UNIVERSITA' DEGLI STUDI DEL PIEMONTE ORIENTALE

"Amedeo Avogadro"

UNIVERSITÀ DEL PIEMONTE ORIENTALE

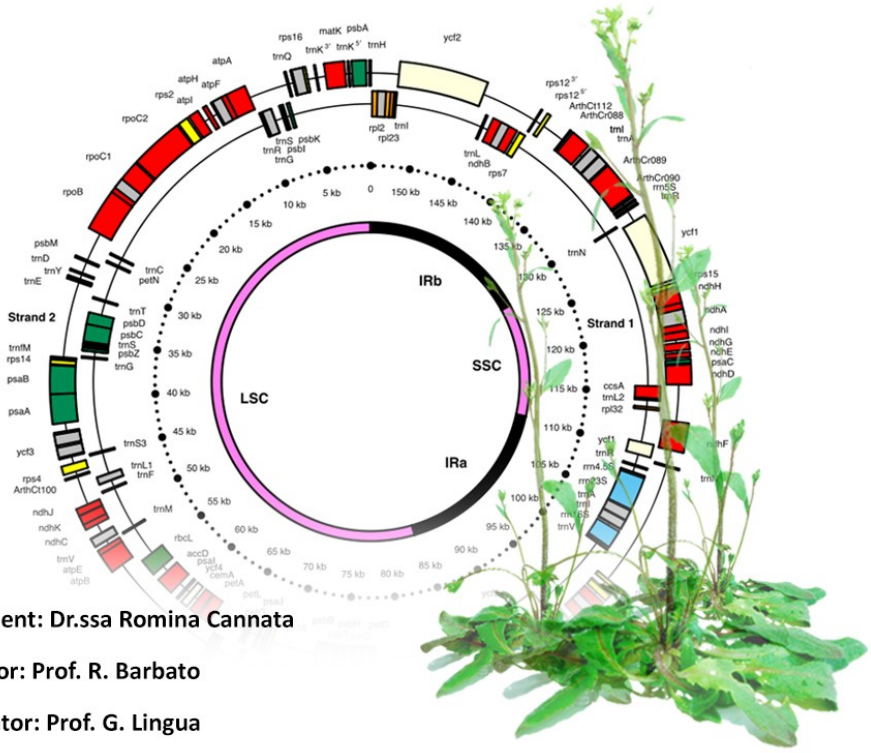
DIPARTIMENTO di SCIENZE ed INNOVAZIONE TECNOLOGICA
Corso di Dottorato di Ricerca in CHEMISTRY & BIOLOGY

Energy, Enviromental and food sciences

XXXI ^ ciclo

"The role of different photoprotection mechanisms in preventing photoinhibition of Photosystem II"

SSD: BIO/04



PhD student: Dr.ssa Romina Cannata

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DOTTORATO DI RICERCA

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LIST OF ABBREVIATIONS

APS Ammonium persulphate

ATP Adenosine triphosphate

BCIP 5-bromo-4- chloro-indolyl phosphate

Chl Chlorophyll

HEPES: N-2-hydrossiethilpiperazine N'-2-etansulphonic acid

LHCII Chl containing light-harvesting complex of PS II

NADP Nicotinamide adenine dinucleotide

NBT Nitro blue tetrazolium

OEC Oxygen-evolving complex

P_{680} Primary electron donor in PS II

PQ Plastoquinone

Pheo Pheophytin, localized in D1 protein

PS I Photosystem I

PS II Photosystem II

Q_A The first quinone electron acceptor in PS II

Q_B The second quinine electron acceptor in PS II

SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis

TemedN,N,N- tetramethylethylendiamine

TMBZ Tetramethyl benzidine

Tricine N-[2-hydroxy-1,1- bis(hydroxymethyl) ethyl]glycine

Tris Tris-[hydroxymethyl]amino-methane

Abstract

Light is fundamental for photosynthesis. However, when in excess, it may produce *reactive oxygen species* (*ROS*) which, in turn, may damage the Photosystem II reaction center D1 protein. In order to avoid this, photosynthetic organisms have developed different mechanisms able to protect their photosynthetic apparatus. The term *photoprotection* is used to define all these process. Higher plants have evolved different protective mechanisms which are: the thermal dissipation through *NPQ* depending on PSBS protein, phosphorylation of LHCII to ensure efficient distribution of light energy between photosystems (*state transition*) depending on STN7/TAP38 kinase/phosphatase system and *Cyclic Electron Flow/ Photosynthesis Control (CEF/PC)* around PSI depending on PGR5/PGRL1A/B proteins. However, how this mechanisms affect D1 turnover is not know. Much of current knowledge about photoprotection comes from studies with knockout mutants of *Arabidopsis thaliana* in which respective key genes have been inactivated. In this work, this genetic approach was extended by generating, by crossing, high order mutants where two (*ΔSL, ΔS5*) or all three (*ΔSL78*) photoprotection mechanisms have been eliminated.

These mutants have been characterized and their light sensibility investigated, by using fluorescence (PSII), absorbance changes (PSI), protein phosphorylation and turnover of D1 protein by immunoblotting. Although all the mechanisms seems to be important in plants photoprotection, the sensibility of PSII to light is more marked in mutants where CEF/PC is absent and this is paralleled by an increase in D1 turnover. PSI is also highly damaged by light, but, if lincomycin is present, it resulted protected. In *ΔS* mutant the sensibility to light, in terms of PSII efficiency, is not as marked as it could be expected but the D1 turnover is very high.

1. INTRODUCTION

1.1 Oxigenic Photosynthesis: general aspects

The overall process whereby plants, algae and prokaryotes use light energy to synthesize organic compounds is called photosynthesis. This process is important because it provides food and biomass as well as fossil fuel; moreover it is the only source of molecular dioxygen required for respiratory activity by aerobic organisms. Molecular dioxygen is released as a by-product of the photosynthetic oxidation of water and, over billions of years, oxygenic photosynthesis has altered the composition of Earth's atmosphere, from anoxygenic to oxygenic enabling the development of aerobic life forms. The present life on Earth is entirely dependent on photosynthesis.

All the reactions that convert solar energy into chemical energy can be represented by the overall reaction:

$$6\ CO_2 + 6\ H_2O \rightarrow C_6H_{12}O_6 + 6\ O_2$$

Photosynthesis takes place in *chloroplast*s, specialized plastids surrounded by a double membrane system called outer and inner envelope. Inside these membranes there is a complex internal membrane system known as thylakoid membrane.

In a typical plant chloroplast the thylakoids are organized in *grana* (granal thylakoids) a system of appressed membranes, whereas the stromal thylakoids are unstacked. Thylakoids are all connected and the space inside the thylakoid membranes is called lumen whereas the liquid medium surrounding the thylakoids is called stroma.

The photosynthetic process occurs by two coordinated and sequential phases:

- *light reactions* (in thylakoid membranes) which produce O_2, ATP and NADPH;

- *Calvin cycle* which reduces CO_2 to carbohydrates and consumes ATP and NADPH produced by the light reactions: this is located in the soluble compartment of the chloroplast called stroma.

The photosynthetic electron transfer chain, located in the thylakoid membranes, is organized as a number of different multi pigment-protein complexes called *Photosystem II* (PSII), *Photosystem I* (PSI), *Cytochrome* b_6f, and *ATP synthase*; the water soluble protein *plastocyanin* links cytochrome b_6f to PSI whereas *plastoquinone* links PSII to cytochrome b_6f.

The electron transfer chain (ETC) takes place in the thylakoidal membranes and the complexes mentioned before play a definite role in this process.

Photosystem complexes contain the antenna proteins to which *Chl* (a and b) and β-carotene are bound, making the LHC complexes with the function of capturing excitation energy and passing it to the reaction center by a mechanism based on resonance energy transfer (Raven *et* al., 2002). In the reaction center there is a *chlorophyll a,* P_{680} for the PSII and P_{700} for PSI, named according to the wavelength of maximum bleaching following their oxidation.

The PSII captures photons and use their energy to extract electrons from water molecules and these can be used in several ways. By removing electrons from water, the water molecules are broken into the dioxygen gas and hydrogen ions, which are used for the ATP synthesis. The remaining electrons are passed down through a chain of electron-carrying proteins and part of the energy is conserved in form of electrochemical gradient, obtained by pumping hydrogen ions across

the membrane. Finally, the electrons, which got an additional energy boost at the level of PSI, are placed on NADPH used in all biosynthetic reductive reactions.

On the donor side a strong oxidant is generated by the photo-oxidation of P_{680} that is able, using the Ca, Mn Cluster, to extract electrons from water.

On the acceptor side, electrons are collected on a plastoquinole pool.

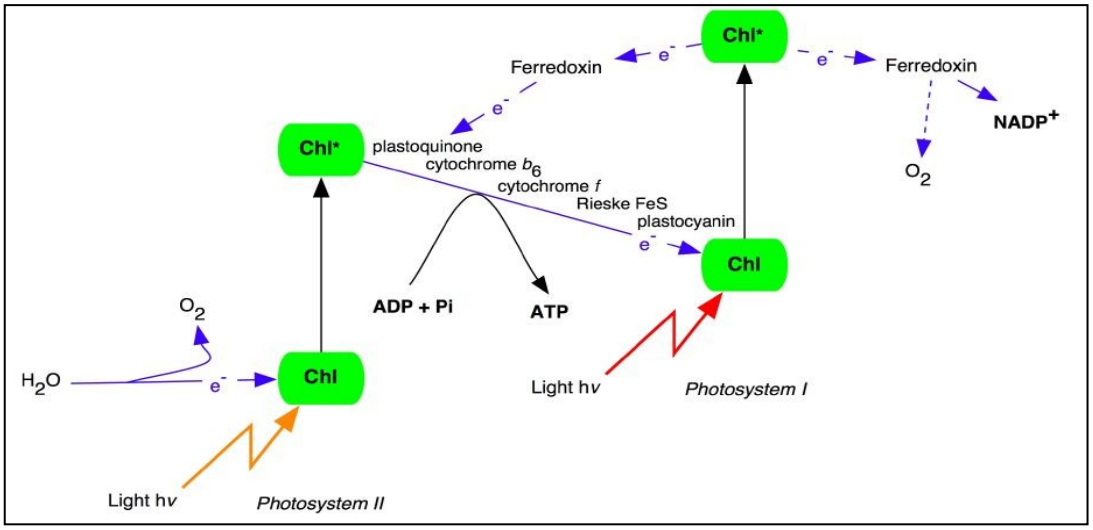
The Cytochrome b_6f Complex is able to re-oxidase the plastoquinoles and then reduces oxidized plastocyanin. The photo-oxidized P_{700} of the PSI gets electron from plastocyanin and, once reduced, reduces ferredoxin at its stromal side. Finally, to obtain NADPH, ferredoxin is oxidized by Ferredoxin-$NADP^+$Reductase (FNR). The redox potential relationships between the involved electron acceptors and donors are described by the Z scheme, a schematic representation of the linear electron flow (Fig. 1-2).

This representation is an energy diagram for electron transfer during "light reactions" and shows the pathway of electron transfer from water to $NADP^+$ by which plants transform light energy into chemical energy as reduced NADPH and ATP.

ATP synthesis is due to the proton gradient formed across the thylakoid membrane. This driving force is developed by the proton transfer into lumen by the water splitting process and by plastoquinone that is deprotonated by *Cyt* b_6f on the luminal side.

The distribution of the protein complexes involved in light reaction is not homogeneous in the thylakoid membranes: PSII is localized in the stacked grana regions whereas PSI and ATP synthase in the stroma-exposed thylakoid membranes (Anderson and Andersson, 1982; Chow *et* al., 1991; Albertsson, 2001; Danielsson *et* al., 2004; Chow *et* al., 2005). Cyt b_6f complexes are evenly distributed throughout the thylakoid membranes (Albertsson, 2001).

Fig.1: The Z-Scheme of photosynthesis in plants (Allen J. F. , 2004)



1.1.2 Photosystem II

The solar water-splitting protein complex, photosystem II (PSII), catalizes one of the more energetically demanding reaction in nature by using the light energy to drive a catalyst capable of oxidizing water.

The water oxidation reaction is catalyzed by the tetramanganese-calcium-oxo (Mn_2Ca-oxo) cluster in the oxygen evolving complex (OEC) of PSII which cycles through five light-driven charge-storage or S-state intermediates (S_0-S_4) as it accumulates charge equivalents to split water.

The PSII is a multi-protein complex of plants, algae and cyanobacteria embedded in the thylakoid membrane of these organisms.

The detailed structure of PSII has been elucidated, in the past years, by X-ray crystallography (Zouni *et* al., 2001; Ferreira *et* al., 2004; Iwata and Barber, 2004; Loll *et* al. 2005). The structure gives insight into the evolution of photosynthetic

organisms showing existence of common ancestors of both photosystems (Grotjohann *et* al., 2004; Nelson and Ben-Shem, 2005).

This photosystem is composed by 30 protein subunits (Shi and Schröder, 2004; Nelson and Yocum, 2006) that are organized in a reaction center (RC) that contains the D_1 and D_2: these proteins bind the P_{680} chlorophyll and all the other cofactors needed for the PSII electron transport. In addition to the RC there is a light harvesting complex (LHCII) composed by approximately 250 chlorophyll molecules able to absorb light energy and transfer it to the RC by a mechanism called resonance energy transfer (Föster energy transfer).

The electrons from P_{680} are transferred to the PSII electron transfer components that are bound by D_1 and D_2 protein. The primary acceptor is a pheophytin, a molecule which transfers electrons received from P_{680} to Q_A and Q_B (plastoquinone molecules); Q_B is oxidized by an integral membrane complex, the *Cyt* b_6f*,* able to transfer electrons to from plastoquinol to plastocyanin and in the meantime protons are translocated from stroma to lumen forming the proton gradient important for the ATP synthesis.

1.1.3 Photosystem I

PSI is one of the most intricate membrane complexes in Nature and is comprised of two complexes: a reaction center (P_{700}) and a light –harvesting complex LHCI, similar to PSII structure. This photosystem is one of the pigment-protein complexes of photosynthetic electron transfer chain, which is capable of light energy conversion. The primary acceptor in PSI is a special molecule of *chlorophyll a* called A_0 whereas P_{700} is the primary electron donor. Other electron carriers

involved in the pathway of electron transfer through PSI are a phylloquinone (A_1), three different Fe-S center (F_x, F_a and F_b), and the Ferredoxin (Fdx), a soluble iron-sulfur protein.

The last one is a strong reductant (-420 mV) located in the stroma of chloroplast and in its reduced form is able to reduce $NADP^+$ through the aid of an intermediate enzyme, the *ferredoxin-NADP$^+$ reductase* or *FNR* (Buchanan *et* al., 2003).

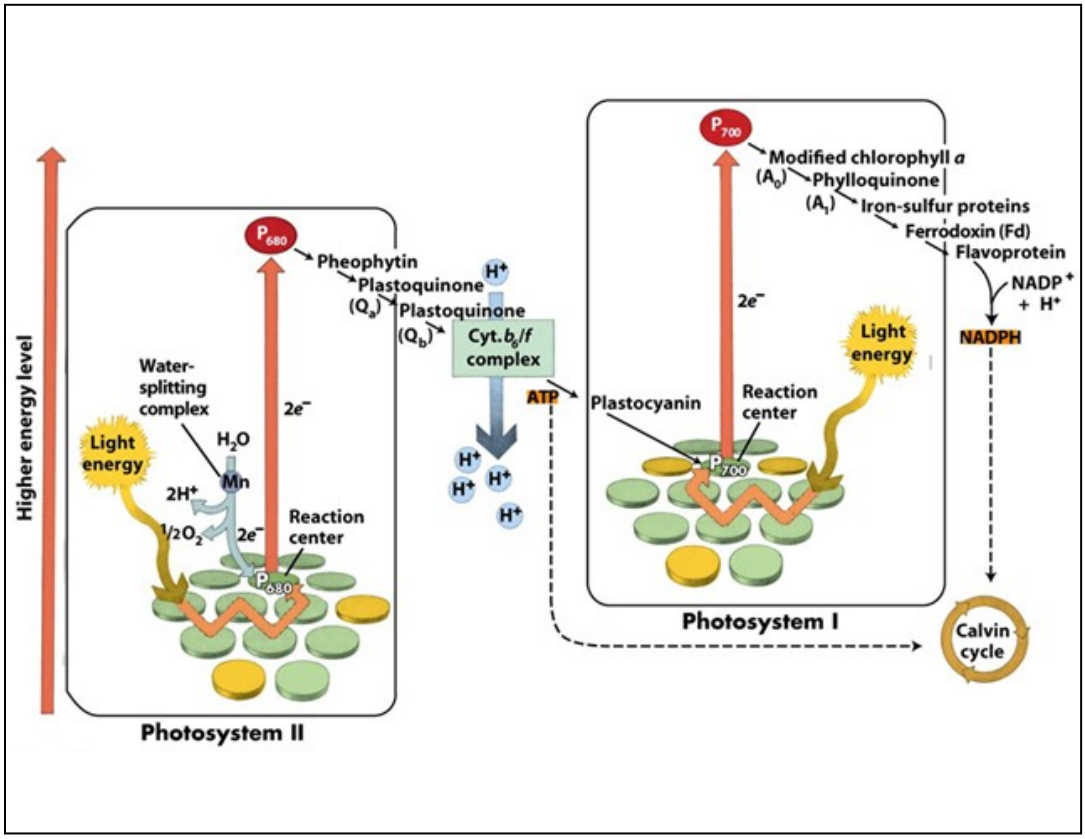


Fig.2: Schematic representation of PSII and PSI (Raven P. H et al, Zanichelli 2002)

1.1.4 Linear electron transport chain

The main pathway of the photosynthetic light reactions is represented by the *linear electron transport chain* involving the three major thylakoid membrane protein complexes, PSII, Cytb_6f and PSI.
These three cooperate in order to reduce nicotinamide adenine dinucleotide phosphate ($NADP^+$) (Hill *et* al., 1960) through electrons from water molecules.

The first electron donor of PSII is the chlorophyll P_{680} which receives the excitation energy captured by LHCII and the primary charge separation happens between P_{680} and Pheophytin, the primary electron acceptor.
Then electrons are transferred to a primary and secondary quinone acceptors, Q_A and Q_B.
Q_B is double-reduced by a subsequent electron transfer (Q_B^{2-}) and, binding two proton from the stroma in been converted to plastoquinol. In the next step electrons pass through the Cyt b_6f complex and one electron is transported to oxidized P_{700} via *plastocyanin*, a small soluble protein and, simultaneously, two protons are released into the thylakoid lumen.
At the same time the chlorophyll in the reaction center, P_{700}, is excited by light absorbed by LHCI and the $NADP^+$ is reduced by electrons transferred through several electron carrier via ferredoxin-$NADP^+$-oxidoreductase.
During these processes of linear electron transport a ΔpH is developed through the thylakoid membrane because protons are pumped from the stroma to the lumen and is used to drive the synthesis of ATP by the ATP synthase.

1.2 Light and Photosynthesis

Under natural condition plants often receive more light, in term of excitation energy than they can use to drive photochemistry.

Once a photon is absorbed by an antenna pigment molecule there are three pathways by which this excited state decays: this energy can be used to drive photochemistry, it can be dissipated as heat through the Xantophylls Cycle or emitted as fluorescence.

In healthy plants only the 2-5% represents Chl fluorescence, about 80% of this energy is used in the photochemical pathway and the rest is dissipated as heat (Niyogi *et* al., 1998; Govindjee, 2005; Gruszecki *et* al., 2006; Lazar, 2006; Szabó *et* al., 2005).

These three process occur in competition , such that any increase in the efficiency of one will result in a decrease in the yields of the other two.

Each of these process has a quantum yield and the sum of quantum yield of fluorescence (ΦF), heat (ΦD) and photochemistry (ΦP) is always one (energy conservation law):

$$(\Phi F)+(\Phi D)+(\Phi P)=1$$

It has been demonstrated that to manage the changing in the light environment the percentage wherewith these events occur can change, in particular, an increasing fraction of energy dissipated by heat has been observed when light intensity increases (Külheim *et* al., 2002; Gruszecki *et* al., 2006; Lazar, 2006).

A non-invasive method to measure the photosynthetic reactions in vivo is represented by the analysis of the yield of fluorescence at different light

environment level and this measure may also result in information about the function of the electron transport and heat dissipation.

1.3. Photoprotection

1.3.1 Light stress and photoprotection

Light energy is very important for the photosynthetic process. Absorbed solar energy may be defined as excessive when it exceeds the capacity of photosynthesis to use it for assimilation (Demmig-Adams and Adams, 1992; Niyogi *et* al., 1998).

An excess of absorbed light may produce a damage to plants because *reactive oxygen species* (*ROS*) may be generated. These molecules may produce photodamage to the photosynthetic protein complexes (Barber and Andersson, 1992) and, in order to avoid this, all plants have developed several mechanisms to manage the excess absorbed energy. ROS are important signaling molecules in plants and they can have deleterious effects on photosynthesis and other leaf processes that will reduce growth and plant fitness but they also lead plant cells to death due to interaction of these reactive molecules with the cell membranes (Murchie and Niyogi, 2011).

Photoprotection is the term used to define the mechanisms by which plants protect themselves from excess of absorbed light; these include the thermal dissipation through *NPQ* (Holt *et* al., 2004) mainly depending on PsbS protein, phosphorylation of LHCII to ensure efficient distribution of light energy between

photosystems (*state transition*) depending on STN7/TAP38 kinase/phosphatase system and the *Cyclic Electron Flow/ Photosynthesis Control (CEF/PC)* around PSI depending on PGR5/PGRL1A/B proteins.

1.3.2 Physical Photoprotection

In addition to all the molecular mechanisms trough which plants avoid photodamage, there are a lot of strategy that helps plants to manage an excess of light. These mechanisms includes paraheliotropic leaf orientation and leaf folding, by alteration of whole-leaf light absorption, enhanced reflectance through leaf hairing (Ripley *et* al., 1999), reflective epicuticular wax layers (Robinson *et* al., 1993) and a lot of morphological adaptations like small leaf size, thick leaves, etc.
Inside cells another process that allowed the physical photoprotection is represented by the chloroplasts movements to reduce light absorption.
Chloroplast movements towards the anticlinal cell walls can occur in sudden high light exposure within minutes (Chow *et* al., 1988; Park *et* al., 1996; Briggs and Christie, 2002; Wada *et* al., 2003). Mutant plants lacking the chloroplast movement response are more susceptible to photoinhibition than wild type plants (Kasahara *et* al., 2002).
Photosynthetic organisms also have screening compounds like anthocyanins, betalains and rhodoxanthin that are pigments able to decrease the light absorption (Weger *et* al., 1993; Smillie and Hetherington, 1999; Steyn *et* al., 2002). Other screening compounds , like flavonoids and hydroxycinnamic acids are situated in the plants cuticle and act against UV radiation (Kolb *et* al., 2001; Markstädter *et* al., 2001).

1.3.3 Photoprotection mechanisms and mutants

An excess of light could be harmful because plants are unable to manage it when it exceeds the capacity of photosynthesis to use it for the assimilation: the lifetime of the singlet state of the excited chlorophyll is extended and this leads to the formation of a triplet state. In this situation the energy from this triplet state reacts with oxygen generating singlet oxygen, a reactive oxygen species (ROS).
The de-excitation of singlet chlorophyll may follow different pathways:

- driving photochemistry
- return to the ground state by emission of fluorescence
- by thermal dissipation

1.3.3.1 Non Photochemical Quenching

The major component of photoprotection is the last one, known as NPQ or Non-Photochemical Quenching.
Non-Photochemical Quenching is a regulatory process that maintain the balance between the utilization and the dissipation of the light energy that plants are able to absorb in order to minimize the photo-oxidative damage reducing the generation of reactive oxygen species (Niyogi *et* al., 2000). This photoprotection mechanism could be described as an increase heat dissipation of absorbed photons when the absorbed light exceeds the capacity of plant to drive photosynthesis.

High light exposure represents a problem for plant health and NPQ is a molecular adaptation that represent the fastest response of the photosynthetic membrane to excess light (Demming-Adams *et* al., 2014, Ruban, 2016).

This parameters refers to the ability to protect the PSII reaction centers (RCII) by a rapid regulation of light harvesting complex.

There are different processes that contribute to the induction and relaxation of non photochemical quenching has three components:

- qE energy-dependent quenching
- qT state transition quenching;
- qI photoinhibitory quenching (Quick and Stitt, 1989).

The major one in *A. thaliana* is qE, or *NPQ*, and it depends on the formation of a transmembrane proton gradient.

qE, the major rapidly reversible NPQ component, reflects a key molecular protective process in the photosynthetic membrane of higher plants and algae, which enables rapid adjustment of light harvesting efficiency to incidental light intensity. (Ruban, 2016)

When the thylakoid lumen pH is low, the violaxanthin-depoxidase enzyme is activated: this lumen- localized enzyme catalyses the conversion of violaxanthin to zeaxanthin. One the other hand, the low thylakoid lumen pH led to the protonation of proteins that are involved in the qE formation like PSBS. (Holt *et* al., 2004; Cogdell, 2006).

It has been hypothesized that protonation activates a binding site for zeaxanthin (Nyogi *et* al., 2000).By isolating and characterizing mutants of *A. thaliana* lacking

qE it was shown that qE requires PSBS in addition to a low thylakoid lumen pH and the presence of depoxidazed xanthophylls like zeaxathin.

Recently it has been discovered that PSBS protein is a dimer more stable at low pH and acidification seems to cause a conformational change associated with alteration in luminal intermolecular interactions (Fan *et* al., 2015).

PSBS acts like a switch that is triggered by ΔpH and not like a quenching site (Ruban *et* al., 2012).

Recent studies demonstrate that an overexpression of PSBS lead to an increase in qE (Kromdijk *et* al., 2016).

When a plant is transferred from dark to light NPQ is induced in the time scale of seconds and the accumulation of protons in the lumen of the thylakoid membranes is the initial event in the induction of NPQ (Horton *et* al., 2005; Szabó *et* al., 2005).

The npq1 mutant and npq2 mutant are defective in the Xanthophylls Cycle (npq1 mutant lacks violaxanthin de-epoxidase and npq2 mutant lacks zeaxanthin epoxidase), whereas *npq4-1* mutant has a normal xanthophylls cycle but lacks the ΔpH induced conformational change (zeaxanthin-dependent) in thylakoids membrane.

In order for PSBS to function as the site of qE, the presumed pigments bound to PSBS would have to be coupled to one or more chlorophylls in the LHCII system.

In this work I used *npq4-1* mutant, called *ΔS* mutant. (Niyogi *et* al., 2000).

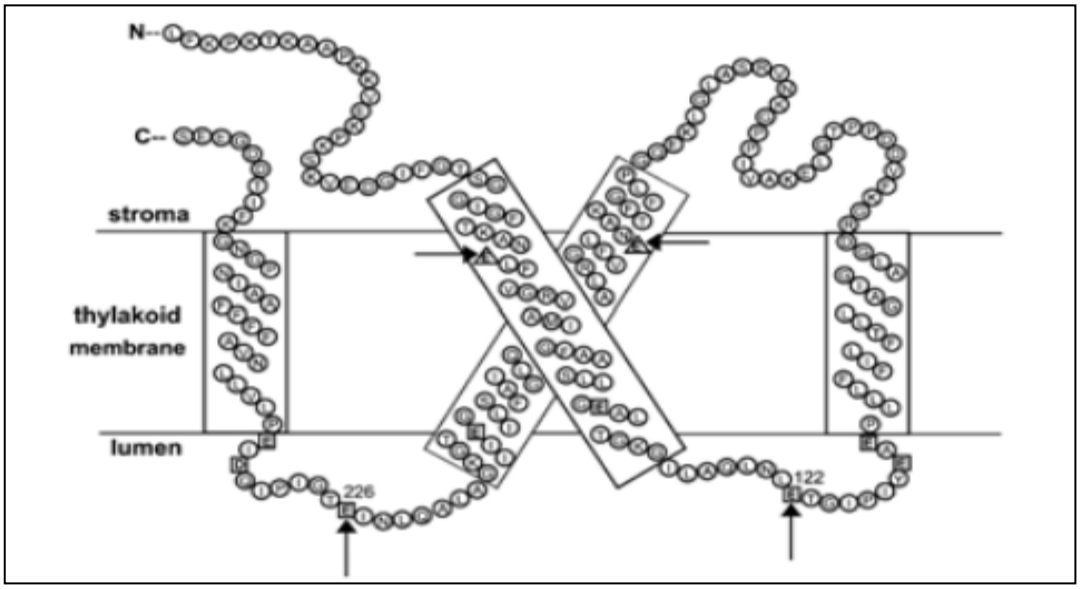


Fig.3: PSBS protein structure with indicated the two protonable lumenal glutamates (E122/E226) (Li et al., 2004).

1.3.3.2 Cyclic Electron Flow/Photosynthesis Control

As described before, during photosynthesis, two photoreaction centers, located in the chloroplast and more specifically in the thylakoid membrane, PSI and PSII, use light energy to generate ATP and NADPH. There are two modes of electron flow, of which the linear electron flow (LET) is driven by PSI and PSII, generating ATP and NADPH, whereas the Cyclic Electron Flow (CEF)/Photosynthesis Control only generates ATP and is driven by PSI alone (Shikanai *et* al., 2007; Shikanai T., 2014).
The electron distribution between the two photosystems is regulated by different environmental and metabolic conditions that lead to an adjustment of ATP/NADPH ratio: LEF is favored when an highly active Calvin cycle form an efficient sink for electrons from PSI, whereas CEF predominate when the dark reactions of photosynthesis are down regulated, during high light or low CO_2

conditions (Clarke and Johnson, 2001; Joliot and Joliot, 2002, 2006; Golding and Johnson, 2003; Golding *et* al., 2004; Dal Corso *et* al., 2008).

CEF starts with light dependent excitation of P_{700}, the primary electron donor of PSI, and electron is transferred to Fd. Once oxidized P_{700}^{+} is reduced by electrons from PQ pool via *Cyt* b_6f and PC. Electrons from Fd must eventually be donated to PQ to complete the cycle. It can take place by two different pathways: the NDH-dependent pathway plays a major role in cyanobacteria, its contribution in C3 plants is still ambiguous but it has been shown to be essential in photoprotection (Endo *et* al., 1999), and the Fd-dependent pathway (known as "CEF around PSI) bypasses NADPH and may play a role in the acidification of the C3 thylakoids lumen (Mi *et* al., 1995; Ravenel *et* al., 1994; Asada *et* al., 1993; Joe¨t *et* al., 2002; Dal Corso *et* al., 2008).

In the Cyclic Electron Flow ATP is produced via proton-motive force and ΔpH contribute to the downregulation of PSII by NPQ.

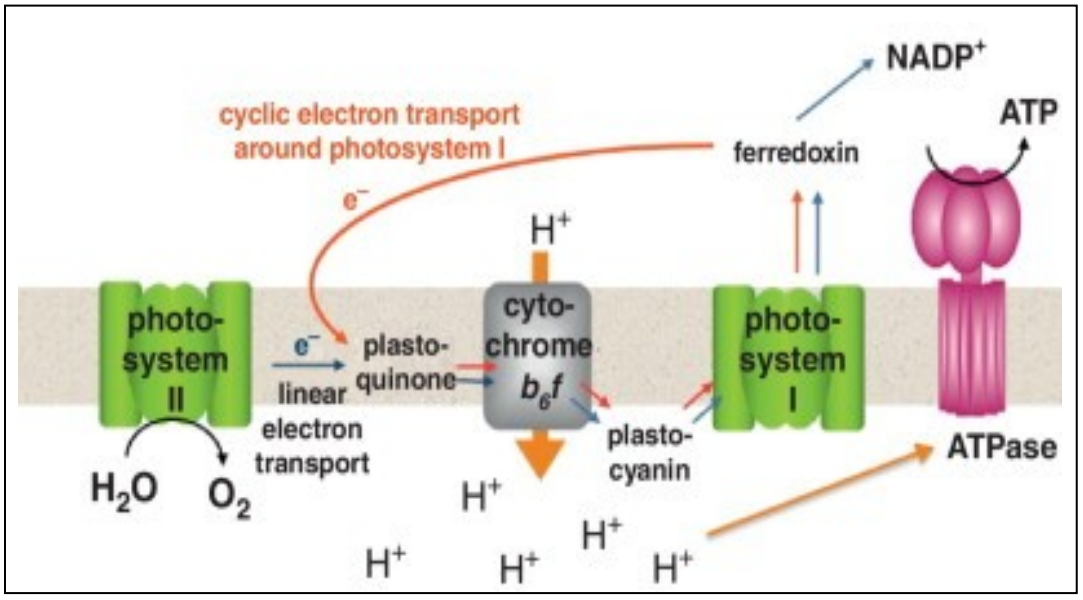


Fig.4: Schematic representation of the cyclic electron flow (Shikanai T., 2014)

Two components are involved in this alternative pathway: Proton Gradient Regulation (PGR5) and Proton Gradient Regulation Like 1 (PGRL1).

When the activity of Calvin cycle is reduced PGR5 is involved in the generation of ΔpH that induce thermal dissipation through the Xantophylls Cycle. PGR5 is important because through this pathway is able to reduce the photoinibition of PSI by limiting the over-reduction of the acceptor side of PSI.

In pgr5 mutants the absence of PC/CEF leads to an insufficient ΔpH generation that is reflected in a reduced induction of thermal dissipation under excessive light conditions.

The small amount of NPQ in pgr5 may be possibly due to photoinhibition and/or the movement of peripheral light –harvesting antennae from PSII to PSI (Shikanai *et* al., 2002).

The other component of the CEF is the integral thylakoid protein PGRL1 and mutants lack this protein show a malfunction in CEF similar to pgr5 mutants. These two protein interact physically and are associate with PSI (Colombo *et* al., 2016).

PGRL1 is encoded by two homologous genes, PGRL1A and PGRL1B.

In this mutants the induction of NPQ is reduced like pgr5 mutant and both have a similar behaviour (Dal Corso *et* al., 2008; Pesaresi *et* al.,2016).

1.3.3.3 State Transitions

In stn7 and stn8 mutants this process is undetectable because they lack these proteins and the dephosphorylation process is not present.

In *A. thaliana* this process requires a protein kinase called STN7. This is really important because is involved in LHCII reversible phosphorylation associated with the relocation of LHCII and the redistribution of excitation energy between the two photosystems.

STN7 is responsible for the phosphorylation of LHCII in *Arabidopsis* (Bellafiore *et* al., 2005), whereas, another one, STN8, is requires for the quantitative phosphorylation of PSII core proteins.

STN7 is activated by the overreduction of PQs and phosphorylates part of LHCII.

In the dark, plant thylakoids are in State I, where LHCII is bound to PSII. When the plastoquinone pool becomes reduced in the light, a conformation change in Cyt b_6f activates LHC kinases, and the LHCII of PSII becomes phosphorylated.

Phosphorylated LHCII units have decreased affinity to PSII and they move to PSI resulting in State II (Lunde et al., 2000; Aro and Ohad 2003; Kanervo *et* al., 2005; Dekker and Boekema 2005).

Under oxidizing conditions the LHCII kinase become inactivated and a phosphatase, TAP38 dephosphorylates LHCII which return to PSII. (Pribil *et* al., 2010).

State transition is also considered a component of non photochemical quenching (Takahashi, 2009), qT.

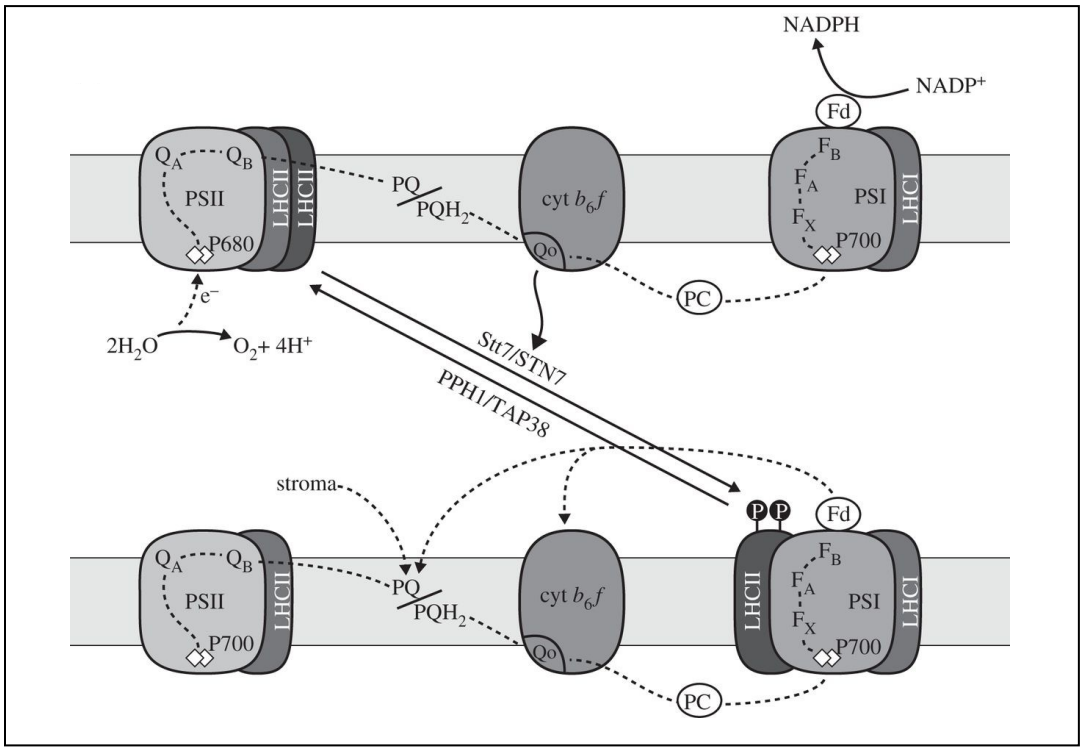


Fig.5: Schematic representation of state transitions (Rochaix, J.D. et al., 2012)

1.3.4 Photohinibition and D1 protein turnover

Light is essential for photosynthesis, but it can also damage PSII in the light induced process called photohinibition. When there is an imbalance between the PSII photodamage and its repair cycle, the term photohinibition is used.

High light condition results in a damage to RC of PSII and this process occurs in any light intensity and the rate constant of photoinhibition is directly proportional to the light intensity (Jones and Kok , 1966; Tyystjärvi and Aro, 1996; Santabarbara *et* al., 2002; Nishiyama *et* al., 2005).

This term is used to indicates a decrease of the quantum yield of PSII when the rate of damage is faster than the rate of repair (Nishiyama *et* al., 2006).

The D1 protein of PSII, under natural conditions is the primary target of photodamage but the exact mechanism by which this event occur is still unclear.

There different ideas about this process: D1 turnover seems to involve the disintegration of Mn clusters in PSII leading to an energy imbalance and consequently to cleavage of the D1 protein by proteases (Kato *et* al., 2015).

Other studies argue the hypothesis that this damage is due to an increase proton motive force which leads to an elevated electric field, rather than lumen acidification, which in vivo increased PSII charge recombination rates, producing singlet oxygen and subsequent photodamage (Davis G. A. *et* al., 2016).

More recent studies have discovered that, in addition to the rapid D1 protein turnover as consequence of photodamage, also subunits of *Cyt* b_6f and NAD(P)H dehydrogenase (NDH) complex are characterized by a fast turnover .

Mutants carried out defects in these two complexes have shown enhanced sensibility linked to an increase photodamage partially correlated to the Cyclic Electron Flow (Li *et* al., 2018).

Studies demonstrate that during an entire sunny day all the population of the D1 protein is replaced (Ohad *et* al., 1984; Aro *et* al., 1993; Melis, 1999; Andersson and Aro, 2001; Chow and Aro, 2005). The repair cycle process occurs in different steps: the phosphorylated PSII dimeric complexes monomerized and monomers migrates to the stroma-exposed thylakoids where damaged D1 proteins are dephosphorylated and then degraded by proteases (Van Wijk *et* al., 1996; Baena Gonzales *et* al., 1999; Aro *et* al., 2005; Aro *et* al., 2015). Phosphorylation of the PSII core proteins facilitates the migration of the damaged PSII from the grana to the stroma-exposed membranes, where proteolisis of the D1 damaged protein is

due to the activity of FtsH and Deg proteases (Huesgen *et* al., 2006; Aro *et* al., 2015).

The re-synthesis of this protein is regulated at the levels of translation initiation and elongation and after the co- translational insertion in the tylakoid membrane of PSII center (Zhang and Aro, 2002), the protein is then processed by the removal of the C-terminal extension of the pre-D1 protein (Taylor *et* al., 1988; Diner *et* al., 1988; Fujita *et* al., 1995). Finally, the new reassembled PSII migrates back to the grana tylakoids membranes where it forms a functional dimer of PSII. The D1 protein is the only component of the PSII that is not recycled.

It has been demonstrated that the D1 protein damage is proportional to the light intensity (Park *et* al., 1995; Tyystjarvi *et* al., 1996) and the inhibition of PSII complexes seems to play a role on the protection of PSI by decreasing the redox pressure.

Photoinhibition does not lead to loss of PSII activity unless the rate of damage is higher than the rate of repair (Tikkanen *et* al., 2014). To measure photoinhibition one approach is to inhibit the D1 protein turnover using antibiotics like lincomycin (Mulo *et* al., 2003). This is a translation inhibitor, such as streptomycin or chloramphenicol, able to inhibit the synthesis of plastidial-encoded protein like D1. In the presence of lincomycin, the repair cycle does not take place and after illumination the residual amount of protein can be measured by western blot in order to quantify the extent of damage.

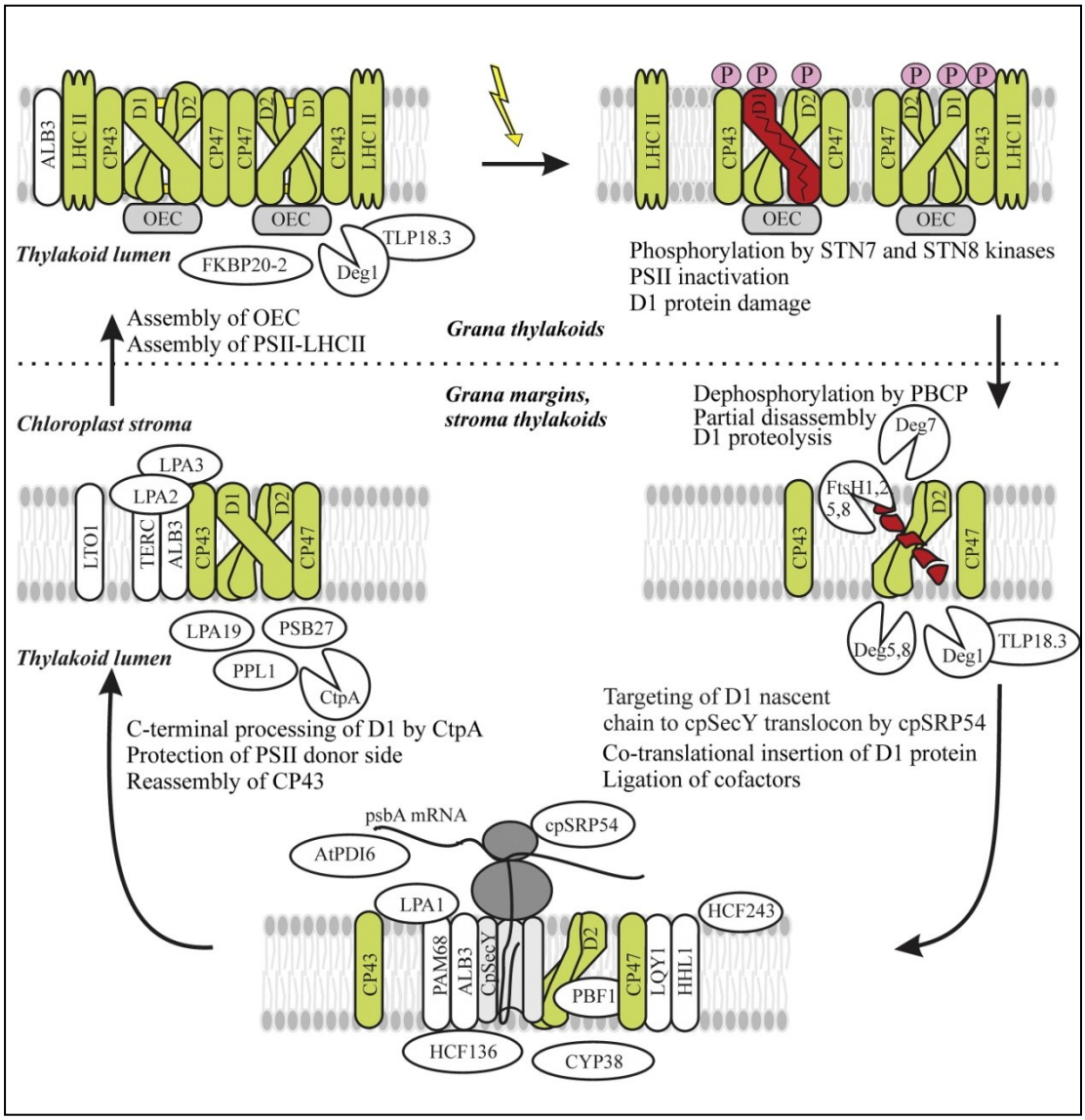


Fig.6: PSII photoinhibition-repair cycle (Aro, E.M. et al., 2015)

1.3.5 Chl Fluorescence and fluorescence measurements

During the photosynthetic process the excitation energy is transferred to the reaction centers of both photosystems where the photosynthetic energy conversion starts. This energy can follow different pathways: the photochemical pathway is in competition with other two represented by the thermal dissipation and the Chl fluorescence.

The latter refers to a fluorescence emission originated from deexcitation of excited chlorophyll molecules.

In a healthy plant the photochemical pathway is predominant and up to 80% of absorbed energy is used in this route , 2-5% is re- emitted as Chl fluorescence and the remaining faction dissipated as heat.

Fluorescence is a very informative and may be measured in a number of different ways, such as prompt fluorescence (i.e. by PEA), fluorescence decay (i.e. by single turnover flash), PAM fluorimetry and so on, each of which giving information on different aspects of energy transfer, dissipation and photochemistry.

Prompt fluorescence: prompt Fluorescence is measured normally after a dark adaptation. In this condition all the reaction centers are in the reduced state ("open"). Actinic light is then switched on and fluorescence rising directly measured (usually up to 1 sec) during light irradiation.
PAM fluorimetry: in a typical PAM experiment a saturating flash is applied (3000 µmol m^{-2} sec^{-1}) and the reaction centers are oxidized. A strong fluorescence increase is observed reaching a maximum value called Fm, the reaction center are now in the "closed" form.

A non saturating actinic light is then applied to activate photosynthesis afterwards some saturating flashes are fired on the top of this light to achieve full reduction of the primary electron acceptor Q^A. This technique allows to calculate quenching parameters such as YII and NPQ.

The PAM instrument is not only suited only for evaluation of PSII state via Chl fluorescence, but also to analyze other aspects of photosynthesis, such as energy conversion in PSI (via dual wavelength P700 measurements (Klughammer and Schreiber, 2008).

After a dark adaptation the level of ground fluorescence , indicated as F_0 is measured: a measuring light is applied and this parameter reached. Then a saturating flash is applied which allows to fluorescence to reach its maximum level represented by the Fm parameter: in this case too, it is possible to obtain Fv/Fm, the maximum quantum efficiency of PSII as described before.

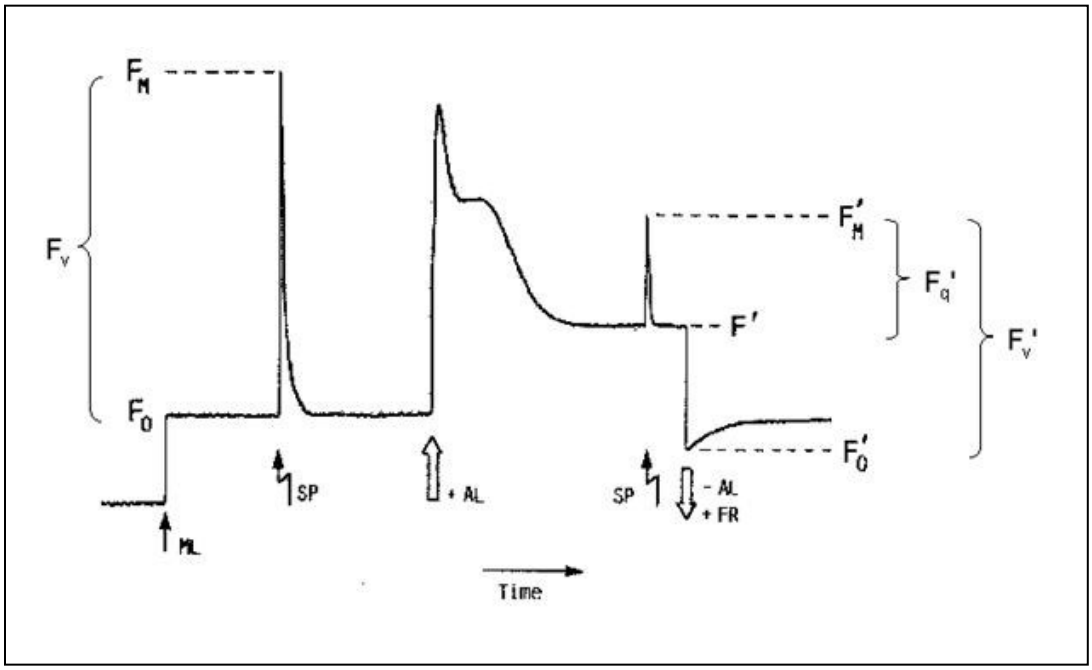


Fig.7: Schematic representation of chlorophyll fluorescence measurement by the saturation pulse method (Van Kooten& Snell, 1990).

Fluorescence decay: fluorescence decay in microsecond to second time scale, allows the evaluation of electron transfer at the acceptor side of PSII and charge recombination with the donor side. In a typical experiment, a short saturating flash (µseconds) is administrated and fluorescence decay measured. The experimental curve may be simulated by a sum of three different components, two exponential and one hyperbolic respectively describing by Vass *et* al., 1999.

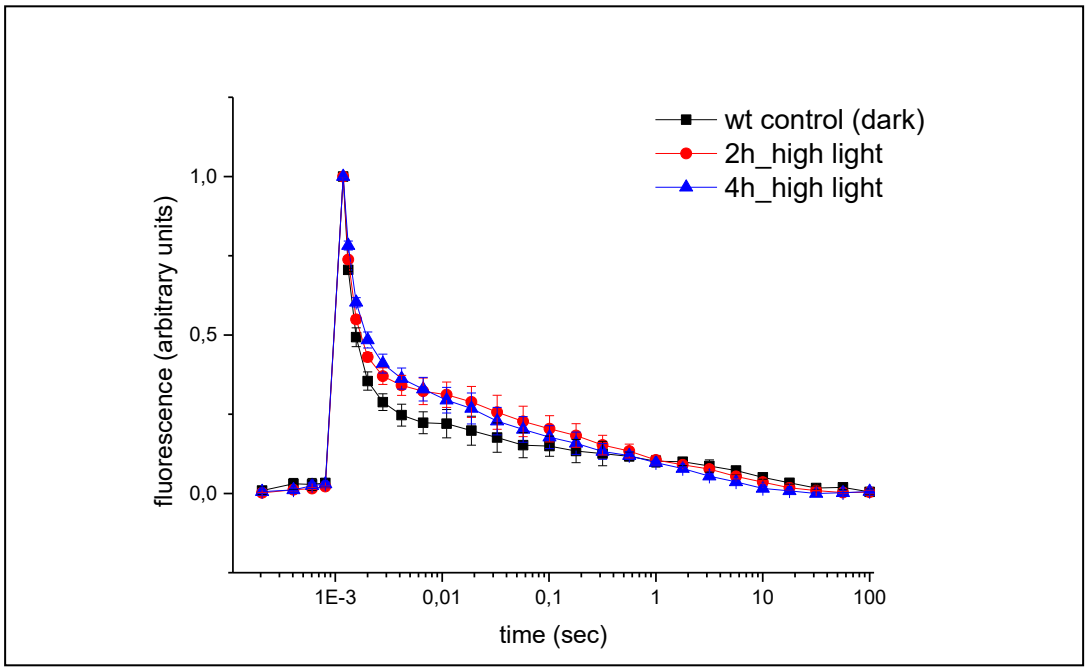


Fig. 8 : Example of fluorescence decay experiment in wt

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2. OUTLINE OF THE THESIS

Although light is essential for photosynthesis, when in excess, it may damage the photosynthetic apparatus, leading to a phenomenon known as photoinhibition. Photoinhibition was thought as a light-induced damage to photosystem II; however it is now clear that even photosystem I may become very vulnerable to light. One main characteristic of light induced damage to photosystem II (PSII) is the increased turnover of the reaction center protein, D1: when rate of degradation exceeds the rate of synthesis, loss of PSII activity is observed. With respect to photosystem I (PSI), an excess of electrons, instead of an excess of light, may be very dangerous. Plants possess a number of mechanisms able to prevent, or limit, such damages by safe thermal dissipation of light energy (energy-dependent quenching, qE), slowing-down of electron transfer through the intersystem transport chain (Photosynthesis Control, PSC) in co-operation with the Proton Gradient Regulation (PGR) proteins, PGR5 and PGRL1 and, at least to some extent, redistribution of light between photosystems (state transition, qT), collectively called as short-term photoprotection mechanisms. In the last years, genes coding for proteins behind these biophysical photoprotection mechanisms have been identified, offering the possibility to study these topics in well-defined genetic materials. In order to extend this genetic approach to the study of photoprotection, a number of higher order mutants were generated during this study by crossing genotypes carrying defects in each of the short-term photoprotection mechanisms. In this way, in a well defined genetical contest we found that mutants carrying a defect in the ΔpH-dependent photosynthesis-control are characterized by photoinhibition of both photosystems, irrespectively of whether qE or state transitions defects were present or not in the same individual, demonstrating the primary role of PSC in photoprotection. Moreover,

mutants with a limited capability to develop a strong qE, were characterized by a high turnover of the D1 protein.

The aim of my study consists in the attempt to clarify the role of different photoprotection mechanisms involved in stability of PSII, of PSI and how these mechanisms are correlated with the turnover of D1 protein, the primary target of PSII photodamage.

3. MATERIALS AND METHODS

3.1 Mutants

Arabidopsis thaliana mutants were grown in a growth chamber under a photon flux density of 90-110 µmol m^{-2} sec^{-1}in 8 h light/ 16 h dark regime at 19-22°C.

The control plant was *Arabidopsis thaliana* wild type ecotype Columbia-0 (Col 0).

A. thaliana mutant lines in the Columbia-0 (Col-0) background were located in the T-DNA Express Database (http://signal.salk.edu/cgi-bin/tdnaexpress) and obtained from the European Arabidopsis Stock Center.

The *pgrl1A/B* (ΔL) double mutant is a CEF/PC mutant that was generated by Dal Corso *et* al (2008). Is an insertion mutant obtained from publicly available T-DNA insertion collection and each lacked the respective transcript. The double mutant was generated by crossing single *pgrl1A* and *pgrl1B* knockouts and screening the resulting F2 generation for homozygous double mutants.

By crossing single *pgrl1A* and *pgrl1B* knockouts and screening the resulting F2 generation for homozygous double mutants.

The *psbS* (ΔS) mutant, which is unable to form NPQ, was created by Niyogi *et* al. (2005) by inducing a short deletion generated by fast-neutron bombardment of seeds which result in an unstable PSBS protein not detected in the thylakoid membrane.

The *pgr5* (Δ5) mutant, who lacks the Cyclic Electron Flow/Photosynthesis Control, was described by Shikanai *et* al (1999) and the mutation consists in a nucleotide transition (from G to A) leading an aminoacidic substitution from glycine to serine (at position 130).

The *stn7/8* (Δ78) double mutant lacks two protein kinases : STN7 and STN8 that are involved in *state transition*s.

During this work, these mutants were used to generate high order mutants and in particular ΔSL, ΔS5 and ΔSL78. The ΔS5 and ΔSL double and triple mutants were generated by crossing respective mutants defective in one protection mechanism to obtain F1 generation from which genotypes containing both mutations were identified by PCR.

The quintuple mutant ΔSL78 lacks all the photoprotection mechanisms described before: it is unable to develop NPQ, lacks cyclic electron flow around PSI/Photosynthesis Control and also lacks state transition. High order mutants, like ΔSL78, were generated by crossing the ΔSL mutant with the Δ78. Recombinants for all five genes were identified by western blot.

3.2 Treatment with lincomycin and light

The D1 protein turnover was determined by comparing its degradation rate in the presence or absence of lincomycin (1mg/ml). *Arabidopsis thaliana* mutants leaves were incubated overnight with the antibiotic and then were irradiated with a light intensity of 500 μmol m^{-2} sec^{-1} for different periods of time (0, 1, 2 and 4 hours). Samples were collected at the appropriate irradiation time and thylakoids were immediately isolated as described.

3.3 Isolation of thylakoids

In order to isolate thylakoid membranes, samples of *A. thaliana* mutants leaves were frozen in liquid nitrogen and were homogenized in mortar to obtain a fine powder using a buffer composed of:

- 50 mM HEPES-NaOH pH 7.2
- 5 mM $MgCl_2$
- 15 mM NaCl
- 0.4 M sucrose

The homogenate was filtered through eight layers of cotton cloth, then membranes were pelletted by centrifugation for 10 min at 3000 g at 4 °C. Pellets were resuspended in a second buffer composed of:

- 50 mM HEPES-NaOH pH 7.2,
- 5 mM $MgCl_2$
- 15 mM NaCl

After this operation the homogenate was spun down at 4500 g for 10 min.
Thylakoids were finally re suspended in:

- 50 mM HEPES-NaOH pH 7.2
- 5 mM $MgCl_2$
- 15 mM NaCl
- 0.4 M sucrose

The chlorophyll (Chl) concentration was measured with the protocol proposed by Arnon, 1949.

3.4 Chlorophyll measurement

The concentration of chlorophyll was estimated by the method of Arnon (1949), by measuring spectrophotometrically the absorbance at 720, 663 and 645 nm of chlorophylls extracted with 80% acetone.

The following formulas were used:

$$\text{Total chlorophyll [chl (µg / ml)]} = 20.2\,(A645-A720) + 8.02\,(A663-A720)$$

$$\text{Chlorophyll a [chl (µg / ml)]} = 12.7\,(A663-A720) - 2.69\,(A645-A720)$$

$$\text{Chlorophyll b [chl (µg / ml)]} = 22.9\,(A645-A720) - 4.68\,(A663-A720)$$

Thylakoids samples were immediately used or stored at –80 °C after being flash frozen in liquid nitrogen.

3.5 Polyacrylamide gel electrophoresis and immunoblotting

Polyacrylamide gel electrophoresis and immunoblotting SDS–PAGE was as described by Barbato *et* al (1992).

After electrophoresis, gels were either stained with Coomassie Blue R-250 or blotted to nitrocellulose membranes (Dunn, 1986). For immunodetection, membranes were saturated with 5% (w/v) skimmed milk and probed with antibodies to thylakoid proteins anti-D1, anti-PSBS (Barbato *et* al., 1992), anti-PGR5 (Munekage *et* al., 1999), anti-PGRL1A (Dal Corso *et* al., 2008), anti-STN7 and anti-STN8 (Barbato *et* al., unpublished). Antibody to Pthr were from Zymed and used as reported by (Rimtamäki *et* al., 1997).

3.6 Fluorescence decay

Decay of flash-induced chlorophyll fluorescence was measured by the double modulation fluorometer FL-3500 (PSI, Brno, Czech Republic) and data were analyzed as described by Vass *et* al (1999). Multicomponent deconvolution of the measured curves was done by using a fitting function with two exponential components and one hyperbolic component, according to Vass *et* al (1999):

$$F_{(t)} - F_0 = A_1\exp(-t/T_1) + A_2\exp(-t/T_2) + A_3/(1+t/T_3) + A_0$$

where $F_{(t)}$ is the variable fluorescence yield, F_0 is the basic fluorescence before the flash, A_0 to A_3 are the amplitudes and T_1 to T_3 are the time constants. Very slowly decaying fluorescence is described by a constant A_0 amplitude.

3.7 State transitions measurement

State transition were measured with a pulse amplitude modulated fluorometer (Dual-PAM-100 Walz). Leaves were exposed to a flash of saturating white light (800 ms) in order to measure F_m value. After that were illuminated with 100 µmol m^{-2} sec^{-1} (blue light PSII) for 15min. Far red light is turned on and the maximum fluorescence yield in State 1 was determined. This far red light was then switched off and the fluorescence recorded for 15 min, after which the maximum fluorescence yield in State 2 was determined.

To calculate the relative change in fluorescence the following formula was used :

$$F_r=[(F_{i'}-F_i)-(F_{ii'}-F_{ii})]/ (F_{i'}-F_i)$$ (Jensen *et* al., 2000)

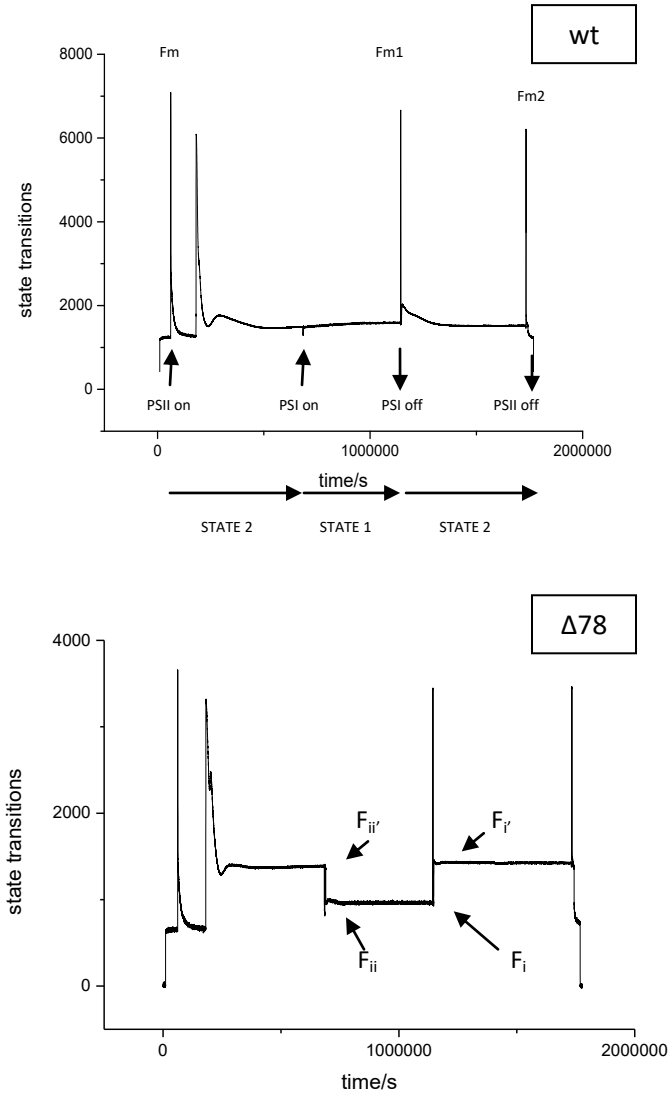

Fig. 9 : Example of state transition experiment in wt and Δ78

3.8 Pulse Amplitude Method measurements

One widely used technique to study Chl fluorescence is the pulse amplitude modulated (PAM) fluorometer. In this study the Dual PAM 100 (Walz) was used.

In PAM measurements the measuring beam is modulated and only the modulated fluorescence signal is detected while the illumination applied during the fluorescence measurement to influence the PSII function is filtered from the signal, in this method is possible to measure fluorescence emission in presence of background light.

The complementary quantum yield of PSII have been incorporated in the user software of Dual-PAM-100 fluorometer and are indicate as Y(II), Y(NPQ) and Y(NO) for the PSII and Y(I), Y(NA) and Y(ND) for PSI (Klughammer *et* al., 2008).

Y(II) represent the fraction of energy photochemically converted in PSII, Y(NO) is the fraction of non regulated energy dissipation and Y(NPQ) for the energy dissipation as heath by regulated NPQ mechanisms.

For PSI, P_{700} absorbance changes provide similar information as chlorophyll fluorescence changes provide on PSII and is possible doing absorbance measurement in two wavelength: 830 nm and 875 nm. The parameter used for the PSI are :

Y(I): quantum yield of photochemical energy conversion;

Y(ND): quantum yield of non-photochemical energy dissipation due to donor side limitation;

Y(NA):quantum yield of non-photochemical energy dissipation due to acceptor side limitation.

3.9 Plant Efficiency Analyzer

Chlorophyll *a* fluorescence analyses were carried out with a Hansatech Photosynthetic Efficiency Analyzer (Handy PEA, Hansatech Ltd., Norfolk, England). Maximal oxidation of the PSII primary acceptor Q_A was achieved by 30 min dark adaptation in a leaf clip. Fluorescence signals were recorded up to 1 s, with a data acquisition rate of 10 µs for the first 2 ms and every 1 ms thereafter, with a 12 bit resolution, as described by Strasser *et* al (1995).

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4.RESULTS

4.1 Mutants characterization

As reported in Fig.10 a-h, bringing CEF/PC, NPQ and ST mutations in the same genotypes did not affect growth in a significant manner, at least under our experimental conditions. ΔSL and ΔS5are rather similar in dimension, and their biomass accumulation might be slightly higher respect to Δ5 and ΔL mutants, likely due to the absence of a significant NPQ. At the same way ΔS displayed bigger dimension than wt. Δ78 and ΔSL78 mutants have similar dimension as reported in Fig.10 g-h.

Fig.10: Arabidopsis thaliana genotype: a) wt, b) ΔS, c) ΔL, d) Δ5, e) ΔSL, f) ΔS5, g) Δ78 and h) ΔSL78.

Biochemical characterization of photoprotection mutants is reported in Fig. 11a, and Fig. 11b where results of immunoblots with antibodies to PSBS, PGRL1A, PGR5, STN7 and STN8 proteins are shown.

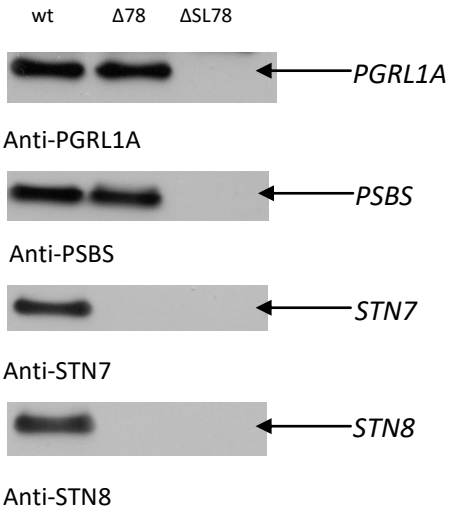


Fig.11a: immunoblot with antibodies to PGRL1A, PSBS, STN7 and STN8 of thylakoids isolated from different genotypes.

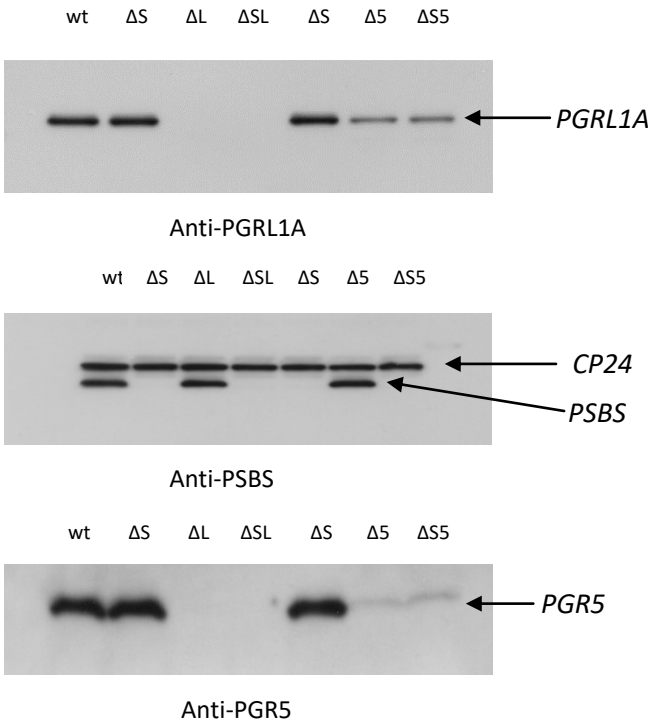


Fig.11b: immunoblot with antibodies to PGRL1A, PSBS and PGR5 of thylakoids isolated from different genotypes.

Biochemical characterization of photoprotection mutants is reported in Fig. 11a, and Fig. 11b where results of immunoblots with antibodies to PSBS, PGRL1A, PGR5, STN7 and STN8 proteins are shown. The anti-PSBS antibody showed the absence of the PSBS protein in ΔS, ΔS5 ,ΔSL, ΔSL78 genotypes, the antibody to PGRL1A showed the absence of both Pgrl1A and B proteins in ΔL, ΔSL and ΔSL78 mutants, and the PGR5 antibody showed the absence of the corresponding protein in Δ5 and ΔS5 mutants. Worth of note that about the 25% of the PGRL1A protein is still present both in the Δ5 and ΔS5 mutants (Dal Corso *et* al., 2008). On the contrary, PGR5 protein was detected neither in ΔL nor in ΔSL mutants.

As expected, STN7 and STN8 proteins are absent from Δ78 and ΔSL78 mutants.

The photosynthetic performance of the mutants was carried out by Dual-PAM100, checking both PSI and PSII properties. PSII photochemistry was not affected in any genotypes, as Y(II) light curves from all mutants were essentially identical (Fig.12a). NPQ is regularly formed in the wild type and in Δ78 mutant but is almost absent in the ΔS mutant. In Δ5 and ΔL genotypes, significant NPQ is formed only when actinic light is at least 400 µmol $m^{-2}sec^{-1}$ in intensity and reached about 40% of that observed in the wild type.

At variance, in ΔS5, ΔSL and ΔSL78, NPQ is almost undetectable as in the single ΔS mutant (Fig.12b).

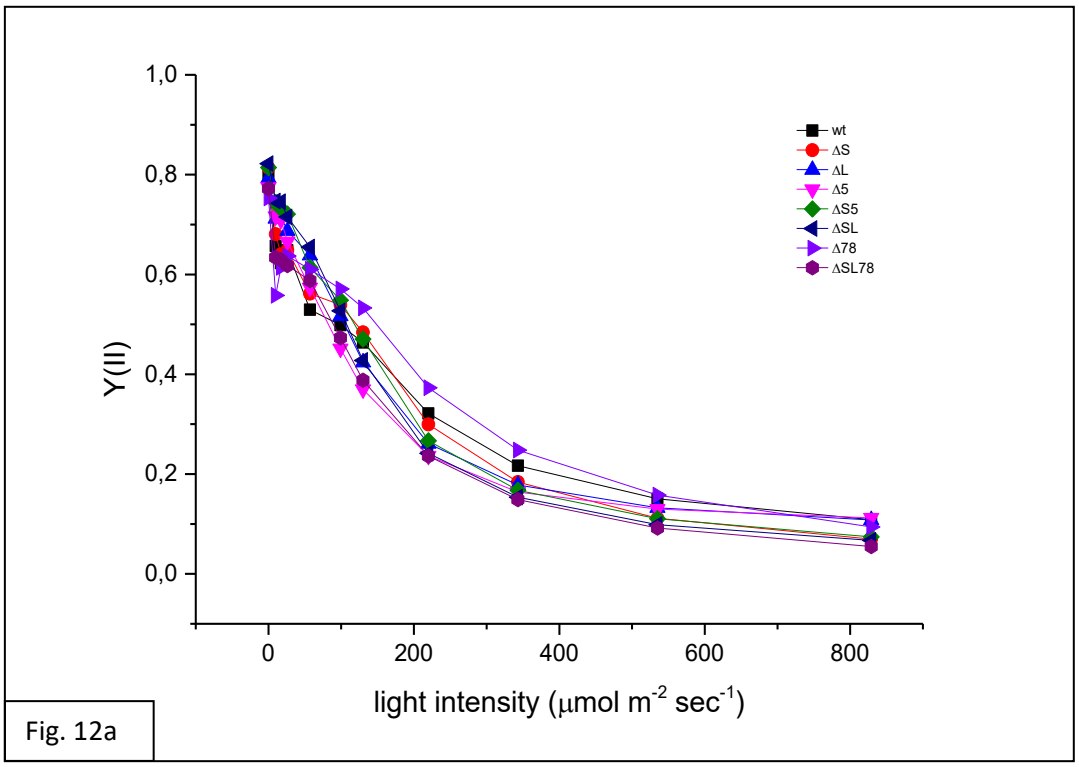


Fig. 12a

Fig.12a: Y(II) light curve from different mutants (determined from grow light plants)

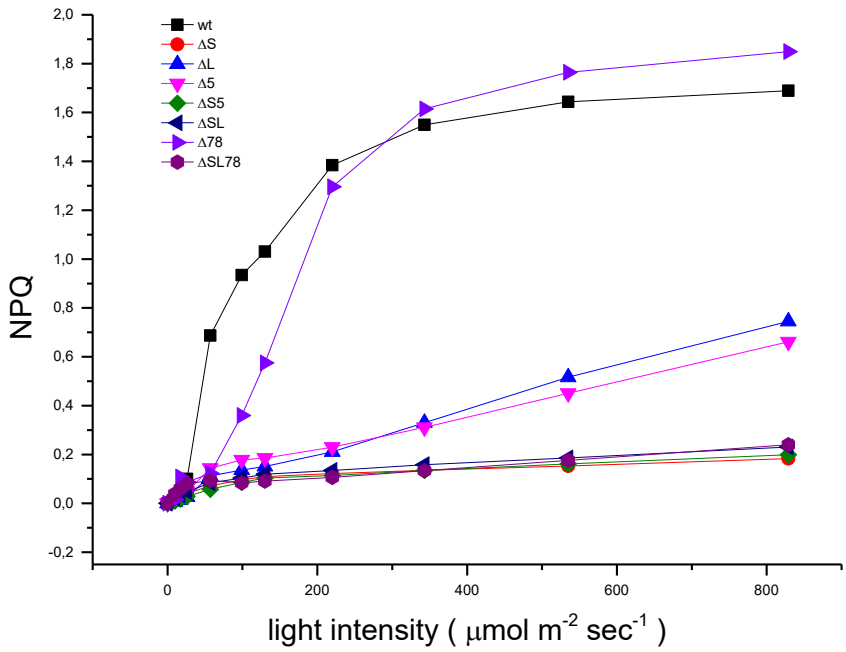


Fig. 12b

Fig.12b: NPQ light curve from different mutants (determined from grow light plants)

The Y(NO), or even the F/F_m parameter, is taken as an indication of the amount of Q_A in a reduced state, Q_A^-. This is known to be quite high in the ΔS mutant (Fig. 12c), as well as in the ΔSL, ΔS5 and in the quintuple mutant ΔSL78. In the Δ5 and ΔL mutants, a decrease of this parameter is observed at light intensities above 400 $\mu molm^{-2}sec^{-1}$, when the PSBS-dependent NPQ quenching started to occur. In ΔS5 and ΔSL mutants, this effect is not observed. The Δ78 mutant has a behavior similar to the wild type.

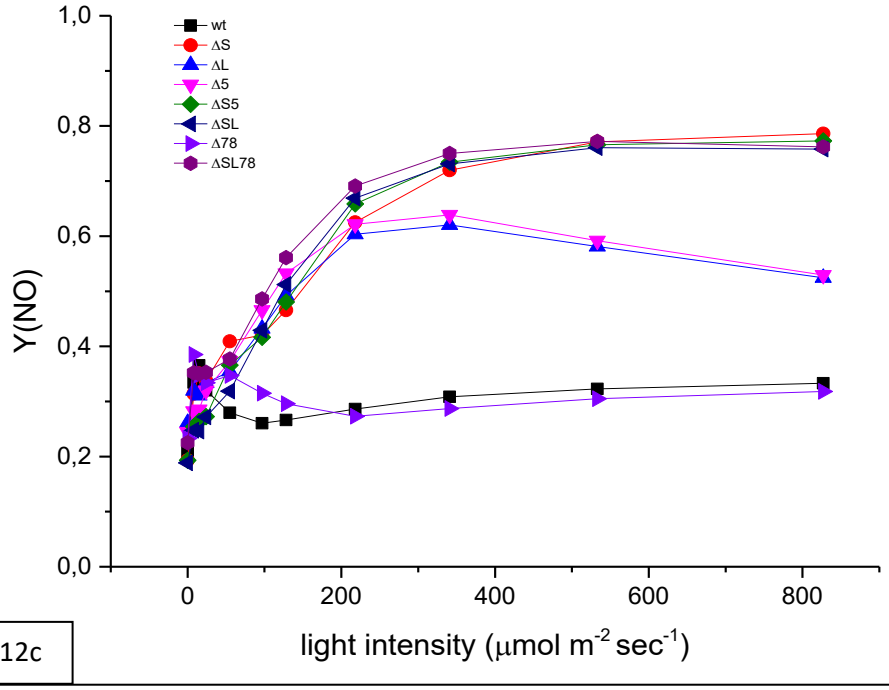


Fig. 12c

Fig. 12c: Y(NO) light curve from different mutants (determined from grow light plants)

In Fig. 13 a-b-c, PSI characterization is reported. Here, ΔS and Δ78 mutants are almost identical to wild type, with maximum quantum yield for PSI photochemistry in low light; accumulation of P_{700}^{+} in high light is also observed, indicating that in these genotypes a normal Photosynthesis Control is operative. In contrast, in all other genotypes, PSI photochemistry is efficient only in very low light whereas, at higher light intensity, the incapability to photo-accumulate P_{700} in the oxidized form (Y(ND) near zero became evident. As a consequence, PSI is blocked by over-reduction of the acceptor side, detected as a strong increase in the Y(NA) parameter.

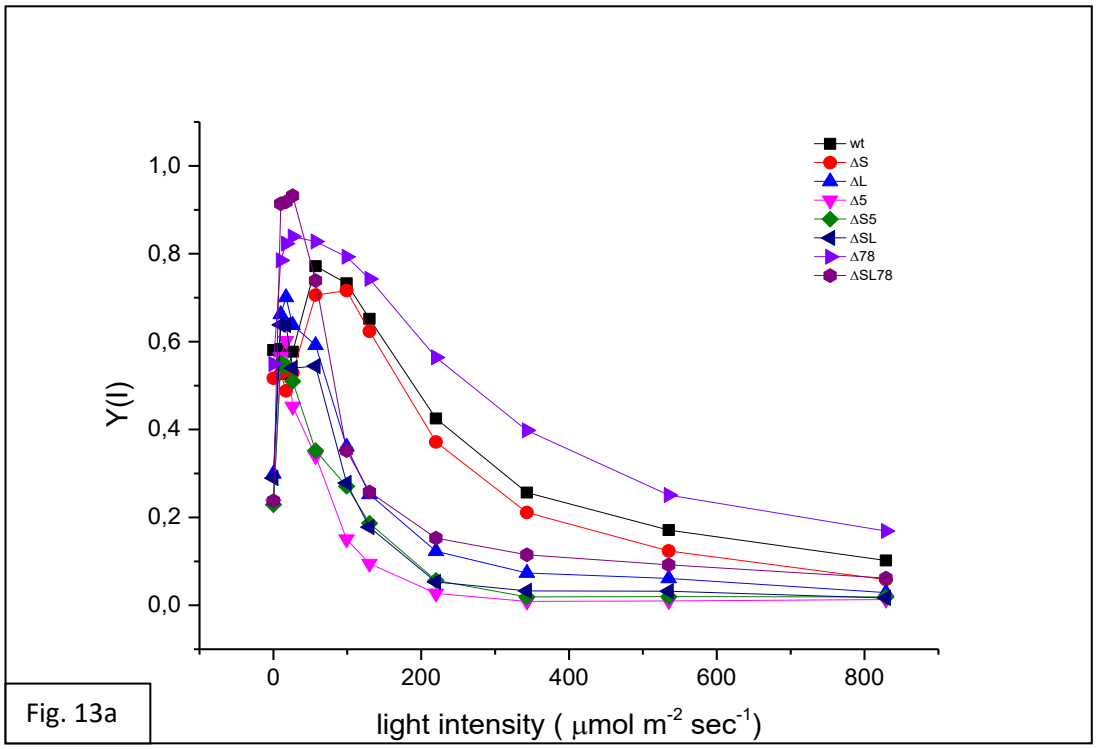


Fig. 13a

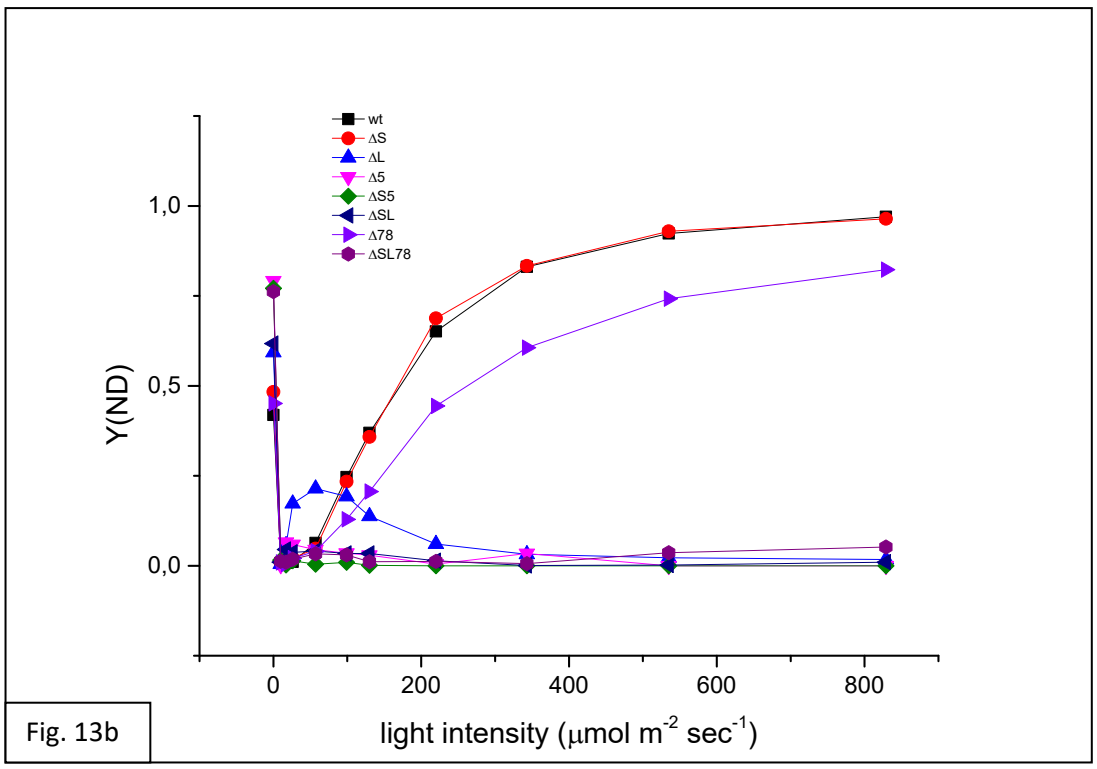


Fig. 13b

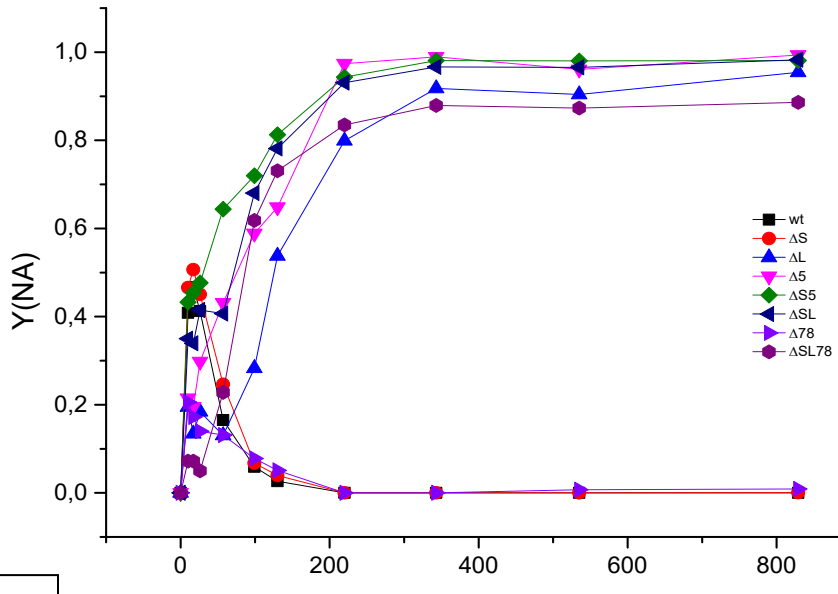
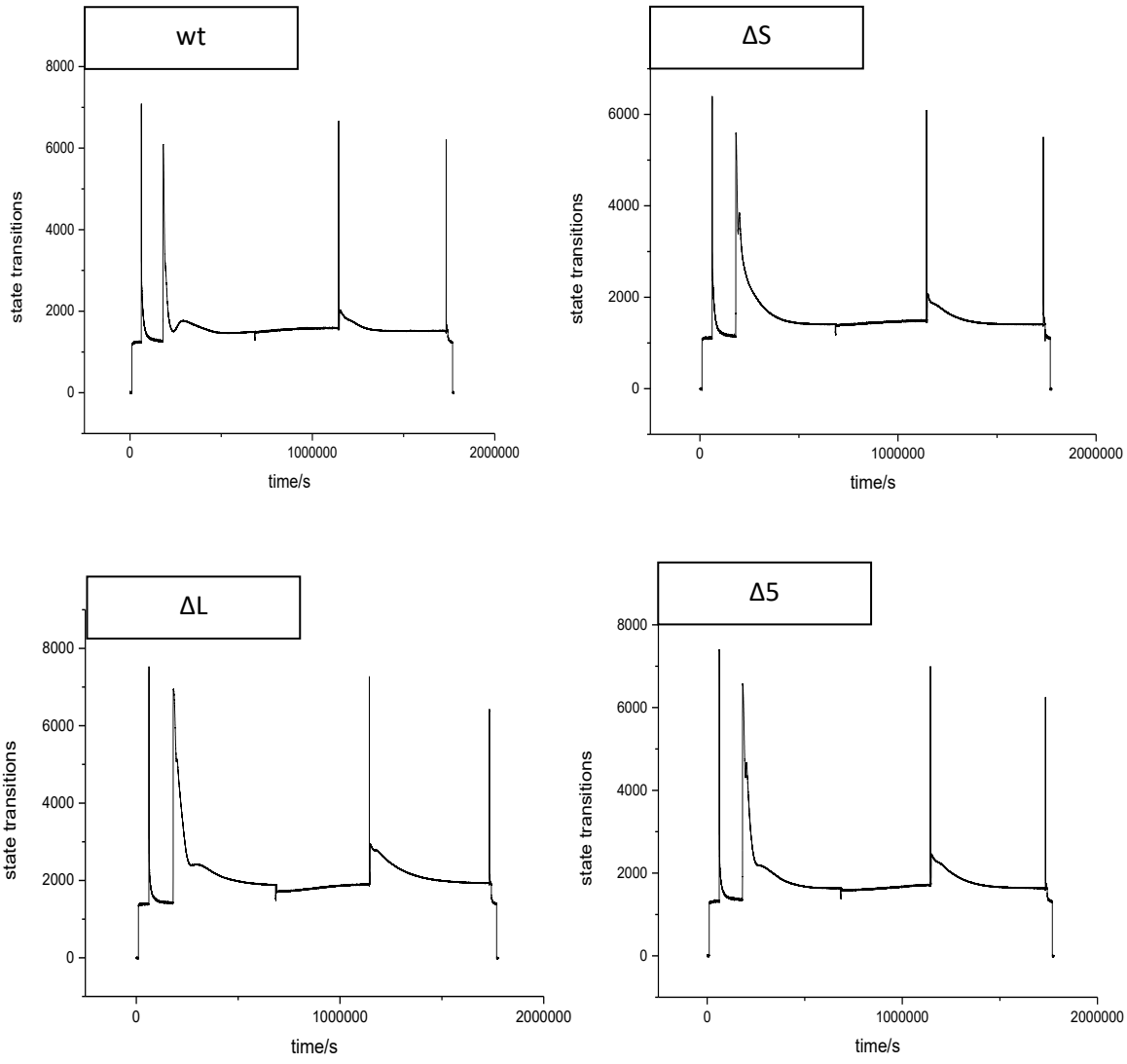


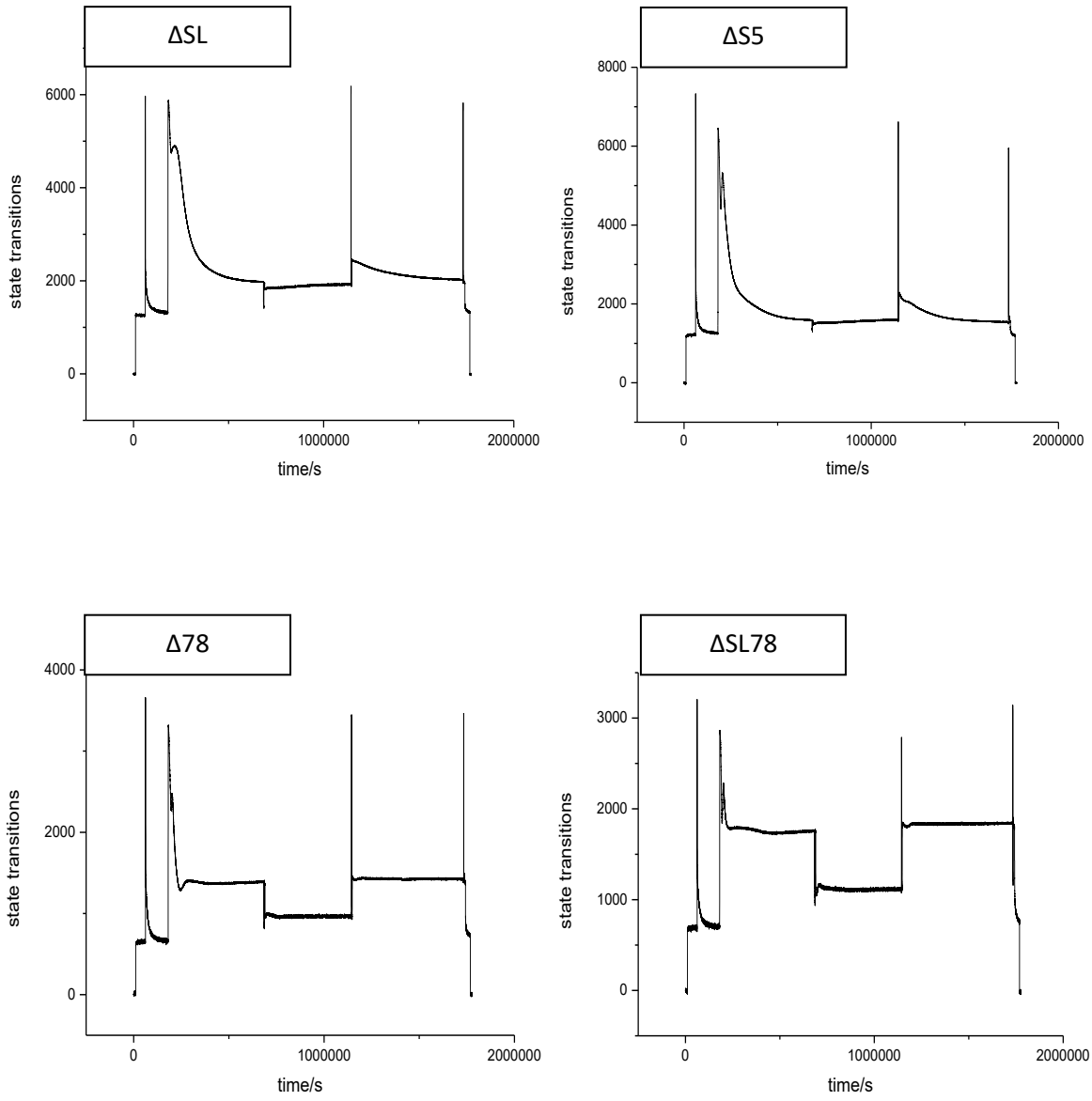
Fig. 13c

Fig.13: a), Y(I); b), Y(ND); c), Y(NA): light curves from different mutants(determined from grow light plants).

State transitions can be detected as a differential changes in fluorescence from PSII when leaves are exposed alternately to light favoring PSII and light favoring PSI (Lunde *et* al., 2000). Plants lacking STN7 and STN8 were highly deficient in state transitions as evidenced in the fluorescence change compared to wt plants. When PSII light is on, and the PSII is favored, the redox conditions in the thylakoids change and result in activation of a protein kinase. The kinase phosphorylates main light harvesting complex (LHCII) and the mobile antenna is detached from PSII. In our Δ78 and ΔSL78 mutants the absence of state transitions is due to the absence of the kinases as shown in Fig. 11a.

State transition, evaluated as described by Nature were detected in wild type and ΔS but not in Δ78 as reported previously. State transitions were also modified in both ΔL and Δ5 (Pesaresi *et* al.,2016), ΔS5 and ΔSL and totally absent in ΔSL78 (Fig. 14g-h). The relative extent of state transitions in different genotype is reported in Tab. 1





<u>Fig.14:</u> Measurement of state transition between state 1 and state2 from different genotypes; wild type (a),ΔS mutant (b), ΔL mutant(c), Δ5 mutant (d),ΔSL mutant(e),ΔS5 mutant(f),Δ78mutant(g)and ΔSL78 (h). Dark-adapted leaves were exposed to light favoring PSII (blue) and light favoring PSI (far-red).

Tab. 1: State transitions percentage of all genotypes compare to wild type.

Genotype	**wt**	**ΔS**	**ΔL**	**Δ5**	**ΔSL**	**ΔS5**	**Δ78**	**ΔSL78**
%	*100*	*96*	*85*	*92*	*79*	*91*	*7*	*9*

As expected, in the ΔSL and ΔS5 mutants, the lack of NPQ induction and the absence of Photosynthesis Control are present in the same genotype, whereas in the ΔSL78 the absence of all photoprotection mechanisms occurs.

4.1.1 Mutants lacking CEF/PC are very sensitive to high light

High order mutants possessed several properties that in principle, could make them more sensitive to light. In order to assess this possibility, plants were irradiated with 130, 500 and 1000 µmol $m^{-2}sec^{-1}$ of white light for 2 hours and the effect of light treatment evaluated by F_v/F_m measurements.

The result of this experiment is reported in Fig. 15. From this figure, it is clear that wild type, Δ78 and, surprisingly, ΔS mutant were more resistant to light than any other genotype here investigated. In particular, Δ5 and ΔL mutants were very sensitive to light. High order mutants, i.e. ΔS5, ΔSL and ΔSL78 displayed a light sensitivity similar to that of Δ5 and ΔL mutants. Thus, it seems that the effect of mutation on CEF/PC confers enhanced light sensitivity, irrespectively of whether a strong NPQ and full state transitions are developed or not.

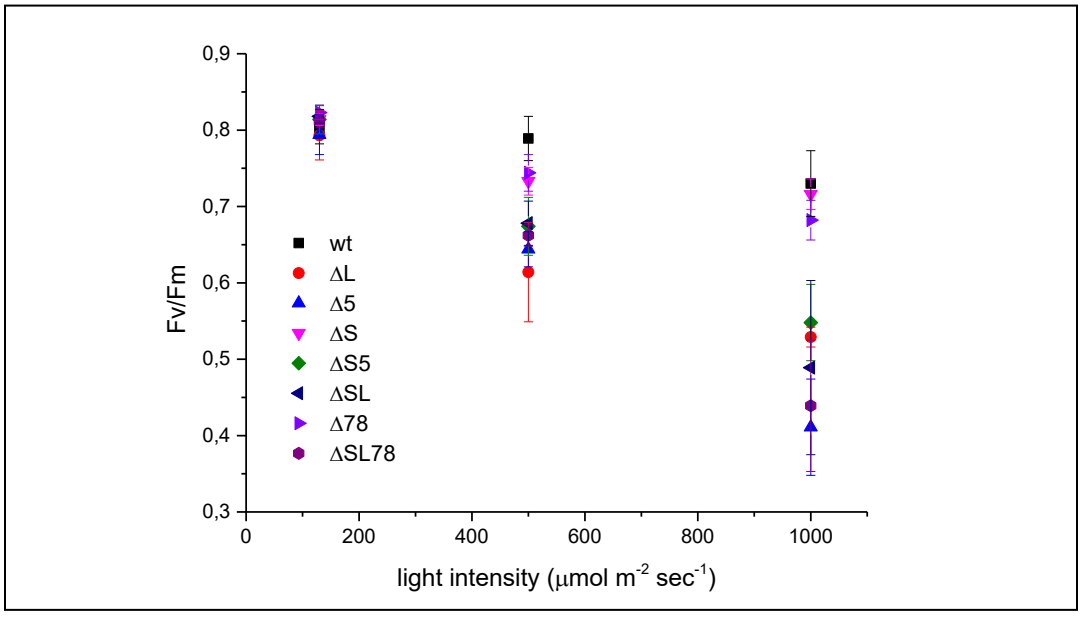


Fig.15: Fv/Fm values from wt, ΔS, ΔL, Δ5, ΔSL, ΔS5,Δ78 and ΔSL78 mutants treated with 130, 500 and 1000 µmol m^{-2} se^{-1} for 2 hours.

4.2 Fluorescence decay measurement

To further characterize light induced damage to PSII we used fluorescence decay in the 10^{-4}-10^{2} time scale. The same was then carried out in samples which had been irradiated for either 2 o 4 hours. The result of this experiment is reported in Fig. 16 a-h. When a leaf is illuminated with a single-turnover saturating flash, the reduction of Q_A with an electron extracted from the donor side of PSII occurs, leading to increased fluorescence yield. Subsequent dark reoxidation of Q^-_A resulted in the relaxation of fluorescence yield exhibiting three main decay phases (Vass *et* al., 1999). In the case of wild type, the fast phase, A_1, contributes to approx. 80% of total amplitude of decay with a time constant T_1 of approx. 300 μs, and arises from the reoxidation of Q^-_A by plastoquinone molecules bound to the Q_B site before the flash. The middle phase, A_2, approx. 10% relative amplitude and T_2 of approx. 17ms, originates from Q^-_A reoxidation by plastoquinone molecules in centers where the Q_B site was empty at the time of the flash. Finally, the slow phase, A_3, approx. 10 % relative amplitude and $T_3$of approx. 4 s, arises from a back-reaction of the S_2state of the water-oxidizing complex with Q^-_A, which is populated via the equilibrium between Q^-_A/Q_B and Q_A/Q^-_B

A similar distribution of amplitudes and time constants is observed also for all other genotypes, ensuring that under standard growth conditions, PSII is working in a very similar if not identical manner.

However, when plants are treated with high light, a totally different situation occurred. In wild type, irradiation with 2 or 4 hours, produces a small decrease of total amplitude (A_1+A_2+A_3, about 10%) due to a decrease of the A_1 phase. At

variance, there is a slight increase of both A_2 and A_3 phases. Time constants T_1 and T_2 remain in the order of 0.3 ms and 20-30 ms, irrespectively of irradiation time, whereas a strong shortening of T_3 is detected. A similar situation is observed in both ΔS and Δ78 mutants. At variance, a totally different situation is observed with mutants containing defects in CEF/PC. First of all, a loss of a total amplitude of about 25% is observed in all genotypes, due to a marked loss of A_1 amplitude (Tab. 2). As an example, in ΔL, A_1 varied from 85% of the dark control to the 31% of the 4-hours irradiated sample. This decrease is paralleled by a marked increase in both the A_2 and A_3 phases. As far as time constants are concerned, it may be observed a strong increase of T_2 in Δ5 and ΔS and a strong shortening in high order mutants, pointing to a modification of Q_B site. A perturbation of the donor side is also likely to occur, as the A_3 showed a marked decrease paralleled by a decrease of time constants.

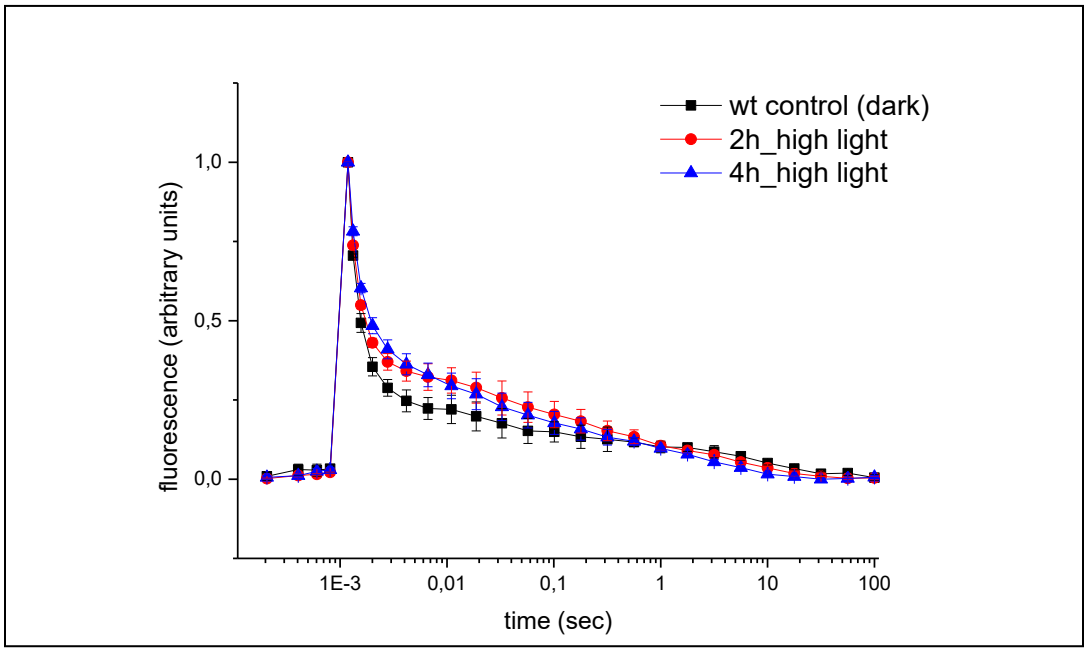


Fig.16a: Fluorescence decay of wt plant

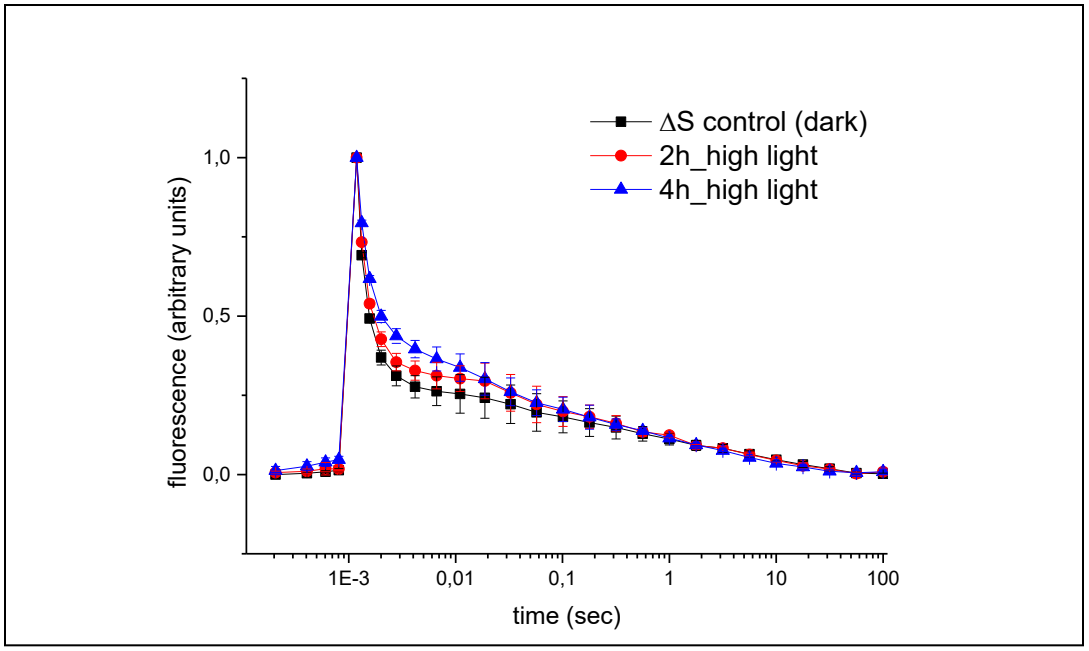


Fig. 16b: Fluorescence decay of ΔS mutant.

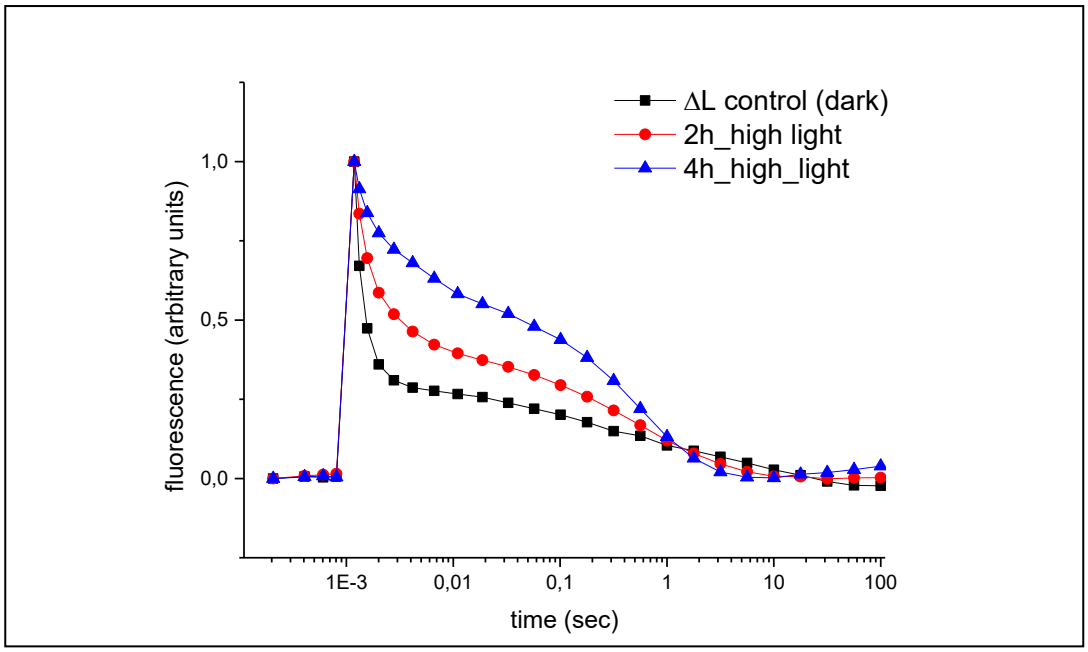


Fig. 16c: Fluorescence decay of ΔL mutant.

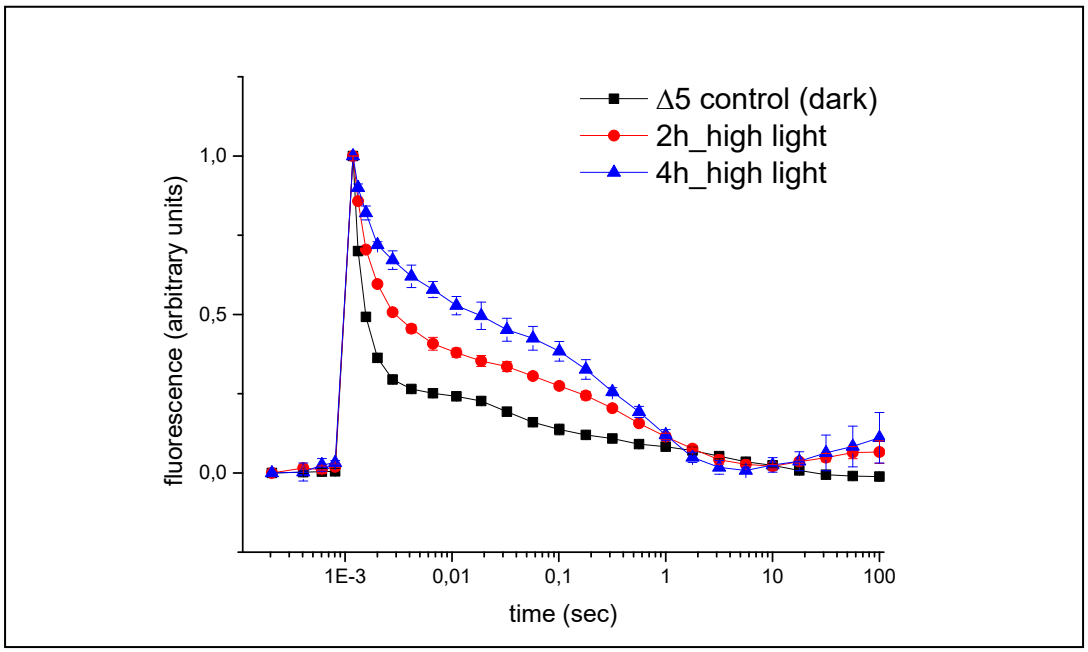


Fig. 16d: Fluorescence decay of Δ5 mutant.

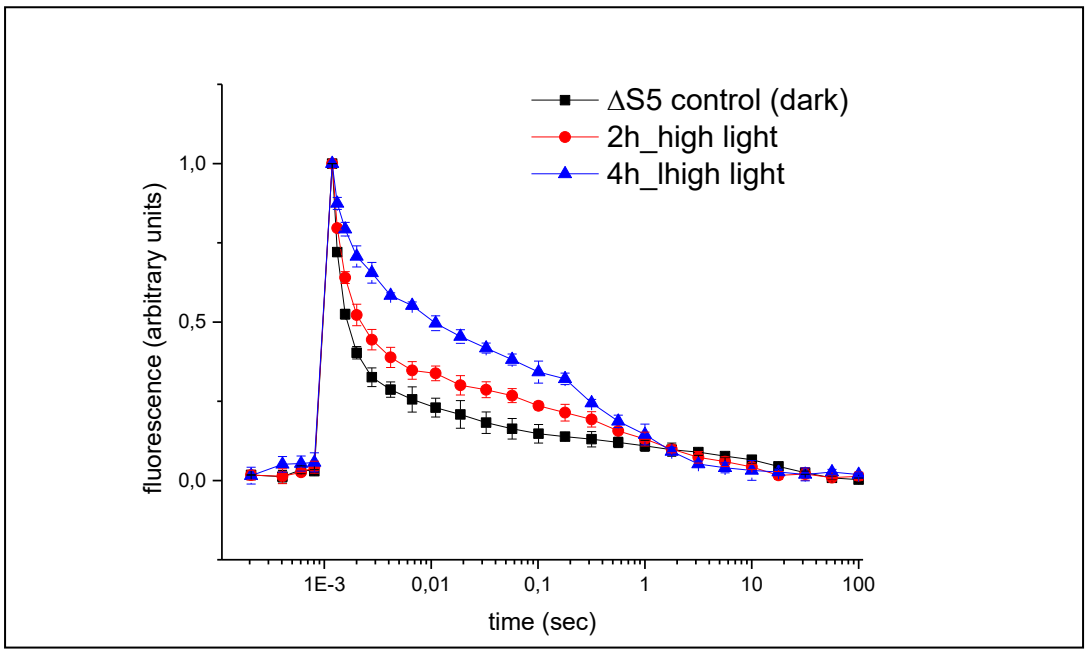


Fig.16e: Fluorescence decay of ΔS5 mutant.

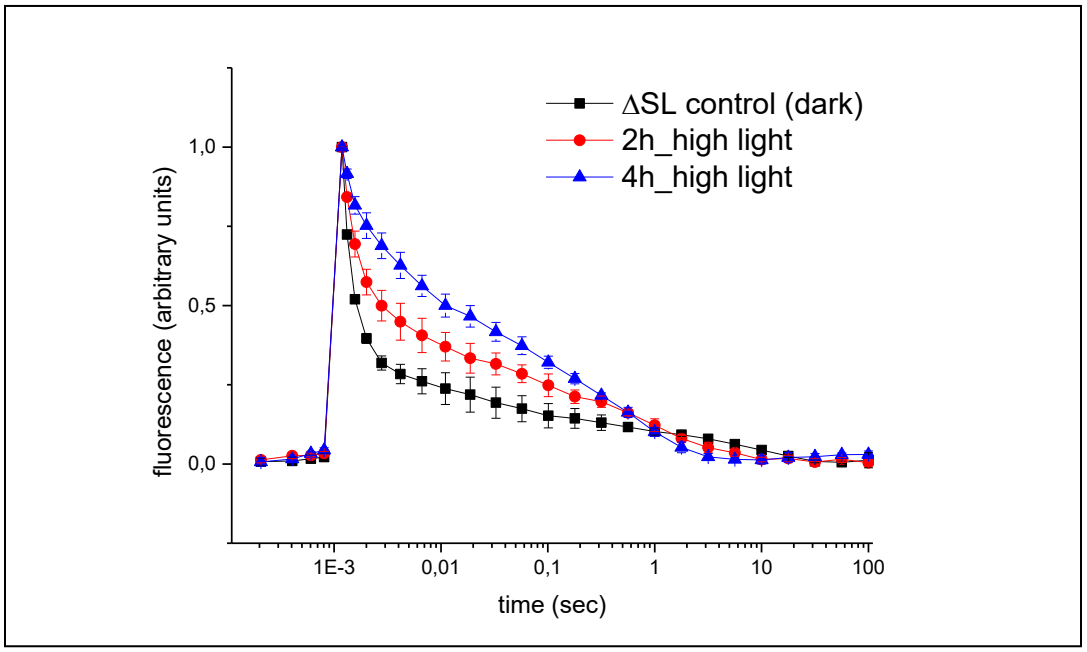


Fig. 16f: Fluorescence decay of ΔSL mutant.

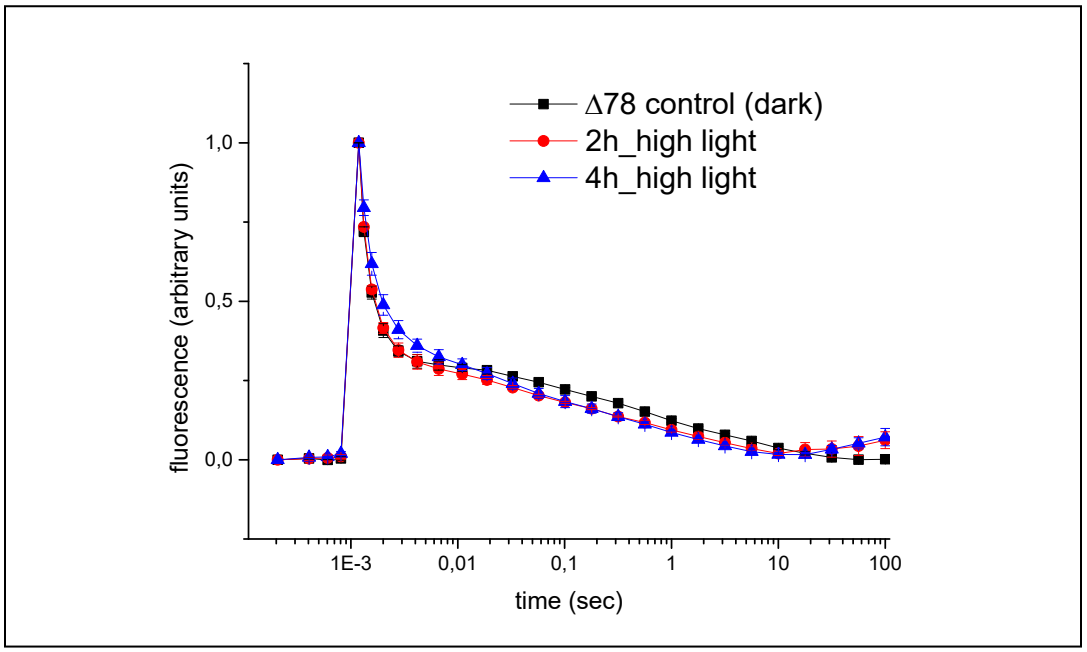


Fig.16g: Fluorescence decay of Δ78 mutant.

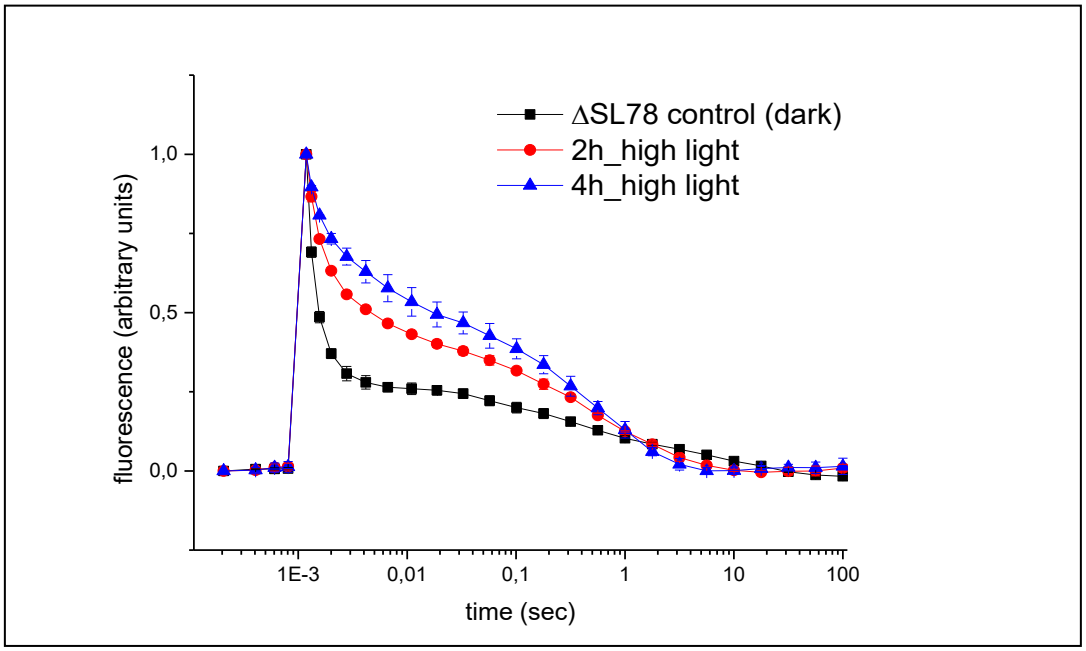


Fig. 16h: Fluorescence decay of ΔSL78 mutant.

Tab.2: Amplitude and time constants for different genotypes irradiated for 2 or 4 hours.

Genotype/irradiation time (h)	Total amplitude	fast phase $t_{½}$(µs) (amp (%)	middle phase $t_{½}$(ms) (amp(%)	slow phase $t_{½}$(s) (amp(%)
wt/0h	100	309±18 (82±3.4)	17±4 (9.5±0.9)	4.2±1.6 (8.8±0.8)
wt/2h	96	307±18 (76±3.3)	29±6 (12±0.9)	1.2±0.3 (12±0.9)
wt/4h	89	353±39 (70±2.9)	14±2 (16±1.1)	0.9±0.2 (14±0.7)
ΔS/0h	100	283± 18 (81±4.9)	26±8 (8±0.1)	2.1±0.7 (11±0.8)
ΔS/2h	93	313±19 (77±3.4)	37±9 (11±1.0)	1.8±0.6 (12±0.9)
ΔS/4h	85	353±21 (69±2.5)	19±3 (16±0.9	1.1±0.2 (15±0.7)
ΔL/0h	100	258±16 (85±4.5)	51±16 (8±0.1)	2.4±0.8 (12±0.9)
ΔL/2h	81	312±25 (54±2.0)	3.7±0.2 (17±1.4)	0.43±0.03 (29±0.4)
ΔL/4h	70	316±65 (31±3.2)	665±32 (41±0.3)	0.005±0.0.003 (28±1.9)
Δ5/0h	100	301±16 (81±3.2)	36±7 (11±0.8)	2.84±1.0 (8±0.7)
Δ5/2h	78	362 ±66 (54±4.1)	3.3±1.1 (20±4.0)	0.28±0.04 (26±1.0)
Δ5/4h	71	387±15 (38±6.7)	533±83 (34±2.6)	0.005±0.003 (32±5.4)
ΔSL/0h	100	277±16 (82±3.8)	50±15 (8±1.3)	2.97±1.06 (10±0.1)
ΔSL/2h	87	300±25 (65±2.6)	3.4±0.7 (15±1.8)	0.50±0.05 (20±0.1)
ΔSL/4h	74	330±54 (37±2.8)	3.9±0.7 (20±2.0)	0.33±0.02 (43±0.1)
ΔS5/0h	100	303±23 (78±4.0)	13±2.8 (13±1.1)	4.81±1.89 (9±0.1)
ΔS5/2h	89	327±36 (64±3.5)	5.0±1.2 (16±2.0)	0.62±0.01 (20±0.1)
ΔS5/4h	77	363±49 (41±2.8)	6.6±1.0 (22±1.5)	0.37±0,03 (37±0.1)
ΔSL78/0h	100	292±18 (81±4.1)	65±2.6 (7±1.2)	2.19±0.87 (12±1.2)
ΔSL78/2h	78	387±31 (51±1.8)	5.5±0.8 (17±1.3)	0.44±0.03 (32±5.3)
ΔSL78/4h	75	332±64 (37±3.5)	5.0±0.1 (20±2.1)	0.33±0.03 (43±0.1)
Δ78/0h	100	307±20 (78±3.6)	27±10 (7±1.0)	1.30±0.33 (15±0.1)
Δ78/2h	98	246±27 (73±4.7)	2.8±0.9 (13±2.5)	0.24±0.05 (15±0.1)
Δ78/4h	92	342±40 (68±3.9)	5.4±1.9 (14±2.3)	0.22±0.06 (18±1.30)

4.3 Prompt fluorescence

In Fig. 17 a-h, the effect of light treatment on chlorophyll fluorescence transients on different genotypes is reported. A general decrease of fluorescence is detected, which is particularly pronounced on CEF/PC mutants. In addition, a marked modification on the shape of transients is observed in these mutants. A double normalization step (to F_0 and F_m) was performed and fluorescence plotted as relative V_{OP} (Fig. 18a-h). From this figure, little effect id observed in wt, ΔS and Δ78 genotypes, whereas a marked difference is observed in all other mutants. The most sensitive seems to be the ΔSL78 mutant in which, after 2h of irradiation, a full damage is induced and no other modification of the transient was observed on prolonged irradiation time. The observed differences were further highlighted by calculating the relative difference ΔV_{OP} among light treated and dark adapted plants. In this case a broad and strong band peaking at about 3 ms is detected in all CEF/PC mutants. Further analysis was carried out by normalization between 0 and 0.3 ms (Fig.19 a-h) and between 0 and 2 ms (Fig. 20 a-h), which allowed to understand whether or not a K and L band are respectively induced by light treatment. A positive K step is induced in ΔL and Δ5 mutants and, to a less extent, in the ΔS5 mutants, indicative of a damage to the donor side of the photosystem. In other CEF/PC mutants this band is not so evident, whereas it is almost absent in wt, ΔS and Δ78.

This data were then subjected to JIP analysis and presented as spider plot (Fig. 21 a-h). From these figures it may be observed that in wt, ΔS and Δ78, light treatment did not affect significantly any of the considered parameters. At variance, in CEF/PC genotypes most of parameters are strongly affected. F_0 showed a marked increase in all CEF/PC mutants, whereas little or no effect was

observed in wt, ΔS and Δ78 genotypes. In parallel, a decrease in F_m is observed, which brought about a marked decrease of F_v/F_m. Mo, i.e. the at which reaction center are closed and DIo/RC, the amount of energy dissipated as heath, fluorescence or transferred to other systems, increase dramatically, indicating a damage to PSII. In general, all considered parameters tend to indicate that in CEF/PC mutants, PSII is much more sensitive to light than in wt, ΔS and Δ78.

Fig. 17 a, b, c, d: Fluorescence transient of wt, ΔL, Δ5 and ΔS normalized between F_0 to F_m

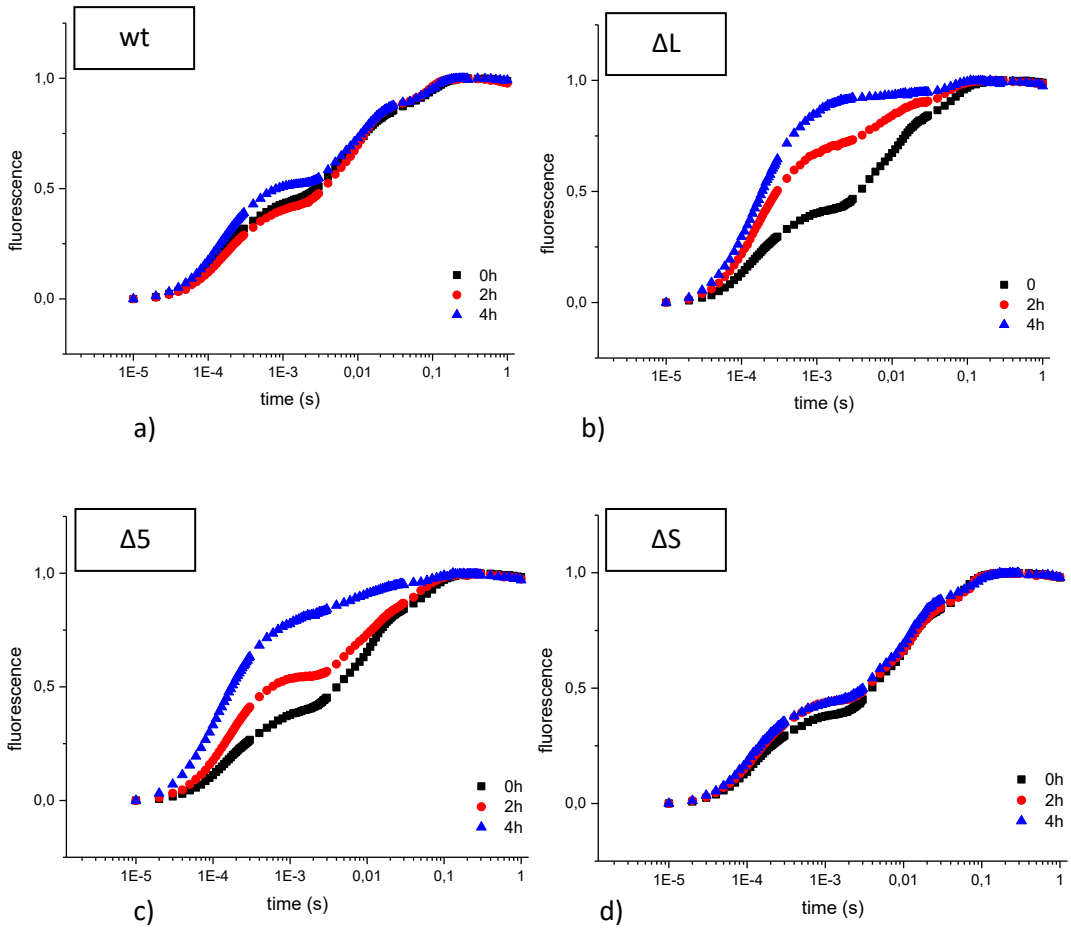
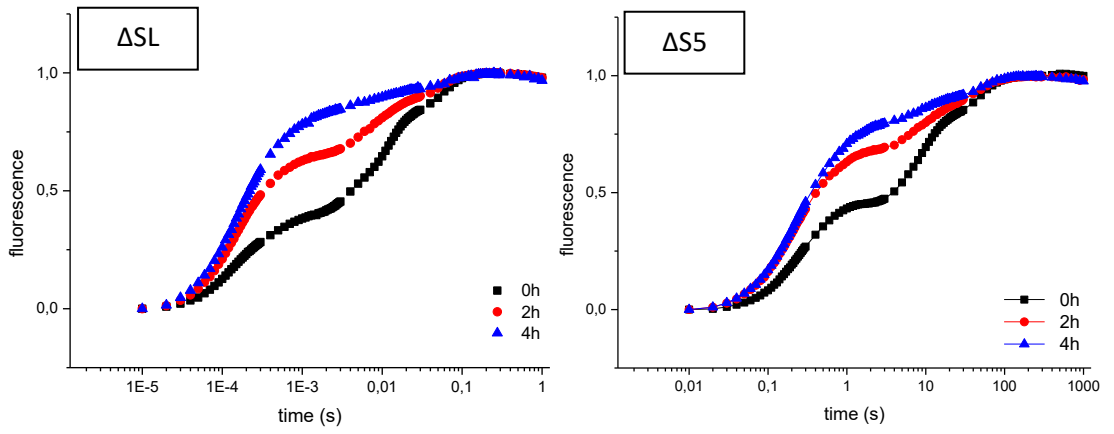
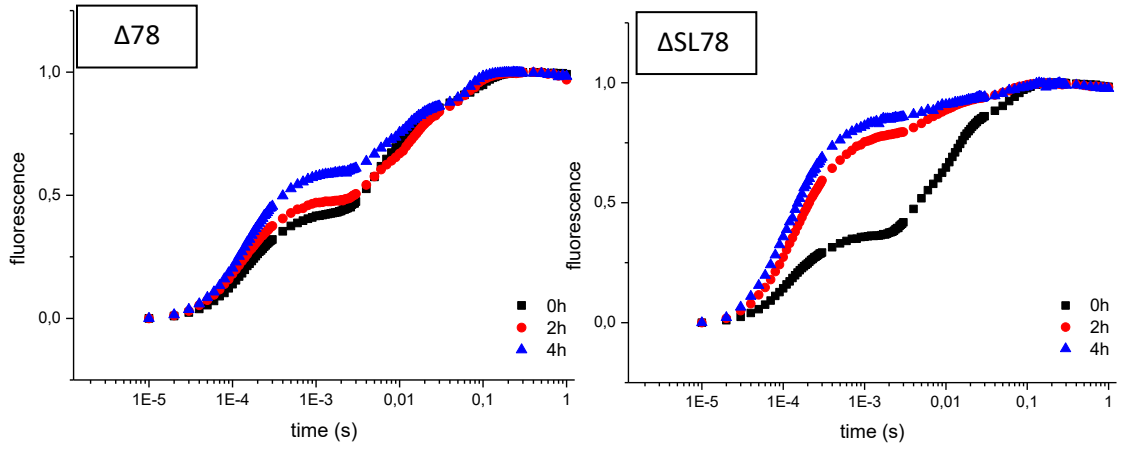


Fig. 17 e, f, g, h: Fluorescence transient of ΔSL, ΔS5, Δ78 and ΔSL78 mutant normalized between F_0 to F_m



e) f)



g) h)

Fig. 18 a, b. c, d: relative difference ΔV_{OP} among light treated and dark adapted plants in wt, ΔL, Δ5 and ΔS

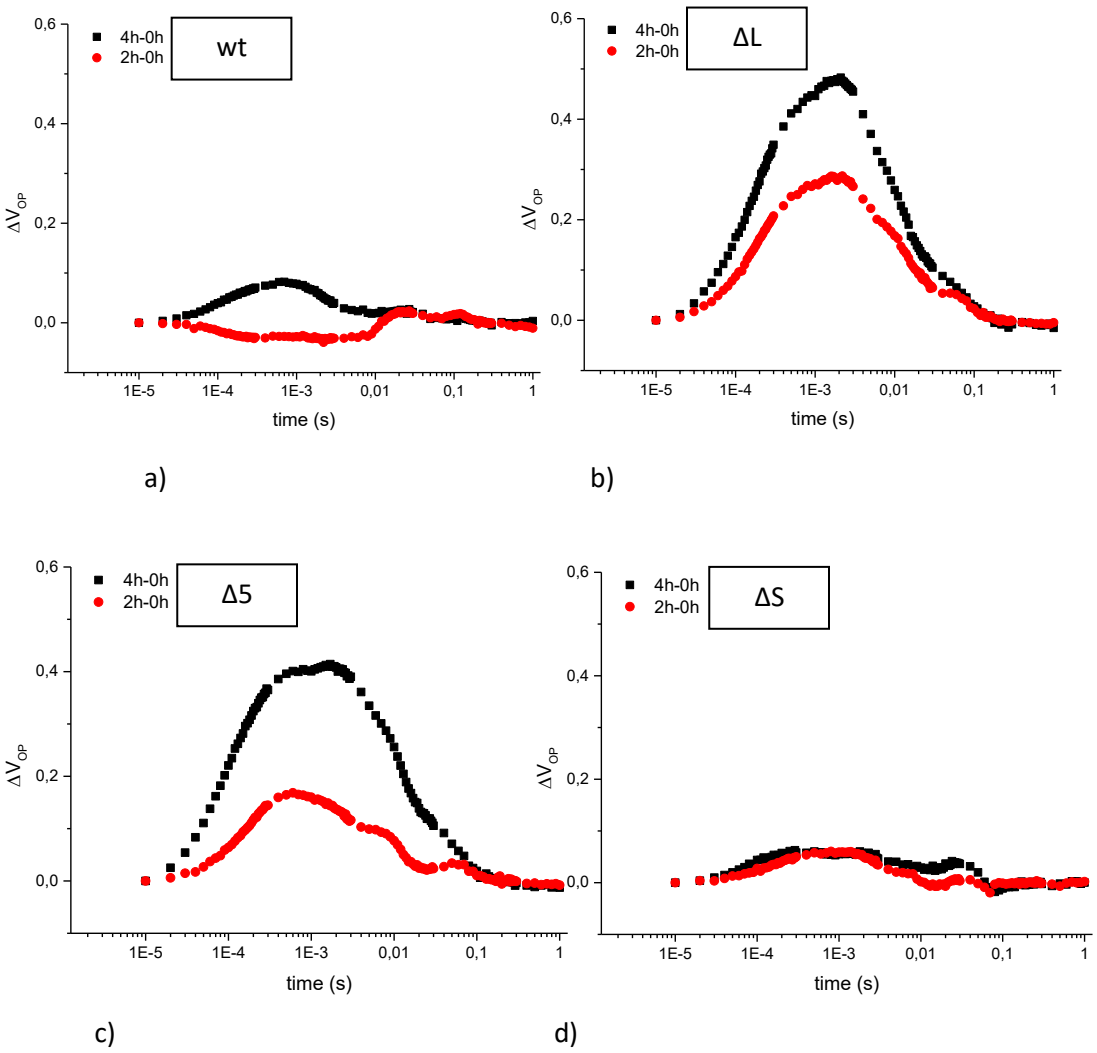


Fig. 18 e, f, g, h: relative difference ΔV_{OP} among light treated and dark adapted plants in ΔSL, ΔS5, Δ78 and ΔSL78 mutants

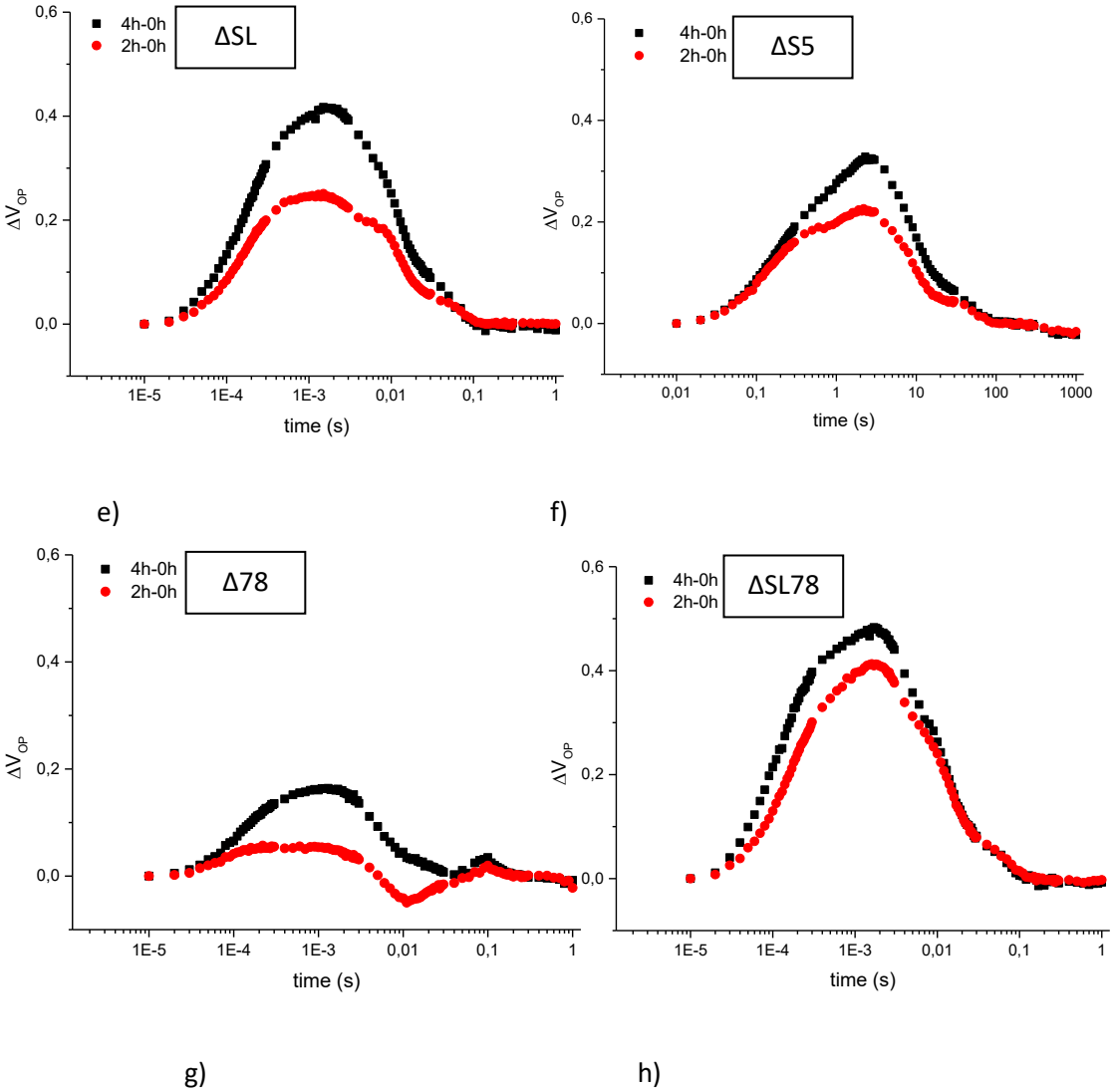


Fig. 19a: Fluorescence transient of wt normalized between 0 and 0.3 ms

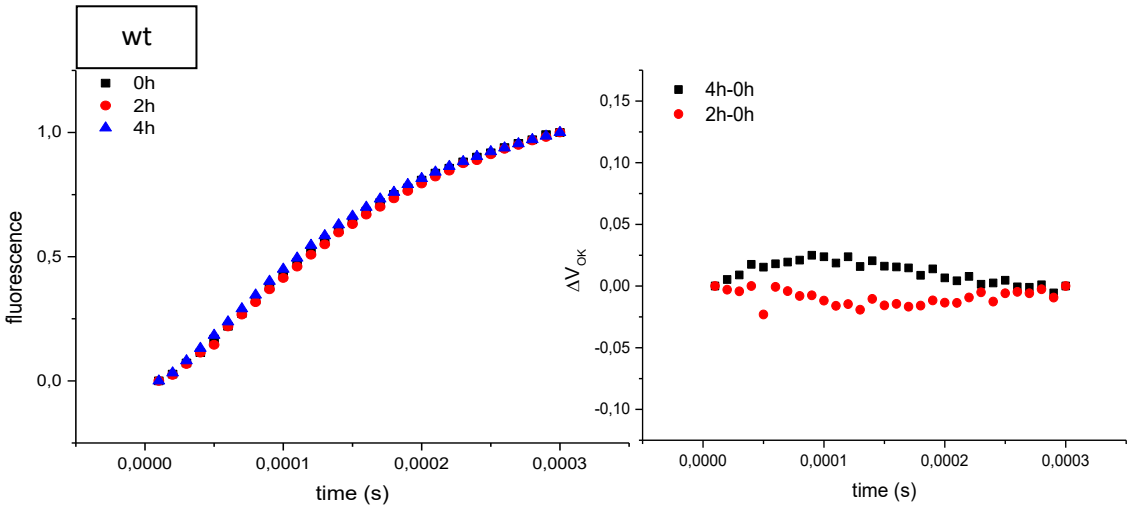


Fig. 19b: Fluorescence transient of ΔL mutant normalized between 0 and 0.3 ms

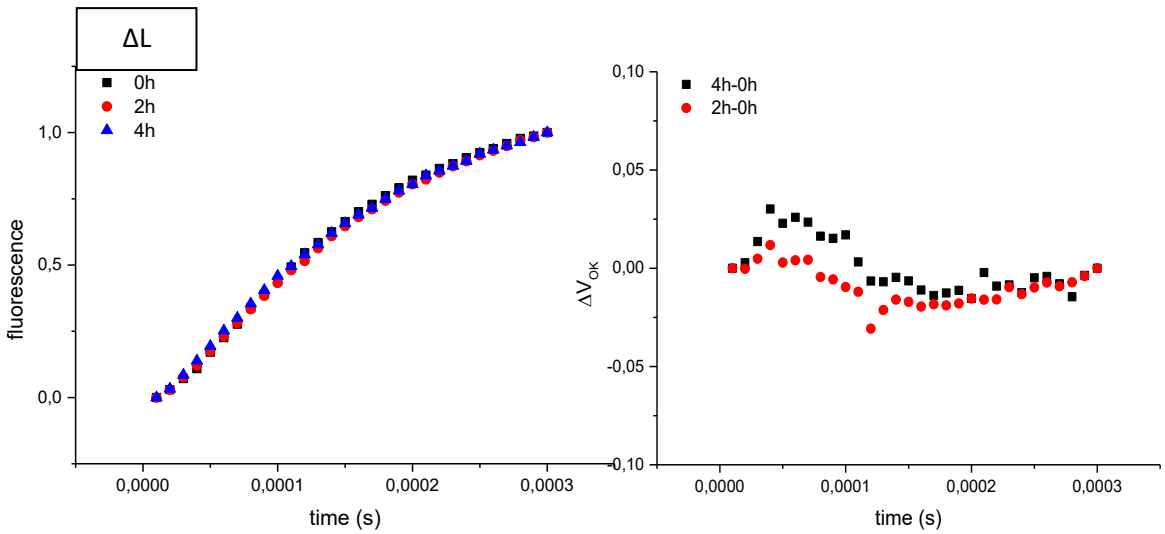


Fig. 19c: Fluorescence transient of Δ5 mutant normalized between 0 and 0.3 ms

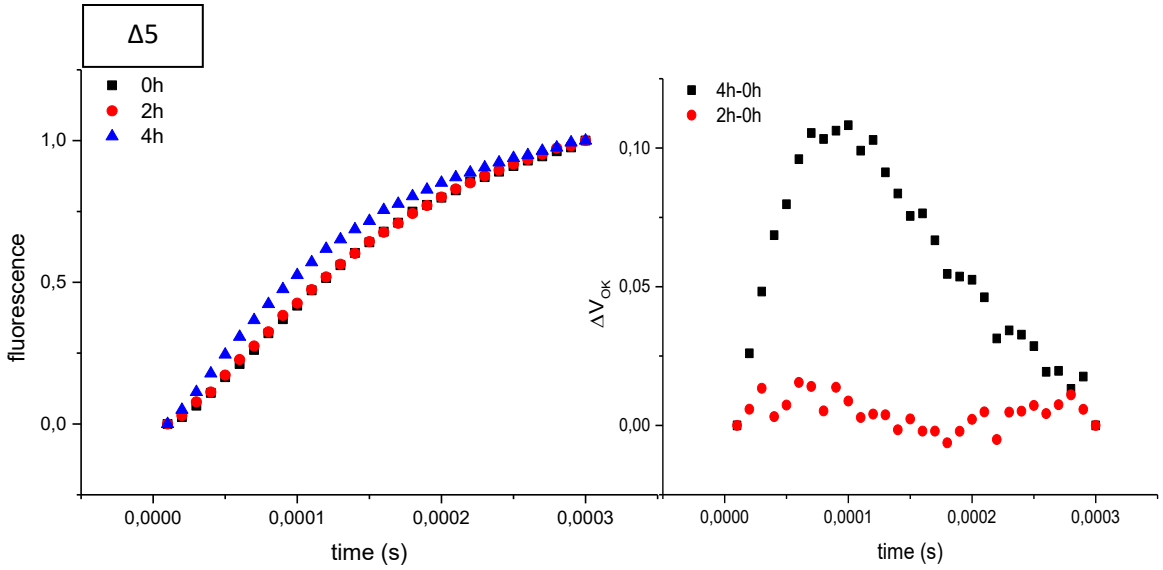


Fig. 19d: Fluorescence transient of ΔS mutant normalized between 0 and 0.3 ms

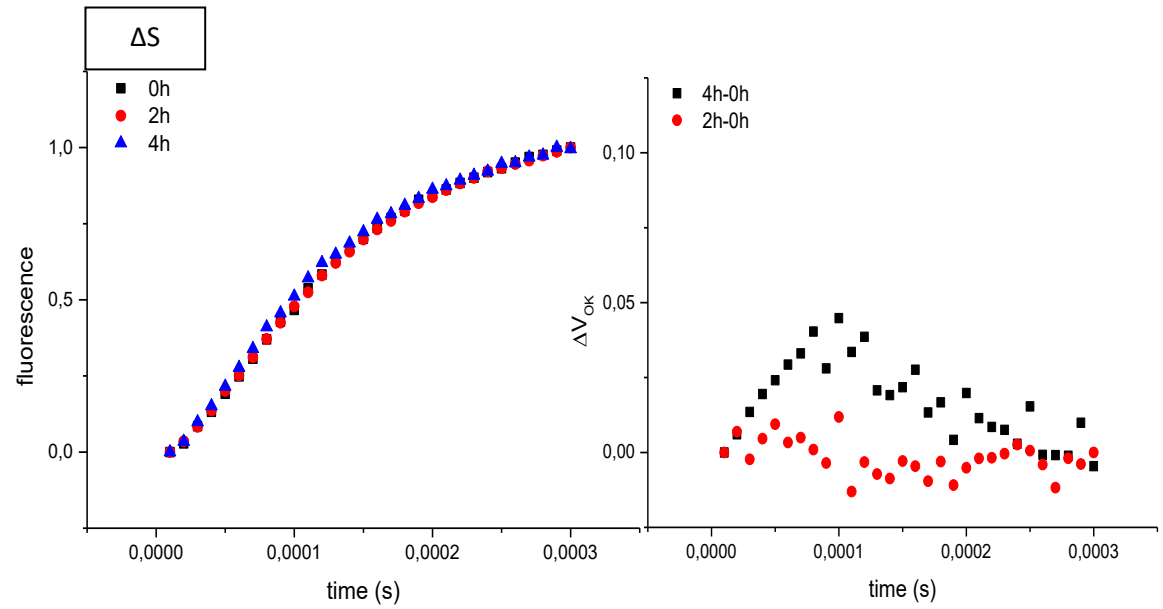


Fig. 19e: Fluorescence transient of ΔSL mutant normalized between 0 and 0.3 ms

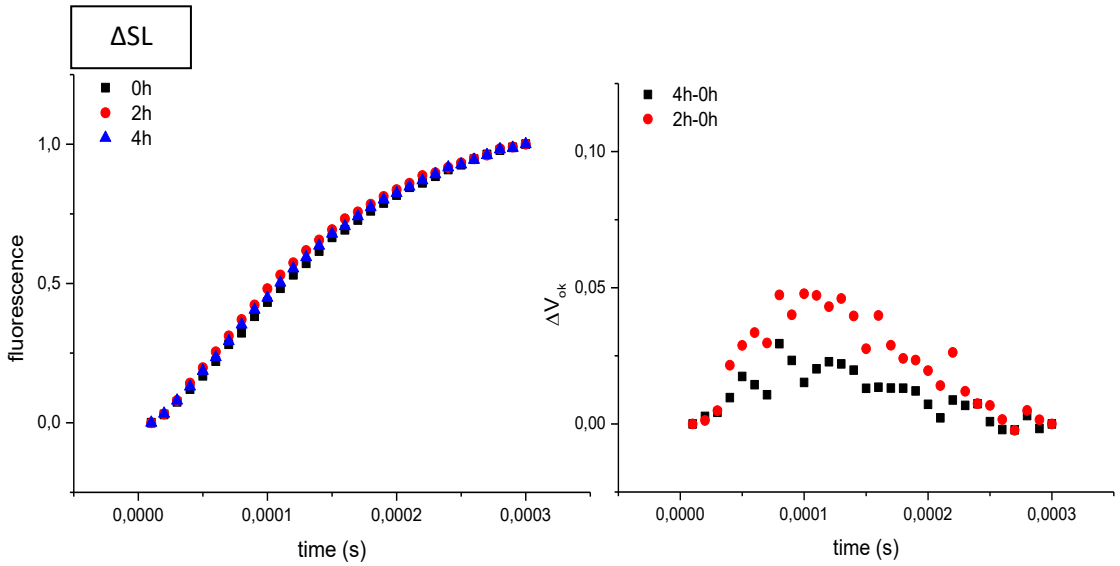


Fig. 19f: Fluorescence transient of ΔS5 mutant normalized between 0 and 0.3 ms

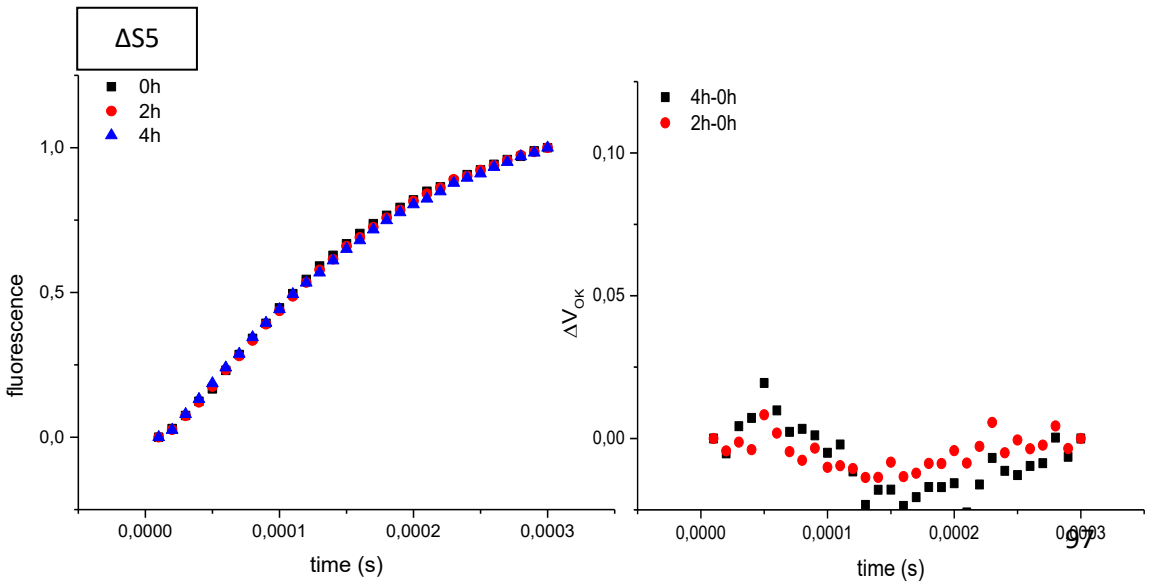


Fig. 19g: Fluorescence transient of Δ78 mutant normalized between 0 and 0.3 ms

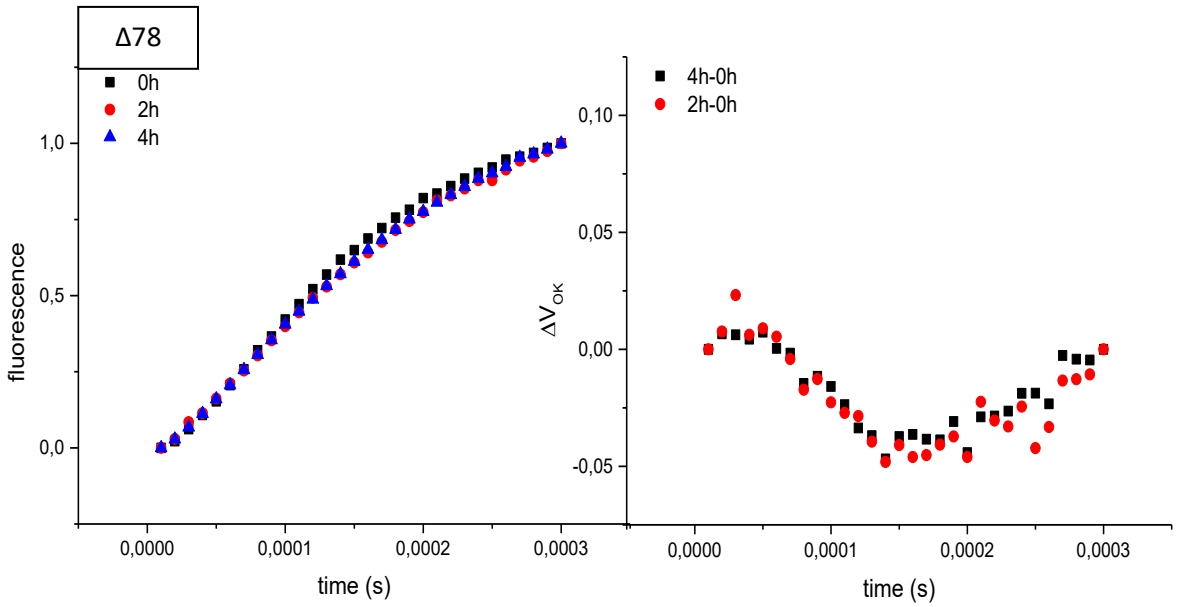


Fig. 19h: Fluorescence transient of ΔSL78 mutant normalized between 0 and 0.3 ms

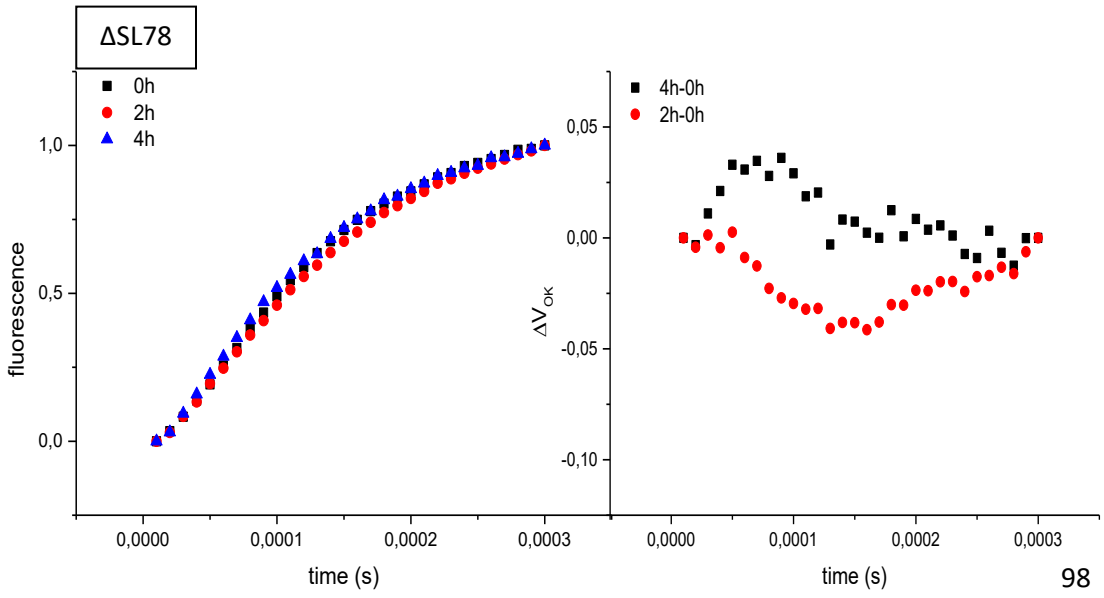


Fig. 20a: Fluorescence transient of wt normalized between 0 and 0.2ms

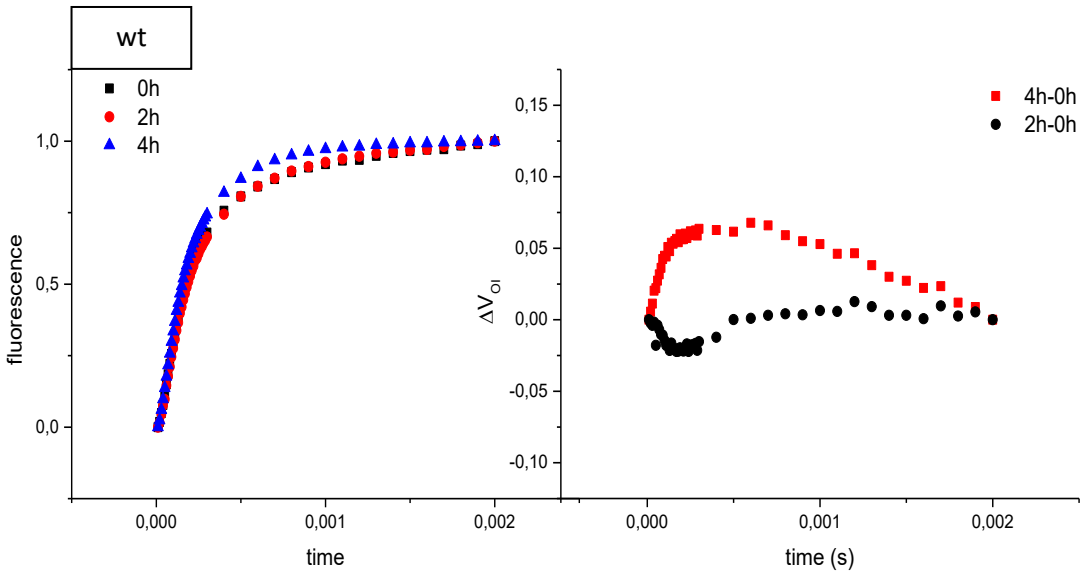


Fig. 20b: Fluorescence transient of ΔL mutant normalized between 0 and 0.2ms

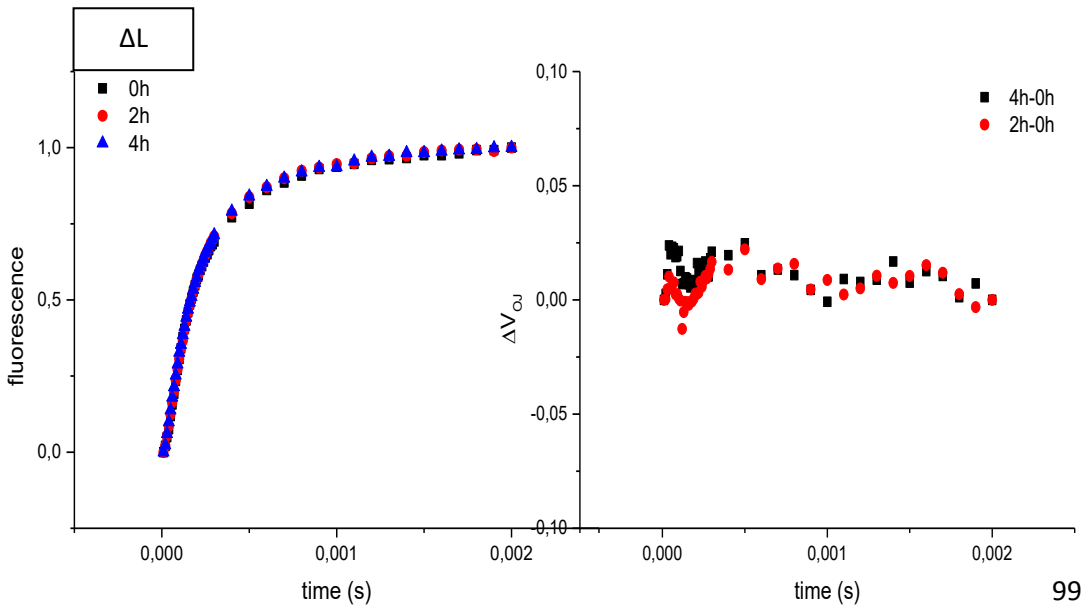


Fig. 20c: Fluorescence transient of Δ5 mutant normalized between 0 and 0.2ms

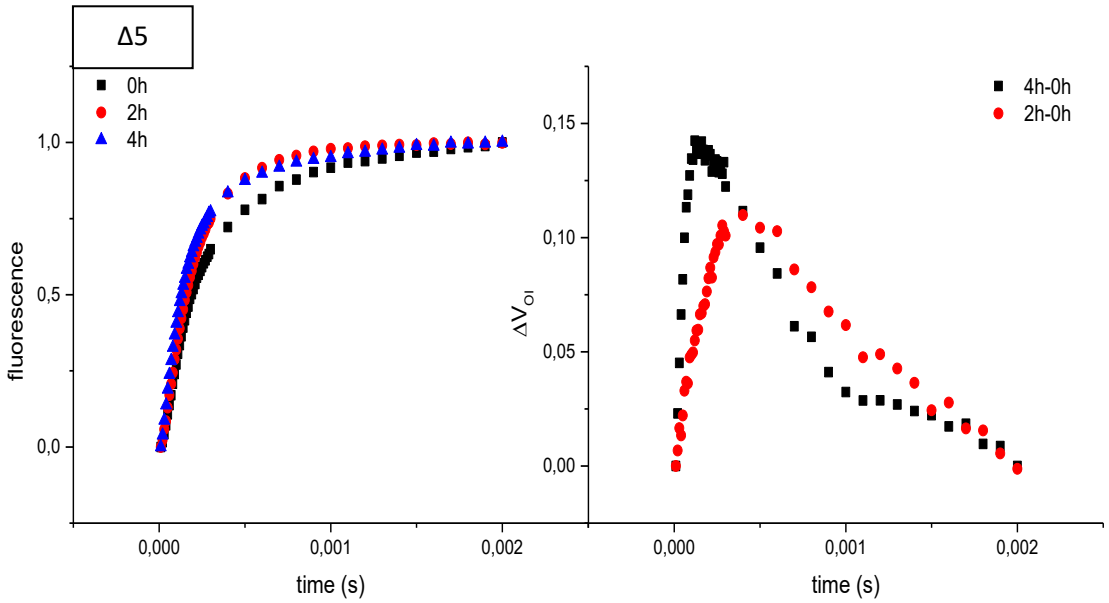


Fig. 20d: Fluorescence transient of ΔS mutant normalized between 0 and 0.2ms

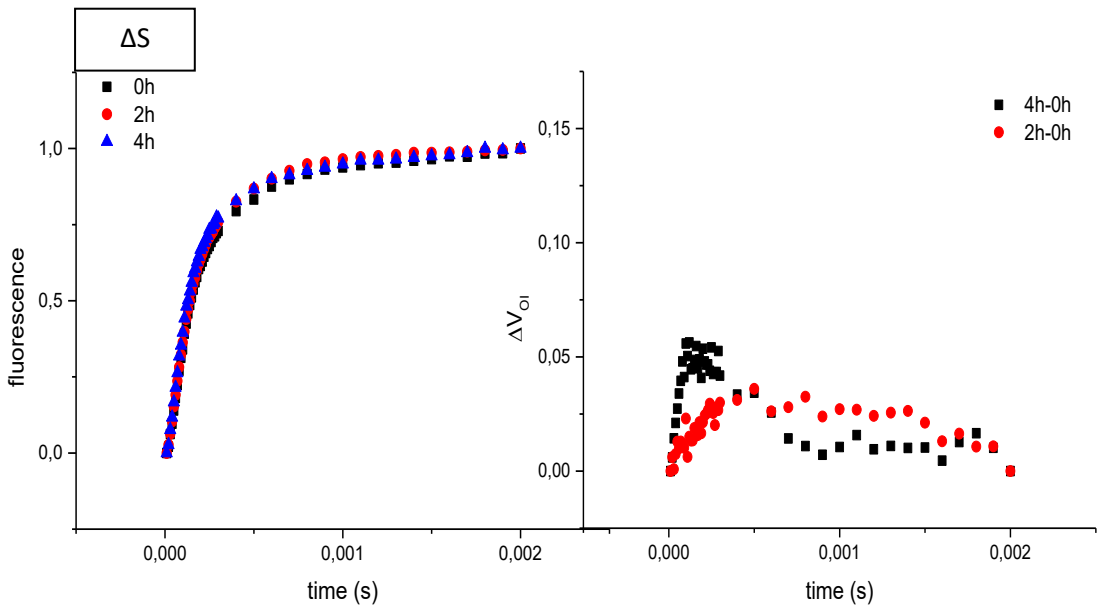


Fig. 20e: Fluorescence transient of ΔSL mutant normalized between 0 and 0.2ms

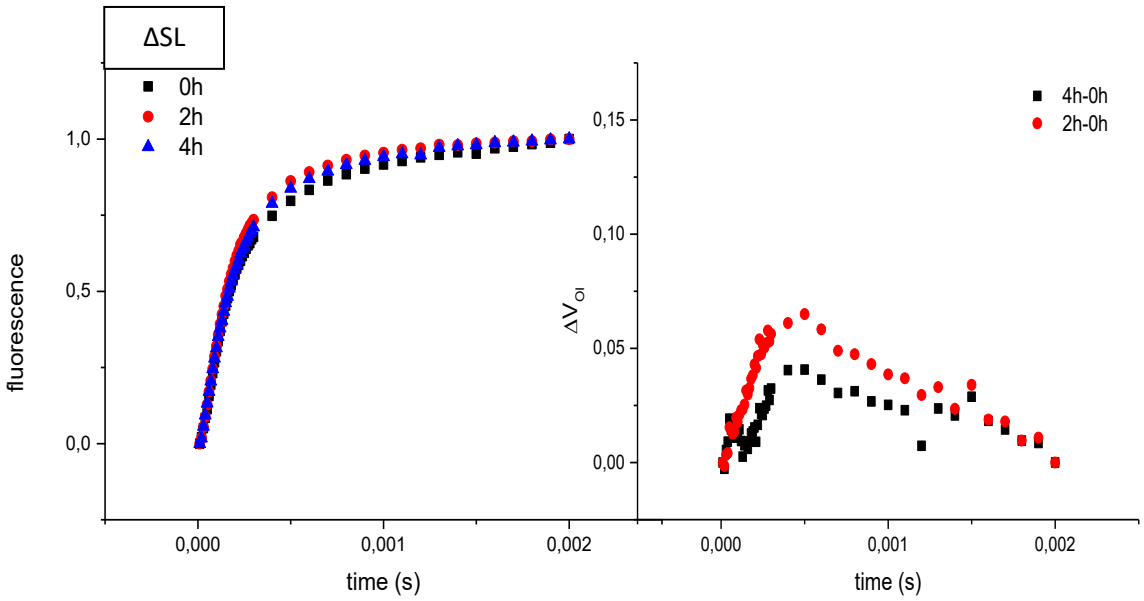


Fig. 20f: Fluorescence transient of ΔS5 mutant normalized between 0 and 0.2ms

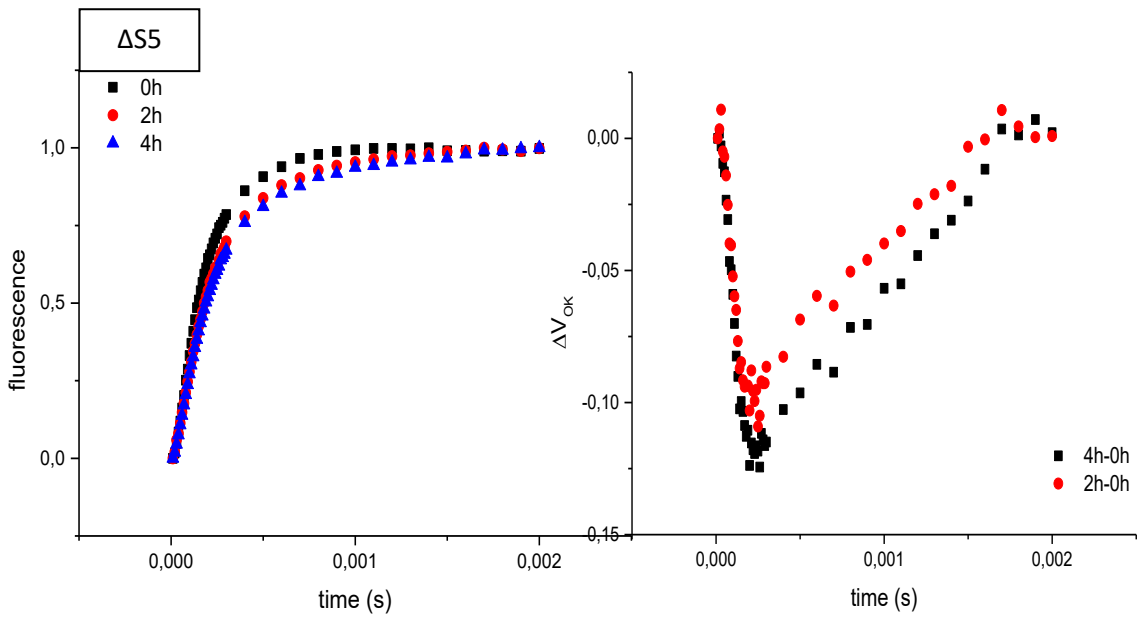


Fig. 20g: Fluorescence transient of Δ78 mutant normalized between 0 and 0.2ms

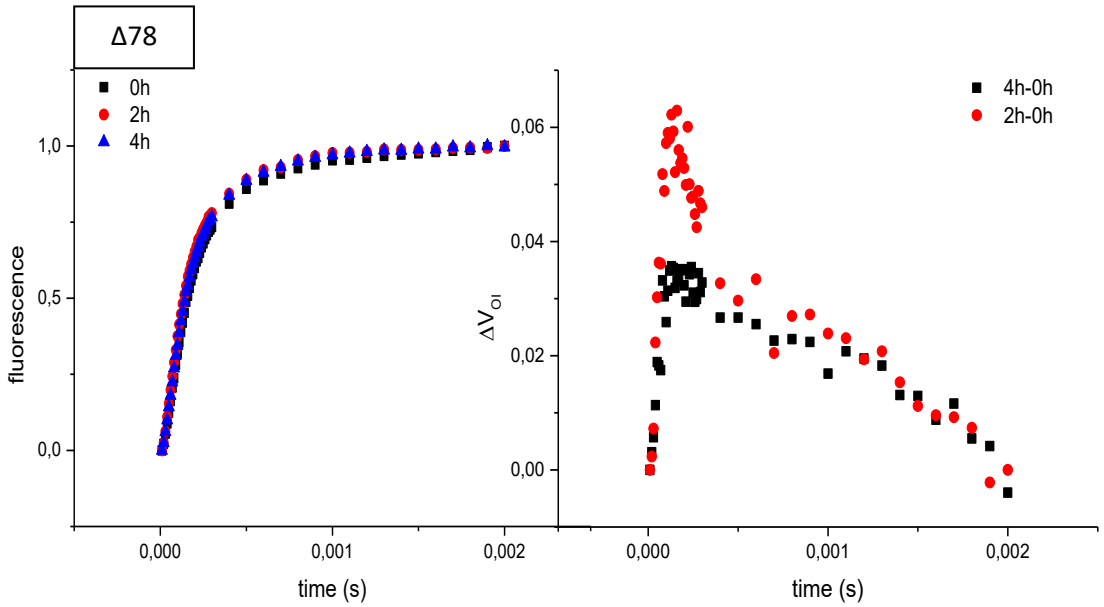


Fig. 20h: Fluorescence transient of ΔSL78 mutant normalized between 0 and 0.2ms

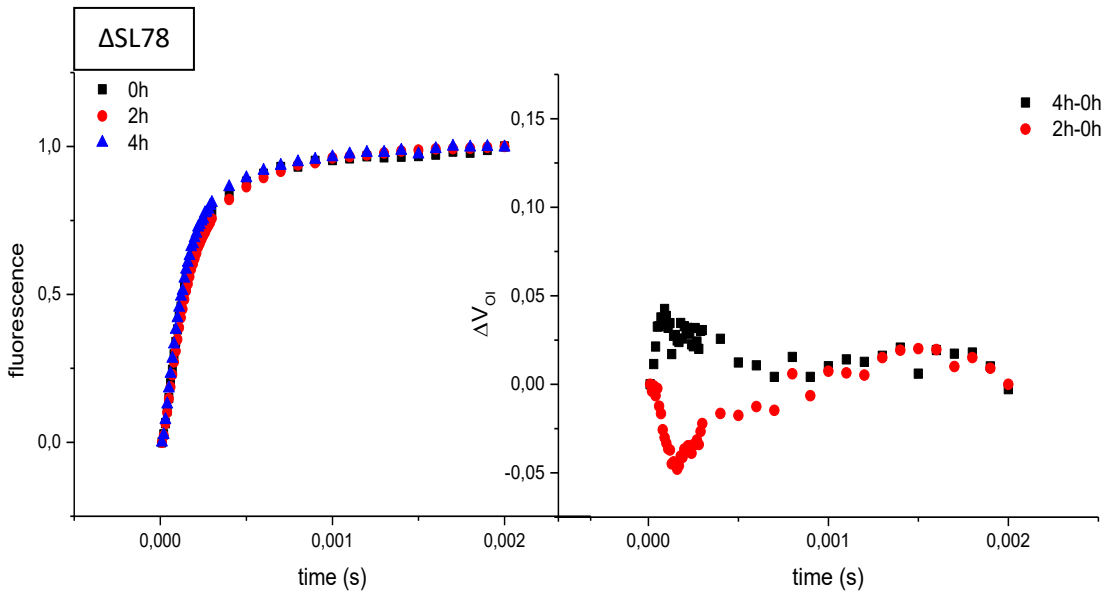


Fig. 21a: JIP analysis by spider plot with wt values

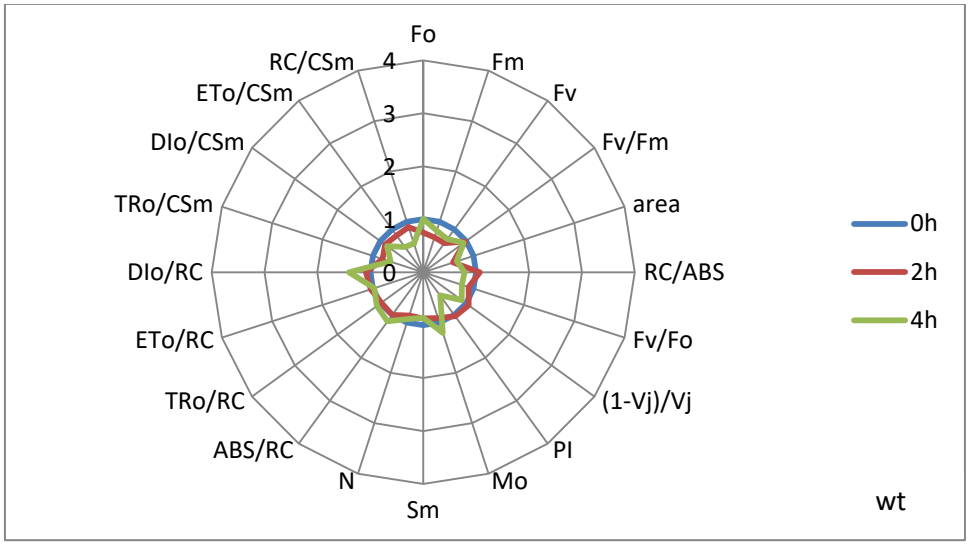


Fig. 21b: JIP analysis by spider plot with ΔL values

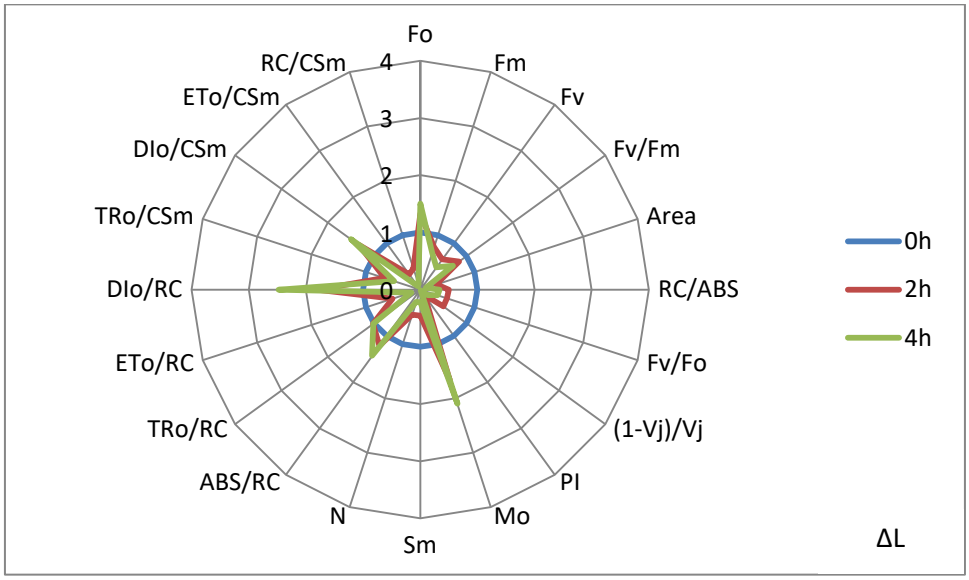


Fig. 21c: JIP analysis by spider plot with ΔS values

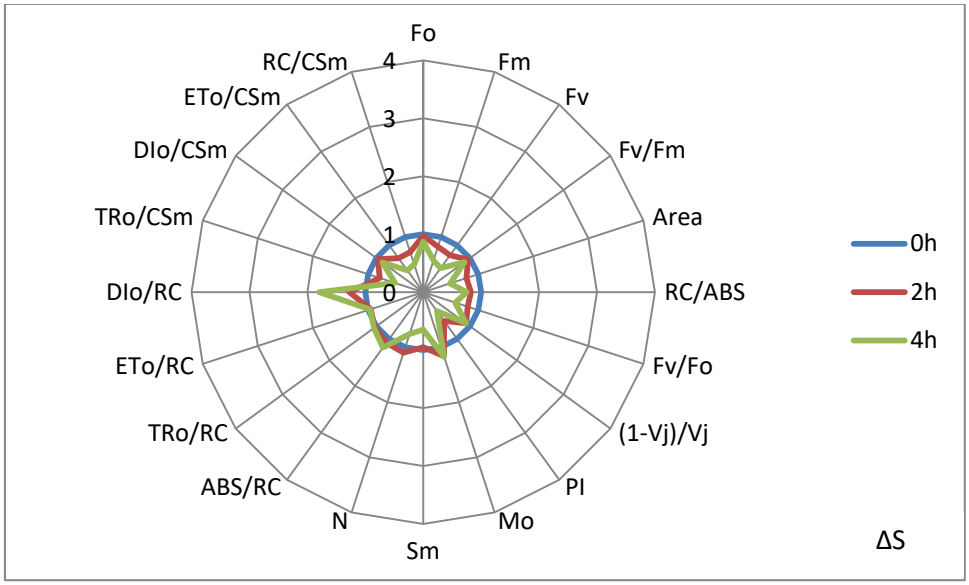


Fig. 21d: JIP analysis by spider plot with Δ5 values

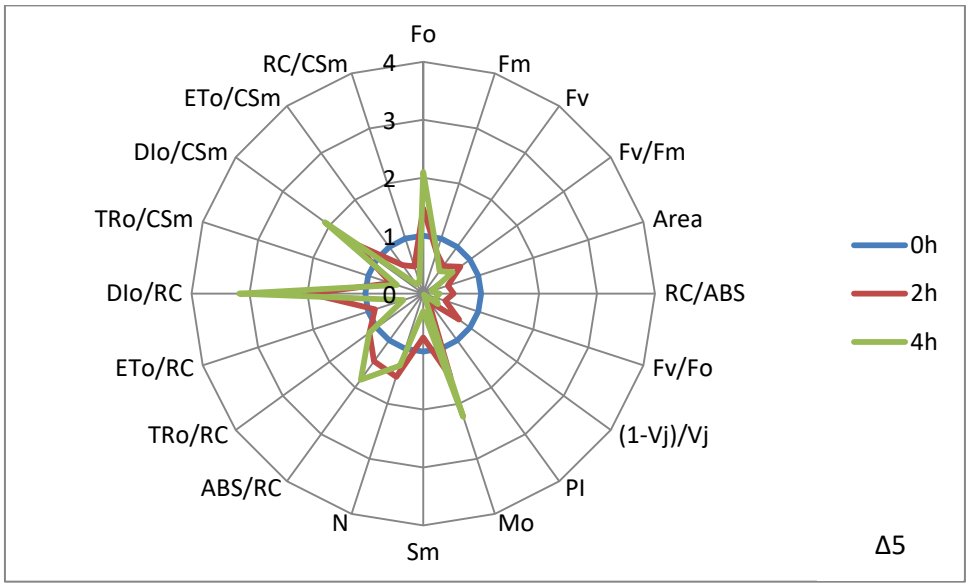


Fig. 21e: JIP analysis by spider plot with ΔSL values

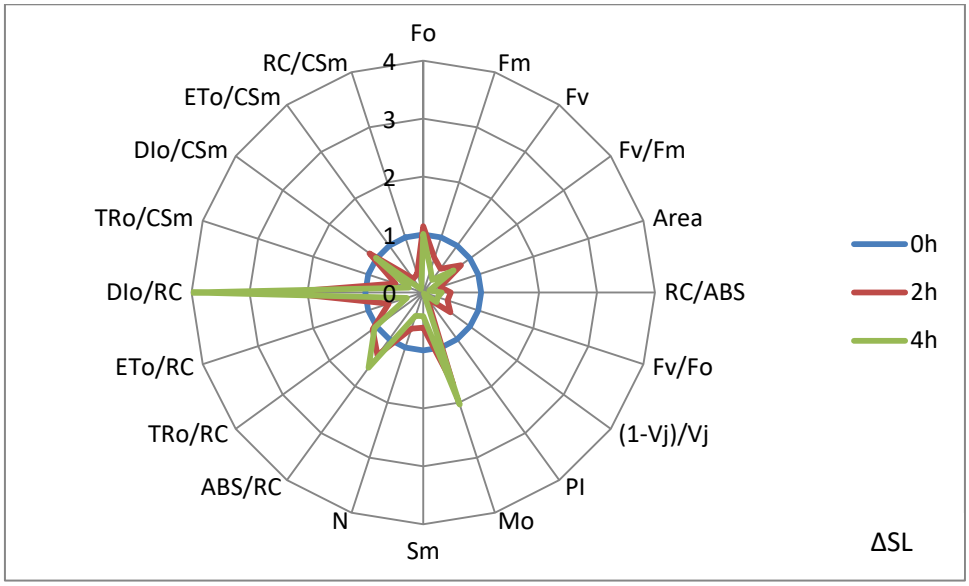


Fig. 21f: JIP analysis by spider plot with ΔS5 values

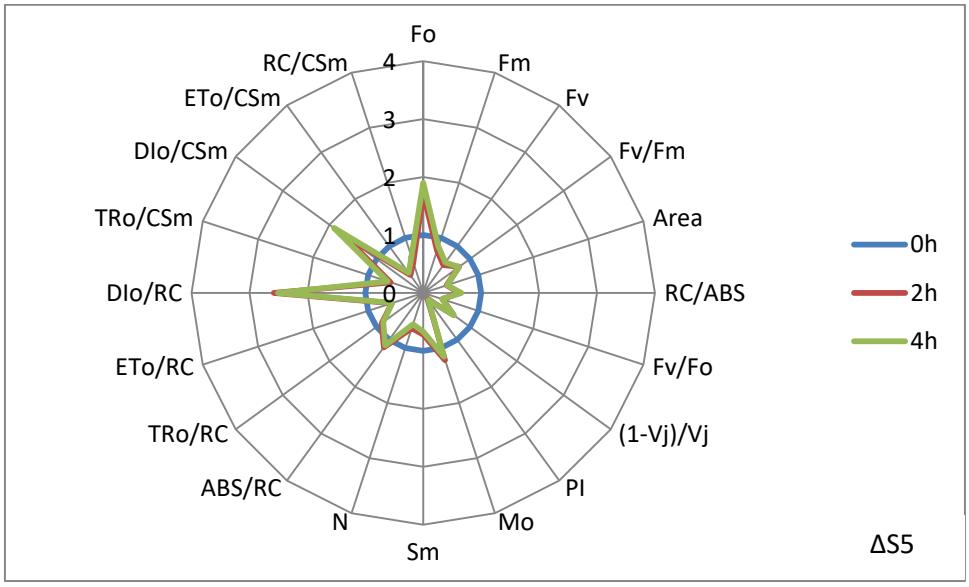


Fig. 21g: JIP analysis by spider plot with Δ78 values

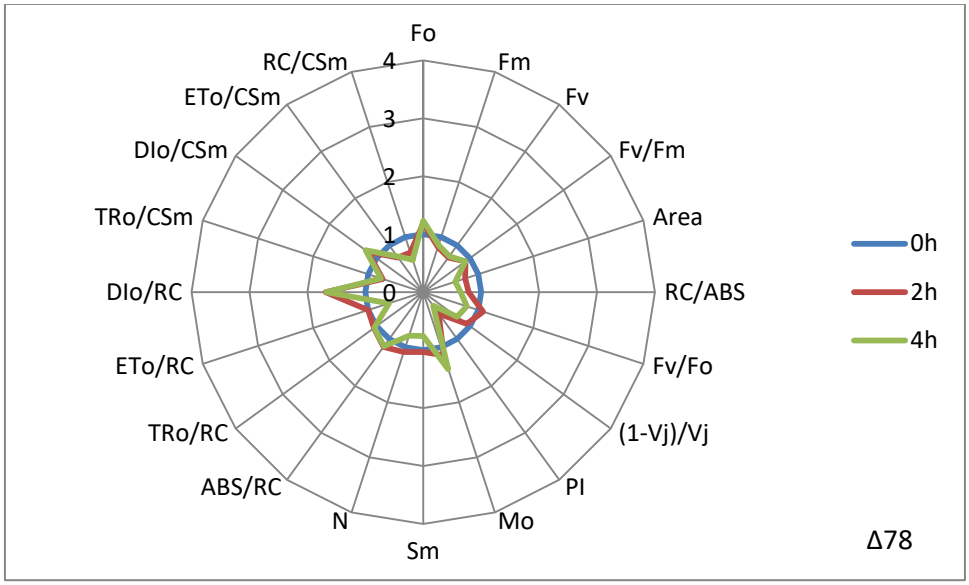
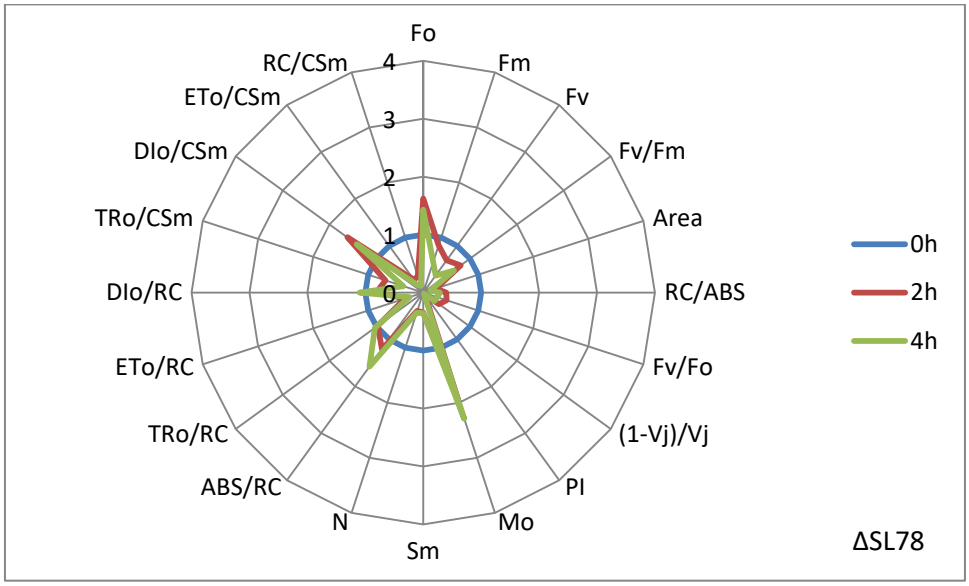


Fig. 21h: JIP analysis by spider plot with ΔSL78 values



4.4 NPQ and CEF mutants show an increased turnover of the D1 protein and is paralleled by a loss of Fv/Fm

Turnover of D1 protein was studied by incubating overnight leaves of different genotypes in 1 mg/ml of lincomycin and then exposed to light (either GL or HL) for different period of times. The remaining amount of D1 protein was then measured by immunoblotting (Fig. 22 a-b). From the figure, it is clear that in absence of lincomycin the level of D1 protein is not affected, neither in GL nor in HL. However, when leaves were pre-treated with the antibiotic, the amount of D1 protein clearly decreased in HL, whereas in GL decrease of D1 was less obvious. In any case, in all mutants, the decrease of D1 was much more marked than in wild type. Loss of D1 was particularly marked in ΔS, Δ5, ΔL and ΔSL78 (b),while it could be slightly less pronounced in the ΔS5, ΔSL (a) and Δ78 (b) genotypes.

Fig.22 a: western blot of thylakoids isolated from different mutants. Plants were treated with grow light (GL) or high light (HL) for the indicated periods of time in presence or absence of lincomycin.

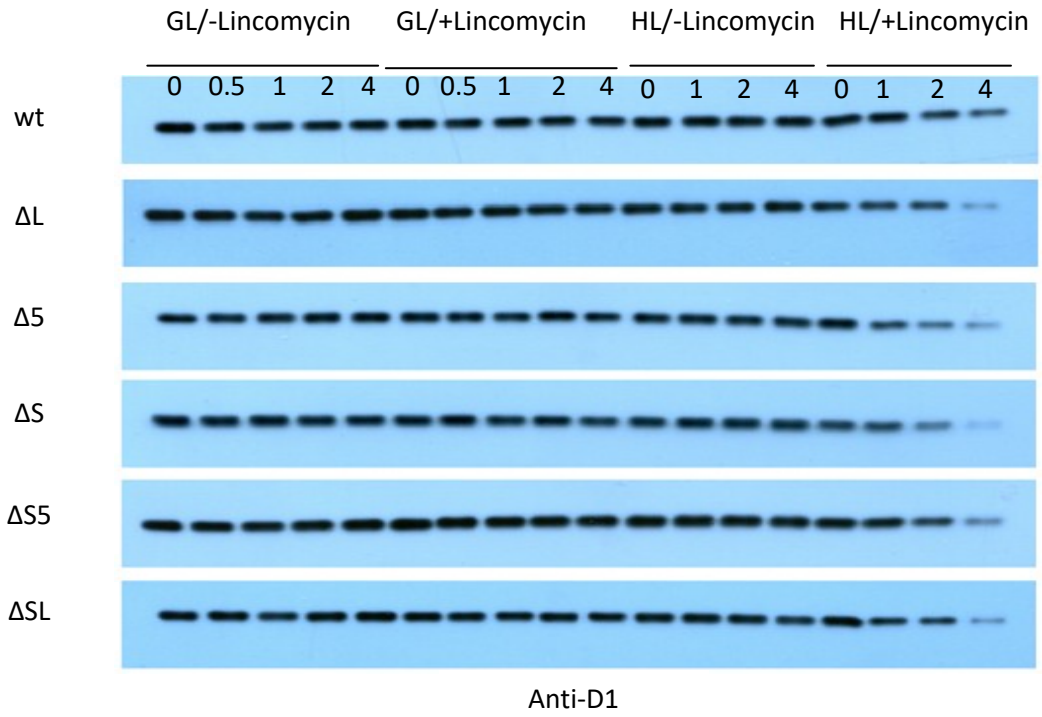
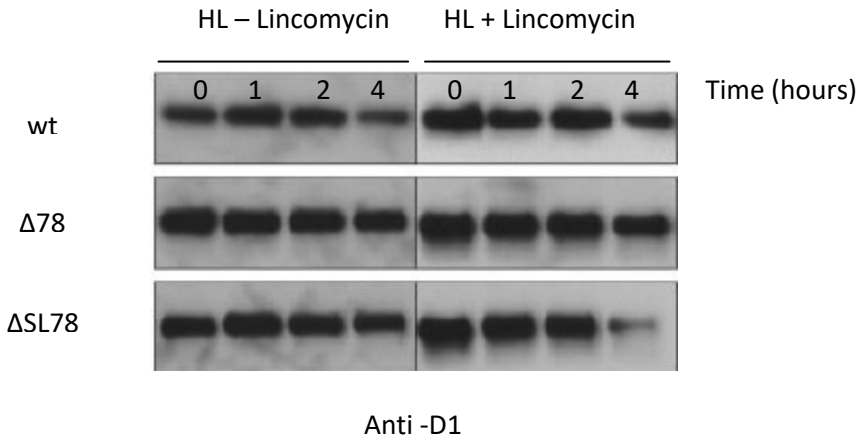


Fig.22 b: western blot of thylakoids isolated from different mutants. Plants were treated with high light (HL) for the indicated periods of time in presence or absence of lincomycin.



4.5 Preinactivation of PSII prevents damage to PSI in CEF/PS mutants

In order to evaluate the impact of PSII photoinhibition on PSI integrity, Y(II) and Y(I) parameters were measured from dark-adapted and HL-treated (2 and 4 hours) plants, in either absence or presence of lincomycin (Fig. 23a-h and Table 2). In wt plants, the Y(II) parameter slightly decreased by increasing the length of exposure to HL, while addition of lincomycin led to a marked drop of Y(II) values (0,46 after 4 h of HL + Lin *vs* 0,83 of dark-adapted leaves, see Table 2). On the other hand, Y(I) values remained higher than 0,80 at the different HL regimes and even in the presence of lincomycin. An almost identical behavior was observed in ΔS and Δ78 leaves, whereas PGR-devoid mutants were highly sensitive to high light conditions. In particular, Y(II) values were around 0,60 upon 4 h of exposure to high light with respect to 0,74 of wt value under the same conditions (Fig. 23 and Table 2). In addition, PSI activity was found to be under the limit of detection in Δ5, ΔSL and ΔS5 thylakoids under the same conditions. Interestingly, the addition of lincomycin to the high-light treatment restored PSI activity to values higher than 0,40 in PGR-devoid mutants, while PSII efficiency dropped to values even lower than 0,20, as in the case of ΔS5 and ΔSL78 leaves. Overall, these findings indicate that in the absence of the ΔpH-dependent photosynthesis control, a marked inhibition of PSII activity is beneficial to prevent PSI inactivation, highlighting further the primary importance of photosynthesis control in photoprotection of PSI.

Fig. 23a: Photodamage to PSI and PSII either in the presence or absence of lincomycin in wt plant.

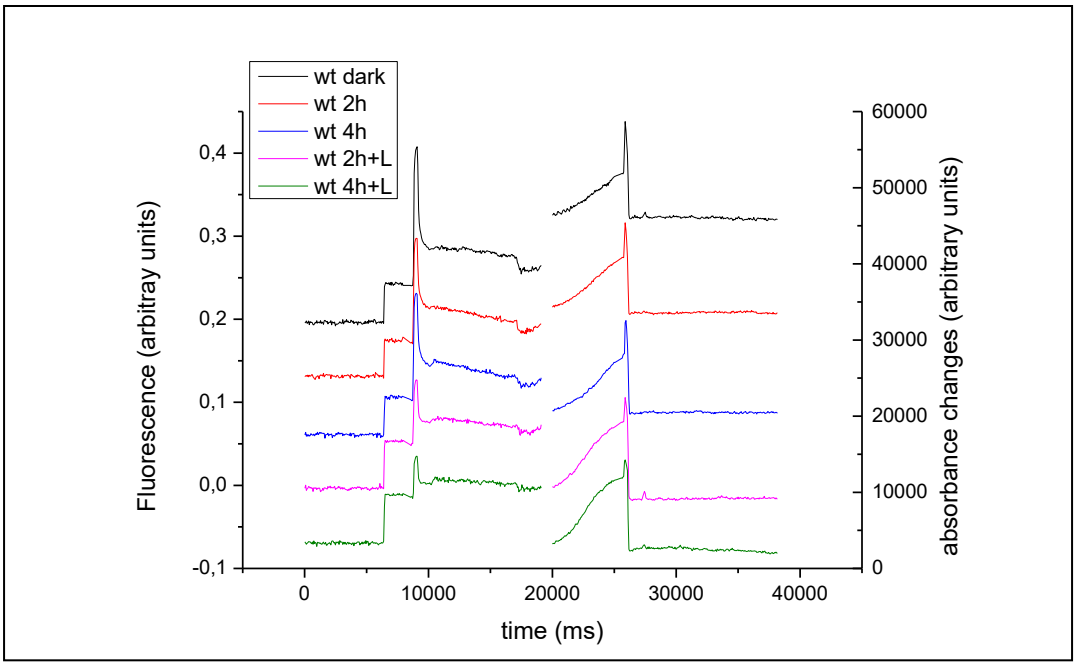


Fig. 23b: Photodamage to PSI and PSII either in the presence or absence of lincomycin in ΔS mutant.

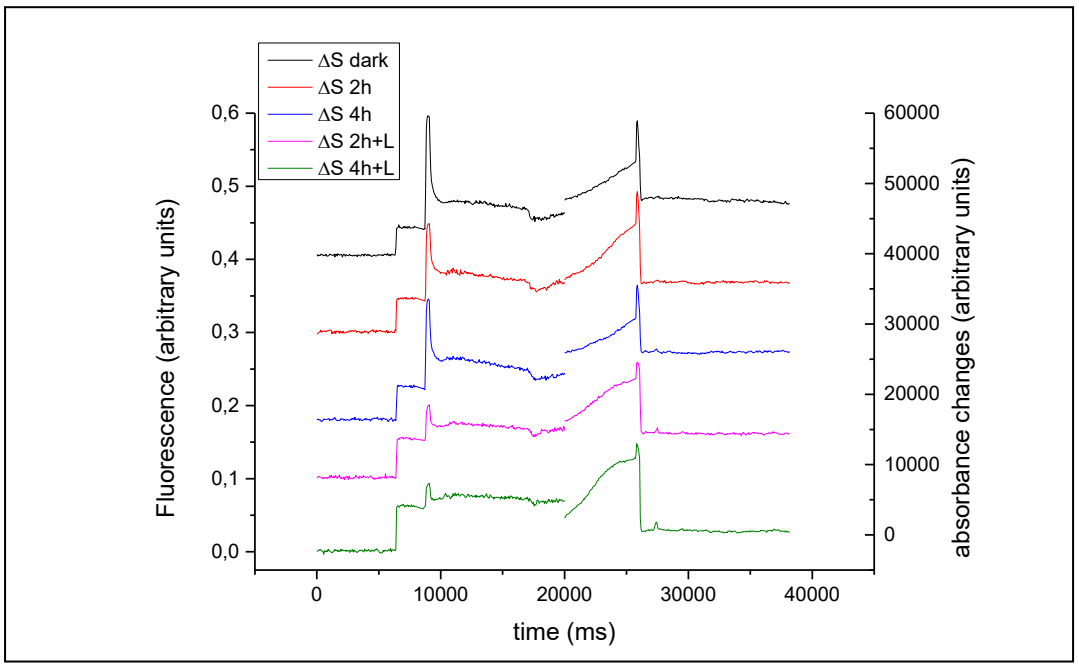


Fig. 23c: Photodamage to PSI and PSII either in the presence or absence of lincomycin in ΔL mutant.

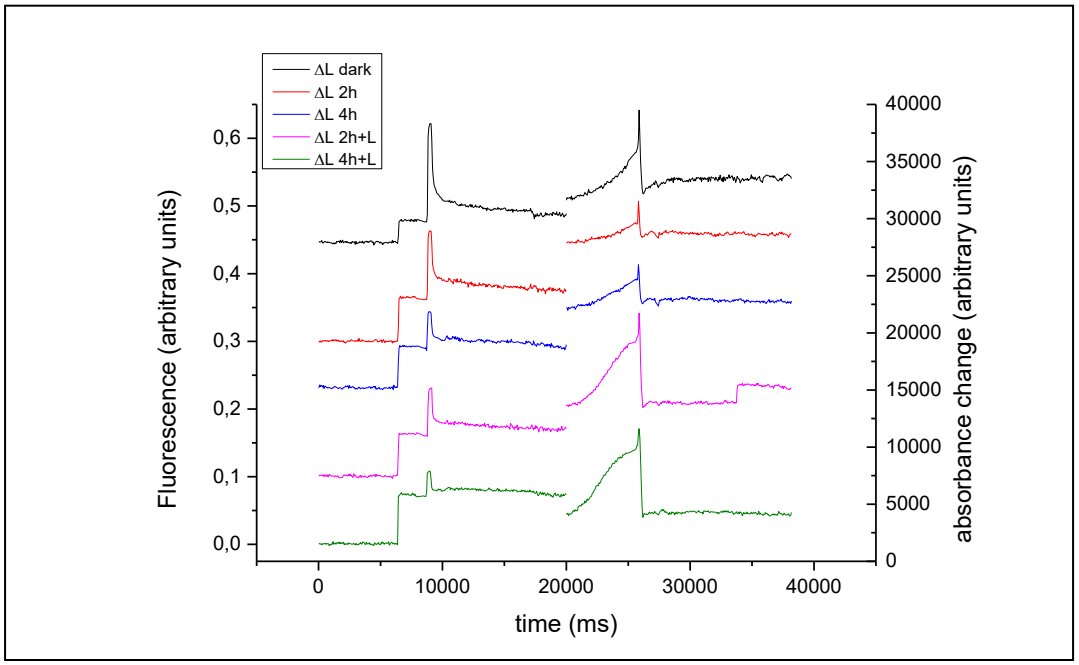


Fig. 23d: Photodamage to PSI and PSII either in the presence or absence of lincomycin in ΔLS mutant.

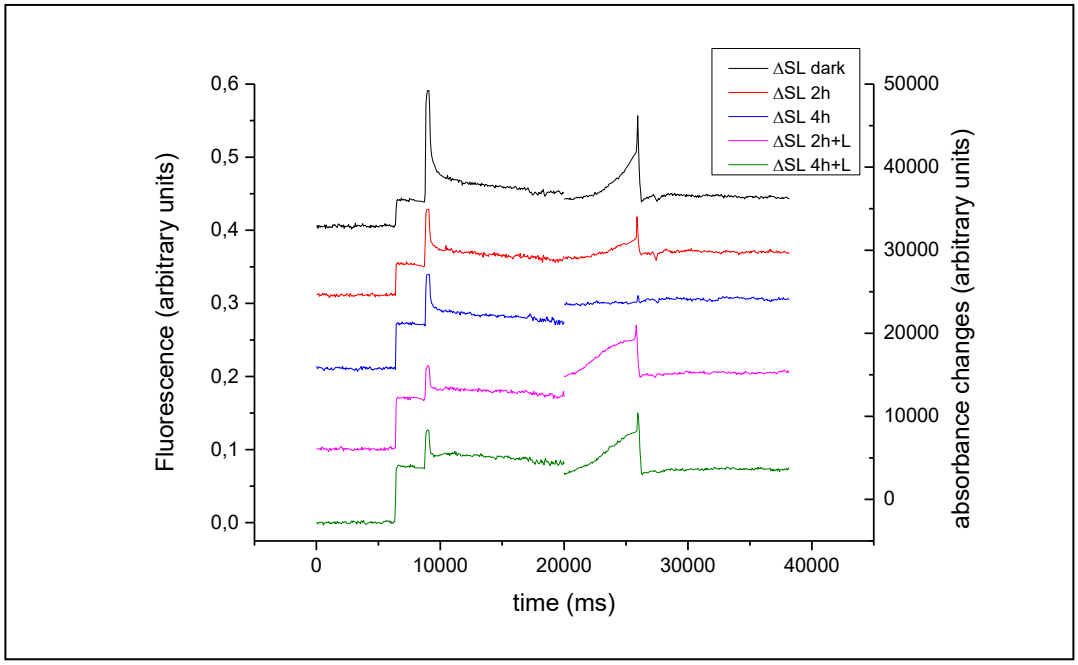


Fig. 23e: Photodamage to PSI and PSII either in the presence or absence of lincomycin in Δ5 mutant.

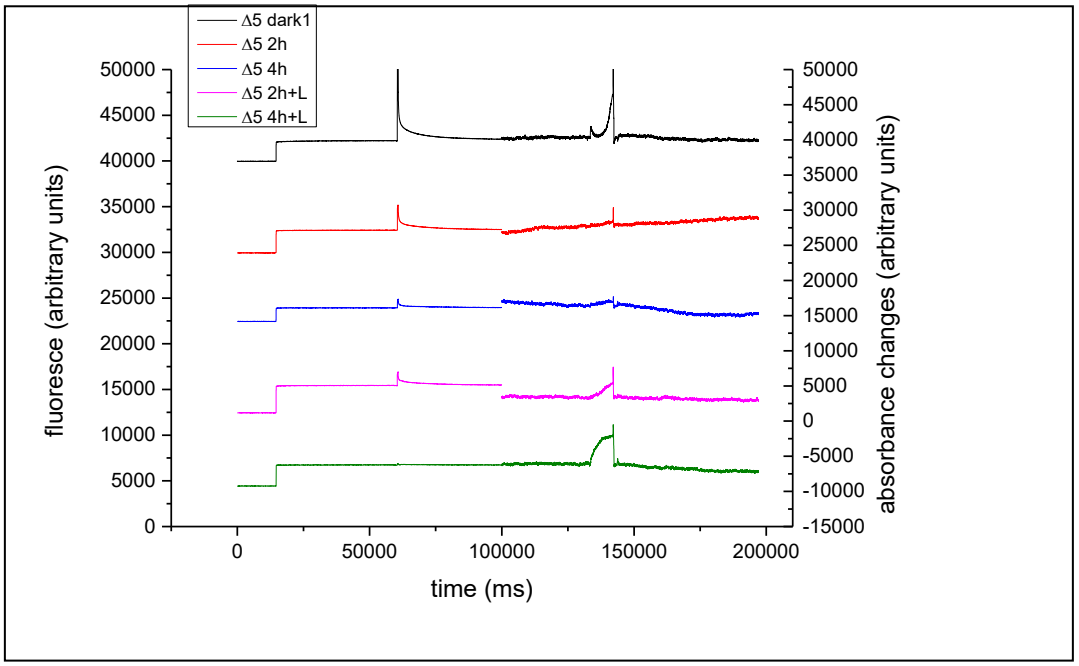


Fig. 23f: Photodamage to PSI and PSII either in the presence or absence of lincomycin in ΔS5 mutant.

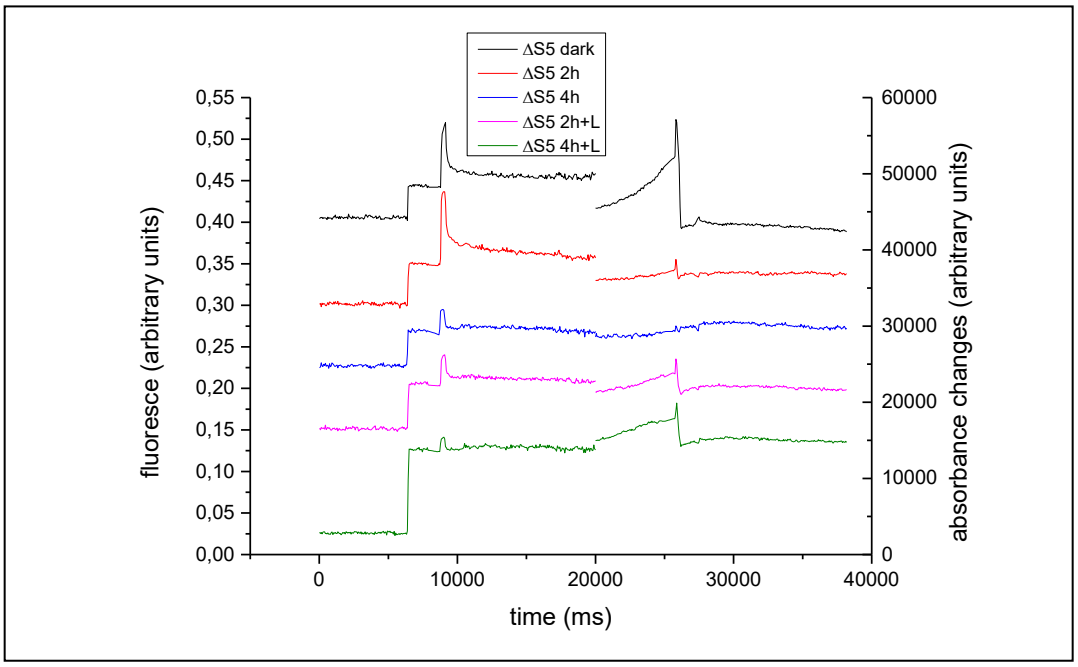


Fig. 23g: Photodamage to PSI and PSII either in the presence or absence of lincomycin in Δ78 mutant.

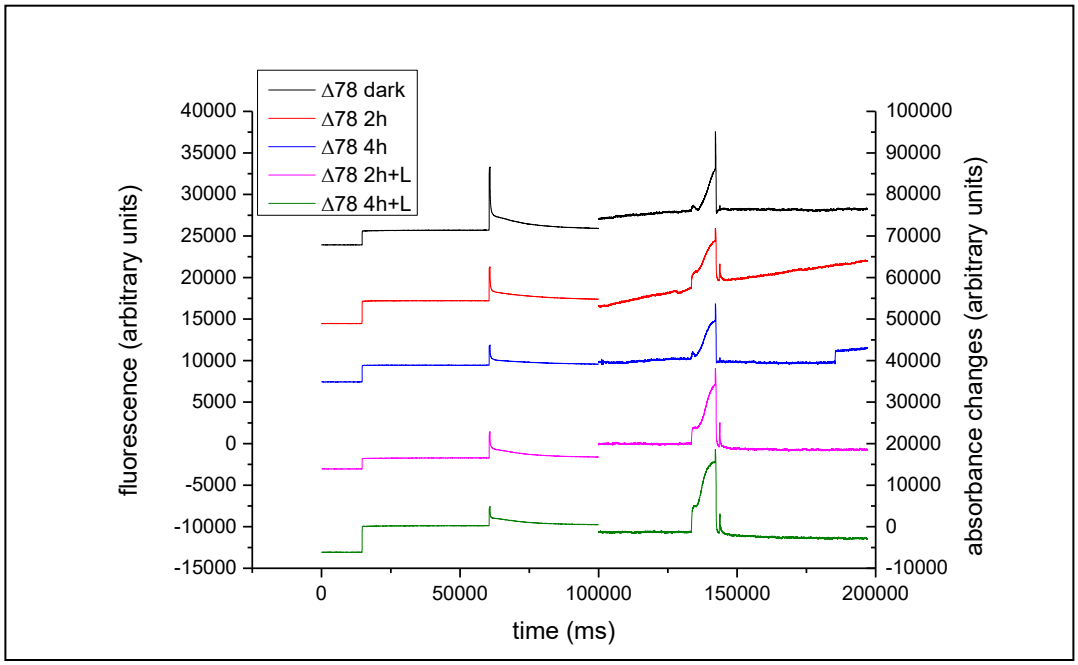
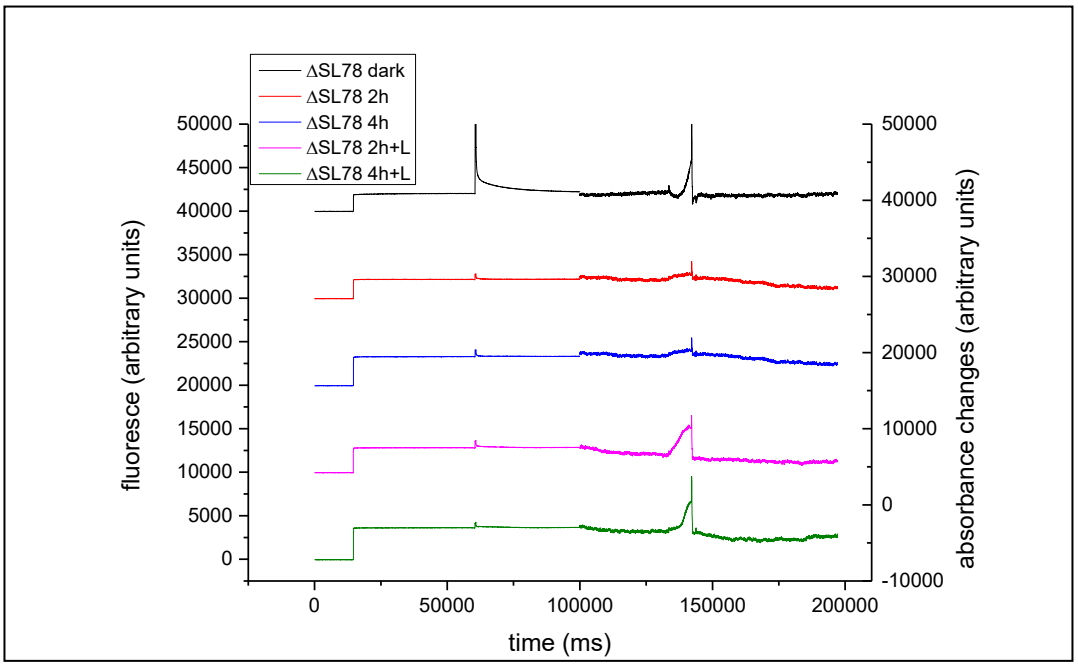


Fig. 23h: Photodamage to PSI and PSII either in the presence or absence of lincomycin in ΔSL78 mutant.



Tab. 2: Photosynthetic efficiency of PSII and PSI measured in wt and mutants after dark adaptation and exposure for 2 and 4 hours to high-light (HL) in either absence or presence of lincomycin. Chl fluorescence emission and P700+ absorbance were recorded to monitor PSII and PSI activity, respectively. Photosynthetic efficiency related to PSII and PSI (Y(II) and Y(I), respectively, were calculated as reported in Materials and Methods. Measurements were performed in triplicates, average values ± s.d. are indicated.

	DARK		2h HL		4h HL		2h HL+Lincomycin		4h HL+Lincomycin	
	Y(I)	Y(II)	Y(I)	Y(II)	Y(I)	Y(II)	Y(I)	Y(II)	Y(I)	Y(II)
wt	0,83±0,02	0,92±0,04	0,75±0,04	0,86±0,06	0,74±0,01	0,80±0,03	0,57±0,03	0,82±0,05	0,46±0,01	0,83±0,05
ΔS	0,80±0,01	0,87±0,05	0,72±0,03	0,83±0,05	0,71±0,03	0,83±0,05	0,48±0,03	0,87±0,03	0,33±0,02	0,83±0,03
ΔL	0,80±0,01	0,68±0,06	0,61±0,03	0,42±0,06	0,57±0,02	0,40±0,02	0,46±0,04	0,69±0,03	0,29±0,04	0,64±0,03
Δ5	0,78±0,02	0,77±0,05	0,67±0,05	0,24±0,01	0,63±0,03	N.D.	0,43±0,02	0,53±0,01	0,23±0,01	0,47±0,04
ΔSL	0,81±0,02	0,72±0,03	0,59±0,02	0,32±0,04	0,56±0,01	N.D.	0,48±0,05	0,61±0,05	0,37±0,05	0,54±0,04
Δ55	0,81±0,01	0,74±0,04	0,66±0,02	0,23±0,02	0,57±0,01	N.D.	0,35±0,02	0,53±0,02	0,17±0,02	0,42±0,03
Δ78	0,84±0,02	0,87±0,05	0,71±0,01	0,78±0,05	0,71±0,01	0,84±0,05	0,41±0,01	0,81±0,03	0,36±0,01	0,85±0,03
ΔSL78	0,83±0,02	0,83±0,04	0,66±0,05	0,32±0,03	0,59±0,03	0,12±0,03	0,28±0,01	0,49±0,02	0,19±0,01	0,49±0,05

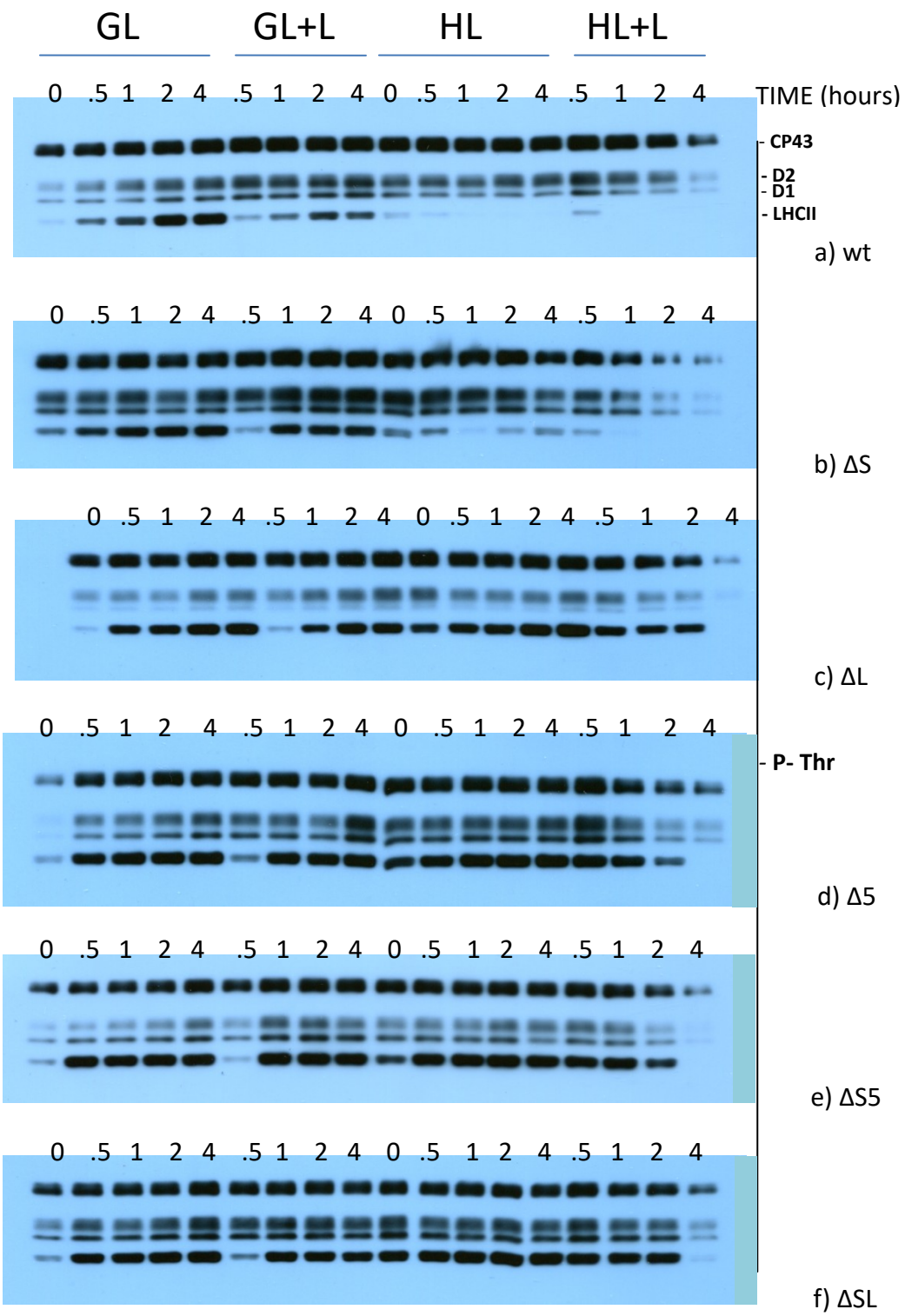
4.6 Phosphorylation studies

In Fig. 24 a-e the phosphorylation pattern of thylakoid proteins for wild type and mutants is reported. In wild type, increasing irradiation time with GL brought about a general increase in phosphorylation of all main phosphoproteins, i.e. LCHII, D_1 and D_2, whereas, in our experimental conditions, CP_{43} is already strongly phosphorylated in our experimental conditions. Exposure to HL (lanes 11-14) did not bring about any further increase in phosphorylation level. The presence of lincomycin did not affect the phosphorylation pattern strongly, even though the final phosphorylation level of LHCII in the presence of lincomycin is lower than in its absence. Moreover, for irradiation time of 4h (lane 10), a decrease in phosphorylation is clearly detected for LHCII. In HL and in presence of lincomycin, a progressive dephosphorylation of all P-protein is observed (lanes 15-18). In the ΔS mutant, the phosphorylation pattern is similar to that of wild type, with an increase in phosphorylation in GL with longer irradiation time (Fig. 24b, lanes 1-5). When lincomycin was present, phosphorylation of LHCII decreased at 4h irradiation, as in the wild type (lane 10). In HL, a similar pattern to wild type was observed. In the ΔL mutant (Fig. 24c), CP_{43} is already phosphorylated in the dark (lane 1), whereas LHCII, D_1 and D_2 are almost completely dephosphorylated. When light is turned on, the level of LHCII phosphorylation increased strongly, whereas that of D_1 and D_2 in a much less pronounced way (lanes 1-5). In HL, the behavior of the mutant is different from that of wild type or ΔS, as the phosphorylation level of LHCII is even stronger than in GL. On the contrary, phosphorylation of D_1 and D_2 did not proceed to the same extent. When lincomycin was present, it has a similar effect as described for wild type and ΔS: in GL there is an initial increase in LHCII phosphorylation but a decrease at 4h irradiation, in HL there is a progressive decrease in phosphorylation. In the ΔSL mutant (Fig. 24f), an even stronger

phosphorylation is observed both in GL (lanes 1-5) and HL (lanes 11-4). When lincomycin is present, again a transient increase in LHCII phosphorylation is observed in GL, whereas in HL, a dephosphorylation is observed at 4h irradiation. In Δ78 and ΔSL78 mutants, no phosphorylation signal was detected (not shown).

Fig. 24 a-f: Thylakoid protein phosphorylation pattern. Thylakoid membranes were isolated from wt, single and multiple mutants, fractionated onto SDS-PAGE, transferred onto nitrocellulose membranes and probed with a polyclonal anti-phosphothreonine antibody. Levels of phosphorylation of CP43, D2, D1 and LHCII are shown over time (0-to-4 hours) upon exposure to optimal growth light (GL) and high-light (HL) conditions. Lincomycin treatment was performed overnight in the dark where indicated (+ Lin).

Fig. 24


GL
GL+L
HL
HL+L
0 .5 1 2 4 .5 1 2 4 0 .5 1 2 4 .5 1 2 4
TIME (hours)
- CP43
- D2
- D1
- LHCII
a) wt
b) ΔS
c) ΔL
- P- Thr
d) Δ5
e) ΔS5
f) ΔSL

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5. DISCUSSION

Light induced inactivation of PSII causes enhanced degradation of the D1 protein, while the PSII recovery relies on *de novo* synthesis of D1. Under PSII photoinhibitory conditions (high light), activity and stability of PSI is not affected, unless high light treatment is performed in cold environment (Sonoike K. , 2011) or in mutant backgrounds lacking the PGR5/PGRL1 complex, in which the ability to form a normal ΔpH and activate the photosynthesis-control is not working properly (Munekage, Y. N. *et* al., 2008, Suorsa M. *et* al., 2012).

A large number of molecular processes have been suggested to function as protection mechanisms against an excess of light. Among those, the most relevant consists in the formation of the qE component of NPQ. Nevertheless, several authors argued that qE could have only a little role in the direct photoprotection of PSII, while could be important for the PSII recovery (Takahashi, S. and Badger, 2011).

The data obtained from PsbS-depleted mutants are in line with these findings, as ΔS plants show sensitivity to high light similar to wild type. Accordingly, in a very recent study is reported that ΔS mutant, after 10 h irradiation with 1500 µmol photons m^{-2} s^{-1} showed a F_v/F_m ratio of about 0.48-0.50 whereas for the wild type the ratio was about 0.58-0.60. However, the fact that high light irradiation, combined with lincomycin treatment, led to the enhanced degradation of D1 in ΔS mutant, indicated that, in mutant background devoid of PSBS, the turnover of D1 is constitutively higher. A similar effect on D1 turnover in PsbS-less mutant was previously reported (Roach, T. *et* al., 2012). Thus, the loss of PSII activity is not observed as long as the rate of damage does not exceed the rate of repair (Aro, E.-M., *et* al., 1993, Li, L. *et* al.,2018). From these observations, we can suggest that the ability to engage a full qE may actually act as a signal aimed to regulate the D1 turnover rather than (or besides to), as a direct photoprotection mechanism

meant to prevent D1 degradation. From a redox point of view, a reduced level of qE correlates with a higher accumulation of centers with a reduced Q_A. As Q_A-Q_B is in equilibrium with Q_AQ_B-, it could be expected that in PsbS-less mutants a higher fraction of centers could accumulate a semi-reduced secondary quinone acceptor, which, according to previous works (Keren, N. *et* al., 1997, Greenberg, B. M. *et* al., 1989), could play a role as a photosensitizer for enhanced degradation of the D1 protein. Thus, the redox state of the plastoquinone, regulated by the extent of qE, could serve as a sensor for turnover of D1 in near-physiological conditions. Mutants with defect in building up proper trans-thylakoidal pH gradient, such as Δ5 and ΔL, show enhanced degradation of D1, similarly to ΔS mutant. However, in PGR-devoid mutants, the treatment with high light caused a strong inactivation of PSII, even in the absence of lincomycin. As they are able to engage about 40% of the qE observed in wild type and are much more sensitive to light than PsbS-less plants (where the extent of qE is near zero), we conclude that, at least in our experimental conditions, the qE component of NPQ does not act as an efficient photoprotection mechanism.

Furthermore, unlike wild type and ΔS plants, the photosynthesis-control depleted mutants are not able to photo-accumulate P_{700}+, as their Y(ND) is near zero at any light intensity due to the low values of both thylakoid proton gradient (ΔpH) and proton motive force (pmf) they can develop (Sato, R. *et* al., 2019). At the same time, they are characterized by the over reduction of PSI acceptors, observed as an increase of Y(NA). Thus besides PSII, PSI is also photodamaged in these mutants, likely because of impairment of iron-sulfur clusters. As no additive phenotypic effects are observed between the photosynthetic characteristics of ΔpH mutants (Δ5 and ΔL) and the ones of higher order mutants (ΔS5, ΔSL, ΔSL78), it can be concluded that the short-term light adaptation is mainly depending on

the Photosynthesis-Control rather than qE, and/or state transitions. Accordingly, mutants lacking of NDH-dependent CET such as crr2-2, crr-3, crr4-2 but still able to photo-accumulate P_{700}+, are more light resistant than the Δ5 mutant, deficient in CET and unable to photoaccumulate P_{700} in the oxidized form (Munekage, Y., *et* al., 2004, Kono, M. & Terashima, I. ,2016).

It is noteworthy that PSI photodamage in PGR-depleted mutants can be markedly reduced through the inhibition of PSII activity, as a consequence of the fact that the amount of electrons injected in the intersystem transport chain is decreased. This indicates that the photosynthesis-control is the main regulator of photosynthetic electron transport and that PSII photoinhibition is the very last option to reduce PSI photodamage (Tikkanen, M. *et* al., 2014). On the other hand, damages to PSI are relevant in inducing inactivation of PSII: the acceptor side of PSII becomes over-reduced and this, in turn, increases the rate of charge recombination with formation of P_{680} and PSII inactivation (Nanba, O. & Satoh, K., 1987, Vass, I. & Styring, S., 1992). In addition, the absence of ΔpH-dependent photosynthesis-control affects the value of pmf, and this could alter the electron transfer between Q_A and Q_B, promoting PSII inactivation (Sato, R. *et* al., 2019).

Overall, it appears clear that the ΔpH-dependent Photosynthesis Control is essential for safeguarding the entire photosynthetic electron transport chain in the thylakoid membrane, and its failure induces a rapid and coordinated inactivation of both PSII and PSI. ΔpH-dependent Photosynthesis Control thus maintains the optimal balance between the two main power-units of the photosynthetic apparatus.

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"Thylakoid Electron Transfer in Salicornia veneta under Different Salinity Levels: a Fluorescence-based Study"

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"Higher order photoprotection mutants reveal the importance of ΔpH-dependent photosynthesis-control in preventing light induced damage to both photosystem II and photosystem I"

Roberto Barbato, Luca Tadini, Romina Cannata, Carlotta Peracchio, Nicolaj Jeran, Azfar Ali Bajwa, Virpi Paakkarinen, Marjaana Suorsa, Eva Mari Aro, Paolo Pesaresi

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“La fine di un viaggio è solo l’inizio di un altro”

J. Saramago

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