting complex II (LHCII) is responsible for delivering excitation energy to PSII. The proteins of both the complexes are encoded by the Lhc super-gene family (Jansson, 1994) The 10 most commonly expressed Lhc genes encode polypeptides with molecular weights between 20-30 kDa, and the high degree of sequence homology among them indicates a common evolution (Green and Pichersky, 1994). All the Lhc polypeptides share significant sequence homology and significant conservation amongst chlorophyll binding residues with a predicted structure comprising 3 transmembrane helices (Green *et al*., 1991). These polypeptides have also tendency to replace one another, which is an evidence of robust molecular design of these antenna complexes and hence their functional significance (Ruban *et al.,* 2003).

1.6.1 LHCI

Four light-harvesting proteins (Lhca1-4) with protein masses of around 25 kDa form LHCI. These are arranged to form two heterodimers (LHCI-730 and LHCI-680) that bind asymmetrically to the RC, via associations between a number of different subunits. The crystal structure of 4.4 Å resolution has revealed 56 chlorophylls bound by this complex (Ben-Shem *et al*., 2003). Biochemical work has also proposed that each individual Lhca protein binds 12-14 chlorophyll *a* and *b* molecules in total, along with 2-3 lutein, 1-1.5 violaxanthin and 1-1.5 β-carotene molecules (Klimmek *et al*., 2005). In addition to the chlorophylls bound directly by Lhca polypeptides, 10 chlorophylls are considered to be localised between the PSI core and LHCI proteins. These ‘gap’ chlorophylls are suggested to optimize energy transfer from LHCI to the core and also to stabilise the structural

interaction between core and antenna. The chlorophyll *a/b* ratio has also been found as higher in PSI-LHCI complexes, which is around 10 (Morosinotto *et al*., 2005) as compared to PSII particles with this ratio as 2 (Ruban *et at*., 1999). The red shifted fluorescence emission spectrum of PSI is caused by a few so-called 'red' chlorophylls of antenna, which have an energy level lower than the RC. These 'red' chlorophylls are able to transfer excitation energy 'up-hill' to the reaction centre although this does not affect the efficiency of energy trapping by the core (Gobets *et al*., 2001).

The LHCI-730 dimer (named after the 77K fluorescence emission maximum) is formed by association of the Lhca1 and Lhca4 polypeptides, while Lhca2 and Lhca3 form the LHCI-680 dimer (Klimmek *et al*., 2005). A recent study has suggested LHCI structure as rigid, as the docking sites for the individual polypeptides are highly specific, except for Lhca4 being replaced by Lhca5 (Wientjes *et al*., 2009). The antenna serves to capture light and funnel it to the PSI core. The amount of LHCI bound to the PSI core has been demonstrated to fluctuate with various light conditions (Bailey *et al*., 2001), however another study has suggested that the LHCI polypeptide content is not affected by environmental conditions (Ballottari *et al*., 2007). PSI is also the binding site for phosphorylated LHCII following the state 1 to state 2 transitions (Lunde *et al*., 2000).

1.6.2 LHCII

The LHCII polypeptides bind chlorophyll *a*, chlorophyll *b* and xanthophylls (Melis and Andersson, 1983), and these are found to be intrinsic membrane proteins. LHCII is made up of four distinct complexes: the major trimeric LHCIIb complex and the three minor monomeric CP24, CP26 and CP29 complexes. LHCII complexes were conclusively resolved and identified using native deriphat-PAGE (Peter and Thornber, 1991). Trimeric LHCIIb was found to be comprised of various combinations of three very similar polypeptides; Lhcb1 (28 kDa), Lhcb2 (27 kDa) and Lhcb3 (25 kDa). The monomeric minor antenna complex polypeptides are Lhcb4 (29 kDa), Lhcb5 (26 kDa) and Lhcb6 (24 kDa), which are also called as CP29, CP26 and CP24 respectively (Peter and Thornber 1991; Jansson, 1994). It is considered that the atomic structures of Lhcb4-6 will be similar to that

of a monomer of LHCIIb, as there has been found a high degree of sequence homology among the six Lhcb proteins (Jansson, 1994; Green and Kuhlbrandt, 1995). These polypeptides have tendency to substitute each other, as shown by the replacement of two main components by another protein to attain the normal assembly and function, this is an evidence of robust design of antenna and hence its importance in highly efficient photosynthesis (Ruban *et al.*, 2003). All these complexes increase the capacity of PSII for light capture, the minor antenna forming a link between the PSII core and the LHCII outer antenna. This function of the light-harvesting antenna is subject to regulation by a number of processes including state transitions and nonphotochemical quenching, both discussed in more detail below.

1.6.2.1 The major LHCII antenna (LHCIIb)

Early biochemical work has shown that trimeric LHCIIb (often called as LHCII) has a molecular weight of 72 kDa and a chlorophyll *a*/*b* ratio of 1.33 and that each monomer binds 3.5-4 xanthophylls in total, this includes 2 molecules of lutein, 1 of neoxanthin and 0.5-1 of violaxanthin (Peter and Thornber, 1991; Ruban *et al.*, 1999). The Lhcb1, Lhcb2 and Lhcb3 polypeptides usually occur in a ratio of about 8:3:1 and join to form trimers which are not unique in composition (Jansson, 1994; Jansson, 1999). Among these three polypeptides, only Lhcb1 can form homotrimers and it is also present in all heterotrimeric forms such as Lhcb1(2)/ Lhcb2, Lhcb1(2)/ Lhcb3 and Lhcb1/Lhcb2/Lhcb3 (Jackowski *et al.*, 2001). The first structural information about the monomeric subunit suggested 3 transmembrane helices to constitute each subunit (Burgi *et al.*, 1987), which was later confirmed by the structural model of trimeric LHCII resolved at 6 Å by electron crystallography of 2-Dimensional crystals, however there was insufficient evidence for the precise location of the chlorophyll molecules (Kuhlbrandt and Wang, 1991). The structure was further refined by the same method (Kuhlbrandt *et al.*, 1994) to a 3.4 Å resolution, this time elaborating the interaction between two of the three membrane-spanning α-helices. An additional short helix was also located at the interface between the membrane and the lumen. Pigments, 12 chlorophylls and 2 xanthophylls, were also located in the centre of the

complex. The positions for chlorophyll *a* and chlorophyll *b* were not distinguished in this model but only designated by considering their energy transfer efficiencies. Later, the 3-Dimensional structures of LHCII from spinach at 2.72 Å resolution (Liu *et al*., 2004), and from pea at 2.5 Å (Standfuss *et al*., 2005) provided a detailed account of protein and pigment orientation. The spinach structure confirmed the binding of 14 chlorophyll molecules and 4 xanthophylls by each monomeric LHCII, which was in conformity with earlier biochemical results suggesting 13-15 chlorophyll *a* and chlorophyll *b* molecules (Peter and Thornber, 1991) and 3-4 xanthophylls (Ruban *et al*., 1999). The structure is shown in Figure 1.8.

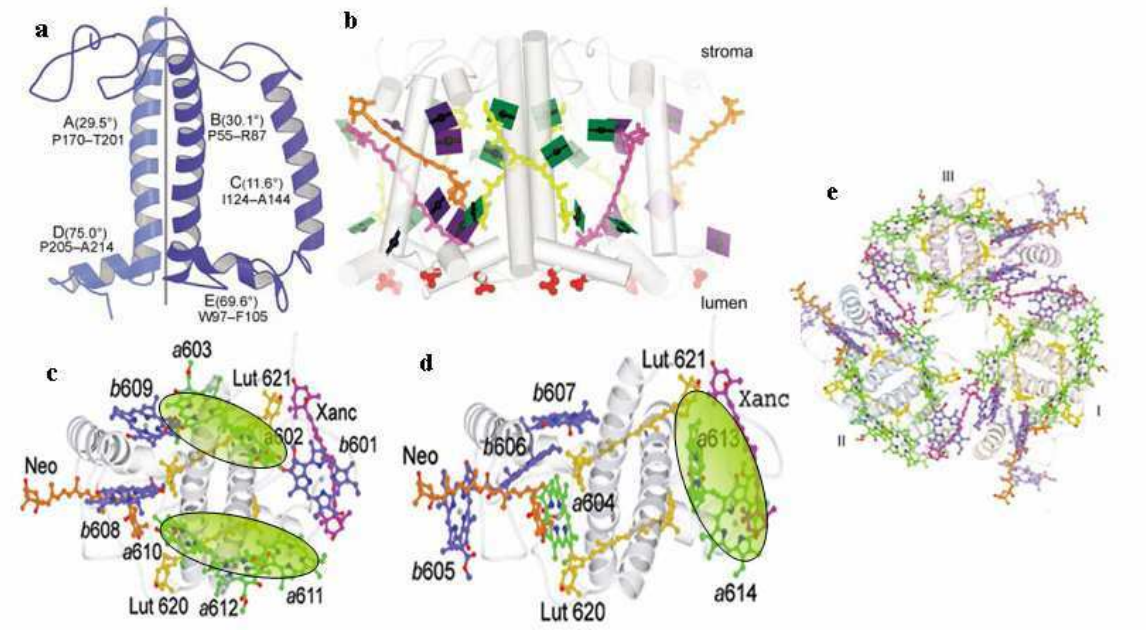


Figure 1.8 Structural model of the LHCII obtained by X-ray crystallography (a). Side view of monomer showing the pigments: lutein (yellow), neoxanthin (orange), violaxanthin (purple), chlorophyll a (green), chlorophyll b (blue) (b). Pigment pattern in an LHCII monomer at the stromal and lumenal sides, respectively, displaying the strongly coupled chlorophyll clusters shown by green ovals (a610-a611-a612, a602-a603 and a613-a614) (c,d). Top view of LHCII trimer (e),(Liu *et al*., 2004).

All the 14 chlorophyll molecules of monomer were identified as 8 chlorophyll *a* and 6 chlorophyll *b*. The 4 xanthophylls were designated as 2 all-*trans* luteins in the form of a

cross-brace, 1 molecule of 9-*cis* neoxanthin localised in a highly selective binding site and 1 all-*trans* violaxanthin at the monomer-monomer interface. The chlorophylls were located at specific binding sites for either chlorophyll *a* or chlorophyll *b* molecules, with no mixed binding sites available for both (Liu *et al*., 2004). The chlorophylls formed two layers within the membrane, one layer of 8 chlorophylls in the proximity of stroma and the other one of 6 chlorophylls near the lumen. In the trimer, chlorophylls form two rings on the stromal side, the inner one considered to be responsible for energy transfer between monomers (Gradinaru *et al*., 1998) while the outer one thought to broaden absorption of light energy and to transfer energy to the RC (Liu *et al*., 2004).

The chlorophylls on the lumenal side were suggested to function upstream of the stromal chlorophylls (Liu *et al*., 2004). Three clusters of strongly coupled chlorophyll *a* molecules were found, to which energy could be transferred rapidly from chlorophyll *b* (Figure 1.8.c.d): the *a*610-*a*611-*a*612 trimer and the *a*602-*a*603 and *a*613-*a*614 dimers (Novoderezhkin *et al*., 2005). The *a*610-*a*611-*a*612 cluster facilitated a good connection with other PSII subunits as it was found at the periphery of the LHCII trimer. This peripheral chlorophylls' cluster, together with the adjacent lutein (lut 620) constituted the terminal emitter domain, which was proposed as the possible energy quenching site in LHCII (Wentworth *et al*., 2003; Pascal *et al*., 2005).

This structural model not only confirmed the presence of the three transmembrane helices (A-C), and the α-helix along the lateral plane of the membrane (D), but also revealed a new, short amphipathic helix (E) inclined to the membrane plane by 30^{o}. In the native membrane, LHCII is arranged as a trimer (Figure 1.8.e), and the trimerisation region was found to cover both the N-terminal and C-terminal domains, the stromal end of helix B and some residues of helix C. Trimer stability in the crystal structure seemed to be dependent on the presence of a phosphatidylglycerol (PG) molecule (Liu *et al*., 2004; Standfuss *et al*., 2005), confirming the earlier reports (Remy *et al*., 1982; Nussberger *et al*., 1993) which suggested role of this lipid as significant for the stability of trimer, while monomerisation of this trimer could be achieved by hydrolysis of PG with phospholipase A2 (Remy *et al*.,

1982). Another lipid digalactosyl diacylglycerol (DGDG) was found to join the adjacent trimers through van der Waals forces (Nussberger *et al*., 1993; Liu *et al*., 2004).

The high resolution crystal structure also confirmed the biochemical results for xanthophyll binding. Earlier biochemical studies proposed four xanthophyll binding sites for LHCII, two internal ones for lutein (L1 and L2) bound tightly within the complex, a neoxanthin binding site (N1) and one peripheral violaxanthin binding site (V1) bound only loosely (Ruban *et al*., 1999). Lutein had been found to perform various structural and functional roles like *in vitro* correct folding and stability of LHCII protein structure (Plumley and Schmidt, 1987; Croce *et al*., 1999; Phillip *et al*., 2002) and non-photochemical quenching (Lokstein *et al*., 2002). Contrarily the neoxanthin molecule had not been found essential for protein folding (Croce *et al*., 1999), although a photoprotective role for it was suggested in protection of the chlorophyll against photodamage (Standfuss *et al*., 2005). The neoxanthin was located in a chlorophyll *b* rich region close to helix C, a site which was also previously designated to be selective for it. Thus, both lutein and neoxanthin were considered to be functional in light harvesting and photoprotection (Kuhlbrandt *et al*., 1994). The loosely bound violaxanthin was considered to be significant in non-photochemical quenching (Demmig-Adams, 1990) through the xanthophyll cycle but its light harvesting role was not clear.

1.6.2.2 CP29 (Lhcb4)

CP29 is the largest of the minor antenna complexes, containing about 257 amino acids, with a molecular weight of 29 kDa (Peter and Thornber, 1991; Bassi, 1996). This complex is always found as a monomer and is the product of the Lhcb4 genes (Jansson, 1994). There are three Lhcb4 genes in *Arabidopsis*, two (Lhcb4.1 and Lhcb4.2) with similar expression levels while the third (Lhcb4.3) with lower expression level (Jansson, 1999). CP29 has been found to bind 8 chlorophyll molecules in total, with 6 chlorophyll *a* and 2 chlorophyll *b* (Sandona *et al*., 1998; Bassi *et al*., 1999). Isolation by native deriphat PAGE found approximately 3 xanthophylls: one lutein molecule, 0.77 neoxanthin and 1.54 violaxanthin (Peter and Thornber, 1991). However, this ratio of carotenoid binding was disputed, as *in*

vitro pigment reconstitution of recombinant CP29 produced in *Escherichia coli* showed that only 1 lutein and 1 violaxanthin could bind this complex (Bassi *et al*., 1999). The work using isoelectric focussing by Ruban *et al*. (1999) showed that there were two types of violaxanthin that are differentially bound to the complexes, one tightly bound which cannot be de-epoxidised, and the other loosely bound available for de-epoxidation. CP29 has been proposed to be functional in light harvesting along with a regulatory role. Its capacity to bind dicyclohexylcarbodiimide (DCCD) indicates that this protein can be protonated and for this a putative protonation site has been located on the lumenal loop (Pesaresi *et al*., 1997). These results together with the evidence of the relative abundance of violaxanthin in CP29 as compared to the LHCII suggest a significant role for the former in non-photochemical quenching. Moreover, under certain photoinhibitory conditions, this complex can also be phosphorylated in the N-terminal domain (Bergantino *et al*., 1995).

However, antisense inhibition studies do not support an essential role of CP29 in photoprotection and suggest a more likely function in the coordination of the major antenna (Andersson *et al*., 2001). Evidence for this hypothesis has been provided by the observation that in absence of CP29, no intact PSII-LHCII supercomplexes could be prepared from the thylakoid membrane proposing a crucial stabilizing role for CP29 (Yakushevska *et al*., 2003).

1.6.2.3 CP26 (Lhcb5)

This minor antenna protein is about 247 amino acids in length with a molecular weight of 26 kDa protein, which binds pigments to form the CP26 complex (Peter and Thornber, 1991, Bassi, 1996). It binds 9 chlorophyll molecules, including 6 chlorophyll *a* and 3 chlorophyll *b*, with three chlorophyll *b* specific binding sites (Croce *et al*., 2002). Regarding xanthophylls, CP26 has been found to bind 1 neoxanthin, 2 luteins and 0.5 violaxanthin. Ruban *et al*. (1999) reported 7-8 molecules of chlorophyll *a* and 3 of chlorophyll *b* along with a molecule each of the three xanthophylls lutein, neoxanthin and violaxanthin, a scheme later confirmed by the data of Wehner *et al*. (2006). However, these results contrast with the findings of reconstitution work carried out by Sandona *et al*.

(1998), which suggest existence of only 2 binding sites L1 and L2 in CP26, occupied by lutein and by violaxanthin, respectively. On the basis of this, a key role has been suggested for violaxanthin at L2 in non-photochemical quenching (NPQ) (Dall'Osto *et al*., 2005). CP26 shares the most sequence homology with Lhcb1 among the three minor complexes (Jansson *et al*., 1999), and consequently it has been found to join with Lhcb3 to form trimers substituting for LHCII trimers in antisense plants lacking both Lhcb1 and Lhcb2, which is also indicative of functional robustness of the light harvesting antenna (Ruban *et al*., 2003).

1.6.2.4 CP24 (Lhcb6)

CP26 is the smallest of the Lhcb proteins which is about 210 amino acids long, with a molecular mass of 24 kDa (Morishige *et al*., 1990). This minor complex is considered to bind 5 each of chlorophyll *a* and chlorophyll *b* molecules (Peter and Thornber, 1991; Pagano *et al*., 1998), thus possessing the lowest chlorophyll *a*/*b* ratio among all the antenna complexes. It has also been found to bind only two xanthophylls, lutein and violaxanthin, hence possibly no neoxanthin (Bassi *et al*., 1993, Ruban *et al*., 1999). Reconstitution work has also suggested in conformity the absence of neoxanthin in this complex, with lutein and violaxanthin occupying L1 and L2 sites respectively (Sandona *et al*., 1998; Wehner *et al*., 2006). CP24 has also been found to play regulatory and structural roles similar to the case for CP29. In the plants with an antisense Lhcb6 gene and knock-out Lhcb6 mutants, absence of CP24 complex resulted in partial inhibition of NPQ and disruption of the macroorganisation of the PSII-LHCII supercomplexes (Kovacs *et al*., 2006).

1.6.2.5 Other LHC-related proteins

Other than the light-harvesting complexes of the two photosystems, there are also many 'LHC-like' proteins, sharing some sequence homology with LHC proteins (Grimm *et al*., 1989; Jansson *et al*., 2000). These include the one-helix proteins (OHP), the two-helix stress enhanced proteins (SEP), the three-helix early light induced proteins (ELIP) and the

four-helix PsbS protein. ELIPs appear to bind lutein and chlorophyll *a* and these stress-induced proteins are are produced under high light conditions (Adamska *et al*., 1999) along with SEPs and HLIPs (high light inducible proteins) (Heddad and Adamska, 2000). The light harvesting antenna complexes of photosynthetic organisms are considered to be evolved from one-helix proteins which first underwent gene duplications forming a four-helix complex (PsbS-like) and then by the loss of one helix present three three-helix proteins were evolved (Green and Pichersky, 1994; Montane and Kloppstech, 2000).

1.6.2.6 PsbS

The PsbS protein has been found to be essential for the rapidly reversible component of NPQ (Li *et al*., 2000). This membrane protein is widely distributed among plants, both angiosperms and gymnosperms (Schultes and Peterson, 2007), moss (*Phycomitrella patens*) and in two green algae (*Chlamydomonas reinhardtii* and *Volvox carteri*) (Anwaruzzaman *et al*., 2004). The precursor polypeptide is encoded by nuclear *psbS* gene and comprises of 274 amino acids. This hydrophobic protein has four transmembrane helices (Figure 1.9).

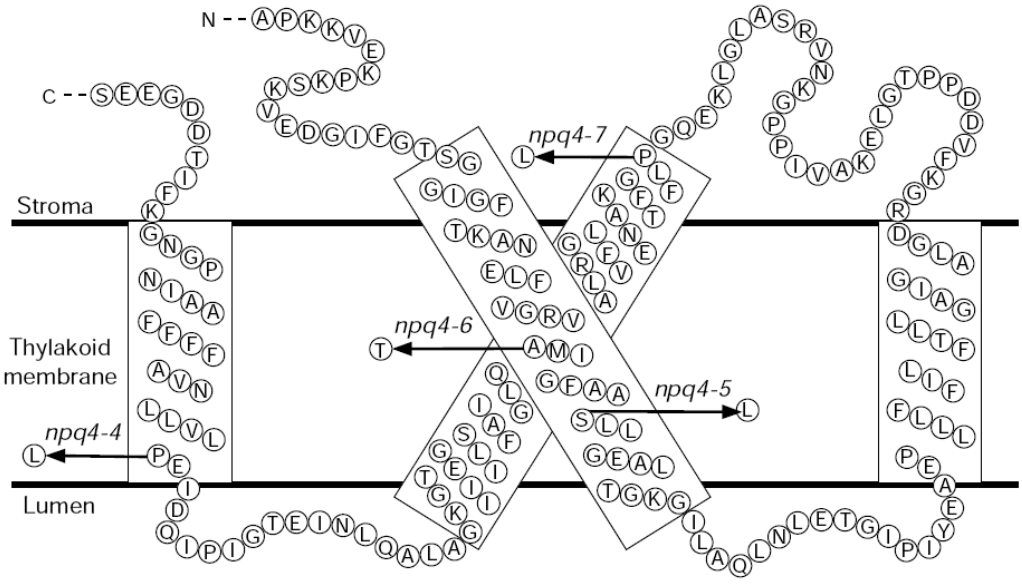


Figure 1.9 Schematic representation of the PsbS protein. The positions of single amino acid mutations are indicated (Li *et al*., 2000).

Despite its similarity with other pigment-binding proteins, PsbS has been shown to lack potential chlorophyll binding histidines and asparagines residues (Green and Pichersky, 1994), however certain dual motifs have also been located in it similar to those associated with chlorophyll binding sites of LHCII (Schultes and Peterson, 2007). Various conflicting reports of pigment-binding properties of this protein makes it controversial, however it has been shown as stable in the absence of pigments (Funk *et al*., 1995) and has also been found to loosely and transiently bind zeaxanthin *in vitro* (Aspinall O'Dea *et al*., 2002), by means of glutamate residues. However, in another study the binding of zeaxanthin to PsbS has been doubted (Bonente *et al*., 2008). The exact location of this protein in the thylakoid membranes has also been found as elusive, as it has been found associated with multiple locations (Teardo *et al*., 2007) in the grana as well as stroma lamellae. The distribution of PsbS seems to be highly dependent on the environmental conditions. It has also been shown that the PsbS protein can exist in dimeric forms depending on the light and pH (Bergantino *et al*., 2003), thereby monomers associate with LHCII and dimers with the PSII core. Forward genetics studies helped to identify the significant role of PsbS in photoprotection (Li *et al*., 2000), which will be discussed later in section 1.10.4.

1.7 Macromolecular organisation of the photosystem II

The first description of macromolecular structure of PSII from barley came from Peter and Thornber (1991) who employed the deriphat-PAGE native gels to find two copies each of CP26 and CP29 and an LHCIIb trimer for each dimeric PSII core. The similar isolation approach was used to produce an oligomeric LHCIIb complex of approximately 250kDa molecular mass which was found more heavily phosphorylated than the PSII associated LHCIIb (Peter and Thornber 1991). The evidence of oligomeric LHCII association and the fact that the minor complexes CP26 and CP29 remained attached to the core complex despite LHCIIb dissociation led the way to the proposal of a model describing the PSII organisation (Peter and Thornber, 1991). This model depicted association of up to 4 peripheral LHCIIb trimers on both sides of a central dimeric PSII core complex, with the help of 'linker' minor complexes CP24, CP26 and CP29. These findings were further

supported by 2D-electrophoresis of detergent solubilised PSII particles and cross-linking studies which also confirmed existence of PSII as a dimer core connected with the LHCIIb trimers on the periphery of the complex, by the help of minor antenna CP29, CP24 and CP26 (Bassi and Dainese, 1992; Jansson, 1994).

Further details of the macro-organisation of PSII and its associated light-harvesting antenna were revealed by the techniques of electron microscopy (EM) and single particle analysis of mildly solubilised PSII-enriched particles from spinach (Boekema *et al*., 1995). The PSII supercomplex was observed in the shape of a rectangle, which was proposed to be a dimer formed from two PSII cores (C) and two strongly bound LHCII trimers (S), along with two copies each of CP29 and CP26 monomers. This unit supercomplex was referred to a C2S2. However, there was no information about the Lhcb3 containing trimer and the Lhcb6 gene product. More gentle approach was employed in a later study on partially solubilised membranes to isolate the PSII particles by gel filtration chromatography. This time all the three minor complexes were located and an extra pair of moderately bound LHCII trimers (M) symmetrically associated within the supercomplex was also found. This new supercomplex was called C2S2M2 complex.

Further detailed analysis revealed binding of a third type of loosely bound LHCII trimers (L) (Boekema *et al*., 1999) (Figure 1.10). However, no supercomplex with six LHCII trimers bound (C2S2M2L2) has been observed, and even it has been found as case of extremely low frequency to observe a supercomplex with five trimers (C2S2M2L) attached to it. The *in situ* particle analysis on grana membranes gently solubilised by using the mild detergent n-dodecyl-α,D-maltoside showed that mainly the C2S2M supercomplexes form the basic motifs of the large semi-crystalline membrane domains in spinach. By analyzing the pairs of membranes with large-spaced crystalline macrodomains, it was shown that PSII complexes in one layer face the LHCII complexes in opposite layer (Boekema *et al*., 2000a), this suggests that the organisation of the supercomplex membranes is arranged in a way to enhance transfer of energy between the layers (Dekker and Boekema, 2005). An assembly of 7 LHCII trimers to form an oligomer has also been shown to represent the

native structure in the LHCII-only domains of grana at the peripheral margins (Dekker *et al*., 1999).

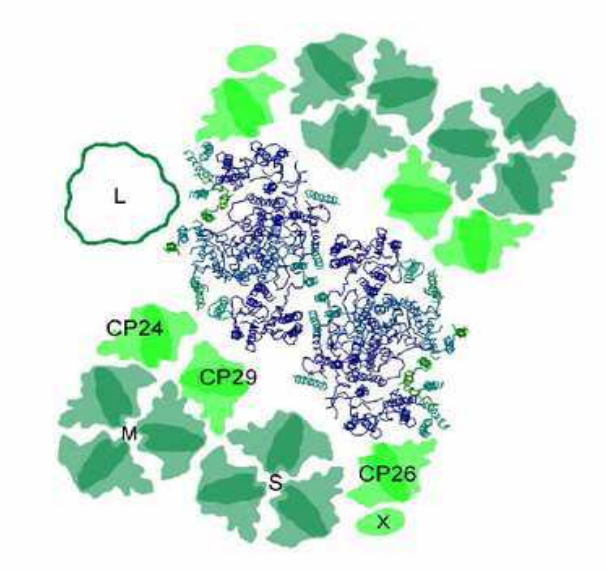


Figure 1.10 Top view of the spinach C2S2M2 supercomplex (Dekker and Boekema, 2005). "S" and "M"refer to strongly and moderately bound LHCII, respectively. "L" indicates the loosely bound trimer,found only in spinach. The central part indicates the protein backbone in the membrane-intrinsic part of the PSII core complex (calculated from the structure of the PSII core complex from *S. vulcanus*). Also shown the minor complexes CP29, CP26 and CP24. "X" denotes a possible small peripheral subunit according to fitting and a comparison of slightly different types of supercomplexes (Boekema *et al*., 1999b).

Similar C2S2M2 type supercomplex composition was also observed in *Arabidopsis* in the form of a larger unit cell (Figure 1.11c). The highly ordered semi-crystalline fragments (Yakushevska *et al*., 2001) were aligned and an average of such 450 crystal fragments was revealed as a unit cell as shown within the rectangular marking in Figure 1.11a. A density map was used to locate the likely positions of LHCII S and M trimers (shown as yellow) and the minor complexes, CP29, CP26 and CP24 (shown as green) in Figure 1.11b, which was further modeled to draw an individual isolated complex (Figure 1.11c) (Yakushevska *et al*., 2001).

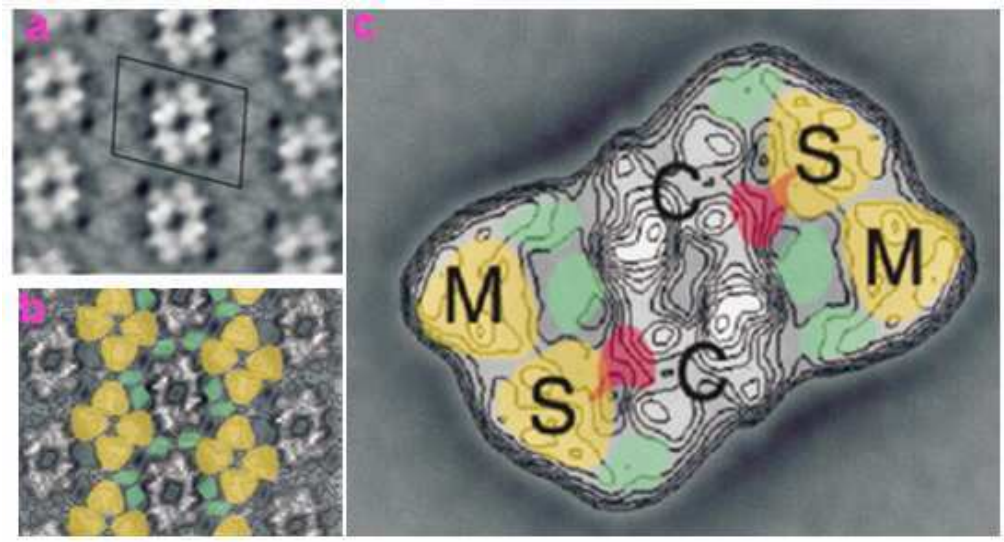


Figure 1.11 Electron Microscopy images of grana fragments from *Arabidopsis* showing PSII-LHCII macrostructure, containing C2S2M2 PSII-LHCII supercomplex as the basic motif. (Yakushevska *et al*., 2001). Sum of 450 aligned crystal fragments; the unit cell is indicated by the rectangular shape (a). Image of (a) showing the S and M trimers (yellow) and CP29, CP26 and CP24 (green) in (b) and a marked C2S2M2 supercomplex in (c).

The location of minor, monomeric light-harvesting proteins in the macromolecular organisation was determined by studies involving cross-linking and antisense plants of these proteins. Cross-linking studies revealed CP29 and CP47 in close contact to one another on one side of the supercomplex while CP26 and CP43 in proximity to each other on the other side (Hankamer *et al*., 1997). Studies of antisense plants lacking either CP26 or CP29 revealed the exact positions of the minor complexes within the supercomplex by process of elimination (Yakushevska *et al*., 2003). CP24 (Lhcb6) was located near CP29 (Lhcb4) as revealed by study of antisense plant of CP29, while a decrease in the level of CP24 was also observed in this plant (Andersson *et al*., 2003). A later study of the knockout mutant lacking CP24 confirmed the positioning of CP24 and further identification of its role in binding the M trimer to the supercomplex (Kovacs *et al*., 2006). CP29 has been considered as essential for the formation of supercomplexes, as no supercomplexes are recovered following membrane solubilisation in the absence of this protein (Yakushevska *et al*., 2003). Contrarily, the supercomplexes remain intact in the absence of

CP26, although an increase in the solubility of thylakoid membranes and the resulting instability of supercomplexes is also noticed (Yakushevska *et al.*, 2003).

In case of major light harvesting proteins, absence of Lhcb3 protein has been found to have little effect on trimer stability or macro-organisazation as compared to wild type (Damkjaer *et al.*, 2009). Interestingly, the supercomplexes without other two major light harvesting proteins Lhcb1 and Lhcb2, isolated from antisense *Arabidopsis* plants (asLhcb2) have also been found to maintain apparently unchanged macrostructure as that observed in case of wild type plants (Ruban *et al.*, 2003). These antisense plants are devoid of both major LHCII proteins, but this appears to be compensated by an enhanced expression of the minor antenna protein Lhcb5 (CP26), without any change in levels of other Lhcb proteins. The LHCIIb trimers seems to be replaced by minor Lhcb5 forming trimers with Lhcb3, binding at both the S and M binding sites. Though, these trimers were found less stable than those in the wild type plants. The plants also show the same extent of grana stacking as the wild type and similar photosynthetic characteristics, although non-photochemical quenching is reduced (Andersson *et al.*, 2003). An increase in photosystem I antenna size was also observed in these antisense plants, implying a concerted compensatory response in the composition of both photosystems as result of loss of the phosphorylated LHCIIb. All this demonstrates the significance of PSII and LHCII macro-molecular organisation and assembly to maintain light-harvesting and electron transport, by means of extreme plasticity at the level of its composition and hence functional robustness (Ruban *et al.*, 2006).

1.8 Acclimation and adaptation to the light environment

In nature, plants face large temporal and spatial variations in the intensity and quality of light. For instance, plants exposed to direct sunlight receive more than hundred times higher daily photon flux as compared to that available for plants growing under the deep shade in the tropical forest. Similarly, light spectral quality varies between full sunlight and shade conditions, with reduced red light in the latter by virtue of filtering by the canopy (Anderson and Osmond, 2001). These fluctuations in light environment necessitate the development of an entire multilevel network of acclimations and adaptations in plants to

regulate light harvesting, ranging from systemic to molecular level. At systemic level changes are slow and involve leaf orientation (Björkman and Powles, 1987) and morphological measures to curtail light absorption by glossy cuticle, salt deposition, air-filled trichomes (Ruban, 2009). Changes at cellular level, like chloroplast movements (Chow *et al*., 1988), are relatively fast and can affect light absorption by 10-20% (Brugnoli and Björkman, 1992).

The changes at molecular level are most profound, and these can be categorised as long-term photoacclimation and short-term adaptation.

1.8.1 Photoacclimation

Photoacclimation can be defined as adjustment of photosynthesis to different light conditions by altering the composition of the leaf (Murchie *et al*., 2002, 2005; Walters, 2005). The long-term photoacclimation is mainly developmental change, considered to be regulated by complex light-induced gene expression at transcriptional, translational and post-translational levels. Photoacclimation has been demonstrated to be adapted at both leaf and chloroplast levels (Murchie and Horton, 1997). These processes result in morphological and anatomical changes at leaf level to optimise light absorption, such as changes in thickness and orientation of leaves. At chloroplast level, the compositional and structural changes in thylakoid membrane are brought about, which are mainly changes in antenna size and alteration of the ratio between PSI and PSII (Murchie and Horton, 1998). The PSII antenna size is reduced under high light intensity due to proteolysis (Anderson and Aro, 1997). Similarly high light can alter the ratio between various PSI and PSII units, for instance by inactivating a subpopulation of PSII reaction centres (Anderson *et al*., 1988).

1.8.2 Photoprotection

The short-term photoprotection is not generally under gene control. These adaptations are meant to counteract fast fluctuations in the light environment, like diurnal variations in the light quality and quantity, sun flecks and canopy effect (Anderson and Osmond, 2001).

1.8.2.1 Photoprotection under low light: state transitions

Changes in the spectral quality of light environment influence the activity of photosynthetic electron transport. In order to compensate for the frequently occurring imbalance in the photosystems excitation, plants have evolved a short-term adaptation mechanism, known as state transitions (State 1-State 2 transitions) (Bonaventura and Mayers, 1969). The need for state transitions arises from the fact that the reaction centres of photosystem I and II have different chlorophyll excited state energies, 700 and 680 nm, respectively. The process of state transitions is based upon LHCII phosphorylation mechanism (Allen *et al*., 1981; Bennett, 1983; Horton, 1983). When the efficiency of PSII to absorb excitation energy is higher than that of PSI, this causes the reduction of the plastoquinone pool. Reduced PQ in turn activates a kinase to phosphorylate some mobile LHCII polypeptides from PSII which can migrate to get attached to PSI complex. This process enhances the PSI efficiency to absorb excitation energy by 20-35% to restore the energy imbalance (Kyle et al., 1983). When PSI gains relatively more excitation energy, the resultant oxidation of PQ this time activates a phosphatase to dephosphorylate LHCII from PSI, which migrate and incorporate back into PSII (Fig 1.12). The use of low-temperature excitation fluorescence spectroscopy has suggested that phosphorylated LHCII is likely to be a trimer in the quenched state. Moreover, this LhcIIb binds and interacts effectively with PSI to enhance light harvesting under limiting light conditions (Ruban and Johnson, 2009).

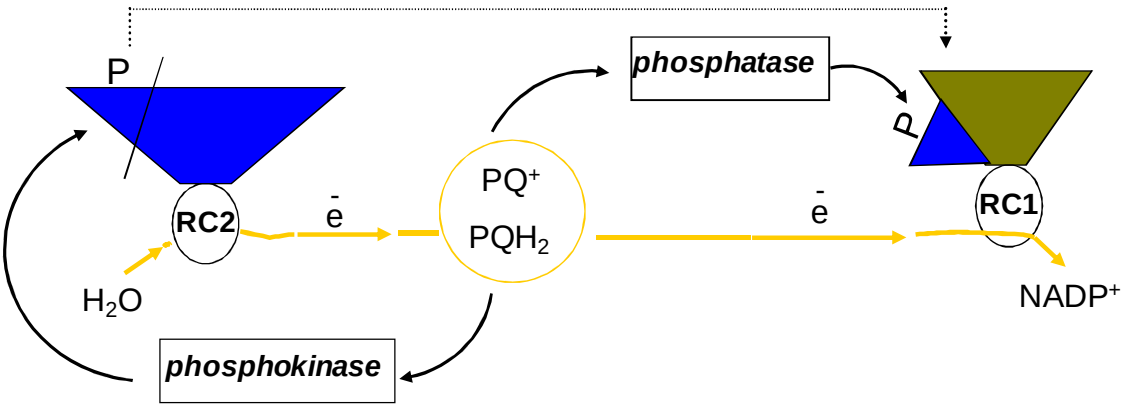


Figure 1.12 The LHCII phosphorylation model of the state transitions (Ruban and Johnson, 2009)

1.8.2.2 Photoprotection under high light: Photoinhibition

Under the conditions of excess light intensity, the balance between excitation energy absorption by light harvesting antenna and its subsequent use in photochemistry is disturbed. This leads to photoinhibition, a sustained decline in the photosynthetic efficiency, mainly associated with the damage of reaction centres (Powles, 1984). The high susceptibility of PSII reaction centres is due to strong oxidation potential of the P680 required to oxidise water. When electron donation to the P680 is less efficient than its oxidation, the powerful oxidant P680+ inevitably starts oxidising and degrading nearest proteins and pigments (Barber, 1995). On the other hand, when acceptor side is less efficient this results in recombination of radical pair to form the P680 triplet. This triplet state P680 is highly prone to combine with molecular oxygen to create highly reactive species of singlet oxygen ($^1O_2$), which in turn can bleach P680 (Telfer *et al*., 1990). Reactive oxygen species can also cause irreparable damage to pigments, proteins and lipids. Therefore a decline in the number of active PSII units and the slow D1 repair both cause a decrease in electron transfer even when excess light is no longer there (Ohad *et al*., 1984). The rate of light energy utilisation can also be perturbed by other environmental factors like low temperature and CO_2 limitation (Horton *et al*., 2001).

In addition to the above mentioned mechanisms at systemic and cellular levels, plants need to regulate their light-harvesting efficiency at molecular level in a dynamic way, so that a balance can be achieved between the absorption and utilisation of light energy to avoid photoinhibition. Thus, under the conditions of excess light, protective mechanisms such as non-photochemical quenching facilitate the de-excitation of the singlet excited chlorophyll molecules to dissipate excess excitation energy as heat, and hence curtailing the formation of triplet states of chlorophylls to avoid photo-oxidative damage (Ruban, 2009).

1.9 The role of carotenoids in photoprotection

In higher plants, carotenoids perform various important functions. These functions can be categorized in four different sections:

1. harvesting the light as accessory pigments by facilitating the absorption in the region of the electromagnetic spectrum where the chlorophyll absorption is low;
2. scavenging of highly reactive triplet chlorophyll and singlet oxygen species;
3. dissipating the excess absorbed energy;
4. stabilising the structure.

As discussed earlier, photoinhibition and photo-oxidative damage of the photosynthetic apparatus are the result of formation of highly reactive ^{3}Chl and $^1O_2$ species. Carotenoids can quench both ^{3}Chl and $^1O_2$ species by energy transfer to form the triplet state of the carotenoid (^{3}Car) (Krinsky *et al.*, 1971), which is accomplished by the non-destructive thermal dissipation of the triplet energy (Mathis 1969, Mathis *et al.*, 1979, Cogdell and Frank, 1987). Such a photoprotective role of carotenoid appears to be universal in chlorophyll-based photosynthetic organisms. On the basis of mutant studies, photoprotective roles have been described in higher plants for β-carotene (Telfer *et al.*, 1994)), lutein (Kuhlbrandt *et al.*, 1994; Pogson *et al.*, 1998; Niyogi *et al.*, 2001), and neoxanthin (Lockstein *et al.*, 2002; Dall'Osto *et al.*, 2007).

These photoprotective roles of carotenoids rely upon their specific chemical structures and electronic properties. Energy level diagrams for carotenoids comprise of at least two singlet excited states denoted as S_1 and S_2 according to their symmetry (Figure 1.13). Although direct transition from the ground state (S_0) to the first excited state (S_1) is forbidden due to symmetry properties, however transition from S_0 to the S_2 excited state can occur. This S_0 to S_2 transition is followed by internal conversion between S_2 and S_1 states from where carotenoids can subsequently return to its ground S_0 state by dissipating the acquired energy as heat or by transferring this energy through resonance to the chlorophyll molecules (Cogdell and Frank, 1987; Ricci *et al*., 1996).

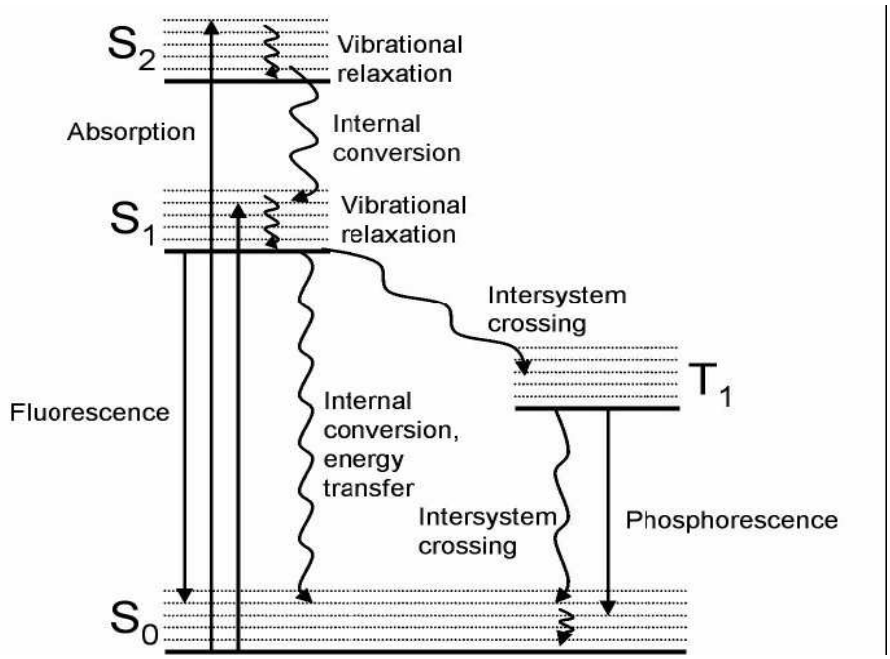


Figure 1.13 Jablonsky diagram of pathways for formation and decay of excited states. S_0 ground state, S_1, S_2,…S_n excited singlet and T_1 excited triplet electronic states. Dotted lines show vibrational levels within each electronic state (Parson and Nagaranjan, 2003).

Variations in the length of the conjugated double bond system of carotenoids have been found to affect their S_2 and S_1 energies, longer the conjugated double bonding system of a carotenoids lower will be its singlet excited state energies. This suggests that a longer

conjugated double bond system is more likely to make a carotenoid reactive to inhibit $^1O_2$ (Edge and Truscott, 1999). The structural differences among the carotenoids are also responsible for other physical properties such as polarity (Ruban *et al*., 1993a).

1.9.1 Xanthophyll cycle carotenoids

The xanthophyll cycle (XC) was discovered by Yamamoto *et al.* (1962) as a reversible process involving the de-epoxidation of violaxanthin to zeaxanthin in two steps by forming another intermediate xanthophyll molecule called antheraxanthin. The XC is located across the thylakoid membrane with the involved xanthophylls bound to the LHC proteins of both photosystems mainly at the peripheral V1 site (Thayer and Björkmann 1992, Lee and Thornber, 1995, Ruban *et al*., 1999) though some fraction of the pool may exist freely in the thylakoid membrane lipid phase under conditions of photo-oxidative stress (Havaux and Tardy 1997, Morosinotto *et al*., 2002). Within thylakoid membranes, the forward de-epoxidation reaction (violaxanthin to zeaxanthin) occurs on the lumenal side and it involves removal of an epoxy group from each of the β-rings of violaxanthin to yield zeaxanthin via formation of a mono-epoxy intermediate antheraxanthin (Yamamoto *et al*., 1962, Hager 1969). The reverse epoxidation reaction (zeaxanthin to violaxanthin) occurs on the stromal side of the thylakoid membrane in which an epoxy group is added to each β-ring occurs, again involving intermediate antheraxanthin in the process (Yamamoto *et al*., 1962).

Violaxanthin de-epoxidation occurs in the light and is catalysed by the nuclear encoded enzyme violaxanthin de-epoxidase (VDE). VDE was first purified from spinach (Arvidsson *et al*., 1996) and then from lettuce (Rockholm and Yamamoto, 1996) and the enzymes from both species have been found with an apparent molecular weights of approximately 43 kDa by the help of SDS-PAGE. VDE requires acidification of the thylakoid lumen to become active, with optimum activity between pH 4.8 – 5.2, whilst becomes completely inactive over pH 6.3 (Eskling *et al*., 1997). VDE also requires the build-up of ascorbate in addition to acidification (Neubauer and Yamamoto, 1994) for maximal activity. The conditions for VDE activity can be mimicked in the dark by the provision of ATP which gets hydrolysed by the thylakoid ATP-ase to form a ΔpH gradient across the thylakoid membrane (Gilmore

and Yamamoto 1992). The activated VDE binds to the membrane at low pH conditions by virtue of a conformational change within it after the protonation of its four key histidine residues (Hager and Holocher, 1994, Gisselsson *et al*., 2004). This enzyme exhibits an even distribution between the grana and stromal lamellae upon membrane binding, with a very low stoichiometry of only one VDE protein per 20 to 100 electron transport chains (Arvidsson *et al*., 1996, Arvidsson *et al*., 1997).

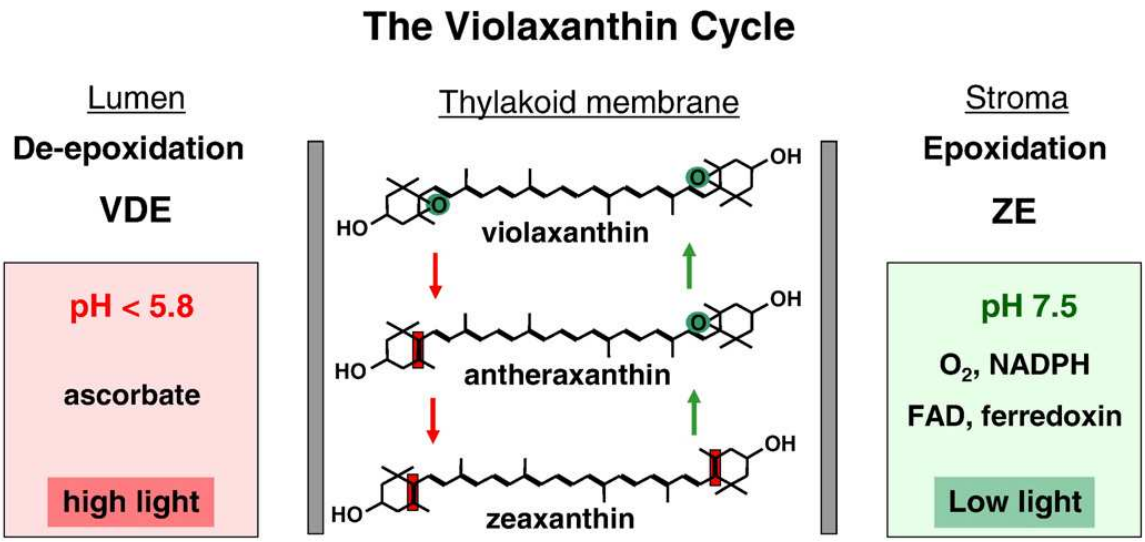


Figure 1.14 The scheme of xanthophyll cycle in higher plants (Jahns *et al*., 2009).

The XC is completed by the reverse reaction, the zeaxanthin epoxidation, which occurs in the dark or under weak illumination. It is catalysed by another nuclear encoded enzyme zeaxanthin epoxidase (ZE). This enzyme has not been purified directly, however cDNA encoding ZE has been extracted from tomato and pepper and then expressed in Escherichia coli to synthesise it successfully (Bouvier *et al*., 1996; Burbridge *et al*., 1997). Similar to VDE, ZE also demonstrates a strong pH dependency, with optimum activity occurring between pH 7.0 – 7.5 (Siefermann and Yamamoto, 1975). Moreover, the epoxidation reaction also requires additional components like molecular oxygen (Takeguchi and Yamamoto, 1968) and the cofactors NADPH (Siefermann and Yamamoto, 1975) and FAD

(Buch *et al*., 1995), together with the presence of ferredoxin or 'ferredoxin' like reductants (Yamamoto, 1999).

Both the enzymes, VDE and ZEP, are members of the plant lipocalin family (Bugos *et al*., 1998) and structurally possess a conserved narrow 'well-like' cavity which enables them to catalyse only all-*trans* xanthophyll substrates but not the 9-*cis* ones (Yamamoto and Higashi, 1978). This 'well like' cavity structure is constituted by an eight stranded anti-parallel β-barrel that is considered to act like hydrophobic binding pocket for the xanthophyll substrates (Bugos *et al*., 1998).

The xanthophyll cycle pool size can vary by four times depending upon growth conditions, as found in field-grown cotton and mazie leaves (Thayer and Björkmann, 1992). Plant growth in high light enhances the demand for de-epoxidation as compared to low light growth conditions (Demmig-Adams, 1990). Thus a prolonged exposure to high irradiance and/or cold induced stress has been demonstrated to increase the size of the xanthophyll cycle pool in a number of plant species (Demmig-Adams 1990, Johnson *et al*., 1994, Verhoeven *et al*., 1999). Despite an increase in de-epoxidation, the level of VDE itself, however, decreases by 15-30% in high light (Eskling and Akerlund, 1998). Though majority of the violaxanthin pool is bound to the antenna complexes at readily accessible sites, not all of it can be converted into zeaxanthin (Ruban *et al*., 1999). The de-epoxidation state (DES) can be calculated as DES=A+0.5 Z/A+Z+V, where A, Z, V are antheraxanthin, zeaxanthin and violaxanthin, respectively. DES has been typically calculated as around 60%, however values as high as up to 90% have also been found specially under stress conditions in certain plant species (Demmig-Adams and Adams, 1992). As a result of light-induced de-epoxidation, the zeaxanthin build up has been shown to have a strong correlation with NPQ (Demmig-Adams, 1990). Further details of the XC role in NPQ will be discussed in section 1.10.4.

In addition to its active role in non-radiative heat dissipation of excitation energy in the PSII antenna, the XC appears to have a number of other important roles as well. The zeaxanthin is evident to play a significant role in protection against the peroxidation and degradation of thylakoid lipids under high light stress conditions (Havaux *et al*. 1991).

Work on an *Arabidopsis* mutant of *npq1*, which is unable to de-epoxidise violaxanthin to zeaxanthin, has demonstrated it as highly prone to lipid peroxidation, pigment loss and photoinhibition, whilst the rate of photosynthesis remains unaffected in this mutant (Havaux and Niyogi, 1999). On the other hand, a two fold increase in the size of the XC pool has also been made possible in another *Arabidopsis* mutant by overexpression of the *chyB* gene encoding the β-carotene hydroxylase enzyme, which is a component of the zeaxanthin biosynthetic pathway (Davison *et al*., 2002). Thus increase in XC pool size enhanced the tolerance to excess light conditions, by means of reduction in lipid peroxidation, along with decline in leaf necrosis and anthocyanin levels (Johnson *et al*., 2007). These evidences highlight a strong link between zeaxanthin and the protection of thylakoid lipids from photodegradation. Moreover, zeaxanthin has also been shown to reduce the membrane fluidity, thus providing a protection against the heat-induced increases in lipid bilayer permeability (Havaux *et al*., 1996). This final piece of evidence suggests a regulatory role of the XC in membrane stability. The XC has also been shown to influence the oligomerisation of the LHCII. Zeaxanthin has shown stimulatory effect on formation of LHCII oligomers with reduction in fluorescence while violaxanthin inhibits such aggregation with enhancement of fluorescence. By these opposing actions of xanthophylls, macro-organisation of LHCII and hence physiological control of light harvesting system can be manipulated by the activity of XC (Ruban *et al*., 1997).

1.10 Chlorophyll fluorescence and non-photochemical quenching (NPQ)

1.10.1 Chlorophyll fluorescence

When a chlorophyll molecule absorbs light energy in the form of a photon, it results in promotion of its electron, present in the conjugated delocalised π-electron system, from the ground state (S_0) to one of number of higher energy levels. This is followed by rapid decay of electron to its first excited singlet state S_1, through the loss of vibrational energy as heat. The electron from S1 state eventually decays back to the ground state (S_0) by either re-emitting the absorbed energy as fluorescence (kF); transferring the energy to a nearby low or non-fluorescent chlorophyll molecule (kT); utilising the energy in photochemistry for

charge separation (kP); or by dissipating the energy in the form of non-radiative heat (kD). Chlorophyll can emit up to 30% of the absorbed light *in vitro*, but *in vivo* this value is reduced to around 3% (Krause and Weis, 1991). The quantum yield of fluorescence (ΦF) of either single chlorophyll or a group of its molecules can be calculated as the fraction of energy remained after competing with all the above processes, as expressed in the following equation:

$$\Phi F = \frac{k_F}{k_F + k_T + k_P + k_D}$$

Equation 1.1 ΦF= chlorophyll fluorescence yield, kF= rate constant for fluorescence, kT= rate constant for energy transfer to an adjacent non fluorescent or low fluorescent chlorophyll, kP= rate constant for photochemical processes, kD= rate constant for heat dissipation.

It is evident from the above equation that a decrease in the fluorescence yield is possible by two processes. The first process involves enhanced photochemical quenching of chlorophyll fluorescence or qP, which is derived from an increase in kP, as a result of photosynthetic electron transport. The second process happens as an increase in the nonphotochemical quenching (NPQ) or qN, which is due to an increase in kT or kD. All the fluorescence changes observed at room temperature result from effects of these two processes on PSII only, since the PSI fluorescence yield is much lower than PSII.

Separation of the photochemical and non-photochemical components of chlorophyll fluorescence quenching has been made possible by employing two techniques. The first technique involves use of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) to block qP, which inhibits electron transport from QA to QB in PSII (Krause *et al*., 1982). In the second method, 'light doubling' technique is employed in which a strong light pulse is used to saturate PSII photochemistry (Bradbury and Baker, 1981). Moreover, the use of modulated fluorescence along with application of the saturating pulses, allows continuous visualization of the fluorescence yield, to facilitate the relative measurement of qP and qN

contributions (Quick and Horton, 1984). The same principle forms the basis of PAM (Pulse-Amplitude-Modulation) fluorimetry (Schreiber, 1986).

Horton and Hague (1988) classified NPQ on the basis of their work involving a range of inhibitors and monitoring the relaxation of NPQ in the dark following illumination. They found it to be comprised of three distinct components: qE (the rapid relaxing phase), qT (slow relaxing phase) and qI (very slow relaxing phase), each representing the regulation of light-harvesting.

The chlorophyll fluorescence quenching analysis is meant to resolve these components and qP (Figure 13). When a dark adapted leaf or chlorophyll containing sample is illuminated with a weak measuring light, a minimum level of fluorescence or F_o is achieved, with all the RCs open at this stage performing photochemistry. When an actinic light (AL) in moderate to excess range (50-2000 µmol photons m^{-2} sec^{-1}) is applied, a surge in fluorescence is observed as the PSII RCs get closed. When exposed to excessive light, photochemistry is reduced to its minimum as all the RCs are closed, thus the fluorescence level reaches its maximum (F_m). This is followed by a decline in fluorescence which is due to an increase in photochemical quenching that begins as the rate of photosynthesis increases, and the slow induction of non-photochemical quenching. The extent of qP and NPQ can be measured here by applying saturating light pulse/s in the presence of actinic light. The maximum fluorescence ($F_{m'}$) attained here by saturating light pulse shows the extent of qP and difference between F_m and $F_{m'}$ is used to calculate the value of NPQ. After switching off the actinic light, recovery of $F_{m'}$ over a variable span of time, ranging from a few seconds to hours, reflects relaxation of various components of NPQ (Horton and Hague, 1988; Walters and Horton, 1991).

The quantum yield of PSII during the actinic light illumination can be calculated as $(F_{m'}-F_s)/F_{m'}$, where $F_{m'}$ and F_s are the maximum fluorescence and the steady state fluorescence during actinic light, respectively. This trace also provides information on the proportion of the light used in photochemistry. qP can be measured by $(F_{m'}-F_s)/F_{m'}-F_o$, where F_o is the minimum fluorescence level at weak measuring light after dark adaptation (Figure 1.15). NPQ is calculated as $(F_m-F_{m'})/F_{m'}$.

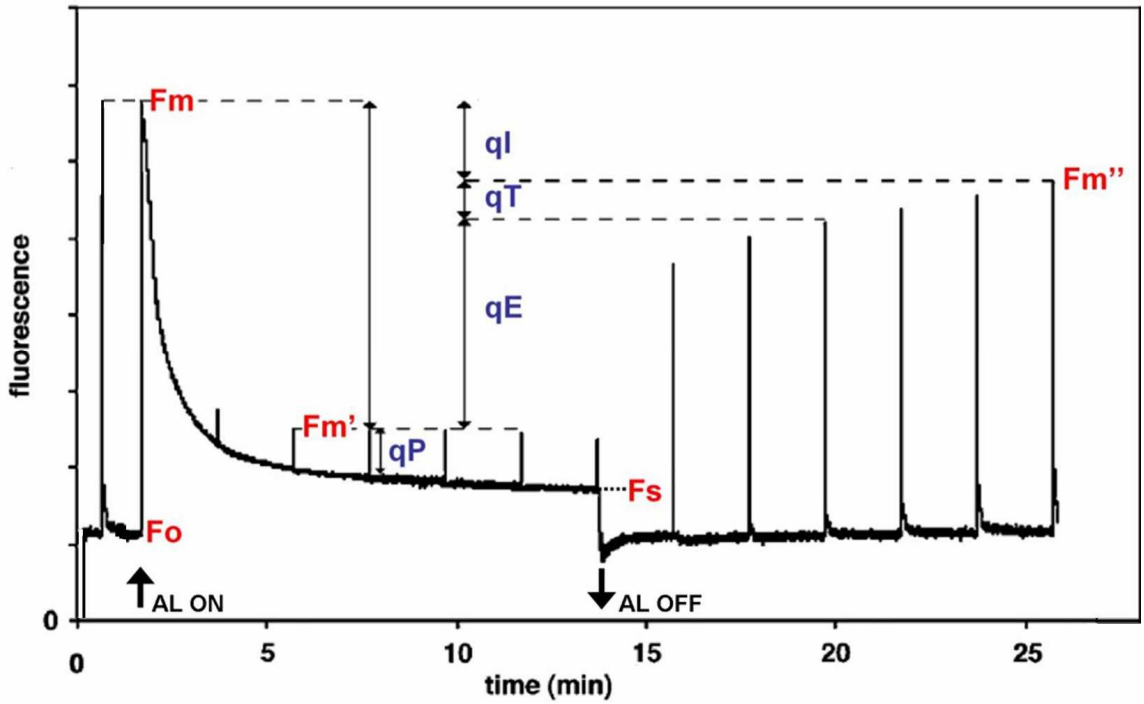


Figure 1.15 A typical fluorescence trace used for quenching analysis. F_o is the minimum and F_m maximum fluorescence. $F_{m'}$ represents maximum fluorescence during actinic light and F_s the steady state fluorescence in actinic light. Fm'' is the maximum fluorescence during dark relaxation. AL=actinic light; qP=photochemical quenching; qE=energy dependent; qT=state transition; qI=photoinhibition components of NPQ (Muller *et al*., 2001).

1.10.2 Non-photochemical quenching of chlorophyll fluorescence

1.10.2.1 Photoinhibition dependent irreversible quenching – qI

The qI or photoinhibitory component of quenching is either irreversible or slowly reversible and may sustain for several hours. It relaxes over a longer period, often with a half time of greater than 10-20 minutes, however part of it can be rapidly reversed on addition of uncoupler nigericin (Ruban and Horton, 1995). Its contribution depends on the degree of light stress. A part of qI occurs as the photodamaged RC quenches fluorescence, reflecting

photoinhibition. However, a significant proportion arises even in the absence of PSII RC activity and is referred to as sustained quenching of the antenna (Gilmore and Björkman, 1994; Horton *et al*., 1996). It has been suggested that qI is a result of structural change caused by illumination (similar to qE), but this change is sustained during the dark for a longer period of time than the one responsible for qE (Ruban and Horton, 1995). qI has also been correlated with the presence of zeaxanthin, which is only slowly epoxidised back into violaxanthin during darkness (Demmig *et al*., 1987; Jahns and Miehe, 1996). Later, it was found that some of this persisting zeaxanthin is bound to the CP26 minor complexes which led to the proposal of minor antenna complexes as the sites for qI (Dall'Osto *et al*., 2005).

1.10.2.2 State transitions dependent quenching– qT

The qT or state transition related component of NPQ has a rather small proportion (15-20%) of the maximum quenching, although its contribution can become major under very low light conditions which promote state transition. It also relaxes slower than qE, with a half time of around 5-10 minutes (Horton and Hague, 1988; Walters and Horton, 1993). This component was first identified as an increase in the rate of non-radiative heat dissipation, which was postulated to be a photoprotective mechanism, that actually quenched both Fo and Fm but unlike qE was not dependent upon the ΔpH (Demmig and Björkman 1987). Horton and Hague first resolved qT and qI by using sodium fluoride (NaF), a thylakoid phosphatase inhibitor. The proportion of reversible quenching found sensitive to NaF was designated as qT. The sensitivity of qT to NaF suggested the involvement of state transitions that were known to depend on phosphorylation of LHCII (Horton and Black 1981). This phosphorylated LHCII is then moved to PSI in order to redistribute the energy between PSII and PSI by striking a balance between the rates of delivery of quanta to the two RCs for maximal photosynthetic efficiency (Allen, 1992). State transitions are inhibited in high light conditions (Aro *et al*., 1993) and are only significant in the adaptation of plants at low light intensities to balance the excitation rates between the two photosystems in order to maintain high quantum efficiency (reviewed in Mullineaux and Emlyn-Jones, 2004). Consistent with this, qT does not make a significant

contribution to quenching under high light. However, it is hard to measure qT exclusively as it overlaps not only with the slower components of qE (Walters and Horton, 1991), it also depends on dark-induced inactivation state of ferredoxin-NADP(+)-reductase (FNR) on the acceptor side of PSI (Schansker *et al*., 2006).

1.10.2.3 Energy dependent quenching – qE

The qE or energy dependent quenching is the largest component of NPQ which is formed during illumination as a proton gradient is developed across the thylakoid membrane (Briantais *et al.*, 1979). The qE is formed rapidly with a relaxation half-time of approximately 30-60 seconds in the dark (Horton and Hague, 1988). It is usually estimated as Fm/Fm'-Fm/Fm'', where Fm'' is the maximum fluorescence after about 10 min of dark relaxation (as shown in Figure 13).

The contribution of nonphotochemical components was first explained as the second wave of fluorescence quenching in the green alga *Chlorella pyrenoidosa*. It was suggested that qE is not dependent on the photosynthetic electron transport rate (Wraight and Crofts, 1970). It was also proposed that alteration in the fluorescence yield might be a result of a change in the arrangement of chlorophyll a molecules under the influence of variable conformation of supporting lamella. Moreover dissociation of non-fluorescing chlorophyll *a* aggregates was considered to be a mechanism for de-excitation of energy from the photosynthetic unit (Papageorgiou and Govindjee, 1968). A decline in chlorophyll fluorescence yield has also been described to be unrelated to photochemistry and rather influenced by high energy state of phosphorylation that may affect the state of chlorophyll *a* molecules in the chloroplasts. The process was thus speculated to be a way to reduce the inactivation of the chloroplasts in excessive light (Murata and Sugahara 1969).

Further investigation of fluorescence quenching explained the inhibitory role of uncouplers to abolish 'energy-dependent' quenching, suggesting its dependence on the trans-thylakoid proton gradient formed during photosynthesis. It was also proposed that chlorophyll fluorescence quenching occurs by an increase in the rate of non-radiative dissipation of the

chlorophyll excited singlet state by means of thermal degradation (Wraight and Crofts, 1970). These results were corroborated by another study to firmly establish a linear correlation between the energy dependent chlorophyll fluorescence quenching (qE) and the intra-thylakoid proton gradient in the broken pea protoplasts (Briantais *et al*., 1979).

Later on, it was discovered that the influx of protons into the lumen is accompanied by efflux of magnesium cations. These simultaneous movements cause certain changes in the thylakoid membrane structure which were proposed to affect both fluorescence and absorption band around 535 nm (Krause, 1973). Krause and Behrend (1986) first proposed a physiological role of qE by discovering the increase in photoinhibition as a direct consequence of abolishing the rapidly relaxing component of NPQ. Horton and Hague (1988) later confirmed by their work on isolated chloroplasts that photoinhibition was inversely proportional to the level of qE. It was suggested that under high light conditions qE impedes the build-up of reduced QA, thereby preventing photoinhibition. It has also been observed that, with the increase in light intensity, a steady decrease in the PSII quantum yield continues, however the level of qP remains high which keeps QA oxidised (Weis and Berry, 1987; Genty *et al*., 1989). This demonstrates that the decrease in quantum yield of PSII is not a consequence of feedback from reduced components of the photosynthetic electron transport system; rather it is result of NPQ that is reflected as an increase in dissipation of the energy absorbed by the antenna. All the above mentioned findings suggest that qE is a regulatory mechanism meant to provide photoprotection.

Another important role of the ΔpH formation in qE is the activation of the xanthophyll cycle, detailed in section 1.8.2. The de-epoxidation of violaxanthin to zeaxanthin has been linked to the level of qE in numerous plant species under various environmental conditions (Demmig-Adams, 1990) as well as in isolated chloroplasts (Gilmore and Yamamoto, 1992). The inhibitor dithiothreitol (DTT) has been used to block both VDE and consequently qE (Bilger *et al*., 1989; Adams *et al*., 1990). Study of NPQ mutants has also provided important insights to understand the mechanism of qE. The *Arabidopsis* mutant of *npq1*, which is unable to convert violaxanthin to zeaxanthin, exhibits much reduced qE. Another mutant *npq2*, which is unable to epoxidise zeaxanthin and hence constitutively

accumulates zeaxanthin, has faster qE induction than in the wild type plants (Niyogi *et al*., 1998).

Spectroscopic studies of isolated thylakoids and leaves have indicated that high light induction of qE correlates with two separate absorbance changes. One absorbance change at 505 nm is considered as a result of the zeaxanthin formation from violaxanthin by de-epoxidation (Yamamoto *et al*., 1972), consequently this change is a quantitative measure of the de-epoxidation reaction (Siefermann and Yamamoto, 1974). The second absorbance change, which is frequently referred to as a light scattering change, has a maximum at 530-540 nm and is designated as Δ535. It is considered to be dependent on pH and the presence of zeaxanthin (Bilger *et al*., 1989). As both pH and zeaxanthin are considered to be significant prerequisites for the induction of qE, thus ΔA535 has been correlated with qE in both leaves and chloroplasts, (Noctor *et al*., 1993; Ruban *et al*., 1993b) and no such change has been observed in npq4 mutants, without PsbS, lacking qE (Li *et al*., 2000). As mentioned earlier, ΔA535 was initially thought to be a light scattering change resulting from a ΔpH-dependent conformational change in the thylakoid membrane (Heber, 1969; Krause, 1973) and it was also considered to be potentially related to aggregation of LHCII (Ruban *et al*., 1993b). Further studies proved that this absorbance change is a "real" one, at least in part, and reflects a change in the electronic absorption of zeaxanthin. Moreover, it was suggested that this change results from the "activation" of zeaxanthin molecules under the influence of some change in their local environment, thus it was regarded as a complex change (Ruban *et al*., 2002b). Change in the local environment of zeaxanthin has been possibly speculated in the form of head-to-tail aggregation (Polivka *et al*., 2002). A similar absorption spectral shift giving rise to a ΔA535 has been replicated *in vitro* upon association between isolated PsbS and zeaxanthin, suggesting that this interaction may be crucial for the functioning of zeaxanthin in NPQ (Aspinall O'Dea *et al*., 2002).

The PsbS protein plays an essential role in qE. The *npq4* mutant, which lacks PsbS, has been found to be deficient in the qE component of NPQ (Li *et al*., 2000a). However, some NPQ still occurs in this mutant but rate of induction and relaxation remains very slow. Correspondingly, the absorbance changes associated with ΔA535 also show slower

formation and smaller amplitude (Johnson and Ruban, 2009). On the basis of these observations, the role of PsbS has been proposed as a promoter of conformational changes within LHCII to bring about NPQ *in vivo* (Horton *et al*., 2000; Horton and Ruban, 2005, Johnson and Ruban, 2009). An in depth study of the role of PsbS has been made possible by a number of site-directed mutations of this protein. Most significant were those in which mutation of certain lumen-facing glutamate residues resulted in the inhibition of qE (Li *et al*., 2002c; Li *et al*., 2004). It was proposed that protonation of these residues is a key event in the pH-dependent induction of qE, which is consistent with DCCD binding to this protein (Ruban *et al*., 1992; Dominici *et al*., 2002). The PsbS over-expressor lines of *Arabidopsis* plants (L17) have also been generated, and these plants exhibited qE of larger amplitude than that found in the wild type (Li *et al*., 2002b). Although this evidence has been used to suggest a direct role of PsbS in NPQ, acting as the binding site for zeaxanthin, however it was subsequently demonstrated that the stimulatory role of PsbS is independent of the presence of zeaxanthin (Crouchman *et al*., 2006). Therefore, the exact mode of PsbS action remains elusive to date. In fact the location of this protein is still ambiguous: some reports suggest its association with either LHCII (Kim *et al*., 1994) or the PSII core (Funk *et al*., 1995; Dominici *et al*., 2002; Bergantino *et al*., 2003); and some others even suggest it as highly mobile within the thylakoid membrane (Nield *et al*., 2000; Yakushevska *et al*., 2001; Teandro *et al*., 2007).

1.10.3 The site of qE

Various studies have attempted to suggest whether qE takes place in the RC or in the light harvesting antenna. Weis and Berry (1987) and later Krieger *et al*. (1992) suggested the PSII RC as site of quenching on the basis of observation that the quantum yield of PSII was correlated with the amount of qE. It was proposed that quenching occurs in the PSII RC itself, due to the increased population of Q_A^-, which facilitates rapid recombination via a back reaction with $P680^+$. This charge recombination and hence RC quenching is promoted by inactivation of electron donation to P680, caused by low pH-induced release of Ca^{+2} from the OEC (Krieger and Weis, 1993). However, there are some results indicating that

quenching process can occur in RC (Finazzi *et al*., 2004), most of the evidence suggests that the qE component of NPQ takes place in the antenna (Horton and Ruban, 1992; Ruban and Horton, 1995; Wentworth *et al*., 2000). Some of these evidence are as follows:

a) the qE-related heat emission occurs within 1.4 μs (Mullineaux *et al*., 1994), significantly faster than the rate of QA^- and $P680^+$ recombination (~120 μs);

b) the 77K PSII fluorescence spectral analysis of photochemical and non-photochemical quenching shows variations of pigment populations in both processes, as qP preferentially quenches the PSII core at 688nm whereas qE quenches at maxima of 683 nm and 698 nm (Ruban and Horton, 1995);

c) the XC carotenoids, which have been implicated to play a significant role in qE (Demmig-Adams, 1990), are only associated with the peripheral light harvesting antennae (Peter and Thornber, 1991; Ruban *et al*., 1999);

d) quenching demonstrated by isolated antenna complexes resembles many features of *in vivo* qE observed in leaves and chloroplasts, such as the kinetics of fluorescence induction, the enhancement by zeaxanthin, and the absorbance changes accompanying the quenching process (Ruban and Horton, 1992; Wentworth *et al*., 2000; Wentworth *et al*., 2001);

e) the qE inhibitor DCCD (Ruban *et al*., 1992) binds antenna polypeptides (Walters *et al*., 1994; Ruban *et al*., 1998a), later on DCCD has also been shown to bind to PsbS protein (Dominici *et al*., 2002).

The above mentioned approaches strongly suggest that qE occurs in the light-harvesting antenna; however the RC could be involved under certain physiological conditions, such as low pH or inhibition of antenna quenching.

1.10.4 The mechanism of qE

Recently evidence has been provided for two distinct types of mechanisms to account for quenching in the antenna. One of the mechanisms suggests zeaxanthin while the other lutein as the quencher of excitation energy, however both assign a substantial role for zeaxanthin in the NPQ either as a direct quencher or allosteric regulator of the process. The first mechanism, suggesting zeaxanthin as the obligatory quencher of chlorophyll excited states, is based on the evidence of strong correlation between qE and the formation of zeaxanthin (Demmig-Adams *et al*., 1989a,b,c; Demmig-Adams, 1990). The essential prerequisite of pH gradient for qE was explained by its role to activate the zeaxanthin binding site within PSII light-harvesting antenna (Gilmore *et al*., 1995).

An idea was proposed to explain correlation between induction of quenching and violaxanthin de-epoxidation, based on the extrapolation of various singlet excited state properties of zeaxanthin in comparison with those of violaxanthin, as depicted in the Figure 1.16 (Owens, 1994). This was later named as the "molecular gear shift model" (Frank *et al*., 1994). As discussed earlier (see section 1.9.1), this model is based on the understanding that chlorophyll can dissipate its excitation energy by transferring it to a carotenoid molecule with the S1 level lower than that of former. Zeaxanthin has lower S_1 level than the chlorophyll Qy band, so it can act as quencher of excited singlet chlorophyll to bring it back into ground state. Since the S_1 level of violaxanthin is higher than that of chlorophyll *a*, which makes it a good candidate as light harvesting pigment. Thus the antenna can be modulated to efficiently harvest light energy or to effectively dissipate excess excitation energy, by converting violaxanthin into zeaxanthin, under conditions with light energy surplus of photochemistry.

Carotenoids exhibit at least two excited states, named as S_1 and S_2. As S_2 to S_1 transition is possible and carotenoids use it as a measure to transfer energy to chlorophylls. The S_1 energy level of carotenoids, zeaxanthin and violaxanthin, relative to the chlorophyll a Qy band can be calculated or extrapolated by using the energy gap law (Frank *et al*., 1994). Thus the S_1 energy levels of violaxanthin and zeaxanthin, in comparison with that of chlorophyll, were found as higher and lower, respectively. Later a relationship was

established between the length of the conjugated double bond chain of the molecule and ability to induce ΔpH dependent quenching in LHCII *in vitro* (Phillip *et al.*, 1996). The de-epoxidation of violaxanthin to zeaxanthin results in increasing the number of conjugated double bonds from 9 to 11, and thereby promotes LHCII quenching. Thus the molecular gear shift model suggests that differences in S_1 energy states between zeaxanthin and violaxanthin are sufficient to account for their roles as quencher and light harvester, respectively. This model also explains the action of violaxanthin as an accessory pigment during low light conditions, whilst its conversion into zeaxanthin makes possible the dissipation of excess energy under high light conditions.

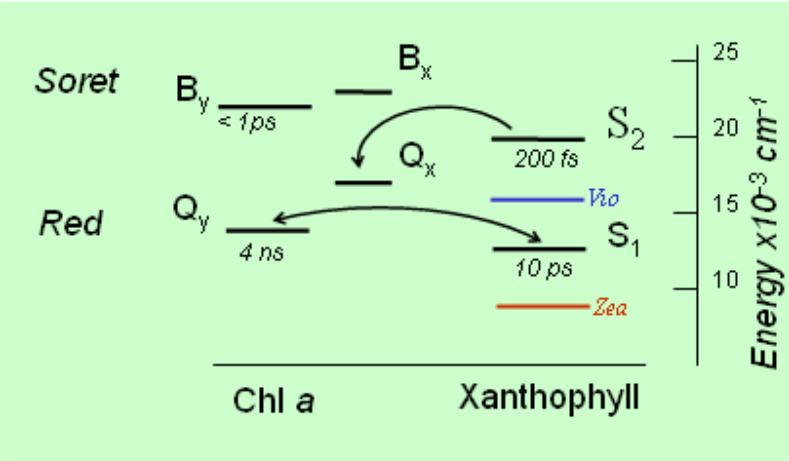


Figure 1.16 The molecular gear shift model for non-photochemical quenching (after Owens, 1994)

Some serious doubts were raised on the validity of molecular gear shift model, when the direct measurements of violaxanthin and zeaxanthin energy levels were performed, using transient absorption (Polivka *et al.*, 1999) and fluorescence (Frank *et al.*, 2000) spectroscopy. These measurements demonstrated that the values of S1 energy states of both xanthophylls were far lower than the extrapolated ones calculated earlier; actually the basic tenet of this hypothesis was negated as both values were found to be lower than the level of chlorophyll a Qy band. A subsequent study, using low temperature fluorescence

spectroscopy, measured the S_1 energy levels of the xanthophyll cycle carotenoids, finding similar values with those previously reported (Frank *et al*., 1994), however it was considered to be a result of significant distortion of the carotenoids, caused by a change in protein at low temperature.

The relationship between the length of conjugated double bond chain and the pH induced quenching in LHCII *in vitro* was used as evidence to support the molecular gear shift model. However, subsequent studies suggested this relationship was likely to be based on differences in carotenoid configuration and not on the S_1 excited state energy level (Ruban *et al*., 1998b; Phillip *et al*., 1996). Direct evidence was obtained, using the artificial conjugated dyad model system, that energy transfer from a chlorin ring of chlorophyll to the S_1 state of a carotenoid, coupled with an internal charge transfer state may cause energy dissipation. Moreover, this study also showed that addition of only one double bond to the conjugated double bond chain of the carotenoid converts the non-quencher carotenoid into a strong quencher (Berera *et al*., 2006).

1.10.4.1 The carotenoid radical cation model

The transfer of electrons between carotenoids and chlorophylls has been presented as a mechanism for chlorophyll fluorescence quenching, as an alternative to singlet-singlet energy transfer. The transient formation of carotenoid cation radicals as a result of photoexcitation has been observed in both bacterial light harvesting complexes (Frank and Brudvig, 2004) and thylakoid membranes (Holt *et al*., 2005). On the basis of these studies, a new model accounting for direct quenching was suggested (Figure 1.17). This model proposes direct quenching of the major light harvesting antenna chlorophylls following the ΔpH-induced formation of a zeaxanthin/PsbS complex (Figure 1.17a). The excitation energy can be transferred from chlorophyll to carotenoids if the lowest excited state of the chlorophyll (Qy band) is higher than the carotenoid excited state. The carotenoid excited energy state was measured by transient absorption spectroscopy of isolated thylakoids, and was theoretically calculated as lower than the chlorophyll Qy band (Ma *et al*., 2003). Subsequently it was proposed that quenching occurs as a result of energy transfer from

chlorophyll to a carotenoid (zeaxanthin) or alternatively by the formation of a chlorophyll xanthophyll heterodimer. The formation of zeaxanthin/PsbS complex was considered to give rise to an exclusive signal depicting qE dependent changes (Holt *et al*., 2004).

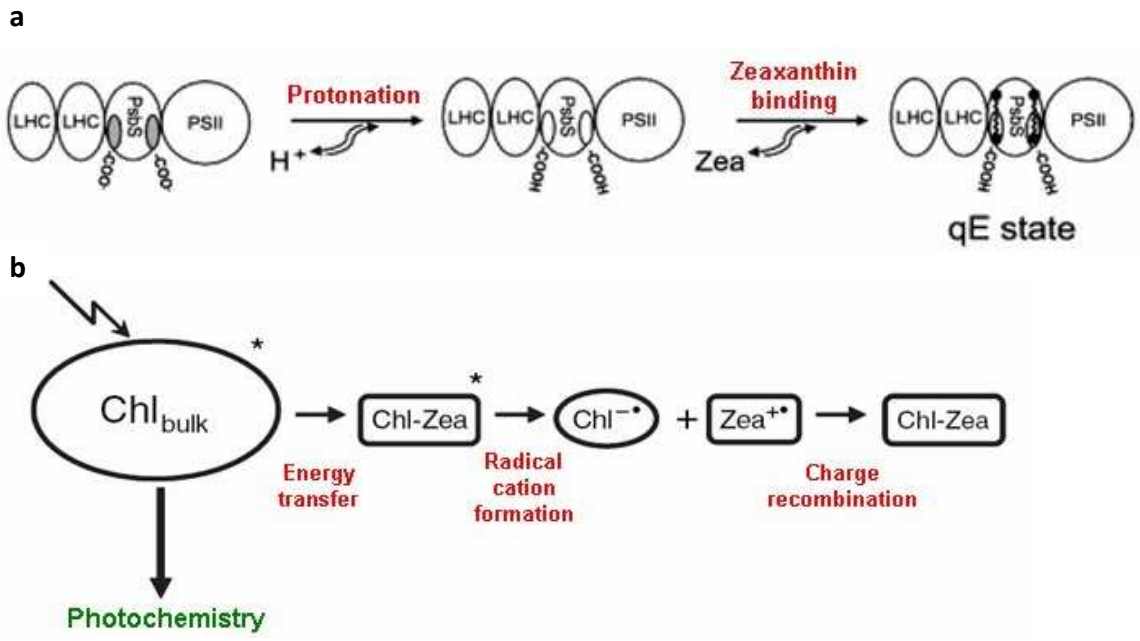


Figure 1.17 The PsbS-zeaxanthin complex model for non-photochemical quenching (a) (Holt *et al*., 2004). The hypothesis for the zeaxanthin cation formation and the dissipation of energy by charge transfer and recombination (b) (Holt *et al*., 2005).

The model suggests that exposure to intense light triggers the formation of zeaxanthin molecules in PSII. This mechanism involves transfer of energy to a chlorophyll-zeaxanthin heterodimer. This heterodimer undergoes charge separation and subsequent recombination, while transiently producing a zeaxanthin radical cation. In charge transfer mechanism, the zeaxanthin molecules interact with excited chlorophylls to dissipate the excess energy, by giving up an electron to the chlorophyll to bring the latter's energy back down to ground state and to turn itself into a cation radical. This zeaxanthin cation radical, unlike an excited chlorophyll, is a non-oxidising agent.

Application of near-infrared absorption spectroscopy demonstrated the appearance of a qE related absorption band (at approximately 1,000 nm), indicating the formation of a carotenoid cation. The signal, which appears only in chloroplasts showing qE and containing zeaxanthin (Holt *et al*., 2005), was interpreted as arising from a chlorophyll-zeaxanthin (Chl-Zea) heterodimer formed when qE is induced (Figure 1.15b). The heterodimer quenches the bulk of chlorophylls (Chl_{bulk}) via the formation of a charge separated ground state Chl^- and Zea^+, which decay further to the ground state by charge recombination. Consistent with this model, it was calculated that zeaxanthin has the lowest ionization potential of the xanthophyll cycle carotenoids (Dreuw *et al*., 2003). A zeaxanthin radical cation was found exclusively at the L2 binding site of all the three isolated minor light-harvesting proteins, CP29, CP26 and CP24, which were either reconstituted *in vitro* or expressed in bacteria. As all the three minor complexes showed quenching, therefore it was suggested that no single antenna protein was specifically required for quenching and these complexes might be the site of qE *in vivo* (Ahn *et al*., 2008; Avenson *et al*., 2008). The effect of this carotenoid radical cation on the excited-state lifetime of the minor antenna complexes has only been demonstrated *in vitro* and found as very small. Moreover, the tightly bound violaxanthin in minor complexes has also been found as inaccessible for deepoxidation. It has been proposed that *in vivo*, under the influence of the ΔpH, a large population of minor complexes can adopt a conformation to account for substantial reduction of excitation lifetime (Avenson *et al*., 2008).

1.10.4.2 The allosteric model

The LHCII aggregation model of qE was first presented by Horton *et al*. (1991) as an alternative to the direct quenching model. It was proposed that protonation causes aggregation of LHCII. As isolated aggregated antenna was found in quenched state, therefore aggregation model was considered to be responsible for the change in antenna underlying NPQ. The extent of aggregation and hence NPQ was found to be controlled by peripheral xanthophylls, violaxanthin and zeaxanthin as inhibitor and promoter respectively (Horton *et al*., 1996). Indeed, earlier studies on isolated chloroplasts already demonstrated

that qE could occur both in the absence or presence of zeaxanthin only with the requirement of different pH. Interestingly, qE was even observed in isolated chloroplasts devoid of zeaxanthin (Rees *et al*., 1989; Noctor *et al*., 1991). These results show that zeaxanthin itself is not the quencher; rather it performs an indirect role to lower the pH requirement for qE. This initial model has been updated (Horton *et al*., 2000; Horton *et al*., 2005) describing 4 LHCII states, depending on the de-epoxidation state of the xanthophyll cycle and the lumenal pH (Figure 1.18).

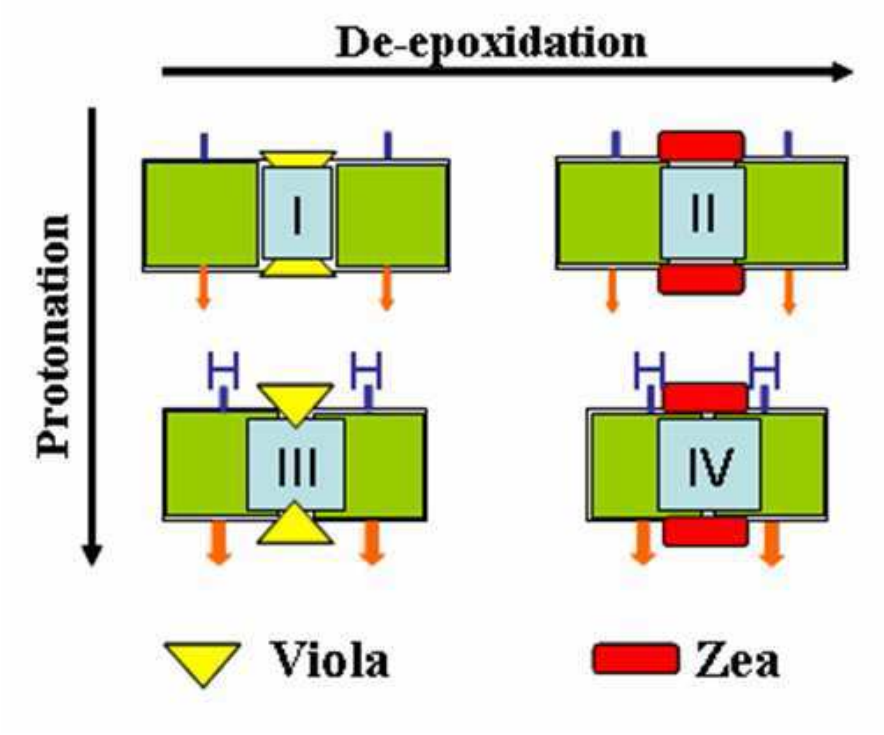


Figure 1.18 The LHCII model for NPQ. LHCII is represented in green; violaxanthin in yellow; zeaxanthin, red; orange arrows indicate energy dissipation; H denotes protonation; (Horton *et al*., 2000; Horton *et al*., 2005).

State I depicts the unquenched, light harvesting state of the antenna complex with bound violaxanthin at no or minimal pH difference between the stroma and the lumen. State III shows a partially quenched state of protonated complex with violaxanthin still bound to it. In the light activated state II, the LHCII is partially quenched in unprotonated and

zeaxanthin bound condition. The final state IV represents the maximum quenching as both protonation and zeaxanthin binding to the LHCII occur. In isolated LHCII, aggregation of the protein and hence quenching can be induced by the removal of the detergent (Ruban *et al*., 1991), by the addition of Mg ions (Arntzen and Ditto, 1976) or low pH (Briantais *et al*., 1979).

Several lines of evidence lend support to the idea that LHCII aggregation was a "first approximation" of the conformational change leading to quenching. However, further investigations revealed that the quenching process in LHCII was not a direct consequence of protein-protein interactions during the process of aggregation, rather a result of intrinsic conformational transition within the monomeric unit of the complex brought about by protonation (Ilioaia *et al*., 2008). This also suggests qE as inbuilt property of LHCII proteins, thus a protein conformational change alters the configuration of bound pigments (Ruban *et al*., 2007).

Evidence has also been found that difference in activity of violaxanthin and zeaxanthin in qE can be a result of difference in their structural rather than in their excited energy levels, as proposed by the molecular gear shift model (Ruban *et al*., 1993a). It has been found that the ability to induce quenching is not merely dependent on the number of conjugated double bonds. Auroxanthin, an isomer of violaxanthin having only 7 double bonds, can induce quenching in the isolated LHCII with even greater efficiency than zeaxanthin (Ruban *et al*., 1998b). The explanation for this observation comes by finding a structural similarity between auroxanthin and zeaxanthin – in both, the head groups are in the same plane with respect to the double bond chain, but they are twisted in violaxanthin. This structural orientation of the head group has therefore been suggested to control the interaction of the xanthophyll molecules with LHCII (Horton *et al*., 1999). This observation is in contradiction to the direct quenching model of qE, but fully endorses the indirect model.

All the PSII light harvesting complexes have intrinsic ability to undergo transition to a highly dissipative state *in vitro* quenching. This may provide a feasible molecular basis to relate conformational changes to qE (Horton *et al*., 1996). However, the minor complexes

have been demonstrated to affect both amplitude and kinetics of quenching more than does the major trimer LHCII (Ruban *et al*., 1996; Wentworth *et al*., 2001). Hence, quenching was proposed as a collective property of the whole antenna, rather than of one particular complex (Horton *et al*., 1996). Studies of various Lhc mutants have provided important insights to this question. These mutants were created either by expression of anti-sense Lhcb genes or T-DNA insertions in Lhcb genes. The first two antisense mutant lines obtained were without Lhcb4 (CP29) and Lhcb5 (CP26), both showed NPQ reduction by only a 30% and 10%, respectively. The mutant lacking CP29, however, was found to have perturbation in macro-organisation with the absence of PSII-LHCII supercomplexes, while slightly unstable supercomplexes were found in case of CP26-deficient mutants (Yakushevska *et al*., 2003). Major effect on NPQ was observed in the plants with antisense and knock-out of Lhcb6 (CP24) with a maximum qE reduction of 60%. The macro-organisation was also drastically altered as only C2S2 complexes were observed, implicating the exclusion of M trimers in addition to CP24. Plants without major Lhcb1 and Lhcb2, forming major constituents of trimeric LHCII, were also created by an antisense Lhcb2 line (Andersson *et al*., 2003). This mutant type also showed a 30% reduction in qE, but macrostructure remained unchanged as Lhcb5 containing trimers replaced trimers with Lhcb1 and Lhcb2 to preserve PSII macro-organisation (Ruban *et al*., 2003). In case of plants without Lhcb3 as a result of gene mutation, the NPQ remained unaffected with only minor deviation in macro-structure from wild type (Damkjær *et al*., 2009). All these results together suggest that no single Lhcb protein can exclusively regarded as the unique site of qE and show, moreover, the significance of the macro-structure of the LHCII antenna system for maximal NPQ *in vivo* (Horton *et al*., 2005; Kovacs *et al*., 2006).

Spectroscopic studies of quenched LHCII help to understand the mechanism of quenching. The significance of the terminal emitter domain, comprising of Chl *a*611, Chl *a*612, Chl *b*608 and lutein 1, has been emphasised by proposing a quenching interaction between the chlorophylls and lutein as a result of conformational change (Wentworth *et al*. 2001). The first elaborate insights into the understanding of quenching mechanism were made possible by the studies of LHCII crystal structure (Liu *et al*., 2004). Later spectroscopic analysis of these crystals by Pascal *et al*. (2005) demonstrated these crystals to be in a quenched state.

The lifetime of these quenched crystals was measured by the help of fluorescence lifetime imaging technique (FLIM) and found as 0.89 ns as against 4.2 ns measured in case of isolated trimers. A characteristic 680 nm band along with another maximum band at around 700 nm was observed in the fluorescence emission spectra, similar to the results found previously in the quenched aggregated LHCII. Similar changes in pigment interactions were also observed between crystals and trimers in the Resonance Raman analysis; the neoxanthin molecule in each monomer in the crystal is twisted relative to its state in the trimer. Similarly, changes were also found in the chlorophyll *b* region of the crystal, indicating formation of an extra hydrogen bond between a formyl group of chlorophyll *b* and *a* water molecule in the crystal (Pascal *et al*., 2005). These observations indicate a change in the LHCII conformation upon its transition to the quenched state. On the basis of pigment orientation in the crystal structure, following three putative quenching sites have been proposed (Pascal *et al*., 2005):

- terminal emitter domain comprises of chlorophylls: Chl *a*611, Chl *a*612, Chl *b*608 and lutein 1 (lut 620);

- neoxanthin domain: neoxanthin, lutein 2 (lut 621), Chl *b*606 and Chl *b*607;

- xanthophyll cycle binding domain (V1): violaxanthin, Chl *a*611 and Chl *a*601;

The difference in interaction strengths of lutein 1 and lutein 2 with adjacent chlorophylls was further elaborated by a subsequent study of crystal structure; suggesting the interaction of lutein 1 molecule with the Chl *a*611 and Chl *a*612 molecules is likely to constitute the potential quenching site (Yan *et al*., 2007). Recently it has been suggested that quenching of excitation energy does not require an intrinsic conformational change, and it can be rather a result of interaction with external pigments *in vitro* or with PsbS *in vivo* (Barros *et al*., 2009). However, studies showing the qE induced by intrinsic conformational change in the absence of LHCII aggregation and hence external pigments *in vitro* (Ilioaia *et al*., 2008) and without PsbS both *in vitro* and *in vivo* (Crouchman *et al*., 2006; Bonente *et al*., 2007, Johnson and Ruban, 2009) are not supportive of the afore mentioned exclusion of LHCII conformational change role in qE.

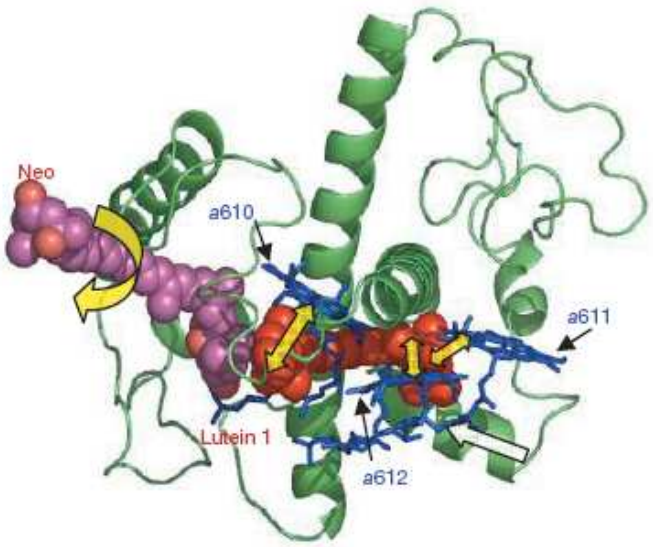


Figure 1.19 Structural model of an LHCII monomer, depicting the molecular mechanism of qE. Lutein 1 (red) is in proximity with chlorin rings of chlorophyll *a* 610, 611 and 612 (blue). The neoxanthin (Neo, pink) twist is shown as curved broad yellow arrow; while the white broad arrow demonstrates the putative movement of lutein 1 towards the chlorophyll cluster (broad yellow arrows) (Ruban *et al*., 2007).

The intrinsically bound xanthophyll at the L1 site, normally lutein, has been proposed as an efficient quencher of chlorophyll excited states (Figure 1.19). Femtosecond transient absorption spectroscopy on the trimeric LHCII aggregates revealed that energy dissipation occurred by energy transfer from chlorophyll *a* to the low-lying excited state of lutein 1. A corresponding change in the conformation of another xanthophyll, neoxanthin, was detected by resonance Raman spectroscopy and correlated with both LHCII *in vitro* quenching and *in vivo* qE. Therefore this quenching mechanism demonstrates a reduction in the chlorophyll excited state lifetime by a magnitude which fully accounts for qE *in vivo*. This molecular insight into the mechanism of quenching re-affirms the allosteric model in which qE is initiated by a conformational change in the LHCII, induced by the light-dependent ΔpH. The conformational change thus gives rise to an increase in the rate of energy transfer from chlorophyll *a* to lutein 1. Considering qE as a heterogeneous process, quenching by lutein 1in LHCII may only be a part, as this model does not rule out

contribution of minor antenna complexes in the quenching, which bind zeaxanthin at internal sites occupied by lutein in LHCII (Ruban *et al*., 2007).

1.11 Project outline

The main focus of the work presented in this thesis is the identification and characterisation of molecular factors that regulate the photoprotective capacity of the photosynthetic membrane and the efficiency of light energy utilisation. The role of two major xanthophylls, lutein and zeaxanthin, which have been implicated to play main role in the NPQ, was investigated. Effect of xanthophyll composition on the regulation of light harvesting was studied using available xanthophyll mutants of *Arabidopsis* and methods of fluorescence and absorption spectroscopy. The *in vitro* biochemical and spectroscopic investigation was carried out to investigate the stability of macro structure of thylakoid membranes obtained from these mutants. Furthermore, LHCII complexes from xanthophyll mutants were isolated and characterised by means of *in vitro* chlorophyll fluorescence quenching and chlorophyll excitation life time measurements. Finally, a double mutant with over-expression of PsbS protein and devoid of zeaxanthin was generated and characterised to further shed light on the role of PsbS and zeaxanthin in energy dependent quenching qE.

Chapter Two

Materials and Methods

2.1 General laboratory chemicals

Chemicals and reagent used for all the analyses and experiments were obtained from *Sigma-Aldrich* unless stated otherwise.

2.2 Plant material and growth conditions

Arabidopsis (*Arabidopsis thaliana* cv *Columbia*) and mutant and transgenic lines derived from it were grown for 8 to 10 weeks in *Sanyo* Versatile Environmental Test Chambers (MLR-351) with an 8 hours photoperiod under a light intensity of 100 μmol photons m^{-2} s^{-1} (also known as μE) and day/night temperatures maintained at 22°C/18°C. The seeds were sown in 19 x 15 cm seed germination trays on *John Innes* seed compost and then were transferred for stratification at 4°C immediately after sowing, for 12 hours (overnight), before being transferred to the growth chambers. Two weeks after planting, the seedlings at 4-6 leaf stage were replanted in 7.5 x 7.5 cm plastic pots in commercial peat free multipurpose compost. Vermiculite, a commercially available naturally occuring non-toxic mica mineral, was also mixed in the compost at 10% (v/v) concentration, to improve water holding capacity and aeration of the compost. The plants for thylakoid and BBY preparation were grown in the plant growth room for 8 to 10 weeks under controlled day/night temperatures of 22°C/18°C and 8 hours photoperiod, provided by a bank of cool white Polylux XL fluorescent tubes (150 μmol photons m^{-2} s^{-1}). The plants for high light acclimation experiment were grown initially under the controlled growth conditions as mentioned above and then well-established potted plants were moved under high light intensity of 700 μmol photons m^{-2} s^{-1} provided by HPI-T400W incandescent lamps (*Philips*), for either short (one week) or long term (four weeks). Plants were hand watered periodically by either sprinkling or sub-irrigation.

The xanthophyll mutants used were as follows: *npq1* (mutated in violaxanthin deepoxidase and therefore unable to synthesize zeaxanthin in excess light); *npq2* (mutated in zeaxanthin epoxidase and constitutively containing zeaxanthin even in low light, while lacking violaxanthin and neoxanthin; Niyogi *et al.*, 1998); *lut2* (lacking the expression of functional

lycopene ε-cyclase and lacking lutein; Pérez-Bueno and Horton, 2008); *lut2npq2* (possessing zeaxanthin as the only xanthophyll; Havaux *et al.,* 2004); and *lut2npq1* (lacking lutein and unable to deepoxidate violaxanthin to zeaxanthin; Niyogi *et al*., 2001). The mutant lines of *Arabidopsis* deficient in PsbS *npq4-1* (Li *et al.,* 2000) and PsbS overexpresser L17 (Li *et al.,* 2002b) were also used. All the mutants were from same background (Col-0) and seeds were obtained from various collections: *npq1*, *npq2* and *npq4* (*Nottingham Arabidopsis Stock Centre*), *lut2* (Patrick Romano), *lut2npq1*, *lut2npq2* and L17 (Kris Niyogi). Plant material was always collected for analyses and sample preparation at the beginning of the photoperiod, after 2 hr of illumination. Fully expanded rosette leaves were used for all measurements.

2.3 Chlorophyll fluorescence induction

Room temperature chlorophyll fluorescence was measured with a pulse amplitude modulated PAM-101 or Dual-PAM-100 Chlorophyll Fluorescence Measuring System (*Heinz Walz, Effeltrich*). All the fluorescence measurements were performed by using DualPAM software. For analysis of NPQ, the plants were adapted in the dark for 30 min prior to measurement. Fluorescence quenching was induced by two periods of 5 min actinic illumination at 700 μmol photons m^{-2} s^{-1}, provided by arrangement of 635 nm LEDs illuminating both the adaxial and abaxial surfaces of the leaf. Each period of actinic illumination was followed by 5 minutes of dark relaxation period. The maximal fluorescence in the dark-adapted state (Fm), during the course of actinic illumination (Fm') and the subsequent dark relaxation period were measured by a 0.8 s saturating (4000 μmol photons m^{-2} s^{-1}) light pulse applied at 1 to 2 min intervals. NPQ was determined as ((Fm-Fm')/Fm'). Fv/Fm was measured at the beginning of the analysis in the dark adapted state. The reversible component (relaxing within 5 min) was assigned to energy dependent NPQ (qE) and was calculated as ((Fm/Fm') – (Fm/Fm''), where Fm'' is the maximal yield of fluorescence after 5 min of dark relaxation following the actinic illumination. To obtain complete inhibition of violaxanthin de-epoxidation, leaves were vacuum infiltrated with a 5 mM dithiothreitol (DTT) solution. For the measurement of state transitions, interference

filters were used to transmit light of 650nm PSII light of 40 µmol photons m^{-2} s^{-1} and 715nm PSI light of 10 µmol photons m^{-2} s^{-1}, using 650FS10-25 and 715FG07 filters respectively. To analyse fluorescence quenching kinetics, a SigmaPlot software curve-fitting procedure (*SPSS*, Chicago, IL) was used.

2.4 Chlorophyll fluorescence lifetime measurement

Time-correlated single photon counting measurements were performed using a FluoTime 200 picosecond fluorometer (*PicoQuant GmbH*). Excitation light was provided by a laser diode at 470 nm with 10 MHz repetition rate. Fluorescence was detected with 2 nm slit width at 685 nm for leaves and 680 nm for isolated LHCII and CP26. The instrumental response function was in the range of 50 ps. For lifetime analysis, FluoFit software (*PicoQuant*) was used. To measure the chlorophyll lifetime in photosynthetic state of the dynamic range (unquenched state, Fm), detached leaves were vacuum infiltrated with 50 µM nigericin to completely inhibit NPQ. The excitation light intensity was carefully adjusted to completely close all PSII reaction centres without causing photoinhibitory quenching of Fm and to be far below the onset of singlet-singlet annihilation. White light intensity of 700 µmol photons m^{-2} s^{-1} was used to induce the NPQ state (quenched state, Fm') *in vivo*.

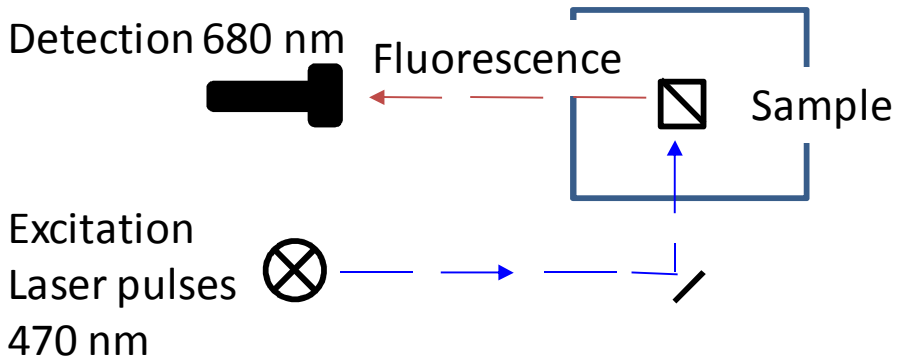


Figure 2.1 Diagrammatic presentation of time-correlated single photon counting setup to measure chlorophyll fluorescence lifetime, using a picosecond fluorometer.

2.5 Preparation of thylakoid membranes

Unstacked thylakoids were prepared by homogenising approximately 50g fresh dark adapted *Arabidopsis* leaves in 300 ml of ice-cold grinding medium (330 mM sorbitol, 10 mM $Na_4P_2O_7x10H_2O$, 2 mM D-iso-ascorbate, pH 6.5) with a Polytron (*Kinematica*). The homogenate was then filtered through four layers of muslin followed by two layers of muslin containing a central layer of absorbent cotton wool. The filtrate was centrifuged using Harrier 18/80 (*MSE*) centrifuge for 10 min at 4000 x g, and the chloroplast-enriched pellet was resuspended in wash buffer (330 mM sorbitol, 10 mM MES, pH 6.5) and again centrifuged for a further 10 minutes at 4000 x g. The pellet was then resuspended in 30 ml of resuspension medium (330 mM sorbitol, 50 mM HEPES, pH 7.6) and osmotically shocked by mixing with 50 ml break medium (10 mM HEPES, pH 7.6) for 30 s to lyse any remaining intact chloroplasts. The osmotic potential was returned to normal immediately by addition of an equal volume of 50 ml osmoticum medium (660 mM sorbitol, 100 mM HEPES, pH 7.6). The thylakoids were centrifuged once again for 10 min at 4000 x g and resuspended in the resuspension medium. Stacked thylakoids were prepared using the same protocol with the addition of 5mM $MgCl_2$ in all the media.

2.6 PSII membrane preparation (BBYs)

Preparation of PSII particles preparations was carried out following the protocol of Berthold *et al*. (1981). Approximately 40g of fresh leaves *Arabidopsis* were homogenised in 300 ml of ice cold grinding medium (330 mM sorbitol, 10 mM $Na_4P_2O_7x10H_2O$, 5 mM $MgCl_2$, 2 mM sodium D-iso-ascorbate, pH 6.5) with 2-3 short bursts from a Polytron. The homogenate was initially filtered through 4 layers of muslin followed by 2 layers of muslin containing in between a central layer of highly absorbent cotton wool. The sample was then centrifuged at 4000 x g for 5 minutes, the supernatant discarded and the pellet resuspended in washing medium (330 mM sorbitol, 10 mM MES, pH 6.5) before centrifugation for 7.5 minutes at 4000 g. The resulting pellet was resuspended in 30 ml of resuspension medium (330 mM sorbitol, 5 mM $MgCl_2$, 40 mM MES, pH 6.5) and osmotically shocked by the addition of 50 ml of breaking medium (5 mM $MgCl_2$, pH 7.6). The osmotic potential was

restored after 30 seconds by the addition of 50 ml of a double osmotic strength medium (660 M sorbitol, 5 mM $MgCl_2$, 40 mM MES, pH 7.6). The thylakoids were then centrifuged for 10 minutes at 4000 x g and the pellet resuspended in stacking medium (5 mM $MgCl_2$, 15 mM NaCl, 2 mM MES, pH 6.3). A 0.5 ml aliquot was used for chlorophyll determination, and the rest of the sample was resuspended to a final chlorophyll concentration of 3 mg/ml in stacking medium. The sample was left on ice in the dark without stirring for a minimum period of 45 minutes to promote membrane stacking. Following this, the sample was then diluted with half its volume of 10 % (v/v) Triton X-100 in stacking medium to give a final detergent concentration of 3.33 % (v/v). The sample was then incubated on ice for 30 minutes with occasional gentle inversions to help membrane digestion. After this step, the digestion was stopped by dilution of the detergent with the addition of at least 6 ml of stacking medium. The sample was then centrifuged for 30 minutes at 30000 x g (4 °C) in a *Beckman* J2 centrifuge using a J2-21 rotor. The pellet was resuspended in particle wash medium (2 mM EDTA, pH 7.5) and again centrifuged as in the previous step (30000 x g, 30 minutes at 4 °C). The supernatant was discarded and the final pellet resuspended in deionised water. Samples were used as required or frozen in liquid nitrogen and stored at –80 °C.

2.7 Sucrose gradient separation

Sucrose density gradient centrifugation was performed for further purification (removing PSI and free pigments) of BBY PSII particles. Seven step exponential sucrose gradients from 0.15 to 1.0 M sucrose were used. Two 60 ml sucrose stock solutions of 0.1 M and 1.5 M in 20 mM HEPES buffer containing 20 mM n-dodecyl β-D-maltoside (pH 8.0) were prepared for a total of six tubes. 1.5 ml of 0.1 M sucrose was pipetted into each of the six tubes (9 ml total). Subsequently, 9 ml of the 1.5 M stock solution was added to the 0.1 M stock and mixed. Again, a total of 9 ml of the latter stock are added to the tubes. This process was repeated six times to form the gradient. 200 to 500 ml of sample were loaded onto each tube and centrifuged at 200 000 x g in a SW41 rotor for 18 h at 4°C.

2.8 LHCII isolation

Major and minor antenna complexes were isolated from unstacked wild-type and mutant thylakoid membranes using non denaturing IEF (isoelectric focussing). The procedure was carried out using a Multiphor II Electrophoresis system (*Pharmacia*) following the protocol of Bassi *et al.* (1991) as modified by Ruban *et al.* (1994). A number of gels and ampholites were used to analyse their suitability, as previously described Ultrodex 50 gel and Ampholine pH 3.5-5.0 carrier ampholite were no more available. Therefore various alternative Sephadex gels (G-50, G-75, G-100, G-150) were used and G-75 was found as most suitable among the available types. Similarly Pharmalytes with various pH ranges like 2.5-5.0, 4.2-4.9, 4-6.5, 3.5-9.5 carrier ampholite were used one with pH 2.5-5.0 was found as an alternative to Ampholine pH 3.5-5.0.

A slurry of volume 100 ml containing 4.6% Sephadex G-75 (*GE Healthcare Bio-Sciences*), 2% Pharmalyte pH 2.5–5.0 (*GE Healthcare Bio-Sciences*), 1% glycine and 0.06% n-dodecyl β-D-maltoside was prepared. Electrode strips were soaked in 2 % (w/v) pharmalyte solution (pH range 2.5-5.0), excess solution was removed with tissue and the strips placed at each end of the glass gel tray with dimensions of 24.5 x 11.0-cm. The slurry was carefully poured into the gel tray, avoiding air bubbles. The tray was then placed on a balance, with a small fan installed about 70 cm above to evaporate the excess water. The set up was left for approximately 2-3 hours in order to evaporate 37g of water. The anode and cathode strips were prepared by soaking the strip in either anode solution (5.6 % (v/v) H_3PO_4) or cathode solution (1 M NaOH). The excess solution was removed with a tissue and the strips were carefully placed at either end of the gel on top of the electrod strips. A 0.1 % (v/v) solution of Triton X-100 was applied to the surface of the Multiphor II cooling plate to ensure good thermal contact with the gel tray. The gel tray was then placed on the cooling plate and the electrodes connected to the electrode strips. Pre-focusing of the gel was carried out at ~8 W (13 mA, 600 V) for 30-60 mins. 2 ml of freshly prepared unstacked thylakoids or PSII particles, with a total chlorophyll concentration of 2.5mg/ml, were used. Then from 10% n-dodecyl β-D-maltoside stock solution, 0.5 ml for thylakoids or 0.3 ml for BBY samples was added and the sample was incubated on ice for 30-60 minutes with

occasional stirring. The sample was centrifuged at 5000 x g for a minute to remove any insoluble material, and then the supernatant was applied 2 cm from the cathode on the pre-cooled and pre-focussed gel using a sample applicator (10 x 2 cm). After sample application the gel was allowed to equilibrate for 3 minutes before the start of focusing. The focusing procedure was carried out for 18 h (overnight) at a constant power of 8W at 4 °C. The initial and final current values were normally ~15 and 5 mA, respectively. Each green band corresponding to the different light harvesting complexes was carefully collected using a spatula. The samples were eluted using a minimum volume of a solution containing 100 mM HEPES (pH 7.6) and 0.01% n-dodecyl β-D-maltoside using a plastic Pasteur pipette. The samples were then loaded onto a desalting column to remove the ampholine, using a desalting buffer (25 mM HEPES and 0.01-0.03 % (w/v) n-dodecyl β-D-maltoside as required).

As mutant LHCII exists in the monomeric rather than trimeric form (Lokstein *et al.,* 2002; Havaux *et al.,* 2004), monomers were prepared from wild-type trimers according to the method described in Nussberger *et al.* (1993), Mutant samples were treated in the same way as a control. For monomer preparation, the trimeric LHCII was treated with phospholipase A2 from bee venom for 48 h at room temperature in the presence of 20 mM $CaCl_2$ in a sterilized eppendorf tube at a chlorophyll concentration of 500 dmol/ml. Immediately after the treatment, the sample was tested for monomerisation by FPLC and absorption spectroscopy. The monomeric LHCII were loaded onto a desalting column as for the trimers onto a desalting column, and then used for analysis.

2.9 Pigment analysis - High Pressure Liquid Chromatography (HPLC)

For analysis of pigments in leaves, 1 cm leaf disks were taken from fully expanded mature leaves and immediately frozen in liquid N_2 and stored at -80 °C until use. Leaf disks were ground in 400 µL cold 100% acetone. The samples were centrifuged at 5000 x g and filtered before collecting in the sample inserts. Pigments from various antenna protein samples were detected by first subjecting to a phase separation- for concentrated major LHCII 40 µl and for diluted minor complexes 200 µl of sample was mixed in mini glass

tube with 340 µl water, 0.5 ml ethanol and 1 mL diethyl ether. The hazy mixture was formed by inverting the tube few times and then allowed to stand. The coloured organic phase on top containing the pigment was then carefully extracted using a fine syringe. The collected sample was taken in a small conical flask and then dried down under a jet of N_2. The dried pigment was resuspended in 200 µL cold 100% acetone and collected in sample inserts. These inserts were placed in brown glass vials and sealed with caps containing PTFE (polytetrafluoroethylene) septa. Pigment composition was determined by HPLC using a LiChroCART RP-18 column (*Merck*) and *Dionex* chromatography system. Two solvents system was used (solvent A: 87% acetonitrile, 10% methanol, 3% 0.1 M TRIS pH 8; solvent B: 80% methanol, 20% hexane). The gradient from solvent A to solvent B was run at a flow rate of 1 mL/min with the following run profile.

0 – 18 minutes: 100% Solvent A

18 – 25 minutes: 0% to 100% Solvent B

25 – 36 minutes: 100% Solvent B

36 – 38 minutes: 100% to 0% Solvent B

38 – 46 minutes: 100% Solvent A

Spectra were recorded between 280 and 750 nm using a *Dionex* PDA-100 photodiode array detector. Each peak was integrated at its optimum absorbance and analysed using *Dionex* Chromeleon software. The system was calibrated using chlorophyll and carotenoid standards of known concentration obtained from *DHI,* Hørsholm (Denmark). The conversion factors allowing the calculation of pigment concentration from the integrated peak area were determined by calibration with the pure pigments.

2.10 Polypeptide analysis

The LHCII proteins were analyzed using immunoblotting essentially as described by Ganeteg *et al.,* (2004). Protein fractions were solubilised in equal volume of 2 x Laemmli buffer, the samples were incubated at 85 °C for 20 min and then proteins were separated by 15% denaturing SDS-PAGE mini gels (Laemmli, 1970), with or without 6M Urea, using Bio Rad Mini Protean Tetra Cell and PowerPac Basic . 10 µL of sample were loaded in each lane, alongside broad-range molecular weight markers 'Benchmark' (*Invitrogen*). Protein bands were stained using Coomassie brilliant blue stain (0.25 % (w/v) Coomassie brilliant blue-R250, 10 % (v/v) methanol, 7 % (v/v) acetic acid, 83 % (v/v) dH2O). Gels were de-stained for 10 hours in 10 % (v/v) methanol, 7 % acetic acid 83 % (v/v) dH_2O.

The PsbS detection was carried out by Western or immunoblotting of thylakoid membranes as described by Jansson *et al.,* (1997). The sample preparation and separation by SDS-PAGE was performed as mentioned above. Sample containing 0.2 to 2 µg of chlorophyll was loaded per lane. Chlorophyll concentration was determined using the method of Porra *et al*., (1989). The gels were blotted onto Hybond nitrocellulose membrane (*Amersham Biosciences*), with the help of Bio Rad Mini Trans-Blot Cell. Primary antibodies for PsbS immunodectection (*Agrisera*), raised in chicken, were detected by a secondary IRDye 680 donkey anti-chicken IgG antibody. The Odyssey Infrared Imaging System with Odyssey software (*LI-COR Biosciences*) was used to obtain high resolution images.

2.11 Determination of chlorophyll concentration

The chlorophyll concentration in various samples was measured following the protocol of Porra *et al.* (1989). Pigments were extracted with 80 % (v/v) acetone and centrifuged at 3000 x g for 5 minutes to remove insoluble material. Sample absorption was measured at 663 nm (A663) and 645 nm (A645) using U2800A UV-Vis spectrophotometer (*Hitachi*). Chlorophyll concentration and chlorophyll *a*/*b* ratio were estimated using the following equations.

[Chl *a*]= 12.7 (A663)-2.69 (A645) (Equation 2.1)

[Chl *b*]= 22.9 (A645)-4.68 (A663) (Equation 2.2)

Total [Chl]= 20.2 (A645)-8.02 (A663) (Equation 2.3)

Chl *a/b* ratio = [Chl *a*] / [Chl *b*] (Equation 2.4)

2.12 Room temperature absorption spectra

Absorption spectroscopy of samples was performed using a U-3310 UV/Visible scanning spectrophotometer. The absorption spectra were recorded for standard measurements from 380-750 nm with a 2 nm slit width and data interval of 1 nm. Data analysis and manipulation was done using UV Solutions software.

2.13 Low temperature fluorescence

Low temperature fluorescence spectroscopy was performed using a Jobin Yvon FluoroMax-3 spectrophotometer with the help of an Optistat DN LN-2 cooled bath cryostat (*Oxford Instruments*). The thylakoid samples were diluted in a medium containing 20 mM HEPES buffer, pH 7.8, 5 mM $MgCl_2$, and 0.33 M sorbitol. The chlorophyll concentration was 12 μg/ml. The excitation light was provided from a Xenon light source. For fluorescence emission spectra, excitation was defined at 435 nm with a 5 nm spectral bandwidth. The fluorescence spectral resolution was 1 nm. In fluorescence excitation measurements fluorescence was detected at 680, 685, 695 and 735 nm for thylakoid and at 680 and 740 nm for LHCII samples. The excitation spectral resolution was 1 nm. The spectra were automatically corrected for the spectral distribution of the exciting light during data acquisition.

2.14 Absorbance changes in leaves

Absorbance measurements were performed, in the 400- to 560-nm range, using an *SLM* Aminco DW2000 dual wavelength spectrophotometer. Leaf samples detached from dark adapted plants and petioles wrapped in moist filter paper were placed in leaf holder. The leaf holder was inserted in the pre cooled (16^{o}C) sample chamber, facing at 45^{o} both the actinic light and the measuring beams. A 250-W tungsten halogen lamp**,** defined by a *Corning* 5-58 Filter, was used to deliver the red actinic light intensity (700 µmol PAR m^{-2} **s^{-1})** with a water filled cuvette in the light path as heat filter along with a long focus lens in between the sample and light source. The photomultiplier was protected by a *Corning* 4-96 filter and a 585nm short pass filter. The instrument slit width was 5 nm and the scan rate was 2 nm/s. For measuring light and recovery spectra, leaf sample was illuminated for 1 min and then kept in dark for 4-5 mins, respectively. These spectra were recorded with two monochromatic beams of 435 and 565 nm, within the range of 410 to 565 nm, at scan rate of 5.0. Filter set-up has been shown in the Fig. 2.2.

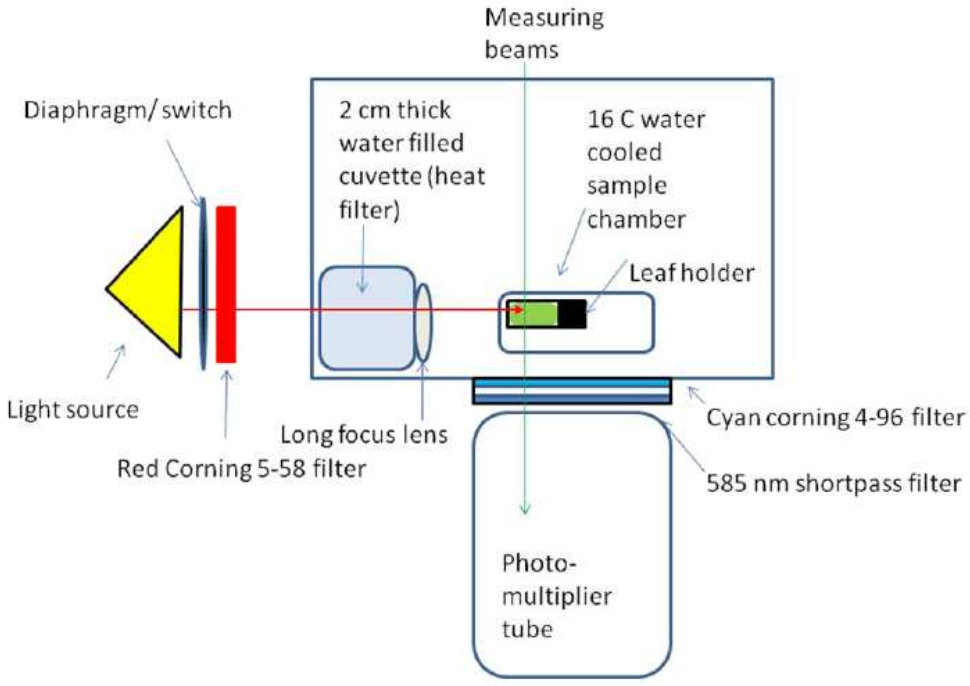


Figure 2.2 Filter setup for Soret region spectra / kinetics.

2.15 Gel filtration- Fast Protein Liquid Chromatography (FPLC)

For FPLC analysis, stacked thylakoid membranes were diluted to a final chlorophyll concentration of 1.0 mg ml^{-1} and solubilised by the addition of *n*-dodecyl α-D-maltoside to three final detergent concentrations of 0.5%, 0.8% and 1.1%. The samples were vortexed thoroughly for 1 min, left to stand on ice for 30 min and then centrifuged for 1 min at 16,000 *g*. The supernatant was then filtered through a 0.54 μm *Minisart* nylon filter and subjected to gel filtration chromatography using a Superdex 200 HR 10/30 column in an *Amersham Biosciences* ÄKTA purifier system run at a flow rate of 0.4 mL min^{-1} using a running buffer containing (20 mM Bis-TRIS, 5 mM $MgCl_2$, 0.03% *n*-dodecyl α-D-maltoside, pH 6.5). Three wavelengths were set in the programme for UV-900, 280 nm for proteins, 670 nm for chlorophylls and 480 nm for carotenoids. By using Unicorn software, composition of supercomplex protein fractions, depicting the stability of membrane macrostructure, was estimated.

2.16 Genetic crossing

Genetic crosses were performed according to standard procedures (Somerville and Ogren, 1982). Fully grown flowering *npq1* and L17 plants were selected. For emasculation, a cluster of buds on the main flowering stem was selected, removing all side branches. All flowers, open buds and tiny central buds were removed, leaving behind only 2-3 closed buds to be emasculated on the floral stem. Using *Dumont* biology tweezers no.5 dumostar (*Agar Scientific*), floral buds were opened to remove all anthers. These floral buds containing only carpels were cross pollinated after 2 days, *i.e.* emasculated *npq1* carpels were pollinated by L17 pollens and *vice versa*. When the tips of siliques turned yellow, they were harvested to be stored in eppendorf tubes for drying out.

Chapter Three

Effects of xanthophyll composition on the regulation of light harvesting in plants

3.1 Introduction

Xanthophylls have been implicated to perform three major roles in the light harvesting antenna, photoprotection against excess excitation energy, structural stabilization of proteins and light harvesting. Any change in the xanthophyll complement of the antenna is therefore likely to affect these functional and structural roles. The xanthophyll composition of each protein member of the LHC family is unique; however the highly conserved secondary structure among these proteins suggests the presence of similar xanthophyll binding sites in all of them. In various xanthophyll mutants of *Arabidopsis thaliana*, a high variability in occupation of the xanthophyll binding sites even under *in vivo* conditions provides a good opportunity to explore the role and need of these xanthophylls. Since binding of xanthophylls has been indicated to be a prerequisite for the stability of LHC proteins (Plumley and Schmidt, 1987), any change in the xanthophyll complement can accordingly alter the organisation of the antenna proteins in the membrane. These changes can lead to structural anomalies as well as variable functional capacities for photoprotection and light harvesting in photosystem II. In this chapter, photoprotective, light harvesting and structural roles of xanthophylls will be elaborated by study of qE and related conformational changes, photochemical parameters and state transitions, respectively. As this study deals with investigating the molecular mechanism of photoprotection in higher plants, therefore functional role of xanthophylls in this regard is studied in detail.

There is strong evidence that the site of photoprotective qE is located in the light-harvesting PSII antenna and that xanthophylls are involved in this function, though the knowledge of the mechanism of energy dissipation remains controversial. Two distinct quenching mechanisms have been suggested, one involving zeaxanthin (type I) and the other lutein (type II). The type I mechanism proposes qE to be obligatorily dependent upon zeaxanthin, which acts as a quencher of chlorophyll excitation energy via the formation of a charge transfer state. Evidence for this quenching mechanism is the formation of a carotenoid radical cation that correlates with the extent of qE showing absorption at approximately 1,000 nm (Holt *et al.,* 2005). Further evidence in this regard was in the form of report of a zeaxanthin radical cation formation which occurs exclusively at the L2 binding site of the

minor antenna complexes (Ahn *et al.,* 2008; Avenson *et al.,* 2008), suggesting reversible insertion of zeaxanthin into this internal site as a prerequisite for quenching. The type II mechanism presents the xanthophyll bound at the L1 site in the major antenna complexes as an effective quencher qE of chlorophyll excited states, controlled by the intrinsic property of LHCII proteins to undergo a conformational change altering the configuration of bound pigments (Ruban *et al.,* 2007; Ilioaia *et al.,* 2008). In this mechanism, the role of zeaxanthin has been suggested as an allosteric modulator of the ΔpH sensitivity.

There is possibility for both the above mentioned mechanisms to contribute to *in vivo* qE, since the quenching occurs in both the presence and absence of zeaxanthin (Adams *et al.,* 1990; Crouchman *et al.,* 2006). Despite the involvement of different xanthophylls operating at discrete sites, both mechanisms have been proposed to evolve similarly as a result of a ΔpH-triggered, PsbS-mediated conformational change (Ruban *et al.,* 2007; Ahn *et al.,* 2008). Here a crucial question arises as whether zeaxanthin-dependent and zeaxanthin-independent qE arise from two different mechanisms (types I and II, respectively) or from the same mechanism (type II). The formation of qE comprises of two components: the initial rapidly formed component is zeaxanthin independent; while the second slowly formed component is zeaxanthin dependent as it correlates with violaxanthin de-epoxidation (Adams *et al.,* 1990; Ruban and Horton, 1999). Both the components of qE relax rapidly in the dark (Adams *et al.,* 1990)

The formation and relaxation kinetics of qE can be investigated to determine whether two mechanisms can account for two components of qE. Here, we test the hypothesis that the two components arise from different mechanisms at two discrete sites: the zeaxanthin-dependent component originates in the minor monomeric antenna by a type I mechanism (Gilmore *et al.,* 1998; Ahn *et al.,* 2008; Avenson *et al.,* 2008), and the zeaxanthin-independent component originates in the major trimeric LHCII by the type II mechanism. The PSII reaction centre quenching has also been suggested to contribute towards the zeaxanthin-independent component of qE, at least under low-light conditions when transient qE is formed (Finazzi *et al.,* 2004). This hypothesis gives rise to some predictions. First, the two components could not compensate for the loss of one another because their

effect would be additive (Niyogi *et al.,* 1998; Pogson *et al.,* 1998) and each of them should contribute a discrete component to the kinetics of qE formation and relaxation. Second, in the mutants lacking lutein, zeaxanthin-dependent component should remain unaltered, while the capacity of the type II mechanism would be reduced. Finally, the two components may be anticipated to correlate with different absorption changes in the Soret region. These absorption changes are result of conformational changes within the PSII antenna upon qE formation and depict the variation in the absorption spectra of bound pigments as a consequence of conformational changes (Ruban *et al.,* 1993a, 1993b, 2002b; Bilger and Björkman, 1994). In this work, the investigation of qE formation and relaxation kinetics, qE-related absorption difference spectra and absolute chlorophyll fluorescence lifetimes has been carried out to test the above mentioned hypothesis. Contrary to the above predictions, the data demonstrates that qE, in both steady and transient forms, is controlled by a single common mechanism within the PSII antenna, irrespective of zeaxanthin.

As mentioned earlier, xanthophylls are also believed to play significant role in structural stability and light harvesting of LHCII antenna. Thereby any change in xanthophyll composition is likely to influence these two roles quite similarly to the effect on photoprotective capacity and dynamics. For this purpose various fluorescence parameters related to photochemistry were also investigated. On one hand, parameters like photochemical quenching (qP), yield of PSII (ΦII) and electron transport rate of PSII (ETR II) indicate towards the light harvesting efficiency of the antenna as a result of any change in the spectral cross section influenced by xanthophyll composition. On the other hand role of a particular xanthophyll in the assembly and stability of antenna can be assessed by state transition parameter, demonstrated by migration and attachment of LHCII antenna under low light conditions. All these methods are non-invasive and insightful, thus can provide reliable and ample information regarding role of xanthophyll in the photosynthetic membrane system.

3.2 Results

3.2.1 Pigment composition of xanthophyll mutants

Xanthophyll mutants were analysed for their pigment composition in comparison to that of the wild type plants. Wild type and xanthophyll mutant plants were grown for 8 to 10 weeks under similar control growth conditions of light and ambient temperature (100 µmol photons m^{-2} s^{-1}, day/night temperature of 22°C/18°C, 8 hr photoperiod). Leaf discs were collected from these plants to analyse their pigment composition by HPLC in both light-treated and dark-adapted conditions. The expected general differences were found between the xanthophyll mutants and the wild-type plants (Table 3.1) as reported previously (Niyogi *et al*., 1998; Havaux *et al*., 2004; Pérez-Bueno and Horton, 2008).

Both antheraxanthin and zeaxanthin were absent in *npq1*, while *npq2* was lacking neoxanthin, violaxanthin and antheraxanthin. Both *lut2* and *lut2npq1* mutants were lacking lutein, whereas in *lut2npq2* all the xanthophylls were absent except zeaxanthin. Absence of any xanthophyll in these mutants was compensated by an increase in the quantity of other xanthophylls, for example all lutein deficient mutants maintained their total xanthophyll contents by an almost similar increase of xanthophyll cycle pool size. All lutein mutants and *npq2* were found to have larger xanthophyll cycle pool than wild type, however no significant difference in pool size was observed between *npq1* and wild type despite the fact that *npq1* lacked antheraxanthin and zeaxanthin. In the internal L1 and L2 Lhcb protein-binding sites of lutein mutants, violaxanthin has been suggested to replace lutein in case of *lut2* and *lut2npq1* mutants, whereas zeaxanthin being the sole xanthophyll in *lut2npq2* substitutes lutein at these sites. Notably, both *lut2* and *lut2npq1* retained significant amounts of their zeaxanthin and antheraxanthin components even in the dark-adapted condition, implying that a fraction of these de-epoxidised xanthophylls is not available for epoxidation. The light-induced de-epoxidation was absent in both *npq1* and *lut2npq1*, whereas 100% de-epoxidation was constitutively present in case of *npq2* and *lut2npq2*.

Plant Type	Neo	Lut	Vio	Ant	Zea	DEPs
Wild-type, dark	5.2 ± 0.5	17 ± 1	4.4 ± 0.2	0.2 ±0.1	0	4±0.9
Wild-type, light	5.1 ± 0.9	16 ± 1	2.0 ± 0.4	0.8 ±0.3	1.7 ±0.4	46±1.2
npq1-dark	5.3 ± 1.1	18 ± 2	4.4 ± 1.1	0	0	0
npq1-light	5.7 ± 0.6	20 ± 1	5.3 ± 0.3	0	0	0
npq2-dark	0	18 ± 1.8	0	0	9.5 ±0.9	100
npq2-light	0	18 ± 1.5	0	0	9.2 ±0.5	100
lut2-dark	4.9 ± 0.8	0	13.7 ±1.4	3.6 ±0.1	1.0 ±0.3	12±2.4
lut2-light	4.8 ± 1.1	0	8.2 ± 0.8	4.5 ±0.9	4.1 ±1.1	38±2.6
lut2npq1-dark	4.9 ± 0.7	0	14.6 ±1.2	2.6 ±0.1	0.9 ±0.8	12±1.9
lut2np1-light	5.2 ± 0.4	0	15.1 ±0.4	2.8 ±0.3	1.2 ±0.2	35±2
lut2npq2-dark	0	0	0	0	15 ±0.8	100
lut2npq2-light	0	0	0	0	15.4±0.5	100

Table 3.1 Pigment composition of wild-type and xanthophyll mutant plants. Leaf discs were collected from plants either dark-adapted for 30 min or light-treated for 10 min at 700 µmol photons m^{-2} s^{-1}. Data are normalized to 100 chlorophyll *a* + *b* molecules and are means ± SE from four replicates. No differences were detected for *npq2*, *npq1*, *lut2npq1*, and *lut2npq2* between light and dark conditions. Neo, Lut, Vio, Ant, Zea and DEPs represent neoxanthin, lutein, violaxanthin, antheraxanthin, zeaxanthin and de-epoxidation state % [(zeaxanthin + 0.5 antheraxanthin)/(violaxanthin + antheraxanthin + zeaxanthin)], respectively.

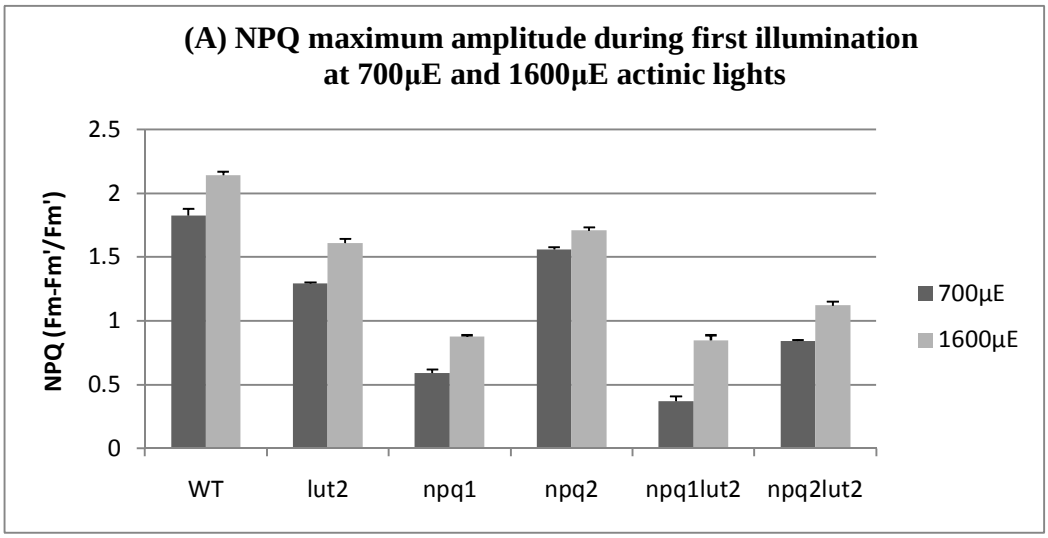
Total chlorophyll contents per unit leaf area of all the genotypes were measured using spectrophotometer and found as similar with no significant differences. However, a significant increase in chlorophyll *a/b* ratio was observed particularly in lutein deficient double mutants (Table 3.2). As Chl *b* is attached particularly to LHCII, so this may provide an evidence of decrease in antenna size in all these mutants.

Plant Type	Total Chl (mg/ml)	Chl *a/b*
WT	3.14 ± 0.22	3.14 ± 0.06
npq1	2.90 ± 0.32	3.18 ± 0.19
npq2	3.05 ± 0.20	3.32 ± 0.16
lut2	2.75 ± 0.38	3.44 ± 0.12
lut2 npq1	3.26 ± 0.25	3.46 ± 0.06*
lut2 npq2	3.11 ± 0.33	3.60 ± 0.15*

Table 3.2 Chlorophyll concentration measurements of wild-type (WT) and xanthophyll mutants. Pigments were extracted with 80 % (v/v) acetone from leaf discs, using UV-Vis spectrophotometer. Mean values of four replicates (±SE) are shown. *= Significantly different to WT (Student's t-test p=0.05).

3.2.2 Effect of xanthophyll composition on qE and related conformational changes

In order to investigate the effect of varying xanthophyll composition on various fluorescence parameters, fluorescence induction curves were recorded on dark adapted wild-type and xanthophyll mutant plants. All of these mutants possess unchanged levels of ΔpH compared with the wild-type plants (Pérez-Bueno *et al*., 2008), the kinetics of NPQ should therefore be affected only by the differences in the xanthophyll contents among these genotypes. Two actinic light intensities, high and moderate (1600 and 700 μmol photons m^{-2} s^{-1}, respectively), were initially used to compare the response of xanthophyll mutants by studying following parameters of PAM chlorophyll fluorescence analysis: qE, qP, qI, quantum yield of PSII, regulated and non-regulated NPQ (ΦII, ΦNPQ, ΦNO, respectively); and electron transport rate of PSII (ETR II). The magnitude of NPQ



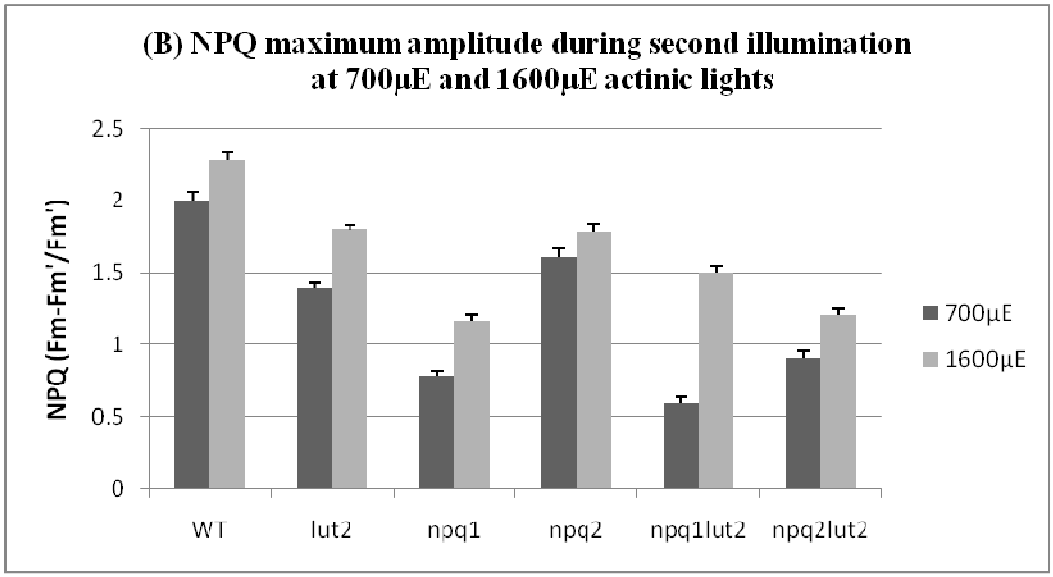


Figure 3.1 Maximum amplitudes of NPQ measured at the end of first 5 min illumination **(A)**, followed by 5 min dark relaxation and then by the end of second 5 min illumination **(B)**. All data are mean ± SE for at least three plants. All data are significantly different to WT (Student's t-test p=0.05).

increased with the use of higher actinic light in all the plants, as high light might be expected to induce higher ΔpH and/or extra zeaxanthin formation. However, use of high actinic light also resulted in the increase of slowly reversible components of NPQ, as

indicated by the effect of uncoupler nigericin (section 3.2.3.5). NPQ amplitude among all the xanthophyll mutants was found lower than that of wild type, at both actinic lights used (Fig. 3.1 A,B).

3.2.2.1 qE Formation Kinetics

To compare the kinetics of both zeaxanthin-independent and zeaxanthin-dependent components of qE, NPQ formation was measured during two successive periods of illumination. The actinic light intensity of 700 μmol photons m^{-2} s^{-1} was selected in order to enhance the reversible qE component of NPQ and to minimize the slowly reversible qI component. During the first illumination, NPQ formation was found as more rapid in *npq2*, *lut2npq2*, and *npq1* than in the wild type, while it was observed as slower in case of *lut2*, however all the xanthophyll mutants showed lower NPQ amplitude than that of wild type. These differences in the kinetics of NPQ formation were found similar to those observed by others (Niyogi *et al*., 1998, 2001; Pogson *et al*., 1998) (Fig. 3.2 A; Table 3.3). The de-epoxidation rates in *lut2* and wild-type plants had been found as virtually identical (Lokstein *et al*., 2002), therefore difference in rates of NPQ formation could not be ascribed to this factor. The NPQ amplitude in *lut2npq1* plants was very small, whilst qE was virtually absent in this double mutant at moderate actinic light; this shows the inhibition of zeaxanthin-independent qE in the absence of lutein and zeaxanthin. Considering this fact, the slower NPQ formation in *lut2* could similarly be assigned to the absence of zeaxanthin-independent qE.

The kinetics of NPQ reformation were also obtained in leaf samples that had previously been subjected to illumination for 10 min at 700 μmol photons m^{-2} s^{-1}, followed by 5 min of dark period in order to achieve qE relaxation. During the second illumination, the NPQ reformation kinetics were accelerated in all the cases compared with the first illumination cycle (Fig. 3.2 B; Table 3.3). The reformation of qE was more rapid in the wild type, taking less than 20 s to reach saturation, and was now even faster than that of the *npq1* mutant. Contrarily, NPQ formation was still at faster rate in the *npq2* as compared to the wild type.

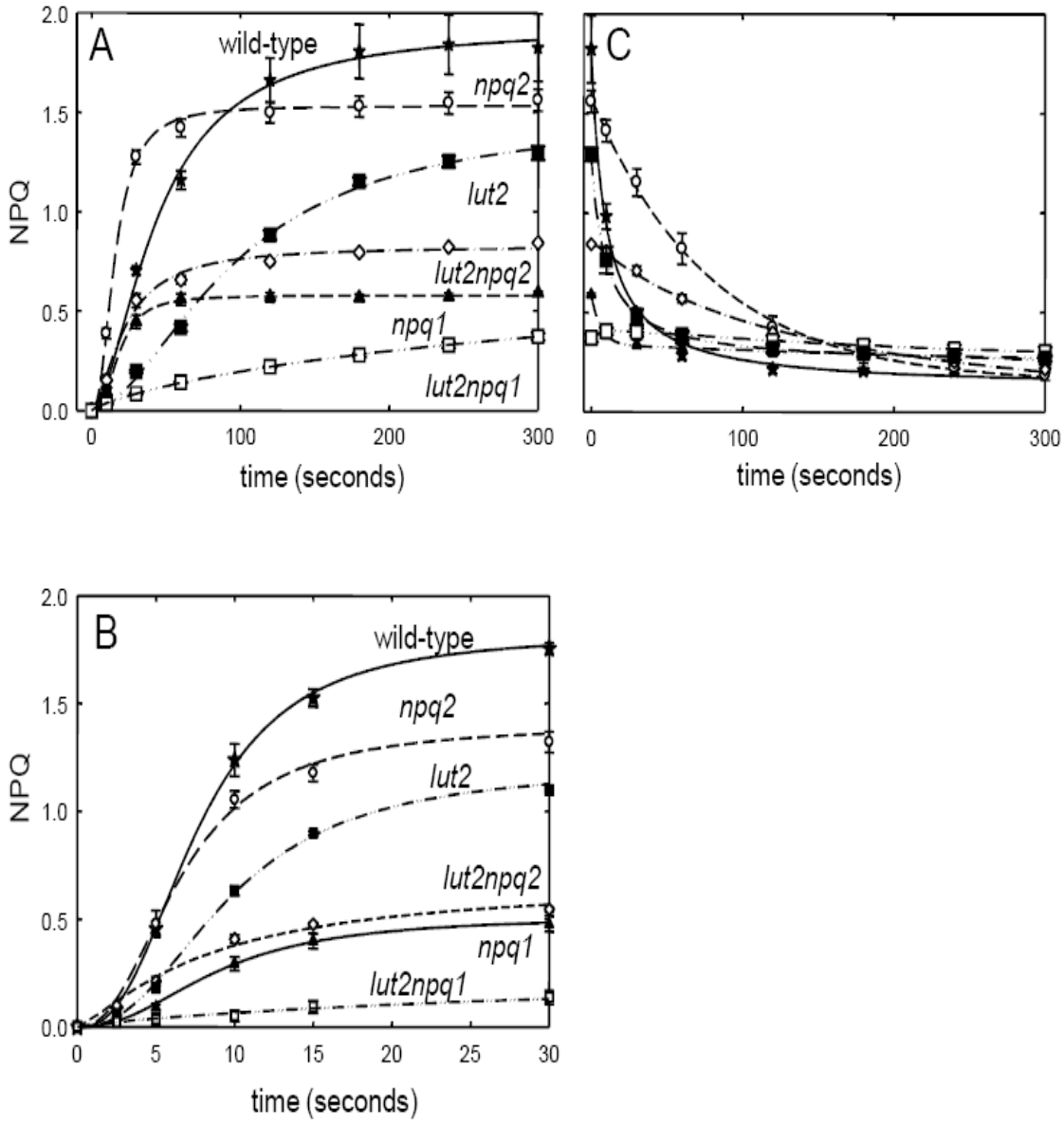


Figure 3.2 Kinetics of NPQ at 700 μmol photons m^{-2} s^{-1} in wild-type and xanthophyll mutant leaves. NPQ formation kinetics in dark-adapted leaves during first 5 min illumination **(A),** followed by 5 min dark relaxation NPQ formation kinetics during second 5 min illumination **(B)** and final NPQ relaxation kinetics during 5 min dark period **(C)**. Average NPQ formation and relaxation curves were fitted in all cases with a Hill function ($y = [ax^b]/[c^b + x^b]$ and a hyperbolic decay ($y = y_0 + [ab]/[b + x]$, respectively. Wild-type (black stars), *npq2* (white circles), *npq1* (black triangles) and *lut2* (black squares), *lut2npq2* (white diamonds). Data is average of 3 experiments ± standard error.

However the effect of lutein replacement by zeaxanthin on qE was intriguing as qE formation in *lut2npq2* was found to be slower and with lower amplitude in both illuminations compared with *npq2*. In the similar way, the lutein replacement by violaxanthin in *lut2* mutant affected qE formation by reducing both rate and amplitude compared with the wild type, despite the fact that there was no difference in de epoxidation state between the two. Therefore in the internal binding sites of the Lhcb proteins, lutein replacement by either violaxanthin or zeaxanthin reduces the rate and amplitude of qE formation, independent of the differences in ΔpH or de-epoxidation state.

Plant	NPQ, Dark Adapted	Formation $t^{1/2}$ (s)	Relaxation $t^{1/2}$ (s)	NPQ Preilluminated	Reformation $t^{1/2}$ (s)
Wild type	1.82±0.1	44±0.9	12.9±1.2	1.75±0.1	7.5±0.4
npq1	0.58±0.1	19±2	5.8±2	0.48±0.1	10.5±0.5
npq2	1.54±0.1	16±0.8	53±5.5	1.32±0.1	6.5±0.1
lut2	1.25±0.1	96±2	10.5±0.5	1.09±0.1	9.5±0.5
lut2npq1	0.37±0.1	120±3	80±3.5	0.35±0.1	16±2
lut2npq2	0.82±0.1	22±1	77±3.4	0.54±0.1	7.5±0.5

Table 3.3 Kinetic parameters of NPQ in wild-type and xanthophyll mutant plants. Half time ($t^{1/2}$) was calculated manually. Data are averages of three independent experiments ± SE, and obtained as for Figure 3.2.

3.2.2.2 qE Relaxation Kinetics

During the dark phase following the first illumination, the kinetics of qE relaxation were also monitored in the wild type and xanthophyll mutants. In all cases, the relaxation of qE could be fitted to a single hyperbolic decay (Fig. 3.2 C; Table 3.3). The mutants with constitutive presence of zeaxanthin, *npq2* and *lut2npq2*, were found to have much slower relaxation kinetics than all others. The rate of relaxation of qE in the *npq1* mutant was measured as twice as fast as in the wild type, as this mutant is without zeaxanthin so qE comprises only of the zeaxanthin-independent component. Contrarily, *npq2* mutant, which contains both zeaxanthin-dependent and zeaxanthin-independent components, relaxed at much slower rate than wild type. The hypothesis regarding involvement of one or two mechanisms in qE can be tested by comparing the relaxation kinetics of *npq1* and *npq2*. If separate type I and type II mechanisms exist, then qE should have relaxed with two components in *npq2*: one - fast zeaxanthin-independent component (similar to *npq1*) and the other - slower zeaxanthin-dependent one. However, defying this prediction, it was observed that the additional constitutive zeaxanthin in *npq2* slowed down the relaxation of all of qE, and not just the zeaxanthin dependent component. Indeed, the rate of qE relaxation in *npq2* was monitored as three times slower than that of the wild type. The relaxation of qE was further slowed down in *lut2npq2* where additional zeaxanthin replaces the internally bound lutein. In contrast, replacement of lutein by violaxanthin in *lut2* resulted in a marginally faster relaxation of qE than in the wild type. In case of *lut2npq1*, as photoinhibitory components have most contribution so the NPQ was slowest to relax. In summary, the amount of zeaxanthin seemed to modulate the rate of qE relaxation, which behaved kinetically as a single process.

3.2.2.3 Transient qE Formation

At low light intensity, qE is transiently formed, which is suggested to form in the PSII reaction centre (Finazzi *et al*., 2004). In this work, effect of alteration in xanthophyll composition on this type of transient qE was also analysed using actinic light of low intensity (100 μmol photons m^{-2} s^{-1}). It was observed that similar to steady state qE

generated at high light intensity, the transient qE formed at low light intensity was also affected by alteration in the xanthophyll composition. The similar dependence on xanthophyll composition was exhibited in transient qE at low light intensity in the form of its strong reduction in *npq1*, *lut2*, and *lut2npq2* mutants compared to wild type and a total absence in case of *lut2npq1* mutant (Fig. 3.3).

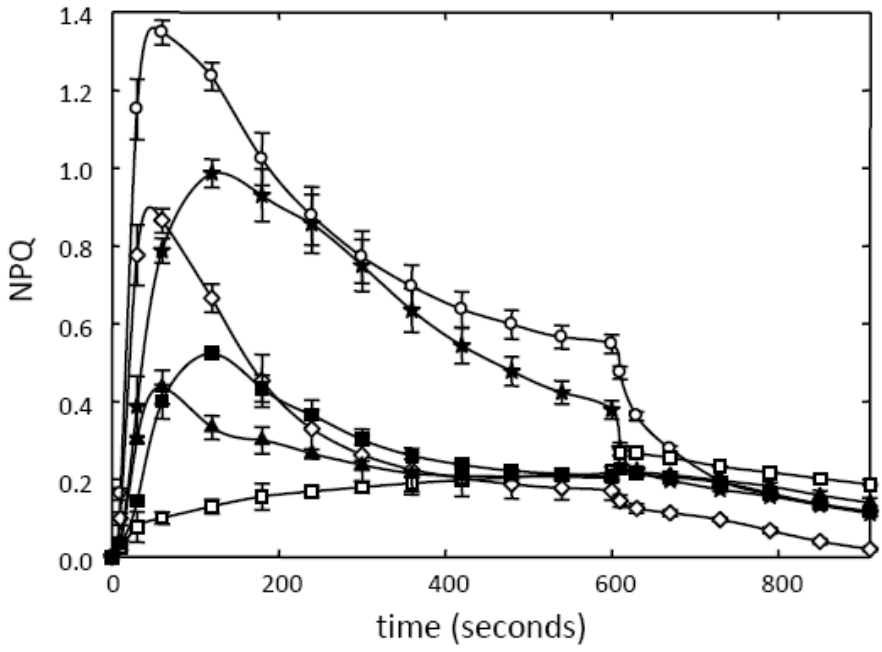


Figure 3.3 Kinetics of transient NPQ at 100 μmol photons m^{-2} s^{-1} in wild type and xanthophyll mutant leaves dark adapted for 30 minutes. Wild type (black stars), *npq2* (white circles), *npq1* (black triangles) and *lut2* (black squares), *lut2npq2* (white diamonds), *lut2npq1* (white squares). Data is average of 3 experiments ± standard error.

3.2.2.4 qE-Related Conformational Changes

A linear correlation has been found between the qE formation and a positive absorption change in leaves at 535 nm (ΔA535), which monitors a conformational change in the PSII antenna that accompanies quenching (Heber, 1969; Bilger and Björkman, 1990; Ruban *et al*., 1993b, 2002b; Bilger and Björkman., 1994). The kinetics of ΔA535 were measured in

the dark adapted leaves of wild type and xanthophyll mutant plants to investigate the qE-related conformational changes. When the ΔA535 kinetics of wild type were compared with those of *npq1*, a smaller amplitude in latter case corresponds to the lower level of qE in this mutant and it confirms that the ΔA535 amplitude is influenced by the de-epoxidation state (Fig. 3.4 A). Similar to qE formation during first illumination, ΔA535 kinetics were formed slower in wild type as compared to the case of *npq1*. In *npq2*, the ΔA535 formation was found faster while relaxation in dark was slower than the corresponding kinetics of wild type, again in agreement with the differences monitored during the qE kinetics. However, the amplitude of ΔA535 in *npq2* was found similar to that of the wild type despite the lower qE amplitude of the mutant.

The ΔA535 signal was completely absent in the *lut2npq1* mutant, which displayed only slowly reversible photoinhibitory component qI without any contribution of qE, also confirms that this signal is only correlated to qE (Fig. 3.4 B). This is also in compliance with the data obtained in the *npq4* mutant lacking PsbS, where no such ΔA535 signal was detected in the absence of qE (Li *et al*., 2000; Ruban *et al*., 2002b). The ΔA535 formation was found faster and relaxation slower in the *lut2npq2* as compared to the corresponding kinetics of the wild type and quite similar to the ones of *npq2*. Once again, a much larger than anticipated amplitude of the signal was detected (like the case of *npq2*), given the fact that the qE amplitude was again less than that of the wild type. The ΔA535 kinetics in *lut2* were detected with lower amplitude and slower formation rate than those of the wild type, in agreement with the smaller qE measured in the mutant. Thus, the discrepancy between the amplitudes of ΔA535 and qE was only observed in case of two mutants with larger de-epoxidation states than that of the wild type.

The absorption difference spectra reveal the nature of the absorption changes that relate to the zeaxanthin-independent component of qE. Any shift in the peak position of various bands provides clues about the involvement of the respective xanthophylls. Thus, the absorption difference spectra of the qE-related conformational changes were recorded in the xanthophyll mutants (Fig. 3.4, C and D).

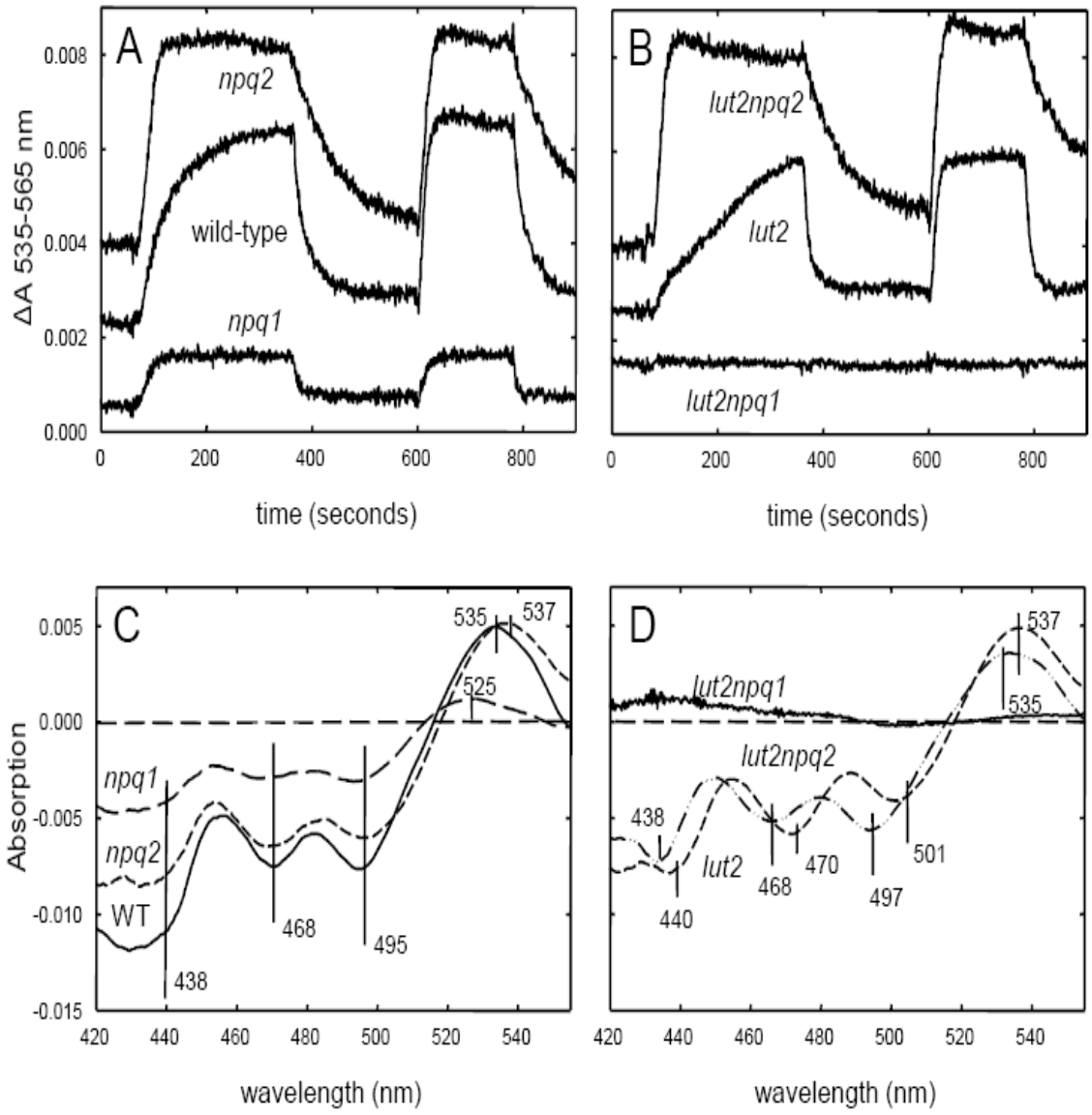


Figure 3.4 Light dependent kinetics of qE related ΔA535 conformational changes at 700 μmol photons m^{-2} s^{-1} and qE absorption difference spectra of wild-type and xanthophyll mutant leaves dark adapted for 30 minutes. (A and B) Kinetics of ΔA535, traces as labeled, 6-10 kinetic traces were averaged, error ± 5%. (C and D) qE absorption difference spectra (5 minutes light- *minus*-5 minutes dark relaxation), Wild-type (solid line), *npq2* (short dashed line), *npq1* (long dashed line), *lut2* (dashed dotted line), *lut2npq2* (medium dashed line), *lut2npq1* (dotted line).

The difference spectra of wild type and *npq1* were found nearly identical to one another with previously observed negative bands at 438, 468, and 495 nm (Fig. 3.4 C). However, the positive band of 535 was blue-shifted to around 525 nm in *npq1*, consistent with the absence of de-epoxidation in this mutant. This positive band of 535 also shifts similarly when the wild-type leaves are infiltrated with dithiotheitol (DTT) to inhibit de-epoxidation (Noctor *et al*., 1991). In correlation with the much reduced qE in *npq1*, all the bands were observed with lesser amplitudes than those found in the wild-type spectrum. In case of *npq2*, the absorption difference spectrum showed few differences from the wild type. A similar amplitude was observed for the positive 535-nm as in the wild type, however a slight red shift to around 537 nm was also detected in this mutant. Interestingly, all the three negative bands in the difference spectrum of *npq2* were of lower amplitude than those of the wild type spectrum, consistent with the lower values of qE in *npq2*. In the 495-nm negative band of *npq2*, a slight red-shift and noticeable broadening was also observed.

In case of *lut2*, the absorption difference spectrum revealed the reduction of all the negative and positive bands in comparison to those in the wild-type spectrum, consistent with the lower qE amplitude in this mutant (Fig. 3.4 D). A slight red shift in the 495-nm negative band to 497 nm was revealed. In the difference spectrum of the double mutant *lut2npq2*, the 535-nm positive band showed similar amplitude to the corresponding bands of *npq2* and wild type difference spectra, with a slight red shift to 537 nm as observed in *npq2*. The 535-band of double mutant was, however, enhanced compared to the corresponding band in the *lut2* mutant difference spectrum, consistent with the similar difference observed in the ΔA535 kinetics data of these two mutants. Once again, negative bands of smaller amplitude were recorded in this double mutant, in agreement with the lower qE amplitude. Interestingly, there was a strong red shift of the 495-nm negative band to around 501 nm. Similar to the kinetics of qE and ΔA535, the absorption difference spectrum of other double mutant *lut2npq1* was found entirely featureless without any negative or positive bands, demonstrating that all the features of the absorption difference spectrum are entirely related to the qE. Interestingly, the transiently forming electro-chromic shift (pigment polarization in membrane only during initial illumination, shown as ΔA 518 nm) was

barely visible in the steady-state spectrum of *lut2npq1*, consistent with a previous study (Li *et al*., 2000).

A similar correlation was also noticed between the qE transiently generated at low light intensity and the transient increase in 535-nm absorption. The absorption difference spectrum of light minus recovery exhibiting the transient absorption change at low light intensity (Fig. 3.5) was found virtually identical to the steady-state absorption difference spectrum observed at high light intensity.

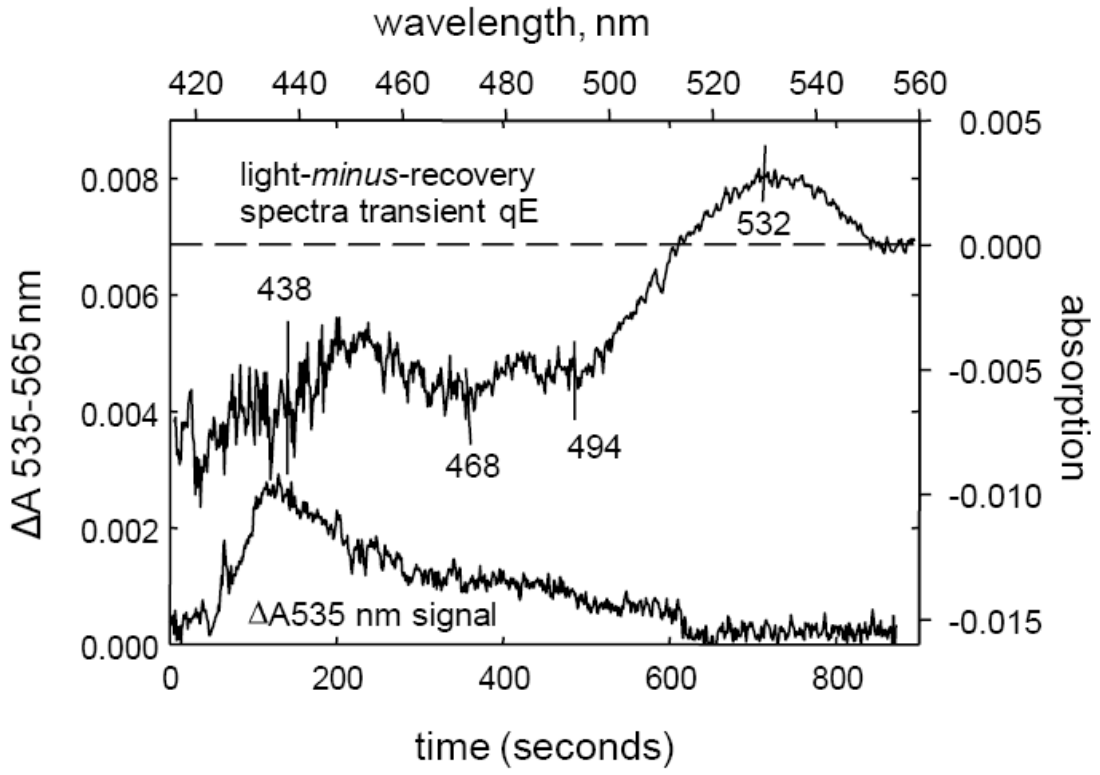


Figure 3.5. Kinetics of ΔA535 conformational changes and qE absorption difference spectra at 100 μmol photons m-2 s-1 measured on wild type leaves previously dark adapted for 30 minutes.

3.2.3 Effect of xanthophyll composition on major photosynthetic parameters of PSII

3.2.3.1 PSII efficiency (*Fv/Fm*):

The intrinsic photosystem II (PSII) efficiency is measured as Fv/Fm. In all genotypes, it was found as >0.8 except in the two zeaxanthin accumulating mutants of *npq2* and *lut2npq2* with lower Fv/Fm values (Fig 3.6, Table 3.4). The lower values of NPQ in *npq2* and *lut2npq2* have also been attributed to the pre-quenching of Fm, the maximum fluorescence in the dark adapted state, due to sustained zeaxanthin-mediated quenching (Dall'Osto *et al*., 2005; Kalituho *et al*., 2006).

To further explore this pre-quenching phenomenon in zeaxanthin-accumulating mutants, chlorophyll fluorescence lifetimes were measured in the leaves of wild type and these mutants in the dark adapted Fm state when all PSII reaction centres were closed by saturating light, along with the use of uncoupler nigericin to block any qE formation. In wild type leaves, the average fluorescence lifetime was 2.0 ns in the dark adapted Fm state, which was consistent with the previously reported values (Gilmore *et al*., 1998). In dark adapted *npq2* and *lut2npq2* leaves, the average fluorescence lifetimes were measured as 1.73 and 1.52 ns, respectively. These shorter than the wild type lifetime values confirm the presence of pre-quenching in these mutants. The respective chlorophyll fluorescence lifetimes were also measured, during the course of actinic illumination Fm' state in the presence of NPQ. In wild-type leaves, an average lifetime of 621 ps was measured with an NPQ value of 2.2, also similar to the previously reported values (Gilmore *et al*., 1998). For *npq2* mutant, the fluorescence lifetime in the Fm' state was slightly longer (637 ps with an NPQ of 1.8) while for *lut2npq2* mutant it was significantly longer (724 ps with an NPQ of 0.95). Thus, *npq2* has similar final extent of Fm' quenching to that in the wild type, this demonstrates that pre-quenching is responsible for the decrease in observed NPQ in this case without any lesion in the NPQ formation. Contrarily, in *lut2npq2*, the final extent of Fm' quenching is much reduced, which shows a real decrease in the amplitude of NPQ in this mutant.

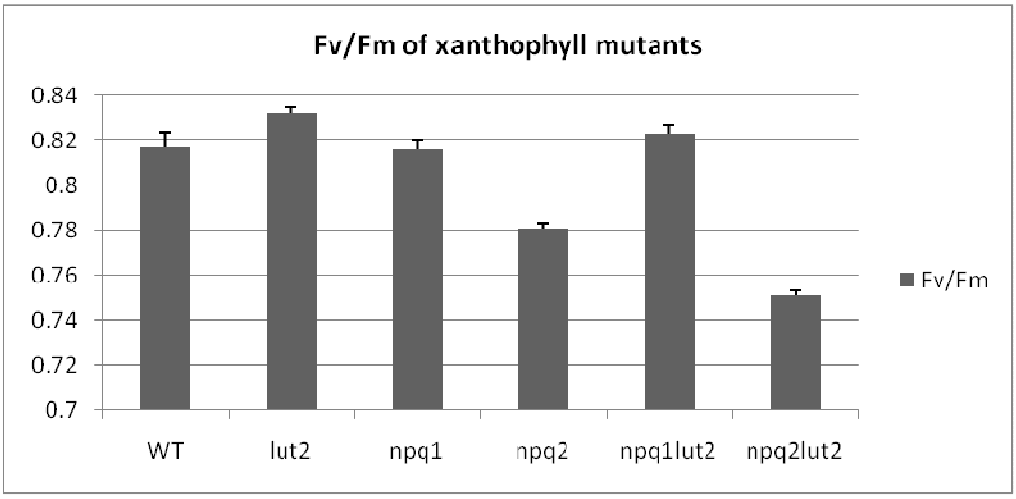


Figure 3.6 Fv/Fm of xanthophyll mutants (measured as Fm-F_0/Fm). All data are mean ± SE for at least three plants.

Plant	F_v/F_m ratio	τFm (ns)	NPQ max	τFm' (ps)
Wild-type	0.82 ± 0.01	2.0 ± 0.02	2.2 ± 0.1	621±13
npq2	0.78 ± 0.01	1.73 ± 0.02*	1.85 ± 0.05	637±32
lut2npq2	0.75 ± 0.01	1.52 ± 0.01*	0.95 ± 0.05	724±8*

Table 3.4 Average chlorophyll fluorescence lifetimes and maximum quantum yield of PSII (Fv/Fm) in wild-type and zeaxanthin accumulating mutants. Average Fm and Fm' state lifetimes of dark adapted leaves from wild-type and xanthophyll mutants under saturating light. Experiments are means ± S.E.M. from 10 replicates. *=Significantly different to wild-type (Student's t-test p=0.005)

3.2.3.2 Photochemical quenching (*qP*):

Utilisation of light energy for photochemical pathway is generally measured in the form of fluorescence quenching and called qP. qP is dependent upon the fraction of open reaction centres (RC). Xanthophylls are believed to act as accessory pigments, complementing the chlorophyll light absorption and hence increasing the spectral cross section of light harvesting antenna.

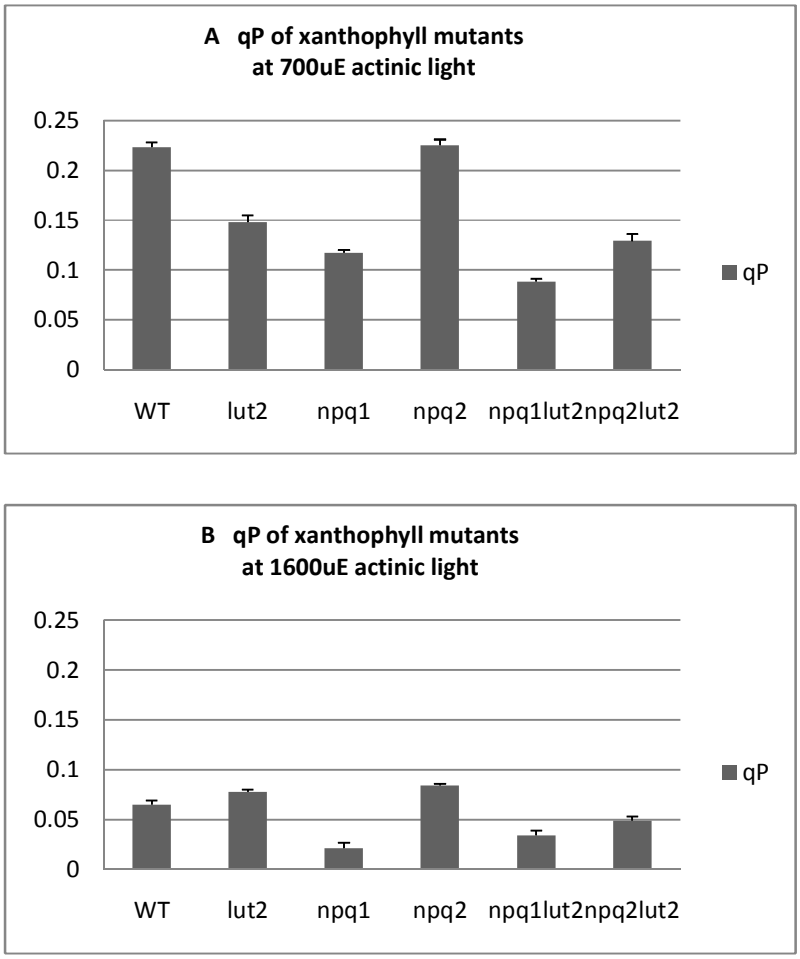


Figure 3.7 (A,B) Photochemical (qP) of xanthophyll mutants at two different actinic lights, calculated as qP= Fm'-Fs/Fm'-Fo'. Data are averages of three independent experiments ± SE. All data are significantly different to WT (Student's t-test p=0.05), except for *npq2* at 700µE.

It was observed that extent of qP was decreased variably in all the mutants, as compared to wild type (Fig.3.7). qP was lowest in both the zeaxanthin deficient mutants of *npq1* and *npq1lut2* while highest qP values were found in *npq2* and wild type. This may indicate towards more reduction of Q_A in the zeaxanthin deficient mutants which also demonstrate inefficient NPQ, while presence of zeaxanthin is likely to reduce excitation pressure in PSII hence leading to higher oxidation of Q_A.

With the increase in actinic light, utilisation of light energy for photochemical pathway decreases as excess energy is dissipated through nonphotochemical quenching. As qP depends on the relative fraction of open reaction centres, so these data may show the less fraction of open RC in all cases when high actinic light is used, diverting most of the light energy for NPQ. Decline in qP also shows the increased reduction of QA upon saturation of linear electron transport from PSII to PSI.

3.2.3.3 Yields of PSII, regulated NPQ and non-regulated NPQ:

A new method has been suggested to estimate photon flux down both photochemical and non-photochemical pathways. In addition to fraction measurement of excitons utilised for photochemistry as quantum yield of PSII (ΦII), remaining fraction going via the NPQ pathway can be calculated as energy-dependent dissipation (ΦNPQ) and other non-regulatory losses due to basal intrinsic dissipation (ΦNO). By this way, the energy absorbed by PSII can be shown as sum of all yields for dissipative processes (Kramer *et al*., 2004).

$$\Phi II + \Phi NPQ + \Phi NO = 1$$

Maximum yields of PSII were measured in case of wild type and *npq2*, which shows the higher fraction of excitons going down the photochemical pathway and hence an enhanced PSII yield in these two genotypes, consistent with their higher values of qP found earlier in this work. The results of regulated NPQ yields also demonstrate a similar value in wild type and *npq2* which is consistent with the outcome of comparative study of chlorophyll fluorescence lifetimes of these two cases. In all other xanthophyll mutants, the yields of both PSII and regulated NPQ are reduced variably. Yield of non-regulated NPQ, indicating

slowly reversible components of NPQ, has been also found lower in wild type and *npq2* as compared to all other xanthophyll mutants. Moreover, highest non-regulated NPQ in *npq1lut2* supports the view of maximum photoinhibition and damage to antenna in this double mutant. Increase in actinic light decreases PSII yield and increases energy-dependent NPQ in all cases (Fig.3.8 A,B).

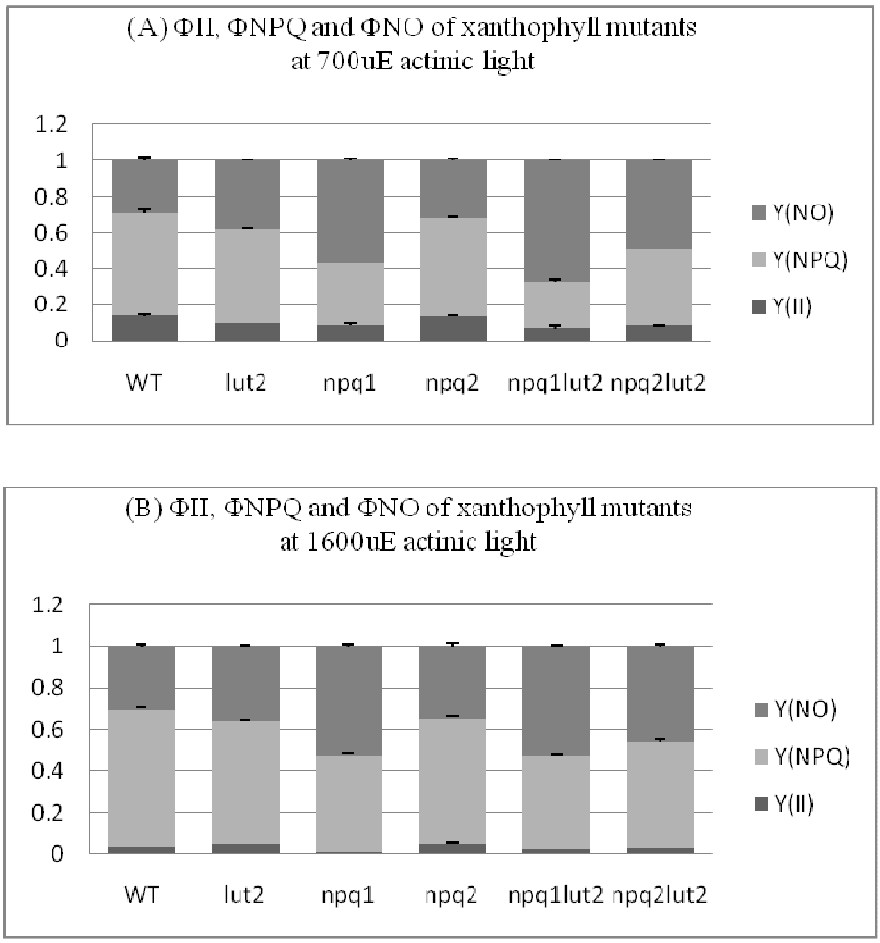


Figure 3.8 (A,B) Yields of PSII, regulated NPQ and non-regulated NPQ shown as ΦII, ΦNPQ and ΦNO respectively, in xanthophyll mutants measured at two different actinic lights, and calculated as ΦII = Fm'- Fs/Fm', ΦNO = 1 / NPQ + 1 + qL [NPQ = Fm-Fm'/Fm' and qL = Fm/Fo-1] and ΦNPQ= 1 - ΦII - ΦNO. All data are mean ± SE for at least three plants.

3.2.3.4 Electron Transport Rate in PSII, ETR (II):

The extent of photochemistry can be demonstrated by electron transport rate in PSII. As utilisation and dissipation of energy are competing processes, so any decrease in ETR (II) can be attributed to increase in NPQ thereby extra available light energy is dissipated (as NPQ) instead of being utilised for photochemistry. Other factors like photoinhibition and smaller antenna size may also affect ETR (II), as in case of *npq1* mutant both NPQ and ETR (II) are reduced. ETR (II) of all the xanthophyll mutants have been affected in the order wild type >*npq2*>*lut2*>*npq1*> *lut2npq2* >*lut2npq1*. Double mutants are more affected than the single ones. Xanthophylls also play significant role in the stability of supercomplexes and macro organisation of the membrane, therefore any structural or organisational disruption may also result in reduction of ETR (II). Increase in actinic light has inversely affected ETR (II) in all cases variably except *lut2* (Fig.3.9), which is difficult to explain as this mutant has not shown any similar increase in qP and PSII yield under higher light.

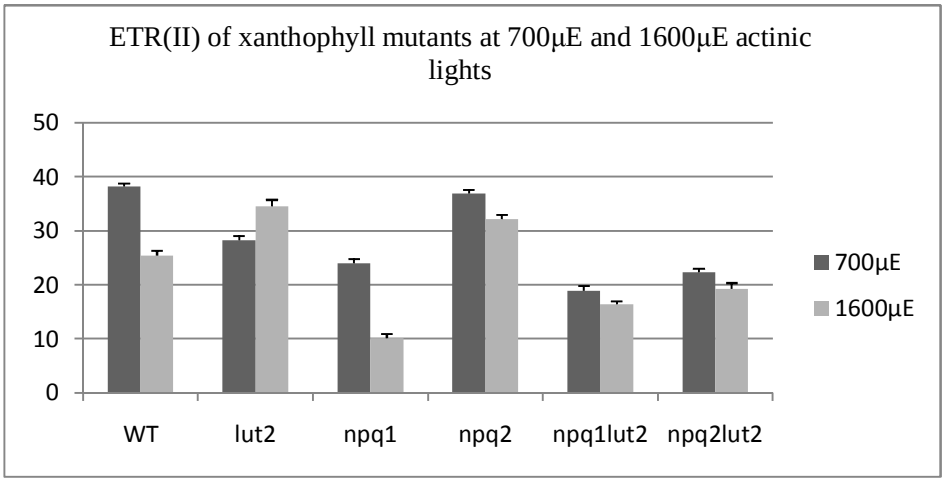


Figure 3.9 Electron transport rate in PSII or ETR(II) of xanthophyll mutants using two different actinic lights and measured as 0.5x ΦII (Yield of PSII) x PFD (photon flux density). All data are mean ± SE for at least three plants. All data are significantly different to WT (Student's t-test p=0.05), except for *npq2* at 700µE.

3.2.3.5 Effect of Inhibitors on xanthophyll mutants:

The uncouplers like nigericin remove ΔpH, so abolish qE and also de-epoxidation. This may help to measure the extent of ΔpH-independent, irreversible and non-relaxing part of NPQ, also called as photoinhibition (qI). No significant difference was observed in the extent of qI between wild type and *lut2*, however the absence of zeaxanthin, as in *npq1*, shows enhancement of qI. In the mutants with constitutive presence of zeaxanthin, *npq2* and *lut2npq2*, photoinhibitory qI is reduced (Fig.3.10 A). This effect of zeaxanthin on photoinhibition may be attributed to its antioxidant nature, as it provides protection against damage to RC's. In case of omission of both zeaxanthin and lutein (*npq1lut2*), all the NPQ seems to be sustained and irreversible by virtue of qI. This may indicate the structural and functional anomalies caused by missing lutein and zeaxanthin in LHCII antenna.

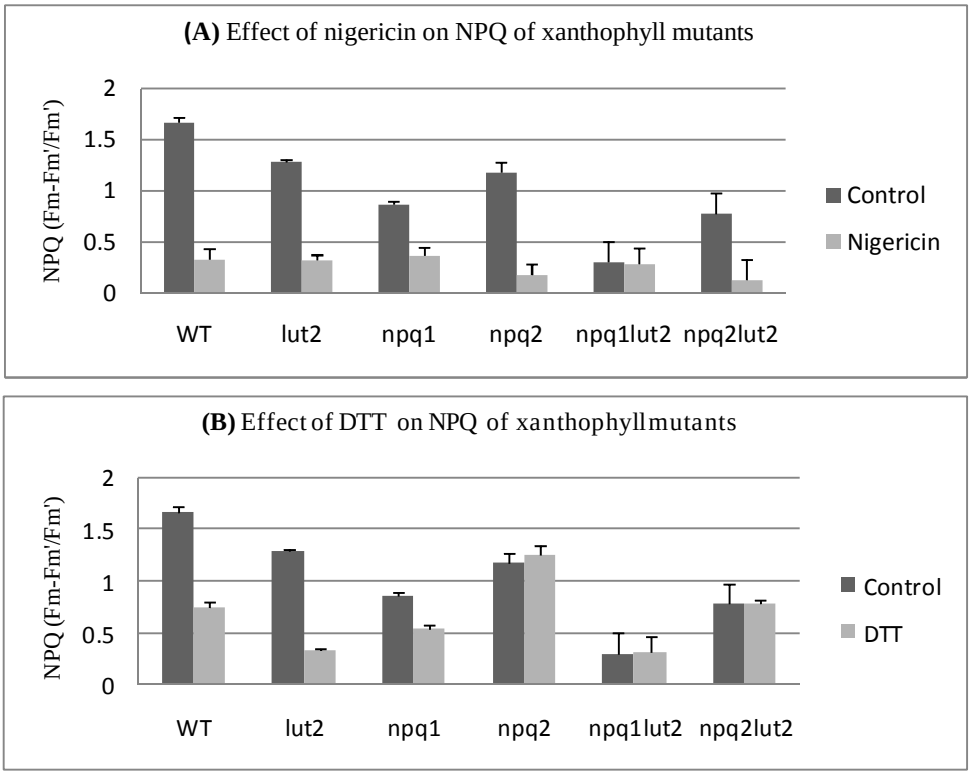


Figure 3.10 (A) Effect of 5mM nigericin and **(B)** 50μM DTT infiltrations on NPQ of xanthophyll mutants, measured using 700μE actinic light. All data are mean ± SE for at least three plants. All data are significantly different to respective controls (Student's t-test p=0.05), except for *npq1lut2* (A) *npq2, npq1lut2* and *npq2lut2* (B).

The use of dithiotheitol (DTT) helps to identify the zeaxanthin-independent and zeaxanthin-dependent components of NPQ, as DTT inhibits deepoxidation. Obviously most affected NPQ levels were observed in those with active xanthophyll cycle (wild type, *lut2*), whilst zeaxanthin containing mutants (*npq2* and *npq2lut2*) remained unaffected. However, effect of DTT on NPQ of *npq1lut2* and even of *npq1* (which were also expected to remain unaffected due to absence of XC activity) shows either additional impact of DTT apart from blocking zeaxanthin synthesis or to inhibit zeaxanthin-independent NPQ in both *npq1lut2* and *npq1* (Fig.3.10 B). This may indicate the role of DTT as a general reductant, because this effect of NPQ in *npq1* is evidence of its non-specific activity.

3.2.4 Effect of xanthophyll composition on state transitions:

Under light limiting conditions, state transitions are meant to optimise light utilisation. Phosphorylation of LHCII complexes causes their migration from PSII to PSI to balance the distribution of excitation energy between the two photosystems (Allen *et al*., 1981; Bennett, 1983; Horton, 1983). As xanthophylls have been implicated to maintain the structural integrity of the antenna complexes, thus changes in xanthophyll composition are predicted to affect the process of state transition as ability of LHCII to migrate and attach are changed. State transition is a short-term adaptation strategy, which can be measured as chlorophyll fluorescence using low light intensity for selective excitation of PSII or PSI. Assessment of state transitions is based upon relative fluorescence measurements. Several parameters characterising this process can be derived (Ruban and Johnson, 2009):

As low light conditions induce an imbalance between the energy absorbed by the two photosystems, thus this imbalance can be measured by removal of far-red light as:

$$\text{Imbalance or IB} = (FsI' - FsI) / F_o$$

Another useful parameter is qS, which shows the efficiency of state transition in a simple way. It demonstrates the optimisation of electron transport with the changing quality of

light. This parameter ranges from 0 to 1, where 1 depicts 100% efficiency of state transition to rebalance electron transport rate after the changes in the spectral quality of light.

$$qS = (FsI' - FsII') / (FsI' - FsII)$$

The results show that state transitions are disturbed by the absence of lutein alone but not of zeaxanthin alone. The level of imbalance and qS are unaffected in the absence of zeaxanthin in *npq1* as compared to wild type. In case of *npq2*, similar qS is also observed however extent of imbalance is almost half of the level of wild type which can be attributed to pre-quenched state or smaller PSII antenna size in this zeaxanthin accumulating mutant. It can also be inferred that presence of zeaxanthin and/or absence of violaxanthin or neoxanthin in *npq2* affect state transitions, as this genotype does not respond to turning on the PS II light. This indicates complete state transition or particular effect of any of these xanthophylls on LHCII function. Interestingly, the results show that both qS and imbalance are reduced in the absence of lutein alone. This may suggest that LHCII antenna structural disturbances can be caused by violaxanthin at lutein-binding sites, but not at its original site. Similarly, in *lut2npq1* values of qS and imbalance are reduced like *lut2*. Moreover, binding of zeaxanthin at lutein-binding sites as in *lut2npq2* has most pronounced impact, as no measurable state transitions and imbalance are observed. This indicates the deleterious structural effect of zeaxanthin at lutein binding sites or protein conformational disturbances caused by absence of lutein, leading to smaller antenna size and/or lack of imbalance between PSII and PSI (Fig.3.11).

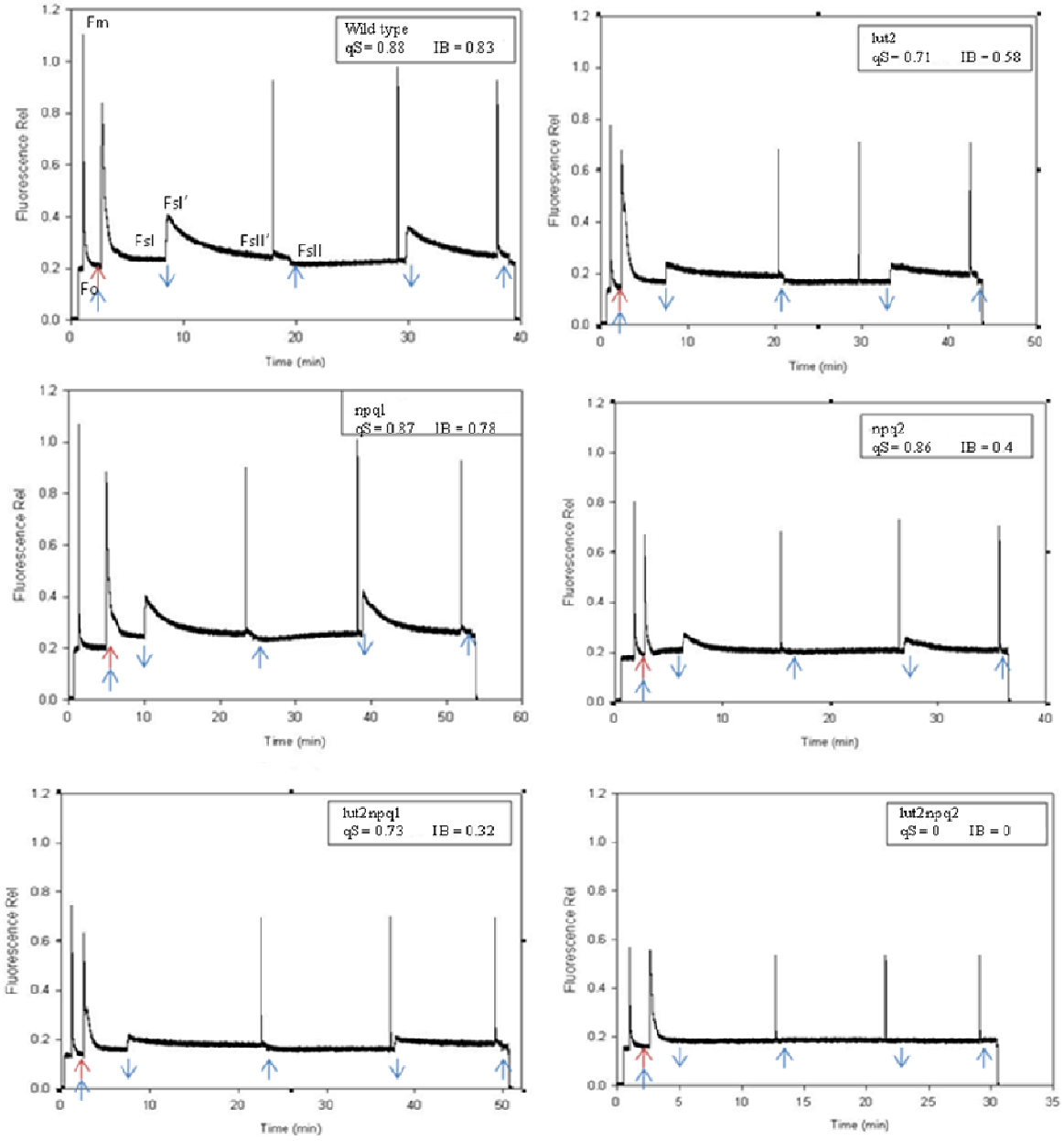


Figure 3.11 State transitions traces of wild type and xanthophyll mutants measured using PAM 101with 25µE PSII and 10µE PSI lights, downward and upward arrows show PSI light off and on respectively. qS is calculated as (FsI´ – FsII´) / (FsI´ – FsII), and IB is measured as (FsI´ - FsI) / F_o. Red and blue arrows show red and blue light, respectively, with light on ↑ and off ↓ . Traces are representative of 3 independent repeats.

3.3 Discussion

In this work, effect of various xanthophyll compositions is studied to perform three major functional and structural roles of photoprotection, light harvesting and stabilization in light harvesting antenna. As this work is focused on photoprotective role of xanthophylls therefore more detailed analysis is carried out in this regard. The hypothesis is tested here that NPQ, with its zeaxanthin-independent and zeaxanthin-dependent components, takes place by two distinct mechanisms. Type I mechanism involves zeaxanthin as quencher, while lutein is considered as quencher in type II mechanism. Both mechanisms have been proposed to occur in different LHCII antenna complexes (Ahn *et al*., 2008; Ruban *et al*., 2007). A thorough investigation of various fluorescence parameters, in particular qE and qE-related absorption changes, was performed using the wild type and xanthophyll mutants with various xanthophyll compositions. The wild type composition of xanthophylls demonstrated not only the maximum qP, ETR II and ΦII but also most efficient qE as compared to all the mutants, implying its suitability for a fully functional NPQ, in which the conformational dynamics are tuned to create maximum flexibility between efficient light harvesting in low light and rapid formation and relaxation of photoprotection in fluctuating light.

Type I mechanism of quenching has been suggested to take place in minor antenna complexes by means of zeaxanthin radical cation formed exclusively at L2 internal binding site. As L2 site is occupied by violaxanthin in the minor complexes therefore a reversible insertion is required at this site for the execution of this radical cation formation event. The biochemical analysis of *lut2* mutant has suggested that extra pool of violaxanthin is attached to internal L1 and L2 sites along with normal peripheral V1 site. Xanthophyll pigment analysis of light and dark states demonstrates that only part of violaxanthin pool, at peripheral site, is de-epoxidised, while majority of it remains inaccessible to de-epoxidase enzyme (Kalituho *et al*., 2006). Moreover, the effect of this *in vitro* cation formation was even found to be quite small to account for *in vivo* qE (Avenson *et al*., 2008).

It can be argued that if type I and type II mechanisms both contribute to qE, then each mechanism would behave like a discrete kinetic component. Contrary to this prediction,

only a single component of qE relaxation was monitored, the rate of which was controlled by the de-epoxidation level. In the type I mechanism, zeaxanthin is regarded as the quencher and its level would not influence the relaxation kinetics. In contrast, type II mechanism proposes zeaxanthin role as an allosteric regulator and it aptly justifies its effect on relaxation kinetics. This effect of zeaxanthin to slow down the qE relaxation has also been reported earlier (Noctor *et al*., 1991). Here in this study, it was observed that the relaxation of qE in *npq1* was faster than that in the wild type, while in *npq2* and *lut2npq2*, qE relaxation was significantly delayed. These relaxation kinetics offer evidence for the allosteric role of zeaxanthin.

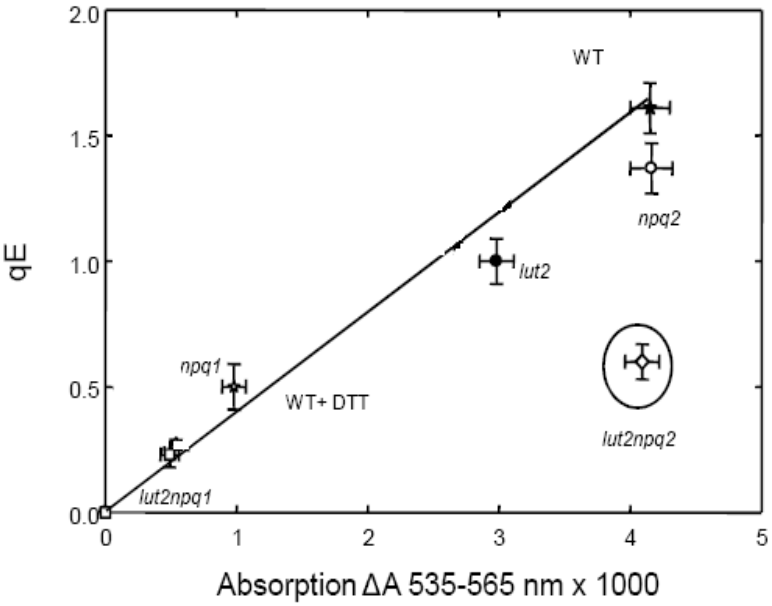
Lower Fv/Fm in zeaxanthin accumulating mutants, *npq2* and *lut2npq2*, was suggested to be caused by pre-quenching of Fm, due to the constitutive presence of extra zeaxanthin (Dall'Osto *et al*., 2005; Kalituho *et al*., 2006). This study confirms this suggestion by measuring the absolute chlorophyll fluorescence lifetimes. In type II mechanism of quenching, the zeaxanthin-binding L2 site in the minor LHCII complexes has been implicated to be the quenching site. In *npq2* and *lut2*np2 mutants, zeaxanthin replaces all of the violaxanthin, including that bound to the L2 site in the minor antenna complexes. In some reconstitution studies, a slight quenching in the similar minor antenna complexes with similar xanthophyll composition to that of these mutants has been reported (Crimi *et al*., 2001; Dall'Osto *et al*., 2005). However, it is important to note that the presence of extra zeaxanthin does not confer any additional capacity for NPQ. Contrarily, NPQ capacity is significantly reduced in *lut2npq2* as confirmed by the lifetime measurements. This suggests that zeaxanthin is not necessarily the best quencher among the xanthophylls.

However, qE formation occurs in the form of two distinct components unlike its relaxation kinetics. Both zeaxanthin-independent and zeaxanthin-dependent components of qE can be discretely observed in the qE formation kinetics of wild type and various xanthophyll mutants. Fast initial zeaxanthin-independent component is shown by *npq1* as the total qE while this component seems to be missing in case of *lut2* mutant which only exhibits second zeaxanthin-dependent component as its sole qE. The perturbed kinetics and lower capacity of NPQ formation in lutein-deficient mutants, also found in the previous studies,

lends some support for the validity of a type II mechanism. The zeaxanthin-independent component of qE was almost completely eliminated while the zeaxanthin-dependent qE was also affected. However, the presence of zeaxanthin-dependent component of qE in the absence of lutein negates the obligatory requirement for lutein, though this component in the lutein mutants is still kinetically different from that of the wild type. It is quite evident that replacement of lutein by either of zeaxanthin or violaxanthin in the internal binding sites of Lhcb proteins negatively affects both the dynamics and amplitude of qE, though quenching still occurs. Therefore, this work adds to the previous work implying the role of lutein for fully functional qE (Pogson *et al*., 1998; Pogson and Rissler, 2000). It can also be suggested from this study that the already known structural destabilization of PSII antenna proteins in the absence of lutein (Lokstein *et al*., 2002; Havaux *et al*., 2004) may impair the intrinsic conformational changes within the individual monomers, which lead to the formation of quenched state.

Further insight into the impact of xanthophyll composition on the conformational changes can be explored by study of the absorption changes correlated with qE formation. Zeaxanthin has been shown previously to enhance long wavelength LHCII 700 nm fluorescence emission bands in the qE state (Ruban *et al*., 1991; Ruban *et al*., 1993a), indicative of LHCII aggregation, which it promotes *in vitro* (Ruban *et al*., 1998b). Here a further evidence of zeaxanthin's ability to promote structural changes within the PSII antenna that lead to the quenching is provided. Constitutive zeaxanthin enhanced the ΔA_{535} signal in *npq2* and *lut2npq2*, despite the fact the qE capacity was no larger. The enhancement of the signal in the zeaxanthin accumulating cases is consistent with its identification by Raman spectroscopy as the source of the band (Ruban *et al*., 2002b). However, in *lut2npq2* a non-linear and significant increase in ΔA535 was observed albeit a reduction in amplitude of qE. All other mutants and wild type demonstrate a linear correlation between the amplitudes of ΔA535 and qE (Fig. 3.12 A). On the other hand if a similar graph is plotted between the amplitudes of ΔA495 and qE shows a linear correlation in all cases (Fig. 3.10 B), which demonstrates an unbroken correlation between qE and change in 495 absorption change, which corresponds to L1 site. It also lends support to the

A



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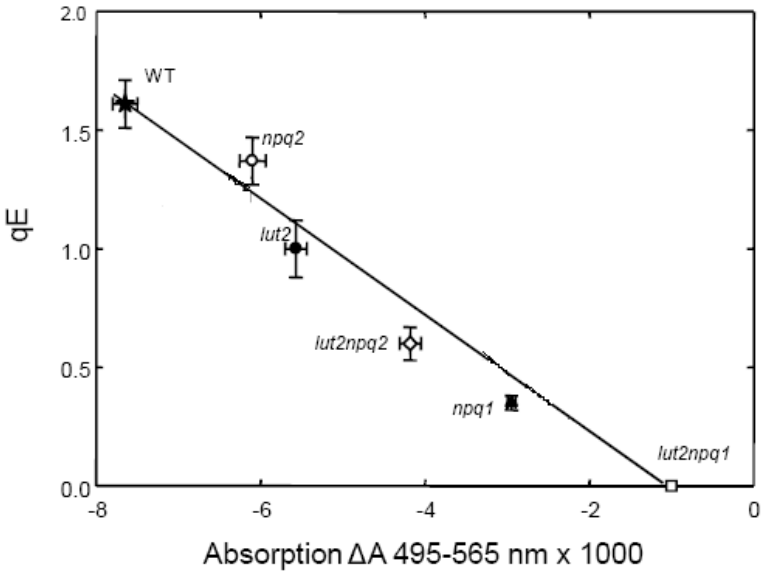


Figure 3.12 Relationship between qE amplitude and qE related absorption changes in the wild-type and xanthophyll mutant leaves. (A) ΔA535 vs. qE, deviation of *lut2npq2* from the linear relationship is highlighted with a circle. (B) ΔA495 vs. qE. Data obtained as in Figures 3.2 and 3.4.

formation of quenching species at L1 site as it directly correlates with qE amplitude in all the cases. The deviation of *lut2npq2* in the form of broken correlation between ΔA535 and qE indicates that the absorption change may not directly correspond to the formation of quenching species, consistent with a previous report (Kalituho *et al*., 2006). Therefore, it can be suggested that ΔA535 monitors the conformational change associated with amplification of qE as a result of de-epoxidation. Moreover, the origin of the red-shifted zeaxanthin accounting for ΔA535 has been attributed to the formation of a dimer (Ruban *et al*., 1993a; Bonente *et al*., 2008) between two zeaxanthin molecules at V1 sites of the two neighbouring LHCII complexes.

In case of *npq1* lacking the zeaxanthin, the position of positive 535 band is shifted to around 525 nm, similar to a previous study involving isolated chloroplasts (Noctor *et al*., 1993). This band cannot be assigned to any other particular xanthophyll in the wake of present understanding. The same 535 band is slightly red shifted to 537 in zeaxanthin accumulating *npq2* and *lut2npq2* mutants, which are in 100% de-epoxidation state at the V1 site. Based on the observation of a broad range of this span, it can be suggested that ΔA535 may be comprised of a mixture of bands ranging from 525 to 540 nm, indicative of the occupancy of the V1 site by different xanthophylls (violaxanthin, antheraxanthin and zeaxanthin) depending upon the de-epoxidation state.

The qE-related absorption difference spectra also exhibit specific negative bands at 495, 468, and 438 nm. Improved resolution of these negative bands provides an insight into the complexity of the variations in pigment configurations upon the establishment of qE. In resonance Raman spectroscopy, light minus recovery difference spectra of leaves and chloroplasts also show resonance only above 500 nm showing exactly the similar positive and negative features of the qE absorption difference spectra, while resonance is lost below 500 nm (Ruban *et al*., 2002b). The 495-nm negative band has been assigned as L1 lutein band (Ruban *et al*., 2000), thereby replacement of lutein by zeaxanthin or violaxanthin in *lut2npq2* and *lut2* mutants caused a red shift in this band. In contrast to the behaviour of the 535-nm band, the amplitudes of these bands were reduced in *lut2npq2* compared with the wild type, therefore being better correlated with qE. Thus, it is suggested that this

observation provides support for the involvement of the L1 domain in qE, as in the type II mechanism.

During transiently formed qE at low light intensity, the analysis of accompanied 535-nm absorption change also suggests that this form of qE also involves similar conformational changes within the PSII antenna, as demonstrated during the steady state qE. The transient qE formed under such low light conditions was previously ascribed to quenching in the PSII reaction centre (Finazzi *et al*., 2004), but here this work indicates that both these types of qE are accompanied by similar absorption change with the same spectrum irrespective of high or low light conditions. Moreover, any alteration in the xanthophyll composition affects the transient qE in a similar fashion as steady-state qE is influenced, adding to previous results (Kalituho *et al*., 2007); hence, in either case the qE was strongly reduced in *lut2* and *lut2npq2* and completely absent in the *lut2npq1* mutant. It has also been suggested that transient qE is dependent on activity of PsbS (Finazzi *et al*., 2004; Kalituho *et al*., 2007), these data strongly suggest that it originates from the same common PSII antenna-based mechanism as steady-state qE in high light. In summary, the evidence that both the zeaxanthin-independent and zeaxanthin-dependent components of qE arise from the same mechanism within the PSII antenna has been strengthened. Both are enhanced by PsbS overexpression (Li *et al*., 2002; Crouchman *et al*., 2006), and both preferentially quench LHCII fluorescence emission bands (Ruban *et al*., 1991). Both components of qE are reduced in amplitude in the absence of lutein (Pogson *et al*., 1998; Niyogi *et al*., 2001). Despite different xanthophyll compositions, qE was found to consistently relax as a single component even though the half-times differed by greater than 15-fold. All of these findings are consistent with a type II mechanism involving the xanthophyll at the L1 binding site, which is activated by the LHCII conformational change that leads to neoxanthin distortion (Ruban *et al*., 2007) and is allosterically regulated by de-epoxidation state at the V1 site (Horton *et al*., 2005). This may take place in some or all of the Lhcb containing antenna complexes (Ruban *et al*., 1996; Mozzo *et al*., 2008). The alternative possibility is that a type I mechanism occurs in both trimeric LHCII and the minor monomeric antenna and that, in the absence of zeaxanthin, a lutein cation can take its place as the quencher. However, irrespective of which mechanism is involved and which

xanthophyll acts as direct quencher, it is abundantly clear from the data shown here that the natural xanthophyll composition of the PSII antenna is essential for a fully functional NPQ. All the xanthophylls have a role to play in the efficient light harvesting in low light and effective photoprotection under fluctuating light conditions. This may indicate the need and purpose of retaining a variety of xanthophylls during the course of evolution in the higher plants.

3.3.1 Hydrophobicity index

A novel approach of hydrophobicity index is applied in this study to determine how xanthophyll polarity collectively tunes the ability of the PSII antenna to undergo a conformational change. Differences in the head group orientation and hydrophobicity between violaxanthin and zeaxanthin have been shown to underlie the differences in their allosteric effects upon quenching in LHCII *in vitro* (Horton *et al*., 1999). Hydrophobicity values for each xanthophyll have previously been determined by their solubility mid-point in water ethanol mixes (Ruban *et al*., 1993b). Therefore, to rationalize the effect of the other xanthophyll binding sites within the PSII antenna upon qE kinetics a *hydrophobicity index* for each mutant was calculated based upon biochemically determined xanthophyll binding and Lhcb protein stoichiometries as shown in Table 3.5 (Ruban *et al*., 1994; Ruban *et al*., 1999; Lokstein *et al*., 2003; Havaux *et al*., 2004; Dall' Osto *et al*., 2005).

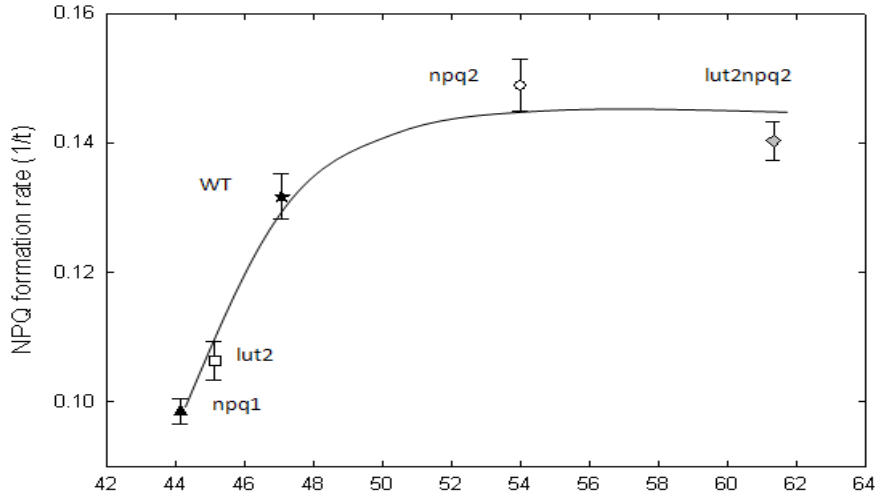
When these calculated hydrophobicity indexes for PSII antenna from each mutant were plotted against the kinetic rates of formation and relaxation a clear correlation was seen (Fig 3.13 A and B). As the hydrophobicity of xanthophyll complement in the PSII antenna increased in a mutant so correspondingly the rate of formation increased and the rate of relaxation decreased in that mutant. It is interesting to note that the dependence for the two processes is very different, a change in hydrophobicity index of 45 to 54 caused an increase in the rate of qE formation of 1.7 times, yet it decreased the rate of qE relaxation by 17 times, a factor of ten difference. This novel finding adds tremendous insight into the role the natural xanthophyll complement plays in tuning the dynamics of the photoprotective switch in antenna function. The allosteric effect of substituting violaxanthin for zeaxanthin

Plant type	LHCIIb stoichiometry	Hydrophobicity index
Wild type	NLLZ	48.75
npq1	NLLV	44.5
npq2	-LLZ	54
lut2	NVVZ	46.25
lut2npq2	-ZZZ	62
lut2npq1	NVVV	42

Table 3.5 Calculation of hydrophobicity index of wild type and xanthophyll mutants on the basis of their respective LHCIIb stoichiometry and hydrophobicity values of constituent xanthophylls, previously determined as the solubility midpoints in the % ethanol/water mixture. Hence, the hydrophobicity values for each xanthophyll were found as: Z (zeaxanthin) 62%, L (lutein) 50%, V (violaxanthin) 45% and N (neoxanthin) 33% (Ruban *et al*., 1993b). The respective values of each plant type were added according to its stoichiometry, e.g. for wild type (NLLZ) = (33+50+50+62/4) and for *npq2* (-LLZ) = (50+50+62/3)

upon the wild-type LHCII xanthophyll hydrophobicity index is clear, allowing reversible change insensitivity of the complex to the PsbS-ΔpH driving force. The significance of disturbing the natural xanthophyll composition by mutation is also clear- a xanthophyll complement is 'too polar' as in *lut2npq1* reduces the sensitivity to the ΔpH to the extent that no qE is possible while one that is 'too hydrophobic' would cause only a slight acceleration of qE formation (3-4 seconds) but significantly retard relaxation (60+ s). Hence in *npq2* and *lut2npq2* the 'positive' dynamic effect on qE formation of having increased zeaxanthin content compared to wild-type is almost certainly mitigated by the 'negative' effect it has upon qE relaxation. A similar conclusion was recently reached in plants overexpressing β- carotene hydroxylase and therefore containing a xanthophyll cycle pool size three times larger than that seen in wild-type (Johnson *et al*., 2008). The dynamics

A



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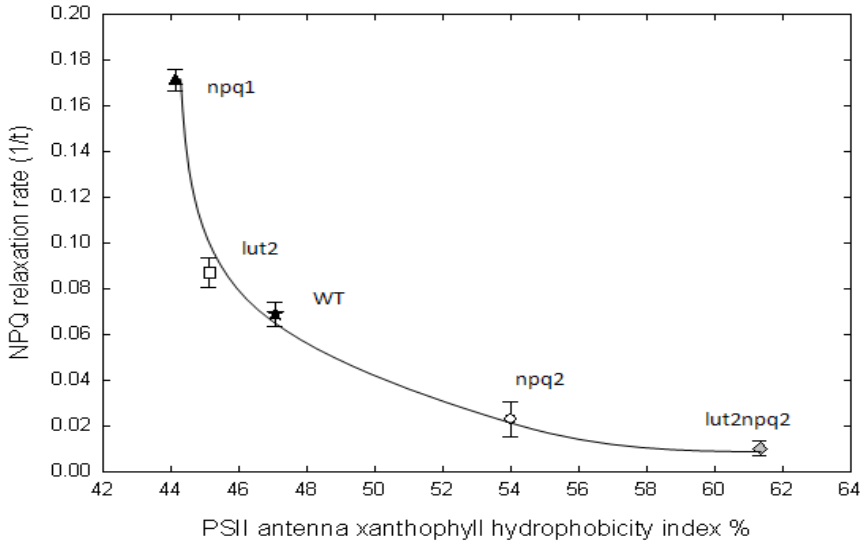


Figure 3.13 Relationship between (A) NPQ formation and (B) relaxation rates (1/t) and PSII antenna xanthophyll hydrophobicity index % of the wild-type and xanthophyll mutants. Data obtained as in Figures 3.2.

of the PSII antenna can therefore be considered to be finely tuned by xanthophyll content to allow the maximum flexibility between allowing rapid induction of photoprotection on one hand and rapid reversal to a light harvesting state on another. The varying xanthophyll complements in each PSII antenna complex would, perhaps, further increase the dynamic flexibility of the process. Thus when sustained periods of illumination cause de-epoxidation at L2 sites within the minor antenna in addition to rapid de- epoxidation at the V1 sites (Farber *et al*., 1997; Wehner *et al*., 2006) the qE phase becomes more and more 'allosterically locked' and thus qE relaxation becomes slower like that seen in diatom algae (Ruban *et al*., 2004). Xanthophyll hydrophobicity may also offer an alternative explanation of their role as structural co-factors of the antenna complexes.

To summarise the data presented in this chapter, this work suggests following outcomes:

1. There exists a single quencher and/or quenching mechanism.
2. Xanthophyll complement affects the kinetics of qE formation and relaxation, and hence is significant for photoprotection.
3. Polarity of xanthophylls collectively tunes PSII antenna dynamics.
4. Xanthophylls also influence the light harvesting capacity and structural stability of antenna.

Chapter Four

Effect of xanthophyll composition on the chlorophyll excited state lifetime in plant leaves and isolated LHCII

4.1 Introduction

Biological regulatory mechanisms involve a switch between two alternative functions, most simply between active and inactive states. An important aspect of this regulatory route is its dynamics ranging from the capacity for maximum activity in one state to minimum in the other. In the course of evolution, biomolecules have optimized this dynamic regulation in order to adapt to their particular set of physiological and environmental circumstances. Albeit this tendency of biomolecules for a dynamic design is apparent it remains poorly explored. Like many regulatory biomolecules, the light harvesting complexes of photosystem II (LHCII) have such type of dual function: they efficiently utilise absorbed light energy for photosynthesis at limiting light intensity and they dissipate excess energy at saturating light for photoprotection. The dynamics of this functional range is controlled by a change in the pathway of energy transfer in LHCII, whilst the light energy is efficiently funnelled down to reaction centres in the photosynthetically-active state it is swayed into a new channel for non-radiative decay of chlorophyll excited states in the photoprotective state. This switch from the light harvesting state into the photoprotective state is termed as nonphotochemical quenching (NPQ) (Horton *et al*., 1996).

LHCII is composed of the major trimeric and minor monomeric complexes. All these complexes variably bind chlorophyll *a* and *b*, and a conserved complement of xanthophylls: lutein, neoxanthin, violaxanthin and zeaxanthin (Liu *et al*., 2004). In major complexes, two lutein molecules are located at L1 and L2 sites in the core of each monomer, both having distinct absorption spectra at the two sites (Ruban *et al*., 2001). The L1 site is located in the proximity of chlorophyll *a*610, *a*611, *a*612, constituting a 'terminal emitter domain' as there is highest probability for excitation energy to be localised in this domain (Novoderezhkin *et al*., 2005). The L2 site is also located near to the chlorophylls *a*602, *a*603, *a*604. The third xanthophyll, neoxanthin, is located in a domain rich in chlorophyll *b*. The neoxanthin is far more polar (Ruban *et al*., 1993) than lutein by virtue of its increased number of oxygenated groups and different conformation. All these three xanthophylls are tightly bound to the LHCII (Ruban *et al*., 1999). The fourth more loosely bound xanthophyll is located at the V1 site near the periphery in the chlorophyll *a*613, *a*614, *b*601

domain (Liu *et al*., 2004; Ruban *et al*., 1999). The V1 site is specified for the binding of the xanthophyll cycle carotenoids. Violaxanthin occupies this site in low light conditions, while as a result of enzymatic de-epoxidation it is replaced by zeaxanthin under excess light conditions causing acidification of the thylakoid lumen. Both violaxanthin and zeaxanthin adopt a distorted configuration *in vivo* (Ruban *et al*., 2002), indicating their interaction with the protein matrix despite loose binding pattern. All these xanthophylls located at different binding sites have structural and biochemical variations in terms of a number of conjugated double bonds and oxygenated groups, cyclic head group orientation and polarity (Ruban *et al*., 1993). On the basis of these differences, variable functional roles of these xanthophylls have been proposed in the light harvesting antenna (Frank *et al*., 1994; Young *et al*., 1997; Ruban, 2009)

Recent studies have suggested the xanthophylls bound to the internal L1 and L2 sites within the complexes are involved in the mechanism of NPQ by quenching chlorophyll excited states via energy transfer to the xanthophyll S1 or via formation of a radical state (Ruban *et al*., 2007; Holt *et al*., 2005; Avenson *et al*., 2008; Ahn *et al*., 2008). This switch to the quenched photoprotective state has also been demonstrated to involve an intrinsic change in the LHCII conformation (Pascal *et al*., 2005; Ilioaia *et al*., 2008), which increases the interaction between the quenching xanthophyll and chlorophyll *a*. This process is triggered by the build up of the trans-thylakoid ΔpH under saturating light i*n vivo* (Briantais *et al*., 1979). Although interactions between complexes in the highly-conserved PSII macrostructure of the grana membranes has been suggested to modulate NPQ (Horton *et al*., 2008), the quenching process itself seems to be intrinsic to the light harvesting complex (Ilioaia *et al*., 2008). The native isolated major and minor LHCII complexes can readily and reversibly switch between the unquenched and highly quenched states by manipulation of detergent concentration and pH in the reaction medium (Ruban *et al*., 1994). The low detergent concentration and low pH induce fluorescence quenching followed by aggregation of LHCII complexes and this *in vitro* process possess many of the same spectroscopic features as NPQ *in vivo* (Ruban *et al*., 2007; Johnson and Ruban, 2009; Ruban *et al*., 1992). Transient absorption studies have revealed that in low detergent aggregated LHCII the excited state of the xanthophyll lutein 1 (S_1) was populated as a

result of chlorophyll excitation (Ruban *et al*., 2007), indicating that this xanthophyll plays a role of the quencher in NPQ process. In addition, a recent two photon excitation study has also demonstrated an enhanced coupling between xanthophyll S_1 and chlorophyll S_1 states in LHCII aggregates (Bode *et al*., 2009).

The intrinsic molecular features of LHCII governing the efficiency and dynamic range of the switching process are not well understood. Since the energy transfer efficiency depends upon the configuration of the quenching xanthophyll and its partner chlorophyll(s), it is predicted that the type of the xanthophylls bound to the internal L1 and L2 binding sites would be important. Various fluorescence measurements presented in the Chapter III have demonstrated that variation in xanthophylls bound to the internal LHCII sites in *Arabidopsis* mutants affect photosynthetic efficiency and NPQ capacity, in agreement with other studies (Pogson *et al*., 1998; Niyogi *et al*., 1998; Pérez-Bueno and Horton, 2008). However, these studies have been based upon the relative fluorescence measurements and hence can only give arbitrary measures of the fluorescence quenching. Therefore, we performed chlorophyll fluorescence lifetime measurements in order to get an absolute value of the excited state lifetime in each of light harvesting and photoprotective states and also to distinguish direct impact of xanthophylls on the intrinsic function of light harvesting complexes from various indirect effects of the mutations in the carotenoid biosynthesis pathways of the *Arabidopsis* mutants used for this study. These measurements also provided additional information on the number and amplitude of fluorescence components. First, we measured the chlorophyll fluorescence lifetime in both the efficient light harvesting and photoprotective (NPQ) states *in vivo*. Then, the similar measurements were also performed on native isolated LHCII major and minor complexes with and without fluorescence quenching mimicking the *in vivo* NPQ. In order to evaluate the impact of specific xanthophylls bound to L1 and L2 internal sites on the LHCII functional dynamics, we used wild type, *lut2* and *lut2npq2* mutants with lutein, violaxanthin and zeaxanthin, respectively, bound into the L1 and L2 internal sites. Another mutant *npq2* was also used which has lutein in L1 and L2 sites and zeaxanthin attached to the neoxanthin-binding site. The LHCII complexes isolated from mutants show similar chlorophyll composition and pigment ratio to the complexes isolated from the wild type plants. The content of peripheral

V1 binding site of xanthophyll cycle carotenoids has been significantly decreased in conformity with the earlier reports (Sandona *et al*., 1998; Ruban *et al*., 1999), indicative of weaker xanthophyll binding or detergent sensitivity. Therefore, here we have investigated exclusively the role of internally-bound xanthophylls in the fluorescence quenching.

4.2 Results

4.2.1 *In vivo* chlorophyll fluorescence lifetime decays in photosynthetic and photoprotective states

Time-correlated single photon counting measurements were performed using a FluoTime 200 picosecond fluorometer. Excitation light was provided by a laser diode at 470 nm with 10 MHz repetition rate. Fluorescence was detected with 2 nm slit width at 685 nm for leaves and 680 nm for isolated LHCII and CP26. The instrumental response function was in the range of 50 ps. For lifetime analysis, FluoFit software (PicoQuant) was used. To measure the chlorophyll lifetime in photosynthetic state of the dynamic range (unquenched state, Fm), detached leaves were vacuum infiltrated with 50 μM nigericin to completely inhibit NPQ. The excitation light intensity was carefully adjusted to completely close all PSII reaction centres without causing photoinhibitory quenching of Fm and to be far below the onset of singlet-singlet annihilation. While light intensity of 700 μmol photons m^{-2} s^{-1} was used to induce the NPQ state (quenched state, Fm') *in vivo*. Figure 4.1 A shows the fluorescence lifetime decays in the form of curves recorded in leaves of wild type, *lut2*, *lut2npq2* and *npq2* in both photosynthetic and NPQ states. LHCII has the ability to switch between these two states- one with long lifetime increasing the chance of energy transfer to neighbouring chlorophyll and eventually the RC and therefore is efficient for light harvesting; the second photoprotective state is inefficient with a short lifetime- and is highly quenched. These curves are semi-exponential presentations: as in case of a single exponential they should be straight lines, but the fact that they are not (particularly for the NPQ quenched states). This suggests that there are several components involved; hence a curve fitting procedure was used. The amplitudes of components resolved in the fluorescence decay are shown in Figure 4.1 B.

In wild type, the average intensity weighted fluorescence lifetime was 1.95 ns in photosynthetic state in the absence of NPQ, consistent with the previous measurements in leaves and thylakoids (Gilmore *et al*., 1995; Gilmore, 2001; Moise and Moya, 2004). Three components were resolved in fluorescence decay in this case. An additional longer component of ~3.0-3.5 ns was not found here, which has previously been reported in several studies involving thylakoids (Wagner *et al*., 1996; Richter *et al*., 1999; Vasil'ev and Bruce, 1998). As this component has also been not observed in another studies on leaves (Moise and Moya, 2004), it can be suggested that it is caused as a result of preparative disruption to the natural system via osmotic shock or detergent effects in thylakoids and PSII particles. This disruption may lead to the formation of free chlorophyll or free/detached antenna components, which can cause a long-living component. Another shorter lifetime component of around 0.35-0.5 ns was also absent here which has previously been reported and is considered to appear only when the excitation energy is not high enough to saturate all PSII reaction centres. In photoprotective state, induced as a result of illumination the average fluorescence lifetime in the wild-type leaves was reduced to 0.61 ns, consistent with a similar decrease reported previously in thylakoids (Gilmore *et al*., 1995). The decays in this state could again be fitted with three components, which show the appearance of a new decay with a lifetime of 0.5 ns and the loss of a 2.3 ns component as the main effect of NPQ. This may demonstrate that NPQ is a process of transformation of one type of antenna state into another, and not a process of emergence of a totally new/additional lifetime state. In both photosynthetic and photoprotective states of all the plant types, a very short (~0.1 ns) lifetime with fairly constant amplitude was observed, which is consistent with the reported lifetime of Photosystem I (Wagner *et al*., 1996; Richter *et al*., 1999; Vasil'ev and Bruce, 1998).

In the *lut2* mutant, with violaxanthin at internal L1 and L2 sites, the average lifetime was significantly longer than in the wild type in both states: in the photosynthetic state its value was 2.17 ns, which was decreased to 0.84 ns in the photoprotective state. The two photosynthetic state components of 1.7 ns and 2.8 ns were replaced by those of 0.6 ns and 1.2 ns in photoprotective state. Thus in this mutant, an increase in lifetime in the photosynthetic state indicates an enhanced light harvesting efficiency, and lifetime increase

in the photoprotective state demonstrates a reduced NPQ capacity. These data are consistent with the slight increase in the maximum PSII quantum efficiency (Fv/Fm) and the decrease in NPQ capacity in the leaves of this mutant compared to the wild type, which has previously been discussed in Chapter III.

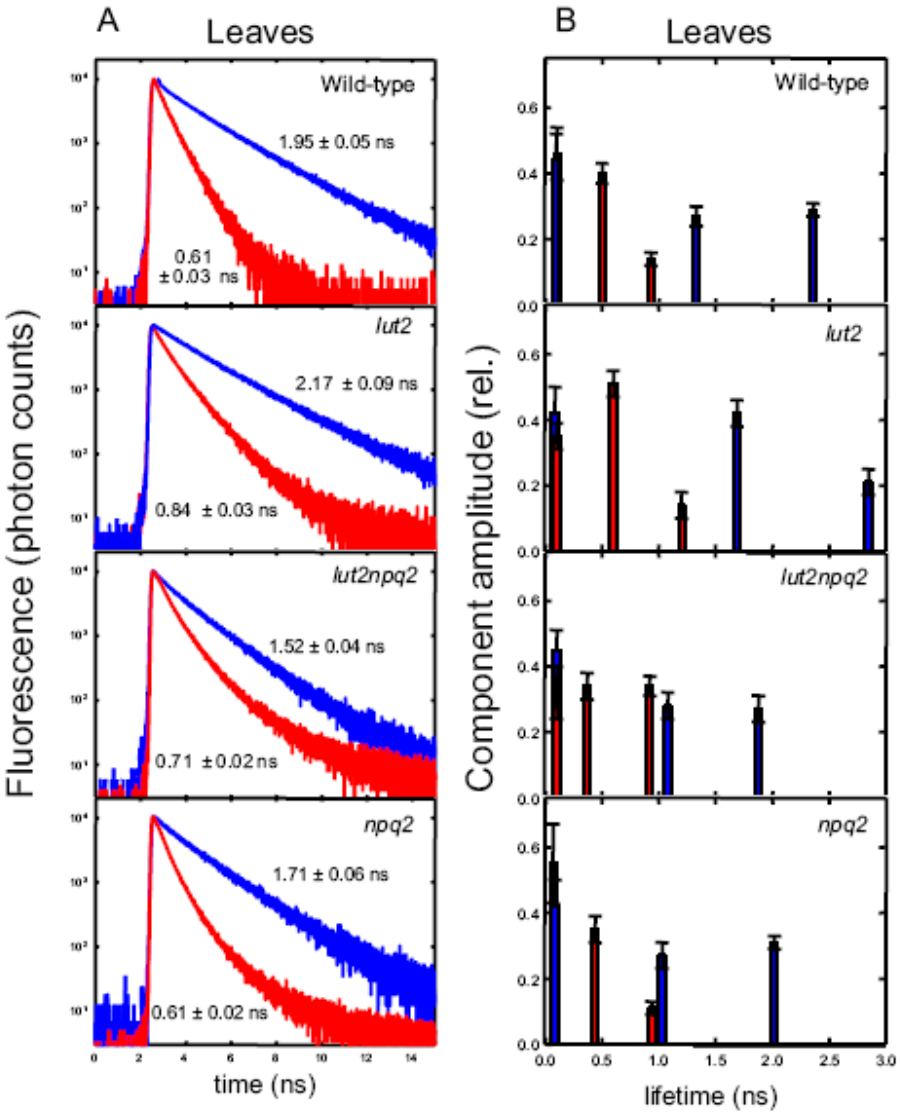


Figure 4.1 Chlorophyll fluorescence lifetime decays (A) and lifetime component amplitudes (B) in leaves from wild type, *lut2*, *lut2npq2* and *npq2* mutant *Arabidopsis* plants. The photosynthetic (unquenched) state (vacuum infiltrated with 50 μM nigericin) appears in blue, the photoprotective (quenched) state (in presence of 700 μM photons m^{-2} s^{-1} light after 10 mins preillumination) appears in red. The intensity weighted average fluorescence lifetime ±SEM (n=10) of each decay is also displayed.

In the *lut2npq2* mutant, with zeaxanthin as the sole xanthophyll, the lifetime in the photosynthetic state was significantly shorter than in the wild type, at 1.52 ns, demonstrating a less efficient light harvesting. This is also consistent with the lower value of Fv/Fm found in this mutant with a constitutive level of energy dissipation (Pogson *et al*., 1998). Interestingly, the decrease in lifetime during photoprotective state was only to 0.71 ns in this zeaxanthin-only mutant, which actually corresponds to its lower NPQ capacity than in wild-type. The increase in average lifetime as compared to wild type is mainly due to the appearance of a higher relative population of the ~0.9 ns lifetime compared to the 0.4-0.5 ns component. These data suggest that replacement of lutein by zeaxanthin undermines both photosynthesis and photoprotection in these leaves. However, *lut2npq2* double mutant not only lacks lutein and violaxanthin but neoxanthin is also absent in it. Therefore, the *npq2* single mutant was analysed to determine the effect of neoxanthin and violaxanthin exclusion while lutein is retained along with zeaxanthin.

In *npq2* mutant the average lifetime in the photosynthetic state was at 1.71 ns which was again lower than that for wild type, but not as low as in *lut2npq2*. However, the lifetime in the photoprotective state was the same as in the wild type, at 0.6 ns. The analysis of decay components of photosynthetically efficient state in this mutant showed a similarity to the *lut2npq2* mutant with the only difference of 2.0 ns component compared to one at 1.8 ns. However, in the photoprotective state, the data are almost indistinguishable from the wild type. This shows that the constitutive presence of zeaxanthin in *npq2* does not confer any additional photoprotective capacity compared to the wild-type. However, the photosynthetic efficiency gets impaired with this combination, consistent with the reduced photosystem II quantum efficiency in this mutant.

All these data recorded in both efficient light harvesting (photosynthetic) and photoprotective states combined from each of the plant types reveals a correlation between the quantum efficiency of PSII and the chlorophyll fluorescence lifetime (Figure 4.2). Moreover, the data shows that in the photosynthetic state both maximum quantum efficiency of PSII and the fluorescence lifetime have values above as well as below the

wild-type levels, which demonstrates the dynamic range in the light harvesting capacity as influenced by various xanthophylls.

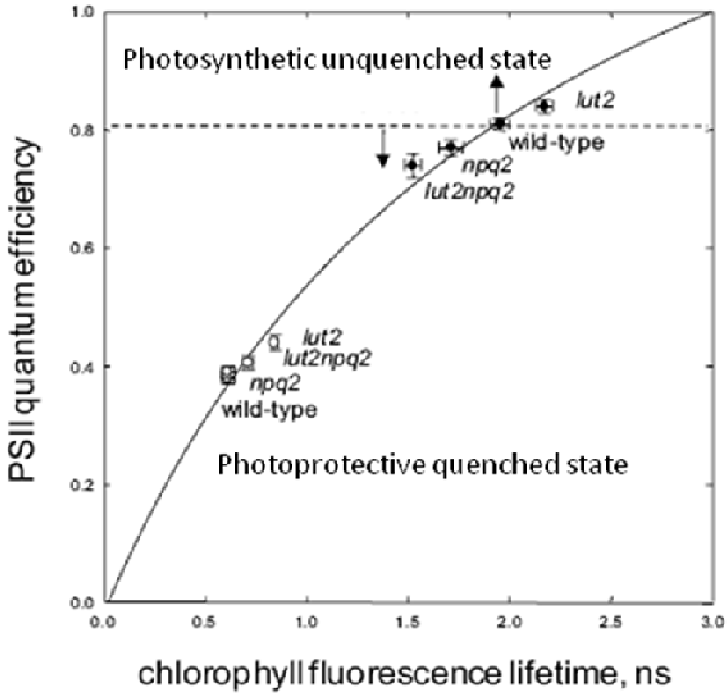


Figure 4.2 Relationship between chlorophyll fluorescence lifetime and photosystem II quantum efficiency measured simultaneously in *Arabidopsis* leaves with different xanthophyll complements in photosynthetic (unquenched) and photoprotective (quenched) states. The arrows highlight how the PSII quantum efficiency can be both increased (by replacement of lutein by violaxanthin in *lut2*) and decreased (by replacement of lutein by zeaxanthin in *lut2npq2*) compared to the wild type level (dashed line).

In the above data, the most efficient photoprotective ability in wild type and *npq2* can also be partly attributed to the presence of zeaxanthin at V1 site, although *lut2* did not show a similar ability despite de-epoxidation state similar to that of wild type. Nonetheless, to further explore the role of only internal bound xanthophyll on lifetime measurements, *in vitro* fluorescence quenching of LHCII major and minor complexes was performed. This can also help to determine whether these observed differences are result of direct

xanthophyll effects on the state of LHCII or other indirect effects, for example altered PSII macrostructure (Lokstein *et al*., 2002)

4.2.2 Isolation and characterisation of major and minor LHCII complexes

Major LHCII and minor CP26 complexes were isolated from unstacked thylakoid membranes prepared from *Arabidopsis* wild type and mutant plants. Detergent (n-dodecyl β-D-maltoside) solubilised thylakoids were fractioned by isoelectric focusing (IEF) and the respective bands were collected as shown in the Figure 4.3.

The reason for selection of minor band CP26 is that CP26 has been presented as unique site of quenching in a recent study with the formation of zeaxanthin radical cation (Ahn *et al*., 2008). The reason for not studying the other two minor complexes was also poor resolution of CP24 and CP29 in *lut2* and *lut2npq2* which may indicate less stability of these complexes in these lutein deficient mutants. The use of new commercially available gel and Pharmalyte materials may also be another reason for the poor resolution obtained for the minor antenna complexes as materials reported in previous studies are no more available. As LHCII in *lut2* and *lut2npq2* mutants exist in monomeric forms rather than trimeric forms (Havaux *et al*., 2004; Lokstein *et al*., 2002), monomers were also prepared from wild type and *npq2* trimers. Mutant samples were also treated in the same way as control.

For the characterisation of these complexes, pigment composition was determined by HPLC (Table 4.1) which showed the absence of lutein in the *lut2* and *lut2npq2* mutants, and its replacement by violaxanthin and zeaxanthin, respectively. In LHCII, the reduction in total xanthophyll bound in *npq2* and *lut2npq2* was mostly due to the absence of neoxanthin. In the case of CP26, there was some additional loss of xanthophyll. The absorption spectra of the samples were also measured and found same as previously published (Figure 4.4). In the case of LHCII from the *lut2npq2* mutant there was a reduction in prominence of the chlorophyll *b* band around 650 nm, consistent with the pigment analyses.

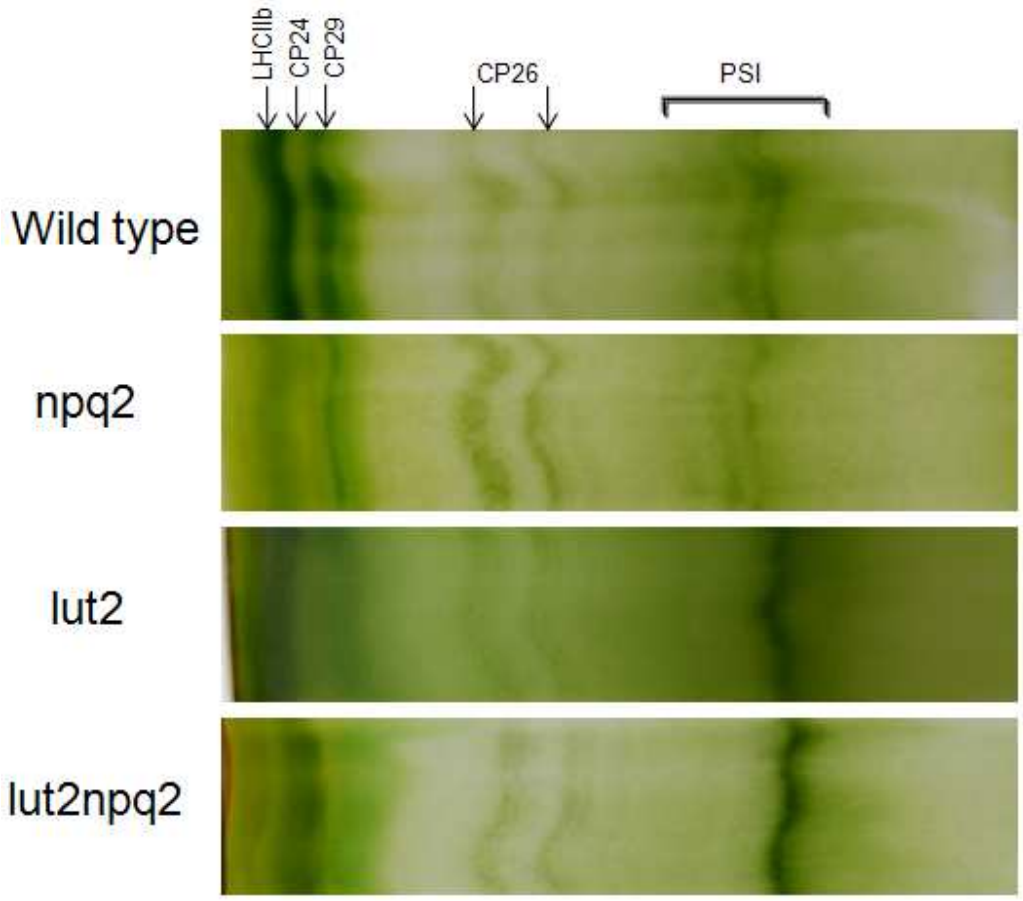


Figure 4.3 Separation of major (LHCIIb) and minor (CP24, CP29 and CP26) light harvesting antenna complexes of PSII in discreet bands from unstacked thylakoids of wild type, *npq2*, *lut2* and *lut2npq2* mutant by isoelectric focusing. Only LHCII and CP26 are well resolved in all the four genotypes.

LHCIIb	Neo	Vio	Ant	Lut	Zea	Xan/ Chl	chl *a*/*b*
Wild-type	61± 1.1	12± 1.6	0	132± 2.6	0	0.22	1.3± 0.1
npq2	0	0	0	131± 4.2	45± 6.6	0.18	1.5± 0.1
lut2npq2	0	0	0	0	120± 9.1	0.14	1.86± 0.1
lut2	53 ± 2.2	100± 9.8	26± 3	0	0	0.20	1.42± 0.1

CP26	Neo	Vio	Ant	Lut	Zea	Xan/ Chl	chl *a*/*b*
Wild-type	59± 2.2	24± 1.6	0	135± 8.6	0	0.24	2.0± 0.1
npq2	0	0	0	92± 4.2	30± 6.6	0.15	2.1± 0.1
lut2npq2	0	0	0	0	94± 0.1	0.11	2.3± 0.1
lut2	51± 5.1	128± 6.7	28± 8	0	0	0.22	2.1± 0.1

Table 4.1 Pigment composition of isolated LHCIIb and CP26. Monomeric LHCIIb and CP26 were prepared by IEF from dark-adapted *Arabidopsis* wild-type and mutant plants. Data are mmoles carotenoids per mole chlorophyll *a* + *b* molecules and are means ± S.E. from four replicates. Neo, Vio, Ant, Lut, Zea, and chl *a*/*b*: neoxanthin, violaxanthin, antheraxanthin, lutein, zeaxanthin, and chlorophyll *a*/*b* ratio. Xan/Chl is the molar ratio of total xanthophylls to total chlorophylls.

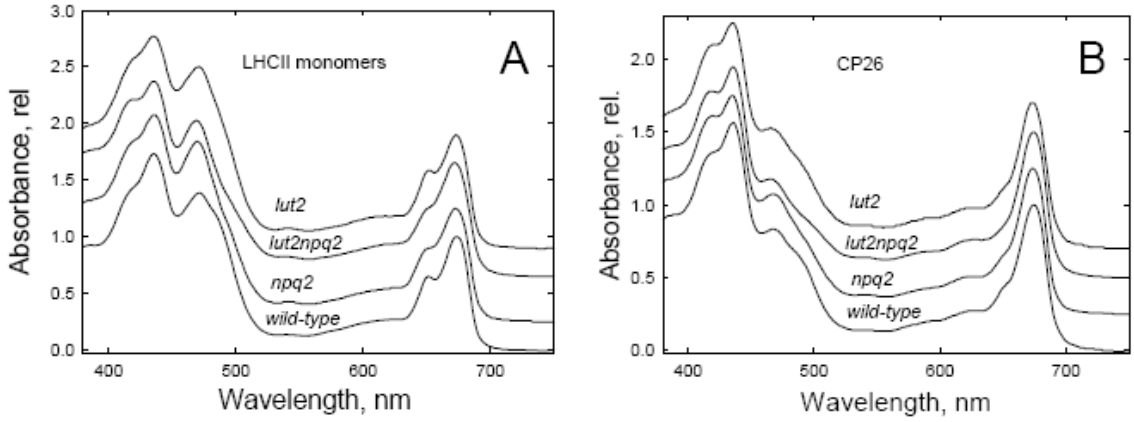


Figure 4.4 Absorption spectra of isolated LHCII (A) and CP26 (B) proteins from the wild-type, *lut2*, *npq2* and *lut2npq2* mutants.

4.2.3 *In vitro* chlorophyll fluorescence lifetime decays of isolated LHCII in unquenched and quenched states

It is known that aggregation of LHCII complexes is inhibited in detergent micelles, thus fluorescence remains unquenched in this condition. Chlorophyll excited lifetime of native LHCII and CP26, isolated from leaves of plants with varying xanthophyll composition, was first compared in detergent micelles. To measure lifetime decays in unquenched state,

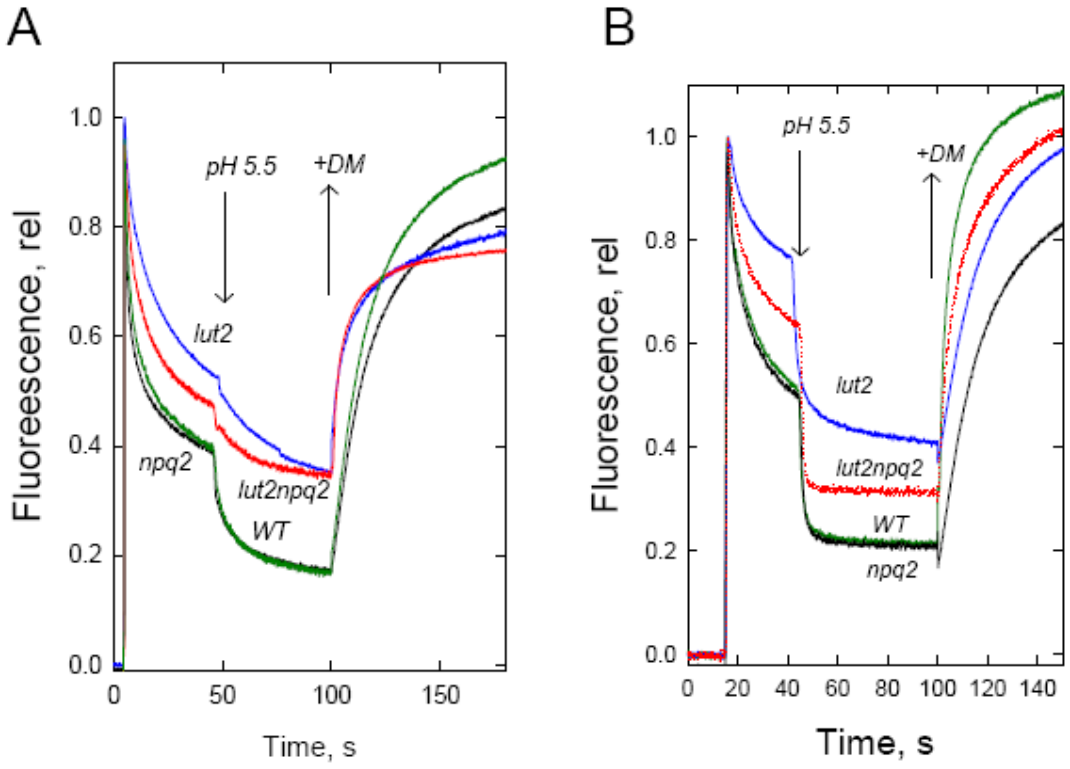


Figure 4.5 *In vitro* fluorescence quenching in isolated (A) LHCII and (B) CP26 from wild-type (green), *lut2* (blue), *npq2* (black) and *lut2npq2* (red) mutants. Samples were diluted into 30 µM DM (LHCII) or 60 µM DM (CP26)/ 10 mM HEPES / 10 mM Sodium Citrate pH 7.8, the pH is then lowered to 5.5 (shown by downward arrow) to induce maximum fluorescence quenching. Recovery from the fluorescence quenching was induced by returning detergent concentration to 200 µM DM (shown by upward arrow with +DM).

isolated complexes were subjected to 200 µM β-DM detergent concentration in a HEPES buffer of pH 7.8. These complexes can also show quenching of fluorescence under low detergent and low pH (5.5) condition (Ruban *et al*., 1994) by forming aggregates, this process mimics many of the features of NPQ *in vivo* (Horton *et al*., 2005). Thereby, the lifetimes were also recorded following the *in vitro* quenching of isolated complexes. For *in vitro* quenching, the isolated complexes were diluted into 30 µM β-DM (LHCII) or 60 µM β-DM (CP26) detergent concentrations in a similar buffer as above. To induce maximum quenching diluted HCl was added to lower the pH to 5.5 after the initiation of spontaneous quenching. Reason for using higher detergent concentration in CP26 quenching was to avoid induction of irreversible fraction of quenching in mutant CP26 samples upon dilution to lower (30 µM β-DM) detergent concentration. This *in vitro* quenching of isolated complexes is a reversible process hence quenching was recovered by returning the detergent concentration to 200 µM β-DM, this recovery also shows that complexes are not denatured during the quenching process (Figure 4.5). Data collected from lifetime measurements in unquenched and quenched states correspond and explain the differences observed *in vivo* during the photosynthetic and photoprotective states, respectively.

The fluorescence lifetime decays recorded in isolated LHCII of wild type, *lut2, lut2npq2* and *npq2* in both unquenched and quenched states are shown in Figure 4.6 A, while the amplitudes of components resolved in the fluorescence decay are shown in Figure 4.6 B. The average lifetime of LHCII from wild type was found as 3.52 ns in unquenched state, while in *lut2* it was measured as 3.7 ns, the longer value observed in latter case was consistent with the *in vivo* result. The extent of *in vitro* fluorescence quenching induced in LHCII from the wild type was much larger than that of *lut2* under identical conditions, consistent with the *in vivo* NPQ data. Accordingly, the average lifetime in the wild type was 0.33 ns as compared to 0.98 ns in case of the mutant. Comparison of the lifetime component amplitudes of the decay curve fits showed that around ~80% of wild type LHCII population was converted to a state with a sub-0.2 ns lifetime, consistent with previous reports (Mullineaux *et al*., 1993; van Oort *et al*., 2007), while in *lut2* LHCII even the shortest lifetime was longer (~0.3 ns) with much less (~55%) conversion at this component and a significant population of a longer 0.9 ns lifetime remaining.

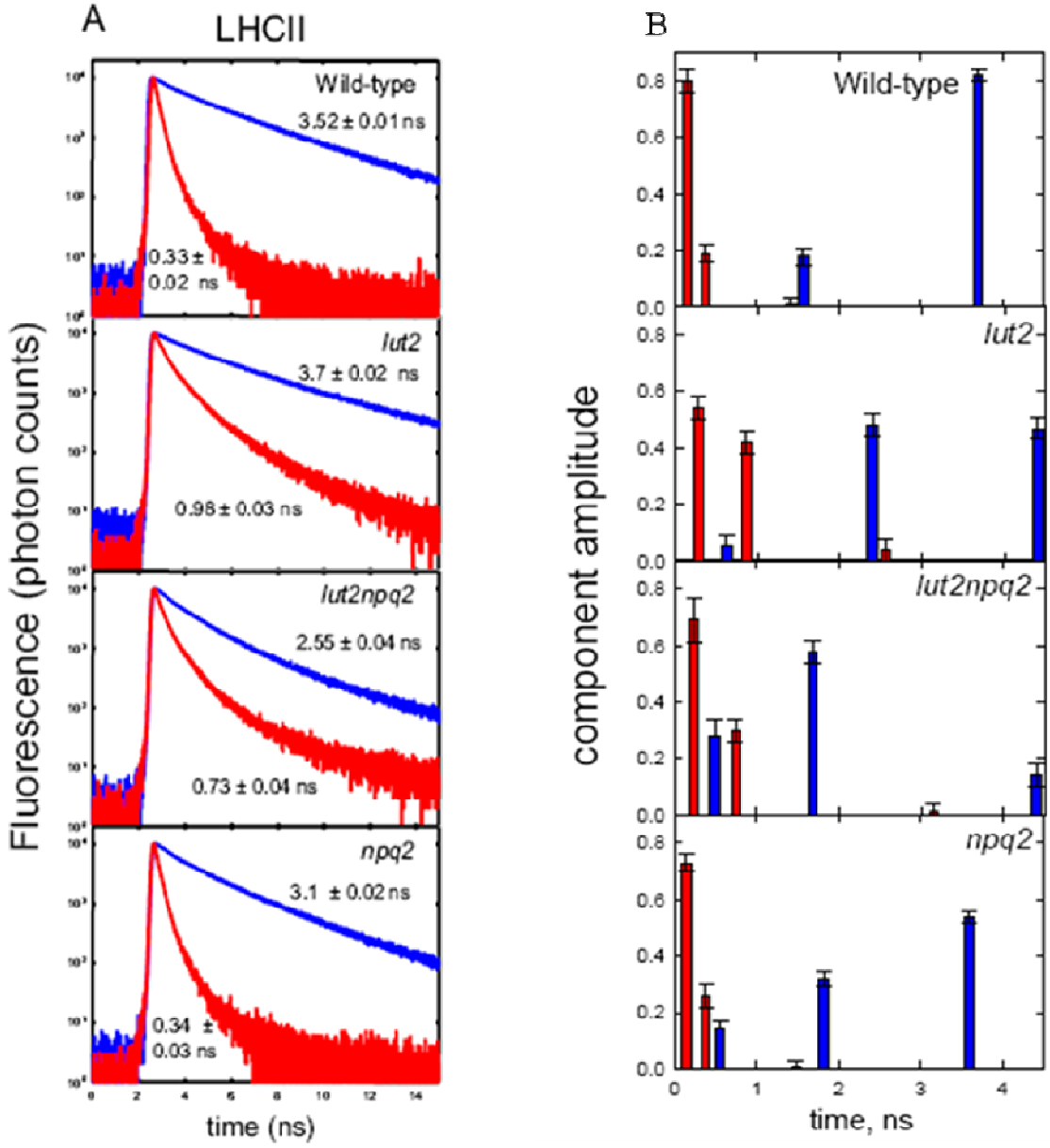


Figure 4.6 Chlorophyll fluorescence lifetime decays (A) and lifetime component amplitudes (B) of isolated monomeric LHCII complexes from wild type, *lut2*, *lut2npq2* and *npq2* mutant *Arabidopsis* plants. The photosynthetic (unquenched) state (in presence of 200 µM β-DM, pH 7.8) appears in blue, the photoprotective (quenched) state (in presence of 30 µM β-DM, pH 5.5) appears in red. The intensity weighted average fluorescence lifetime ±SEM (n=3) of each decay is also displayed.

Similarly, the results of lifetime analysis of LHCII from *lut2npq2* also mirrored those found *in vivo*. The average lifetime was shorter in unquenched state but longer in the quenched state of LHCII which contained zeaxanthin as only xanthophyll. Thus, similar to the *in vivo* finding, zeaxanthin confers reduced light harvesting efficiency without increasing the capacity for energy dissipation. Similar to *lut2*, the component amplitudes in *lut2npq2* also showed less efficient conversion to the shorter lifetimes than in wild-type with a significant population of a ~0.7 ns lifetime.

The LHCII from the *npq2* mutant in unquenched state showed similar but smaller reductions in average lifetime compared to those from the *lut2npq2*. However, the average lifetime of the quenched state of *npq2* was found identical to that of wild type, consistent with the *in vivo* results. Thus the reduction in quenching was only observed when zeaxanthin replaced either lutein and/or neoxanthin in the internal LHCII sites as in *lut2npq2* and *npq2*. Moreover, no impact on photoprotective capacity was observed in the absence of only neoxanthin.

4.2.4 *In vitro* chlorophyll fluorescence lifetime decays of isolated CP26 in unquenched and quenched states

The fluorescence lifetime decays were also similarly recorded in isolated minor complex CP26 of wild type and mutants in both unquenched and quenched states (Figure 4.7 A), along with the amplitudes of components resolved in the fluorescence decay (Figure 4.7 B). This minor antenna complex has been reported to form lutein and zeaxanthin radical cations, which are implicated in the quenching mechanism (Avenson *et al*., 2008; Avenson *et al*., 2009). However, the results obtained here demonstrate a similar pattern to that found for LHCII.

In wild type, once again a significantly shorter average lifetime in the quenched state and only slightly lower value was measured in unquenched state, as compared to the corresponding values of *lut2*. Interestingly, the fluorescence decays obtained for CP26 in unquenched state show 20% contribution from a short lifetime component in wild type and

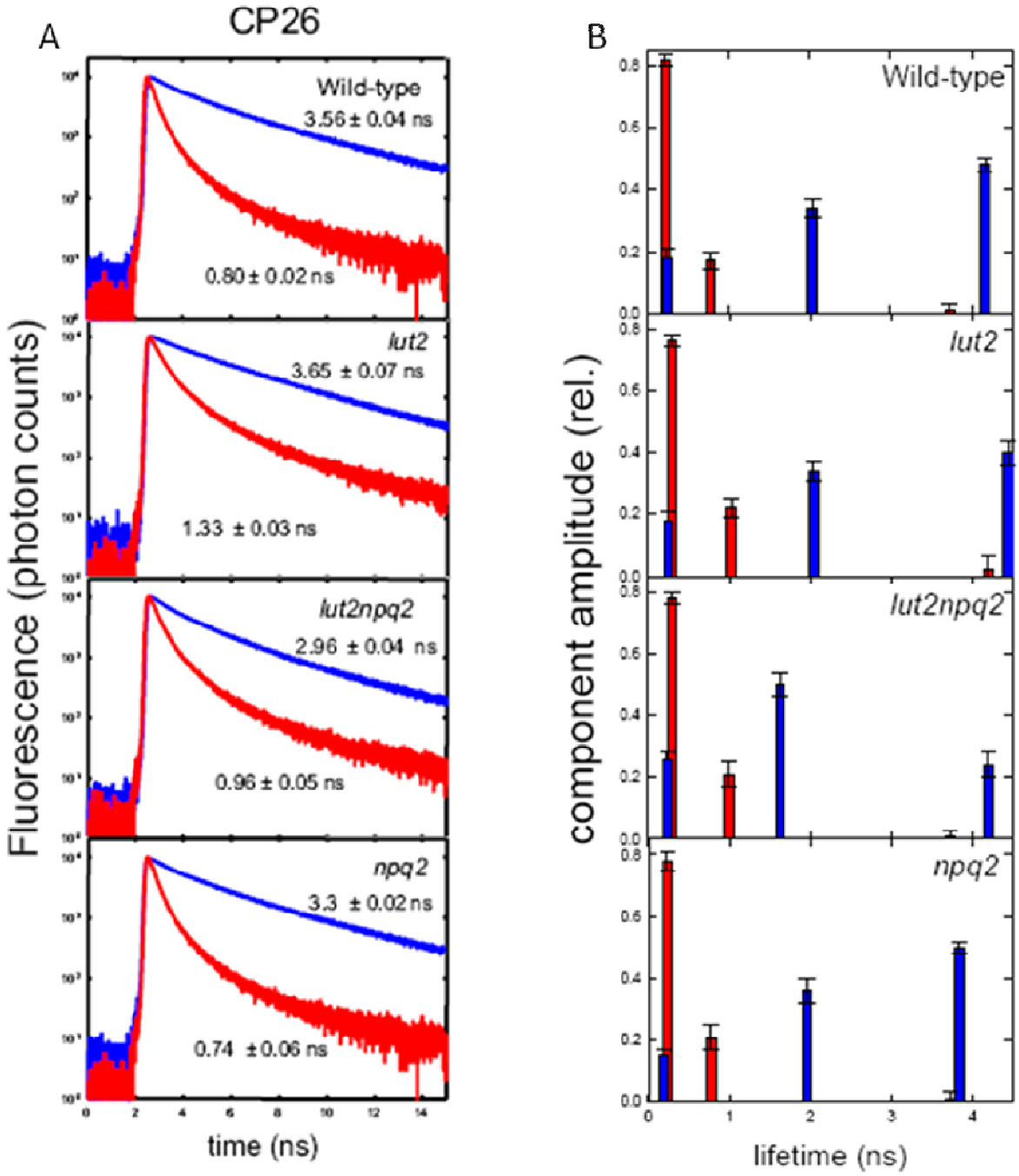


Figure 4.7 Chlorophyll fluorescence lifetime decays (A) and lifetime component amplitudes (B) of isolated CP26 complexes from wild type, *lut2*, *lut2npq2* and *npq2* mutant *Arabidopsis* plants. The photosynthetic (unquenched) state (in presence of 200 µM β-DM, pH 7.8) appears in blue, the photoprotective (quenched) state (in presence of 60 µM β-DM, pH 5.5) appears in red. The intensity weighted average fluorescence lifetime ±SEM (n=3) of each decay is also displayed.

all mutants, indicating some of it was already quenched as reported previously (Avenson *et al*., 2008; Crimi *et al*., 2001). A clear difference between wild type and *lut2* in unquenched state can however be achieved by excluding the short lifetime component, thus considering only the longer lifetime components of wild type at 2.0 ns and 4.2 ns and those of *lut2* at 2.4 ns and 4.5 ns can cause a shift to longer lifetime in the latter case.

In the case of *lut2npq2*, a significant reduction in average lifetime of the unquenched state but only a small increase in that of the quenched state was observed as compared to the wild type. The cause of this reduction in quenching in *lut2npq2* seemed to be the presence of a slightly longer lifetime components of ~0.25 ns and ~1 ns compared to the corresponding components of ~0.2 ns and 0.7 ns in the wild-type.

Similar results were also found in case of CP26 from the *npq2* mutant to those found for LHCII. In the quenched state, almost similar lifetime to that of wild type was observed, while a small decrease in lifetime of the unquenched state. This reduction in lifetime of unquenched CP26 is in fact consistent with the slightly lower fluorescence yield reported earlier in the same complex of *npq2* mutant (Dall'Osto *et al*., 2005).

4.2.5 Room temperature fluorescence spectra of isolated LHCII and CP26

The room temperature fluorescence spectra were also recorded under unquenched and quenched states for both LHCII and CP26 complexes isolated from wild type and mutants (Figure 4.8 A, B). This exercise was performed to see any evidence of denaturation or unfolding of protein complexes in the low detergent and low pH conditions, which would manifest under such conditions in the form of blue-shifted fluorescence arising from uncoupled chlorophylls (Ilioaia *et al*., 2008). No such evidence was found in any of the complexes, spectrum of an LHCII denatured by triton detergent was also recorded for references, as shown in Figure 4.7 A. These spectra also revealed the same characteristic, albeit smaller, red-shift known to accompany quenching in LHCII under low detergent conditions (Ilioaia *et al*., 2008).

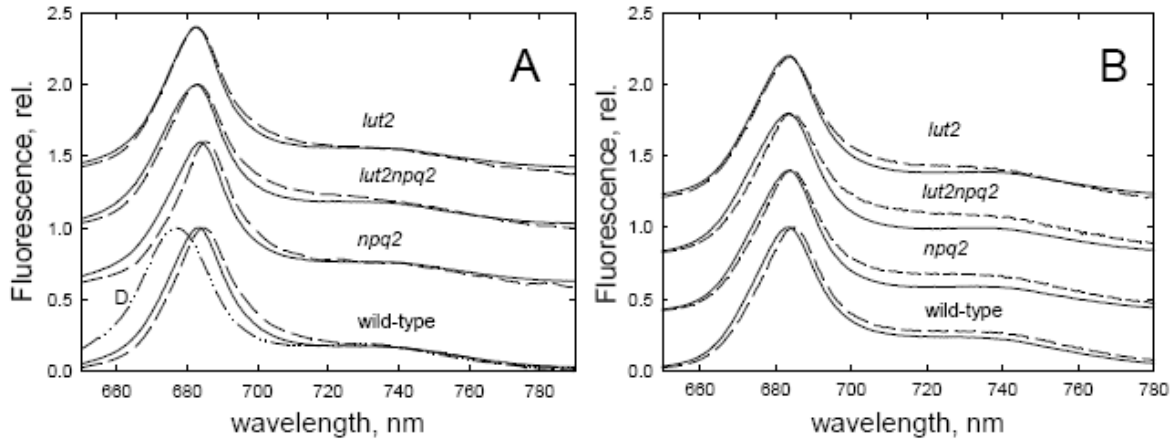


Figure 4.8 Fluorescence spectra of isolated LHCII (A) and CP26 (B) proteins from the wild-type, *lut2*, *npq2* and *lut2npq2* mutants, dashed lines represent samples quenched at 30 μM DM / pH 5.5, solid lines are same samples following restoration of detergent concentration to 200 μM DM. Dashed-dotted spectrum (marked 'D') is that of detergent denatured LHCII achieved by 10 minutes incubation with 3% triton X-100.

4.3 Discussion

The dynamic behaviour of LHCII, ranging from efficient light harvesting at limiting light for photosynthesis to effective excess energy dissipation at saturating light for photoprotection, has been studied for the first time here. Effect of varying xanthophyll composition on the optimisation of light harvesting is analysed in particular, by means of chlorophyll fluorescence lifetime measurements both *in vivo* and *in vitro*. The resulting data also adds to the existing knowledge of the regulation and optimisation of the efficiency of both light harvesting and photoprotection. This dynamic activity demonstrates its optimum range by having maximum light harvesting (or longest lifetime in the unquenched state) and maximum photoprotection (or shortest lifetime in the quenched state) at the same time.

The optimum dynamic range was achieved by having the wild type native complement of internal xanthophylls, with two luteins and one neoxanthin occupying the internal sites. The replacement of lutein with violaxanthin increased the light harvesting efficiency; however

the photoprotective capacity was reduced on the other end, restricting the dynamic range in *lut2*. Therefore the positive impact of increase in the light harvesting efficiency is clearly offset by the reduction in capacity of photoprotection. The replacement of all internal xanthophylls with zeaxanthin resulted in most drastic impact on dynamic activity as both the light harvesting efficiency and photoprotective capacity were jeopardized in this case, as shown in *lut2npq2*. Absence of another internal xanthophyll, neoxanthin, also affected the dynamic range by impairing the light harvesting efficiency, however the photoprotective capacity remained maximum similar to the wild type.

Similar pattern, observed in both *in vivo* and *in vitro* analyses, suggests that the origins of these effects of xanthophyll complement on light harvesting and photoprotection reside in the altered properties of the light harvesting complexes. Remarkably, all the effects on lifetimes of changes in xanthophyll composition observed in leaves were manifested as altered properties of the isolated LHCII. The lifetime of isolated LHCII is not fixed but can be manipulated, by varying detergent concentrations, from ~4 ns down to ~1.0-0.3 ns (Pascal *et al*., 2005; Ilioaia *et al*., 2008; Moya *et al*., 2001; van Oort *et al*., 2007). The *in vitro* quenching induced by low detergent and low pH conditions mimics the NPQ *in vivo*. The similarities between the effects of xanthophyll substitutions on leaves and LHCII suggests that the quenching mechanism is the same in both, consistent with much previous data that highlighted the similarity between NPQ and quenching in LHCII aggregates (Horton *et al*., 1996; Horton *et al*., 2005).

The results described here show that the xanthophyll complement affects the excited chlorophyll lifetimes in both unquenched and quenched states of LHCII antenna complexes. Lutein is clearly an essential cofactor of LHCII and when it is replaced at its internal binding sites by violaxanthin or zeaxanthin the lifetimes either increase or decrease, respectively, in unquenched light harvesting state. However in quenched or photoprotective state, incorporation of violaxanthin and zeaxanthin in place of lutein suggests the fromer two as weaker quenchers than the latter one. It has been suggested that excess energy is transferred by the S1 excited state of chlorophyll *a* to the S1 state of xanthophyll at L1 site and hence dissipated (Ruban *et al*., 2007). The variations in

quenching capacity of these xanthophylls are unlikely to be explained by differences in their excited state energy levels since both violaxanthin and zeaxanthin possess similar S1 lifetimes and energies to lutein when incorporated into these sites in LHCII, which are already below those of chlorophyll *a* (Polívka *et al*., 2002)**.** Thus, the differences in molecular geometry and polarity of each xanthophyll (Ruban *et al*., 1993a) may instead determine how well it is able to interact with the terminal emitter chlorophylls in the L1 binding site. Alternatively, xanthophylls bound at these internal sites may control the structural flexibility needed for the transition between the different states of quenching (Ruban *et al*., 2007; Ilioaia *et al*., 2008). Thus, the identity of the xanthophyll bound to the L1 and L2 sites determines the extent of dynamic range between the quenched and unquenched states, which are believed to originate from intrinsic conformational changes, as originally suggested (Horton *et al*., 2000; Bassi and Caffarri, 2000).

Major and minor LHCII complexes have been shown to undergo *in vitro* quenching by aggregate formation, as there is high degree of structural homology among all these protein complexes. The minor complex CP26 has been reported to aggregate and quench more readily than the LHCII *in vitro* (Ruban *et al*., 1996), however the protein-to-protein interactions and conformational changes cannot be accorded *in vivo*, where these complexes are organised in a strict stoichiometry in the PSII complex (Hankamer *et al*., 1997; Rhee *et al*., 1997). The CP26 has recently been implicated in a reconstituted study as unique site of NPQ whereby quenching occurs by a different mechanism involving a zeaxanthin radical cation (Ahn *et al*., 2008). There is no direct evidence that the minor complex can quench more efficiently than the LHCII *in vivo*. In the present work, the similar behaviour of the minor antenna complex CP26 and LHCII does not lend any support to the above mentioned idea. Indeed, the results shown here provide no evidence for an important direct quenching role for zeaxanthin. The presence of extra zeaxanthin in the *npq2* and *lut2npq2* mutants causes constitutive quenching which is small and does not increase the maximum quenching capacity, in leaves, in LHCII or in CP26; indeed, for the zeaxanthin only mutant it was actually decreased.

In this study, the native isolated LHCII and CP26 complexes seem to be devoid of any peripheral binding xanthophylls at V1 site, as indicated by the pigment analysis. The chlorophyll fluorescence lifetimes measured in these isolated complexes followed the similar pattern as observed during the similar measurements performed *in vivo*, despite absence of peripheral xanthophylls. The well-documented impact of de-epoxidation of violaxanthin to zeaxanthin on enhancement of NPQ (Demmig-Adams, 1990) can therefore be explained by an indirect, regulatory role from the peripheral V1 site, allosterically modulating the pH sensitivity of NPQ, as has been described *in vitro* and *in vivo* (Horton *et al*., 2000). *In vivo*, the quenching state of LHCII is controlled within a range which is less than that achievable in isolated complexes. Hence LHCII acts rather like a dimmer switch, which *in vivo* is never fully turned off or on. In light harvesting state, the lifetime is already reduced to around 2-2.5 ns, consistent with the lifetime of LHCII when incorporated into liposomes (Moya *et al*., 2001), while in photoprotective state, NPQ is formed by further action of the dimmer switch to yield a lifetime of ~0.4-0.6 ns. Resonance Raman analysis also shows that LHCII is in a partially quenched conformation in the absence of NPQ and in an incomplete state of switching at maximum NPQ (Ruban *et al*., 2007). Clearly, external factors in the thylakoid membrane tune the capacity of LHCII to express its potential states: e.g. interaction with neighbouring proteins in the LHCII-PSII macrostructure, such as PsbS (Horton *et al*., 2008). Thus, nature exploits the inherent flexibility of LHCII function by adopting a complement of xanthophylls that optimizes the maximum dynamic range between its light harvesting and photoprotective states. Optimisation of photosystem II quantum efficiency therefore occurs at the level of individual LHCII proteins.

Chapter Five

Dissecting the relationship between PsbS and zeaxanthin in nonphotochemical quenching

5.1 Introduction

Nonphotochemical quenching (NPQ) is a regulatory photoprotective process, by which excess light energy absorbed by the light harvesting antenna of photosynthetic organisms is dissipated as heat (discussed in section 1.10). In algae and plants, the major fraction of NPQ is called qE or energy-dependent quenching, which is rapidly reversible in dark. qE depends upon the thylakoid lumen pH and the presence of specific de-epoxidised xanthophylls (reviewed in Horton *et al*., 1996; Gilmore, 1997; Muller *et al*., 2001). The low-lumen pH (pH 5.2-6.0) is the result of a ΔpH formation across the thylakoid membrane caused by light-driven electron transport (Briantais *et al*., 1979). This lumen acidification is required for activation of the violaxanthin de-epoxidase enzyme (VDE) and protonation of PSII protein(s). The de-epoxidation of xanthophylls required for qE is a part of the xanthophyll cycle in which violaxanthin is de-epoxidised by VDE to yield antheraxanthin and zeaxanthin in excess light (Demmig- Adams, 1990). This zeaxanthin has been suggested to act as an acceptor and hence a direct quencher of excitation energy transferred from chlorophyll excited states (Ma *et al*., 2003; Holt *et al*., 2004).

qE has also been shown to be affected by the level of a photosystem II subunit S (PsbS) protein, as its absence can strongly reduce the extent of qE (Li *et al*., 2000). The site-directed mutagenesis of PsbS has proposed that it is a ΔpH sensor protein possessing two proton-binding lumen-exposed glutamate residues. However, the exact mechanism by which PsbS regulates NPQ is yet to be elucidated. This protein with twofold symmetry has been suggested to be directly involved in NPQ by providing two quenching sites which include two proton-active domains and two zeaxanthin binding sites (Li *et al*., 2004). The evidence for this proposed quenching mechanism was based on the observation of *in vitro* binding of two zeaxanthin to PsbS (Aspinall O'Dea *et al*., 2002). Moreover, this binding of zeaxanthin to PsbS resulted in a strong red shift in the pigment absorption spectrum, which may explain the origin of ΔA535 absorption change from a sub-pool of red-shifted zeaxanthin (Ruban *et al*., 2002a). An inhibitor of qE, dicyclohexylcarbodiimide (DCCD), has also been shown to bind proton active domains of PsbS (Dominici *et al*., 2002). On the basis of these observations, it has been suggested that PsbS binds zeaxanthin and/or protons, both of which are necessary for qE.

Alternatively, an allosteric model for NPQ was put forward to explain the indirect role of both zeaxanthin and PsbS protein in regulation of the quenching site which is localised in the light harvesting antenna complexes (Horton *et al*., 2005). A conformational change within these complexes would result in a change in pigment configuration, creating a quenching interaction between bound pigments. The evidence for such conformational changes has not only been provided *in vitro* in LHCII crystals (Pascal *et al*., 2005), but also in thylakoid membranes *in vivo* by the use of Raman spectroscopy (Ruban *et al*., 2007). Evidence for the indirect role of zeaxanthin as a modulator of NPQ has come from the ΔpH-dependent quenching formed immediately upon illumination in the absence of zeaxanthin (Noctor *et al*., 1991; Ruban *et al*., 1999); this has also been demonstrated in the qE formation kinetics of *npq1* mutant in Chapter 3. Thus, NPQ is not entirely dependent upon the formation of zeaxanthin, as presence of zeaxanthin only lowers the ΔpH requirement for NPQ without increasing the magnitude of it, in pre-illuminated leaves and isolated chloroplasts (Rees *et al*., 1989; Noctor *et al*., 1991, Ruban *et al*., 2001) as well as upon the second illumination of dark-adapted leaves when zeaxanthin is already present (Ruban *et al*., 1999). The latter has also been shown in the qE formation kinetics study of *npq2* mutant in Chapter 3. Zeaxanthin can also promote the induction of quenching in isolated light harvesting complexes by accelerating the rate of formation of the quenched state, indicative of its role to regulate the quenching kinetics (Ruban *et al*., 1994; 1999). In the allosteric model of NPQ, the role of PsbS has been suggested to bind protons and zeaxanthin or to act as a regulator of conformational changes in one or more antenna complexes to cause quenching (Horton *et al*., 2005).

Study of PsbS-lacking mutant *npq4* showed that both components of rapidly forming NPQ, zeaxanthin-independent and zeaxanthin-dependent, were greatly reduced in the absence of PsbS (Li *et al*., 2000). Whilst, the extent of NPQ was increased by almost two fold by over-expression of *psbS* gene in L17 plants with higher levels of PsbS (Li *et al*., 2002c). Interestingly, both the above mentioned plant types showed such a stark contrast in their NPQ despite having no differences in zeaxanthin formation as both possessed normal xanthophyll cycle. However the role of PsbS for both zeaxanthin-independent and zeaxanthin-dependent quenching was only demonstrated in a later comparative study of

L17 and wild type plants, showing similar increase of NPQ, in the presence or the absence of zeaxanthin (Crouchman *et al*., 2006). This study indicated towards the role of PsbS as a trigger of intrinsic conformational change in antenna complexes resulting in NPQ, with zeaxanthin as an allosteric regulator and not the direct quencher. To avoid violaxanthin de-epoxidation, the zeaxanthin-inhibitor dithiotheitol (DTT) was used to measure only zeaxanthin-independent component of NPQ in both types of plants. However, observation of an additional impact of DTT apart from blocking zeaxanthin synthesis in case of *npq1*, as mentioned in Chapter 3, necessitates the use of a natural system for this experiment by enhancing the PsbS levels in absence of zeaxanthin without using any exogenous chemical inhibitor. To achieve this, PsbS can be increased in zeaxanthin-lacking *npq1* mutant by employing physiological measures like acclimation to high light and/or low temperature treatment (Demmig-Adams *et al*., 2006). Similarly, availability of both PsbS over-expressor L17 and *npq1* mutant can also be genetically exploited to create a double mutant possessing both over-expression of *psbS* gene along with mutation in VDE gene.

The work presented in this chapter deals with assessing the role of zeaxanthin as a direct quencher or allosteric regulator by enhancing the level of PsbS in the absence of this xanthophyll. This can also help to dissect the role of both zeaxanthin and PsbS by identifying if quenching can be increased in the absence of zeaxanthin only by the increase in amount of PsbS. As a result of genetic cross between PsbS overexpressor L17 and *npq1* with two copies of the *psbS* gene we also envisage observing the dosage effect of PsbS on NPQ, which has also been demonstrated in an earlier study involving a cross between PsbS-lacking *npq4* and wild type containing two copies of the *psbS* gene (Li *et al*., 2002a). This can help to identify a correlation between PsbS and zeaxanthin-independent and zeaxanthin-dependent components of quenching. The effect of PsbS on NPQ is well established, but its mechanistic role is not clear as it has been suggested either as a direct quencher by binding zeaxanthin (Li *et al*., 2004; Holt *et al*., 2005) or otherwise indirectly as regulator of the conformational change in antenna complexes leading to quenching (Ruban *et al*., 2001; Wentworth *et al*., 2001; Horton *et al*., 2005). The quenching would be expected to be increased by PsbS enhancement only in the presence of zeaxanthin in case

of its direct role, while quenching would be enhanced both in the presence or absence of zeaxanthin in case of indirect role of PsbS.

Furthermore, observation of the kinetics of NPQ formation and relaxation in plants with altered PsbS levels would give insight into the proposed mechanism of qE quenching and support either a direct or indirect role of PsbS in quenching. If PsbS acted as a direct quencher, whilst the level of NPQ would be greater, the rate of formation and relaxation of the quenched state would only be affected by the concentration of the protein in the presence of deepoxidation. However, if PsbS was acting allosterically to regulate the transition to the quenched state, the rate of both formation and relaxation would be expected to differ depending on the concentration of the protein, and these differences would be seen both in the presence and absence of de-epoxidation. The level and rate of non-photochemical quenching in leaves and chloroplasts, both in the presence and absence of de-epoxidation, was therefore analysed in an attempt to establish how PsbS concentration influences both the amount and kinetics of NPQ *in vivo*.

5.2 Results

The fact, that overexpression of PsbS increases the amplitude of NPQ even after using zeaxanthin-inhibitor dithiotheitol (DTT), supported this view (Crouchman *et al*., 2006). Further work is required to elucidate the exact role of PsbS and zeaxanthin in the molecular mechanism of NPQ. To achieve this, PsbS levels can be manipulated by physiological means such as acclimation to high light (Demmig-Adams *et al*., 2006). A similar increase in NPQ in the *npq1* mutant lacking zeaxanthin is also envisaged as a result of enhanced PsbS by high light acclimation, without using DTT. Alternatively, a double mutant can also be generated by crossing the zeaxanthin lacking mutant *npq1* with PsbS overexpressor L17, to obtain a line with potentially higher NPQ level than that of *npq1*. The resultant findings thus can elucidate the role of zeaxanthin as a quencher alone or in combination with PsbS, or alternatively as an allosteric regulator of NPQ.

5.2.1 High light (HL) acclimation to increase NPQ levels

To determine the temporal effect of high light acclimation, *npq1* and wild type plants were exposed to 500 μmol photons m^{-2} s^{-1} high light (HL), with 8h photoperiod at room temperature (24^{o}C) for two acclimation treatments of 8 days and 4 weeks durations. By the end of both HL treatments, plants of same age, *i.e*. 8 weeks, were obtained for further analyses. For 8 days acclimation, 7-week- old plants were used, while 4-week-old plants were used for further 4 week HL acclimation. Prior to HL exposures, plants were grown under controlled conditions of 8h of 100 μmol photons m^{-2} s^{-1} light at 22^{o}C alternating with a 16h dark period at 18^{o}C. Shorter duration of HL exposure (8 days) was found to have more pronounced effect to increase NPQ level while no significant increase in NPQ was observed as a result of prolonged HL exposures (4 week). This may explain the two contrasting strategies of short term photoprotection and long term photoacclimation adapted by plants for the regulation of light harvesting. All the data presented here is therefore obtained from short term (8 days) HL acclimation of plants, as this work is aimed at increasing the extent of NPQ.

Both wild type and *npq1* plants showed no apparent growth differences, when exposed to HL. This shows that both plant types were capable to adapt to the HL and zeaxanthin formation does not limit the energy utilisation and thus plant growth. However, minor changes in leaf morphology were observed in all HL acclimated plants. Leaves with darker colour, coarse texture, curly edges, more thickness and shorter petioles were observed after HL acclimation, in comparison to those grown under controlled growth conditions.

5.2.1.1 Effect of HL acclimation on Pigment composition

5.2.1.1.1 Chlorophyll content

Total chlorophyll concentration per unit leaf area decreased in both wild type and *npq1* after HL acclimation, but no significant differences were observed between both plant types grown at the same light intensity. Moreover, in HL acclimated plants, relatively stronger decrease in chlorophyll *b* than that of chlorophyll *a* resulted in increase of chlorophyll *a/b*

ratio. Since the major light harvesting complexes of PSII (LHCII) are enriched in chlorophyll *b*, this may indicate a likely reduction in the antenna size under HL conditions. The availability of excess light energy eliminates the need for higher capacity of light harvesting and hence a large-sized antenna. Apart from LHCII, some less pronounced changes in other chlorophyll binding components, like minor LHCII complexes, LHCI complexes and reaction centres, have also been reported that may also contribute to the values of chlorophyll *a/b* (Bailey *et al*., 2001).

Plant Type	Total Chl (mg/ml)	Chl *a/b*
Wild type -100μE	2.67 ± 0.33	2.86 ± 0.12
Wild type -500μE	1.58 ± 0.12*	3.28 ± 0.14
npq1 -100μE	2.81 ± 0.20	3.02 ± 0.06
npq1 -500μE	1.63 ± 0.13*	3.35 ± 0.10

Table 5.1 Chlorophyll concentration measurements of wild type and *npq1* grown at 100 μmol photons m^{-2} s^{-1} (control) and 500 μmol photons m^{-2} s^{-1} (high light). Pigments were extracted with 80 % (v/v) acetone from leaf discs, using UV-Vis spectrophotometer. All data are mean ± SE for at least three plants. *= Significantly different to respective controls (Student's t-test p=0.005).

5.2.1.1.2 Xanthophyll composition

To determine the effect of HL acclimation on xanthophyll composition, leaf discs were collected from wild type and *npq1* plants to analyse their pigment composition by HPLC in both light-treated and dark-adapted conditions (Table 5.2). The expected general differences between wild type and *npq1* were found as both antheraxanthin and zeaxanthin were absent in the latter due to inactivity of VDE. The light-induced de-epoxidation was also absent in *npq1*, while in case of wild type it was measured as 46 %. After HL acclimation, xanthophyll cycle pool was significantly increased in wild type, but no significant increase in de-epoxidation was observed as a result of HL acclimation. Interestingly, a similar significant increase in the violaxanthin quantity was also measured in *npq1*, despite the fact that xanthophyll cycle was inactive in this mutant. This increase in xanthophyll cycle pool has been suggested to accumulate in the lipid phase of the

membrane, as the antenna size and hence the number of available xanthophyll cycle-binding sites is expected to be reduced under HL conditions (Kalituho *et al*., 2006).

Plant	**Neo**	**Lut**	**Vio**	**Ant**	**Zea**	**β-Car**	**DEPs**
WT 100μE dark	5.2 ± 0.5	17 ± 1	4.4 ± 0.2	0.2 ± 0.1	0	12.1 ± 1	3 ± 0.9
WT 100μE light	5.1 ± 0.9	16 ± 1	2.0 ± 0.4	0.8 ± 0.3	1.7 ± 0.4	9.8 ± 0.8	46 ± 1.2
WT 500μE dark	4.7 ± 0.5	14.6 ± 2.5	6.0 ± 0.6	0.3 ± 0.1	0	11.9 ± 1.2	4 ± 1.4
WT 500μE light	4.5 ± 0.7	15.2 ± 2.2	2.8 ± 0.8	1.4 ± 0.4	2.4 ± 0.8	10.5 ± 0.6	47 ± 2
npq1 100μE dark	5.3 ± 1.1	18 ± 2	4.4 ± 1.1	0	0	12 ±1.8	0
npq1 100μE light	5.7 ± 0.6	20 ± 1	5.3 ± 0.3	0	0	9.2 ± 0.6	0
npq1 500μE dark	5.0 ± 0.4	17.5 ± 1.7	7.95 ± 0.2	0	0	9 ± 0.6	0
npq1 500μE light	4.8 ± 0.7	18.2 ± 2.4	7.8 ± 0.8	0	0	7.5 ± 0.5	0

Table 5.2 Pigment composition of wild type (WT) and *npq1* grown at 100 μmol photons m^{-2} s^{-1} (control) and 500 μmol photons m^{-2} s^{-1} (high light). Leaf discs were collected from plants either dark-adapted for 30 min or light-treated for 10 min at 1000 μE. Data are normalized to 100 chlorophyll *a* + *b* molecules and are means ± SE from three replicates. Neo, Lut, Vio, Ant, Zea, β-Car and DEPs represent neoxanthin, lutein, violaxanthin, antheraxanthin, zeaxanthin, β-carotene and de-epoxidation state % [(zeaxanthin + 0.5 antheraxanthin)/(violaxanthin + antheraxanthin + zeaxanthin)], respectively.

The additional xanthophyll-binding proteins, like ELIPs, HLIPs and SEPs formed during HL exposure have also been proposed to bind substantial amounts of xanthophylls, as mentioned earlier in chapter I (Adamska, 1997; Adamska *et al*., 1999; Heddad and Adamska, 2000).

5.2.1.2 Effect of HL acclimation on PsbS enhancement

For the confirmation of PsbS enhancement among HL acclimated wild type and *npq1* plants, the western blots were performed using an anti-PsbS primary antibody specific for *Arabidopsis*. Unstacked thylakoids were prepared from the plants grown at HL (500 µmol photons m^{-2} s^{-1}) and at moderate light (100 µmol photons m^{-2} s^{-1}) along with *npq4* and L17 plants as control. Figure 5.1 shows a typical blot where PsbS band of 22 kDa is identified by the help of marker and is confirmed by the absence of this band in PsbS-less *npq4* mutant. The band appears to be noticeably denser in case of HL acclimated plants as compared to those grown at moderate light.

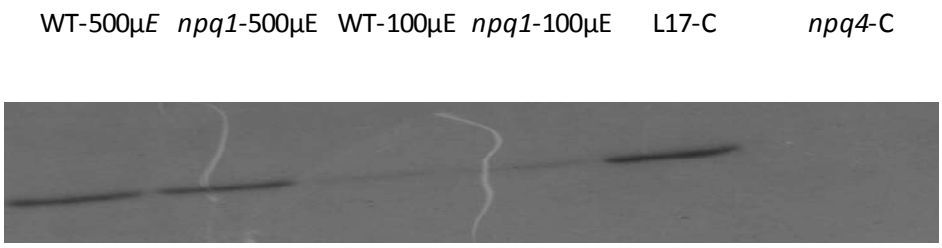


Figure 5.1. Western blot probed with anti-PsbS antibody for wild type (WT) and *npq1* plants grown at 500µE and 100µE. L17 and *npq4* were used as controls, denoted by "-C". Unstacked thylakoids (1µg chl/lane) were run on a 15% SDS PAGE gel and probed with anti-PsbS primary antibody.

5.2.1.3 Effect of HL acclimation on fluorescence parameters

In order to investigate the effect of HL acclimation on various fluorescence parameters, fluorescence induction curves were recorded on dark adapted wild type and *npq1* plants. Three different actinic light intensities (700, 1000 and 2000 µmol photons m^{-2} s^{-1}, also

known as µE) were initially used to compare the response of both plant types as a result of HL exposure by studying the parameter of chlorophyll fluorescence like NPQ and its two components qE, qI.

5.2.1.3.1 NPQ magnitude

Average NPQ for both first and second illumination periods were recorded, as shown in Table 5.3 A and B, respectively. In all the cases, steady state values of NPQ were reached. As a result of HL acclimation, NPQ magnitude was increased in both wild type and *npq1* at all the three actinic light intensities used (Table 5.3 A, B). However, the use of 2000 µmol photons m^{-2} s^{-1} (µE) actinic light caused more pronounced NPQ differences in case of wild type between HL-acclimated plants and controls, as higher actinic light ensures the saturation of photosynthetic electron transport to minimize photochemical quenching, and therefore the contribution of NPQ would be maximum. Contrarily for *npq1*, the use of 700 µmol photons m^{-2} s^{-1} (µE) proved more useful to exhibit clearer NPQ differences between HL acclimated and control plants, as this mutant showed higher contribution of slowly reversible qI component. This may also suggest that *npq1* is unable to cope with HL intensities due to absence of zeaxanthin causing more photoinhibition, which ultimately affects the amplitude and kinetics of NPQ. This data also shows higher level NPQ during the second illumination, in wild type this increase can be attributed to the "light activation" and the formation of zeaxanthin during first illumination which is retained during the short dark period (Demmig-Adams, 1990), while in *npq1* either the first factor or enhanced susceptibility to high light can be responsible as the contribution of qI also increases during the second illumination.

An increase of 34% in NPQ levels was recorded using 700µE actinic light for 8 days HL-acclimated *npq1*. While for wild type of similar HL acclimation, 40% increase in NPQ levels was observed using 2000µE actinic light. Increase in NPQ levels of wild type plants after HL acclimation can be attributed to physiological factors like increase in PsbS concentration and xanthophyll cycle pool. In case of HL acclimated *npq1*, the only apparent

A

Plant	Actinic Light	NPQ 1st Illumination	NPQ 2nd Illumination
Wild type 100 μE	700 μE	1.973 ± 0.15	2.094 ± 0.17
Wild type 500 μE	700 μE	2.207 ± 0.20	2.459 ± 0.22
Wild type 100 μE	1000 μE	2.016 ± 0.17	2.217 ± 0.20
Wild type 500 μE	1000 μE	2.476 ± 0.21	2.638 ± 0.25
Wild type 100 μE	2000 μE	2.237 ± 0.15	2.259 ± 0.19
Wild type 500 μE	2000 μE	2.849 ± 0.20	3.171 ± 0.16

B

Plant	Actinic Light	NPQ 1st Illumination	NPQ 2nd Illumination
npq1 100 μE	700 μE	0.515 ± 0.03	0.568 ± 0.05
npq1 500 μE	700 μE	0.706 ± 0.07	0.762 ± 0.05
npq1 100 μE	1000 μE	0.677 ± 0.05	0.871 ± 0.09
npq1 500 μE	1000 μE	0.750 ± 0.07	1.075 ± 0.10
npq1 100 μE	2000 μE	0.842 ± 0.06	1.028 ± 0.08
npq1 500 μE	2000 μE	0.948 ± 0.08	1.210 ± 0.11

Table 5.3 NPQ magnitudes of wild type (**A**) and *npq1* (**B**) grown at 100μE (control) and 500μE (high light). NPQ was measured by the end of first 5 min illumination, followed by 5 min dark relaxation and then by the end of second 5 min illumination, using three actinic lights of 700, 1000 and 2000 μE. NPQ was calculated as (Fm-Fm')/Fm', where Fm is the maximum dark-adapted fluorescence and Fm' is the maximum fluorescence on application of a saturating pulse during actinic illumination. All data are mean ± SE for at least three plants.

physiological factor causative of NPQ enhancement can be increase in PsbS concentration, as zeaxanthin is missing in this case. This shows that NPQ can be enhanced due to increase

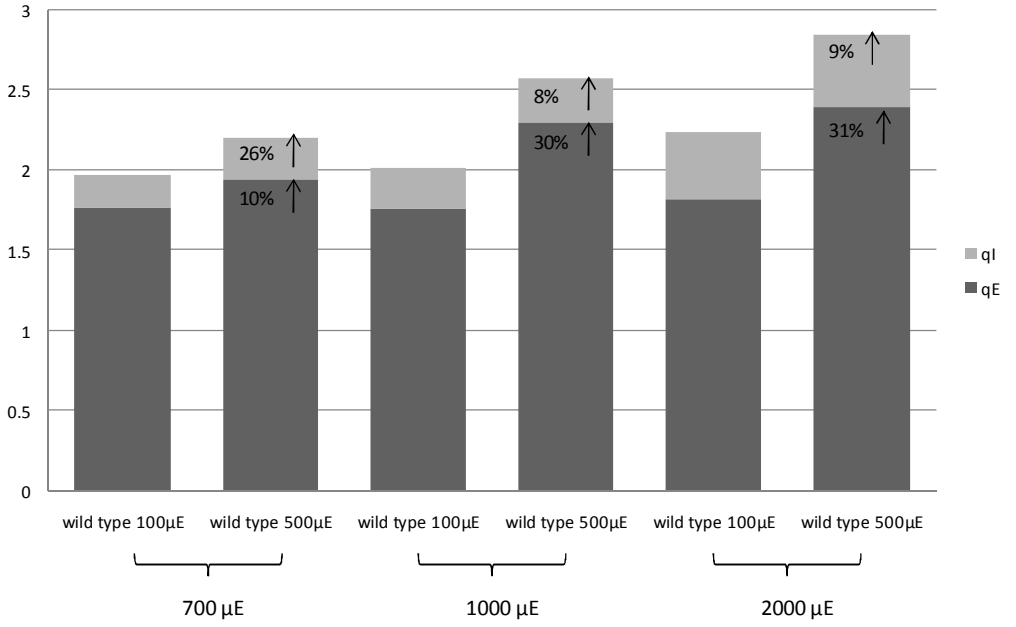
in PsbS, even in the absence of zeaxanthin. This further indicates that role of zeaxanthin in NPQ is unlikely to be direct but an indirect and regulatory one. The magnitude of NPQ increased with use of higher actinic light in both the plant types, as high light might be expected to induce higher ΔpH and/or extra xanthophyll cycle pool. However, use of high actinic light also resulted in the increase of slowly reversible photoinhibitory components of NPQ, which were more pronounced in *npq1* mutant after HL acclimation. The increase in amount of xanthophyll cycle carotenoid violaxanthin in *npq1* as a result of HL acclimation may explain this inhibitory effect on NPQ. Violaxanthin is an allosteric inhibitor (Ruban *et al*., 1996); hence this can explain why under higher actinic light intensities (1000 and 2000 μM) the NPQ is not that enhanced in *npq1* as under lower actinic light of 700 μM.

5.2.1.3.2 qE and qI components of NPQ

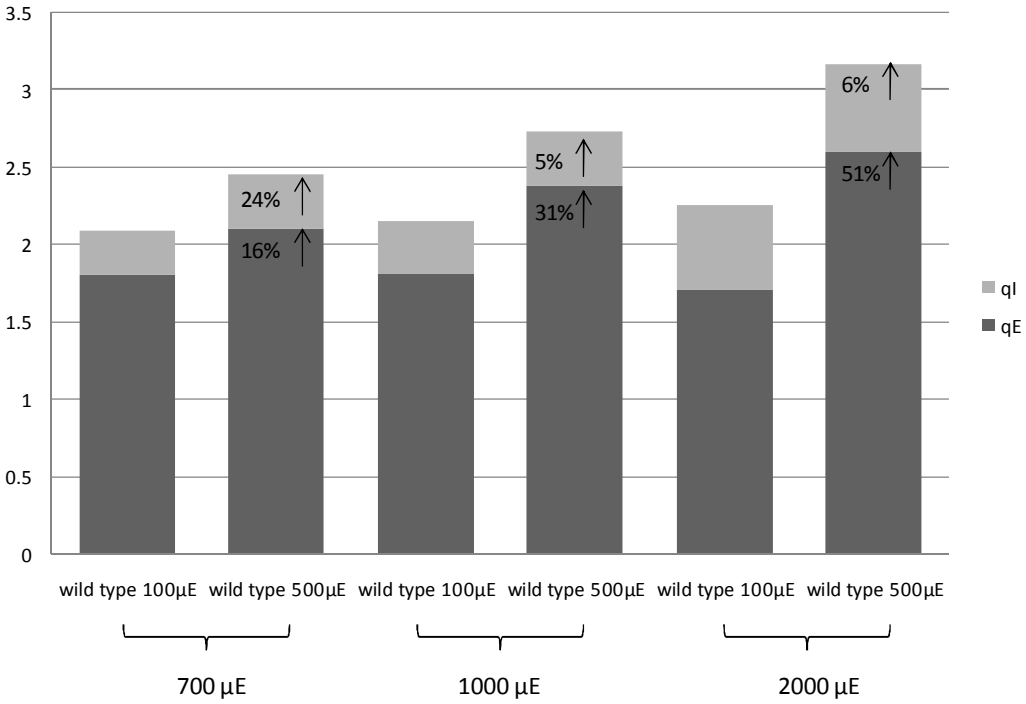
As NPQ comprises of two major components, one rapidly relaxing qE component depends on ΔpH also involving PsbS and zeaxanthin, the other slowly relaxing qI component caused by photoinhibitory effect. The measurement of qE is therefore essential to depict any change in the PsbS levels. Rapidly reversible qE and slowly reversible qI components of NPQ were also measured for both wild type and *npq1* at all the three actinic light intensities.

Figure 5.2 shows the contribution of these two major components of NPQ during first and second illumination each for wild type ((A and B) and *npq1* (C and D). The rapidly reversible qE of wild type plants has higher contribution in total NPQ, whereas as slowly reversible qI of *npq1* forms major part of NPQ, particularly in control plants grown at lower light intensity (80 μE) and upon use of higher actinic light intensities. The data demonstrates that qE, the major component of NPQ, increased variably as a result of HL acclimation at all the three actinic light intensities used in both wild type and *npq1*, which can be attributed to the predominant factor of higher PsbS concentration for the increase in NPQ in case of both wild type and *npq1*.

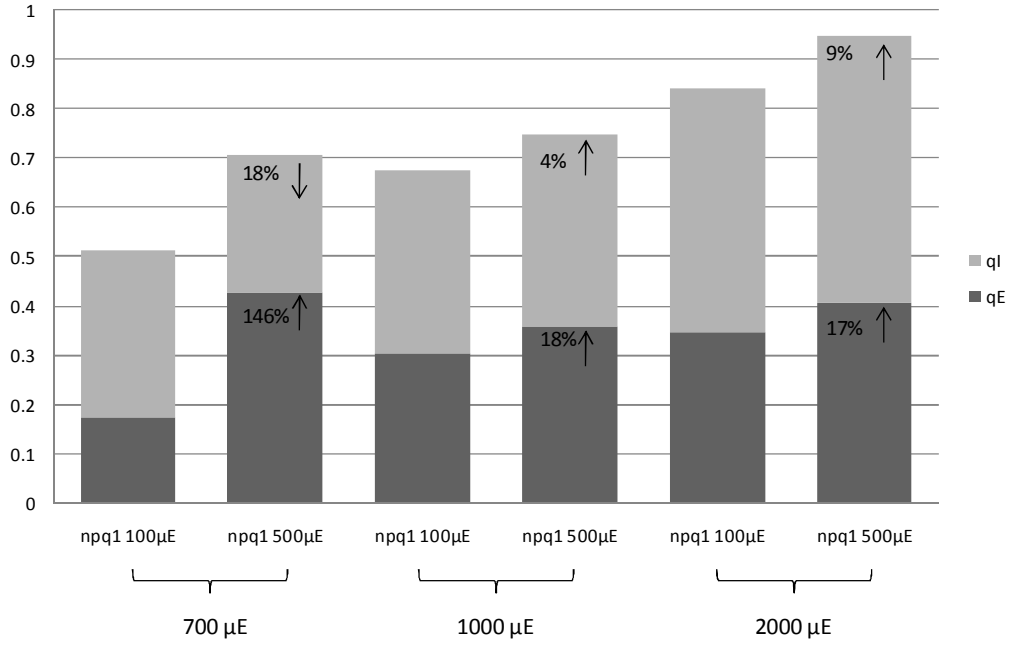
(A) The qE and qI components of NPQ measured for wild type plants acclimated at 2 different light intensities using 3 actinic lights (1st illumination)



(B) The qE and qI components of NPQ measured for wild type plants acclimated at 2 different light intensities using 3 actinic lights (2nd illumination)



(C) The qE and qI components of NPQ measured for *npq1* plants acclimated at 2 different light intensities using 3 actinic lights (1st illumination)



(D) The qE and qI components of NPQ measured for *npq1* plants acclimated at 2 different light intensities using 3 actinic lights (2nd illumination)

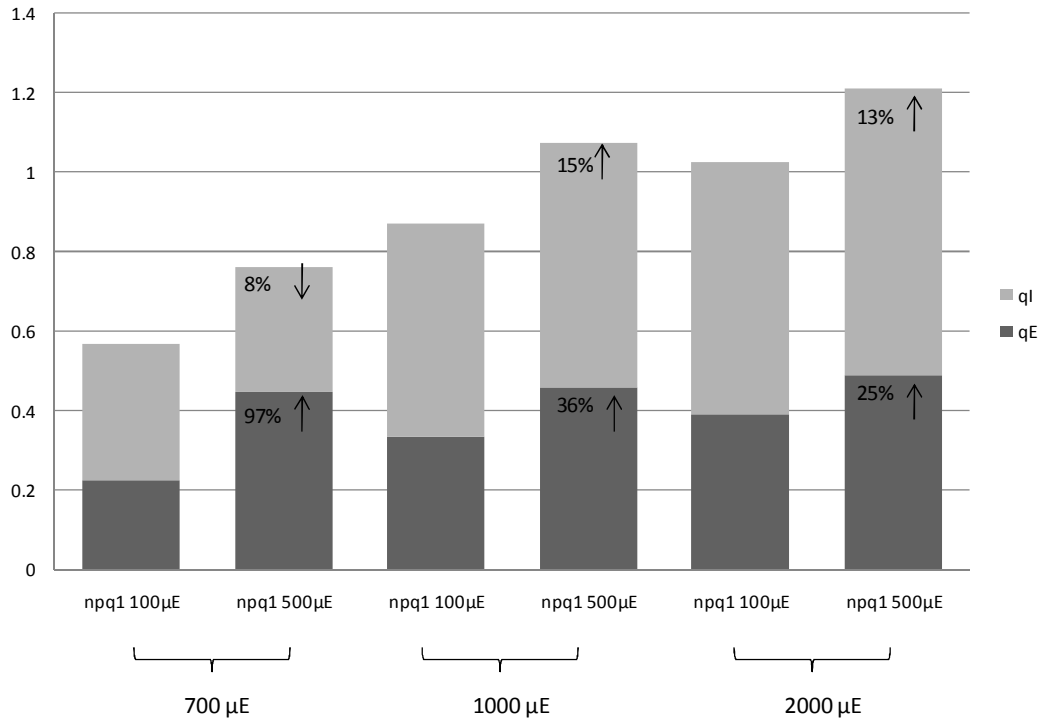


Figure 5.2 Separation of qE and qI components of NPQ during first and second illumination each in wild type (**A, B**) and *npq1* (**C, D**) grown at 100μE (control) and 500μE (high light). Plants were illuminated as described in Table 5.3, using three actinic lights of 700, 1000 and 2000 μE. qE and qI were calculated as (Fm / Fm') – (Fm / Fm") and (Fm – Fm") / Fm", respectively. Fm, Fm' and Fm" are the maximum fluorescence values after dark-adaptation, during actinic illumination and after a period of dark relaxation, respectively. All data are mean ± SE for at least three plants. All data are significantly different to respective controls (Student's t-test p=0.05).

In HL acclimated wild type plants, maximum of 51% increase in qE component was measured using actinic light of 2000 μE intensity, with only 6 % increase in slowly reversible qI component. The qE component also constituted 82% of the total NPQ in this case. Remarkably, in HL-acclimated *npq1* an almost two times maximum increase (97%) in qE component was observed using actinic light intensity of 700 μE, which accounted for 59% of the total NPQ. In contrast to wild type, here a decrease in qI component by 8% was measured in *npq1* as a result of HL acclimation. Though the increase in qI component of HL acclimated *npq1* with use of higher actinic lights was quite similar to that in wild type.

The two fold rise in qE of zeaxanthin-lacking *npq1* mutant can mainly be accredited to enhanced PsbS levels as a result of HL acclimation. Moreover, this enhancement in qE magnitude may also demonstrate that zeaxanthin cannot be a direct quencher in this component of NPQ and PsbS can modulate the conformational change in antenna complexes responsible for quenching without involving zeaxanthin. Similarly, the qE increase in HL-acclimated wild type can also be ascribed to enhanced PsbS levels, as no significant increase in de-epoxidation state was observed in the xanthophyll analysis. The increase in xanthophyll cycle pool may only be responsible to check the photoinhibitory effects as a result of HL exposure, in both wild type and *npq1* which maintained their qI levels to almost same level as in their respective controls. Thereby, both NPQ and its major component rapidly reversible qE can be increased without involving zeaxanthin by enhancing the amount of PsbS as a result of HL acclimation in both wild type and *npq1*.

5.2.1.3.3 Chlorophyll fluorescence quenching traces

For an in depth study to quantify the differences in NPQ as a result of HL acclimation, a detailed analysis of chlorophyll fluorescence formation and relaxation kinetics was carried out. The NPQ formation kinetics were obtained from dark-adapted plants during two successive light cycles of 5 minutes illumination, separated by a dark period of 5 minutes for qE relaxation. The effect of HL acclimation can be compared by the representative fluorescence traces for both wild type and *npq1*, as shown in Figure 5.3 A and B, respectively. In wild type, biphasic quenching was observed as first rapid zeaxanthin-independent induction phase was followed by second slower zeaxanthin-dependent quenching phase. A higher light intensity of 2000µE was used in this case to obtain maximum NPQ differences. In case of *npq1*, quenching with one phase was monitored as only the first rapid zeaxanthin-independent component of qE was formed, due to lack of zeaxanthin in this mutant which is responsible for the second slower component. Lower actinic light intensity of 700µE was used for this mutant to enhance the qE component of NPQ, as there is higher contribution of slowly reversible qI component of NPQ in it as compared to wild type at similar actinic light. This comparative analysis of quenching shows that HL acclimation induces more quenching in both wild type and *npq1*.

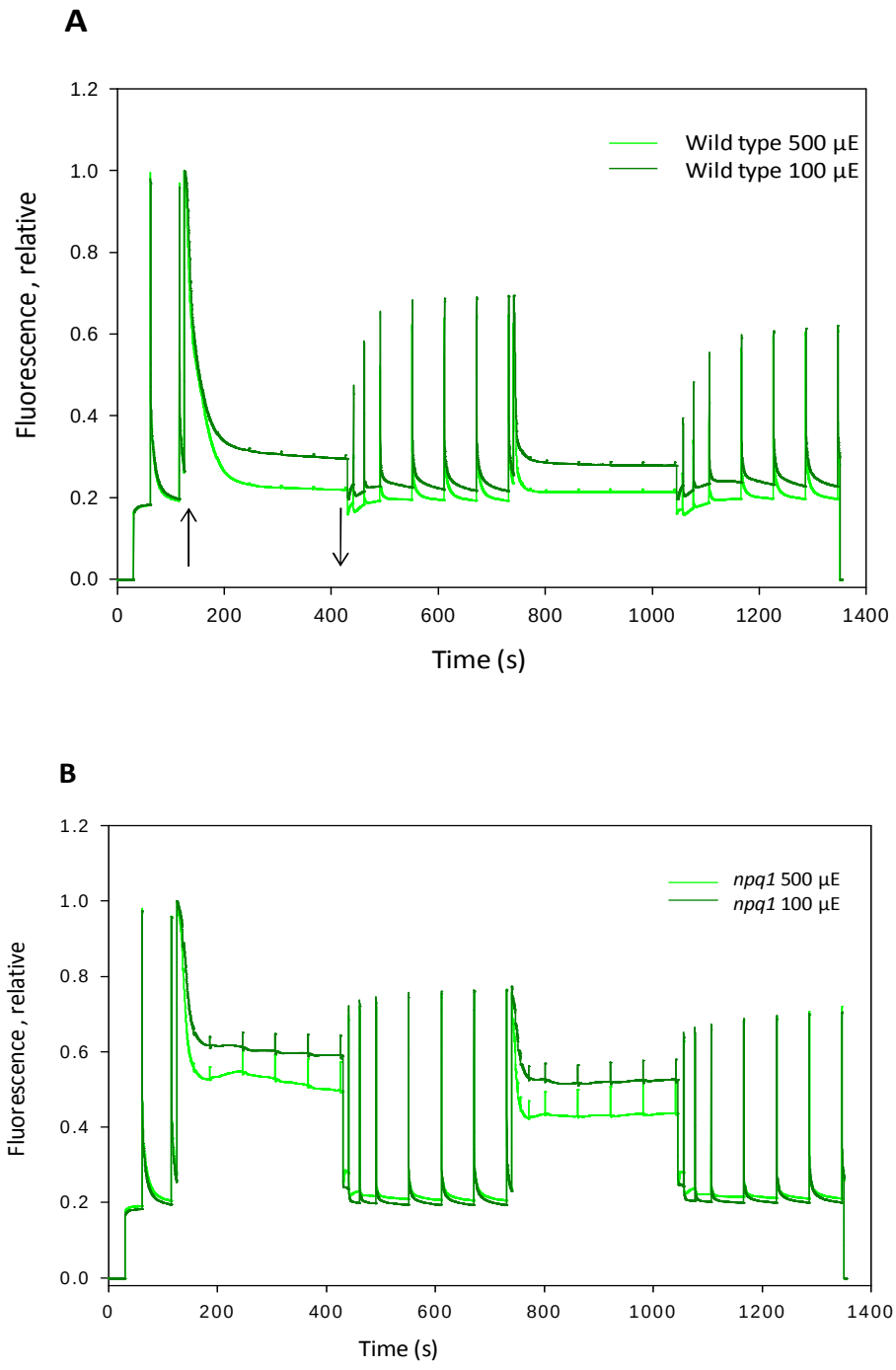


Figure 5.3 Comparison of chlorophyll fluorescence traces of plants grown at 100µE (control) and 500µE (high light) for both wild type (**A**) and *npq1* (**B**). Dark adapted plants were subjected to two cycles of 5 minutes actinic illumination (2000 µE for wild type and 700 µE for *npq1*), each followed by 5 minutes of dark relaxation to allow recovery of the fast qE component of NPQ. ↑ - actinic light on, ↓ - actinic light off, (refer also to figure 1.13).

5.2.1.3.4 NPQ formation and relaxation kinetics

To measure rate of formation and relaxation of NPQ during the chlorophyll fluorescence quenching as shown in comparative traces in Figure 5.2, their respective half times were recorded from kinetics. For statistical fitting of NPQ formation and relaxation kinetics, Sigmoidal Hill 3 and Hyperbolic Decay 3 parameters (Sigmaplot, *Systat Software Inc, USA*) were used, respectively. The half times were measured as the time taken to reach 50% of total NPQ during formation and relaxation.

Figure 5.4 shows that in HL-acclimated wild type, the quenching was formed slower during the first illumination as compared to control, however the relaxation was faster. During first illumination, the kinetics can be limited by de-epoxidation of violaxanthin, and additional complications are brought in by the changes in the qP that result from the induction of carbon assimilation. These factors make it difficult to ascertain the effect of HL acclimation during the first cycle of NPQ formation and relaxation. Thereby, as a result of second illumination, both the presence of zeaxanthin and the "light activation" allow to achieve the maximum NPQ. As discussed earlier the higher levels of NPQ were obtained as a result of HL acclimation. Interestingly, the rate of NPQ formation as a result of HL acclimation was also faster than that of the control. On the other hand, the rate of NPQ relaxation was slower in the HL acclimated wild type plants. Zeaxanthin has been shown to increase the rate of formation and to slow down the relaxation rate, as discussed in Chapter 3. No differences in the final NPQ relaxation levels were observed between the HL-acclimated plants and control.

Figure 5.5 shows similarly the NPQ formation and relaxation kinetics and half times for HL-acclimated *npq1*. It is significant as it may help to understand the effect of PsbS enhancement alone on these kinetics without any zeaxanthin contribution. As a result of xanthophyll cycle activity, zeaxanthin formation has already been demonstrated to accelerate formation kinetics and inhibit relaxation kinetics in the wild type plants. In the absence of violaxanthin de-epoxidation and hence zeaxanthin, the rate of formation was faster in both illuminations as a result of HL acclimation. The half time for relaxation as a result of HL acclimation was faster as fraction of a second, therefore hard to be measured in

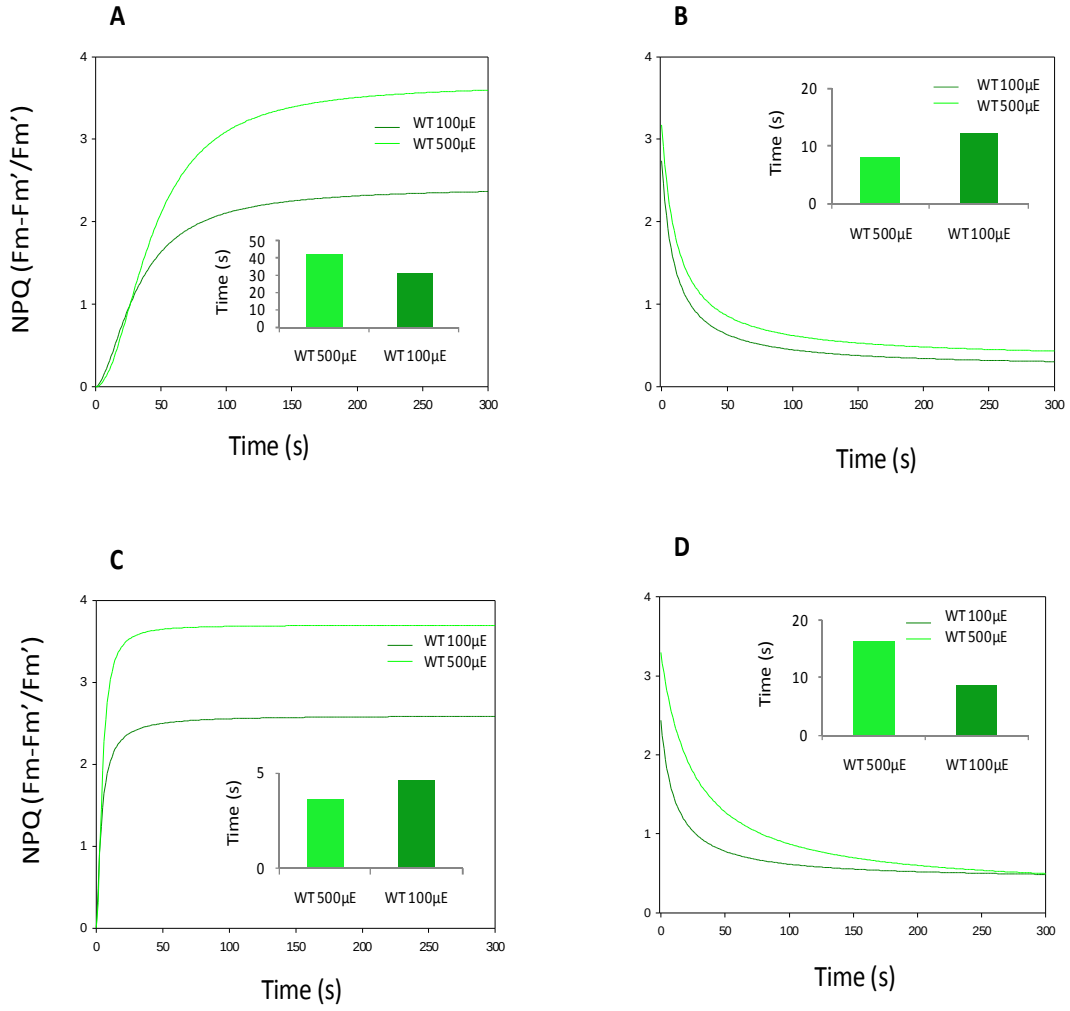


Figure 5.4 Time course of NPQ for wild type (WT) plants grown at 100µE (control) and 500µE (high light). NPQ was calculated as (Fm-Fm')/Fm', where Fm is the maximum dark-adapted fluorescence and Fm' is the maximum fluorescence on application of a saturating pulse during actinic illumination. Plants were illuminated as described in Table 5.3. Half time (t ½) for formation and relaxation of NPQ in small graphs for each case. t ½ was calculated as the time taken for NPQ to reach 50% of total NPQ during formation and relaxation.

A NPQ formation during 1st illumination;

B NPQ relaxation during 1st dark period;

C NPQ formation during 2nd illumination;

D NPQ relaxation during 2nd dark period

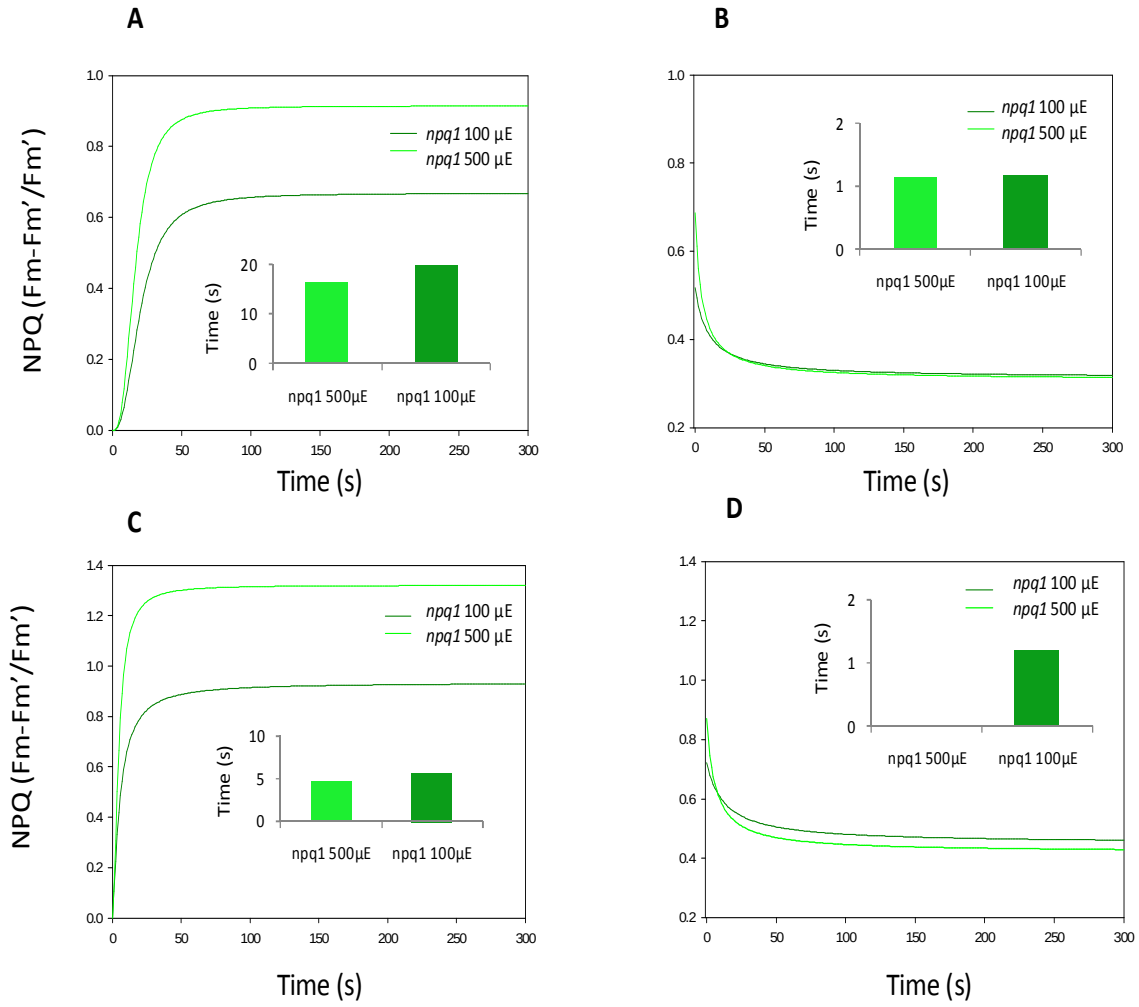


Figure 5.5 Time course of NPQ for *npq1* plants grown at 100µE (control) and 500µE (high light). NPQ was calculated as (Fm-Fm')/Fm', where Fm is the maximum dark-adapted fluorescence and Fm' is the maximum fluorescence on application of a saturating pulse during actinic illumination. Plants were illuminated as described in Table 5.3. Half time (t ½) for formation and relaxation of NPQ in small graphs for each case. t ½ was calculated as the time taken for NPQ to reach 50% of total NPQ during formation and relaxation.

A NPQ formation during 1st illumination;

B NPQ relaxation during 1st dark period;

C NPQ formation during 2nd illumination;

D NPQ relaxation during 2nd dark period

this case, as compared to ~ 1 second in control. The effect of PsbS on quenching has previously been reported by PsbS-less *npq4* mutant, in which the absence of this protein slows down the quenching formation as well as its relaxation in the darkness, as compared to the wild type (Li *et al*., 2000). Similarly, another study involving PsbS over-expressor L17, which contains ~ 4 times the amount of this protein as the wild type, has shown greater extent of quenching induction in comparison to the wild type (Li *et al*., 2002c). However, the effect of PsbS protein on the NPQ relaxation was not clear from this study.

The data shown in the Figure 5.4 may help to gain further understanding of the role of PsbS in the mechanism of NPQ and of the effects of enhancement in the levels of this protein as a result of HL acclimation. This role is independent of factors like violaxanthin de-epoxidation and zeaxanthin, as this mutant lacks zeaxanthin formation by de-epoxidation. Apart from showing the effect of enhanced PsbS to increase the extent of NPQ without involving zeaxanthin, it also indicates towards a regulatory role of PsbS protein as it promotes both the rate of formation and relaxation of NPQ. This regulatory role of PsbS is not similar to that of zeaxanthin, which only promotes the rate of NPQ formation without increasing its extent, and inhibits the relaxation. This data does not support the direct involvement of PsbS as a quencher, since quenching would rely on the association of zeaxanthin-bound PsbS with the quenching complex. This data, however, indicates that PsbS may facilitate the rapid switching between quenched and unquenched conformations.

5.2.2 Genetic Manipulation to increase NPQ levels

The extent of NPQ has been demonstrated to be increased by enhancing the levels of PsbS protein, achieved by over-expression of nuclear gene *psbS*. The L17 overexpressor line, which has been found to possess ~ 4 times the amount of PsbS protein as the wild type, clearly demonstrates a much greater extent of quenching in comparison to the wild type leaf (Li *et al*., 2002c). The absence of this protein on the other hand severely reduces the extent of NPQ, as demonstrated in PsbS mutant *npq4* (Li *et al*., 2000). As discussed earlier in this chapter, the mechanistic role of this protein in the quenching process is not clear, as it is considered to be involved directly in this process by acting as quencher or providing

quenching site by binding protons and zeaxanthin, thus the resultant PsbS-zeaxanthin complex de-excites the singlet excited chlorophyll by energy transfer (Ma *et al*., 2003; Holt *et al*., 2004). Alternatively, PsbS has also been proposed to act indirectly as modulator of the NPQ by controlling the conformational changes in antenna complexes, this model also suggests an indirect allosteric regulator role for zeaxanthin (Horton *et al*., 2005).

The role of zeaxanthin is also not clear in quenching as it has also been considered as direct quencher or indirect regulator in NPQ, as mentioned above. The *npq1* mutant defective in nuclear gene encoding violaxanthin de-epoxidase (VDE) is also available, which is unable to convert violaxanthin to zeaxanthin in excessive light (Niyogi *et al*., 1998). The over-expression of PsbS in this zeaxanthin-lacking mutant can help to elucidate the molecular mechanism of NPQ with respect to the roles assigned to zeaxanthin and PsbS. The single recessive nuclear mutation of VDE gene *npq1* results in absence of zeaxanthin in strong light and the partial inhibition of the quenching of singlet excited chlorophylls in the antenna complexes (Niyogi *et al*., 1998). This gene is located on chromosome 1 of *Arabidopsis* (Bugos and Yamamoto, 1996), at location 2,706,927-2,709,534. *Arabidopsis* has 5 pairs of homologous chromosomes, the chromosome 1 is the longest among all of them with 30,432,563 base pairs (bps) and 5,967 known protein-coding genes. The co-dominant *psbS* gene is also located on chromosome 1 at location 16,874,136-16,875,823. The expression of PsbS protein has been enhanced four times above the wild type level, by transformation of wild type with the *psbS* genomic clone. A 3,198 bp clone fragment with the *psbS* gene under the control of its own promoter was subcloned in an *Agrobacterium tumefaciens* vector to transform *Arabidopsis* with single insertion. The resultant over-expressor line L17 of PsbS has demonstrated a two fold increase in NPQ as compared to that of wild type (Li *et al*., 2002b,c).

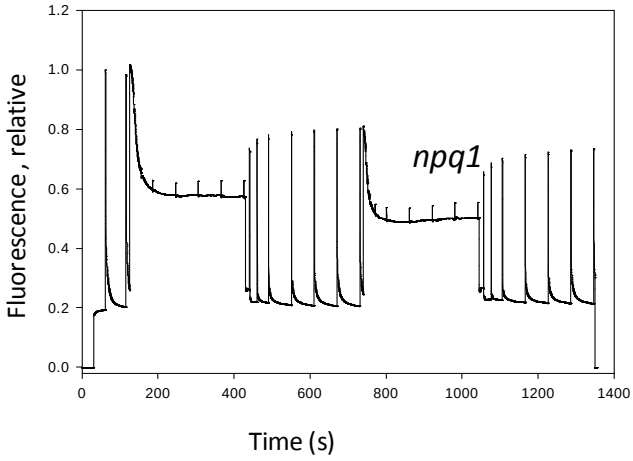
Though insertion of transgene is a random event, however genetic recombination of two genes present on the same chromosome is possible by crossing-over and the frequency of this event depends on the relative distance between the two genes. The longer distance between two linked genes increases the frequency of genetic recombination. The distance between *npq1* and *psbS* transgenes on the chromosome 1 is needed to be long enough to

allow genetic recombination which can be possible considering the total length of this chromosome. Therefore it is likely to obtain a hybrid with both mutant *npq1* and over-expressor *psbS* genes, as a result of cross between *npq1* and L17 parents. To obtain such a mutant deficient in zeaxanthin with over-expression of PsbS, a reciprocal cross was made between *npq1* and L17 first parental lines (P1). However, the selection of *npq1* as female line proved to be more successful and useful, as the crosses involving L17 as female line did not produce viable pods and hence seeds. With *npq1* as female line, screening of the heterozygous hybrids in resultant first filial generation (F1) was also convenient as much higher NPQ was observed in all the heterozygous F1 as compared to *npq1* homozygous plants. A ratio of 7 successful crosses out of total 11 was recorded, yielding heterozygous F1 generation. Four plants were emasculated for each of the genotype to be considered as female lines. Each female line with 3-4 emasculated flowers was cross-pollinated with pollens from opposite male line. Seeds from each attempted cross were germinated to obtain F1 generation, whereby the heterozygous F1 were screened by chlorophyll fluorescence quenching and pigment analysis. All the plants were grown under controlled conditions of 8h of 100µE light at 22^{o}C alternating with a 16h dark period at 18^{o}C.

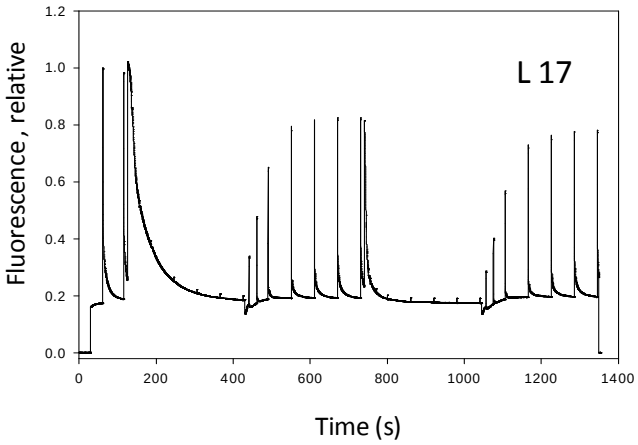
5.2.2.1 Screening and characterisation of F1 generation

The chlorophyll fluorescence quenching was performed in dark adapted P1 and F1 plants (Figure 5.6), using two successive illumination cycles for P1, however only one illumination followed by dark relaxation was used for screening purpose in F1 and subsequent generations. The actinic light intensity of 700 µmol photons m^{-2} s^{-1} was selected in order to enhance the reversible qE component of NPQ and to minimize the slowly reversible qI component. For *npq1*, the quenching was formed in one rapid phase and did not relax fully due to higher contribution of qI in this mutant, as discussed earlier. The NPQ was measured as 0.58 ± 0.05, with ~ 60% of reversible qE in *npq1* P1. The L17 over-expressor line clearly showed a much greater extent of quenching in comparison to the

A


Fluorescence, relative
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
200
400
600
800
1000
1200
1400
npq1
Time (s)

B


Fluorescence, relative
1.2
1.0
0.8
0.6
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0.0
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200
400
600
800
1000
1200
1400
L 17
Time (s)

C

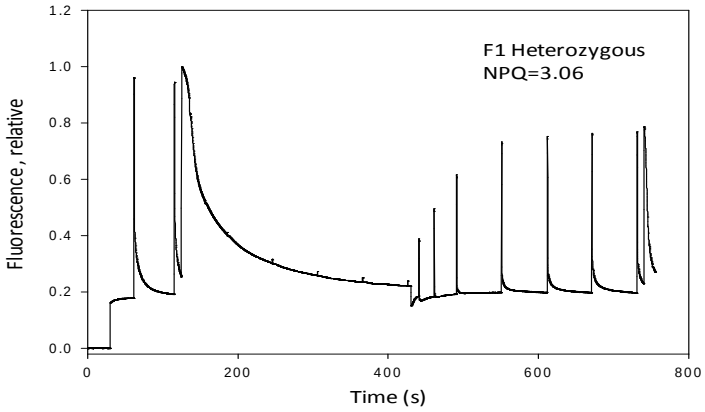
Fluorescence, relative
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
200
400
600
800
F1 Heterozygous
NPQ=3.06
Time (s)

Figure 5.6 Chlorophyll fluorescence traces of dark-adapted *npq1* (**A**) and L17 (**B**) P1 plants, with resultant F1 heterozygous (**C**) plant. The leaves were subjected to actinic light of 700 μE intensity for two cycles of 5 minutes, each followed by 5 minutes of dark relaxation to allow recovery of the fast qE component of NPQ. For screening of F1 heterozygous plants, only one illumination cycle was used.

npq1. The quenching was induced in typical biphasic way like wild type, with initial rapid zeaxanthin-independent component controlled by ΔpH and final slower zeaxanthin-dependent component influenced by de-epoxidation rate. The relaxation of quenching was also faster and to a much higher extent as compared to *npq1*. The NPQ was measured as 3.7 ± 0.1, with ~ 90% of reversible qE in L17 P1. For screening of F1 heterozygote, only first illumination was used and higher magnitudes NPQ of NPQ with biphasic formation kinetics were recorded which resembled to L17 P1. The average NPQ as a result of one illumination cycle was measured as 3.0 ± 0.5 with ~ 90% reversible qE.

Leaf discs were collected from P1 and F1 plants to analyse their pigment composition by HPLC in both light-treated and dark-adapted conditions. The expected general differences were found between *npq1* and L17 (Table 5.4), as reported previously (Niyogi *et al.*, 1998; Li *et al.*, 2002c). Both antheraxanthin and zeaxanthin were absent in *npq1* due to inactivity of VDE. The light-induced de-epoxidation was also lacking in *npq1*, while in case of L17 it was measured as ~ 49%. Despite much higher NPQ levels measured in L17, there have been no significant differences in xanthophyll cycle pool and de-epoxidation state as compared to the wild type. In case of F1 heterozygotes, no significant differences were observed in xanthophyll composition and de-epoxidation state from those of L17 parental line.

Plant	Neo	Lut	Vio	Ant	Zea	β-Car	DEPs
P1-*npq1* dark	5.3 ± 0.3	18 ± 2	4.4 ± 1.1	0	0	12 ±1.8	0
P1-*npq1* light	5.7 ± 0.6	20 ± 1	5.3 ± 0.3	0	0	9.2 ± 0.6	0
P1- L17 dark	5.9 ± 0.2	20.2 ± 1.6	6.0 ± 0.5	0.49 ± 0.1	0	12.9 ±0.4	3.8 ± 0.5
P1- L17 light	5.5 ± 0.5	19.8 ± 0.5	2.4 ± 0.4	0.46 ± 0.1	2.33 ± 0.2	10.6 ± 0.5	49 ± 1
F1- heterozygote dark	6.0 ± 0.2	22.6 ± 1	6.2 ± 0.5	0.6 ± 0.1	0	12.5 ± 1	4.4 ± 0.4
F1- heterozygote light	6.1 ± 0.4	11 ± 1.5	2.2 ± 0.2	0.5 ± 0.2	2.4 ± 0.3	11 ± 1	52 ± 2

Table 5.4. Pigment composition of *npq1* and L17 parental lines and F1 heterozygous lines. Leaf discs were collected from plants either dark-adapted for 30 min or light-treated for 10 min at 1000 µE. Data are normalized to 100 chlorophyll *a* + *b* molecules and are means ± SE from three replicates. Neo, Lut, Vio, Ant, Zea, β-Car and DEPs represent neoxanthin, lutein, violaxanthin, antheraxanthin, zeaxanthin, β-carotene and de-epoxidation state % [(zeaxanthin + 0.5 antheraxanthin)/(violaxanthin + antheraxanthin + zeaxanthin)], respectively.

5.2.2.1.1 Explanation of P1 genetic cross to yield F1 genetic make up

As elaborated in the Figure 5.7, the parent line of *npq1* possesses two copies of recessive *npq1* mutant gene (denoted by "n") along with two copies of *psbS* normal gene (denoted by "l"), both genes located on the same chromosome. The other parental line of L17 has two copies of dominant and normal *NPQ1* gene for VDE (N) with two copies of co-dominant over-expressor *psbS* transgenes (L). Only one set of genes is transferred to the next generation through meiotic gametogenesis, therefore, a putative heterozygote in F1 can be

obtained with a gene combination of “nN lL” where “N” dominant gene will dominate over its recessive counterpart “n” gene to express itself, while “L” over-expressor gene will remain co-dominant with “l” gene to yield dosage effect. As a result of gene expression, the resultant F1 heterozygote would have normal activity of VDE and xanthophyll cycle and hence zeaxanthin concentrations similar to those of L17 parental line. The over-expression of one psbS gene and the other normal gene would result in intermediate PsbS protein concentrations, which are higher than *npq1* but lower than L17. However, it is interesting finding that high NPQ levels were achieved in heterozygote quite similar to those of L17.

The PsbS protein amount has been shown to directly correlate with the NPQ levels, a ~ 4 times increase in PsbS protein level in L17 over-expressor as compared to wild type can almost double the level of NPQ (Li *et al*., 2002c). This increase in NPQ may appear to be smaller than anticipated, as we have measured similar NPQ levels in L17 and heterozygote despite the differences between their *psbS* gene expression. This may suggest that activity of this protein is limited by maximum capacity of plants for NPQ, or it may also be proposed that only a part of this protein is required for NPQ, while the rest of PsbS remains as inactive and excess for this process.

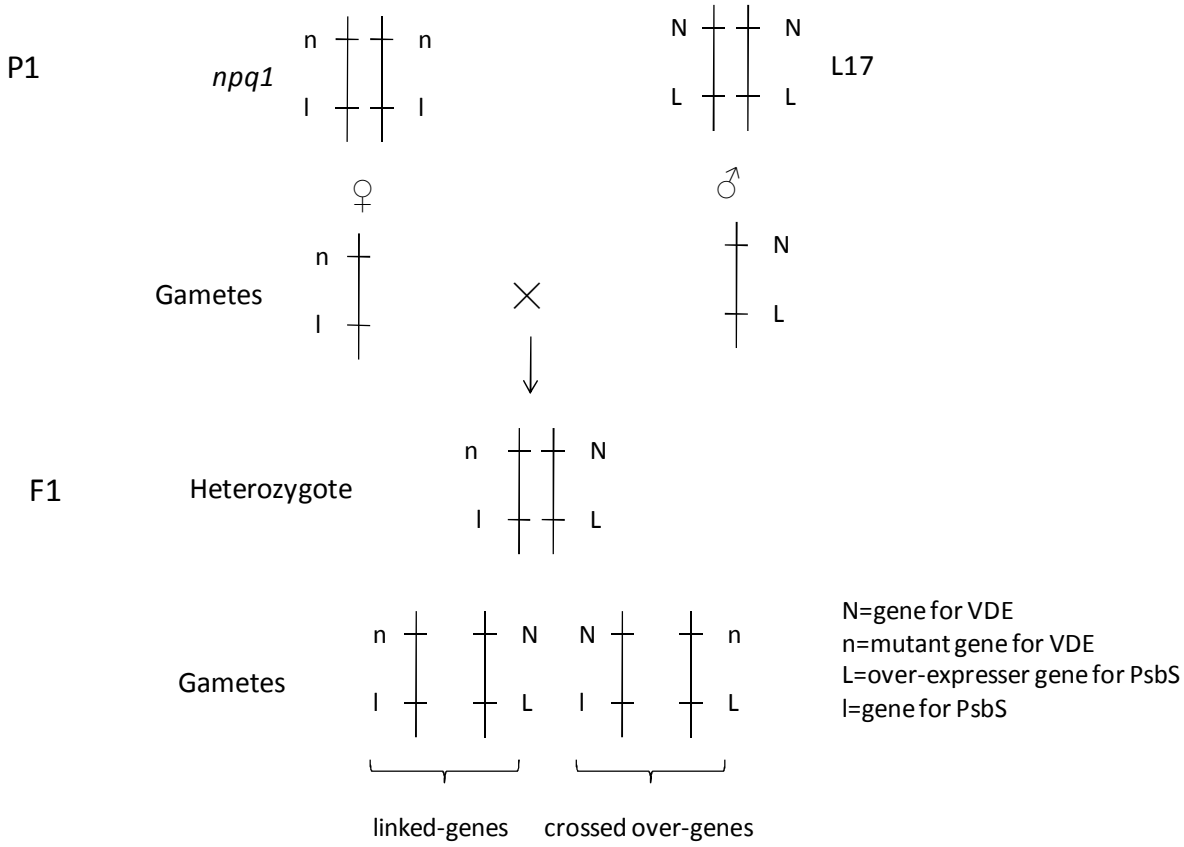

Figure 5.7 Diagrammatic explanation of genetic architecture of the Chromosome 1 of *npq1* and L17 parental lines and F1 heterozygous F1 generation. Gene combination in gametes has also been shown for both P1 and F1 generations, with all possible gene combinations shown for F1 gametogenesis, where "linked gene" combination in the absence of crossing-over and "recombinant gene" combination as a result of crossing-over.

5.2.2.2 Screening and characterisation of F2 generation

The F1 heterozygous generation was allowed to self pollinate to produce seeds, which were sown to obtain F2 generation. During the gametogenesis in F1 generation, the homologous pairs of chromosomes may exchange their genetic material in a process synapsis during prophase 1 of meiosis. The frequency of this crossing-over resulting in genetic recombination depends on the distance between two genes. As discussed earlier, both *npq1* and *psbS* has a long distance between them, so the frequency of genetic recombination would be high in this case. In the Figure 5.8, the F1 gametogenesis has been shown with all possible gene combinations, assuming both occurrence of crossing-over or otherwise.

Possible outcome of all the gene combinations which can be obtained in F2 generation has been shown diagrammatically in Figure 5.8, which involves all the possible gametes produced by F1 generation. Considering only crossed-over gene combinations, it can be predicted that genes will assort independently in F2 genes. In fact, the linked gene combinations cannot be ruled out in this case, as both the genes are located on the same chromosome. For F2 generation, a total of 120 plants were screened again using the combination of two methods, chlorophyll fluorescence quenching and xanthophyll analysis. As shown in Table 5.5, majority of plants showed phenotype like L17 with higher extent of NPQ (2.0-3.4) during one illumination cycle. These plants showed over 90% of reversible qE, similar to L17. Plants having slightly lower NPQ levels (1.3-2.0) with reversible qE ~ 86% of total NPQ were considered as wild type like. There was no clear demarcation between these two groups, most probably due to dosage effect of *psbS* gene. Remarkably, there were no F2 plants in the NPQ range of 1.0-1.3, thus it creates a clear boundary line between all the plants demonstrating NPQ levels higher than 1.3 (L17-like and wild type-like) and all those with NPQ lower than 1.0. The plants exhibiting NPQ levels lower than 1.0 can again be divided into two sub groups, one with very low NPQ along with lowest reversible qE resembling the *npq1* parent, and the other with substantially higher NPQ and more reversible qE than the *npq1*. Only 8 *npq1*-like plants were observed with NPQ 0.55-0.65 and ~ 58% reversible qE. The other sub-group, which can be putative *npq1*L17 double mutants, consisted of 19 plants with NPQ 0.65-1.0 and reversible qE ~ 68%. There was also no distinction between these two sub-groups, again most likely because of the *psbS* dosage effect. The ratio among various F2 sub-groups does not strictly follow the one obtained as a result of Mendelian independent assortment which is unsurprising as statistical probability does not influence the event of crossing-over. Despite this, the ratio obtained here was exactly 8:3:2:1, which is not too deviated from the theoretical 9:3:3:1, considering a comparatively small sample population by statistical standards in this experiment.

The putative *npq1*L17 double mutants from F2 generation were selected to obtain two pure homozygous lines in F3 generation by screening method of fluorescence quenching.

Figure 5.8. Diagrammatic explanation of the possible outcome of gene combination in F2 generation. The expected Mendelian independent assortment ratio among F2 genotypes with all possible gene combinations has also been presented. The putative *npq1*L17 double mutants were finally grown for F3 generation to select pure homozygous lines, with all homozygous plants.

F2 phenotype	Number of plants	NPQ	Reversible qE %
L17-like	65	2.0-3.4	92.2 ± 0.2
Wild type-like	28	1.3-2.0	86 ± 0.6
Putative *npq1*L17 double mutant	19	0.65-1	68 ± 1.4
npq1-like	8	0.55-0.65	58.2 ± 1.9

Table 5.5 Characterisation of F2 generation by quenching phenotypes. NPQ was measured by the end of first 5 min illumination, followed by 5 min dark relaxation, using actinic light intensity of 700 µE. NPQ and qE were calculated as (Fm-Fm')/Fm' and (Fm / Fm') – (Fm / Fm"), respectively. Fm, Fm' and Fm" are the maximum fluorescence values after dark-adaptation, during actinic illumination and after a period of dark relaxation, respectively. Reversible qE % was qE / NPQ x 100. All data are mean ± SE for the respective number of plants in each group.

Availability of a sample population with a broad range of NPQ values with a concomitant variability in reversible qE % provided an opportunity to find a correlation between the values of these two measurements (Figure 5.9), which has not been established before. The extent of NPQ in this population has a range from lowest value of 0.55 to a maximum of 3.4, measured at the end of 5 minutes illumination of dark adapted F2 plants. A concomitant increase in reversible qE % of total NPQ was also noticed which was measured at the end of 5 minutes long dark period following the illumination. A non-linear regression shows a correlation between NPQ and qE % of total NPQ which is rapidly reversible and is measured after 5 minutes of dark period following first illumination. It demonstrates that extent of qE increases with the increase in NPQ; moreover this increase in qE is also dependent on amount of zeaxanthin and PsbS just like NPQ. This correlation also shows that increase in NPQ in F2 generation is due to increase in rapidly reversible qE and not because of photoinhibition.

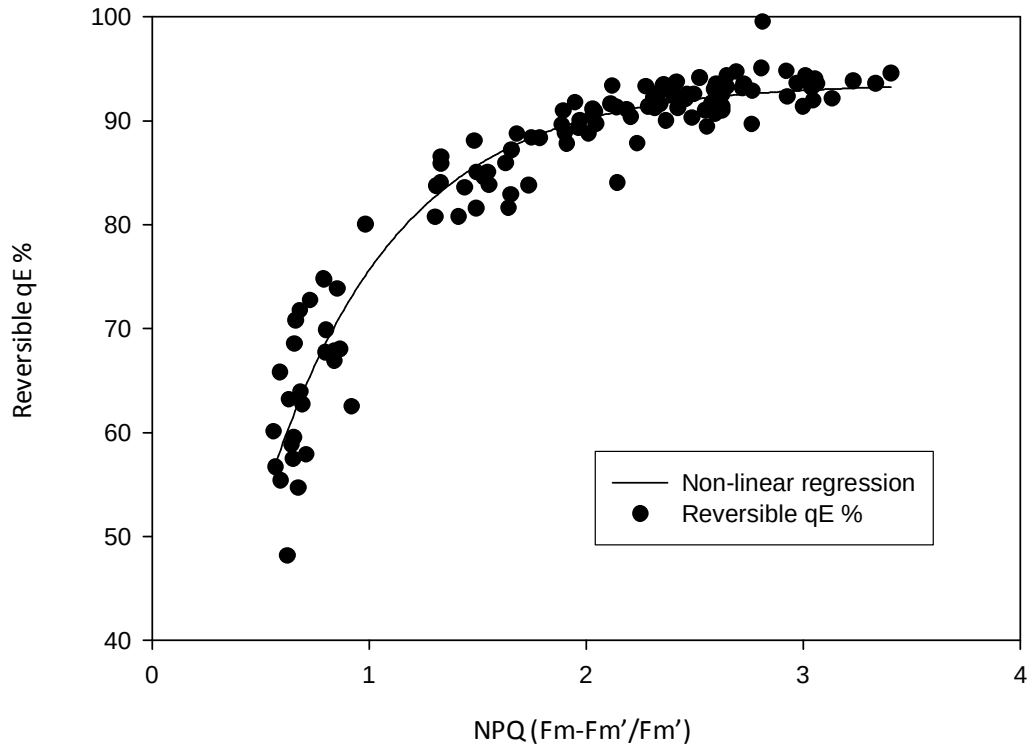

Figure 5.9 Correlation between NPQ and reversible qE % of total NPQ presented in the form of non-linear regression from sample population of F2 generation with NPQ ranging from a lower value of 0.55 to as high as 3.4 and qE % pool from ~ 50% to ~ 100%.

The representative quenching traces of the F2 plants with average NPQ from each of the four sub-groups have been shown in Figure 5.10 for comparison. In all the plants with NPQ levels higher than 1.3, the typical biphasic quenching induction was observed with initial rapid zeaxanthin-independent phase followed by final slower zeaxanthin-dependent phase, quite similar to those of L17 and wild type. On the other hand, all those plants having lower than 1.0 NPQ magnitude, showed only single rapid zeaxanthin-independent phase during quenching formation, controlled by ΔpH. The extent of relaxation upon darkening is distinctly higher and faster in plants with higher NPQ (>1.3) than those with lower NPQ values (<1.0). It is difficult to ascertain any differences in the rate of relaxation between the sub-groups with variable PsbS amounts by these traces, however data in Table 5.5 shows

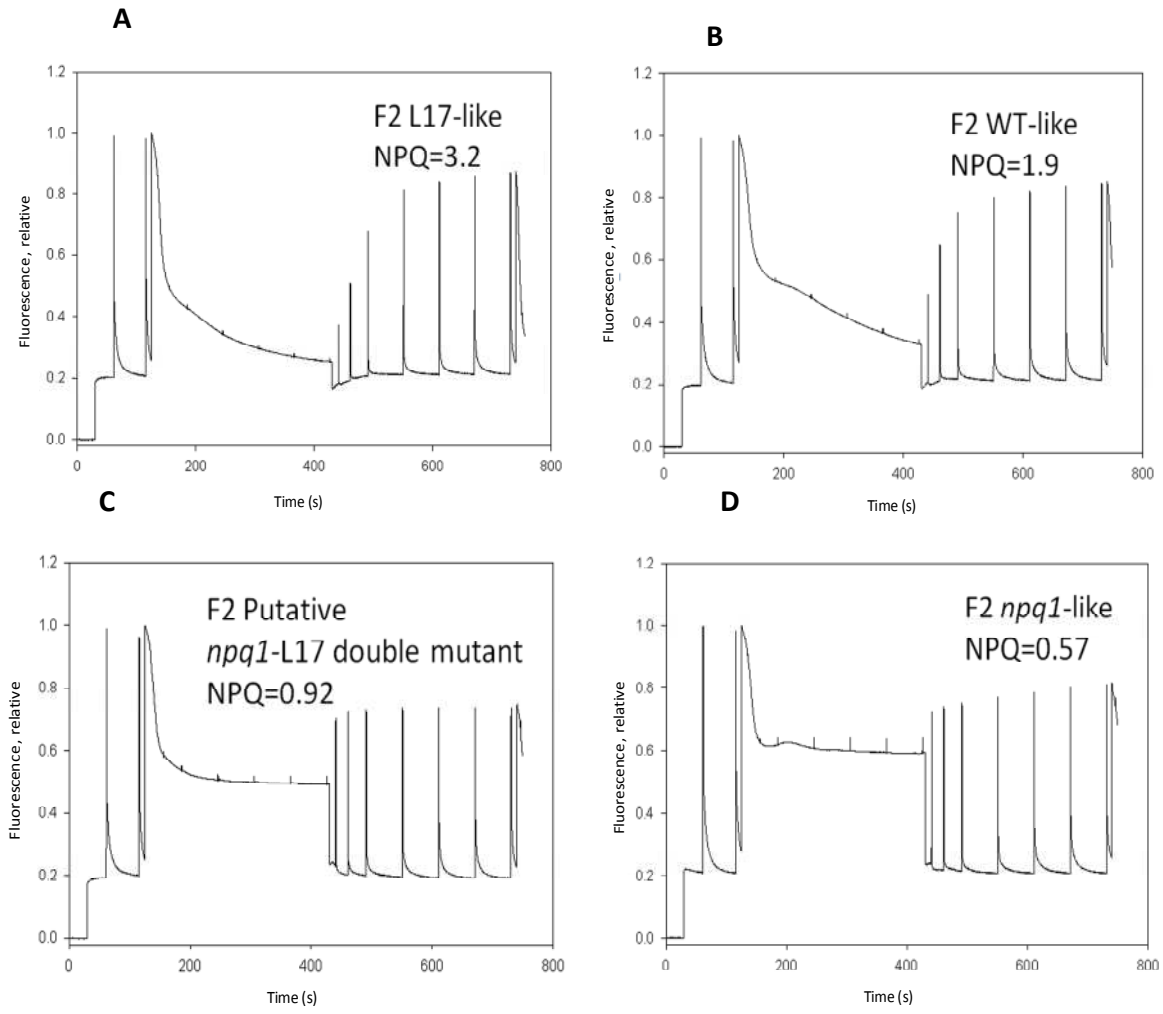

Figure 5.10 Representative chlorophyll fluorescence traces of F2 plants from four sub-groups, L17-like (**A**) and wild type-like (**B**) with biphasic formation kinetics, putative *npq1*L17 double mutant (**C**) and *npq1*-like (**D**) with single phase formation kinetics. The dark-adapted leaves were subjected to actinic light of 700 μE intensity for one cycle of 5 minutes, followed by 5 minutes of dark relaxation to allow recovery of the fast qE component of NPQ. The relaxation during dark period was more reversible in plants with higher NPQ.

F2 phenotype	**Neo**	**Lut**	**Vio**	**Ant**	**Zea**	**β-Car**	**DEPs**
L17-like dark	5.3 ± 1.1	18 ± 2.5	4.4 ± 1.1	0	0	12 ± 1.8	4.0 ± 0.5
L17-like light	5.7 ± 0.6	20 ± 1	5.3 ± 0.3	0	0	9.2 ± 0.6	50 ± 1
WT-like dark	5.3 ± 0.2	20.2 ± 1.6	6.0 ± 0.5	0.49 ± 0.1	0	12.9 ± 0.4	4.4 ± 0.4
WT-like light	5.1± 0.2	19.8 ± 0.5	2.4 ± 0.4	0.46 ± 0.1	2.33 ± 0.2	10.6 ± 0.5	52 ± 2
Putative *npq1*L17 dark	6.1 ± 0.3	22.6 ± 1.8	6.3 ± 0.2	0	0	11.9 ± 0.7	0
Putative *npq1*L17 light	5.0 ± 0.1	11 ± 1.5	5.3 ± 0.3	0	0	9.1 ± 0.1	0
npq1-like dark	5.7 ± 0.5	20 ± 2	6.8 ± 0.8	0	0	9.3 ± 0.5	0
npq1-like dark	4.7 ± 0.2	18.8 ± 2.4	5.1 ± 0.5	0	0	12.3 ± 1.2	0

Table 5.6 Pigment composition of F2 generation for screening of plants lacking antheraxanthin and zeaxanthin. Leaf discs were collected from plants either dark-adapted for 30 min or light-treated for 10 min at 1000 µE. Data are normalized to 100 chlorophyll *a* + *b* molecules and are means ± SE from all the plants available in each sub-group. Neo, Lut, Vio, Ant, Zea, β-Car and DEPs represent neoxanthin, lutein, violaxanthin, antheraxanthin, zeaxanthin, β-carotene and de-epoxidation state % [(zeaxanthin + 0.5 antheraxanthin)/(violaxanthin + antheraxanthin + zeaxanthin)], respectively.

higher extent of relaxation in PsbS over-expressors L17-like and putative double mutant, if compared to wild type-like and *npq1*-like plants, respectively. Another significant result from the average NPQ of each sub-group demonstrates that the proportional increase in NPQ as a result of PsbS over-expression was almost the same. A two fold increase in NPQ was measured in L17-like plants over the wild type-like plants, and similarly in putative *npq1*L17 double mutant over the *npq1*-like F2 plants.

The F2 generation was also screened by measuring the xanthophyll composition. All those plants with NPQ levels lower than 1.0 and monophasic quenching formation were expected to be devoid of both antheraxanthin and zeaxanthin due to *npq1* mutant gene, and all the plants with higher NPQ (>1.3) and biphasic quenching induction were anticipated to possess xanthophyll cycle pigments along with light-induced de-epoxidation. Leaf discs were collected from all the F2 plants to analyse their pigment composition by HPLC in both light-treated and dark-adapted conditions. The data shown in Table 5.6 shows the results confirming above mentioned statements, as both L17-like and wild type-like plants showed almost similar de-epoxidation states along with xanthophyll cycle pool, while putative *npq1*L17 double mutant and *npq1*-like plants demonstrated inactivity of VDE to yield antheraxanthin and zeaxanthin and lacking of de-epoxidation, similar to *npq1* mutant.

5.2.2.2.1 Spectral analysis of F2 generation

The role of PsbS has been suggested to induce a conformational change in antenna complexes which changes the configuration of bound pigments to result in quenching of chlorophyll excitation possibly via chlorophyll-xanthophyll interaction, as discussed in Chapter III in detail. Therefore, the effect of over-expressed PsbS would be predicted to result in more pronounced conformational changes. These conformational changes associated with NPQ can be monitored by accompanying absorption change in the range of 520-540 (Ruban *et al*., 1993; Bilger and Björkman, 1990). This absorbance change consists of a 535 nm band, which has been related to a change in properties of zeaxanthin (Ruban *et al*., 2002). A blue shift in this 535 nm band towards 520 nm in the absence of zeaxanthin suggests that it may arise from other xanthophylls (Noctor *et al*., 1993). The effect of PsbS

on conformational change has also been demonstrated by a study involving *npq4* mutant lacking ΔA535 (Li *et al*., 2000), however a recent work has shown that ΔA535 is only very slow to form and not completely abolished in this mutant (Johnson and Ruban, 2009).

Figure 5.11 shows the light minus recovery absorption spectra for all the four sub-groups of F2 generation, which were recorded in the Soret region (410-565 nm). These spectra also provide information about pigment changes as a result of conformational changes, as discussed in detail in Chapter III. In both L17-like and wild type-like plants (Figure 5.11 A), the presence of de-epoxidation and hence zeaxanthin resulted in a light-minus recovery difference spectrum with the maximum absorption in 535 nm band. The other two sub-groups with lower NPQ values (< 1.0) showed maximum absorption at 525 nm (Figure 5.11 B), which is blue shifted in comparison to spectra of sub-groups with higher NPQ (> 1.3). The reason for this blue shift is absence of zeaxanthin, as mentioned earlier. In addition to this blue shift, the amplitude of the absorption spectra was also reduced in the absence of zeaxanthin, which is consistent with the lower extent of NPQ in plants lacking zeaxanthin. This demonstrates the ability of zeaxanthin to enhance the conformational changes associated with qE in the antenna. The figure also shows the effect of enhanced PsbS on maximum absorption at both 535 and 525 nm, as this protein can increase the absorption in each case by almost similar proportion. The three negative bands were present in all the spectra at 495, 468, and 438 nm; however the amplitude of these bands was smaller in plants with lower NPQ values, consistent with the correlation between absorption changes and the NPQ. This data suggests that the qE-related conformational change can be induced by PsbS independent of zeaxanthin.

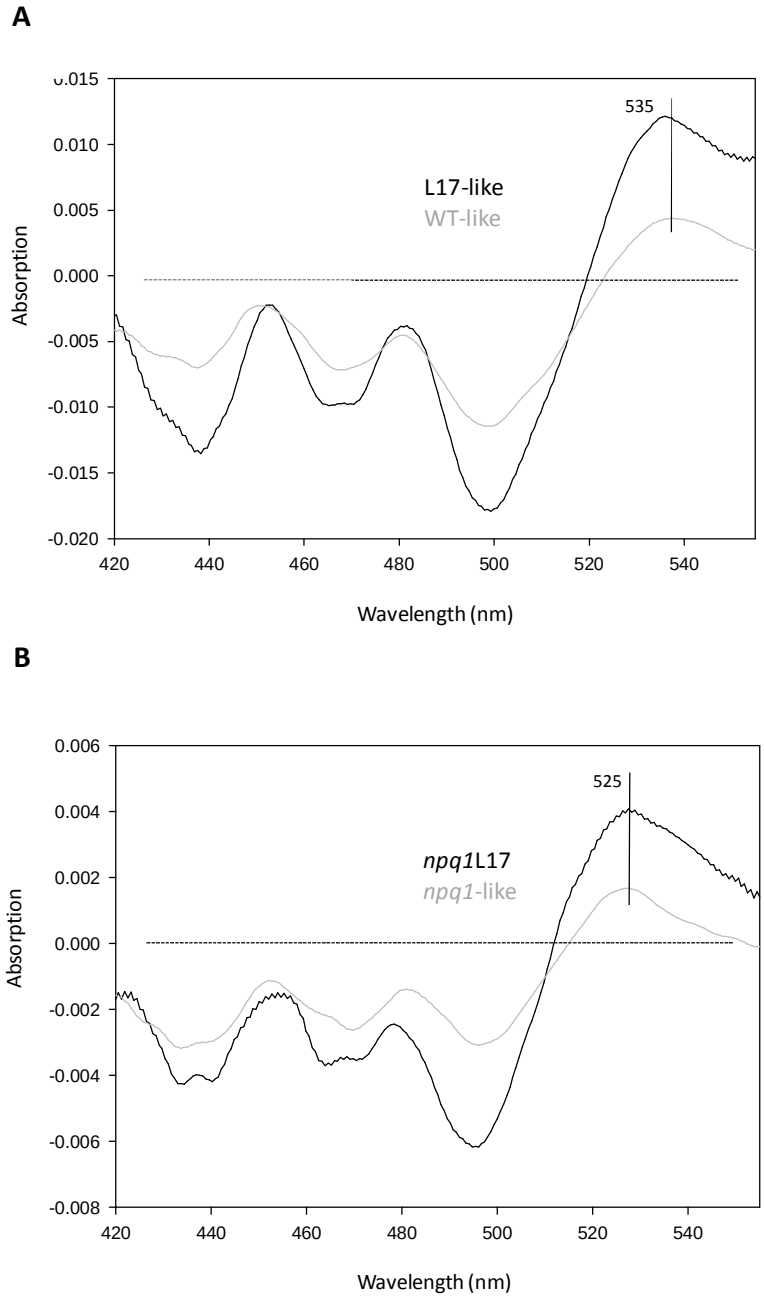

Figure 5.11 Light minus recovery absorption difference spectra of leaves from F2 generation, L17-like and wild type-like (**A**), putative double mutant *npq1*L17 and *npq1*-like (**B**). The spectra were recorded using the Aminco DW2000 spectrophotometer with actinic light intensity 700 μE. Each spectrum is the average of at least 3 separate measurements.

In Figure 5.12, the kinetics of absorbance change during light and dark periods have been shown, in a similar way as chlorophyll fluorescence quenching was measured earlier. The dark-adapted leaves were subjected to actinic light of 700 μE intensity, followed by dark period. The absorbance was measured at 535 nm for the representatives of all the four sub-groups of F2 plants, L17-like and wild type-like (Figure 5.12 A), putative double mutant *npq1*L17 and *npq1*-like (Figure 5.12 B). The absorbance change increased upon illumination and relaxed during the dark period in all cases. The amplitude of absorbance change shows that PsbS over-expression increases the extent of absorbance, and hence the conformational change in antenna complexes. The kinetics of the 535 nm absorbance change also reflects the differences in NPQ kinetics observed earlier. In plants with de-epoxidation (L17-like and wild type-like), the kinetics of 535 absorbance changes are faster to form and slower to relax during the second cycle as compared to the first one because of more zeaxanthin presence in the former cycle. While in case of plants lacking de-epoxidation (*npq1*L17 and *npq1*-like), the differences in kinetics of first and second cycle were not as pronounced as in plants with active de-epoxidation. The effect of PsbS was also apparent in these 535 nm kinetics, as the over-expression of this protein seemed to accelerate both the formation and relaxation rates.

The absorbance measurements revealed that the ΔA535, showing the extent of qE-related conformational change, was enhanced by over-expression of PsbS, both in the presence and absence of zeaxanthin. These results provide support for the NPQ model involving PsbS as modulator of conformational change resulting in quenching and zeaxanthin as allosteric regulator of the process.

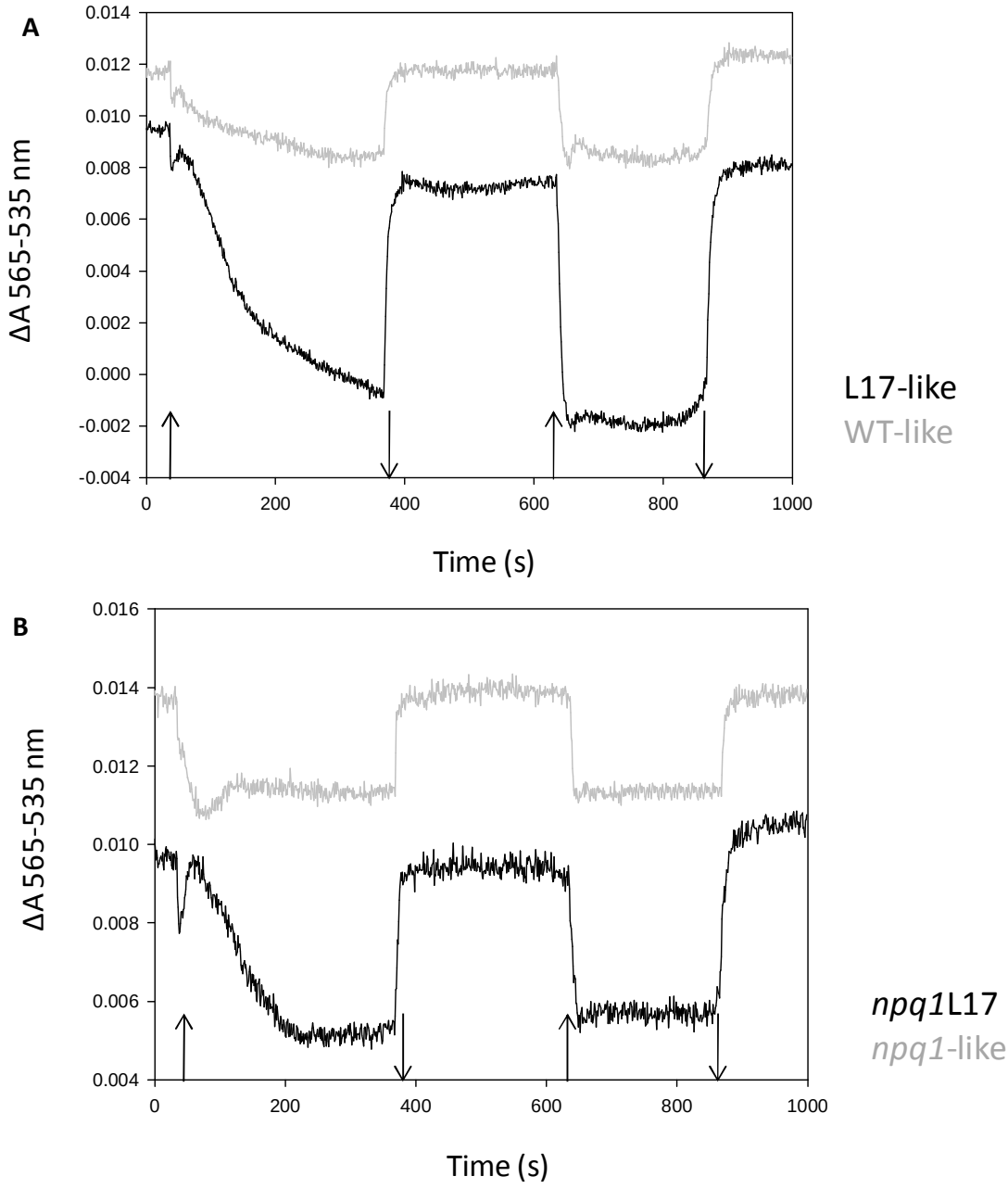

Figure 5.12 Kinetics of light-induced absorption changes in leaves of F2 generation, L17-like, wild type-like (**A**), putative double mutant *npq1*L17 and *npq1*-like plants (**B**). Spectra were recorded using the Aminco DW2000 spectrophotometer with actinic light intensity 700 μE. The absorption change A565 minus A535 nm was used for L17-like and wild type-like plants, while absorption change A565 minus A535 nm was used for putative double mutant *npq1*L17 and *npq1*-like plants. ↑ - actinic light on, ↓ - actinic light off.

5.2.2.2.2 Measurement of PsbS over-expression by western blots

For the confirmation of PsbS over-expression among F2 generation, the western blots were performed using an anti-PsbS primary antibody specific for *Arabidopsis*. Unstacked thylakoids were prepared from all the four sub-groups of F2 plants along with *npq4*, L17 and wild type plants as control. Figure 5.13 shows a typical blot where PsbS band of 22 kDa is identified by the help of marker and is confirmed by the absence of this band in PsbS-less *npq4* mutant. The band appears to be noticeably denser in case of L17-like and putative double mutant *npq1*L17 as compared to wild type-like and *npq1*-like plants. This difference is also comparable to one observed between the L17 and wild type used as controls.

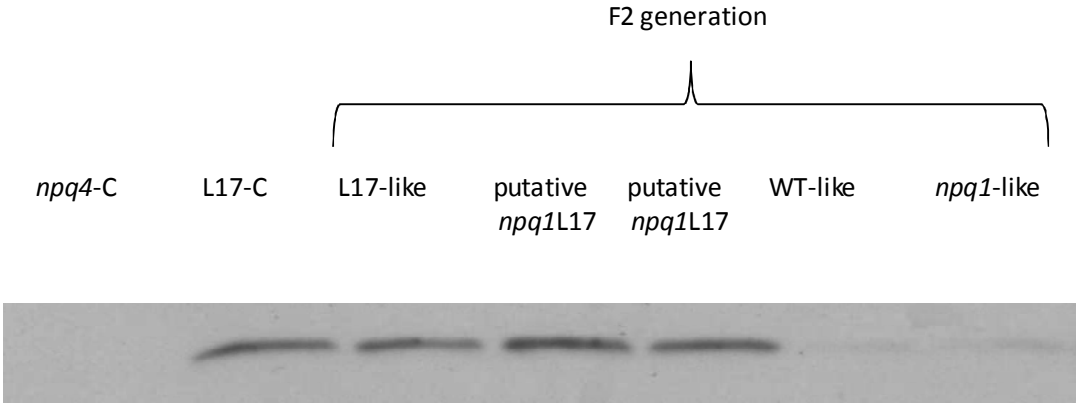

Figure 5.13. Western blot probed with anti-PsbS antibody for F2 generation L17-like, putative double mutant *npq1*L17, wild type (WT)-like, and *npq1*-like plants. L17 and *npq4* were used as controls, denoted by "-C". Unstacked thylakoids (1µg chl/lane) were run on a 15% SDS PAGE gel and probed with anti-PsbS primary antibody.

5.3 Discussion

The aim of the work presented in this chapter was characterisation of the role of zeaxanthin and PsbS in the mechanism of NPQ by dissecting their mutual interaction. This aim was achieved through enhancement of PsbS in the absence of zeaxanthin in the plant *Arabidopsis thaliana*. The work was analysed mainly by chlorophyll fluorescence and absorption spectroscopy. The observations presented in this work clearly demonstrate that an increase in the PsbS contents can enhance NPQ independent of zeaxanthin. This shows

that xanthophyll cycle activity is not an absolute requirement for quenching process, and hence cannot play a direct role in the initial rapid component of NPQ formation controlled by ΔpH. Rather, the data lends support to the view that zeaxanthin acts as an indirect regulator of the process, as it enhances the rate of NPQ formation during illumination, inhibits the rate of relaxation in dark and enhances the extent of the qE-related conformational changes. This work also suggests that PsbS, on the other hand, plays a key role as a kinetic modulator of the NPQ, as it not only increases the extent of NPQ but also seems to accelerate both the formation and relaxation of NPQ, in conformity with the earlier finding (Crouchman *et al*., 2006). The PsbS has also been suggested to affect the macro-organisation of the thylakoid membranes, as its absence induces more rigidity and less flexibility (Kiss *et al*., 2008). A quite recent study has also shown that NPQ is not abolished in the absence of PsbS, it is formed on a much slower timescale, however can reach to the same extent as in the wild type. Moreover this slowly NPQ formation is ΔpH sensitive and only partially zeaxanthin-dependent similar to that in wild type and relaxes in the dark (Johnson and Ruban, 2009).

The activity of PsbS involves binding of protons and/or zeaxanthin, however the mechanistic role of this protein is not well known. This protein has shown to affect the extent and rate of NPQ kinetics, as the over-expression of its gene has shown two-fold increase in NPQ extent (Li *et al*., 2002c) which is quicker to form and relax, as discussed earlier. The enhancement of PsbS amount as a result of HL exposure (Demmig-Adams *et al*., 2006) can also elevate the NPQ. On the basis of these findings, this protein has been proposed to play a direct role as quenching site, by binding of protons and zeaxanthin. Contrarily, the indirect role of PsbS presents it as inducer of the conformational change in antenna complexes leading to quenching (Horton *et al*., 2000), acting as sensor of pH by means of its proton-binding sites (Li *et al*., 2004). In this work, the enhancement in NPQ levels along with qE components was also achieved by HL acclimation both in the presence and absence of zeaxanthin. As mentioned above, this enhancement is believed to be a result of elevated PsbS levels as a result of HL exposure. The enhanced PsbS thus not only increases the extent of NPQ and its rapidly reversible qE component, but also induces faster rates of induction and relaxation. This enhancement of NPQ cannot be explained by

differences in the xanthophyll cycle pool and de-epoxidation state. It was also observed that PsbS enhancement as a result of HL acclimation decreased the extent of slowly reversible qI component part of which is photoinhibitory in nature, again independent of zeaxanthin. This may suggest that protection from photoinhibition is accorded by the larger NPQ in the presence of enhanced PsbS (Li *et al*., 2002).

The enhancement of PsbS was also made possible through genetic manipulation, obtaining a genetic combination of mutant *npq1* and over-expressor *psbS* genes. This double mutant, lacking zeaxanthin and possessing enhanced PsbS, showed an almost similar two-fold increase in the extent of NPQ, as compared to *npq1* mutant. Thus the similar proportional increase in the extent of NPQ both in the presence and absence of zeaxanthin cannot be credited to violaxanthin de-epoxidation yielding the zeaxanthin. The results obtained in this work demonstrate in a more natural system that activity of PsbS is not reliant on zeaxanthin to induce quenching, as it can enhance NPQ equally in the presence and absence of zeaxanthin. This finding contradicts a direct role of zeaxanthin-bound PsbS in NPQ, as proposed by Holt *et al*., 2005.

The activity of PsbS also differs from that of zeaxanthin in two ways,

(a) Firstly, the extent of NPQ can be elevated by PsbS enhancement (L17) while increase in zeaxanthin content does not increase the NPQ level (*npq2*) and rather it results in its decrease due to pre-quenching of Fm level, as discussed in Chapter III.

(b) Secondly, PsbS enhancement induces NPQ formation and relaxation at faster rate, while zeaxanthin increase only enhances the rate of formation and relaxation is inhibited by its increase.

The data presented in this chapter cannot explain a model involving PsbS-bound zeaxanthin as direct quencher of chlorophyll excited state. Both PsbS and zeaxanthin affect the extent and kinetics of NPQ, however their role in this respect is likely to be indirect and regulatory. However the allosteric model for NPQ readily explains the results presented in this chapter. This model suggests quenching as an intrinsic of the antenna protein complexes, where zeaxanthin acts as allosteric regulator and PsbS as modulator of this

process. Protonation of PsbS and/or antenna complexes triggers the conformational change in the antenna. Here PsbS can play its role independent of zeaxanthin, by inducing the conformational change leading to quenching. However, zeaxanthin can regulate this event by lowering the pH requirement for it (Ruban *et al*., 1996). Binding of zeaxanthin by PsbS and/or PsbS antenna complex maintains this conformational change and hence the quenched state. The mechanistic role of PsbS protein appears to be as an effective switch between the unquenched and quenched states of the plant thylakoid membrane, by controlling the dynamics of the macro-organisation of the PSII membranes. The role of zeaxanthin in this switch is to enhance ΔpH sensitivity of NPQ and thus its extent and rate.

Chapter Six

General Discussion

The success and survival of life on earth has been made possible by virtue of oxygenic photosynthesis, and this vital biological phenomenon pedestals on the efficient light harvesting mechanism. The light harvesting antenna is an assembly of pigments and proteins which collects the light energy quanta and transfers it for charge separation and water splitting to the reaction centre. The pigments of antenna are involved in interception and transfer of light energy while the proteins optimise and tune these functions. The role of antenna is dynamic and crucial for light regulation under both low and high light conditions. In case of low light, the antenna increases the excitation rate of the reaction centre to match its turnover rate, for an efficient energy conversion. In high light conditions, the same antenna undergoes a conformational change to channel the excessive and potentially harmful energy into heat dissipation, a process commonly measured and referred to as nonphotochemical quenching. Thereby, the antenna acts like a dynamic switch between photosynthetic and photoprotective states.

The scope of this project was to determine the molecular factors controlling the photoprotective capacity of thylakoid membranes in higher plants. The light harvesting antenna binds about 70% of the photosynthetic pigment molecules, chlorophylls and xanthophylls. The process of light harvesting is mainly performed by chlorophylls, in which a chlorophyll molecule in ground state absorbs light energy to form singlet-excited chlorophyll, the de-excitation of this excited chlorophyll occurs by the release of absorbed light energy in various ways. In photosynthetic state, the excitation energy of chlorophyll molecule is transferred to another acceptor molecule in the electron transport chain. The excess of light energy and chlorophyll over-excitation results in production of triplet-excited chlorophyll by intersystem crossing which can interact with molecular oxygen to generate singlet oxygen, a lethal species for photosynthetic apparatus. This necessitates a need for photoprotective state which enables plants to avoid this oxidative damage by a suite of measures; among these the heat dissipation by means of nonphotochemical chlorophyll fluorescence quenching (NPQ), is believed to be the most significant one. The NPQ is kinetically heterogeneous process which is induced under high light by low thylakoid lumen pH.

The xanthophylls are accessory photosynthetic pigments which are implicated in complementing light absorption, photoprotection and structural stability of antenna. By the help of xanthophyll mutants, this study demonstrated the role of xanthophylls in accomplishing all these three assigned functions. However the main focus here was the elucidation of photoprotective role of xanthophylls in higher plants, which is considered as their essential function. The xanthophylls are considered to limit the destructive reactions of singlet reactive oxygen species either by direct quenching of excitation in antenna or indirectly by eliminating the triplet chlorophyll states. The light harvesting antenna possesses four types of xanthophylls: lutein, neoxanthin, violaxanthin and zeaxanthin. These xanthophylls are oxygen-containing carotenoids with two terminal hydrophilic cyclic groups joined together by hydrophobic long carbon chain (Figure 6.1), possession of both

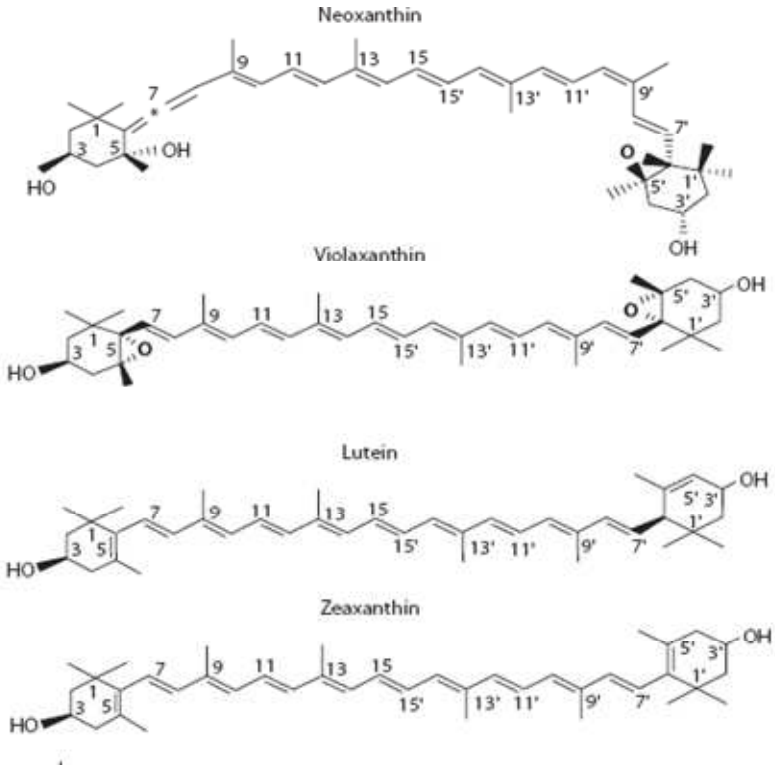

Figure 6.1 Chemical structures of the four xanthophylls, each showing two terminal hydrophilic cyclic groups joined together by hydrophobic carbon chain with conjugated carbon double bonds.

hydrophilic and hydrophobic characteristics makes them similar to detergents and quinones. The photosynthetic xanthophylls vary in their photophysical properties as a result of variations in their symmetry, hydrophobicity, number of oxygen atoms and number of conjugated double bonds which affects their delocalised excited state π-electron energy.

Both zeaxanthin and lutein have been implicated as direct quenchers of chlorophyll excitation in antenna, using ultrafast transient absorption spectroscopic studies (Holt *et al*., 2005; Ruban *et al*., 2007). These two xanthophylls are isomers of each other and are more hydrophobic and less oxygenated as compared to the other two photosynthetic xanthophylls, neoxanthin and violaxanthin. The light harvesting antenna is made up of major trimeric and three minor monomeric pigment binding protein complexes. Lutein, the most abundant xanthophyll pigment of higher plants, occupies exclusively the central L1 binding site of all the antenna complexes. The other central L2 binding site is occupied by lutein in major antenna complexes and by violaxanthin in some of the minor complexes (CP29). The remaining two xanthophylls neoxanthin and violaxanthin bind to N1 and peripheral V1 sites, respectively, in major and most of minor antenna complexes. Violaxanthin loosely bound to peripheral site is reversibly replaced by zeaxanthin in the light by xanthophyll cycle activity; however this replacement has not been demonstrated *in vivo* at internal L2 site of the minor antenna, the suggested site of zeaxanthin cation quenching. The retention of a variety of xanthophylls in light harvesting antenna during evolution points towards their indispensable functional and structural role. It is also interesting to mention here that both lutein and zeaxanthin, which are considered to play a central role in photoprotection in plants, are also present in retinal macula of eye performing there a similar function of filtering the high energy wavelengths of visible light and acting as antioxidants (reviewed in Roberts *et al*., 2009).

Two mechanisms have been proposed for the quenching of excited chlorophyll in plants. One mechanism suggests quenching as intrinsic property of antenna complexes and lutein at L1 site has been demonstrated in major antenna complexes as quencher of chlorophyll excitation energy (Ruban *et al*., 2007). This mechanism suggests zeaxanthin at peripheral binding site V1 as allosteric regulator of the quenching process. The other mechanism

proposes zeaxanthin at L2 site of minor complexes as the direct quencher of excited chlorophyll through charge transfer. Transient zeaxanthin radical cation formation has been demonstrated as evidence in the minor antenna complexes in the reconstituted forms (Holt *et al*., 2005).

In this work, NPQ formation and relaxation kinetics have been studied in the plant types with different xanthophyll composition. From the data it can be inferred that xanthophyll composition affects both the amplitude and kinetics of NPQ, as the absence of any of the constituent xanthophylls decreases the NPQ amplitude along with influencing the kinetics. However, *no single xanthophyll* has been found as obligatory for this process as quenching occurs variably in all the cases. The NPQ formation kinetics consists of an initial rapid zeaxanthin-independent and a second slower zeaxanthin-dependent phase. The zeaxanthin-less mutants show only initial phase, those without lutein exhibit only a second one and those lacking both zeaxanthin and lutein are lacking any rapidly reversible NPQ. This may indicate that NPQ comprises of two discrete mechanisms involving lutein and zeaxanthin as quenchers at two different sites of major and minor antenna complexes, respectively. This must also be reflected in the NPQ relaxation kinetics; however observation of a single component here does not support the assumption of two quenching mechanisms. Moreover, the relaxation kinetics is expected to be influenced by a regulator and not the quencher, and the data in this study suggests zeaxanthin as allosteric regulator of the process. The zeaxanthin–independent quenching is also usually proposed to occur by a different mechanism, for example by transient reaction–centre quenching caused by inactivation of PSII electron transport (Finazzi *et al*., 2004). This work suggests role of xanthophylls in both steady state and transient quenching, both regulated by the same mechanism involving the same conformational changes in LHCII bound pigments.

In addition, the results presented here also suggest that zeaxanthin concentration does not regulate the state of antenna. It is rather de-epoxidation state, the relative proportion of zeaxanthin to violaxanthin, which influences the amplitude of NPQ. The constitutive presence of zeaxanthin though accelerates the NPQ formation; nevertheless it results in decrease in NPQ amplitude due in part to quenching of Fm and also slows down the

relaxation kinetics. It is also interesting that relative proportion of zeaxanthin to violaxanthin cannot enhance the photoprotective capacity beyond wild type level, as studied in one of the over-expressors of xanthophylls sChyB plants (Johnson *et al*., 2007). The saturation of NPQ here can be explained by the limitation of PsbS concentration. Therefore, the de-epoxidation state by means of xanthophyll cycle activity seems to allosterically regulate the conformational state of the LHCII antenna system. Zeaxanthin enhances the conformational change in antenna by promoting the sensitivity to the pH, while violaxanthin allosterically inhibits this change by making LHCII more resistant to pH. It has also been demonstrated in an *in vitro* quenching study that exogenous zeaxanthin promotes both the rate and amplitude of pH induced quenching in isolated antenna complexes, while violaxanthin had an opposite effect (Ruban *et al*., 1996). This stark difference in the activity of these two xanthophylls, which can replace and substitute one another at the same binding site by xanthophyll cycle activity, can be attributed to the differences in their polarities and head group orientations which also influences their binding ability (Horton *et al*., 1999). More hydrophobic zeaxanthin is considered to form stronger interactions with LHCII and had been shown to offer more resistance to extraction by detergent (Ruban *et al*., 1999).

The differences between the NPQ formation kinetics of dark-adapted and pre-illuminated leaf samples can be understood by the differences in the de-epoxidation states. In the dark adapted samples, NPQ formation kinetics is shown as biphasic with initial fast pH dependent phase followed by slower zeaxanthin dependent phase as violaxanthin is slowly de-epoxidised to yield zeaxanthin. Contrarily, NPQ formation is rapid and monophasic during the subsequent illumination because of zeaxanthin presence and light activation in the previous illumination event. Even a relatively low de-epoxidation state of 10-20% suffices to significantly enhance the rate of NPQ formation kinetics. This can be explained by the organisation of antenna complexes acting closely and co-operatively, thus conformational change in one complex may lead to lowering of the activation energy barrier for a similar change in the adjacent counterparts. This may lead to a rapid sequential allosteric transition at rather lower de-epoxidation states. High light acclimation results in reduction of antenna size, thus co-operativity in a small system would likely be enhanced

resulting in further increase in the extent and rate of NPQ. Similarly, the impact of aggregation may also enhance the interaction among these complexes to promote the quenching signal in antenna.

Retention of zeaxanthin and light-activated NPQ capacity also has physiological significance. During intermittent high light exposures which are common in nature due to sun flecks and shading by canopy, plants can dissipate excess absorbed energy instantly and efficiently while maintaining their photosynthetic activity simultaneously. Moreover, this may also act as molecular memory to keep the track of average light conditions in a particular time period. Thus the amplitude and rate of NPQ can be tuned accordingly to match the light conditions without interfering with the linear electron transport and ATP synthesis. Zeaxanthin thus seems to promote interaction between antenna complexes which leads to the quenched conformation, and NPQ is a result of a co-operative progressive event (Horton *et al.*, 2008).

A photoprotective system solely reliant on xanthophylls would have its own disadvantages as the slower kinetics of NPQ formation and relaxation cannot be favourable for efficient photoprotection and effective photosynthesis respectively. Slower NPQ formation can yield into photodamage to the photosynthetic machinery by means of reactive oxygen species, whilst slower NPQ relaxation can hamper photosynthetic activity and cumulative effect of both of these events can ultimately be in the form of lower crop productivity. It is suggested that this disadvantage was overcome by the evolution of PsbS protein in the higher plants. This hydrophobic protein can reversibly bind with either the PSII core or LHCII antenna, which demonstrates its mobile nature in the thylakoid membranes. The evolutionary advantage of this dynamic protein seems to be associated with facilitating the swift transitions between quenched and unquenched states of the antenna. NPQ is not completely abolished in the absence of PsbS; rather it is formed and relaxed over a much longer period of time (Johnson and Ruban, 2009). Presence of this protein also does not seem to limit the photosynthetic activity albeit provides efficient and rapid photoprotection. PsbS is suggested to act like a biological catalyst by lowering the activation energy of the conformational change, as it stimulates the rate of NPQ formation as well as relaxation.

This helps to achieve optimum responsiveness in the light harvesting system towards the fluctuating light exposure without bringing large changes in the xanthophyll pool size.

Like other biological regulatory processes, NPQ is optimised to meet the conflicting demands of environment and development. Both the magnitude and rate of NPQ need to be at optimum levels, so that none of light harvesting and light dissipation modes are jeopardised. This balancing approach explains why plants do not maintain a large xanthophyll cycle pool under the light limiting conditions. A larger xanthophyll cycle pool can make system less responsive by slowing down the transitions between the above mentioned modes (from protective to efficient). The reason for maintaining the xanthophyll cycle is essentially to use light in an efficient way in photosynthesis, which is vital to plant fitness in the field conditions (Kulheim *et al*., 2002). The constitutive presence of zeaxanthin can significantly limit the efficient usage of light in low or fluctuating light by maintaining a perpetual heat dissipation mode (Niyogi *et al*., 1998; Kalituho *et al*., 2007).

Observation of zeaxanthin formation during high light exposure indeed revealed a strong correlation between zeaxanthin and NPQ, which led to the suggestion of its role as quencher in the process. This mechanism also suggests minor complexes as site of quenching which link the light harvesting major complexes with reaction centre; this ideally presents a division of labour among these complexes. Thus light is harvested mainly by major complexes and transferred through linking minor complexes into reaction centre; and in case of excess light the latter complexes become the quenching site. However, it is also interesting to mention here that photoprotection can be considered to be the intrinsic property of all the light harvesting complexes as each of them exhibits quenching *in vitro*. It is *photoprotection* which should be considered to be the "original" function of these complexes instead of *light harvesting*, as the presumed ancestors to all these protein complexes, the cyanobacterial one-helix proteins, were meant for photoprotection and/or pigment metabolism. The light harvesting antenna function could have evolved later, which then led to the diversification of the complexes (Jansson, 2005). The universal binding of conserved lutein at L1 site of all the complexes could indicate a strong correlation with the original photoprotective function. The control of this photoprotective process necessitated

the evolution of an indicative and regulatory event of xanthophyll conversions in the form of xanthophyll cycle.

The binding of different xanthophylls at L2 site can also be presented as indicator of division of labour among the antenna complexes, as lutein of major complexes is replaced by violaxanthin in minor complexes at this particular site. The violaxanthin has been known to play an inhibitory role for quenching process and its conversion into zeaxanthin under high light stimulates the photoprotective quenching of chlorophyll excitation. It has been clearly demonstrated here in this work by xanthophyll pigment analysis of light and dark states that this conversion is not fully accomplished in wild type and a significant pool of violaxanthin (40-50%) persists after light exposure, this suggests that VDE enzyme responsible for this conversion can only bring about partial de-epoxidation, most probably by accessing the peripheral pool of violaxanthin. The replacement of violaxanthin by zeaxanthin at internal L2 binding site has only been demonstrated in reconstitution studies and not *in vivo*. Nevertheless the results obtained in this work with mutants binding zeaxanthin at L2 site clearly show that presence of extra zeaxanthin does not impart any additional photoprotective capacity, rather it impairs the “original” photoprotective role of these complexes.

The antenna complexes have evolved an efficient light harvesting function along with their conserved original photoprotective role. The variety among these complexes might have developed to build up a dynamic structure and macro-organisation of photosystem II, which is considered to play a regulatory role between the two afore mentioned functions. Thereby all the interacting antenna complexes act as a domain to switch from one function to the other. The alteration of antenna composition by genetic manipulation has demonstrated that exclusion of certain minor complexes like CP29 and CP24 disturbs the organisation of the LHCII-PSII super-complexes in the grana membrane (Horton *et al.*, 2008). This structural anomaly also perturbs the amplitude and kinetics of NPQ. However, the antenna composition and organisation also seems to be robust and dynamic in nature as absence of other major and minor complexes does not significantly change the macrostructure and hence the NPQ remains more or less unaffected. There is also evidence for the involvement

of PsbS in the macro-organisation of the antenna as the LHCII-PSII association during restacking of thylakoid has been correlated to the PsbS level (Kiss *et al*., 2007). This suggests that PsbS is likely to have a dynamic role in NPQ by priming the LHCII antenna for conformational change into the quenched state.

The intrinsic conformational change in the major and minor antenna complexes leading to the formation of a highly quenched state has been demonstrated *in vitro*, which provides a molecular basis for link between conformational change and quenching event. Although this event is accompanied by the protein aggregation, quenching itself originates from within each complex as a result of pigment interactions (Pascal *et al*., 2005; Ilioaia *et al*., 2008). The excess of light energy prompts the conformational change in the light harvesting antenna leading to a decrease in the amount of excitation energy delivered to the reaction centre. A new energy dissipative pathway is created in the antenna to achieve this down regulation, which competes with the transfer of energy for photosynthetic activity. The conformational change is induced by acidification of LHCII amino acid residues, as a result of the establishment of the transmembrane proton gradient under excess of light conditions. This also promotes interactions among the complexes leading to aggregate formation, as demonstrated by aggregation of isolated complexes which results in lower fluorescence and shorter excited state lifetime as compared to those in trimeric or monomeric forms (Ruban and Horton, 1992; Mullineaux *et al*., 1992).

Further evidence in this regard has been provided by a pump-probe femtosecond transient absorption spectroscopic study probing the possible molecular cause of the decrease in the excited state lifetime. This has also showed a similar conformational change in LHCII *in vivo* upon formation of NPQ (Ruban *et al*., 2007). The change in conformation is however monitored indirectly by twisting of neoxanthin molecule detected by resonance Raman spectroscopy. This conformational change is suggested to facilitate the energy transfer from chlorophyll *a* to one of the low lying xanthophyll excited states absorbing in 490-495 nm region. The absorption of lutein 1 within same region implies this xanthophyll to be the most likely quencher of the chlorophyll excitation. This is made possible by distortion in the lutein 1 molecule in such a way that it comes close enough to the three chlorophyll *a*

molecules, Chl *a*610, *a*611 and *a*612 (Figure 6.2), of terminal emitter domain to quench the excitation energy. The excitation energy is transferred from the chlorophyll Qy band to the S1 state of the Lutein 1, whereby it is ultimately dissipated as heat via decay to the ground state. A similar distortion in Lutein 1 domain has also been reported in the crystals of LHCII upon dehydration, and this event is also accompanied by a red shift in the chlorophyll emission maxima from 680 nm to 700 nm (Yan *et al*., 2007). The conservation of Lutein 1 in all the major and minor antenna complexes may also validate the particular role of quenching species played by this xanthophyll at this specific position to maintain the original photoprotective function in all the complexes. The data presented in chapter 3 also suggests a direct correlation between ΔA495, a lutein1 0-0 absorption maximum, and energy-dependent quenching, confirming the role of lutein 1 as quenching species.

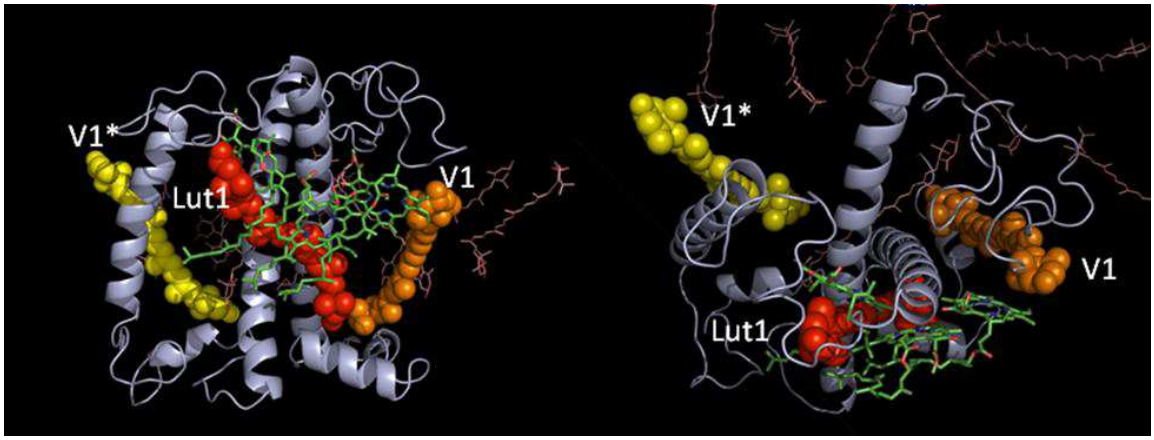

Figure 6.2 Lutein 1 (Lut1) and terminal emitter domain within each LHCIIb monomer (grey). The formation of a quencher is proposed to arise from an interaction between Lutein 1 (red) and chlorophylls *a*610, *a*611 and *a*612 (green) that constitute the terminal emitter domain. The LHCIIb conformational change induced by the ΔpH is proposed to distort lutein 1, bringing it close to the terminal emitter domain. The close proximity of the xanthophyll cycle carotenoid at the V1 site from the same (orange), or adjacent monomer V1* (yellow) has been suggested to allosterically modulate this interaction. (Image created in PyMol).

The regulation of this significant event also necessitates the existence of a specific molecular mechanism. The allosteric model suggests xanthophyll cycle as the regulator of this conformational change, whereby zeaxanthin and violaxanthin act as promoter and inhibitor, respectively. The peripherally bound xanthophyll cycle molecule at V1 binding site may affect the lutein 1 domain within the same monomer or the adjacent monomer in the form of an allosteric effect. It has also been suggested that the thylakoid membranes become thinner, more hydrophobic and dehydrated upon ΔpH formation (Murakami and Packer, 1970), these changes may also involve PsbS which is also a very hydrophobic protein. Such a reduction in volume in the membrane environment of LHCII, could compress the pigments within the structure increasing the interaction between the quencher and chlorophyll. Indeed, the LHCII fluorescence lifetime *in vitro* was found to be very sensitive to even a small change in volume induced by high hydrostatic pressure (van Oort *et al*., 2007). *In vivo* this compression of LHCII structure would be further enhanced by the exchange of the polar xanthophyll violaxanthin, which behaves rather like a detergent, for the hydrophobic zeaxanthin, which behaves more like a lipid, at the V1 binding site (Ruban *et al*., 1997).

It is quite interesting that all the xanthophylls have ability to quench the chlorophyll excitation energy as shown by the *in vitro* quenching and lifetime measurements in Chapter IV, however this ability is not similar. Lutein at internal binding sites has been found as most efficient quencher, whereas its replacement by zeaxanthin and violaxanthin at internal L1 and L2 binding sites impairs the quenching capacity of the complexes. Since the quencher in wild-type LHCII aggregates has been identified as the S_1 state of lutein 1, therefore it can be argued here that the observed differences between the photoprotective capacities upon varying the xanthophyll composition are due to the fact that zeaxanthin and violaxanthin both make 'weaker' quenchers when incorporated into the L1 'quencher' binding site. The differences are unlikely to be explained by differences in excited state energy levels since both violaxanthin and zeaxanthin possess similar S_1 lifetimes and energies to lutein when incorporated into these sites in LHCII, which are already below those of chlorophyll *a* (Polivka *et al*., 2002). It is therefore more likely that the differing polarities and head group orientations of each xanthophyll (Ruban *et al*., 1993; Young *et*

al., 1997) dictate how close the interaction between the xanthophyll and the terminal emitter chlorophylls in the L1 site can be and/ or upon the constriction of the protein matrix itself how much each xanthophyll can be distorted. Potentially, distortion of the xanthophyll can alter its excited state properties (Polivka *et al*., 2002), for instance, by giving rise to an intermolecular charge transfer (ICT) state which could influence the likelihood of energy transfer/ excitonic coupling between the chlorophyll S_1 state and the forbidden xanthophyll S_1 state (van Amerongen and van Grondelle, 2001; Bode *et al*., 2009; Berera *et al*., 2006). Indeed, in both LHCII aggregates and in chloroplasts the excited state energy of the emitting chlorophylls is modified in the NPQ state (Johnson and Ruban, 2009), given that the red shift of the terminal emitting fluorescence correlates with the lifetime.

Violaxanthin was found to be the strongest promoter of the light harvesting mode in LHCII antenna. It can be suggested here that lutein at L1 binding site conserves the original photoprotective characteristic in all the antenna complexes whilst the minor complexes with violaxanthin at L2 site may have evolved to confer additional light harvesting characteristic to the antenna. It can be also be proposed that nature exploits the inherent flexibility of LHCII function by adopting a complement of xanthophylls that optimises the maximum dynamic range between its light harvesting and photoprotective states. Optimisation of photosystem II quantum efficiency therefore occurs at the level of individual LHCII complexes.

The chlorophyll fluorescence lifetime measurements with varying xanthophyll composition have been carried out for the first time in this research. The resulting data furthers our understanding of the regulation and optimisation of the efficiency of both light harvesting and photoprotection. The optimum performance of this regulation, with largest dynamic range, is having the longest lifetime in the unquenched state (giving maximum light harvesting efficiency) and shortest lifetime in the quenched state (giving the most effective photoprotection). Clearly the maximum dynamic range was achieved by having the native complement of xanthophylls (Figure 6.3). Whereas replacing lutein with violaxanthin increased the lifetime of the unquenched state, the capacity of NPQ was reduced, giving a

restricted dynamic range. The advantage of a potential increase in light harvesting efficiency is clearly offset by the reduced level of photoprotection. Similarly, when lutein was replaced by zeaxanthin, the dynamic range was also compromised, since there was a reduced lifetime in the unquenched state without any enhancement in the quenched state.

The origins of these effects of xanthophyll complement on light harvesting and photoprotection were shown to reside in the altered properties of the light harvesting complexes. Remarkably, all the effects on lifetimes of changes in xanthophyll composition observed in leaves were manifested as altered properties of the isolated LHCII. The results also suggest that the xanthophyll complement determines the lifetime of both quenched and

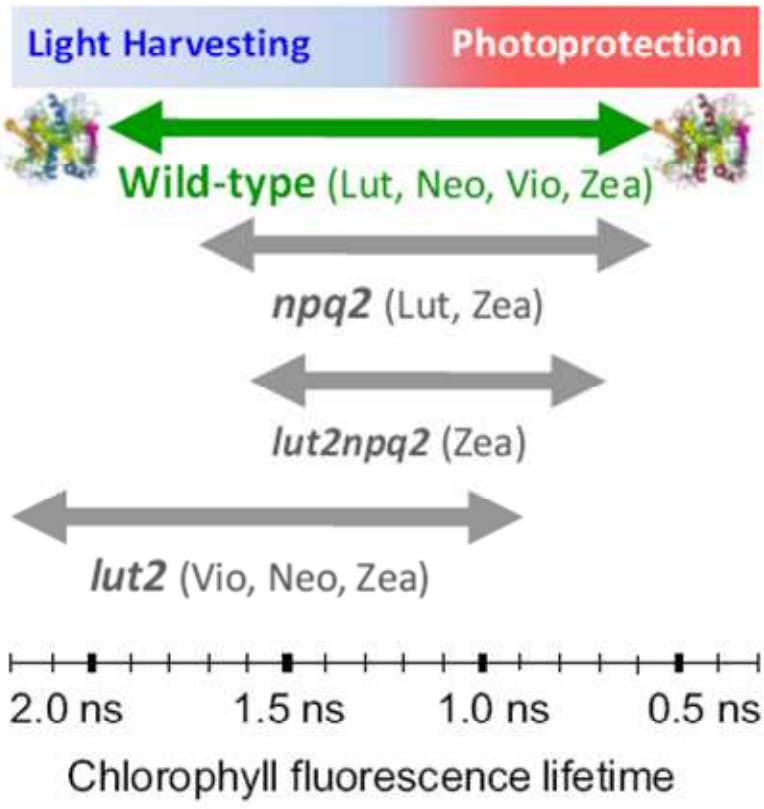

Figure 6.3 Model depicting the relationship between chlorophyll fluorescence lifetime and xanthophyll composition in *Arabidopsis* leaves. The xanthophyll complement of LHCII determines the dynamic equilibrium between light harvesting and photoprotective functional states *in vivo*, which possess different average fluorescence lifetimes. Note in all cases the difference in lifetime between the two states in the mutants is less than in the wild type.

unquenched states. Lutein was clearly shown as an essential cofactor of LHCII and when it is replaced at its internal binding sites by violaxanthin or zeaxanthin the lifetimes either increase or decrease respectively. The similarities between the effects of xanthophyll substitutions on leaves and LHCII suggest that the quenching mechanism is the same in both, consistent with the previous data that highlighted the similarity between NPQ and quenching in LHCII aggregates (Horton *et al.*, 1996; Horton *et al.*, 2005). The similar behaviour of the minor antenna complex CP26 and LHCII does not support the idea that the former is the unique site of NPQ or that quenching in this complex occurs by a different mechanism involving a zeaxanthin radical cation. Indeed, the results presented here in this work provide no evidence for an exclusive direct quenching role for zeaxanthin. The well-documented enhancement of NPQ by de-epoxidation of violaxanthin to zeaxanthin is therefore best explained by an indirect, regulatory role from the peripheral V1 site, allosterically modulating the pH sensitivity of NPQ.

The remarkable reduction in the rapidly reversible component of NPQ in the absence of PsbS protein and the two-fold enhancement of NPQ magnitude achieved by over-expression of this protein, both demonstrate the key role of PsbS in photoprotection (Li *et al.*, 2000; Li *et al.*, 2002). Currently, two models have been proposed which attempt to explain the role of PsbS in NPQ. A brief summary of these two models is as follows:

The first model suggests that direct quenching of chlorophyll excited states takes place by zeaxanthin bound to PsbS. This model was supported by evidence that PsbS is able to bind zeaxanthin (Aspinall O'Dea *et al.*, 2002) and subsequent studies suggested a quenching mechanism of direct energy transfer from chlorophyll to zeaxanthin (Ma *et al.*, 2003; Dreuw *et al.*, 2003; Holt *et al.*, 2004, 2005; Berera *et al.*, 2006). The second is allosteric model which presents PsbS as a biological catalyst meant for lowering the activation energy for the transition from the unquenched to the quenched state (Horton *et al.*, 2000). According to this model, the quenching arises as a result of conformational change within the light harvesting antenna and this transition is regulated by zeaxanthin which acts as an allosteric effector. The initial support for this model came from the observation of aggregation event which accompanies the quenching process of light harvesting proteins

(Horton *et al*., 1991). Both the processes were shown to have a dependency on ΔpH which was found remarkably similar to an enzyme – catalysed reaction (Noctor *et al*., 1991; Horton *et al*., 2000). Moreover it was also demonstrated that this quenching process is independent of zeaxanthin (Noctor *et al*., 1991) and this xanthophyll is only meant to potentiate the process by lowering the pH requirement (Ruban and Horton, 1999).

These two models suggest different mechanistic roles of PsbS and zeaxanthin; however they are by no means mutually exclusive. For instance, it can be possible that zeaxanthin influences both direct quenching in LHCII (Liu *et al*., 2004), probably by accounting for photoinhibitory qI, and the indirect quenching by acting as an allosteric effector (Wentworth *et al*., 2000; Horton *et al*., 2005). The enhancement of rapidly reversible NPQ attained by fortification of PsbS protein both in the presence and absence of zeaxanthin, achieved in the present study, is also strong evidence in support of the allosteric model. The main features of this evidence are outlined below.

It has conclusively been demonstrated in earlier studies that increase in PsbS amount leads to enhancement of the ΔpH-dependent NPQ capacity (Li *et al*., 2000, 2002b, 2002c). This corresponding dosage effect of PsbS on the quenching process supports the critical role of this protein in energy dissipation; however it does not aptly explain its mechanistic role, since the similar effect would be exhibited if PsbS acted in the direct or indirect model.

It has previously been shown by the analysis of leaves and isolated chloroplasts treated with dithiothreitol that over-expression of PsbS can result in the enhancement of NPQ in the absence of de-epoxidation. This was a firm evidence that xanthophyll cycle had no direct role in PsbS – dependent quenching, validating the proposed role of zeaxanthin as an allosteric effector in the indirect quenching model. The use of an exogenous inhibitor of de-epoxidation process can be speculated to have adverse impact on other biological activities; this necessitated the need for development of a more natural system for enhancement of NPQ in the absence of zeaxanthin. This was achieved by increasing the PsbS amount in zeaxanthin-less mutant (*npq1*) by both physiological and genetic means, therefore reversible NPQ was enhanced in similar proportion both in the presence and absence of zeaxanthin. Both the amplitude and rate of NPQ formation was enhanced by increase in

PsbS level, nevertheless both the values were significantly higher in the presence of de-epoxidation. This again presents zeaxanthin as the regulator of the process and not as an absolute requirement. The enhancement of magnitude and level of NPQ, as a result of increased PsbS levels, does not necessarily explain the direct role of this protein, as NPQ still occurs in the absence of PsbS. However, it can be suggested here that PsbS acts to somehow induce conformational changes in light harvesting subunits which leads to the quenching of chlorophyll excited states, whilst the presence of zeaxanthin enhances both the rate and extent of these changes, perhaps by binding PsbS, as has been shown *in vitro* (Aspinall-O'Dea *et al*., 2002).

As PsbS has been suggested as a catalyst for reversible NPQ, thus increase in the levels of this catalyst is expected to enhance the rate of reaction up to certain level. Therefore over-expression of PsbS in L17 plants allows the conformational changes to happen more efficiently, which results in a faster transition to the quenched state. However, an increase in PsbS level of 4 times in the over-expressor line as compared to wild type only resulted in a NPQ increase by 2 fold, which may indicate that either the reaction (NPQ) has reached its ceiling or the activity of the catalyst (PsbS) is limited by substrate concentration (zeaxanthin and/or antenna proteins). On the other hand, the direct quenching model has no explanation for these changes in kinetics, since in the absence of de-epoxidation there would be no PsbS–zeaxanthin quenching complex and thus the rate of quenching would be expected to remain unaffected. As mentioned earlier, this model suggests zeaxanthin–independent quenching to occur by a different mechanism of transient type, which takes place in reaction centre due to inactivation of PSII electron transport (Finazzi *et al*., 2004).

Further analysis of NPQ kinetics also demonstrated that PsbS over-expression also enhanced the rate of relaxation in the dark. However, the presence of zeaxanthin made this recovery slower as compared to that in the absence of de-epoxidation. This observation indicates towards a key role played by PsbS in the NPQ kinetics independent of zeaxanthin. Thus it can be suggested that PsbS acts as a reversible switch between the quenched and the unquenched states, facilitating both the rapid induction and relaxation of quenching by inducing conformational changes in the thylakoid membrane. This activity to induce the

formation of the quenched state is in an allosteric fashion. The presence of zeaxanthin appears to enhance the function of PsbS by increasing both the magnitude and rate of NPQ formation; however it inhibits the rate of relaxation of quenching.

The differences in kinetics between wild type and *npq1* mutant plants have also been demonstrated in plants which accumulate PsbS during acclimation to growth at high irradiance. High light acclimated plants had approximately twice the level of PsbS protein and exhibited an increase in the rate of NPQ activation in both the presence and absence of zeaxanthin. The control plants grown under normal lower light conditions showed lower levels of PsbS and an associated small NPQ which was slower to form. This indicates that high light exposure demands the need the need for a large, rapidly forming NPQ which can be met by the accumulation of PsbS. Conversely, low light conditions require avoidance of unnecessary wastage of absorbed energy in the form of quenching, and this is achieved by reducing the level of PsbS protein. The relaxation kinetics were also observed as faster in the presence of enhanced PsbS levels, thus it would be reasonable to assume that the requirement for a rapidly forming quenched state also requires the corresponding ability to relax back to the unquenched state equally rapidly, so that an unnecessary dissipation of harvested energy can be avoided. This proposed role of PsbS as a "molecular switch" between the quenched and unquenched transitions is consistent with the field studies which showed the essential requirement of PsbS for plant fitness under conditions of fluctuating light (Kühlheim *et al*., 2002). A recent field study of plants with different levels of PsbS expression has demonstrated that this photoprotective protein also affects metabolite profiles as up to ten-fold differences were measured in the concentrations of some carbohydrates and amino acids among these plants. Moreover, transcriptomes of such plants were also varied as certain stress-related genes for the jasmonate biosynthesis were up-regulated in the PsbS-lacking mutants, when subjected to herbivory. Ironically, these profile changes were not observed under constant laboratory conditions (Frenkel *et al*., 2009). It would be interesting to employ a similar ecophysiological approach towards the study of xanthophyll mutants as the xanthophylls have only been implicated so far for the regulation of light harvesting in controlled conditions, while their physiological role to influence metabolite and transcriptome profiles under field conditions is yet to be explored.

Further analysis of the effects of PsbS level on NPQ capacity and kinetics is needed to clarify the role of PsbS in the transition between the light harvesting and energy dissipation states. It would also be interesting to study mutants which over-express PsbS along with enhanced pool of xanthophyll cycle, as this would allow better understanding of the maximum potential of NPQ and further investigation of the zeaxanthin-dependent quenching mechanism.

The measurement of NPQ kinetics in two illumination cycles allowed making certain interesting observation as the rate of formation and capacity of NPQ was always enhanced during the second illumination after a period of dark relaxation, even in the absence of zeaxanthin as this promoting effect is mainly attributed to the zeaxanthin formation. The increase in NPQ was shown to be of reversible nature (qE) and not photoinhibitory (qI). These differences were small however significant with reference to the previously thought influence of the de-epoxidation state (Demmig-Adams, 1990; Ruban *et al*., 1993). This observation suggests that the thylakoid membrane may undergo zeaxanthin-independent transition during illumination which is "memorised" during the dark period and "prime" the system for quenching during the subsequent illumination period. This increase in the NPQ capacity during the second illumination period may suggest that that PsbS is involved in such a process, by inducing the conformational change in antenna complexes which does not fully relax during the dark period. The proposed priming of thylakoid membranes can be understood further by in depth study of NPQ extent and kinetics in the plants with PsbS over-expression subjected to various regimes of pre-illumination.

Absorption changes in the broad region of 520 nm to 540 nm have been correlated with the conformational changes in the thylakoid membrane which lead to the quenching (Noctor *et al*., 1993; Ruban *et al*., 1993, 2002). The spectral analysis of leaves also show that the magnitude and kinetics of 535 nm absorbance changes were enhanced in the PsbS over-expressor, suggesting that the conformational changes, which lead to quenching, result from PsbS-dependent alterations in the light harvesting antenna. This is a further support for the proposed role of PsbS in acting as a "molecular switch" facilitating the transition between the unquenched and quenched states of the photosynthetic apparatus, which is

most likely proceeded by induction of conformational changes in the membrane. Moreover, these absorbance changes were also enhanced in the presence of zeaxanthin similar to the fluorescence quenching, providing further support to the model where zeaxanthin regulates the rate of formation and extent of the quenched state, by acting like a positive effector.

It can also be interesting to mention here that the amplitude of 535 nm absorbance change does not apparently match the extent of NPQ level, as at least 3 times increase in absorbance change was observed against the 2 times greater NPQ increase in L17 as compared to the wild type. This discrepancy may be explained by assuming that the 535nm absorbance change does indeed arise as a result of PsbS binding zeaxanthin (Aspinall O'Dea *et al.*, 2002; Ruban *et al.*, 2002). Since the availability of PsbS protein is much higher in the over-expressor as compared to normal wild type levels, therefore obviously more zeaxanthin molecules would bind to the protein to get activated, resulting in the more pronounced increase in the ΔA535. However, this increase in the amount of PsbS-zeaxanthin associations would not necessarily be expected to lead to a similar increase in the level of NPQ, as it can be limited by the amount of available antenna complexes with the ability to quench chlorophyll excited states.

In summary, the PsbS-zeaxanthin binding event was not confirmed in this study by observation of the absence of linear correlation with an increase in NPQ. The enhancement of the similar absorbance change in the absence of zeaxanthin also demonstrates that this change may also be solely dependent upon PsbS, although its origin is not well understood. The involvement of more than one xanthophyll has also been speculated in the 520 nm to 540 nm absorbance region, possibly lutein. Thus, it can be suggested that absorbance change depicting conformational change is result of pigment interactions between chlorophyll and lutein, also proposed as a mechanism for energy dissipation (Ruban *et al.*, 2007). Further investigation of the origin of this broad absorbance band can provide insight into the changes taking place in the thylakoid membrane during the quenching process and the role of PsbS to influence these changes.

Since PsbS appears to equip plants with the advantage of large extent of NPQ with rapid formation and relaxation rates, it can be considered as a strategy to optimise the

photoprotective capacity of plants. The constitutive expression of higher PsbS level can be significant at high light intensities, as revealed by high light acclimation studies (Bailey *et al.*, 2004; Ballottari *et al*., 2007), the enhanced photoprotective capacity can actually be a real disadvantage at low light intensities, where unnecessary wastage of absorbed light energy can easily be carried on by heat dissipation. Thus a delicate balance must be reached whereby protection from harmful excess excitation is avoided, but not at the expense of photosynthetic capacity. It seems therefore that the most crucial function of PsbS is to provide flexibility in photosynthesis under fluctuating light conditions, and to allow control over photoprotective capacity during acclimation.

Chapter Seven

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