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UNCOVERING A MONOCOT-SPECIFIC MECHANISM OF PHOTOPROTECTION: HIGH LIGHT-INDUCED PHOSPHORYLATION OF THE MONOMERIC ANTENNA PROTEIN CP29

(BIO/04)

Tutor:

Ch.mo Prof. BASSI ROBERTO

Co-tutor:

Dott. BETTERLE NICO

Ph.D. Student:

ROSA ANTHONY

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Summary

Summary

Photosynthesis is a crucial process for the formation and maintenance of life on Earth, and it involves the use of light energy to fix CO_2 and form O_2 and biomass. This process is carried forth by photosynthetic organisms, which evolved multi-subunit pigment-binding complexes, namely Photosystem II (PSII) and Photosystem I (PSI). These photosystems are the key players in light harvesting and charge separation for the transport of electrons to form reducing power and ATP, both required for the fixation of CO_2. But more than often light may result either too scarce or in excess with respect to plants photosynthetic capacity, and may result dangerous, leading to the formation of Reactive Oxygen Species (ROS) and photo-inhibition, which in turn leads to compromised plant growth and losses in crop productivity. Thus, it became necessary for photosynthetic organisms to evolve mechanisms capable of preventing or limiting such damages, and at the same time allow them to regulate the amount of light they can harvest, depending on light intensity and quality. Among the various mechanisms these organisms can exploit, State Transitions and Non-Photochemical Quenching (NPQ) have been of pivotal importance in the field of photosynthesis research in the last decades. While the former involves the transition of harvesting antenna complexes between photosystems to optimize energy distribution in low light conditions, the latter is necessary for the thermal dissipation of excess energy in high light condition, in order to avoid photo-damage to the photosynthetic apparatus. Furthermore, to maintain the photosynthetic apparatus always functional, algae and plants have evolved repair mechanisms for the turnover of ROS-damaged proteins. To regulate all of these mechanisms properly, reversible phosphorylation of the photosystems subunits represents an important factor. This process is carried out by kinases and phosphatases, which act on different substrates and in different conditions. STN7 and STN8 kinases phosphorylate respectively LHCII and PSII core complexes and they are active in different conditions. While the former is active mainly in low light, the latter acts in excess light conditions. Their activity is counteracted by a set of phosphatases, respectively, TAP38/PPH1 and PBCP.

Up until recent years, there has been no evidence of a correlation between phosphorylation of antenna proteins in high light and heat dissipation. Just recently a connection between the two has been reported in the model monocot

Oryza sativa, where CP29 phosphorylation occurs and has a positive effect on NPQ and ROS protection, but the mechanism still needs to be fully clarified.

During my Ph.D. thesis, I focused on unravelling the high light-induced phosphorylation of the minor antenna protein CP29, in order to determine the actors involved in this process in monocots and better understand its involvement in NPQ, with the aim of transferring this mechanism to dicots, which lack this photo-protective trait. If this could effectively be obtained, increase in photoprotection could be inserted in species important to the food industry, in order to improve crop productivity, for which abiotic stresses such as high light represent a major issue.
Furthermore, characterization of this mechanism is of great importance to better understand the mechanisms underlying photoprotection in plants. Determination of the kinase/phosphatase pair responsible for the regulation of CP29 phosphorylation, and the changes that this modification implies on the protein's conformation, could shed light on the role of this component in the short-term responses plants exploit to defend themselves from high-light induced photo-damage.

In Chapter 1, in order to determine the kinase/phosphatase pair responsible for the high light-induced phosphorylation of CP29, we analysed an *stn8* mutant of rice, model for monocot plants, where phosphorylation of the PSII core is absent. A previous study conducted in our laboratory demonstrated the independence of this mechanism from STN7 kinase. It was reasonable to suggest as a candidate STN8, given that this protein is generally active in high light, although phosphorylation of an LHC protein had not been reported previously for this protein. Furthermore, we set out to determine if the phosphatase involved in this process was PBCP, whose Arabidopsis orthologous was reported to have an opposite role to that of STN8. Given the lack of the appropriate mutant in rice, this was accomplished through the expression of a recombinant form of the rice phosphatase and subsequent analysis *in vitro.* Our work clearly demonstrated that indeed STN8 and PBCP counteract in regulating CP29 phosphorylation in high light. Results of this work have been recently published.

As previously mentioned, CP29 phosphorylation in high light is unique to monocots and is promoted by the STN8 kinase. Through a bioinformatic analysis we compared the sequences of CP29 and STN8 of various monocots and dicots. Surprisingly, CP29 showed a very high similarity among the various species considered, and the monocot site of phosphorylation, a threonine residue, was maintained in dicots as well. On the other hand, STN8 showed differences between the two groups: while the catalytic domain remained conserved, the N-terminal region was much longer in monocots. This led us to believe that this difference could be responsible for the different substrate specificity observed between monocot and dicot's STN8. Indeed, expression of the rice kinase in *A. thaliana* mutants lacking the STN8 led to restoration of PSII core phosphorylation and to phosphorylation of CP29 as well. This additional mechanism led to slight increases in NPQ in the complemented lines when subject to high light intensities, indicating that this protein could indeed have a specific role in enhancing photoprotection.

Expression of the rice STN8 kinase in Arabidopsis performed in Chapter 2 led to the phosphorylation of CP29, but to better determine the individual contribution of the minor antenna from that of the PSII core phosphorylation, we decided to prepare different versions of CP29 of Arabidopsis, carrying mutations at the phosphorylated threonine site to suppress it and to mimic its constitutive activation. This was performed in Arabidopsis given the lack of availability of mutants in rice compared to the rich library present for Arabidopsis, and the ease with which the former is genetically manipulable. These constructs were used to co-transform knockout mutant lines of CP29 together with *Os*STN8, in order to reproduce the high light-induced phosphorylation mechanism described in chapter 2. Obtaining these lines would allow discerning the effect of CP29 phosphorylation on photoprotection from that of the PSII core phosphoproteins. Furthermore, we set out to perform *in vitro* analysis on the phosphorylated and dephosphorylated form of CP29, both from rice and Arabidopsis, in order to determine whether or not conformational changes are induced on the protein, such as those observed for CP29 of maize, leading to a

better understanding of how CP29 acts in high light conditions. Work is still ongoing on this subject, and future perspectives regarding *in vivo* and *in vitro* analyses are discussed in the chapter.

Introduction

Oxygenic photosynthesis

One of the most important reactions on Earth is termed photosynthesis, it consists in the conversion of solar energy to chemical energy, and it is carried out by a number of organisms, mainly plants, algae, and a number of bacteria. The importance of this process lies in the fact that it is the basis for all life on our planet, as it is responsible for the production of oxygen in our atmosphere and the fundamental basis of our food chain.
Oxygenic photosynthesis involves the conversion of water and carbon dioxide to oxygen and organic molecules such as carbohydrates.

Overall reaction: $nH_2O + light + nCO_2 \rightarrow (CH_2O)_n + nO_2$

The overall process of photosynthesis can be divided into two phases, commonly known as "light" and "dark" phases: the light phase, in which water is split into oxygen, protons and electrons using light as energy to bring forth the reaction; the dark phase, so called because it does not directly involved the energy derived from the sun, in which the NADPH and ATP formed during the light phase are used to reduce CO_2 into carbohydrates through the Calvin-Benson cycle.

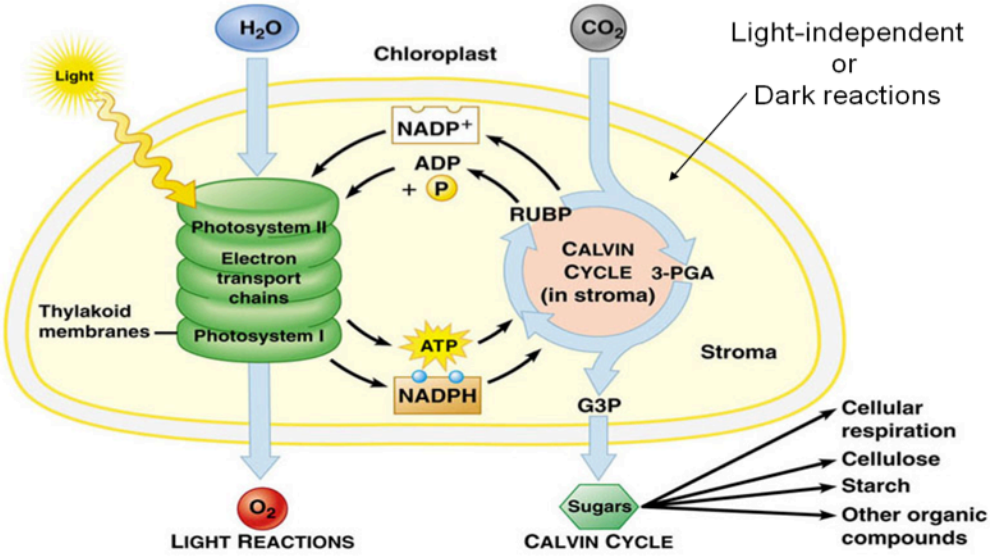


Fig. 1. ***Simplified representation of light and dark reactions in photosynthesis***

In photosynthetic organisms this process of light conversion to organic compounds takes places in specific organelles known as chloroplasts.

Light and dark reactions occur in separate areas of the chloroplast, and this is due to the complex structure of the organelle itself: two outer membranes, known as envelope, delimit an aqueous space known as stroma, where the dark phase takes place. Inside the stroma sits a third membrane structure where the light phase takes place, named thylakoids, which enclose a second aqueous solution termed *lumen*. Thylakoid membranes show two different domains: a stacked formation which is termed **grana**, which is interconnected to other stacked thylakoids through a single pair of membranes termed **stroma lamellae.**

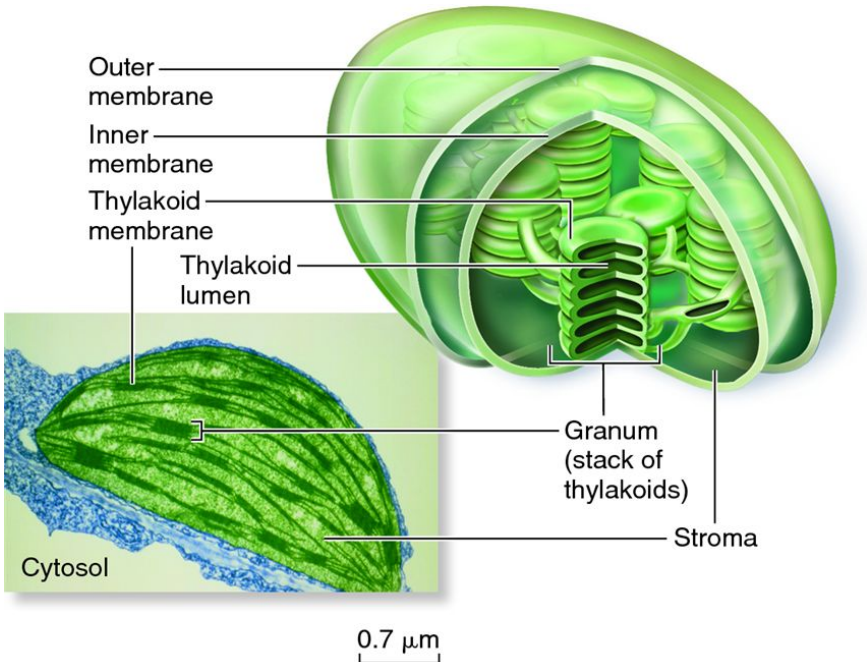


Fig. 2. ***Schematic organization of the chloroplast and its membrane layers, with its various components (top). Transmission electron microscopy image of the chloroplast (bottom).***

Oxygenic photosynthesis can be traced back to an endosymbiotic event occurred circa 1-1,5 billion years ago, originated by the endocytosis of a cyanobacterium by an ancestral eukaryotic cell. This single evolutionary event gave rise to all other organisms, such as plants, algae and diatoms, which perform oxygenic photosynthesis.

The complexity of the chloroplast organization in higher plants is further shown by the communication network established between the two genomes,

necessary for the formation of the systems involved in the photosynthetic process. The organs involved in photosynthesis in land plants are the leaves, and each cell composing the leaf contains about 100 chloroplasts. Each chloroplast contains ~ 100 copies of its circular genome of about ~120-160kb, and which encodes for only about 5% of the amount of proteins necessary for the photosynthetic apparatus assembly, with the rest being encoded by the nucleus and subsequently imported inside chloroplasts.

Light phase

Reaction:

$$\text{light} + 2H_2O \rightarrow O_2 + 4H^+ + 4e^-$$

As mentioned above, the light phase of photosynthesis takes place in the thylakoid membranes, where four embedded complexes are capable of capturing light energy and transforming it into ATP and reducing power (NADPH). These four major protein complexes are: Photosystem II (PSII), Cytochrome b6f (Cyt-b6f), Photosystem I (PSI), ATP synthase (ATPase). Among the thylakoid membranes, these complexes are heterogeneously distributed: while PSII is mainly present in grana partitions, PSI and the ATP synthase are mostly present in the stroma lamellae. Cyt-b6f in turn is homogenously distributed along all partitions of the thylakoid membranes, as demonstrated by freeze fracture electron microscopy.

The first event occurring in the light phase of photosynthesis is the absorption of solar energy by pigment molecules located in the thylakoid membranes. These pigments are bound to both photosystems, both to the central core, known as reaction center (RC), and to the protein antenna system surrounding the RC, known as Light Harvesting Complex (LHC), and act in absorbing light and transferring excitation energy to the core. The two photosystems act in series, forming what is called Z-scheme, as shown in the image below (Fig. 3)

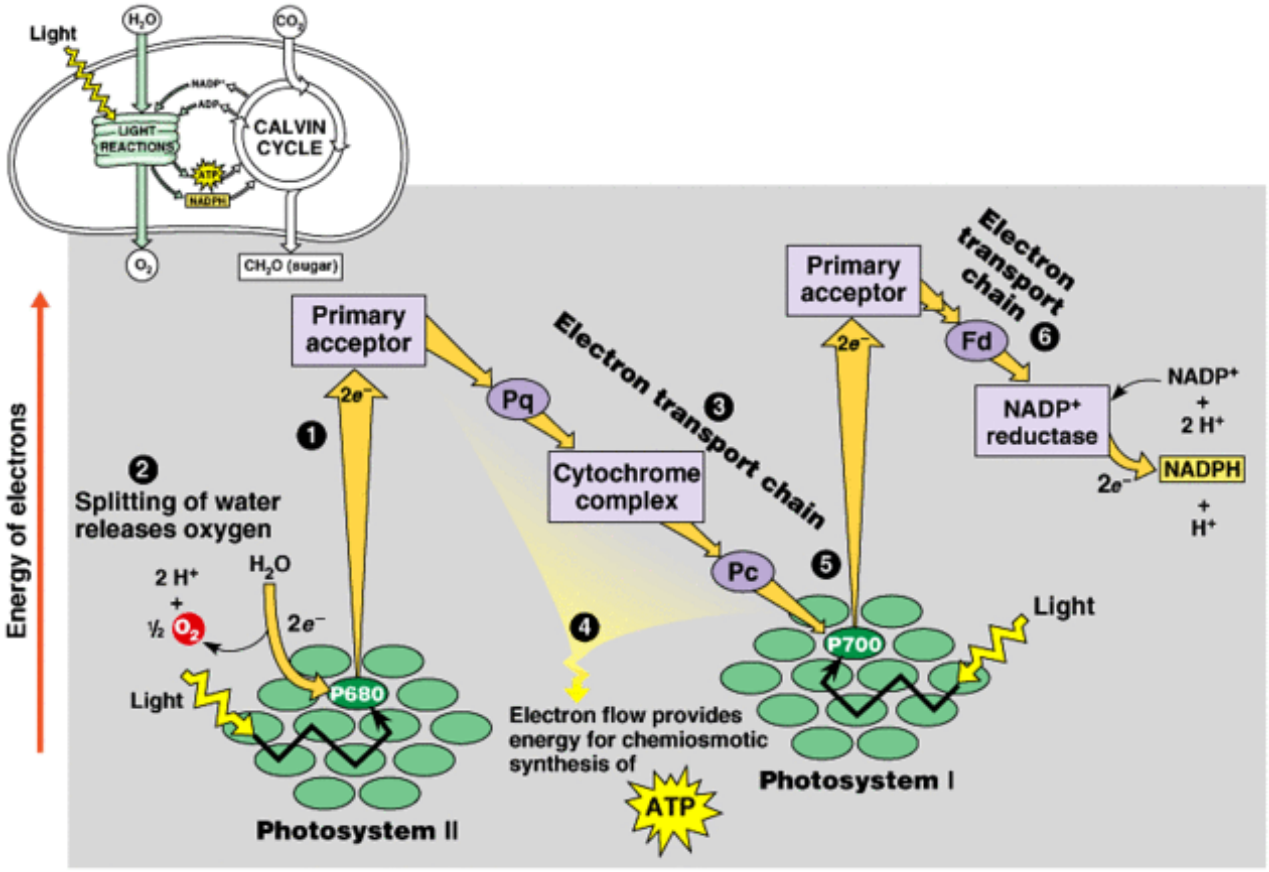


Fig. 3. ***Z-scheme of the electron transport chain in photosynthesis. All cofactors involved in electron transport chain from water to NADP⁺ are shown.***

Light absorbed is transferred in an ordered way between the different pigments forming the antenna system, probably through a mechanism known as Forster's transfer. The energy transfer occurs in an energetically down-hill manner, moving from Chl b (647nm) to Chl a (663nm) molecules until they reach the reaction centers of either PSII, P680 (which absorbs at 680nm), or PSI, P700 (which absorbs at 700nm).

When P680 is excited it releases an electron to Pheophytin acceptor (Pheo) towards the stromal side of the thylakoid membrane, which is then donated to Q_A, a monovalent quinonic acceptor, and then to a divalent plastoquinone (PQ) molecule. With the second photocycle PQ takes up two protons from the stroma and forms plastoquinol (PQH_2), which diffuses inside the thylakoid membrane to the Cyt-b6f complex, where it is oxidized. Through this process P680 becomes a strong oxidant ($P680^+$), which is capable of taking electrons from water through a tyr residue (Yz) and the Oxygen Evolving Complex (OEC), leading to the formation of O_2 and the release of protons in the lumen, causing acidification of the thylakoid lumen, and neutralizing $P680^+$ back to P680.

The electrons donated to the Cyt-b6f complex are later transferred to a copper-containing protein, plastocyanin, with the simultaneous release of two additional protons in the inner thylakoid space (lumen). Only one electron at a time is donated to plastocyanin, with the other one being recycled back to PSII. This is part of what is called the Q cycle: for every two electrons transported to PSI, four protons are translocated to the lumen.

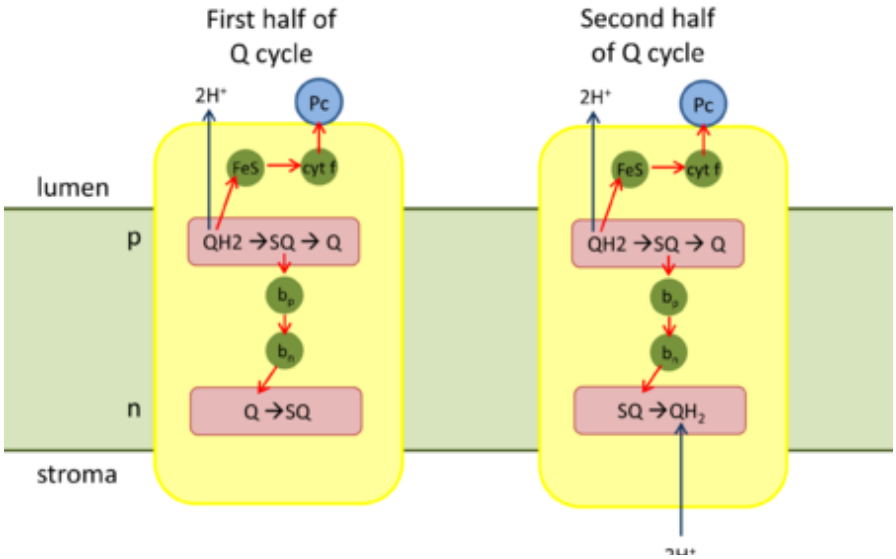


Fig. 4. ***Schematic representation of the PQ pool cycle***

PSI reaction center P700, as in the case of PSII, contains a special pair of chlorophylls, which upon excitation donate an electron to ferredoxin (FD) on the stromal side of the thylakoid membrane. Two electrons from ferredoxin are necessary for the $NADP^+$ oxidoreductase to convert $NADP^+$ to NADPH, which is

released in the stroma. $P700^+$, the oxidized form of P700, is reduced by the electrons arriving from plastocyanin. While PSII has a quantum yield efficiency of about 0.83, PSI shows a quantum yield of almost 1 (0,93). As mentioned earlier, the mobilization of electrons through the transport chain leads to the formation of an electrochemical potential gradient caused by the accumulation of protons in the luminal side of the thylakoid membranes. This gradient is used in order to power the ATPase, which pumps protons back to the stroma while transforming ADP + Pi into ATP, which is used among other things in the Calvin-Benson cycle to fix CO_2. The fourth protein complex of the electron transport chain is a multimeric complex, with both stromal (CF_1) and transmembrane regions (CF_0), driven by proton motive force: CF_0 drives protons from the lumen to the stroma, coupled to CF_1 which synthesizes/hydrolyzes ATP. There are 4 subunits composing CF_0: I, II, III, IV (in a probable stoichiometry of 1:1:14:1, with the 14 subunits forming a ring-like structure). Some subunits are stationary such as I, II, IV, α, β and δ, while others are rotary, like III, ε and γ, and the whole CF_1 – CF_0 complex acts as a rotary proton motive force (Fig. 5).

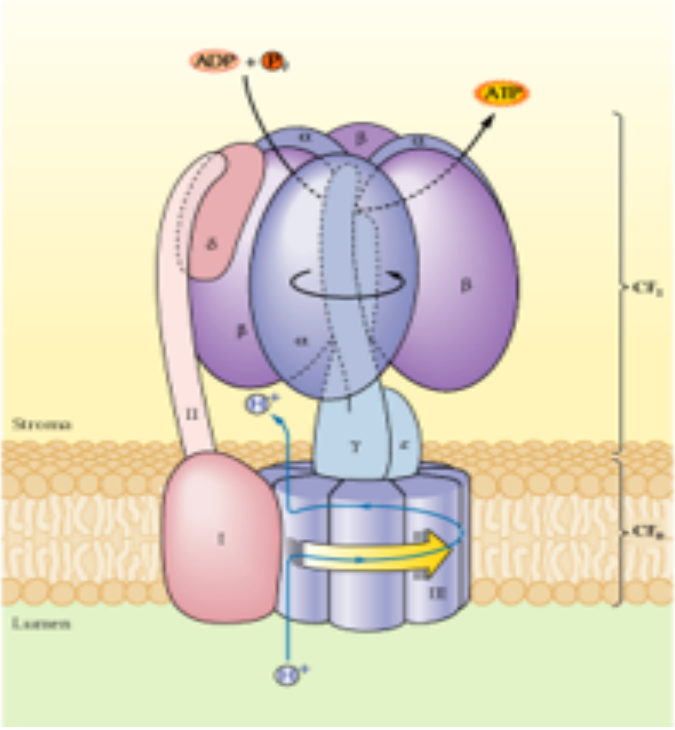


Fig. 5. A simplified model of the ATPase structure and function

The dark phase

Reaction: $3CO2 + 3RuBP + 6ATP + 6NADPH \rightarrow 6GAP + 6ADP + 6NADP + 6Pi$

$5\ GAP + 3ATP \rightarrow 3\ RuBP + 3ADP$

Via the Calvin-Benson cycle CO_2 is fixed to carbohydrates, a process carried out consuming the ATP and NADPH formed during the light reactions, thus regenerating ADP and $NADP^+$. The first step of the cycle consists in the combination between CO_2 and a 5-carbon sugar, ribulose 1,5-bisphosphate, in a reaction catalyzed by Rubisco (ribulose 1,5-bisphosphate carboxylase/oxygenase). The intermediate that is formed is a 6-C sugar that splits immediately into two molecules of 3-phosphoglycerate. Following phosphorylation (with consumption of ATP) and subsequent reduction, a 3-C molecule is formed, namely glyceraldehyde 3-phosphate (GAP). Every three molecules of CO_2 fixed to ribulose 1,5-bisphosphate, 6 molecules of GAP are formed. Of these six molecules, however, only one is used for metabolic processes, as the other five are used in order to regenerate ribulose 1,5-bisphosphate, with further consumption of ATP (Fig. 6).

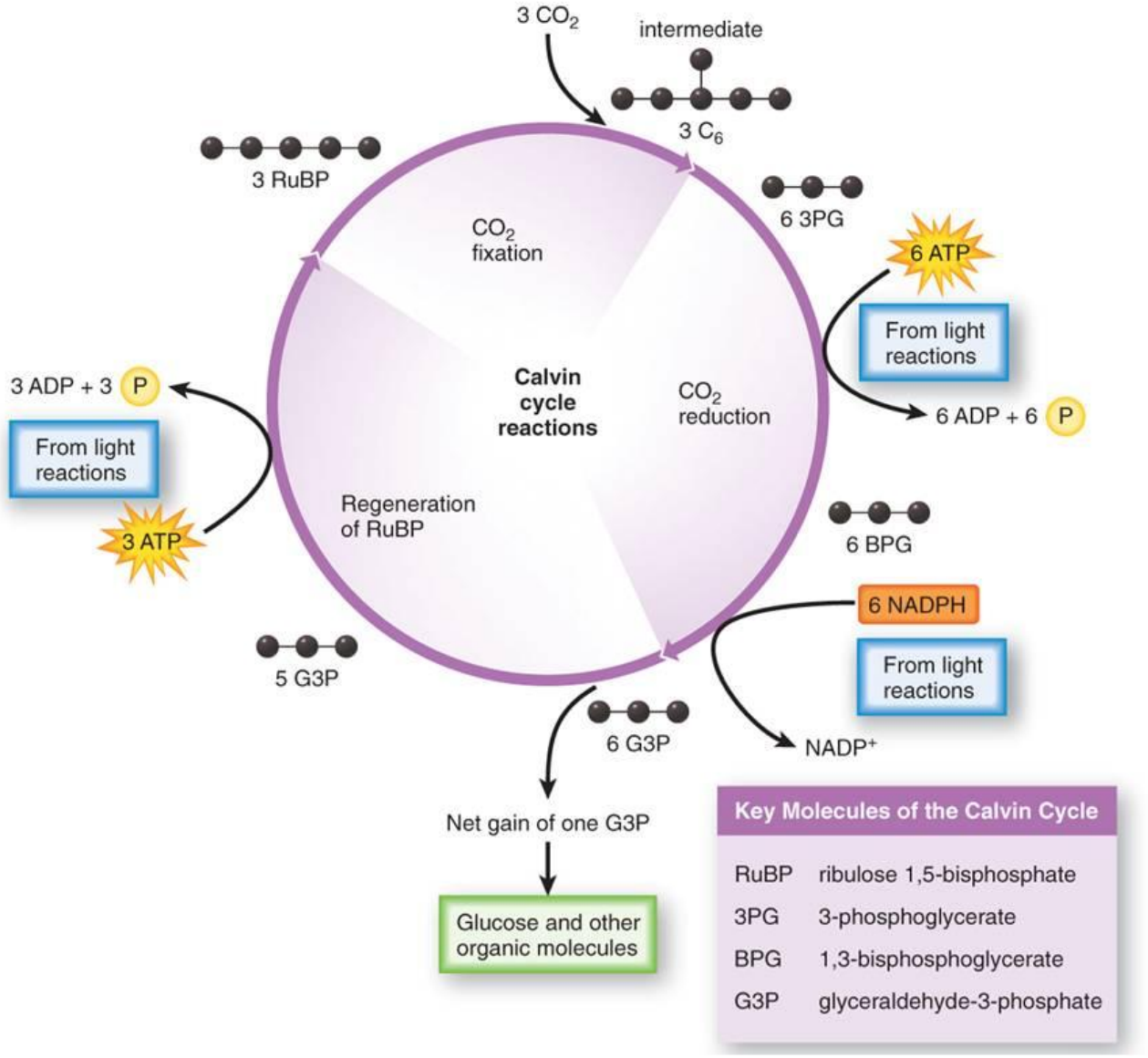


Fig. 6. ***Calvin-Benson cycle representation divided in its three steps, with the intermediates formed and cofactors utilized.***

Light Harvesting Pigments

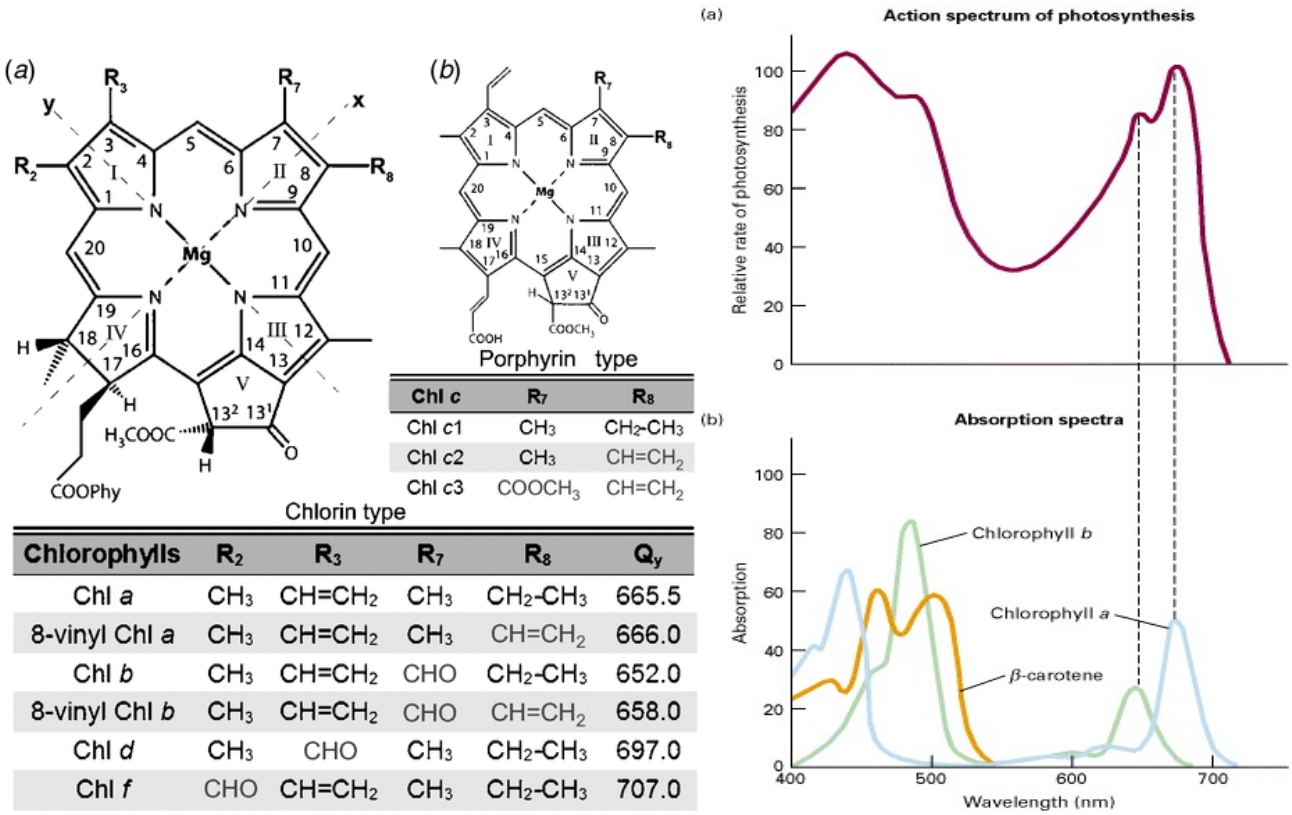
As previously mentioned, photosynthesis begins with the absorption of light energy by pigments that are located in the thylakoid membrane. There are very few pigments involved in the photosynthetic process, the most well-known of which are chlorophylls (Chls), but there are also Carotenoids (Cars), and in certain algae and bacteria, bilins. These pigments all have in common within their chemical structure an alternating series of carbon single and double bonds, which form a conjugated π-electron system (Johnson, 2016). They are the main

actors responsible for light absorption, charge separation and energy transfer to the reaction centers of both photosystems.

Chlorophyll

Chlorophylls are constituted of 4 pyrrole rings (designated I to IV), ligated into a tetrapyrrole ring, which harbours in its center a magnesium atom (von Wettstein et al., 1995). Biosynthesis of the molecules takes place in the chloroplast and the process can be divided into 4 steps: (1) synthesis of ALA (5-aminolevulonic acid), a non-protein amino acid which is formed from the intact carbon skeleton of glutamate. (2) two molecules of ALA are condensed to yield porphobilinogen, catalyzed by ALA-dehydratase. Four porphobilinogen are then converted into hydroxymethylbilane, the ring is closed and the acetyl and propionyl groups of the D ring are isomerized, leading to formation of uroporphyrinogen III; (3) subsequent oxidations which lead to protoporphyrin IX; (4) there is a branch point which gives rise to chlorophyll by insertion of the Mg^{2+} to the tetrapyrrole ring and esterification of Chlorofyllide *a* with phytol. There are different types of chlorophylls, the most important of which is chlorophyll *a.* This molecule allows photosynthesis to occur, and all photosynthetic organisms contain this type of chlorophyll (except photosynthetic bacteria, where a related pigment bacteriochlorophyll is present instead). Chlorophyll *a* and *b* are the most commonly distributed, but other chlorophylls have been observed in nature: Chl *c1* and *c2,* Chl *d,* which is found in cyanobacteria and red algae, and the most recent discovered Chl *f*, which has been found only in wet environment cyanobacteria (Behrendt et al., 2015). The most distributed chlorophylls in vascular plants are chlorophyll *a* and *b,* which differ from one another as Chl *b* contains an aldehyde in the second pyrrole ring, derived from oxygenation of Chl *a.* The ability of chlorophylls to absorb light in the visible region of the spectrum is due to the high number of conjugated double bonds present in their structure. Moreover, the absorption spectra of the two Chl does not overlap in solution, increasing the efficiency of light harvesting since the spectral range in which these two Chls can absorb is wider. Two bands compose the absorption spectrum of Chls: the Qy transition band which

corresponds to the red-most band, with peaks around 640 for chlorophyll *b* and 670 for chlorophyll *a.* This indicates the transition of an electron from S_0 to S_1. In the blue-violet region there is another strong band, called "Soret" band, which corresponds to a higher transition state, and has a maximum at 430nm for Chl *a* and 460nm for Chl *b.* Plants seem green due to the characteristic of their absorption spectra. The structure of chlorophylls, in particular the presence of a Mg ion at the center of the porphyrin ring, allows for chlorophylls to interact with nucleophilic amino acids present in proteins, such as histidine. For this reason chlorophylls are found bound to thylakoid membrane proteins, resulting indispensable for the correct folding of certain photosynthetic proteins, such as LHC proteins (Paulsen et al., 1993).



Chl *c*	R_7	R_8
Chl *c*1	CH_3	CH_2-CH_3
Chl *c*2	CH_3	$CH{=}CH_2$
Chl *c*3	$COOCH_3$	$CH{=}CH_2$

Chlorophylls	R_2	R_3	R_7	R_8	Q_y
Chl *a*	CH_3	$CH{=}CH_2$	CH_3	CH_2-CH_3	665.5
8-vinyl Chl *a*	CH_3	$CH{=}CH_2$	CH_3	$CH{=}CH_2$	666.0
Chl *b*	CH_3	$CH{=}CH_2$	CHO	CH_2-CH_3	652.0
8-vinyl Chl *b*	CH_3	$CH{=}CH_2$	CHO	$CH{=}CH_2$	658.0
Chl *d*	CH_3	CHO	CH_3	CH_2-CH_3	697.0
Chl *f*	CHO	$CH{=}CH_2$	CH_3	CH_2-CH_3	707.0

Fig. 7. ***Chemical composition of chlorophylls (left) with substitutions indicated for each typology of chlorophyll (left). Action spectrum of photosynthesis (right A) and absorption spectra of chlorophyll a, b and β-carotene***

Carotenoids

Carotenoids comprise a large family of C40 tetraterpenes and are synthesised by all photosynthetic organisms, aphids, bacteria and fungi alike. Carotenoids are essential for energy capture from the solar emission spectrum. Of the many naturally occurring carotenoids, less than 50 play a light-harvesting role in photosynthetic organisms (Polivka and Frank, 2010). Their colours range from pale yellow to a reddish brown depending upon the number of conjugated double bonds along the carbon backbone, as well as other cyclic and oxygenic modifications (Cazzonelli et al., 2011). Carotenoids and their oxidative and enzymatic cleavage products called apocarotenoids are crucial for various biological processes in plants, such as assembly of photosystems and light harvesting antenna complexes for photosynthesis and photoprotection, and regulation of growth and development. They absorb light across a broader range of the spectral region in which sun irradiates maximally, and this energy is then transferred to chlorophylls. In particular, in plants, they are bound to pigment-protein complexes of the antenna system (LHCII). Carotenoids are synthesized in all plastids, but the level of accumulation varies widely among the different types of plastids (Ruiz-Sola and Rodríguez-Concepción, 2012). Chromoplasts typically accumulate the highest amounts of carotenoids, but high carotenoid levels are also found in the chloroplasts of photosynthetic tissues. Chloroplasts have a remarkably similar carotenoid composition in all plants, with lutein (45% of the total), β-carotene (25-30%), violaxanthin (10-15%) and neoxanthin (10-15%) as the most abundant carotenoids (Britton, 1993). In plants, carotenoids are divided into two different classes: non-oxygenated carotenoids known as carotenes and oxygenated molecules known as xantophylls. Carotenes (mainly b-carotene) are enriched in the photosystem reaction centres, whereas xanthophylls are most abundant in the light-harvesting complexes (Niyogi et al., 1997).

The first step in carotenoid synthesis is the formation of the C40 compound phytoene, by the head-to-head condensation of two molecules of GGDP (geranylgeranyl diphosphate) by phytoene synthase. Phytoene is then subjected to a series of four sequential desaturation reactions, by two separate enzymes,

to yield lycopene. Lycopene is then cyclized to β-carotene by two β-cyclizations or to α-carotene by one β- and one ε-cyclization. Carotene serves as the precursor for hydroxylation (twice) to lutein, the most abundant carotenoid in green plant tissues. β-Carotene is subjected to a series of hydroxylation and epoxidation reactions to yield the other common leaf xanthophylls: zeaxanthin, antheraxanthin, violaxanthin and neoxanthin (Fig. 8).

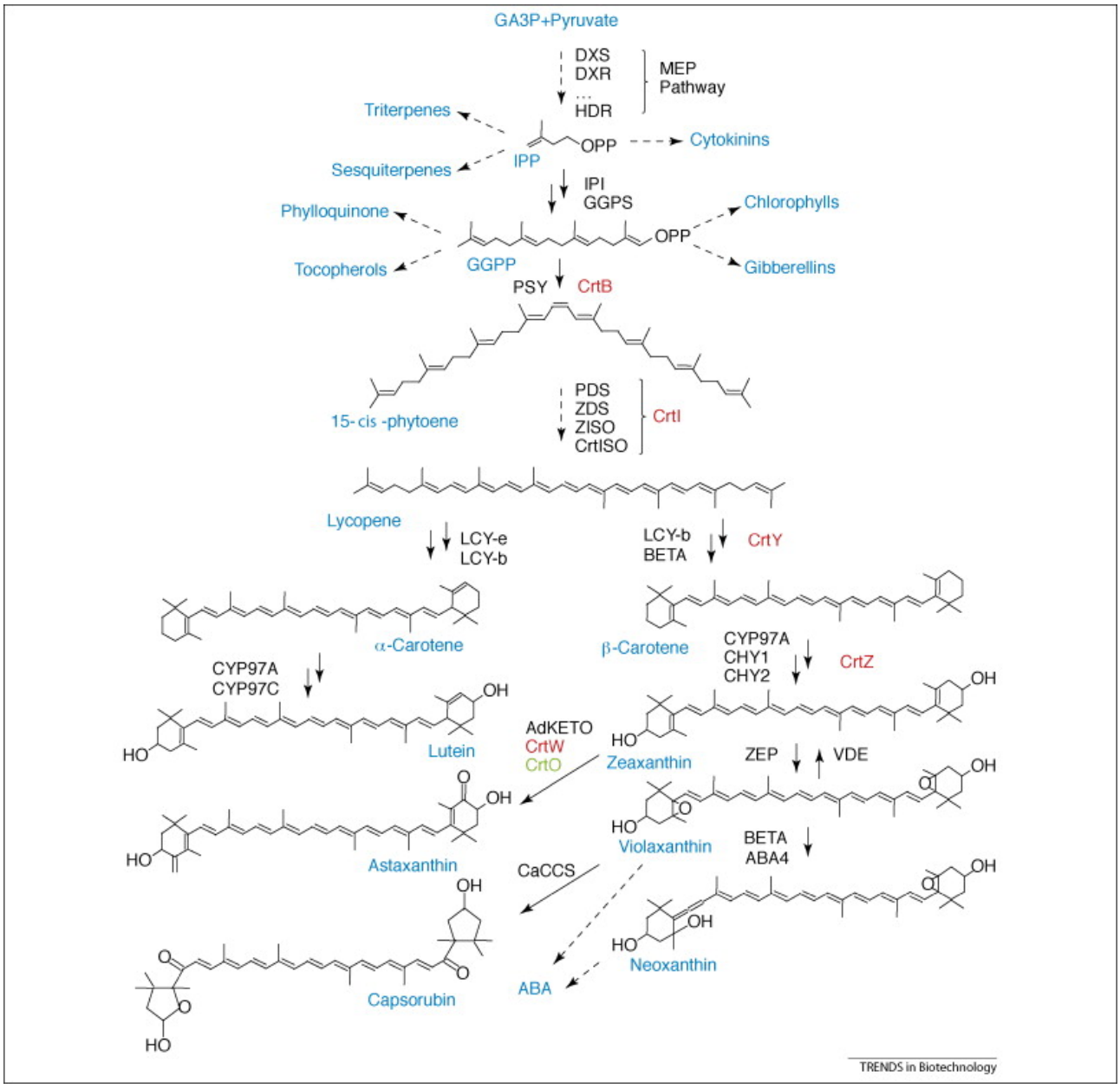


Fig. 8. ***Biosynthetic pathway of carotenoids in higher plants with all the enzymes involved (Giuliano et al., 2008).***

The structure of carotenoids, specifically the conjugated double bond system, determines their photochemical property. They have a very high extinction coefficient in the 400nm-550nm region, allowing the dipole-dipole transition from S_0 to S_2. This characteristic of Cars allows for an increase in light-absorption capacity of Chl-binding proteins in the blue spectral region. To carry out this function, Cars are positioned in close proximity and position to Chls. Indeed, Cars are located in specific binding sites in pigment-protein complexes of photosystems. This relationship renders proteins dependent towards Cars for their proper folding and activity, such as in the case of LHCs, which rely on xantophylls for the correct formation of the complex (Niyogi et al., 1997; Dall'Osto et al., 2006). While carotenes are bound especially to the two photosystems, with the most abundant being α- and β-carotenes in higher plants, xantophylls are found associated to the antenna complexes (Bassi et al., 1993; Caffarri et al., 2001; Ruban et al., 1999), represented mainly by four xantophylls in plants: Neo, Viola, Zea, Lut. The importance of Cars in the photosynthetic apparatus is not limited to structural and light-absorption properties, but they are also involved in photoprotection from oxidative stresses. In particular, Cars are natural antioxidants capable of dissipating excited states of chlorophyll (Chl*) and scavenging of Reactive Oxygen Species (ROS), which might arise from the reaction of Chl* with molecular oxygen. Each carotenoid plays a role in different photo-protective mechanisms: Lut, which binds to the specific site L1 in all light harvesting complexes, is involved in the quenching of harmful triplet states of Chl* (^{3}Chl*); Neoxanthin, whose specific binding site is N1 in LHCII, binds also to LHCB4 and LHCB5, protruding with their allene groups and actively scavenging superoxide (Liu et al., 2004); the reversible conversion of Viola to Zea (violaxanthin cycle) via the intermediate Antheraxanthin is essential for the adaptation of plants and algae to different light conditions and allows a reversible switch of photosynthetic light-harvesting complexes between a light-harvesting state under low light and a dissipative state under high light (Jahns et al., 2009). Zea plays a central role in different photo-protective mechanisms in chloroplasts: it contributes essentially to the dissipation of excess excitation energy in the antenna of PSII and

additionally acts as antioxidant in the lipid phase of the thylakoid membrane or the protein/lipid interface.

Light absorption: Photosystem I and II

Among the complexes involved in the reactions of photosynthesis, the actors that catalyse light-driven electron transfer to form ATP and reducing power are two photosystem complexes, named PSI and PSII. These two complexes are structurally different, but show similar moieties, specifically two: 1) both photosystems bind exclusively Chl a and carotenes to form a **core complex (RC)**, where charge separation occurs; 2) a **peripheral antenna system**, composed of Chl binding proteins which harvest and transfer energy to the core complex (RC). Genes called *psa* and *psb*, respectively for PSI and PSII, encode the components forming the core complexes, which are widely conserved during evolution. These gene products are encoded either by the nuclear or chloroplast genomes. RCs are not homogenously distributed among the thylakoid membranes: while PSII is mainly located in the grana membranes, PSI is mostly located in the stroma lamellae membranes. The antenna systems on the other hand are much more variable among photosynthetic organisms. In green algae and land plants, the polypeptides forming the antenna complexes are encoded by genes of the LHC superfamily: these proteins have three transmembrane helices and contrarily to RCs, they bind both Chls *a* and *b* as well as xantophylls (Green and Durnford, 1996). PSII light harvesting proteins are denominated LHCb, while PSI antenna proteins are denominated LHCa, and they are both encoded by the nuclear genome (Jansson, 1999). In *A. thaliana* 10 isoforms have been identified, four of which are associated to PSI (LHCA1-4) and six to PSII (LHCB1-6). A further distinguish can be made among LHCBs: while LHCB4-6 are present as monomers, LHCB1-3 proteins form trimeric complexes, termed the major PSII antenna complex (Jansson, 1999).

Photosystem I

Photosystem I (PSI) is a multisubunit protein complex which generates the most negative redox potential in nature and is extremely efficient in its utilization of light for electron transport, from plastocyanin on the lumenal side to ferredoxin on the stromal side of the thylakoid membrane (Jensen et al., 2007) (Fig. 9). It emerged as a homodimeric structure containing several chlorophyll molecules over 3.5 billion years ago, and has optimized its photoelectric properties ever since (Amunts and Nelson, 2008). Being one of the most elaborated membrane complexes, PSI operates with the unprecedented photochemical quantum yield of close to 1.0 (Amunts and Nelson, 2009). PSI structure has undergone various rearrangements during evolution, beginning from a homodimeric reaction center with 11 transmembrane helices (TM) to evolve into the current 11 heterodimer found in plants and cyanobacteria today (Nelson and Ben-shem, 2005). The P700 RC contains two homologous polypeptides (PsaA and B) and approximately 80 Chl molecules. The PSI RC, isolated from eukaryotes and cyanobacteria, catalyses light-induced plastocyanin (PC)-ferredoxin (Fd) oxido-reduction and contains multiple protein subunits, approximately 100 Chl molecules and up to 14 different polypeptides, named PsaA-PsaP. The largest complex, the PSI RC and LHCI, contains approximately 200 Chl molecules (Nelson and Yocum, 2006). The stromal subunit of PsaC carriers the two terminal iron-sulfur clusters and it participates with PsaD and PsaE in the docking of ferredoxin. PsaF and PsaN are necessary for the binding of plastocyanin and for providing excitation energy transfer from LHCI to the core complex (Boekema et al., 2001). PsaJ is a hydrophobic protein located close to PsaF and plays a role in the stabilization of this subunit conformation (Fischer et al., 1999). PsaH, PsaI, PsaL, and PsaO form a cluster of integral membrane proteins, placed on one side of the core, where they are involved in interactions with LHCII during state transitions (Lunde et al., 2000; Zhang and Scheller, 2004). PsaG is homologous to PsaK; it is situated on the opposite side of PsaK and contributes most of the contact surface area for association with LHCI (Nelson and Yocum, 2006).

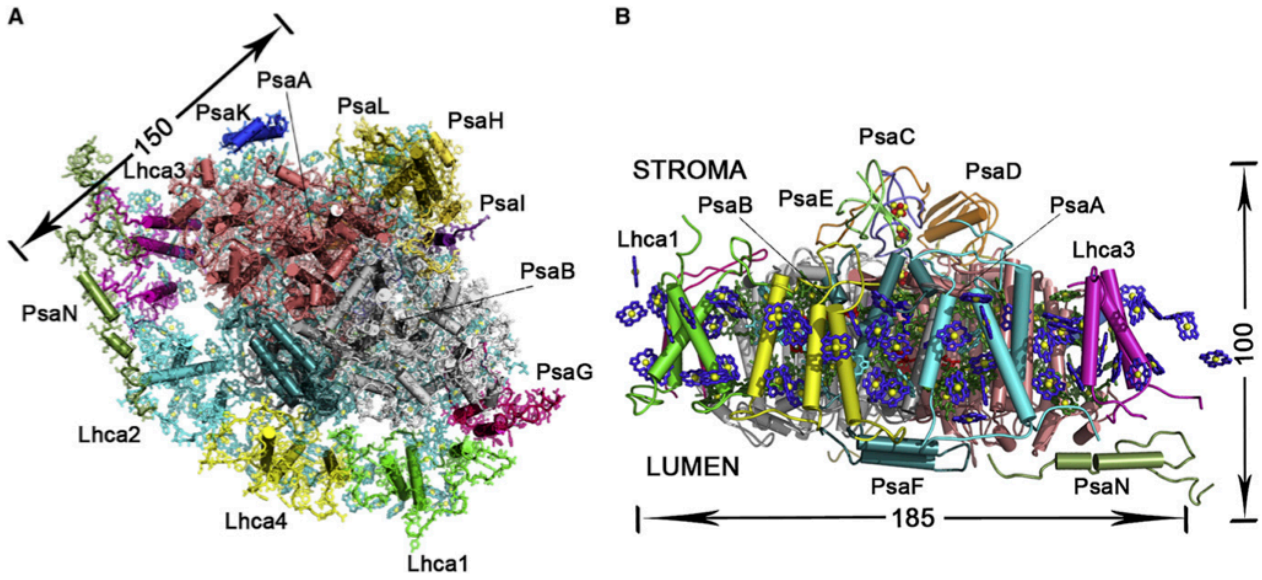


Fig. 9. ***Photosystem I structure. A View from the lumen. Chlorophylls and amino acids are shown in transparency. B View perpendicular to the membrane normal. Chlorophylls of LHCI are blue, gap chlorophylls are cyan, the rest of RC chlorophylls are green. The PsaC/D/E ridge forms a ferredoxin docking site. PsaF involved in the binding of plastocyanin. PsaN is found to interact with Lhca2/3 (Amunts and Nelson, 2009).***

PSI antenna complex

Four membrane-bound subunits Lhca1–Lcha4 bind cooperatively to the PSI core complex and form LHCI, the peripheral antenna of PSI (Jensen et al., 2007). The genes encoding the antenna complexes of PSI of plants and green algae are members of the *LHC* multigene family, which also includes the antenna complexes of PSII. The four LHCI subunits form two dimers arranged in series to create a half-moon-shaped belt docked on the F subunit side of the RC, specifically LHCA1-LHCA4 and LHCA2-LHCA3 (Ben-Shem et al., 2003). Some species, such as *A. thaliana*, have only one gene for each Lhca1–4 subunit, but have in addition two homologous genes encoding a fifth and sixth Lhca protein (Lhca5 and Lhca6). Lhca5 and Lhca6 are highly homologous to the Lhca1–4 proteins. Lhca5 accumulates to sub-stoichiometric amounts with respect to PSI. However, it is more abundant under certain conditions like high light (Jensen et al., 2007). Data for Lhca6 are scarce, but the Lhca6 mRNA profiles match that of Lhca5 and several of the one- and two-helix proteins homologous to the Lhc proteins, with yet unknown functions (Klimmek et al., 2006). More recently, LHCA6 polypeptide has been detected and together with LHCA5 it is required

for the formation of the full-size NAD(P)H dehydrogenase-PSI supercomplex formation (NDH-PSI supercomplex) (Peng and Shikanai, 2011).

Photosystem II

PSII is a large homodimeric transmembrane protein complex found in the thylakoid membranes of higher plants, cyanobacteria and green algae, which functions as a water-plastoquinone oxidoreductase, transferring electrons from water to PQ producing oxygen (Linke and Ho, 2014) (Fig. 10). This aspect renders Photosystem II a key component in the formation of life on Earth. Energy from absorbed photons is transferred from large chlorophyll antennae to the reaction center, where a special assembly of two chlorophyll molecules, known as P680, is found. Excitation of P680 leads to charge separation, where an electron is transferred to a pheophytin, the plastoquinone QA and finally to plastoquinone QB. In the course of two charge separation events, QB is reduced and protonated twice to give the plastoquinol QBH2 that leaves the acceptor side of PSII, to be replaced by a plastoquinone molecule from the plastoquinone pool of the thylakoid membrane. The oxidized P680 is reduced by a nearby tyrosine residue, Tyr161 of the D1 subunit designated YZ, which in turn oxidizes a Mn4CaO5 cluster, which is the catalytic center for water splitting. Once four electrons have been extracted from the Mn4CaO5 cluster, two water molecules are split into four protons and one oxygen molecule (Shen, 2015). In all of the PSII structures now available from different organisms, it can be seen that membrane-spanning helices of the PsbA (D1) and PsbD (D2) RC subunits are in close proximity to one another and RC chromophores (comprising six Chls, two Pheo *a*, the plastoquinones QA and QB, and the redox-active tyrosines YZ and YD) are bound to these subunits, as expected (Nelson and Yocum, 2006). Surrounding the D1 and D2 subunits are the CP47 and CP43 subunits (the gene products of *psbB* and *psbC*, respectively) (Shen, 2015). 14 Chl *a* are bound to PsbB, and 16 are bound to PsbC, and together they form the inner antenna of the photosystem II. In addition to these 4 large subunits, there are 13 low-molecular-weight transmembrane subunits: PsbE, PsbF, PsbH, PsbI, PsbJ, PsbK, PsbL, PsbM, PsbT, PsbX, PsbY, PsbZ, and Psb30. Furthermore, there are 3

membrane-peripheral, extrinsic proteins associated with the lumenal side of PSII and are necessary to maintain the oxygen-evolving complex (OEC), and in algae and higher plants they are: PsbO, PsbP and PsbQ (Nield and Barber, 2006).

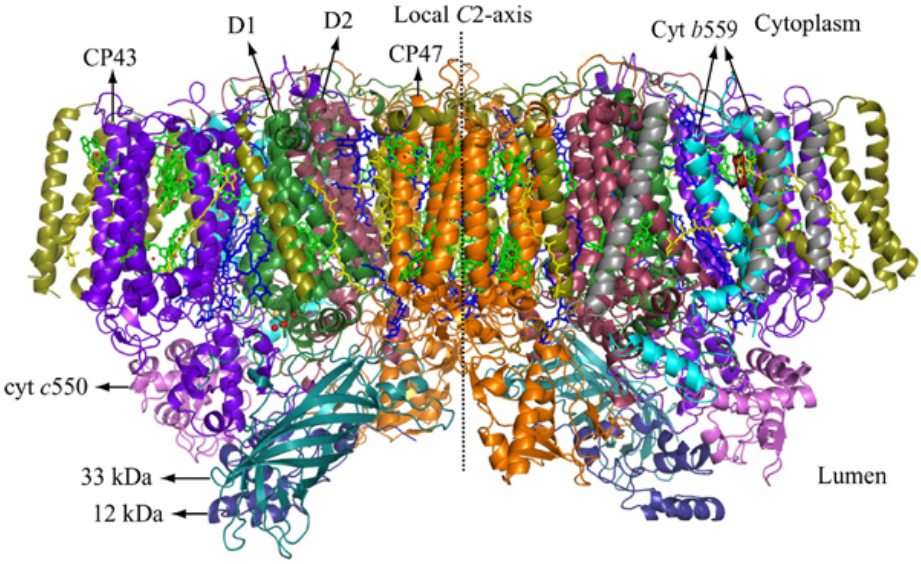


Fig. 10. ***3-D structure of Photosystem II complex from T.elongatus.***
View of the PSII dimer perpendicular to the membrane plane (Umena et l., 2011).

Photosystem II antenna complex

Two different class of light harvesting complexes form the PSII peripheral antenna system: the **trimeric major light harvesting complex** (also termed **LHCII**) and the **monomeric (or minor) antennae.** All light-harvesting outer antenna proteins are encoded by the nuclear genome, whereas the majority of RCI and RCII proteins are encoded by the chloroplast genome (Eberhard et al., 2008). Antenna complexes bind Chl *a, b* and xantophylls, and carry out two major functions: 1) to transfer excitation energy to the photosynthetic reaction centers; 2) to serve as a safety valve for the thermal dissipation of excess absorbed light energy (Govindjee, 2002). All LHCs share a highly homologous protein sequence and a very similar folding. The major LHC complex, LHCII (Fig. 11), for which two high-resolution structures are available, exemplifies a typical LHC architecture. In higher plant LHCII, the most abundant light-harvesting complex is composed of 3 gene products (*Lhcb1–3*) organized as heterotrimers (Drop et al., 2014). The three polypeptides, however, are not equimolar, with LHCB1 found in larger amounts (Caffarri et al., 2004; Dekker and Boekema,

2005). One LHCII monomer binds a total of 18 pigments: 6 Chls *b*, 8 Chls *a* and 4 xanthophyll molecules: luteins (Lut 1 and Lut 2), violaxanthin (Vio) and neoxanthin (Neo) (Liguori et al., 2015). In the trimeric complex each LHCII monomer is composed of 3 transmembrane α-helices. Both the N-terminal and the C-terminal peptides are exposed respectively to the stroma and to the lumen side. Two helices, named A and C, participate to Chl binding since they possess a characteristic 'LHC motif' in which a Glu from one LHC motif binds a Chl *a* molecule via a salt bridge to the Arg of the other: this characteristic of chlorophyll stabilizes the two central helices.

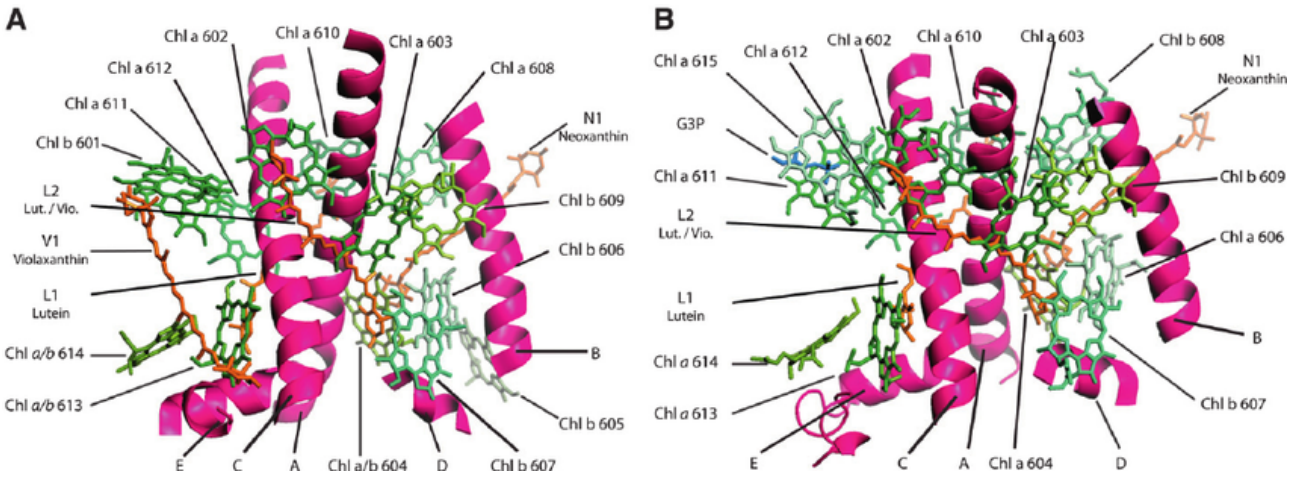


Fig. 11. ***Molecular model of LHCII monomer and Lhcb4 showing chlorophyll and xanthophyll chromophores bound to different binding sites. The model (A–B) has been drawn based on the crystal structures of LHCII trimer (A) and Lhcb4 (B). Pink, polypeptide; green, chlorophylls; orange, carotenoids; blue, lipids. (Ballottari et al., 2012).***

Luteins are bound in the hook-like extension at the stromal end of transmembrane helix 4 by the characteristic DPLG sequence. Electron crystallography indicates that two lutein molecules take on a crossbrace formation and are bound at sites L1 and L2 associated with the two membrane-spanning α-helixes (Snyder et al., 2004).

The third xanthophyll, neoxanthin (Neo) is located in the Chl *b*-rich region around helix C in the Car binding site N1. A Tyr residue in the lumenal loop has been found to form hydrogen bound with Neo, stabilizing N1 site (Caffarri et al., 2007; Hobe et al., 2006).

The fourth site (V1) is at the periphery of the monomeric subunits, and it accommodates violaxanthin, lutein, or zeaxanthin depending on light conditions. All carotenoids except for the one bound to the V1 site are involved in light-harvesting and singlet energy transfer (Mozzo et al., 2008).

The other class of light harvesting complexes are the **minor antenna** and consist in three Chl *a*/*b* binding proteins, namely: **LHCB4** (CP29)**, LHCB5** (CP26) and **LHCB6** (CP24)**.** They are encoded respectively by *LHCB4, LHCB5* and *LHCB6,* and are highly homologous to each other and to the other proteins of the LHC superfamily. LHCB5 and LHCB6 are encoded by single genes, while three genes encode for LHCB4 in *A. thaliana.* Of the three minor antennae LHCB4 and LHCB5 have orthologs in higher plants, while LHCB6 has only been found in organisms associated to a land environment, while absent in species such as *Chlamydomonas reinhardtii.* Minor antennae bind about 10% of total Chls and 20% of Viola of the total complex (Nield et al., 2000b).

In *A. thaliana*, **CP29** is encoded by three highly conserved genes: *Lhcb4.1*, *Lhcb4.2*, and *Lhcb4.3*. Two genes, *Lhcb4.1* and *Lhcb4.2*, have no significant difference in expression level, whereas *Lhcb4.3* is expressed at a lower level and also differs from the first two by lacking a large part of the C-terminal domain (Chen et al., 2013). As a protein, CP29 has an amino acidic length 256-258 in its mature form, resulting as the largest protein of the peripheral antenna. The first X-ray structure of minor light-harvesting complex CP29 was obtained by Pan et al. (2011) at 2.80-Å resolution (Fig. 12). It is characterized by three membrane-spanning helical regions connected by both stroma and lumen-exposed loops and two amphipathic helices exposed on the thylakoid lumen surface. The crystal structure of CP29 contains 13 chlorophyll-binding sites and 3 species of Cars: Lut, Viola and Neo, each occupying a separate site (Pan et al., 2011). The 13 chlorophyll-binding sites are assigned as eight chlorophyll *a* sites, four chlorophyll *b* and one putative mixed site occupied by both chlorophylls *a* and *b.* CP29 is necessary for PSII organization and crucial in the formation of the PSII-LHCII supercomplex (de Bianchi et al., 2011). LHCB4 is normally located in PSII complex between the outmost LHCII protein and the inner CP43 antenna, a

position that ensures a good energy flow toward the reaction center (Caffarri et al., 2009). Furthermore, there have been an increasing number of results indicating a role of CP29 in photoprotection (Ahn et al., 2008, de Bianchi et al., 2011, Betterle et al., 2014).

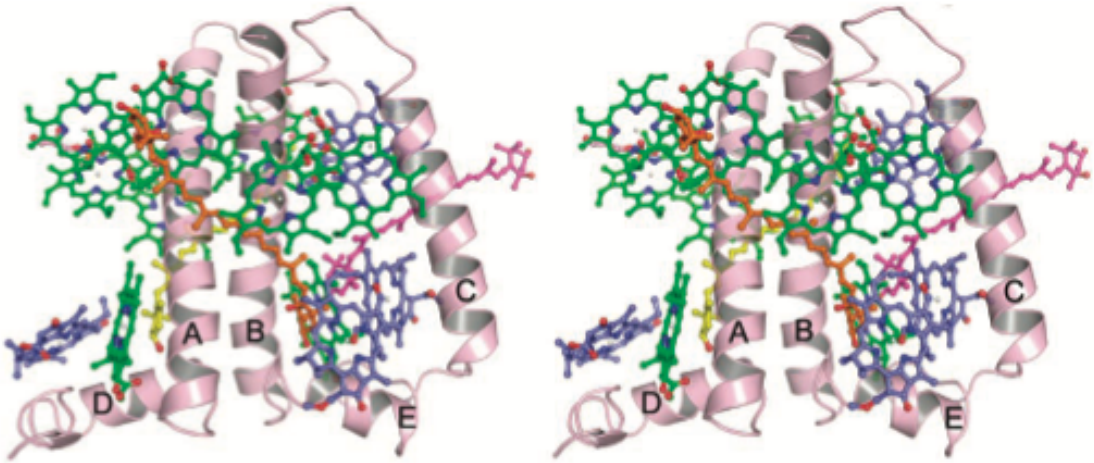


Fig. 12. ***Stereo view of the overall structure of CP29. View in parallel with the membrane plane. For clarity, the Chl phytyl chains are not shown. Green, Chl a; blue, Chl b; yellow, Lut; orange, Vio; magenta, Neo; light pink, G3P (Pan et al., 2011).***

When compared to LHCII, CP29 does not bind any pigments at Chls *b*601 and *b*605 sites that are located at the periphery of the LHCII monomer. Instead, a new chlorophyll-binding site, Chl *a*615, which is absent in LHCII, has been discovered on the surface of CP29 and close to the previous Chl *b*601 site in LHCII. Moreover, in the conserved chlorophyll-binding sites 609 and 614, CP29 binds Chl *a* and Chl *b*, respectively, whereas LHCII binds Chl *b* and Chl *a* instead. (Pan et al., 2011) (Fig. 13). The carotenoid-binding sites differ as well between the two structures, in particular at site L2, where LHCII binds Lut whereas CP29 binds Viola. In addition, CP29 does not contain the fourth Car-binding site at the monomer-monomer interface of the LHCII trimer (V1 site, binding a xanthophyll cycle Car).

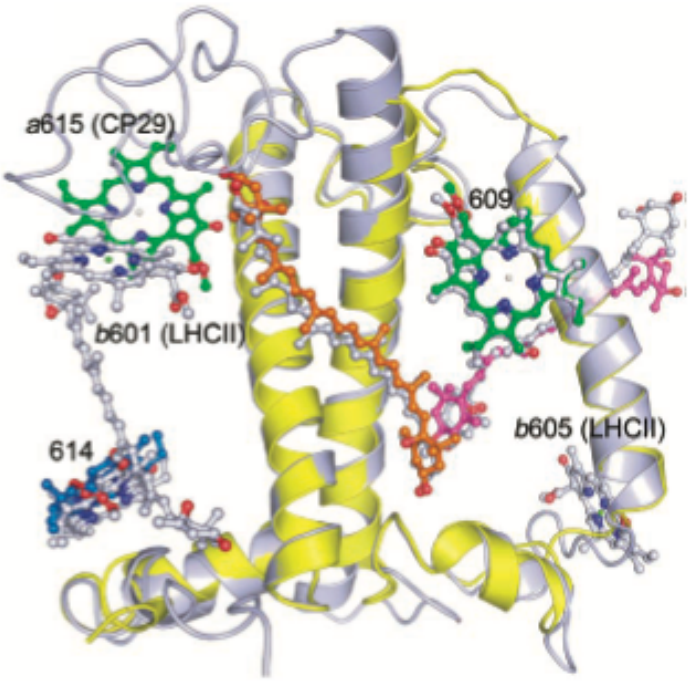


Fig. 13. ***Structural comparison between CP29 and LHCII. Superposition of the two structures based on Cα of their helices C. View along the membrane plane. Both the apoprotein and pigments of LHCII are shown in grey. The apoprotein of CP29 is shown as a yellow ribbon, and its pigments are shown as ball-and-stick models with the same color designation as in Figure 12 (Pan et al., 2011)***

Under high light conditions LHCB4 adopts different conformations in order to photo-protect PSII system from excess light. The pigment cluster Viola (Zea)–a603–a609 may dissipate excess energy through a charge-transfer mechanism (Ahn et al, 2008). Alternatively, another pigment cluster, a615–a611–a612–Lut, may quench the Chl excited states in a way similar to what Lut620–a611–a612 does in LHCI (Mozzo et al., 2008). Furthermore, CP29 is phosphorylated among various stressful conditions and its reversible phosphorylation is mainly involved in photoinhibition recovery (Bergantino *et al*., 1995) and state transitions (Tikkanen *et al*., 2006). Phosphorylation of CP29 as a response to environmental cues is a widespread phenomenon in monocotyledonous plants, but not in dicotyledonous plants (Chen et al., 2013). The identified phosphorylation site of maize CP29 protein has been localized at Thr112 by peptide mapping analysis (Testi et al., 1999), while in *A. thaliana* the known phosphorylation sites in CP29 (LHCb4.2) have been localized to Thr37, Thr109, and Thr111 by MS sequencing. In *C. reinhardtii* six phosphorylation sites have been identified and they all differ from those found in higher plants (Turkina et al., 2006; Vener, 2007). Thus, CP29 protein phosphorylation in thylakoid membranes may be involved in a number of responses to the changing environment.

LHCB5 (CP26) is a 26 kDa protein of LHC family. It shows a 48% identity to LHCII and binds 10 chlorophylls, 7 Chl *a* and 3 Chl *b*, and 2-3 xanthophylls (Croce et al., 2002). Protein refolding *in vitro* with pigments suggests that lutein has a primary role in the folding and stability of the complex, whereas violaxanthin and zeaxanthin have a negative effect on folding yield and stability, respectively (Ballottari et al., 2009). CP26, as LHCB1 and CP29, presents a Tyr residue suggested to stabilize the third carotenoid-binding site, N1 (Caffarri et al., 2007) and it has a role in the slowly activated NPQ component qI (Dall'Osto et al., 2005).

LHCB6 (CP24) is the most recent member of LHC family evolved after the splitting between land plants and algae (Koziol et al., 2007) and it is the smallest due to the lack of the major part of the C-terminal region of the protein. The *in vitro* reconstitution of this protein has shown that it binds 10 Chl molecules and two xanthophylls (Pagano et al 1998). CP24, differently from other Lhcb proteins doesn't bind neoxanthin; indeed the Tyr residue involved in N1 stabilization is absent (Caffarri et al., 2007). Due to the position of CP24, which sits between trimeric LHCII and PSII, mutants of *A. thaliana* lacking this protein showed reduced efficiency in energy transfer (Kovács et al., 2006). Mutants have a limitation in electron transport due to restricted electron transport between QA and QB, which retards PQ diffusion; a lower pH gradient is formed across the grana membranes and a reduced capacity for NPQ. It has been proposed that LHCB6 with other antenna proteins is site for the formation of the zeaxanthin-radical cation (Avenson et al., 2008).

PSII Supercomplexes

Over the last decades an effort has been made to determine the structure of PSII complexes, single subunits and antenna proteins. Of equal interest, though, has been the determination of the way in which these complexes form larger associates, known as supercomplexes. A variable number of peripheral antennae can associate with dimeric PSII core complex to form the so called PSII-LHCII supercomplex. Around the plant PSII core, the most abundant antenna complex

is trimeric LHCII which contains three apoprotein subunits LHCB1, LHCB2 and LHCB3, and form $LHCB(1)_3$, $LHCB(2)_3$ homotrimers or mixed LHCB1/LHCB2/LHCB3 heterotrimers with variable stoichiometry of three subunits (Standfuss and Kühlbrandt, 2004; Jackowski et al., 2001). Together with the LHCB trimeric antenna the monomeric ones are present as well, CP29, CP26 and CP24. The location of the large core subunits was assigned by crosslinking experiments (Harrer et al., 1998) and confirmed by EM on solubilized membranes of plants lacking individual antenna complexes (Yakushevska et al., 2003). Caffarri et al., (2009) were able to obtain a larger complex from *A. thaliana* at 12 Å resolution, allowing to better understand the position of peripheral antenna proteins. The larger supercomplex observed contains a dimeric core (C2), two LHCII trimers (trimer S) strongly bound to the complex on the side of CP43 and CP26, and two more trimers, moderately bound (trimer M) in contact with CP29 and CP24. This complex is known as the $C_2S_2M_2$ supercomplex (Dekker and Boekema, 2005). The S-LHCII trimer is composed of the Lhcb1 and Lhcb2 gene products, while the M-LHCII trimer consists of Lhcb2 and Lhcb3 gene products. In spinach besides S and M trimers another type of trimer loosely bound and so called L trimer has been described (Boekema et al., 1999). Recent studies suggest that LHCII L have a “peripheral” position and can migrate between PSII and PSI to balance the excitation level of two photosystems in response to light fluctuations (Galka et al., 2012).

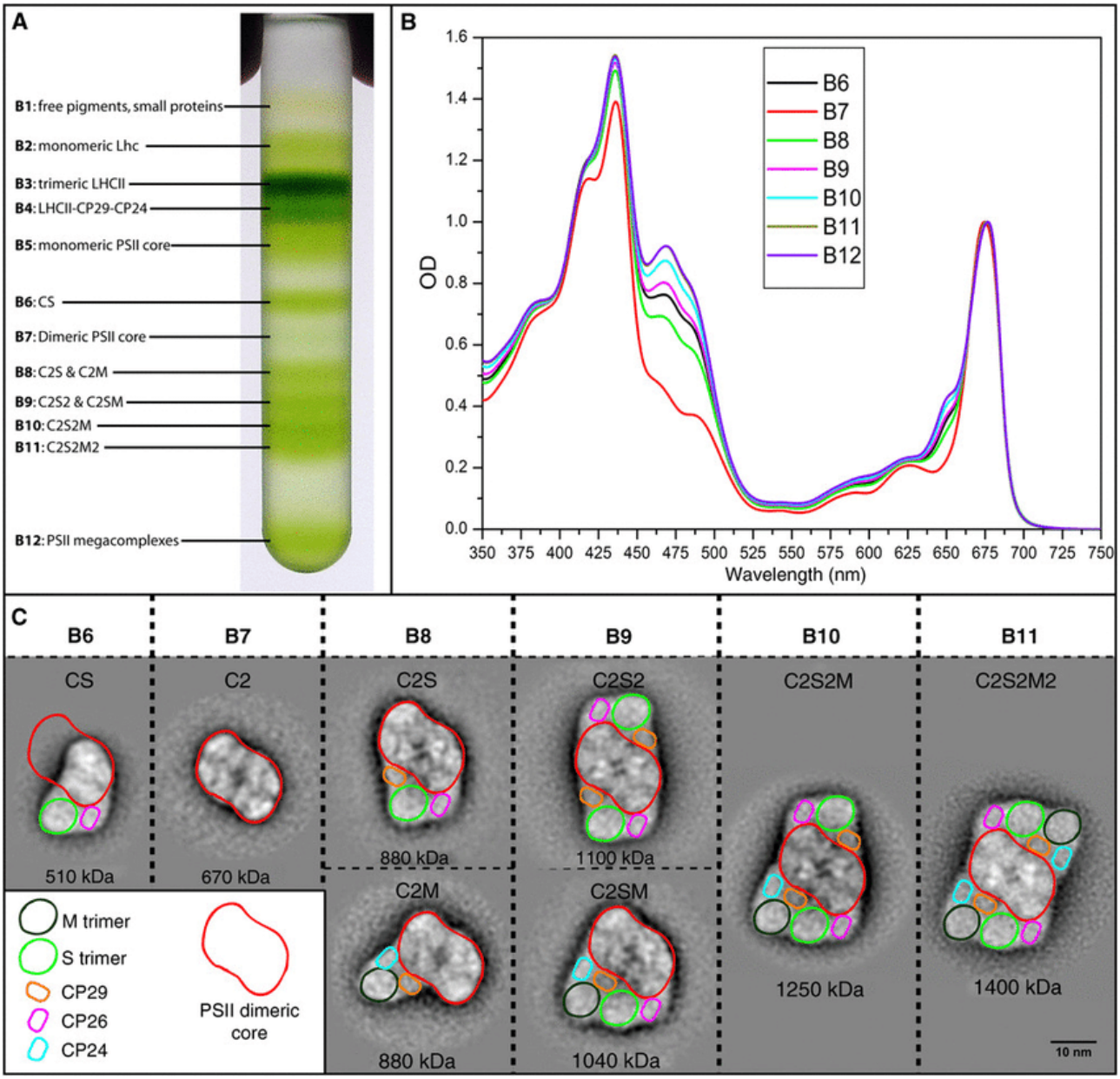


Fig. 14. ***Isolation and characterization of the PSII supercomplexes. (A) Sucrose gradient of solubilized membranes, showing 12 green bands. The content of each band is indicated on the basis of earlier work (Caffarri et al, 2001) (B1–B5) and this work (B6–B12). (B) Absorption spectra of bands 6–12. The spectra are normalized to the maximum in the red region. B11 and B12 are almost superimposed. The Chls b content, which is proportional to the antenna content, is deducible from the intensity of the bands at 470 and 650 nm. (C) EM analysis of the supercomplexes. The projections obtained for bands 6–12 are shown. B6 contains a newly identified supercomplex formed by a monomeric core, one LHCII S trimer and the minor antenna CP26. Contours representing the different complexes are superimposed. Also note that the position of the M trimer in the absence of trimer S (C2M in B8) is different. C, core; S, LHCII trimer strongly bound; M, LHCII trimer moderately bound (see text). The molecular weight of each particle, calculated on the basis of the protein content as determined by EM and SDS–PAGE, is also reported (Caffarri et al., 2009).***

ROS formation and photoprotection

Although light is essential for photosynthesis, it can also be harmful for plants ad the photosynthetic apparatus when in excess. In fact, light intensity and spectral quality are highly variable in space and time depending on various factors such as daytime, season, geography, climate, position of the leaf within the canopy, but also on plant growth conditions such as temperature and nutrients availability. In certain conditions, light energy absorbed may exceed the capacity for photochemistry utilization, leading to the generation of reactive oxygen species (ROS), among others, which in turn can damage the photosynthetic apparatus. This process of damage caused by excess light is termed "photoinhibition", and the three major sites where these dangerous molecules are produced are: LHC of PSII, PSII reaction center and the acceptor side of PSI. In the LHC upon absorption of blue light, an electron from the ground state is raised to a higher energy state and the energy is rapidly dissipated non-radiatively as heat mainly by internal conversion, and the electron rapidly relaxes to the first excited state. Absorption of a red photon causes Chl to enter directly the singlet excited state (^{1}Chl*).

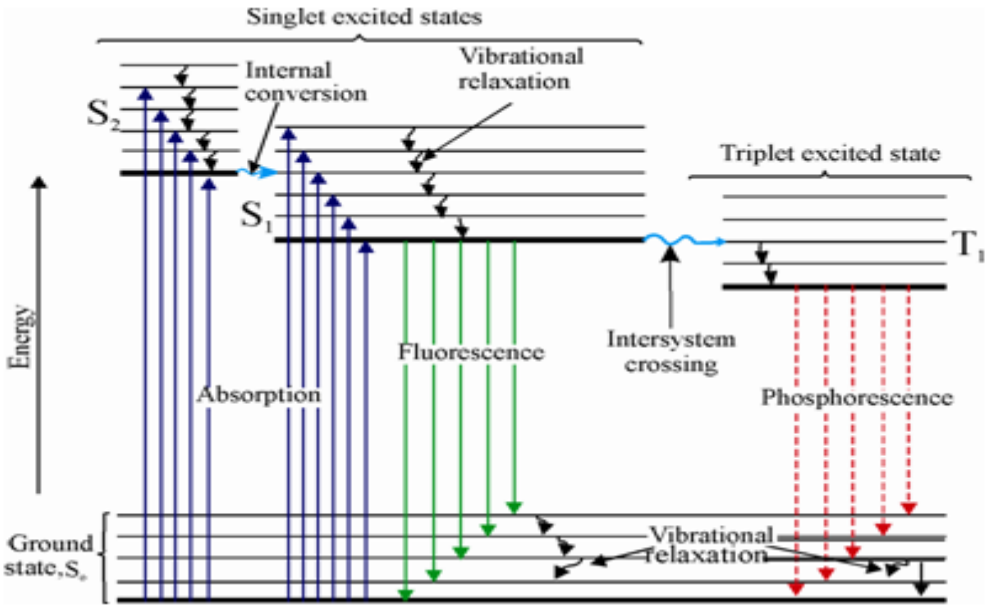


Fig. 15. ***Representation of the Jablonsky energy diagram (https://www.quora.com/What-is-the-Jablonski-diagram).***

Once Chl is excited, the excitation energy can relax to the ground state via different pathways: it can be emitted as fluorescence, it can be transferred to the reaction center where it drives electron transport (photochemistry), or it is dissipated non-radiatively under the form of heat (NPQ). Both photochemistry and NPQ reduce the amount of energy that can be emitted as fluorescence. Finally, another possible route for excited ^{1}Chl* is the conversion to ^{3}Chl* through intersystem crossing (ISC). ISC is a slow process and therefore its rate becomes relevant only when the lifetime of ^{1}Chl* increase, which, in turn, depends on the saturation of photochemical reactions. This usually occurs in excess light conditions when excitation energy accumulates in the antennae, and photochemical reactions and heat dissipation are not able to process this state fast enough, increasing ^{1}Chl lifetime and ISC probabilities.
Lifetime of ^{3}Chl* is much longer than that of ^{1}Chl*, in the ms range, and it can react with triplet oxygen ($^3O_2$) to form singlet oxygen ($^1O_2$), a highly dangerous ROS that can cause oxidation of proteins, pigments and lipids (Tardy and Havaux 1996).
As mentioned previously, one of the major sites of ROS formation is the PSII. After primary charge transfer, $P680^+$ and $Pheo^-$ species are formed; $Pheo^-$ is reconverted to Pheo after electron transfer to Q_A, while $P680^+$ is reconverted to P680 through Tyr oxidation. When in excess light conditions, though, Q_A is fully reduced and electron transport is impaired, which can lead to recombination between $P680^+$ and $Pheo^-$, producing $^3P680^*$, which in turn can generate singlet oxygen leading to damage of D1 core protein (Aro et al., 1993). During evolution, plants have evolved various mechanisms in order to prevent ROS formation and photoinhibition.

Photo-protective mechanisms

There are basically two classes of responses that plants can exploit in order to protect themselves from excess energy and consequent ROS production. These two classes are based on the time-scale on which these responses take place:

- short-term photo-protective mechanisms
- long-term photo-protective mechanisms

The main difference between these two classes lies in the fact that long-term responses rely on changes in gene expression and *de novo* synthesis of proteins, while short-term responses activate more quickly and generally involve quenching mechanisms to avoid ROS formation and dissipate excess energy.

State Transitions

Changes in light conditions require rapid adaptation on behalf of photosynthetic organisms. For plants and algae this is particularly important, given that they have evolved mechanisms to collect energy when in limiting conditions and dissipate excess energy when light exceeds their capacity.

Light in limiting conditions requires a system capable of optimizing the harvesting and distribution of solar energy. To do so, photosynthetic organisms employ what are generally referred to as State Transitions. This necessity arises from the fact that PSII and PSI have different light absorption properties and distinct light harvesting systems, which influence their relative performance. While PSII preferentially absorbs in the blue and red region, PSI has a broad absorption peak in the far-red region. The ratio between red/far-red, which can change in canopy conditions or through shading, affects photon absorption of the two photosystems. Given that they act in series, it is crucial for photosynthetic organisms to keep energy distribution balanced in order to optimize energy flow efficiency. Over excitation of PSII leads to reduction of PQ and the Cyt b6f complex, which activates the kinase acting on LHCII. Phosphorylation of LHCII induces dissociation from PSII and migration to PSI, known as "**state 2**". P-LHCII associated to PSI increases its antenna system cross-section, thus re-equilibrating energy distribution between the two photosystems. On the other hand, a preferential excitation of PSI leads to the oxidation of the PQ pool, inactivation of the kinase followed by dephosphorylation of LHCII. This is referred to as "**state 1**".

In *C. reinhardtii* the kinase responsible for LHCII phosphorylation is Stt7, while Stl1, a related kinase, is responsible for the phosphorylation of PSII core proteins (Depege et al., 2003; Vainonen et al., 2005). These two kinases are conserved in land plants, where they are called STN7 and STN8, respectively

(Bellafiore et al., 2005; Bonardi et al., 2005). Stt7/STN7 kinase presents two Cysteine residues at its N-terminal domain, which are essential in regulating its activity, even though they are site outside of the catalytic domain and interacts directly with the Rieske protein of the Cyt-b6f (Shapiguzov et al., 2016). It was proposed that in high light these Cys residues are reduced, inactivating the enzyme (Rintamaki et al., 2000). The activity of Stt7/STN7 kinase is countered by the activity of the LHCII phosphatase PPH1/TAP38, part of the PP2C-type phosphatase family, which was independently discovered by two groups (Pribil et al., 2010; Shapiguzov et al., 2010). PPH1/TAP38 is responsible for the dephosphorylation of LHCII, but not of the PSII core proteins, and is mainly associated to the stroma lamellae of the thylakoid membranes.

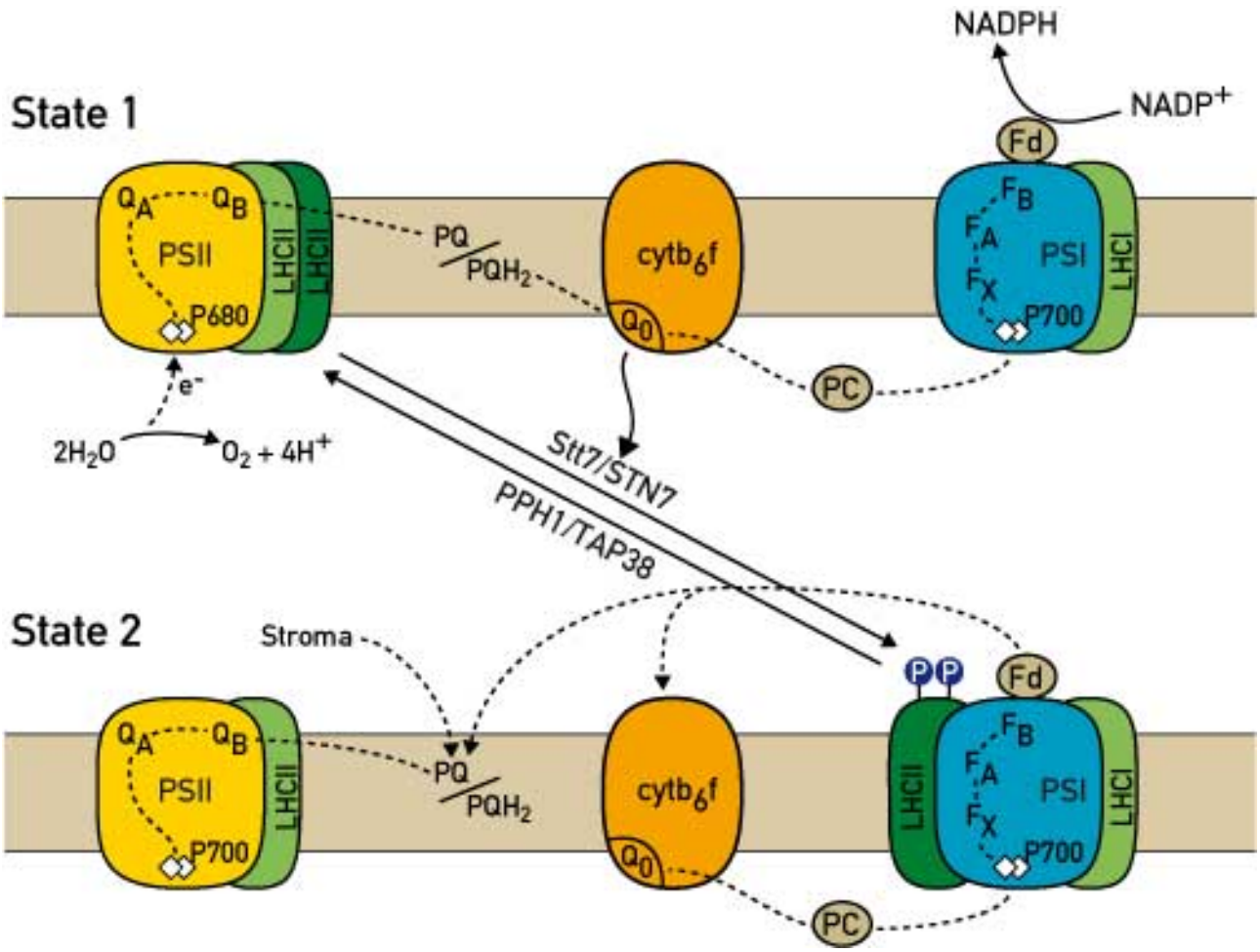


Fig. 16. ***Scheme of LHCII state transitions with the kinase and phosphatase involved (Rochaix 2010).***

Non-Photochemical Quenching

As mentioned previously, excited ^{1}Chl* can lead to the formation of ^{3}Chl*, which in turn can react with oxygen to form ROS, products that are harmful for the photosynthetic apparatus. In order to prevent this, photosynthetic organisms employ a set of inducible mechanisms, collectively referred to as Non-Photochemical Quenching (NPQ), which dissipate excess energy absorbed by PSII under the form of heat (up to 75% of photons) (Frank et al., 2000), and can be monitored as a quenching of Chl leaf fluorescence (Genty et al., 1989).
At least three major components can be identified as constituting the NPQ response, according to the kinetics of their rise in the light and decay in the darkness (Horton and Hague, 1988).

- qE (energy quenching), the predominant component of NPQ. It is rapidly turned on and off, in a matter of seconds to minutes, and it is triggered by the change in pH (ApH) that occurs across the thylakoid membranes when exposed to high light
- qI, defined as photoinhibitory quenching, the slowest component of NPQ, and it has been attributed to the damaged PSII centres present in high light. This response is around 60 minutes long
- the third component, namely qT or qZ, was originally attributed to state transitions, and more recently to the binding of Zea to the LHC proteins. It has a half-time of about 10-20 minutes.

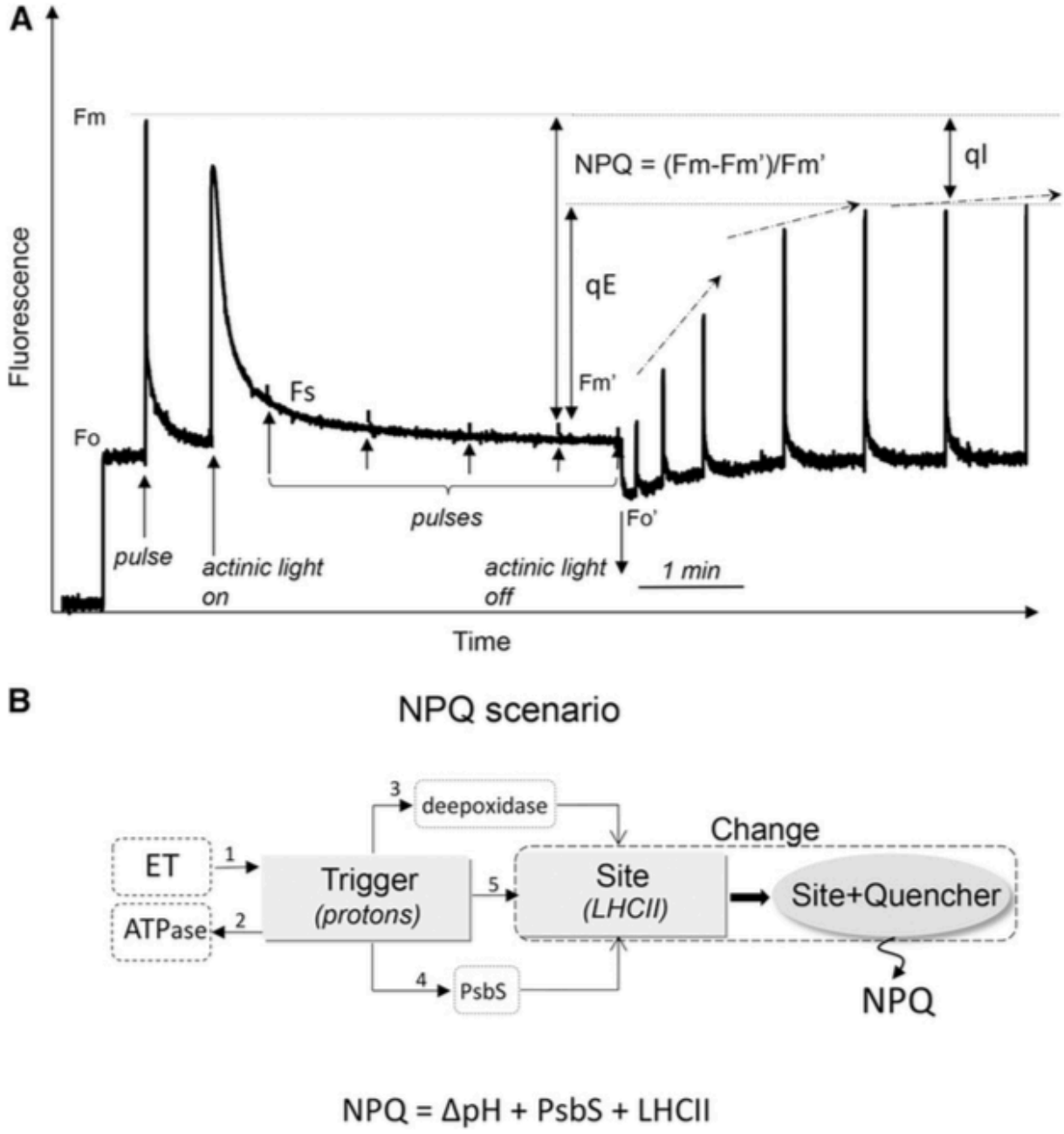
qE

When absorbed sunlight exceeds the capacity of the plant to fix CO_2, a proton gradient builds up across the thylakoid membrane due to the electron transport chain, resulting in acidification of the thylakoid lumen which immediately triggers qE. This process is swiftly reversible, allowing for plants to respond within seconds to changes in light intensity, which naturally fluctuates during a cloudy day for example (Muller et al., 2001). This was confirmed through the use of uncouplers of the ApH such as nigericin, which collapses the pH gradient and prevents activation of NPQ (Shikanai et al., 1999). Responses to changes in the

pH, i.e. over-acidification of the lumen, involve a protein that belongs to the family of LHCs, named PSBS. Interestingly, PSBS presents four transmembrane helices, compared to the three found in LHC proteins (Richard et al., 2000), and is characterized by the absence of most of the highly conserved Chl binding sites in its sequence (Dominici et al., 2002). Another peculiarity of PSBS is the presence of two lumen-exposed glutamate residues, Glu^{122} and Glu^{226}, which bind DCCD (N,N'-dicyclohexylcarbodiimide) a protein-modifying agent that covalently binds to protonatable residues in hydrophobic environments (Jahns et al., 1988). Mutation of each of these residues in *A. thaliana* led to a 50% decrease in both qE and DCCD binding capacity, while the double mutation of both residues led to a mutant with no qE, much similar to the PSBS-less mutant *npq4* (Li et al., 2004). The result suggested that these residues are protonated upon lumen acidification and induce the PSBS mediated qE activation. Exact localization of PSBS within the PSII supercomplexes has yet to be defined, although the most recent report by Correa-Galvis et al. (2016) revealed that in higher plants in the dark, PsbS is localized around PSII supercomplexes, while in the NPQ state, PsbS begins to interact with various LHCII antenna components, with preferential binding to the major trimeric LHCII complex. Immunoaffinity and immunoprecipitation experiments showed that PSBS interacts with many different photosynthetic complexes (as CP29, LHCII, PSI, or Cyt-b6/f complexes), leading to the model suggesting that PSBS might be mobile in thylakoid membranes (Teardo et al. 2007).

An additional factor needed for qE activation is Zeaxanthin. The amount of Zea synthesized via the xanthophyll cycle is highly correlated with the level of qE (Demmig-Adams 1990). Mutants that are unable to convert Viola to Zea have been isolated and show a lower level of qE. Although Zea is generally necessary for maximal qE in vivo, it is not sufficient. In mutants that accumulate it constitutively, qE must still be induced by a low pH (Niyogi 1999). This demonstrates that the low pH has an additional role in qE, besides activation of the xanthophylls cycle. Another xanthophyll that impacts on qE is Lut. While *A. thaliana lut2* mutant lacking lutein has less qE than wild type, double mutants lacking bot Lut and Zea show no qE at all (Pogson et al., 1998; Niyogi et al., 2001).

The mechanism through which PSBS interacts is still unknown. In one proposal, PSBS would promote LHCII aggregation, whereas the level of quenching is regulated by de-epoxidation state of the site V1 (Kiss et al., 2008). A second hypothesis is based on the two different forms of PSBS, suggesting that the dimer-to-monomer transition might uncover the Zeaxanthin-binding site on the PSBS surface, so PSBS monomer could transiently bind Zeaxanthin and bring this pigment in close contact with either LHCII or minor LHCB proteins forming a Chl-Zea heterodimer (Bergantino et al., 2003). LHCB proteins appear to be ideal candidates for the role of quenching sites; the ch1 mutant of A. thaliana that lacks Chl b, thus leading to degradation of LHC proteins, exhibits a strongly reduced capacity of NPQ in the presence of both Lute and Zea, suggesting that LHCs are needed for the quenching events (Andrews et al. 1995). Down-regulation of LHCB1 showed a decrease in qE while down-regulation LHCB2 and knockout of LHCB3 did not significantly decrease NPQ amplitude or slow down its kinetics (Andersson et al. 2003, Damkjaer et al. 2009, Pietrzykowska et al. 2014). qE is also affected in CP24 and CP29 knockout plants (Andersson et al. 2001, de Bianchi et al. 2008), although qE was not abolished completely. A third observation about the triggering of qE is that PSBS action is able to affects the rigidity of grana membranes and the readjustment of the antenna organization that might result in the formation of quenching sites. High light induce dissociation of antenna from PSII core and the formation of two different quenching site; the Q1 site reflecting the functional detachment of part of the antenna of the PSII supercomplex, and the Q2 quenching site that is located in the antenna that remains attached to the PSII core under HL conditions (Miloslavina et al. 2011). Thus the unquenched conformation of LHCB proteins is stabilized by their inclusion in this large complex, while its dissociation by PSBS would allow transition to the quenching state, also promoted by Zea binding. Thus the unquenched conformation of LHCB proteins is stabilized by their inclusion in this large complex, while its dissociation by PSBS would allow transition to the quenching state, also promoted by Zea binding (Betterle et al., 2009).



NPQ = ΔpH + PsbS + LHCII

Fig. 17. ***A Typical PAM fluorescence trace of an Arabidopsis leaf showing induction and relaxation of NPQ. B Model of NPQ development (NPQ scenario) showing key factors triggering and regulating the process (for more details, see the text). The formula for the minimum component requirement for NPQ is shown below the diagram (Ruban et al., 2016).***

NPQ components qI and qT/qZ

Photoinhibitory quenching, or qI, has been associated to a kinetic component whose relaxation is far slower than the decay of trans- thylakoid pH gradient upon light to dark transition and was attributed to processes involving damage of PSII, implying a reduction of the quantum yield of photosynthetic electron transport (Krause, 1988).

The intermediate kinetic component initially termed as qT, was presumed to be associated to state1-state2 transitions, attributable to the shift of LHCII from PSII to PSI and consequential decrease of PSII fluorescence. Study with the *stn7* mutant, which lacks the kinase for LHCII and is thus impaired in state transitions, showed an unaltered amplitude in the kinetic components of NPQ (Bellafiore et al., 2005; Nilkens et al., 2010). Further evidence is the fact that state 2 is not obtainable in high light as the kinase is inhibited and LHCII in a dephosphorylated state.
Another term for this component was qZ, intended as the Chl^{-}/Zea^{+} depending quenching, given that Zea modulates, upon binding to LHC, the amplitude of the intermediate component of NPQ relaxation. Although this could be true, the component has half-relaxation time of 10-20 minutes while the Zea decrease in the dark is much more slower with a half-time higher than 1 hour. Recently, studies performed in KO line lacking blue light photoreceptors phototropins (*phot2* mutant) in *A. thaliana* antenna show that the intermediate phase of NPQ kinetics strongly depends on the chloroplast avoidance movement, and has been termed qM (Cazzaniga et al., 2013).

Damaged PSII turnover

In spite of the amount of photoprotective mechanisms that plants can employ, damage to the photosynthetic apparatus is inevitable, and the main target of photo-oxidative damage, as mentioned previously, is the PSII reaction center. To restore PSII functionality, photosynthetic organisms have evolved a mechanism to degrade damaged D1 protein and reassemble newly synthesized PSII proteins

(Aro et al., 1993). A key component in this process was suggested to be the phosphorylation of the PSII core phosphoproteins, namely D1, D2, CP43 (Tikkanen et al., 2008). This mechanism is regulated by a kinase, STN8 (Bonardi et al., 2005; Vainonen et al., 2005), and phosphatase, PBCP (Samol et al., 2012), as shown in the model proposed in figure 18. Recent studies on *stn7*, *stn8* and *stn7stn8* mutants have revealed that PSII core protein phosphorylation does play a significant role in PSII repair cycle during photoinhibition by facilitating an efficient degradation and migration of damaged PSII reaction center proteins from grana membranes to the stroma lamellae membranes (Nath et al., 2013). Indeed, when leaves of both wild type and doublt mutant *stn7stn8* were treated with lincomycin prior to the exposure to HL illumination, in order to prevent the synthesis of the new copies of the D1 protein, a rapid disassembly of PSII supercomplexes was observed only in the wild type suggesting that the *stn7stn8* double mutant is impaired in the efficient disassembly of PSII supercomplexes (Tikkanen et al., 2008). What remains still unclear is the role of LHC proteins phosphorylation in the PSII repair mechanism. As mentioned previously, LHCII is phosphorylated in low light by the STN7 kinase, which is inhibited in its activity in high light. However, phosphorylation of the minor antenna CP29 has been reported as well, mainly in monocots such as maize, rice and barley, with phosphorylation occurring in photoinhibitory conditions such as cold stress or high light irradiance (Bergantino et al., 1994; Liu et al., 2009; Betterle et al., 2015). Recently, the possibility of STN7 being the kinase involved in this mechanism was ruled out (Betterle et al., 2015). CP29 high light induced phosphorylation has been observed in dicots as well, e.g. Arabidopsis, but at very low levels, analysed through MS spectrometry, and it was found to be dependent on STN7 kinase activity, as opposed to what as been observed in monocots (Fristedt and Vener, 2011). However, analysis on PSII supercomplexes using *stn7, stn8* and *stn7stn8* mutants showed a role of the phosphorylated minor antenna in dissociation of PSII supercomplexes to PSII dimers and monomers, supporting the idea of an involvement in PSII repair mechanism. Finally, report from Betterle and co-workers (2015) demonstrated for the first time a mechanistic correlation between CP29 phosphorylation and NPQ enhancement in rice, indicating a possible role for the protein in other

photoprotection mechanisms as well.

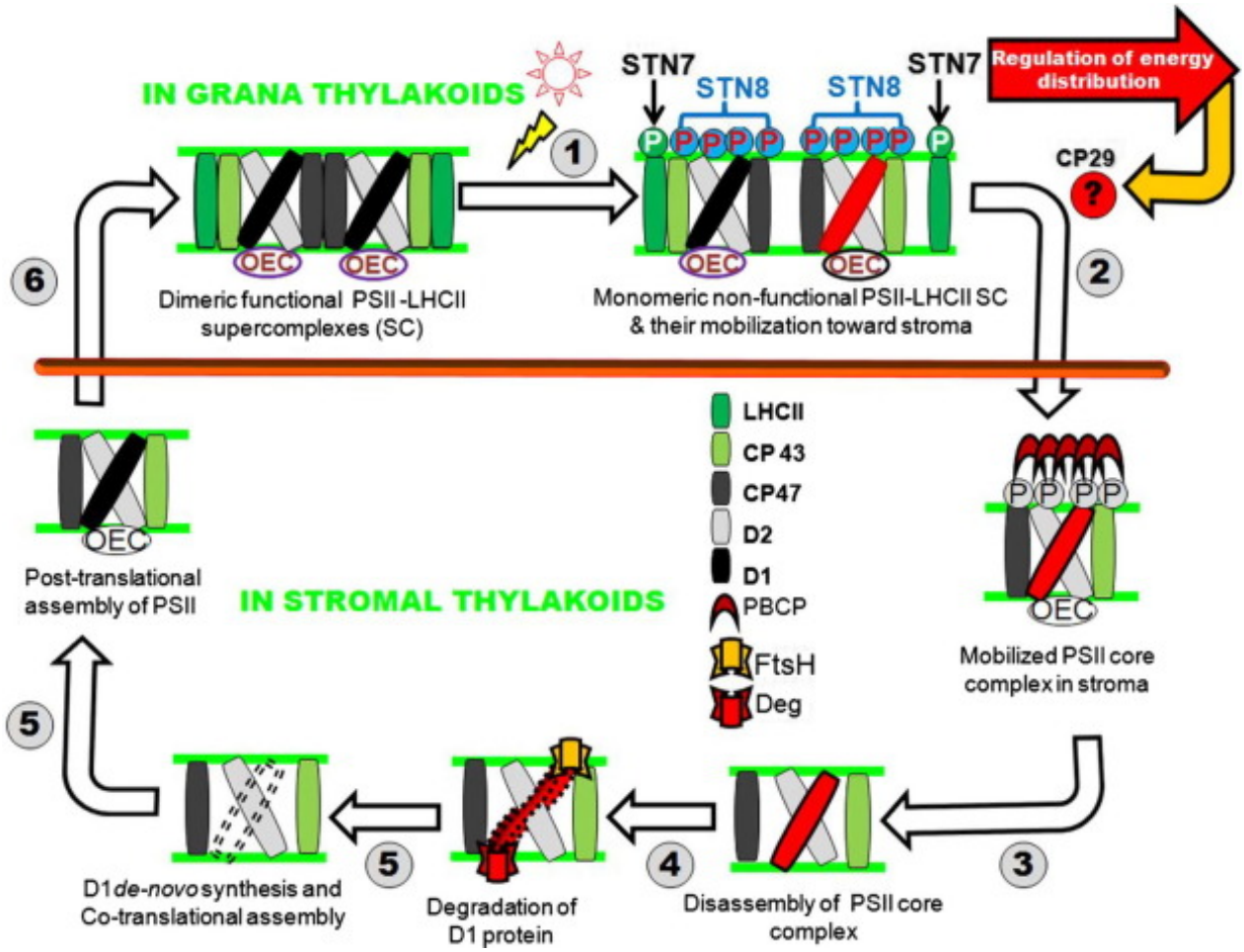


Fig. 18. ***Schematic representations of sequential events in PSII repair cycle during HL illumination. Numbers 1–7 in circles briefly explain the sequential events during the PSII repairs cycle. (1) Phosphorylation of LHC II and PSII core proteins by STN7 and STN8 kinases, respectively under light. (2) Mobilization of non-functional PSII complex toward stroma region. (3) Dephosphorylation of PSII core proteins by PBCP phosphatases. (4) Proteolytic degradation of photo-damaged D1 protein by D1 specific proteases. (5) Co-translation and de novo synthesis of pre-D1 protein to be inserted into the PSII complex. (6) Post-translational modification of pre-D1 protein. (7) Reassembly of dimeric functional PSII complexes in grana (Nath et al., 2013).***

Long-term photoprotective mechanisms

Long-term photoprotective mechanisms are employed by plants when they are subject for long periods to stressful conditions. The general behaviour is a switch from an energy conserving state typical of limiting light conditions to a more photoprotective mode, and this is done through regulation of gene expression and modifications to the plant architecture and composition. A typical example is the modification of *LHC* genes expression or LHC proteins degradation (Escoubas et al., 1995), yielding a reduction of light-harvesting antenna size. Moreover, up-regulation of enzymes involved in the Calvin cycle or ATPase and electron transport components.

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Chapter 1

The STN8 kinase-PBCP phosphatase system is responsible for high-light-induced reversible phosphorylation of the PSII inner antenna subunit CP29 in rice

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The STN8 kinase-PBCP phosphatase system is responsible for high-light-induced reversible phosphorylation of the PSII inner antenna subunit CP29 in rice

Nico Betterle[1,†], Roshan Sharma Poudyal[2,‡], Anthony Rosa[1], Guangxi Wu[2], Roberto Bassi[1,*] and Choon-Hwan Lee[2,*]
[1]Dipartimento di Biotecnologie, Università di Verona, Ca' Vignal 1, Strada le Grazie 15, Verona 37134, Italy,
[2]Department of Molecular Biology, Pusan National University, Busan 609-735, Korea.

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*For correspondence (e-mails roberto.bassi@univr.it or chlee@pusan.ac.kr).
N.B., R.S.P. and A.R. contributed equally to this article.
[†]Present address: Department of Plant & Microbial Biology, University of California, 111 Koshland Hall, Berkeley, CA, 94720-3102, USA.
[‡]Present address: Center for Plant Aging Research, Institute for Basic Science, Daegu, 42988, Korea.

SUMMARY

Reversible phosphorylation of thylakoid light-harvesting proteins is a mechanism to compensate for unbalanced excitation of photosystem I (PSI) versus photosystem II (PSII) under limiting light. In monocots, an additional phosphorylation event on the PSII antenna CP29 occurs upon exposure to excess light, enhancing resistance to light stress. Different from the case of the major LHCII antenna complex, the STN7 kinase and its related PPH1 phosphatase were proven not to be involved in CP29 phosphorylation, indicating that a different set of enzymes act in the high-light (HL) response. Here, we analyze a rice *stn8* mutant in which both PSII core proteins and CP29 phosphorylation are suppressed in HL, implying that STN8 is the kinase catalyzing this reaction. In order to identify the phosphatase involved, we produced a recombinant enzyme encoded by the rice ortholog of *At*PBCP, antagonist of *At*STN8, which catalyzes the dephosphorylation of PSII core proteins. The recombinant protein was active in dephosphorylating P-CP29. Based on these data, we propose that the activities of the *Os*STN8 kinase and the antagonistic *Os*PBCP phosphatase, in addition to being involved in the repair of photo-damaged PSII, are also responsible for the HL-dependent reversible phosphorylation of the inner antenna CP29.

Keywords: high-light illumination, protein phosphorylation, photosystem II, CP29, STN8 kinase, PBCP phosphatase, Rice.

INTRODUCTION

Plants are exposed to rapid changes of their environment resulting in abiotic-type stresses that account for most of the productivity loss in crop species and primarily target the photosynthetic apparatus (Navabpour *et al.*, 2003). Indeed, the efficiency of mechanisms that regulate photosynthesis is critical for plant productivity due to their ability to minimize oxidative damage from over-excitation resulting from fluctuations in light intensity and/or changes in spectral composition of light within canopies (Nelson and Ben-Shem, 2004).

State transitions (STs) and non-photochemical quenching (NPQ) are two major mechanisms for the rapid and reversible acclimation of the photosynthetic electron transfer chain to changing light. Indeed, (i) STs operate in limiting light by balancing the excitation energy delivery to photosystem I (PSI) versus photosystem II (PSII), allowing for optimal photon use and avoiding plastoquinone (PQ) over-reduction and photoinhibition (Rochaix *et al.*, 2012); and (ii) NPQ, through its main component qE (Horton *et al.*, 1996; de Bianchi *et al.*, 2010; Niyogi and Truong, 2013), catalyzes the thermal dissipation of energy absorbed in excess. STs are inhibited in high light (HL; Rintamaki *et al.*, 1997), whereas excess light activates qE through thylakoid lumen acidification. This acidification occurs due to the inhibition of ATP synthase by lack of its ADP substrate, when the catalytic rate of the Calvin–Benson cycle is exceeded. Over-excitation of PSII versus PSI activates STs by reducing PQ (Allen and Staehelin, 1992) and promoting the phosphorylation of stroma-exposed threonine residues in trimeric LHCII by the STN7 kinase (Bellafiore *et al.*, 2005; Rochaix *et al.*, 2012; Betterle *et al.*, 2015). In *Arabidopsis thaliana*, a fraction of the phosphorylated LHCII, enriched

in LHCB2 (Pietrzykowska *et al.*, 2014; Crepin and Caffarri, 2015; Longoni *et al.*, 2015), diffuses from grana partitions, where it participates to the PSII antenna system, towards stroma-exposed membranes where it increases PSI antenna size (Bassi *et al.*, 1988; Galka *et al.*, 2012). The STN7 activity is inhibited in HL through the reduction of a sulfur bridge (Shapiguzov *et al.*, 2016). Upon inactivation of the kinase, the PPH1/TAP38 phosphatase (Pribil *et al.*, 2010; Shapiguzov *et al.*, 2010) reverses the process.

Besides LHCII, other PSII subunits including the PSII core proteins CP43, D1 and D2, are phosphorylated and this is fundamental for the turnover of components damaged by oxidative stress (Aro *et al.*, 1994). This second type of phosphorylation, which is not prevented by HL, is catalyzed by the STN8 kinase (Bonardi *et al.*, 2005; Nath *et al.*, 2013a) and reverted by PBCP phosphatase (Samol *et al.*, 2012). An additional HL-dependent phosphorylation event is restricted to monocots and consists of phosphorylation of the CP29 subunit of PSII, which connects the LHCII-M trimer to PSII core complex (Bergantino *et al.*, 1995, 1998; Hwang *et al.*, 2003; Pursiheimo *et al.*, 2003; Caffarri *et al.*, 2009; Chen *et al.*, 2009; Liu *et al.*, 2009). CP29 phosphorylation enhances NPQ (Mauro *et al.*, 1997; Betterle *et al.*, 2015) and is STN7-independent (Betterle *et al.*, 2015), whereas the kinase/phosphatase gene products involved are yet unknown. The functional redundancy for PSII core protein phosphorylation between STN7 and STN8 kinases is reduced in rice with respect to Arabidopsis (Nath *et al.*, 2013a), leading to the hypothesis that STN8 might be involved in CP29 phosphorylation.

In this report, we analyze wild-type (WT), *stn8* knockout, STN8 overexpressor and *stn7* rice genotypes to show that STN8 is the kinase responsible for CP29 phosphorylation. We also identify the enzyme involved in CP29 dephosphorylation. These findings open the way to the transfer of said process from monocots to dicots with the aim of increasing crop resistance to abiotic stresses.

RESULTS

Previous work on CP29 phosphorylation showed that it was independent from STN7 (Betterle *et al.*, 2015). Moreover, the early observation that HL enhanced CP29 phosphorylation rather than inhibiting it suggested that STN8 might be the kinase involved in this activity.

In order to verify this hypothesis, we used the *stn8* rice mutant (Nath *et al.*, 2013a), and compared its CP29 phosphorylation behavior with that of other mutant lines recently described (Nath *et al.*, 2013a; Betterle *et al.*, 2015). Mutant rice lines *stn7*, *stn8*, *pph1*, a complemented line overexpressing STN8 (OE-STN8) and their respective WT cultivars were either maintained in the dark (D) or exposed to excess light (HL, 1500 μmol photons m^{-2} sec^{-1}, 28°C) for 60 min, after which thylakoid membranes were purified and their polypeptides analyzed by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) followed by immunoblot using anti-CP29 antibody (Figure 1). Two bands were detected, and the slow migrating one corresponded to phosphorylated CP29 (P-CP29) as previously described (Bergantino *et al.*, 1995; Hwang *et al.*, 2003; Betterle *et al.*, 2015). The upper band was present in HL-treated samples with a similar abundance, whereas it was absent in the sample from *stn8* despite HL treatment, implying that STN8 and not the STN7 kinase was involved in the formation of P-CP29 in rice.

Indeed, HL treatment (1500 μmol photons m^{-2} sec^{-1}, 30 min 24°C) induced the appearance of a new sharp Coomassie-stained band with an apparent molecular weight (MW) of 30 kDa in WT samples, which was absent in the *stn8* genotype (Figure 2a). Immunoblot analysis using anti-CP29 antibody showed two bands of, respectively, 30 and 28 kDa in the sample from HL-treated WT. In contrast, samples from dark-adapted (D) WT and from *stn8*, either D or HL, only showed the low MW form of CP29. The intensity of the 28-kDa band was decreased by approximately 50% in HL WT versus D as quantified from both the Coomassie-stained gel and the anti-CP29 antibody reaction.

Immunoblot with anti-phosphothreonine (P-Thr) antibody on the same samples revealed three major bands at, respectively, 40, 33, 30 kDa apparent MW, corresponding to P-CP43, P-D2 and P-D1, according to a previous report (Nath *et al.*, 2013a). The intensity of the two lower MW bands was strongly increased in the HL versus D WT samples, whereas no such increase was detected in the *stn8* samples (Figure 2b). Immunoblot analysis using antibodies

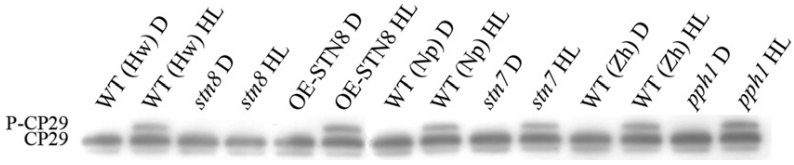

Figure 1. The STN8 protein kinase of rice is required for CP29 phosphorylation.
Immunoblot analysis of thylakoids isolated from *Oryza sativa* wild-type (WT; Hw, cv. Hwayoung; Np, cv. Nipponbare; Zh, cv. Zhonghua 11) and mutant plants recently described (Betterle *et al.*, 2015; Nath *et al.*, 2013a). Before thylakoid isolation, leaves were dark-adapted for 12 h (D) and then treated with white light [high light (HL); 1500 μmol photons m^{-2} sec^{-1}] for 60 min. Tris-Gly sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) 10% acrylamide plus urea 2 M; Immunoblot was performed using anti-CP29 polyclonal antibody (Agrisera); 2 μg total chlorophylls (Chl) per lane.

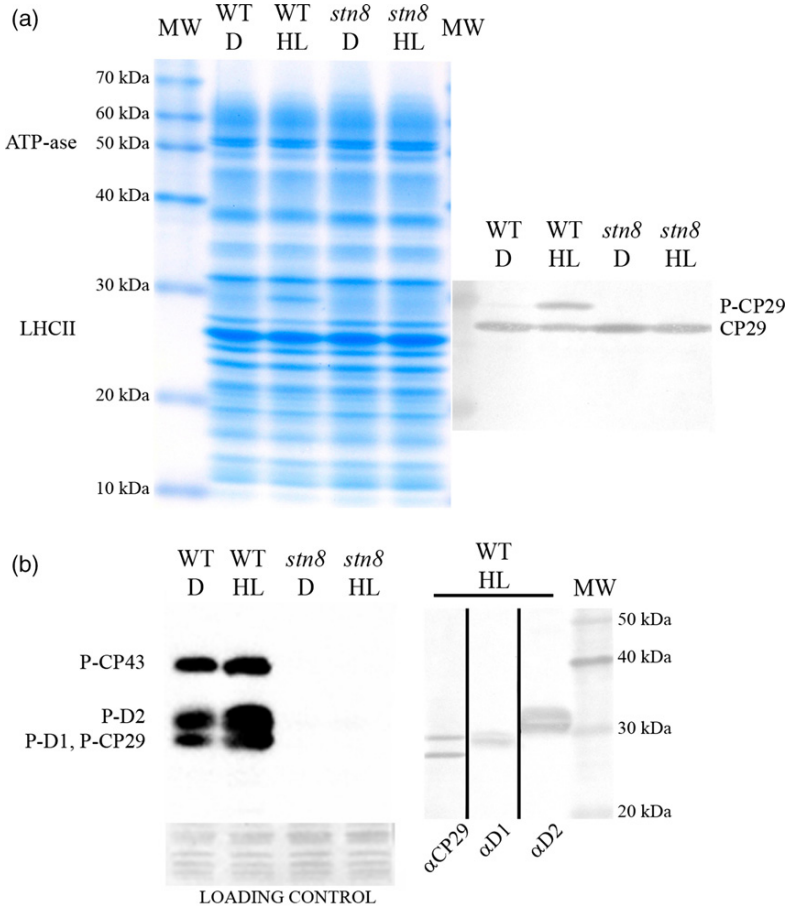

Figure 2. The STN8 protein kinase is responsible for both photosystem II (PSII) core subunits and CP29 phosphorylation.
(a) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblot analyses of thylakoids isolated from wild-type (WT) and *stn8 Oryza sativa* plants. Before thylakoids isolation, WT and *stn8* mutant leaves were dark-adapted for 2 h (dark) and then treated with white light [high light (HL); 1500 μmol photons m^{-2} sec^{-1}] for 30 min. Tris-Gly SDS–PAGE 12–18% acrylamide plus urea 3 M; Immunoblot was performed using anti-CP29 polyclonal antibody; 3 μg total chlorophylls (Chl) per lane.
(b) Analysis of thylakoid phosphoproteins using anti-phosphothreonine (P-Thr) antibody (Cell Signaling). Before thylakoids isolation, WT and *stn8* mutant rice leaves were treated as previously described in (a). A detail of Red Ponceau-stained filter before immunoblot analysis, corresponding to the LHCII migration region, is shown as loading control. Antibodies against CP29, D1 and D2 were used to identify the electrophoretic migration of CP29, D1 and D2 proteins from thylakoids of HL-treated WT leaves. [Colour figure can be viewed at wileyonlinelibrary.com].

specific for CP29, D1 and D2 showed that P-CP29 co-migrated with P-D1 (Figure 2b). In order to verify whether the low-MW anti-P-Thr-reactive band could be P-CP29 co-migrating with P-D1, we fractionated thylakoid proteins by sucrose-gradient ultracentrifugation upon solubilization with β-dodecylmaltoside (DDM), as previously reported (Betterle *et al.*, 2015), and probed separately monomeric LHC proteins (including CP29) and PSII core components, which are characterized by a different sedimentation velocity in the gradient (Figure 3a). The polypeptides from the upper, Chl *b*-enriched, gradient band were depleted of PSII core components, whereas the lower, Chl *a*-enriched gradient band contained PSII core complexes as shown by their reactivity to anti-D1 and anti-D2 antibodies while lacking CP29 (Figure 3b). When probing the two bands from the gradient with anti P-Thr antibody (Figure 3c), three bands were detected in the PSII core fraction with a migration pattern similar to that observed in HL-treated thylakoids. Thus, these bands corresponded to P-CP43, P-D1 and P-D2. Instead, the monomeric antenna fractions from HL samples contained a single P-Thr reactive band with a 30-kDa apparent MW. No signal was detected in the dark-adapted sample (Figure 3c). We conclude that P-CP29 and P-D1 co-migrated in SDS–PAGE from whole thylakoid samples, clarifying why the HL-dependent phosphorylation of CP29 remained undetected in previous work with the rice *stn8* mutant (Nath *et al.*, 2013a).

The involvement of STN8 kinase in CP29 phosphorylation and the upper limit of 50% P-CP29 versus unphosphorylated CP29 suggested a fraction of this protein is not accessible to the kinase. We thus proceeded to determine its distribution among thylakoid domains. To this aim, we isolated intact chloroplasts and thylakoids from dark-adapted WT rice plants and, upon SDS–PAGE, detected STN8 by immunoblot analysis with anti-STN8 antibody. Thylakoids from *stn8* plants were also included as a control of the anti-STN8 reaction (Figure 4). The STN8 gene product was detected with the same intensity in chloroplasts and thylakoid samples consisting with STN8 being fully bound to the thylakoid membrane. A faint background signal was present in the sample loaded with thylakoids from *stn8* rice plants, although with slightly higher apparent MW. To determine STN8 protein accumulation level, compared with STN7, in greenhouse-grown plants, his-

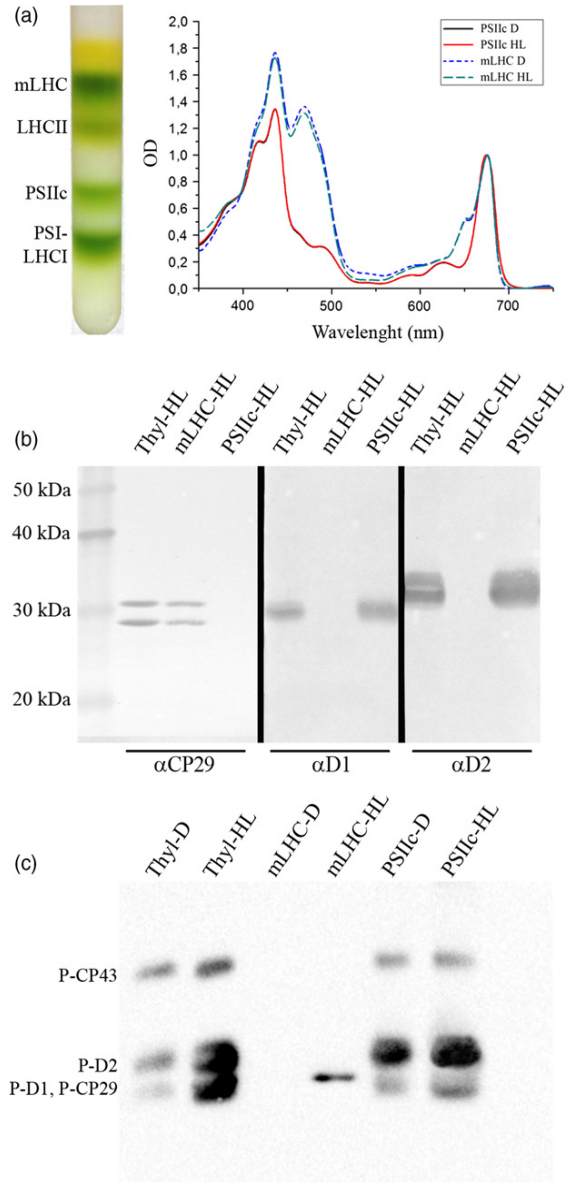

Figure 3. Demonstration of CP29 phosphorylation detection by anti-phosphothreonine (P-Thr) antibody.
(a) Sucrose gradient fractionation of mildly solubilized thylakoids and absorption spectra in the visible region. Thylakoids were isolated from wild-type (WT) rice leaves dark-adapted or treated with white light [high light (HL); 1500 μmol photons m^{-2} sec^{-1}] for 30 min, and then monomeric antenna proteins (mLHC) and photosystem II (PSII) core (PSIIc) fractions were purified upon sucrose gradient ultracentrifugation of β-dodecylmaltoside (b-DDM) solubilized thylakoids (Thyl).
(b) Immunoblot analysis of thylakoids and sucrose gradient isolated fractions. Antibodies against CP29, D1 and D2 were used; 0.75 μg total chlorophylls (Chl) per lane with thylakoids samples, 0.25 μg total chlorophylls (Chl) per lane with mLHC and PSIIc fractions. Tris-Gly sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) 12% acrylamide plus urea 3 M.
(c) Analysis of thylakoid phosphoproteins using anti-phosphothreonine (P-Thr) antibody (Cell Signaling). Samples used were collected from solubilized thylakoids from dark-adapted leaves or treated with HL as shown in (a). [Colour figure can be viewed at wileyonlinelibrary.com].

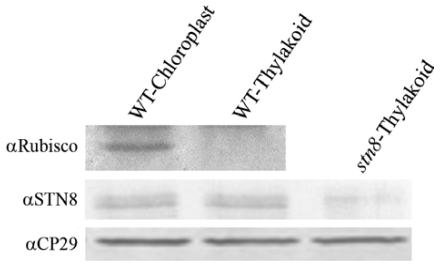

Figure 4. Subcellular localization of STN8 kinase in rice.
Determination of STN8 localization by immunoblot analysis on isolated chloroplasts or thylakoids. Chloroplasts and thylakoids were isolated from dark-adapted wild-type (WT) rice leaves. Immunoblot analyses have been performed using anti-Rubisco, anti-STN8 and anti-CP29 antibodies. The presence of Rubisco reflected the presence of stromal components, while CP29 was a marker for thylakoid complexes. Thylakoids from *stn8* plants were used as a control for reliable STN8 detection, because of the presence of an aspecific band with similar molecular weight (MW); 1 μg Chls per lane.

tagged STN8 and STN7 were expressed in *Escherichia coli* and recovered as inclusion bodies. Recombinant proteins were then purified by affinity chromatography in denaturating condition, and the purity of the recombinant proteins was determined by SDS–PAGE (Figure 5a). Coomassie binding capacity of STN7 and STN8 was normalized based on the content in basic residues and aromatic residues (Syrovy and Hodny, 1991). Different amounts of the recombinant proteins were loaded together with rice WT thylakoids, and an immunoblot analysis was performed using antibodies specific for the two kinases (Figure 5b and c). The signal obtained from the recombinant proteins was used to generate a calibration line to determine the relative amount of the two kinases in thylakoid membranes based on the intensity of immunoblot signals yielded by the recombinant proteins and by the endogenous kinases in thylakoid samples. We obtained a content of STN7 in thylakoids approximately twice as high with respect to STN8. An additional factor is the distribution of these enzymes among thylakoid domains: to assess thylakoid domain distribution of STN8 and STN7 kinases, we proceeded to solubilize stacked thylakoid membranes by using increasing concentrations of α-DDM, a treatment that preferentially solubilizes stroma-exposed thylakoid membranes and allows to harvest fractions of paired grana membrane discs of different diameter by differential centrifugation (Morosinotto *et al.*, 2010). Consistent with previous reports (Pinnola *et al.*, 2015), the Chl *a*/*b* ratio decreased in the pellet fraction by increasing the detergent concentration (Table 1). The SDS–PAGE analysis of these fractions showed that ATPase, which is a protein complex restricted to stroma membranes (Staehelin and Arntzen, 1983), could not be detected in the collected membranes upon treatment with high α-DDM concentrations (Figure 6),

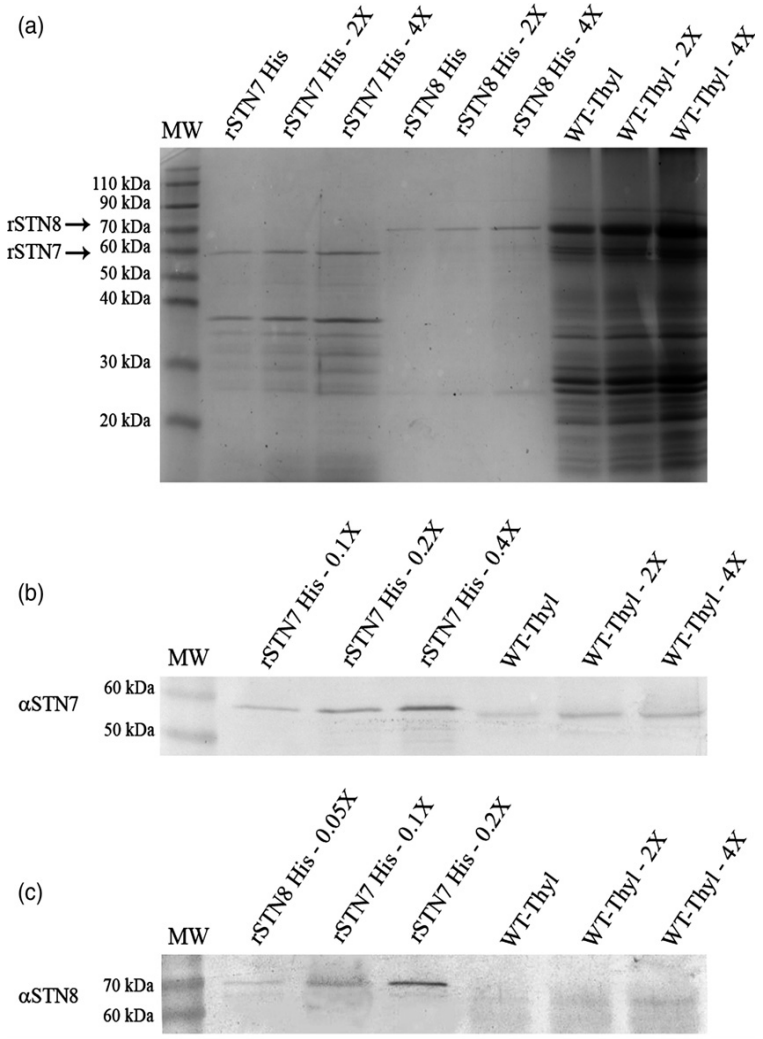

Figure 5. Determination of STN7 and STN8 kinases relative amount in isolated thylakoids.
His-tagged proteins were purified by affinity chromatography and checked in Tris-Glycine sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Immunoblot analyses were performed using anti-STN7 and anti-STN8 antibodies on these fractions and on thylakoid (WT-Thyl) samples isolated from wild-type (WT) plants; 0.75, 1.5 and 3 μg Chls per lane were, respectively, loaded for WT Thyl, WT Thyl – 2X and WT Thyl – 4X. Quantification analysis was performed with a second independent preparation of thylakoids giving a similar result.

Table 1 Determination of chlorophyll *a*/*b* ratio of isolated membranes

	WT-Thyl	WT-pellet 0.09%	WT-pellet 0.18%	WT-pellet 0.37%	WT-pellet 0.75%	stn8-Thyl
Chl *a*/*b*	3.4	4.1	3.8	3.2	2.8	3.4

Thylakoids from WT plants (WT-Thyl) and pellet fractions were obtained with differential solubilization, as previously described (Morosinotto *et al.*, 2010; Pinnola *et al.*, 2015). The spectrum of the 80% acetone-extracted pigments was analyzed by fitting the spectra of the individual pigments in acetone as previously described (Croce *et al.*, 2002). *stn8* thylakoids were used as control.
WT, wild-type.

whereas LHCII polypeptides were enriched. The efficiency of the fractionation was further confirmed by the increased detection of CP43 and CP29 as markers for PSII, and depletion of LHCI, a marker for PSI. In particular, CP29 was progressively enriched in the pellet fractions, whereas LHCI content declined. Detection of STN7 and STN8 kinases in these fractions using anti-STN7 and anti-STN8 antibodies (Figure 6) showed that STN7 was fully depleted from membranes collected upon treatment with α-DDM as in the case of ATPase. The pattern obtained with anti-STN8 antibody was, however, different because a detectable level of STN8 was present in the pellet even at higher α-DDM concentrations, a condition where the membranes are highly enriched in PSII core subunits and thus correspond to the inner region of grana partitions (Pinnola *et al.*, 2015). Thylakoids from *stn8* plants were also included as a control of the anti-STN8 reaction. Thus, both STN7 and STN8 mainly partitioned in stroma-exposed membranes, as evaluated by the ready solubilization of a large fraction of both antigens at low α-DDM concentrations. However, whereas further solubilization rapidly led to STN7 depletion, a significant fraction of

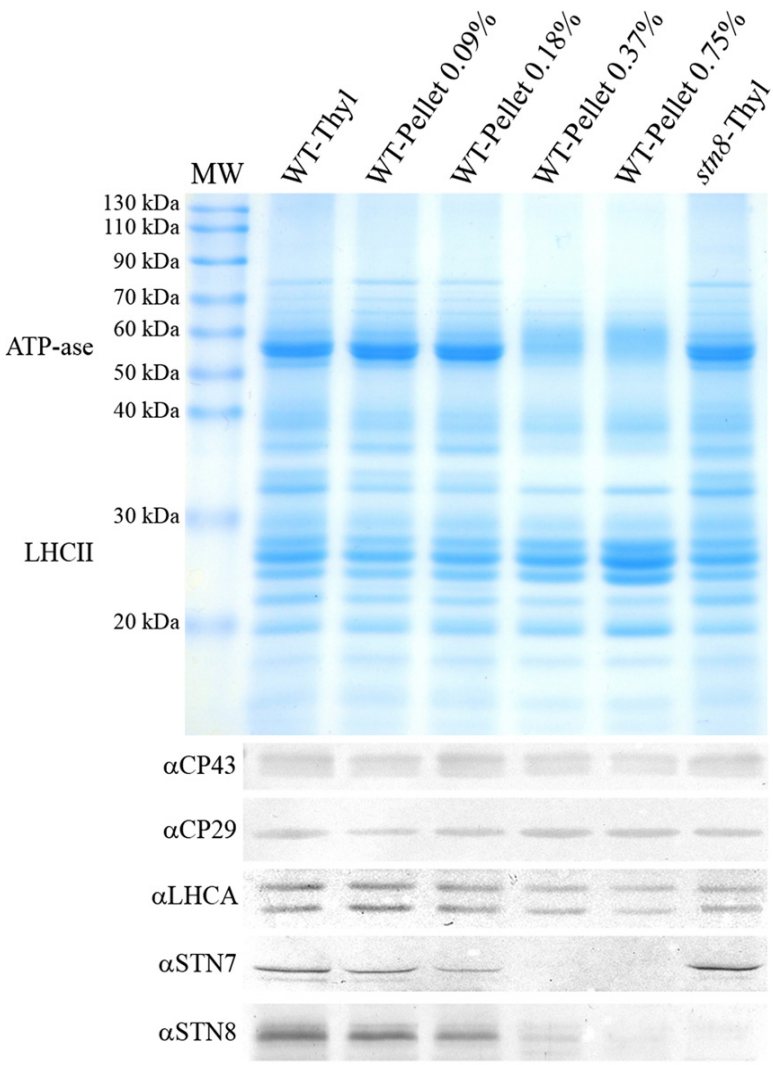

Figure 6. Distribution of STN8 kinase in thylakoid membranes.
Fractionation of wild-type (WT) thylakoid membranes as previously reported (Morosinotto *et al.*, 2010) through solubilization with increasing amounts of α-dodecylmaltoside (a-DDM; Pellet-X%, where X% represents the final concentration of the detergent), followed by centrifugation for separating solubilized membranes from unsolubilized ones. The obtained fractions were used for the determination of STN8 and STN7 kinase localization in thylakoid membranes, using the analyses with antibodies against CP43, CP29 and LHCA as indicators for grana or stroma exposed membranes enrichments. Immunoblot analyses were performed using anti-STN7 and anti-STN8 antibodies; 1.5 μg Chls per lane. Fractionation analysis was repeated giving a similar result. [Colour figure can be viewed at wileyonlinelibrary.com].

STN8 was still detected in fractions corresponding to grana-partitions-derived regions. We calculated the content of STN8 in solubilized membranes by densitometry analysis of the corresponding Western blot signal (Figure 6), normalized to the amount of STN8 present in the insolubilized thylakoids. We obtained a figure of 18 and 3% STN8 detectable in the membranes solubilized with the two highest α-DDM concentrations with respect to its content in whole thylakoids. STN7 was no longer detectable in these two solubilized fractions.

It was recently reported that STN7 and PPH1 are involved in phosphorylation of LHCII but not of CP29 in rice (Betterle *et al.*, 2015; Figure 1). We thus proceeded to verify whether dephosphorylation of CP29 was catalyzed by PBCP phosphatase, responsible for PSII core components dephosphorylation in *A. thaliana* (Samol *et al.*, 2012). Because a knockout line of the PBCP rice ortholog is not available, we proceeded to the preparation of recombinant *Os*PBCP. To this aim, we identified a putative sequence in rice (LOC_Os01g07090) based on the phylogenetic analysis of *At*PBCP orthologs (Samol *et al.*, 2012). The mature coding sequence was amplified from cDNA *Oryza sativa* cv. Nipponbare, excluding the transit peptide sequence deduced through bioinformatic analysis with ChloroP. An N-terminally HIS-tagged PBCP (rPBCP) was expressed in *E. coli*, followed by affinity chromatography in denaturing conditions. Upon verification of eluted fractions with SDS–PAGE (Figure 7a), fraction rPBCP-EL.3 was refolded *in vitro* according to a previously described protocol (Pribil *et al.*, 2010). The phosphatase activity of rPBCP was verified using para-nitrophenylphosphate (pNPP), as previously described (Samol *et al.*, 2012). The specific activity was enhanced by the presence of dithiothreitol (DTT), Mn^{2+} cation and α-DDM (Figure 7b). On this basis we conclude that an active recombinant *Os*PBCP was successfully obtained.

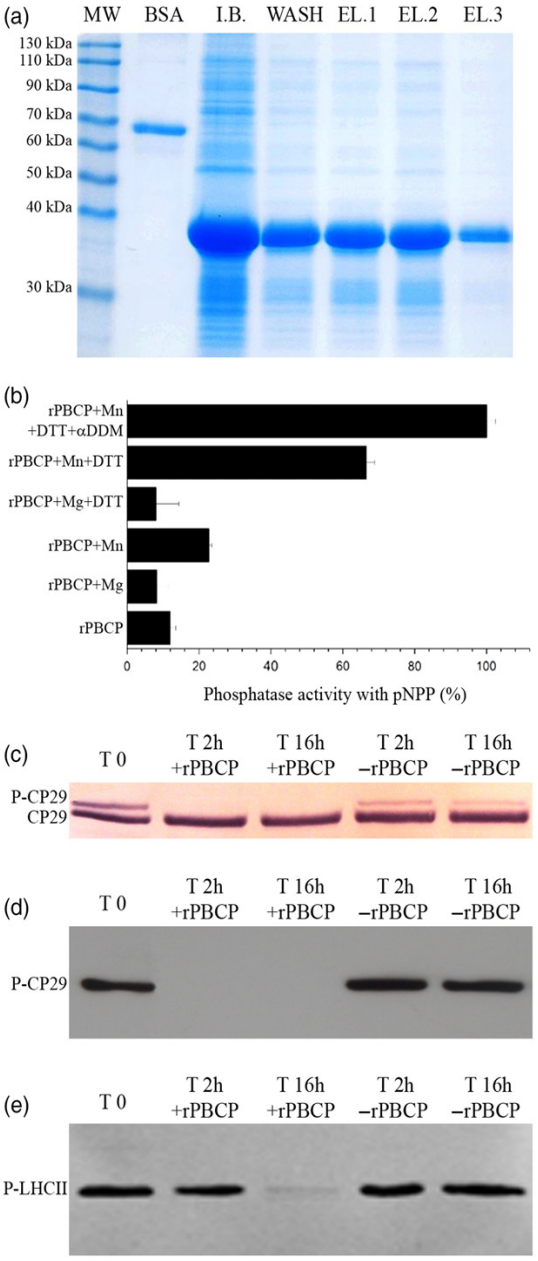

Figure 7. Recombinant PBCP of rice is able to dephosphorylate P-CP29.
(a) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of fractions resulted from affinity chromatography for purification of recombinant PBCP-HisTag in denaturant condition; 1 μg bovine serum albumin was loaded as a reference.
(b) *In vitro* spectrophotometric assay of recombinant PBCP activity upon *in vitro* refolding. The dephosphorylation of para-nitrophenylphosphate (pNPP) was measured by the increase in absorption at 405 nm in 1-mL reaction volumes containing 1 μg recombinant PBCP, 300 mM NaCl, 50 mM HEPES, pH 8.0, 10 mM pNPP, and the additives indicated. Error bars represent the SD of triplicate measurements.
(c) Thylakoid membranes were collected from high light (HL)-treated leaves of wild-type (WT) plants. Samples (1 μg Chls) were finally incubated for 2 h/overnight in the presence/absence of purified recombinant PBCP (1 μg). Immunoblot analysis was performed using anti-CP29 antibody.
(d) Monomeric antenna complexes were purified by sucrose gradient fractionation of solubilized thylakoids used in (c). Samples (0.25 μg Chls) were treated as above. Immunoblot analysis was performed using anti-phosphothreonine (P-Thr) antibody.
(e) Trimeric LHCII complexes were purified as in (d), using thylakoids isolated from leaves treated with photosystem II (PSII)-enriched light. Samples (0.25 μg Chls) were treated as above. Immunoblot analysis was performed using anti-P-Thr antibody. [Colour figure can be viewed at wileyonlinelibrary.com].

We then proceeded to verify if rPBCP was active specifically on P-CP29. To this aim we used as substrate thylakoids isolated from HL-treated rice WT plants. The substrate was incubated for different time lengths either with or without rPBCP. After incubation, the reaction mixtures were precipitated in cold acetone and loaded on an SDS–PAGE followed by immunoblot analysis with the anti-CP29 antibody. rPBCP was effective in dephosphorylating P-CP29 contained in thylakoids as judged from the complete depletion of the P-CP29 band upon 2 h treatment (Figure 7c) with a corresponding accumulation of CP29. Dephosphorylation activity was also detected in samples without the addition of rPBCP, possibly due to the presence of the endogenous enzyme *Os*PBCP in traces. Also, we solubilized HL-treated thylakoids with α-DDM and purified monomeric antenna complexes, including P-CP29, by sucrose gradient ultracentrifugation. This monomeric LHC fraction was used as the substrate for rPBCP, whose activity was followed by using anti-P-Thr antibody, capable of recognizing P-CP29 (Figure 7d). Upon 2 h treatment with rPBCP, P-CP29 was totally absent, while no dephosphorylation activity was detected in the samples incubated without the recombinant enzyme. The specific activity of rPBCP against P-CP29 versus P-LHCII was also determined. WT rice leaves were treated with PSII-specific light for inducing LHCII phosphorylation (Pesaresi *et al.*, 2009), and trimeric LHCII was purified by sucrose gradient centrifugation. Assay of P-LHCII with anti-P-Thr showed that 2 h incubation of P-LHCII with rPBCP yielded a very low decrease in P-LHCII (Figure 7e), if any. The P-LHCII signal was still evident upon overnight treatment, although decreased. Thus, the activity of rPBCP on P-LHCII was far lower than on P-CP29.

DISCUSSION

High light-induced phosphorylation of CP29 was reported in monocots, including corn, rye, barley and rice (Bergantino *et al.*, 1995; Mauro *et al.*, 1997; Hwang *et al.*, 2003; Pursiheimo *et al.*, 2003; Chen *et al.*, 2009, 2013; Liu *et al.*, 2009); yet, the identity of the kinase is unknown. Despite *Os*CP29 (LHCB4) is a member of the LHC protein family, sharing high homology to LHCII including a similar structural organization (Liu *et al.*, 2004; Pan *et al.*, 2011), it is not phosphorylated by the STN7 kinase (Betterle *et al.*, 2015), responsible for activity on LHCII (Bellafiore *et al.*,

2005). This is surprising as CP29 and LHCII are near-neighbors within PSII supercomplexes (Bassi and Dainese, 1992; Boekema *et al.*, 1999; Caffarri *et al.*, 2009). The second-best candidate for the role of CP29 kinase is the STN8 kinase because it is the only known threonine kinase enzyme, besides STN7, associated to maize thylakoids (Friso *et al.*, 2010). Moreover, differently from STN7, it is not inhibited by HL (Bonardi *et al.*, 2005; Vainonen *et al.*, 2005), a property consistent with CP29 phosphorylation occurring under excess light (Bergantino *et al.*, 1995). Indeed, STN8 was shown to act on non-homologous and structurally unrelated substrates, i.e. the different PSII core proteins (Bonardi *et al.*, 2005; Nath *et al.*, 2013a).

The comparison of *O. sativa* WT and thylakoid kinase mutants, either dark-adapted or HL-treated, clearly showed that P-CP29 could not be detected in the absence of STN8 kinase. This result was evident upon analysis by SDS–PAGE followed by immunoblot (Figure 1) or by Coomassie stain (Figure 2a). Due to the slower migration of rice P-CP29 with respect to CP29 in SDS–PAGE gels (Bergantino *et al.*, 1998; Hwang *et al.*, 2003; Chen *et al.*, 2009; Liu *et al.*, 2009), CP29 phosphorylation in rice was previously unrecognized (Nath *et al.*, 2013a): in fact, P-CP29 co-migrates with heavily phosphorylated D1 and D2 subunits that form diffuse bands over the ~30 kDa MW range (Figure 2b). Here, we show that the combined use of a CP29 antibody and a high-resolution gel system allows for a clear detection of P-CP29 (Figure 2a). Moreover, a complete separation among P-PSII core subunits versus P-CP29 can be obtained by a pre-fractionation of the thylakoid membranes using sucrose gradient ultracentrifugation (Figure 3a). This technique allowed to separate solubilized complexes of different MWs; indeed, PSII core is a complex of 300 kDa including P-CP43, P-D1 and P-D2, instead P-CP29 is a smaller monomeric holoprotein of ~40 kDa including chlorophyll and carotenoid co-factors (Figure 3a–c). We conclude that the long-seeked CP29 kinase can be indeed identified with *Os*STN8.

CP29 is not the only substrate of *Os*STN8. Rather, this kinase retains the main substrates previously determined for the ortholog enzyme in *A. thaliana*, namely CP43, D1 and D2 (Bonardi *et al.*, 2005; Vainonen *et al.*, 2005), as shown by the lack of P-CP43, P-D1 and P-D2 bands in immunoblots from HL-*stn8* thylakoids (Figure 2b). The inclusion of CP29 as a substrate for *Os*STN8 may depend on differences in the kinase or in the structure of CP29. *Os*CP29 is encoded by a single gene in rice (Loc_Os07 g37240), whereas three different orthologs are present in *A. thaliana* (LHCB4.1, AT5G01530; LHCB4.2, AT3G08940; LHCB4.3, AT2G37140). It was shown by sequence alignments that the three isoforms of *At*CP29 are highly conserved to the rice ortholog gene, and even the phosphorylation site of *Os*CP29, threonine-82, is maintained (Betterle *et al.*, 2015), suggesting changes in the kinase structure are responsible for the wider substrate specificity. This is supported by two evidences: (i) only traces of P-CP29 were found in Arabidopsis as a result of STN7 kinase activity (Tikkanen *et al.*, 2006; Fristedt and Vener, 2011); (ii) *Os*STN8 and *At*STN8, although sharing high similarity in the C-terminal catalytic domain, are divergent in their N-termini, a domain suggested to be involved in substrate binding (Betterle *et al.*, 2015).

A further substrate-related observation concerns the P-CP29/CP29 ratio that can be obtained with different durations of the HL treatment and was observed to tend to 1:1 without exceeding this value even upon long exposure. This seems a general feature in several species, including corn (Bergantino *et al.*, 1995) that cannot be easily rationalized. One hypothesis is based on the dimeric structure of PSII supercomplexes (Santini *et al.*, 1994; Boekema *et al.*, 1999) and assumes that the kinase might have access to one monomer only. Verification of this hypothesis is difficult, as even the mildest solubilization of thylakoid membranes cannot preserve interactions between PSII and the STN7 or STN8 kinases (Lemeille *et al.*, 2009). We observed that the level of CP29 phosphorylation in HL-treated WT and overexpressing STN8 plants was equal (Figure 1), implying that the amount of STN8 kinase was not a limiting factor and suggesting the topological distribution of the kinase could be important. It should be considered that STN8 requires the reduction of cytochrome b_6f (cyt b_6f) for its activation: indeed, no phosphorylation of CP29 was observed in the presence of DCMU or DBMIB (Pursiheimo *et al.*, 2003; Betterle *et al.*, 2015), two compounds blocking electron transfer from PSII to cyt b_6f, suggesting a mandatory interaction between STN8 and reduced cyt b_6f. The latter was recently shown to be distributed not only in the stroma lamellae but also in the grana domain (Johnson *et al.*, 2014), where the STN8 substrate (CP29) is also present. The kinase access to 50% of CP29 could be explained by the localization of the kinase within a specific (sub)domain of grana partitions that hosts PSII supercomplexes including CP29 in contrast with non-CP29 PSII complexes that can be also found in stroma-exposed thylakoid domains and agranal chloroplasts (Bassi *et al.*, 1995). However, the fraction of CP29-depleted PSII complexes was shown to be very low (Santini *et al.*, 1994). The thylakoid content of STN7 was twice as high as STN8 (Figure 5) and was excluded from grana stacks, showing a behavior similar to the ATPase. Instead, a significant fraction of STN8 was located in preparations of grana partitions in agreement with previous reports in *A. thaliana* (Wunder *et al.*, 2013; Figure 6). This different distribution was evident even upon treatment with the highest detergent strength that trims away grana margins (Morosinotto *et al.*, 2010; Pinnola *et al.*, 2015). Thus, the presence of STN8 in grana domains, different from STN7, is consistent with the

presence of both its activator and its substrate. Although present in grana membranes, *Os*STN8 is mostly removed by DDM final concentrations of 0.75%, implying the center of grana partitions was strongly depleted of both kinases (Figure 6). Indeed, the mobility of PSII complexes within grana membranes appears to be limited (Kirchhoff, 2014). We suggest that the major reason for limitation of P-CP29 phosphorylation level to 50% is the lack of *Os*STN8 from the center of grana disks.

As for the difference between monocots and dicots, the finding that *Os*STN8 was localized in similar thylakoid domains as *At*STN8 supports the conclusion that phosphorylation of CP29 in rice is allowed by some critical differences in the sequences of Arabidopsis and rice STN8, as previously suggested (Betterle *et al.*, 2015), rather than by their differential localization in thylakoid membranes.

Phosphorylation events are regulated by antagonistic kinase/phosphatase pairs; thus, we attempted identification of the phosphatase acting on P-CP29. PPH1 phosphatase was recently shown not to be involved in the process of CP29 dephosphorylation (Betterle *et al.*, 2015). We verified the hypothesis that the enzyme antagonist to STN8 activity for PSII core phosphorylation, namely PBCP in *A. thaliana* (Samol *et al.*, 2012), was involved. The ortholog maize gene product (gene model GRMZM2G003096_P01) was found to be intrinsic of thylakoid membranes, as for maize PPH1 ortholog (Friso *et al.*, 2010). Because no additional phosphatases were detected by proteomic analysis (Friso *et al.*, 2010), the hypothesis appeared to be well grounded. The ortholog sequence from rice expressed in *E. coli* (Figure 7a) showed an activity strictly dependent on the presence of both Mn^{2+} cation and DTT, which could be significantly increased in the presence of α-DDM (Figure 7b), in agreement with previous reports with *At*PBCP (Samol *et al.*, 2012). We observed that recombinant *Os*PBCP (rPBCP) was highly active and capable of fully dephosphorylating P-CP29 in thylakoid membranes isolated from HL-treated WT rice leaves (Figure 7c). Dephosphorylation activity of rPBCP on P-CP29 was far higher than on P-LHCII (Figure 7d and e), suggesting P-CP29 rather than P-LHCII is its physiological substrate. These results further supported the assessment that *Os*PBCP protein is not only responsible for PSII core subunit dephosphorylation, as previously observed in *A. thaliana* (Samol *et al.*, 2012), but it is also responsible for P-CP29 dephosphorylation in monocots. Although the absence of a rice *pbcp* mutant does not allow to prove that beyond any doubt, this conclusion is consistent with the observation that the phosphorylation of substrates in thylakoids is regulated by a specific kinase and phosphatase pair, as observed for the regulation of: (i) P-LHCII, involving STN7 and PPH1/TAP38 (Rochaix *et al.*, 2012); (ii) the phosphorylation of PSII core subunits, involving STN8 and PBCP (Nath *et al.*, 2013b).

In conclusion, CP29 phosphorylation is involved in photoprotection (Mauro *et al.*, 1997; Betterle *et al.*, 2015) and caused a protein conformational change (Croce *et al.*, 1996). Based on our data, we hypothesize that the activity of STN8 kinase is necessary for the repair of photodamaged PSII though phosphorylation of D1 subunit of PSII, a step essential for the PSII repair cycle (Pesaresi *et al.*, 2011; Tikkanen and Aro, 2012; Nath *et al.*, 2013b). In addition, the phosphorylation of CP29, which occurs in the same photoinhibitory conditions, ensures the maintenance of a quenched state of the PSII antenna system (Mauro *et al.*, 1997). This is useful to minimize reactive oxygen species production during the disassembling and repair of damaged PSII. In order to confirm this hypothesis for the function of CP29 phosphorylation, the analysis of a mutant depleted of CP29 phosphorylation site will be of the utmost importance.

EXPERIMENTAL PROCEDURES

Plant materials and growth condition

Oryza sativa stn8 mutant was obtained from a T-DNA insertional mutant library (Nath *et al.*, 2013a), whereas *stn7* and *pph1* mutants were, respectively, obtained from *Oryza Tag Line* and RMD rice mutant libraries (Betterle *et al.*, 2015). Plants were grown in a greenhouse at 35/28°C (day/night) with natural light during warm seasons. Leaves detached from rice plants were dark-adapted for 2 h and then treated with HL (1500 μmol photons m^{-2} sec^{-1}) using halogen lamps filtered by a 1-cm water layer to remove infrared radiation. To induce LHCII phosphorylation, dark-adapted leaves were treated for 1 h with orange light at 100 μmol photons m^{-2} sec^{-1} (30 W warm white fluorescent lamps filtered through Lee Filters 105 Orange) as previously reported (Pesaresi *et al.*, 2009).

Membrane isolation

Functional chloroplasts/thylakoids were isolated as previously described (Casazza *et al.*, 2001). Grana membranes were prepared according to a previous report (Morosinotto *et al.*, 2010).

Sucrose gradient fractionation

Membranes corresponding to 150 mg chlorophyll were washed with 5 mM EDTA and then solubilized with 0.6% β-DDM (Figure 3) or α-DDM (Figure 7). Solubilized samples were then fractionated by ultracentrifugation in a 0.1–1 M sucrose gradient containing 0.03% of the detergent used for thylakoid solubilization and 20 mM HEPES, pH 7.5 (SW60 Ti Rotor, 5 h 30 min at 60 000 rpm, 4°C).

Pigment composition

Chlorophyll *a*/*b* ratio of isolated membranes was analyzed by fitting the spectrum of the 80% acetone-extracted pigments with the spectra of the individual pigments in acetone as described previously (Croce *et al.*, 2002).

Gel electrophoresis and immunoblotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis was performed with the Tris-Glycine buffer system (Laemmli,

1970), with the addition of 2 M urea to the running gel to separate phosphorylated and unphosphorylated CP29 polypeptides. For Western blot analysis, chloroplast or thylakoid samples were loaded on SDS–PAGE and electroblotted on nitrocellulose membranes or polyvinylidene fluoride membranes (0.45 μm pore size; Millipore). Proteins were detected using homemade anti-CP29, anti-CP43, anti-LHCA, anti-Rubisco, anti-STN7 or anti-STN8 antisera, all raised in rabbit, and an alkaline phosphatase-conjugated secondary antibody (Sigma-Aldrich, St. Louis, MO, USA). In the cases of anti-STN7 and anti-STN8 antisera, recombinant *A. thaliana* mature STN7 and STN8 were provided by Prof. Jean David Rochaix (University of Geneva) and used for rabbit immunization. For immunoblotting analysis using anti-CP29 antibody (Agrisera, Vannas, Sweden), thylakoid samples were isolated as previously described (Oh *et al.*, 2009). For detection of phosphoproteins, anti-P-Thr polyclonal antibody (Cell Signaling, Danvers, MA, USA) was used. Signal amplitude was quantified using GelPro 3.1 software (Bio-Rad, Hercules, CA, USA).

Expression of recombinant proteins

OsPbcp gene sequence was identified upon alignment of *AtPbcp* gene sequence (Samol *et al.*, 2012) with rice genome. Chloroplast transition peptides of *Stn7* (LOC_Os05g47560), *Stn8* (LOC_Os05g40180) and *Pbcp* (LOC_Os01g07090) gene products were determined using ChloroP method. cDNA sequences of the predicted mature proteins were cloned into pDEST17 (Invitrogen, Waltham, MA, USA), a Gateway compatible vector to allow for N-terminal 6x-His tagging, and expressed in *E. coli* strain BL21. Recombinant proteins were purified using a protocol described previously (Paulsen *et al.*, 1993) and resuspended in denaturant solution (Tris 20 mM pH 7.4, NaCl 300 mM, urea 6 M). Denatured proteins were loaded in Ni-Sepharose column, and elution of tagged proteins was performed in a solution containing Tris 20 mM pH 7.4, SDS 0.2% and 200 mM imidazole. Denatured rPBCP was reconstituted as recently described (Pribil *et al.*, 2010). Subsequently, 1 μg phosphatase was incubated together with thylakoid membranes corresponding to 1 μg total chlorophylls. The dephosphorylation reaction was performed in 100 μL containing 0.03% (w/v) α-DDM, NaCl 100 mM, 10 mM $MnCl_2$, 10 mM DTT, 20 mM HEPES (pH 7.5), at 25°C for 2 h and overnight. The reaction mixture was precipitated in cold acetone and then loaded on a SDS–PAGE. Immunoblot was finally performed using anti-CP29- or anti-P-Thr-specific antibodies, as described above.

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Chapter 2

Expression of rice STN8 in Arabidopsis thaliana reproduces high light-induced phosphorylation of the monomeric antenna protein CP29

Abstract

Reversible phosphorylation of thylakoid proteins is a widespread phenomenon among photosynthetic organisms when subject to changing light conditions. It has been demonstrated that in monocots, as opposed to dicots, upon high light exposure the minor antenna CP29 is phosphorylated (P-CP29) enhancing NPQ and reducing singlet oxygen production. Analysis of rice *stn8* mutant (Chapter 1) showed that in addition to that of the PSII core proteins, CP29 phosphorylation was suppressed as well, thus proving that STN8 is the kinase involved in CP29 phosphorylation in monocots. In dicot plants, CP29 phosphorylation was reported to occur at a far lower extent and in different sites compared to monocots.

In order to verify the possibility to induce a robust phosphorylation of CP29 even in dicots, and increase their resistance to abiotic stresses, we transformed *stn8* mutant of *Arabidopsis thaliana* with the *OsStn8* gene. The expression of rice STN8 kinase determined not only the restoration of the PSII core proteins phosphorylation, but in addition CP29 was strongly phosphorylated under high light conditions, as opposed to the wild type strain. Furthermore, we observed that the introduction of this process in Arabidopsis was associated to a slight increase in NPQ with respect to WT.

Introduction

Reversible phosphorylation of thylakoid membrane proteins is a way for photosynthetic organisms to cope with changing light conditions. More specifically, phosphorylation of either the major antenna system LHCII or the PSII core occur in different lighting conditions. Indeed, LHCII phosphorylation is occurring in a low light state in order to balance the rate of photon absorption by the two photosystems (State Transitions, ST), regulated in land plants by the kinase/phosphatase pair STN7 and TAP38/PPH1, respectively (Bellafiore et al., 2005; Pribil et al., 2010; Shapiguzov et al., 2010), while the PSII core phosphorylation occurs in high light conditions, allowing for the turnover of

damaged core proteins, in particular D1, mediated by STN8 kinase and PBCP phosphatase (Bonardi et al., 2005; Samol et al., 2012).

Among the proteins that are phosphorylated in high light, namely D1, D2 and CP43, an additional protein is phosphorylated, the minor antenna protein CP29, a mechanism that had been first discovered and characterized in maize (Bergantino et al., 1995). Since then, this phenomenon has been characterized in various species, from C3 to C4 plants (Bergantino et al., 1998; Pursiheimo et al., 2001; Chen et al., 2009), always in connection to environmental cues (Chen et al., 2013). Monocots show many unique putative phosphorylation residues for CP29. In fact, only two threonine residues are conserved in all plants, while monocotyledons have many serine and tyrosine residues, sites of possible phosphorylation, which are absent in dicots (Chen et al., 2013). CP29 phosphorylation has been observed in dicots as well (Tikkanen et al., 2006), but the phenomenon occurs at very low level and not easily detectable as in the case of monocots. Indeed, *Arabidopsis thaliana* CP29 can be phosphorylated, but at very low levels and at different threonine residues compared to monocots (Fristedt and Vener, 2011). Phosphorylation of maize CP29 was demonstrated to induce a protein conformational change, thus leading to the hypothesis that phosphorylated CP29 (P-CP29) might be involved in photoprotection (Croce et al., 1996). The same phosphorylation event as that observed in maize was proven to occur in rice as well and a recent study has proven for the first time a mechanistic connection between CP29 phosphorylation and NPQ, so far believed to be independent processes (Betterle et al., 2015).

In this chapter, we analyse Arabidopsis mutants lacking native STN8 kinase transformed with the *OsStn*8 gene. These transformed lines showed a robust CP29 phosphorylation, implying that it was possible to introduce a monocot-specific photo-protective mechanism into a dicot.

Results

Bioinformatic comparisons of CP29 and STN8 proteins from different monocots and dicots

In order to elucidate the difference in behaviour between monocots and dicots, involving CP29 phosphorylation, primary sequences of the minor antenna protein from different species were compared (Fig. 1A). Of particular importance was the determination of whether the phosphorylation site identified in rice, Thr-83 (Betterle et al., 2015), conserved with maize CP29 (Bergantino et al., 1998), was present even in Arabidopsis sequences. As shown in figure 1A, similarity between the sequences is very high, from 80 to 90%, with the biggest differences clustering mainly in the N-terminal region. Interestingly, the residue of interest is maintained among the different species, indicating that the lack of high light phosphorylation of CP29 in dicots is probably due to the kinase's structure, rather than depending on differences in the antenna protein itself. To evaluate this, we compared the sequence of the STN8 kinase of the various monocots and dicots considered for CP29 alignment (Fig. 1B). While the N-terminal region was much longer in monocots compared to *A. thaliana* (Fig. 1B), the catalytic domain retains a high homology among the various species, beginning around residue 200, as shown for *Oryza sativa* (Fig. 1C). The longer N-terminal domain might be responsible for the different substrate specificity of the monocot kinase, and we expressed rice STN8 kinase in Arabidopsis *stn8* mutants for verifying that. The expression of the heterologous enzyme should not only restore PSII core phosphorylation, but it could also induce a strong CP29 phosphorylation.

```
A.t.4.1  -MAATSAAAAAASSIMGTRVAPGIHPGSGRFTAVFGFGK---KKAAPKKSAKKTVTTDRP    56
A.t.4.2  MA--ATSTAAAASSIMGTRVVSDISSNSSRFTARFGFGT---KKASPKKA--KTVISDRP    53
C.s.     MAATSTAVAAASSSIMGTRVVSDIHSGSSRFTARFGFGT---KKAAPKKA--KKVITDRP    55
B.d.     ---MASSVAAAASTFLGTRLA-DPAPQNGRIVARFGFGLGRKKAPAKKA-SRAPPTTDRP    55
H.v.     ---MASSVAAAASTFLGTRLA-DPRPQNGRIVARFGFGK--KKAPP-KK-AKAPPTTDRP    52
O.s.     ---MASSVAAAASTFLGTRLA-DPAPQSGRIVARFGFGGGKKAA-AKKAARPSAPTTDRP    55
Z.m.     ---MASSVAAAASTFLGTRLA-DPAPQNGRIVARFGFGLGGKAKPAPKKVAKTSTSSDRP    56
         ::::.***:*:::***:. .    ..*:.* ****         *        :***

A.t.4.1  LWYPGAISPDWLDGSLVGDYGFDPFGLGKPAEYLQFDIDSLDQNLAKNLAGDVIGTRTEA   116
A.t.4.2  LWFPGAKSPEYLDGSLVGDYGFDPFGLGKPAEYLQFDLDSLDQNLAKNLYGEVIGTRTEA   113
C.s.     LWFPGATSPEYLDGSLVGDYGFDPFGLGKPAEYLQFDLDSLDQNLAKNLYGEVIGTRTEE   115
B.d.     LWFPGAQAPEYLDGTLVGDYGFDPFGLGKPAEYLQYDPDSLDQNLAQNLAGEVIGTRFED   115
H.v.     LWFPGAQAPEYLDGTLVGDYGFDPFGLGKPAEYLQYDVDSLDQNLAQNLAGEIIGTRFED   112
O.s.     LWFPGAVAPDYLDGSLVGDYGFDPFGLGKPAEYLQFELDSLDQNLAKNNAGEIIGTRFET   115
Z.m.     LWFPGAVAPDYLDGSLVGDYGFDPFGLGKPVEYLQFELDSLDQNLAKNEAGGIIGTRFES   116
         **:*** :*::***:***************.****:: *********:*  * :**** *

A.t.4.1  ADAKSTPFQPYSEVFGIQRFRECELIHGRWAMLATLGALSVEWLTGVTWQDAGKVELVDG   176
A.t.4.2  VDPKSTPFQPYSEVFGLQRFRECELIHGRWAMLATLGAITVEWLTGVTWQDAGKVELVDG   173
C.s.     VDPKSTPFQPYSEVFGLQRFRECELIHGRWAMLATLGALTVEWLTGVTWQDAGKVELVDG   175
B.d.     ADIKSTPLQPYAEVFGLQRFRECELIHGRWAMLATLGALTVEWLTGVTWQDAGKVELVDG   175
H.v.     ADVKSTPFQPYAEVFGLQRFRECELIHGRWAMLATLGALTVEWLTGVTWQDAGKVELVDG   172
O.s.     GEVKSTPFQPYTEVFGLQRFRECELIHGRWAMLATLGALSVEWLTGVTWQDAGKVELVDG   175
Z.m.     SEVKSTPLQPYSEVFGLQRFRECELIHGRWAMLATLGALSVEWLTGVTWQDAGKVELVDG   176
          : ****:***:****:**********************::********************

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C.s.     SSYLGQPLPFSISTLIWIEVLVVGYIEFQRNAELDSEKRLYPGGKFFDPLGLASDPEKKA   235
B.d.     SSYLGQPLPFTITTLIWIEVLVIGYIEFQRNAELDPEKRLYPGGSYFDPLGLAADPEKKE   235
H.v.     SSYLGQPLPFTITTLIWIEVLVIGYIEFQRNAELDPERRLYPGGSYFDPLGLAADPEKKE   232
O.s.     SSYLGQPLPFSISTLIWIEVLVIGYIEFQRNAELDPEKRLYPGGSYFDPLGLASDPEKKE   235
Z.m.     SSYLGQPLPFSISTLIWIEVLVIGYIEFQRNAELDPEKRLYPGGSYFDPLGLAADPEKKE   236
         **********:*:*********:************ *:******.:*******:****.

A.t.4.1  QLQLAEIKHARLAMVAFLGFAVQAAATGKGPLNNWATHLSDPLHTTIIDTFSSS-    290
A.t.4.2  QLQLAEIKHARLAMVGFLGFAVQAAATGKGPLNNWATHLSDPLHTTIIDTFSSS-    287
C.s.     QLQLAEIKHARLAMVAFLGFAVQAAATGKGPLNNWATHLSDPLHTTIIDTFSSS-    289
B.d.     RLQLAEIKHARLAMVGFLGFAVQAAATGKGPLNNWATHLSDPLHTTIFDTLGS--    288
H.v.     TLQLAEIKHARLAMVAFLGFAVQAAATGKGRLNNWATHLSDPLHTTIFDTFGSS-    286
O.s.     RLQLAEIKHARLAMVAFLGFAVQAAATGKGPLNNWATHLSDPLHTTIFDTFSSSS    290
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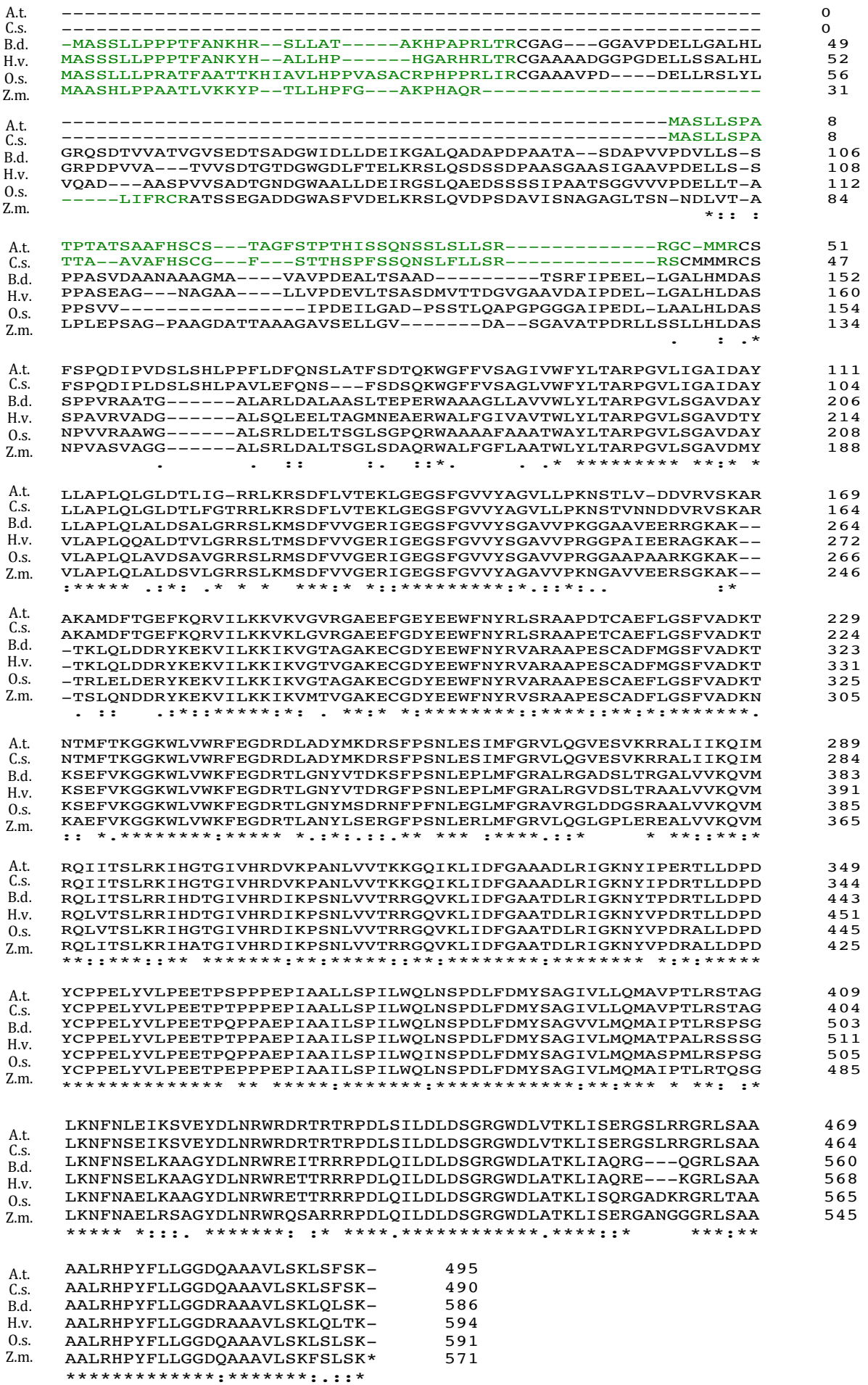
A.t.
C.s.
B.d.
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O.s.
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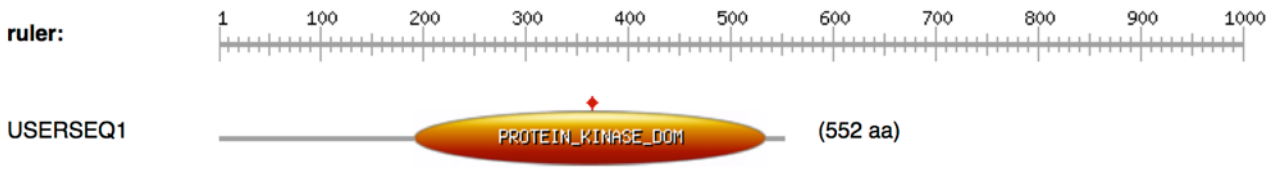

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PDELLTAPPSVVIPDEILGADPSSTLQAPGPGGGAIPEDLLAALHLDASNPVVRAAWGALSRLDEL
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RVARAAPESCAEFLGSFVADKTKSEFVKGGKWLVWKFEGDRTLGNYMSDRNFPFNLEGLMFGRAVR
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VPDRALLDPDYCPPELYVLPEETPQPPAEPIAAILSPILWQINSPDLFDMYSAGIVLMQMASPMLR
SPSGLKNFNAELKAAGYDLNRWRETTRRRPDLQILDLDSGRGWDLATKLISQRGADKRGRLTAAAA
LRHPYFLLGGDQAAAVLSKLSLSK

Fig. 1: Sequence analysis of CP29 and STN8 from monocots and dicots. A Multiple alignment of CP29 sequences from *Arabidopsis thaliana* isoform 4.1 *(A.t.4.1),* isoform 4.2 *(A.t.4.2), Camelina sativa (C.s.), Brachypodium distachyon (B.d.), Hordeum vulgare (H.v.), Oryza sativa (O.s.), Zea mays (Z.m.).* Green highlight represents predicted signal peptide sequence using ChloroP. Red highlighted Threonine residue (T) represents conserved amino acid, site of STN8 high light-induced phosphorylation in rice (Betterle et al., 2015); **B** Multiple alignment of STN8 sequences from different species as in figure 1A*;* **C** ScanProsite prediction of the kinase domain of *Os*STN8 (top), highlighted in yellow in the sequence (bottom), from 192-534, with a protonatable site (red diamond) at residue 365.

Expression of *Oryza sativa* STN8 kinase in *Arabidopsis thaliana stn8* mutants

We complemented two different mutants of *Arabidopsis thaliana*, *stn8* and *stn7stn8,* with STN8 obtained from rice. The full coding sequence of *OsStn8* was amplified from *Oryza sativa* spp. Japonica genomic DNA and introduced in a binary gateway vector pH7WG2 under the control of the constitutive promoter CaMV 35S. The resistant lines, once grown in soil for two-to-three weeks, were screened through Western Blot analysis using an anti-STN8 polyclonal antibody (Fig. 2). As mentioned previously, *Os*STN8 showed a longer N-terminal with respect to *At*STN8, and in fact the predicted molecular weight of *Os*STN8 was of 60 kDa compared to 50 kDa of *At*STN8. For this reason, the anti-STN8 antibody

showed a different recognition pattern between the two different kinases, where *At*STN8 shows a single band at apparent molecular weight of around 50kDa, *Os*STN8 showed a more complex profile, with a strong band falling at around 60kDa, as predicted, and a number of bands at lower molecular weight, with the smallest one at the same level as *At*STN8. This led us to presume that in *A. thaliana* the processing of the protein is different from rice, or, most likely, some proteolytic activity might occur giving rise to different forms of the rice kinase expressed.

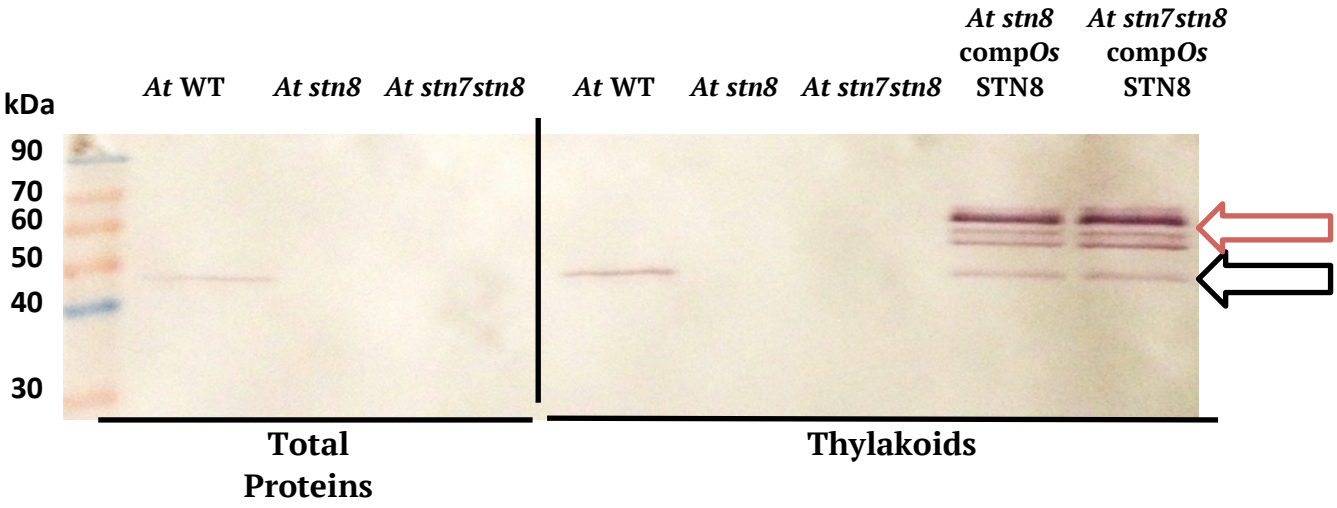


Fig. 2: Screening of *A. thaliana* mutants complemented with *Os*STN8. Western blot analysis using an anti-STN8 antibody on total proteins and thylakoid samples isolated from *A. thaliana* leaves harvested directly from the greenhouse. Comp*Os*STN8 represents plants transformed with *OsStn8* gene. Black arrow indicates the apparent molecular weight of *At*STN8, while the red arrow indicates expected apparent molecular weight of *Os*STN8. Loading: 1µg of chlorophylls.

Phosphorylation pattern and regulation of *Os*STN8 in complemented *A. thaliana stn8* mutant lines

To determine whether the expression of the rice kinase restored PSII core phosphorylation in *A. thaliana* mutant lines, we conducted an immunoblotting on the second generation of the transgenic lines in figure 2, utilizing an anti-phosphothreonine antibody (P-THR), which recognizes phosphorylated threonine residues as in the case of D1, D2, CP43 (Rochaix et al., 2012). While

PSII core phosphorylation occurred normally in WT, it was absent in the case of *stn8* mutant. Indeed, *stn8* showed the phosphorylation of only LHCII, since STN7 kinase is still present in this line (Fig. 3A). On the other hand, as expected, *stn7stn8* mutant showed no sign of phosphorylation both in the cases of PSII core and LHCII proteins. Lines complemented with *Os*STN8 showed a restored phosphorylation pattern of PSII core subunits, indicating that the heterologous kinase was functional (Figure 3A). Moreover, the phosphorylated band associated to P-D2 and P-D1 was broader in the complemented lines with respect to other samples. This observation rose the suggestion that another protein migrating closely to D1 and D2 could be phosphorylated as well. Indeed, we checked the electrophoretic migration of CP29 and we observed that it was very close to D1 and D2 (Figure 3B).

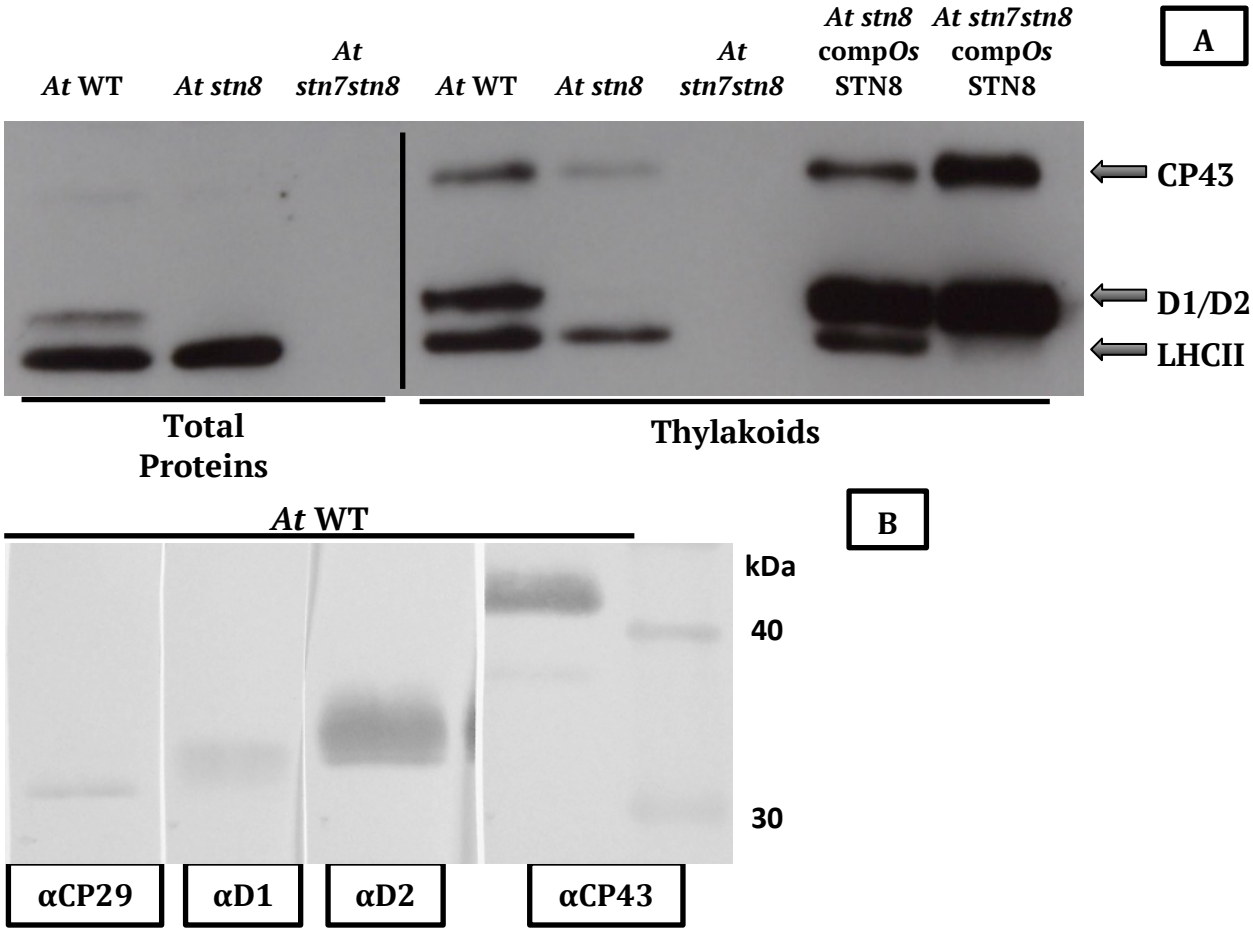


Fig. 3: Phosphorylation pattern of *A. thaliana* mutants complemented with *Os*STN8. **A** Western blot analysis using an anti-phosphothreonine antibody on the same samples as in figure 2. Loading: 1µg of chlorophyll per lane. **B** Western blot analysis of *A.t.* wild type thylakoid samples using anti-CP29, anti-D1, anti-D2 and anti-CP43 antibodies, to evaluate electrophoretic migrations of PSII core subunits and CP29.

Thus, we further investigated if the heterologous expression of *Os*STN8 led to phosphorylation of CP29 as well, and if this process was regulated as in rice. Leaves from Wild type and *stn8* comp*Os*STN8 were previously adapted to dark (D), further treated in high light (HL) at 1300 µmol photons m^{-2} s^{-1}, and recovered in the dark for 1 hour after high light illumination (Rec). Thylakoid samples were isolated from leaves, solubilisation of thylakoids with 1% DM and pigment proteins were fractionated by sucrose gradient ultracentrifugation. The monomeric LHC fraction (mLHC) was collected from the sucrose gradient (Fig. 4A). To exclude the possibility of contamination of the mLHC fractions by PSII core subunits, those samples were initially checked by western blot analysis using anti-D1 and anti-CP29 antibodies (Fig 4B). D1 was absent from the monomeric LHC fraction utilised while CP29 was detected, indicating that the preparation showed no contamination from PSII core proteins. An anti-P-THR immunoblot was performed following SDS-PAGE analysis of the monomeric antenna fraction samples in order to determine whether CP29 was phosphorylated or not in the complemented lines. As shown in figure 4C, the mLHC samples from wild type Arabidopsis were devoid of phosphorylated proteins, whether in D, HL or Rec, while the complemented lines showed a band at apparent molecular weight of 30kDa, which corresponded to CP29, having previously confirmed absence of PSII core subunits. Furthermore, P-CP29 was reduced upon recovery for 1 hour in the dark, indicating that CP29 phosphorylation was regulated as in rice, with the kinase's activity inhibited in low-to-dark lighting conditions, and the related phosphatase active in dephosphorylating the protein.

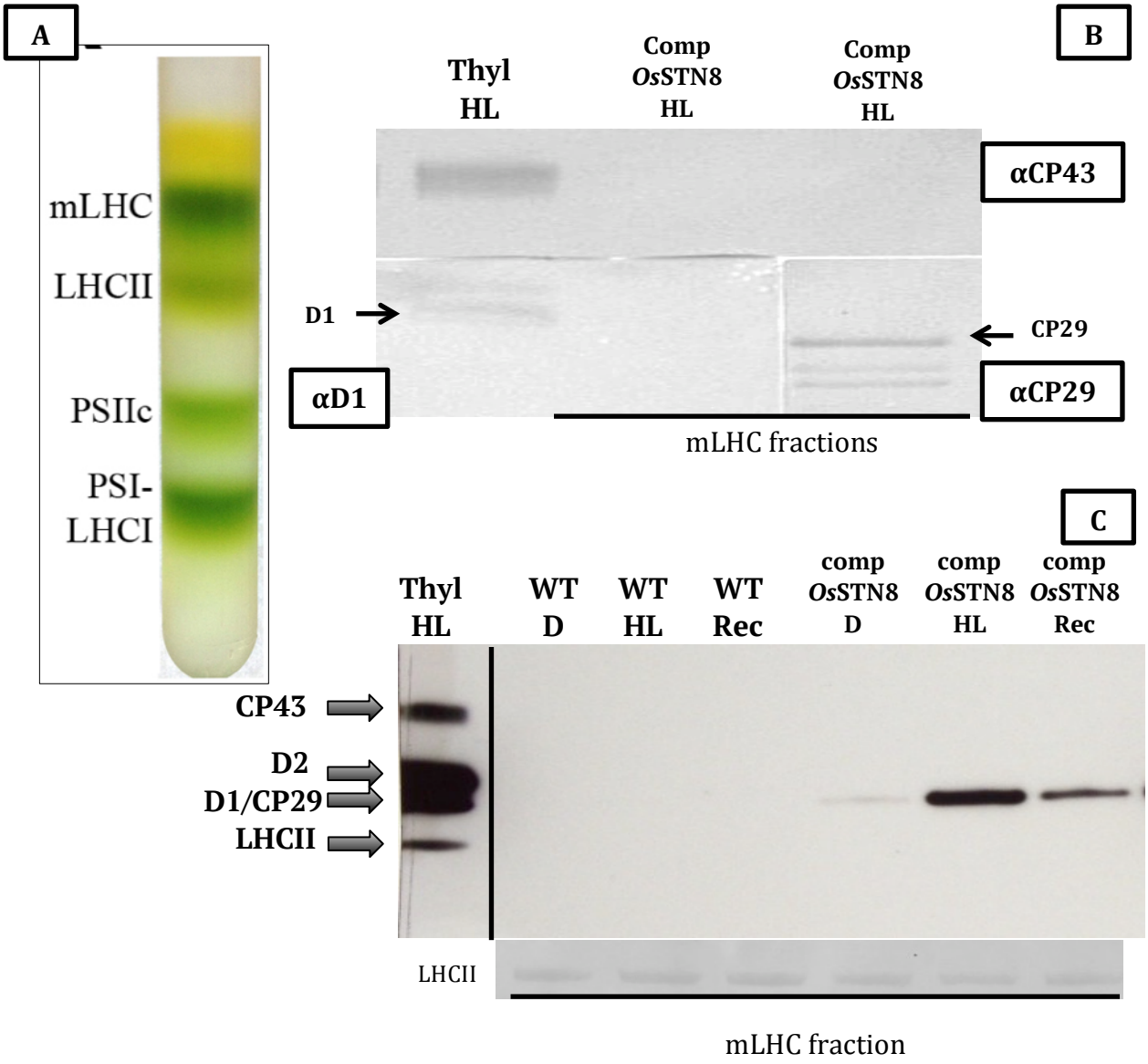
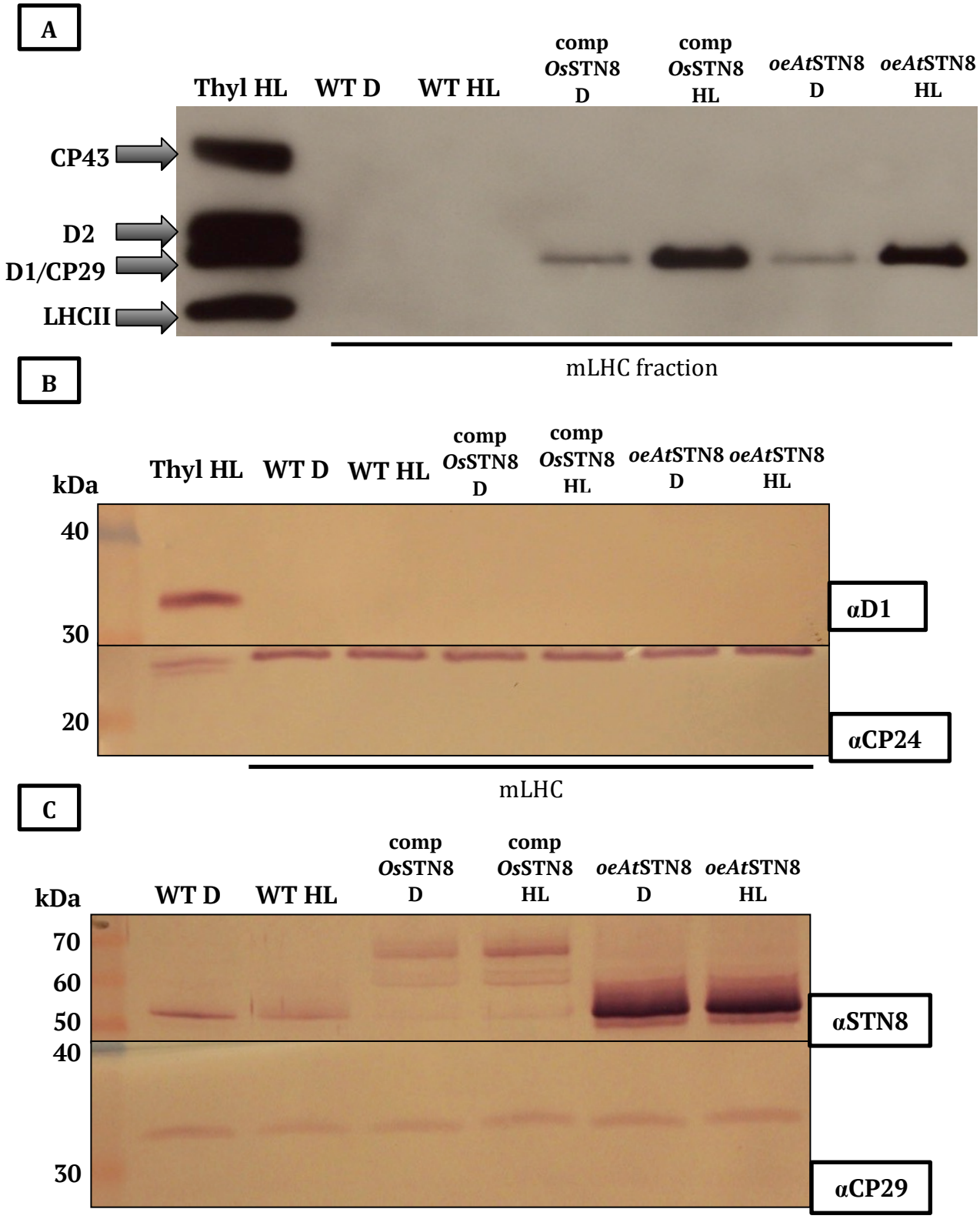


Fig. 4: Evaluation in monomeric LHC antenna fractions of CP29 phosphorylation. A Sucrose gradient fractionation of Arabidopsis thylakoid membranes solubilized with β-dodecylmaltoside. The monomeric antenna fraction (mLHC) was collected to evaluate CP29 phosphorylation in wild type and comp*Os*STN8 samples; thylakoids corresponding to 100µg of total chlorophylls were loaded. **B** Western blot analysis using an anti-CP43, anti-D1 and anti-CP29 antibodies on the collected monomeric LHC fractions (mLHC) . WT: wild type; comp *Os*STN8: *A.thaliana stn8* complemented with *Os*STN8; D: dark treatment for 1-hour; HL: high light treatment at 1300µmol photons m^{-2} s^{-1} for 30min. Thyl: thylakoids. Loading: 0,4µg of chlorophylls for mLHC; 0,8µg of chlorophylls for thylakoids. **C** Western blot analysis (top) and ponceau staining (bottom) using an anti-phosphothreonine antibody on the monomeric LHC fractions isolated from a sucrose gradient from *A.thaliana*. WT: wild type; comp *Os*STN8: *A.thaliana stn8* complemented with *Os*STN8; D: dark treatment for 1-hour; HL: high light treatment at 1300µmol photons m^{-2} s^{-1} for 30min; Rec: recovery, high light treatment followed by dark recovery for 30 min. Thyl: thylakoids. Loading: 0,4µg of chlorophylls for mLHC; 0,8µg of chlorophylls for thylakoids.

Analysis of an *A. thaliana stn8* mutant line overexpressing *At*STN8

Complemented lines showed phosphorylation of CP29. Nevertheless, the significance of this finding was hampered by the fact that expression levels of *Os*STN8 were much higher with respect to the levels of wild type Arabidopsis STN8. To evaluate whether overexpression of the kinase could be the reason for CP29 phosphorylation, we compared our complemented lines with those overexpressing the *At*STN8 kinase of Arabidopsis, kindly provided by Prof. Leister. Leaves from these plants were previously incubated in the dark for an hour and then treated in HL (1300 µmol photons m^{-2} s^{-1}). Thylakoids and mLHC fractions were isolated from wild type, *stn8*, comp*Os*STN8 and line oe*At*STN8 as in Figure 4A. P-THR immunoblot was performed following SDS-PAGE on both thylakoids and mLHC samples, as shown in figure 5A. Overexpression of *At*STN8 led to phosphorylation of CP29, as with the overexpression of *Os*STN8. We checked for D1 contamination and CP24 level (Fig. 5B), observing that no contamination of D1 occurred in mLHC samples and the loading was the same in all samples as judged by the signal with anti-CP24. Finally, an anti-STN8 and anti-CP29 immunoblot was performed on the thylakoid preparations to evaluate expression levels for the kinase and the monomeric antenna (Fig. 5C), showing that *At*STN8 expression was much higher in the *Atoe*STN8 line than in the WT sample. This data suggested that the presence of P-CP29 observed in *stn8* mutant lines complemented with the rice kinase could be a consequence of high, non-physiological, levels of STN8 expression rather than suggesting CP29 as a physiological substrate of this kinase. To verify this possibility, we proceeded to select lines of *A. thaliana* with different expression levels for the *At*STN8 kinase, as for *Os*STN8, and determined their capacity to phosphorylate CP29 in HL conditions.


A
Thyl HL
WT D
WT HL
comp OsSTN8 D
comp OsSTN8 HL
oeAtSTN8 D
oeAtSTN8 HL
CP43
D2
D1/CP29
LHCII
mLHC fraction
B
kDa
Thyl HL
WT D
WT HL
comp OsSTN8 D
comp OsSTN8 HL
oeAtSTN8 D
oeAtSTN8 HL
40
30
20
αD1
αCP24
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kDa
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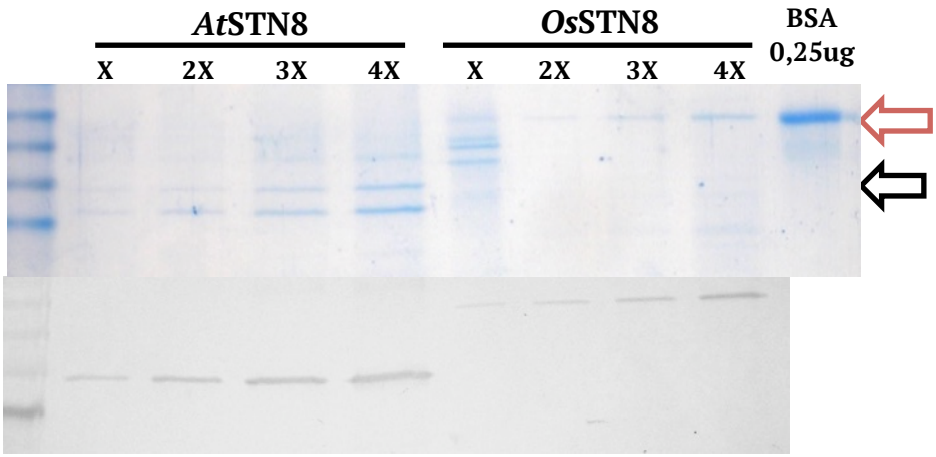
WT HL
comp OsSTN8 D
comp OsSTN8 HL
oeAtSTN8 D
oeAtSTN8 HL
70
60
50
40
30
αSTN8
αCP29

Fig. 5: Western blot analysis of thylakoids and mLHC of *A.thaliana* lines overexpressing STN8. A Western blot analysis using an anti-phosphothreonine antibody against monomeric LHC fractions of *A.thaliana* wild type, *stn8* complemented with *Os*STN8 (comp*Os*STN8) or *At*STN8 provided by Prof. Leister's laboratory (*oeAt*STN8); D: dark treatment 1hr, HL: high light treatment at 1300µE for 30 minutes, Thyl: thylakoids. Loaded: 0,8µg of chlorophylls for thylakoids, 0,3µg for mLHC. **B** Western blot analysis using anti-D1 (top) and anti-CP24 (bottom) antibodies against monomeric antenna fractions of *A.thaliana* wild type, comp*Os*STN8 and *oeAt*STN8; D: dark treatment 1hr, HL: high light treatment at 1300µE for 30 minutes Thyl: thylakoids. Loaded: 0,8µg of chlorophylls. **C** Western blot analysis using anti-STN8 (top) and anti-CP29 (bottom) antibodies against thylakoid samples of the same lines treated in dark or high light. Loaded: 0,8µg of chlorophylls.

Specificity of the STN8 antibody versus recombinant *At*STN8 and *Os*STN8

As mentioned in the previous paragraph, the necessity to generate *A. thaliana* lines expressing different levels of rice or native kinase arose from the observation that overexpression of *At*STN8 led to phosphorylation of CP29 to levels unobserved in WT. This brought us to question whether or not phosphorylation of CP29 by *Os*STN8 was dose-dependent *in vivo*. Before selecting lines of *A. thaliana* with different levels of rice or Arabidopsis STN8, it became necessary to titrate the efficiency of the anti-STN8 antibody in detecting *Os*STN8 compared to *At*STN8. To do so we amplified *Os*STN8 coding sequence from rice genomic DNA devoid of its predicted signal peptide and inserted it in the pDEST17 expression vector (Invitrogen). This vector was designed for the expression of the heterologous protein in *Escherichia coli* fused to a 6X HisTag at the N-terminal domain, facilitating its purification through affinity chromatography. Regarding *At*STN8, the expression vector was kindly provided by Prof. Clermont. The coding sequence of *At*STN8 was inserted in a pMAL vector and the heterologous protein was expressed in *E. coli* fused to maltose-binding protein (MBP). Fusion protein was purified with an amylose resin and *At*STN8 was cleaved from MPB by treating with TEV protease and DTT. Both the purified heterologous STN8 proteins were loaded at different concentrations on SDS-PAGE and immunoblot was performed with the anti-STN8 antibody on the same samples (Fig. 6). The affinity of the two proteins to Coomassie was determined through evaluation of the number of binding residues specifically

Arg, Lys and His, which is 15% higher for rice STN8, given that a 6Xhistag was added as well. Recognition of the rice kinase by the antibody was evaluated to be approx 50% as efficient as with *A. thaliana* STN8 (Table 1).



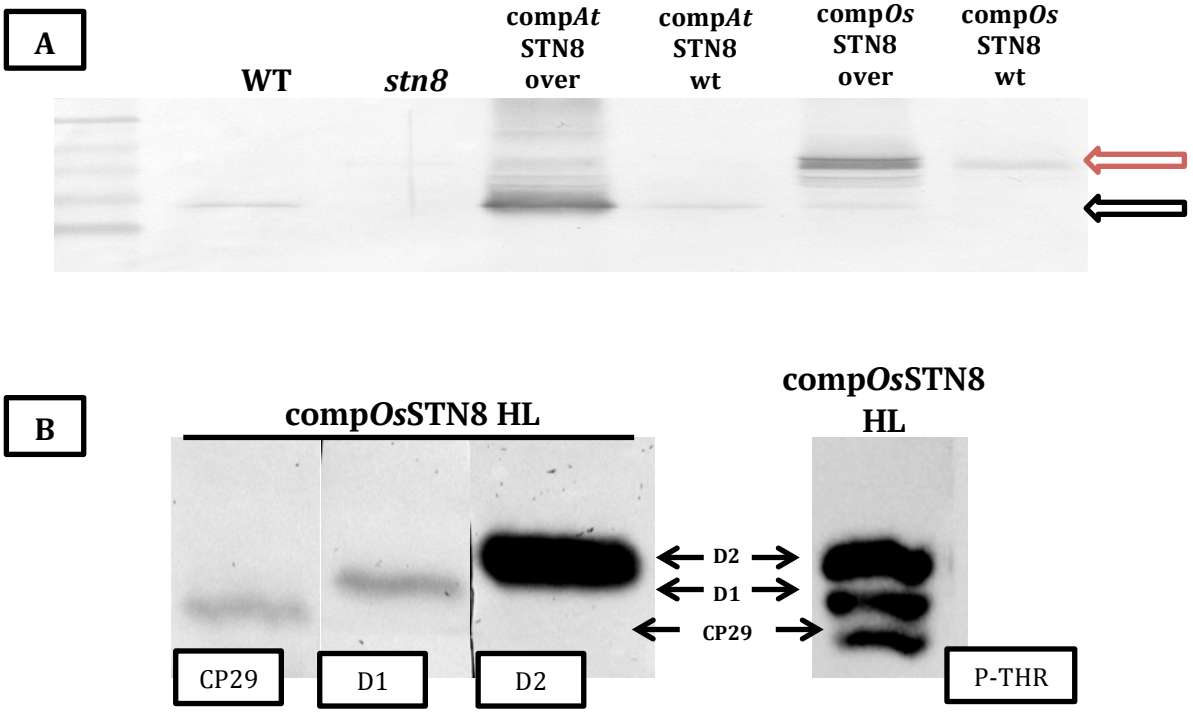
	A. thaliana	*O.sativa*
Average	**2,5708**	**1,0624**
St.Dev.	**0,0924**	**0,0952**

Fig. 6: STN8 antibody specificity to *At*STN8 and *Os*STN8 recombinant proteins. Tris-glycine SDS-PAGE (top) and western blot (bottom) analyses of purified *At*STN8 and *Os*STN8 recombinant proteins. A polyclonal anti-STN8 antibody produced in rabbit was used for Western Blot. Various dilutions of the recombinant proteins were loaded (1X, 2X, 3X, 4X) together with 0,25µg of bovine serum albumin (BSA) standard in order to calculate protein concentration. Red arrow indicates apparent molecular weight of *Os*STN8 recombinant protein; black arrow indicates apparent molecular weight of *At*STN8 recombinant protein.

Table 1: SDS-PAGE and western blot densitometry results. Values shown are the ratio of western blot signal over Coomassie signal. The average and standard deviation of four different dilutions are reported.

Selection of *Os*STN8 and *At*STN8 complemented lines of *A. thaliana stn8* mutants to evaluate dose-dependency of CP29 phosphorylation

A. thaliana stn8 mutant lines were separately complemented using two different constructs, Gateway destination vector pH7WG2 carrying either the rice kinase or *A. thaliana* STN8 full coding sequences, comprehensive of signal peptides. These lines, at generation T3, were selected for overexpression and for those expressing the same level as the wild type of *A. thaliana* (Fig. 7A). On the selected lines, we performed a Western Blot analysis utilising an anti-P-THR antibody following SDS-PAGE on a gradient gel (2M urea-tris-tricine 12%-18% aa). Such gel system allowed separating of CP29 from D1, thus avoiding the need for mLHC fraction preparation (Fig. 7B). Thylakoids were isolated from wild type, *stn8* and complemented lines leaves, after dark-incubation (1hour) and 40 minutes of HL treatment (1300 µmol photons m^{-2} s^{-1}) (Fig. 7C). As can be observed, while in both STN8 over-expressors CP29 was phosphorylated, in the case of the lines expressing wild type-like levels of kinase, only rice STN8 was capable of phosphorylating CP29 in HL while no signal was detected in the case of comp*At*STN8.



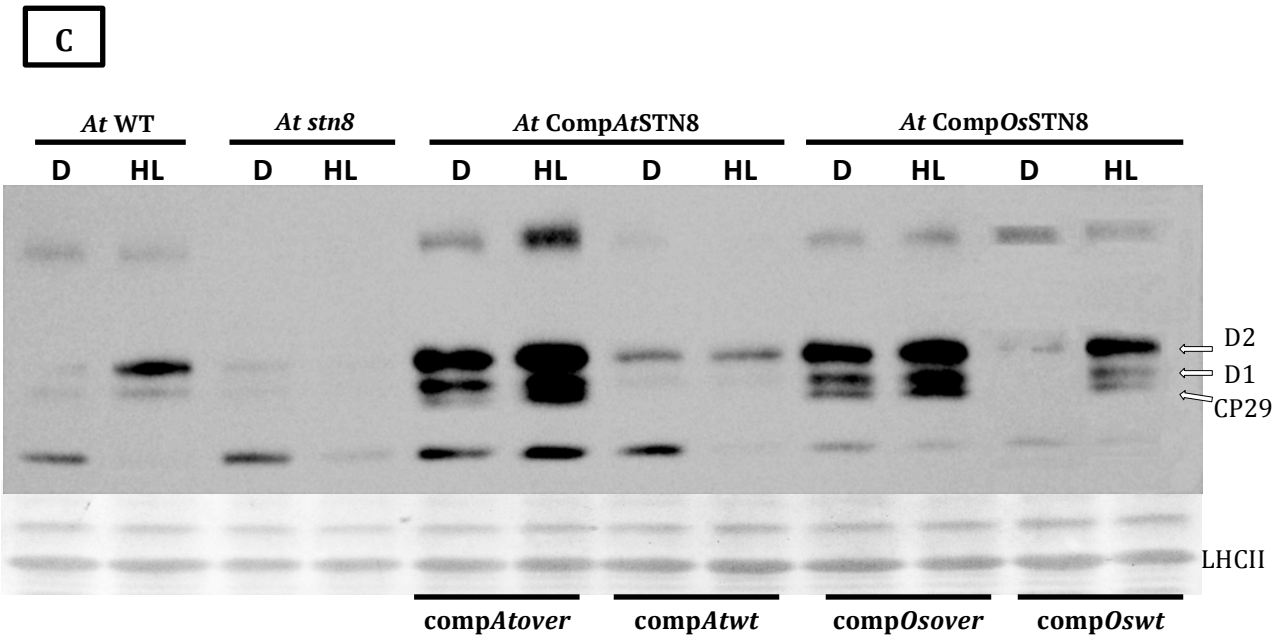


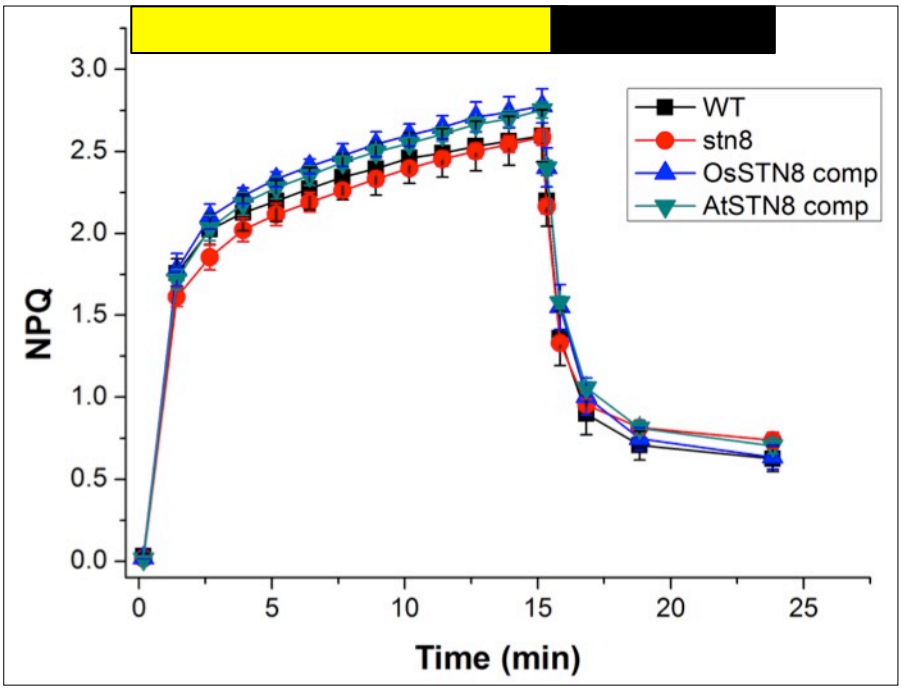
Fig. 7: Dose-dependence of CP29 phosphorylation by *At*STN8 and *Os*STN8. A Western blot analysis using an anti-STN8 antibody against thylakoids prepared from leaves treated for 30 minutes at 1500µmol photons m^{-2} s^{-1} after 1-hour incubation in the dark. Samples are *A.thaliana* wild type, *stn8,* and two different lines of comp*At*STN8 and comp*Os*STN8 expressing either the same level of kinase as wild type (comp*At*STN8 and comp*Os*STN8wt) or over-expressors (comp*At*STN8 and comp*Os*STN8over)**.** Red arrow indicates expected apparent molecular weight of OsSTN8. Black arrow indicates apparent molecular weight of AtSTN8. Loaded: 0,8µg of chlorophylls. **B** Western blot analysis using anti-CP29, anti-D1, anti-D2 and anti-P-THR antibodies following SDS PAGE on 2M urea-tris-tricine gradient gel (12%-18%). Loaded: 1,5µg of chlorophylls **C** Western blot analysis using an anti-phosphothreonine antibody (top) and Ponceau staining (bottom) against the same samples as above, treated for 1-hour in the dark (D) or for 30 minutes at 1500µmol photons m^{-2} s^{-1} after 1-hour dark incubation (HL). Loaded: 1,5µg of chlorophylls for thylakoids.

NPQ measurements of the *stn8 A. thaliana* complemented lines in different conditions

Analysis of the rice mutant of *stn8* showed a clear decrease in NPQ (Poudyal et al., 2016), as opposed to what has been observed for *A. thaliana stn8*, where the mutant underwent quenching as the wild type (Wunder et al., 2013), indicating a difference between the two species in response to high light stress. Since NPQ activity on isolated functional chloroplasts of the *Osstn8* mutant was significantly decreased (Betterle et al., 2015) we hypothesized this could be due

to lack of CP29 phosphorylation. To determine whether or not expressing *Os*STN8 in *A.t.* could affect photoprotection, we performed NPQ measurements on the lines overexpressing STN8 (comp*Os*STN8 and comp*At*STN8), exhibiting CP29 phosphorylation (Fig. 7C). Indeed, these lines showed slightly higher NPQ values compared to wild type and *stn8* (Fig. 8A). In order to further assess the contribution of CP29 phosphorylation in heat dissipation, we performed NPQ measurements using increasing actinic light intensity, as previously described by Wunder et al. (2013). Non-Photochemical Quenching at five different white light intensities, delivered in steps of 15 minutes is shown in figure 8B. WT and *stn8* showed the same NPQ upon treatment with strong actinic light, instead *Os*STN8 over-expressor shows an increase in NPQ with all the tested light conditions (Fig. 8B). On the other hand, the comp*Os*STN8wt line, which phosphorylates CP29 less strongly than comp*Os*STN8over (Fig. 7C), behaves as the wild type at low light intensities (200-400µmol photons m^{-2} s^{-1}), and increases slightly further on as light intensities increase.

A



B

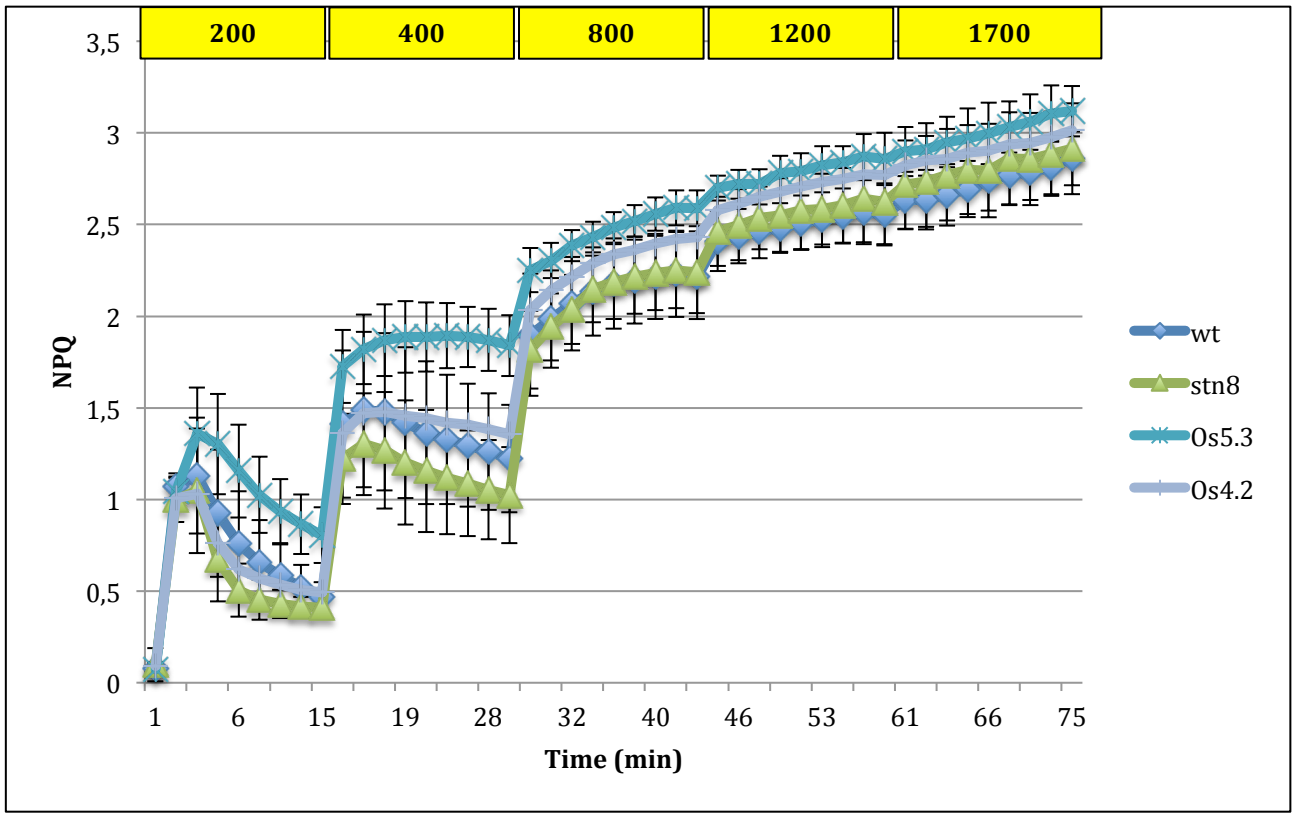


Fig. 7: A NPQ measurements on *A.thaliana* wild type, *stn8,* comp*At*STN8 and comp*Os*STN8 lines. Leaves were subject to actinic light for 15minutes and recovered 10 minutes in the dark. Actinic light: 1500µmol photons m^{-2} s^{-1}. Saturating light: 4500µmol photons m^{-2} s^{-1}. **B** NPQ measurements on *A.thaliana* wild type, *stn8* and comp *Os*STN8 expressing the same level as wild type kinase (OsWT) and overexpressor (OsOver). Light intensity was increased every 15 minutes. Actinic light: 200, 400, 800, 1200, 1700µmol photons m^{-2} s^{-1} (represented in yellow boxes). Eight replicates per genotype were used for the

Discussion

Phosphorylation of CP29 (P-CP29) was reported for the first time in maize (Bergantino et al., 1995) upon a combined treatment of high light and cold. After this first report, other authors described this post-translational mechanism in other higher plants such as rye (Pursiheimo et al., 2001), barley (Bergantino et al., 1998; Morosinotto et al., 2006; Chen et al., 2009). Thus, CP29 phosphorylation was so far detected only in monocots, but never in dicot plants. CP29 phosphorylation in the model dicot plant *Arabidopsis thaliana* was first reported by Tikkanen and co-workers (Tikkanen et al., 2006) in response to strongly reducing condition as dependent on STN7 activity, alike the case of LHCII when eliciting state transitions in low-light condition (Rochaix et al., 2012). The dependence of P-CP29 from STN7 activity was further supported by Fristedt and co-workers (Fristedt et al., 2011), yet these reports showed very low levels of CP29-P, detectable only by using a highly sensitive MS approach. Moreover, CP29 in Arabidopsis was phosphorylated at residues different than Thr-83 detected in monocots (Bergantino et al., 1998). Reiland and co-workers (2011), however, attributed CP29-P upon high light treatment to STN8 kinase activity.

In chapter 1 we described that STN8 is responsible for the strong, high light-dependent phosphorylation of CP29 in monocots, in agreement with the reported STN8, but not STN7 activity, in high light conditions (Vainonen et al., 2005; Rochaix et al., 2012, Rintamaki at al., 1997). CP29 phosphorylation in maize occurs on Threonine-83 (Bergantino et al., 1995) a residue conserved in rice (Betterle et al., 2015). Surprisingly, a multiple alignment between rice and Arabidopsis CP29 protein sequences, among other species, revealed that the phosphorylation site identified in rice and maize was conserved also in Arabidopsis despite low phosphorylation, if any (Fig. 1A). In fact, comparison of the CP29 primary sequence from different species showed a very high level of identity suggesting the differences in the CP29 phosphorylation in monocots vs dicots was rather to be due to differences in the kinase rather than in the substrate. Indeed, when comparing STN8 sequences of the same species, a visible difference among the various kinases could be highlighted. While the

catalytic domain is highly conserved between species (Fig. 1C), the N-terminal domain appeared to be much longer (about 100 amino acids) in monocots compared to dicots (Fig. 1B). This finding prompted us to investigate whether or not this difference in kinase structure was the reason for a difference in substrate specificity. The overexpression of *Os*STN8 in Arabidopsis *stn8* mutant showed that the heterologous protein was functional in restoring PSII core phosphorylation (Fig. 3A). Furthermore, *Os*STN8 also led to P-CP29 accumulation in response to high light. Indeed, we ruled out the possibility that the P-Thr reaction could be due to (Fig. 4B) co-migration of PSII core subunits. Light dependence of CP29 was similar to that in rice (Fig. 4C), suggesting that *Os*STN8 is sufficient to replicate the onset of CP29 phosphorylation in Arabidopsis while the resident phosphatase activity, likely PBCB (Samol et al. 2012) is sufficient for P-CP29 de-phosphorylation upon dark recovery. It is worthy to notice that P-CP29 in *A. thaliana* complemented lines did not show a difference in migration with respect to CP29, as observed in rice. This could be due either to the type of gel system employed, which does not separate well the phosphorylated form as in rice, or to the properties of the protein, which may be slightly different between the two species, leading to a different behaviour upon SDS-PAGE analysis.

The detection of P-CP29 in the *At*STN8 overexpressing lines rather than in the *Os*STN8 over-expressors only (Fig. 5) casted doubts on the hypothesis that structural differences between monocot and dicot kinases was responsible for the physiological differences among taxa. However, CP29 phosphorylation by *At*STN8 was strongly dose-dependent, as shown by the behaviour of different lines expressing different levels of the kinase (Supplementary Figure 1). Thus, when the two STN8 proteins from *A.t.* and *O.s.* were expressed to the same level, only the latter was effective in accumulating P-CP29 in *A. thaliana* to a level compared to monocots (Fig. 7C). We conclude that the changes in primary sequence found in *Os*STN8 respect to *At*STN8 are responsible for the increased affinity of the kinase for CP29. It is likely that the variable sequence (i.e. residues 37 to 110 in Fig. 1) corresponds to the domain responsible for the interaction with the substrate while the 10 kDa increase in molecular mass may well

corresponds to a wider structure accommodating more diverse substrates with respect to the dicot enzyme, including CP29 in addition to D1, D2 and CP43.
Previously, overexpression of the phosphatase PBCP (Samol et al., 2012) caused increased dephosphorylation of PSII core but also of LHCII, the substrate of PPH1/TAP38 phosphatase (Pribil et al., 2010). Here, overexpression of STN8 enzymes led to loss of both substrate specificity and regulation, with PSII core subunits and CP29 being constitutively phosphorylated, even when incubated in the dark (figure 7C). This suggests a low constitutive STN8 activity, which in WT is counteracted by PBCP phosphatase, preventing accumulation of P-PSII in the dark. The constitutive PSII core phosphorylation is consistent with its role in PSII disassembly and repair (Mattoo and Edelman, 1987), which proceeds at higher rate in HL but is still active in the dark.
Previous work showed that *A. thaliana* wild type and *stn8* mutant express the same NPQ dependence on actinic light intensity Wunder et al., (2013). In rice, however, the *stn8* mutant showed a lower NPQ with respect to wild type with a distinct dependence on light intensity (Poudyal et al., 2016). This suggests that P-CP29 accumulation in HL could be the reason for increased NPQ activity in rice. Consistently, inhibition of CP29 phosphorylation in rice led to a decrease in photoprotection (Betterle et al., 2015). The P-CP29 dependent increase in photoprotection appears not to be restricted to monocots when P-CP29 can be reproduced as suggested by the study by Wunder et al., (2013), showing increased NPQ in *oeAt*STN8 with respect to wild type and *stn8* plants. This difference can be attributed to P-CP29 in light of the results of figure 7C, showing an increased NPQ activity, particularly at low actinic light in the samples where P-CP29 is known to be accumulated: the over-expressors, both *Os*STN8 and *At*STN8 and the comp*Os*STN8 line with WT level of expression (Fig. 8A-B). The effect of P-CP29 on NPQ appeared to be lower in the complemented lines than in rice. It is reasonable to think that P-CP29 in Arabidopsis has a different interaction with other components contributing to photoprotection with respect to monocots. Also, although similarity being very high, CP29 in Arabidopsis and rice are not identical: it is possible that substitution might enhance the efficiency of the response to phosphorylation in terms of changing CP29 structure towards the quenching state. This has been suggested to consist

into an increased interaction between two chlorophyll ligands, namely Chl A5 and Chl B5, thus allowing for the formation of a radical cation towards heat dissipation of excess excitation energy (Ahn et al., 2008). Further biochemical analysis on isolated CP29 proteins from monocots and dicots is needed in order to confirm that the phosphorylation-dependent spectroscopic changes previously observed in *Zm*CP29 are also present in the protein from Arabidopsis.

Conclusions and perspectives

In this work we have shown that a monocot photo-protective mechanism, such as the high light-induced phosphorylation of CP29, can be transferred to a dicot, specifically *A. thaliana.* The possibility to transfer photo-protective mechanisms from one class of plants to another could represent a valid strategy in improving plants fitness to environmental stresses. However, before such an important result can be considered as assessed and transferable to other species, a phenotypic characterization of growth rate in response to different environmental conditions must be performed with particular reference to challenging conditions such as cold stress and highly variable light conditions that severely limits plant productivity.

While the increased NPQ activity appears at the present state as the best candidate to explain the increased photoprotection from P-CP29, other physiological mechanisms might contribute and should be evaluated. Among these is the effect on PSII repair and turnover, which might well have an impact on photoprotection efficiency. However, the recent finding that CP29 phosphorylation does not increase dissociation of PSII supercomplexes, believed to be a step in the repair cycle, reinforces the focus on CP29 as a major site of energy dissipation and its regulation.

Materials and methods

Plant material

A. thaliana Col-0 plants were all grown in control conditions for 3 weeks with a 16-h photoperiod at a photon flux density of circa 20 μmol m^{-2} s^{-1}, at constant temperature of ~22°C. *stn8* mutant was obtained from the laboratory of Prof. J.D.Rochaix. Leaves were harvested from 3-weeks old plants, and prior to thylakoid isolation they were incubated for 1 hour in the dark, and further treated to high light (1300μmol photons m^{-2} s^{-1}) unless otherwise indicated.

STN8 complemented lines

STN8 construct was generated through insertion of cDNA sequence of the predicted mature protein *Os*STN8 (LOC_Os05g40180.1) in a pEARLYGate100 Gateway® vector. *At*STN8 expression was obtained through insertion of cDNA sequence of the predicted mature protein (LOC_At5g01920.1) in the same vector as for *Os*STN8. Transformation was performed as described in Zhang et al., (2006).

Thylakoids Isolation and Pigment Binding Complexes Purification

Thylakoids were purified from *A. thaliana* leaves as described in Rintamaki et al. (1996) with minor modifications. Leaves were harvested and macerated in extraction buffer (50mM HEPES-NaOH, pH 7.5, 300mM sucrose, 5mM $MgCl_2$, 1mM Na-EDTA, 10mM NaF, 1% (w/v) bovine serum albumin and 0.005M ε -aminocaproic acid, 0.001 phenyl-methylsulfonyl fluoride and 0.001 benzamidine as protease inhibitors). After filtration through Miracloth filters, samples were precipitated by centrifugation at 1500g for 5 min at 4°C and then resuspended in hypotonic buffer (10mM HEPES-NaOH, pH 7.5, 5mM sucrose, 5mM $MgCl_2$, 10mM NaF and protease inhibitors) and pelleted at 3000*g* for 3min. After centrifugation thylakoids were resuspended in a buffer containing 0.4M sorbitol,

0.015M NaCl, 0.005M $MgCl_2$, and 0.01M HEPES-KOH, pH 7.5. This procedure was performed in the dark at 4°C. Finally, thylakoids were either used directly or frozen in liquid nitrogen and stored at – 80°C until use.
For separation of pigment binding complexes 100µg of thylakoid membranes were washed with 5mM EDTA and resuspended at a final concentration of 1 mg/ml in 10mM Hepes, pH 7,5. Samples were then solubilised at a final concentration of 0,5 mg/ml adding 1,2% α-DM and 10mM Hepes, pH 7,5 and vortexing for 1min. After 10min of ice incubation, thylakoids membranes were centrifuged at 15.000g for 10min to eliminate not solubilised materials. Fractionation occurred upon by ultracentrifugation on a 0,1 to 1 M sucrose gradient containing 0,03 α-DM and 10mM Hepes, pH 7,5 (SW60 Ti Rotor, 5h 30min at 60,000rpm, 4 °C). The green bands were harvested using a syringe needle.

Fluorescence analysis

In vivo chlorophyll fluorescence of *A. thaliana* leaves WT, *stn8* and the various complemented lines expressing the different kinases was measured at room temperature on Fluorcam imaging fluorometers (Photon system instrument), with saturating light at 4000µmol m^{-2} s^{-1} and various actinic lights: at 1500µmol m^{-2} s^{-1} for the first measurement (Fig. 8A), and 15-minute step increasing lights: 200, 400, 800, 1200, 1700µmol m^{-2} s^{-1}. Before measurements, plants were dark-adapted for 1 hour at room temperature. Parameters Fv/Fm and NPQ were calculated as (Fm – Fo)/Fm and (Fm –Fm ′)/Fm′ (Demmig-Adams et al., 1996). Data are presented as means ± SD of at least three independent experiments.

SDS-PAGE and Western Blot

SDS-PAGE analyses were performed as described by Laemmli (1970) or with the Tris-Tricine buffer system (Schägger and von Jagow, 1987). For the separation of P-CP29 from P-D1, a Tris-Tricine gel with a 12%-18% gradient was utilised, and urea (2M) was also added into the running solution. Polypeptides, following SDS-PAGE, were transferred onto a nitrocellulose blotting membrane (Millipore)

using a Mini Trans-Blot cell (Bio-Rad) and detected by use of homemade polyclonal antibodies, except for anti-phosphothreonine (Cell Signaling).

Protein expression in Escherichia coli

Chloroplast transition peptide of *Stn8* (LOC_Os05g40180) gene product was determined using ChloroP method. cDNA sequence of the predicted mature protein was cloned into pDEST17 (Invitrogen), a Gateway compatible vector to allow for N-terminal 6x-His tagging, and expressed in *Escherichia coli* strain BL21. Recombinant protein was purified using a protocol described previously (Paulsen et al., 1993) and resuspended in denaturant solution (Tris 20mM pH 7.4, NaCl 300mM, Urea 6 M). Denatured proteins were loaded in Ni- Sepharose column and elution of tagged proteins was performed in a solution containing Tris 20mM pH 7.4, SDS 0.2 % and 200mM Imidazole. *AtStn8* construct was kindly provided from the laboratory of Prof. Goldschmidt-Clermont, expressing the coding sequence of *AtStn8* in a pMAL vector for the fusion of the protein of interest to Maltose Binding Protein (MBP). MBP was linked via a TEV protease site for cleavage post-purification. *At*STN8 was purified using an amylose resin and MBP cleaved using TEV protease.

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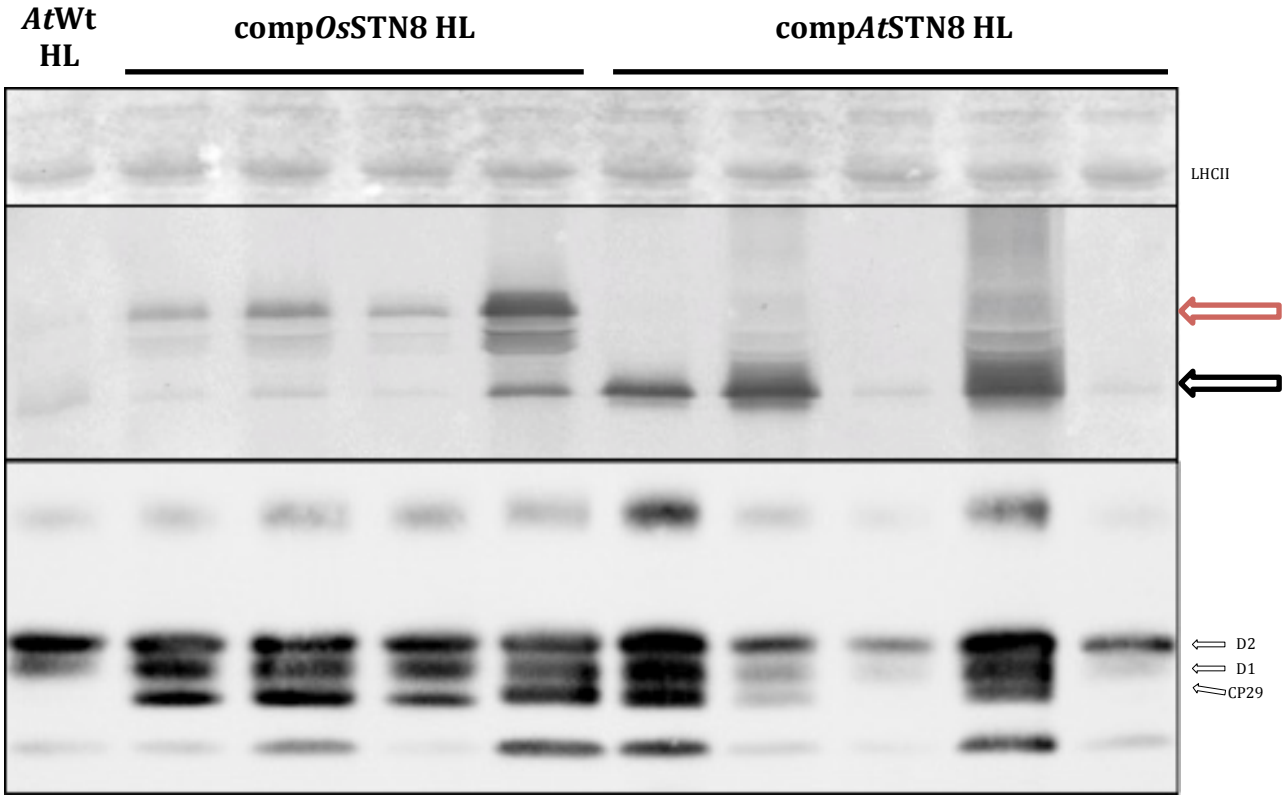
Supplementary Figure 1

Dose-dependent phosphorylation of CP29 in A. thaliana compAtSTN8 lines compared to the specific phosphorylation occuring in A. thaliana compOsSTN8 lines.



Supp. Fig. 1: CP29 phosphorylation in lines expressing different levels of *At*STN8 and *Os*STN8. A Ponceau staining (top) and Western blot analysis using anti-STN8 (middle) and anti-phosphothreonine antibodies (bottom) against thylakoids prepared from leaves treated for 30 minutes at 1500µmol photons m^{-2} s^{-1} after 1-hour incubation in the dark. Samples are *A.thaliana* wild type, and different lines of comp*At*STN8 and comp*Os*STN8 expressing various levels of the two different kinases comp*At*STN8 and comp*Os*STN8). Red arrow indicates expected apparent molecular weight of OsSTN8. Black arrow indicates apparent molecular weight of AtSTN8. Loaded: 1,5µg of chlorophylls.

Chapter 3

Characterization of phosphorylated CP29 contribution to photoprotection through in vivo and in vitro approaches

Abstract

Phosphorylation of the minor antenna protein CP29 was proven to be involved in the enhancement of photoprotection in response to environmental cues in different monocot species. The mechanism by which this phenotype is obtained consists into an increased ability to trigger energy dissipation, particularly at low and moderate light intensity. In turn, this effect has been connected to the capacity of phosphorylating CP29, a property of monocots only, as previously shown in *Oryza sativa* and in *Zea mays*, where it provided resistance to cold stress. Since dicots lack CP29 phosphorylation, their productivity could benefit from integrating CP29 phosphorylation. To verify this hypothesis the *Os*STN8 kinase, catalyzing CP29 phosphorylation, was expressed in *A. thaliana* and yielded into CP29 phosphorylation as well as into increased NPQ (Chapter 2). In this work we analyzed the effects of expressing *Os*STN8 in *A. thaliana* on photoprotection. Since *Os*STN8 catalyzed both CP29 and PSII core phosphorylation, we aimed to distinguish photoprotection effects from the two events. To this aim we constructed an Arabidopsis genotype that, although expressing *Os*STN8, yet cannot undergo CP29 phosphorylation, by complementing ko*lhcb4* mutants of *A. thaliana* with CP29 from Arabidopsis or rice, either in their native form or mutated at Thr-83 residue to suppress phosphorylation. Physiological analysis of plants phosphorylated at both CP29 and PSII core or at PSII core only will define the specific function of PCP29 *in vivo*. This work was complemented by isolation of CP29 either phosphorylated or not and its spectroscopic characterization with the aim to assess whether the conformational changes of P-CP29 vs CP29 are conserved in the protein from the two species.

Introduction

Photosystem II is the preferential target of photo-inhibition (Kock, 1956) because its acceptor size cannot be protected by carotenoid cofactors owing to the strongly oxidizing reactions involved in oxygen evolution (Telfer et al., 1994). Environmental stresses are the major responsible for productivity loss of

crops (Cramer et al., 2011) and optimization of photoprotection mechanisms has been shown to be effective in increasing plant and algal productivity in the field (Kromdijk et al., 2016) or in photobioreactors (Berteotti et al., 2016). Photosystem II forms supercomplexes together with light harvesting complexes which enhances its cross section for photon absorption and strongly contribute to photoprotection by means of their large and diversified xanthophyll complement (Dall'Osto et al. 200x) and through complex mechanisms of regulation that balance the concentration of chlorophyll excited states with the capacity for energy use in electron transport. Among these, state1-state2 transitions (Goldschmidt and Bassi, 2016), xanthophyll cycle (Dall'Osto et al., 2010) and Non-Photochemical Quenching (Niyogi et al., 2015) are prominent. The PSII antenna system is made of trimeric LHCII complexes connected to the dimeric PSII core through the monomeric LHCs called CP24, CP26 and CP29. These monomers have a special role in photoprotection as judged from the enhanced photosensitivity of deletion mutants (De Bianchi et al., 2008, 2010; Dall'Osto et al., 2017). Properties of monomeric LHCs have been studied by mutation analysis *in vitro* upon reconstitution of the apoproteins expressed in bacteria with pigments (Bassi et al., 1999, Ahn et al., 2008; Ballottari et al., 2012; Pagano et al., 1998; Passarini et al., 2009; Betterle et al., 2010). Despite the large body of knowledge obtained, limitations of the *in vitro* system have emerged due to the lability of (part of), the chromophores revealed crystal structure (Pan et al., 2008) respect to those binding in recombinant CP29 or for the study of ΔpH dependent functions (Ahn et al., 2008), which require an intact thylakoid system. Thus, *in vivo* analysis of monomeric Lhcs is of crucial importance in order to understand their role in photoprotection.

In Chapter 2, we complemented Arabidopsis *stn8* mutant lines with the kinase from rice, which restored the phosphorylation of PSII core and enhanced NPQ activity. However, unambiguous discrimination of the specific contribution of P-CP29 with respect to that of P-PSII core could not be obtained.

In this chapter, we have complemented Arabidopsis *lhcb4* knockout mutants with WT and mutant forms of CP29, from both rice and Arabidopsis lacking the 83 Threonine and therefore unable to undergo phosphorylation. In a complementary approach P-CP29, from both rice and Arabidopsis, was isolated,

using different strategies, in order to verify that phosphorylation-dependent changes were indeed the same previously reported in *Zea mays* CP29 (Croce et al., 1996).

Results

Generation of *A. thaliana* and *O. sativa* different CP29 constructs

To shed light on the individual contribution of P-CP29 on photoprotection in dicots, we transformed *lhcb4* mutants of *A. thaliana* for expressing different versions of *At*CP29 or *Os*CP29, in both their native forms and mutated at the Thr-83 site. These lines needed also to be transformed with another vector for the expression of *Os*STN8 in order to obtain high light induced phosphorylation, as described in Chapter 2. In the following the individual constructions will be described together with their scope:

***At*CP29**: the full *At*Lhcb4.1 genomic sequence was cloned into a Gateway binary vector, pK7WG2, under the control of its native promoter and terminator. Initially, 35S was utilized as promoter, but expression levels of the complemented lines were very low. To this end, we utilized the full genomic sequence of *At*CP29, as the construct was already present at the time of transformation. This construct will be indicated as ***At*CP29 WT**. In addition to the native gene sequence of *At*CP29, we designed other two versions of the sequence by editing the codon ACC encoding Thr-83 site to GCC for Thr→Ala mutation, the aim of which is to suppress the *Os*STN8-dependent CP29 phosphorylation. This construct will be indicated as **atCP29 NP** (No Phosphorylation)**.** A second mutation consisted in editing the codon ACC for Thr-83 site to GAG for Thr→Glu mutation. Mutation of a Ser/Thr site to Glu has been effective, in cases, in mimicking phosphorylation (Symington et al., 2007) thus blocking the protein conformation to the phosphorylated state irrespective of phosphatase activity. This construct will be indicated as ***At*CP29 CP** (Constitutive Phosphorylation)**.**

***Os*CP29**: the sequence encoding the rice CP29 protein was used to replace the *At*Lhcb4.1 sequence in the same Gateway vector pK7WG2, under the control of

the 35S promoter. This construct will be indicated as ***Os*CP29 WT.** Mutations were introduced in this sequence similar to the case of atCP29 to produce a construct ***Os*CP29NP** (Thr→Ala) to suppress phosphorylation. A similar construct **osCP29WT-His** encoded a phosphorylatable form of *Os*CP29 carrying a 6X Histag at the C-terminus of the protein to facilitate purification for *in vitro* analysis. All constructs are shown in figure 1.

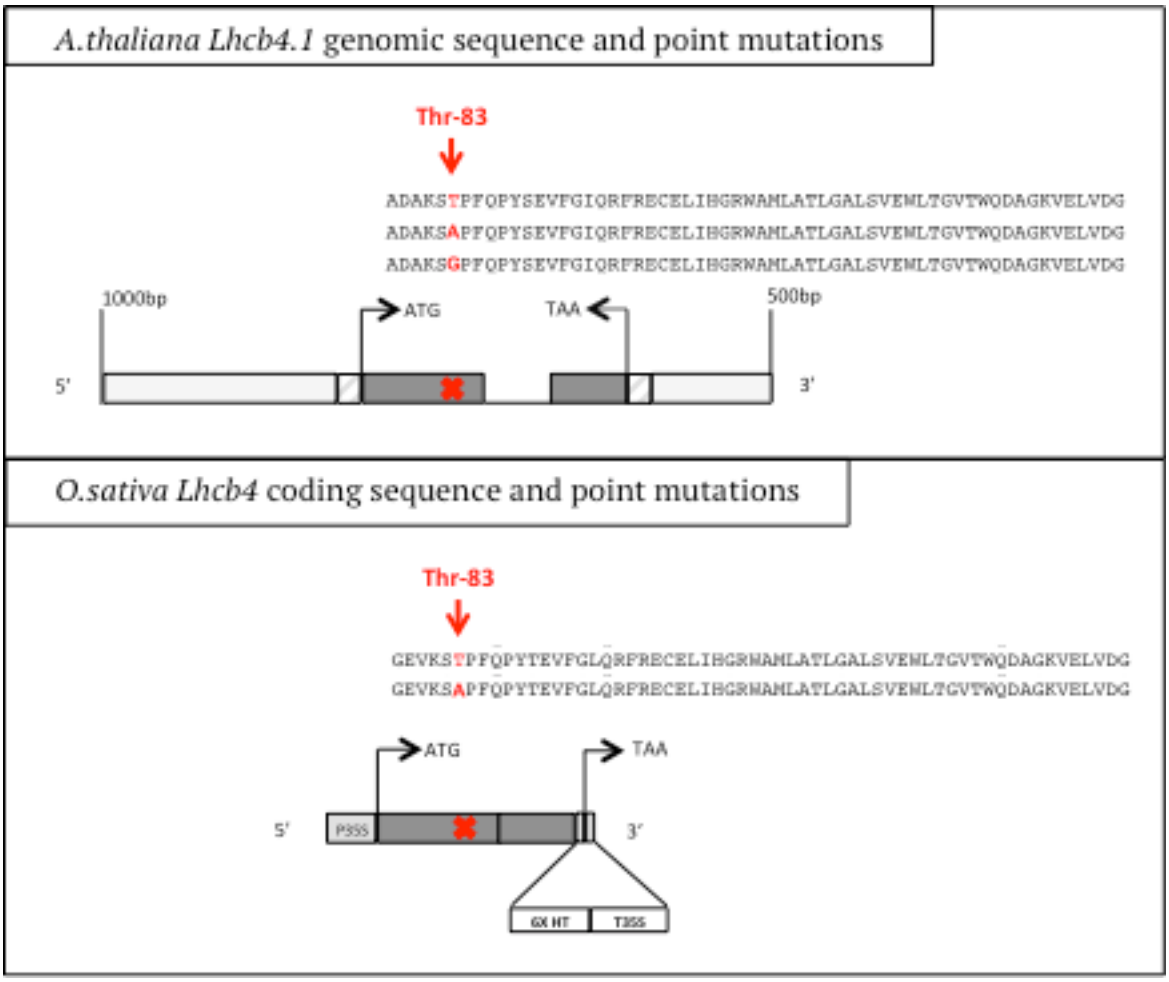


Fig. 1: *AtLhcb4.1* and *OsLhcb4* sequences utilized for the complementation of *lhcb4* mutants. A *A. thaliana Lhcb4.1* genomic sequence utilized for the complementation of *lhcb4* mutants. The gene is under control of its native promoter (1000bp before the 5'UTR) and terminator (500bp after 3'UTR). The sequence was inserted in a pK7WG2 Gateway destination vector. Original sequence and mutations are represented on the gene and protein sequence (red cross and letters, respectively): Thr 83→Ala (A) and Thr-83→Glu (G). **B** *Oryza sativa Lhcb4* coding sequence. The gene is under the control of the 35S promoter of the pK7WG2 vector. Sequence for the expression of a 6X Histag at the C-terminus of the protein was inserted for purification. Original sequence and mutations are represented on the gene and protein sequence (red cross and letters, respectively).

Co-transformation of *Atlhcb4* plants with *Os*STN8 and *At*CP29/*Os*CP29

A. thaliana lhcb4 mutant lines were co-transformed with the *Os*STN8 construct previously generated (Chapter 2) and the *At*CP29 either WT, NP or CP forms. Unfortunately, co-transformation was unsuccessful and we only succeeded in selecting lines expressing solely *At*CP29 (but not *Os*STN8) (Fig. 2) that could further be transformed with the construct encoding *Os*STN8. This procedure yielded three different lines, namely: **comp*At*CP29wt**, where the native form of the protein is expressed; **comp*At*CP29NP**, which is mutated at the Thr-83 site to Ala; **comp*At*CP29CP**, where Thr-83 is mutated to Glu. At mid February 2017, lines co-expressing both the minor antenna protein together with *Os*STN8 are not available. As can be observed in figure 2, wild type Arabidopsis was loaded as a reference, and comp*At*CP29wt and comp*At*CP29NP complemented lines show the same expression levels of CP29. Similarly, comp*At*CP29CP shows expression levels comparable to wild type, and yet it migrates at a higher apparent molecular weight with respect to wild type CP29. Since P-CP29 shows a slower migration with respect to CP29 in SDS-Urea PAGE (Bergantino et al., 1995) this is an indication, although preliminary, that the Thr>Glu mutation affected the CP29 conformation similar to phosphorylation.

***Os*CP29 transformation**. Similar to the case of *At*CP29, mutant lines of *A. thaliana lhcb4* were co-transformed with *Os*STN8 and *Os*CP29, both in its wild type form and mutated at Thr 83→Ala, added of a 6X Histag at the C-terminus. After selection *in vitro*, western blot analysis using anti-STN8 and anti-CP29 antibodies was performed in order to evaluate protein expression. Again, as for *At*CP29 co-transformation was unsuccessful. Western blot analysis of Arabidopsis lines expressing *Os*CP29 is shown in figure 2. Lines expressing *Os*CP29 in its tagged form show lower levels of expression with respect to wild type, and the anti-CP29-reactive band migrated at a higher apparent molecular weight due to the addition of the 6X Histag (Fig. 2). Plants independently expressing *Os*STN8 and *Os*CP29 were selected in order to obtain stable lines to be used in crossings to obtain transgenic lines expressing both heterologous *Os*CP29 and *Os*STN8 proteins.

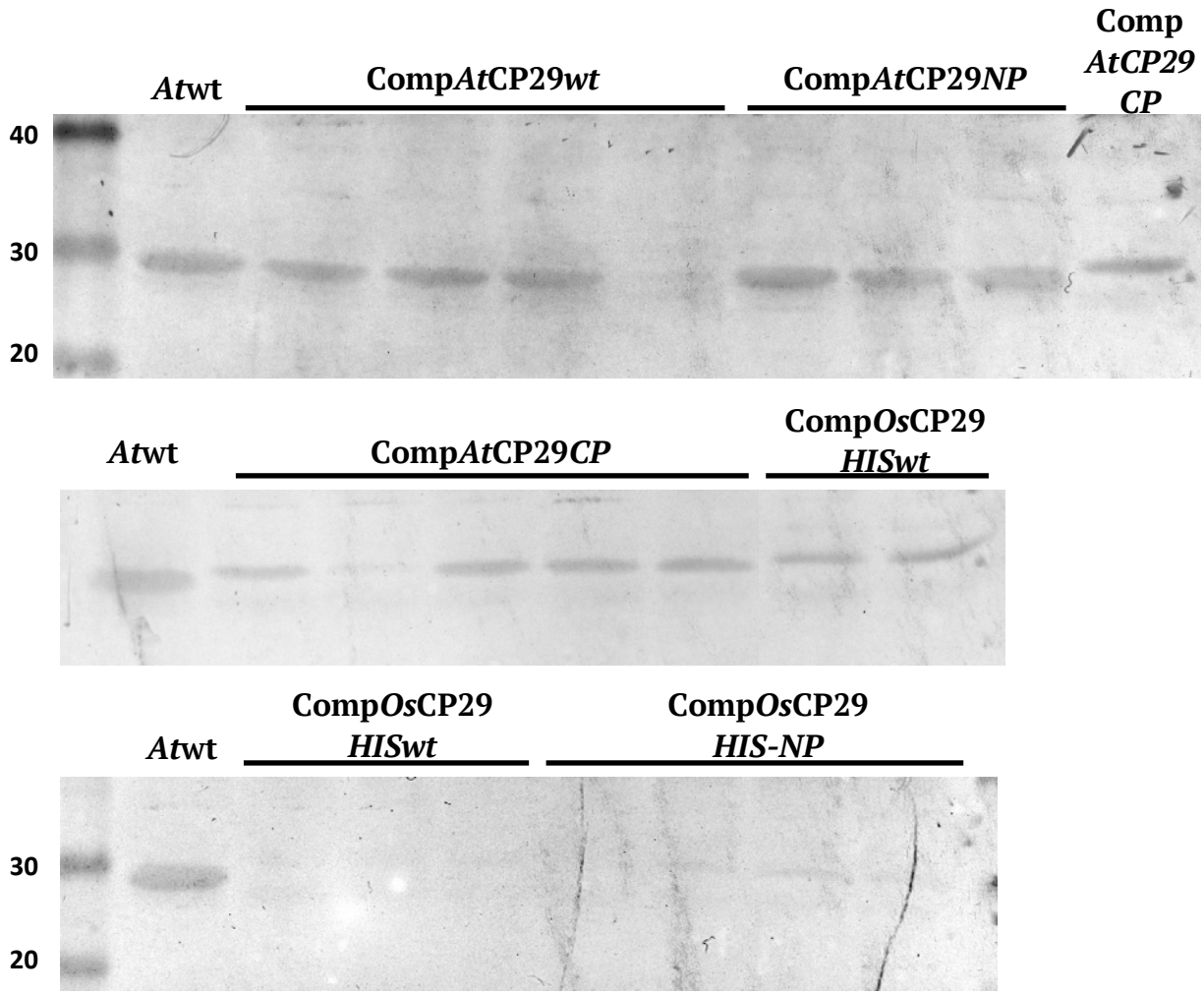


Fig. 2: Western blot analysis of lines complemented with *At*CP29 and *Os*CP29. Western blot analysis using an anti-CP29 antibody on total protein extracts from *A. thaliana* wild type (*At*wt) and *lhcb4* mutant complemented with: *At*CP29wt (comp*At*CP29wt), *At*CP29NP (comp*At*CP29NP), *At*CP29CP (comp*At*CP29CP), *Os*CP29wt added of a 6XHistag (comp*Os*CP29HISwt), *Os*CP29NP added of a 6XHistag (comp*Os*CP29HIS-NP). Leaf disks were macerated in 60µl Loading Buffer and 20µl were loaded.

Transformation of double and quadruple *A. thaliana* mutants with *Os*STN8

Together with phosphorylated *Os*CP29, we set out to obtain purified P-*At*CP29 in order to determine if phosphorylation of the minor antenna protein obtained in dicots by expressing the rice kinase led to similar conformation changes in Arabidopsis. Given the difficulty in obtaining transgenic lines heterologously co-expressing both CP29 and STN8, we decided to transform a set of *A. thaliana* mutant lines kindly provided by the laboratory of Prof. Goldschmidt-Clermont. The mutant lines transformed were: a double mutant, *A.t. stn8pbcp,* lacking the PSII core kinase and phosphatase, and two different quadruple mutants, *A.t. stn8pbcpstn7pph1.1* (*quad 1.1*) and *stn8pbcpstn7pph1.3* (*quad 1.3*), which differ in the isoform of the LHCII phosphatase retained. Double mutant lines were complemented with *Os*STN8 or *At*STN8 in order to obtain phosphorylation of the minor antenna protein by overexpression of the kinase, and lack of the PBCP phosphatase should allow constitutive phosphorylation. For the same reason, the two quadruple mutant lines were transformed with *Os*STN8, but in this case both PSII core and LHCII phosphatases, PBCP and TAP38/PPH1.1 respectively, were absent. All samples were treated in the dark for 1 hour (D), then treated to high light for 1 hour (HL, 1300µmol photons m^{-2} s^{-1}) and further recovered in the dark for 30 minutes. As shown in figure 3A, CP29 was phosphorylated in all lines upon HL treatment as expected. However, the only case in which CP29 remained constitutively phosphorylated upon HL treatment plus dark recovery was the complementation of *quad1.1*, (Fig. 3A). Thus this line was chosen to attempt isolation of P-CP29. The monomeric LHC fractions of thylakoid preparations from wild type and *quad1.1* comp*Os*STN8 lines treated in high light were isolated through sucrose gradient fractionation in order to obtain a P-CP29-enriched fraction, to further purify through preparative isoelectrofocusing. As shown in figure 3B, native absorption spectra reveal no substantial differences in composition between the two preparations. The samples were further analyzed through Coomassie and western blot using an anti-phosphothreonine antibody (Fig. 3C). Wild type mLHC fraction shows no sign of CP29 phosphorylation, while the *quad1.1* comp*Os*STN8 line shows a band at 30kDa, the expected molecular weight of P-CP29.

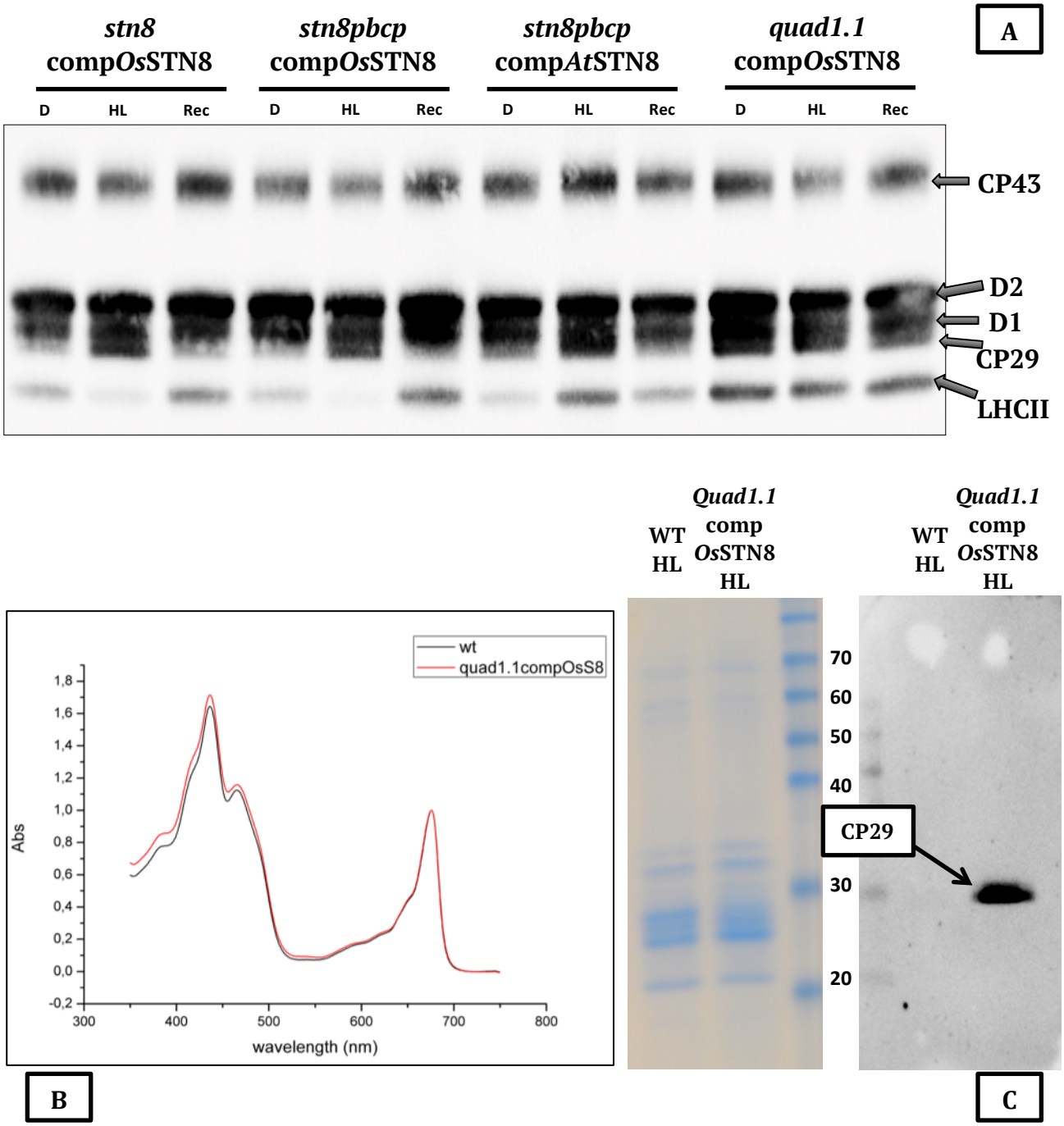
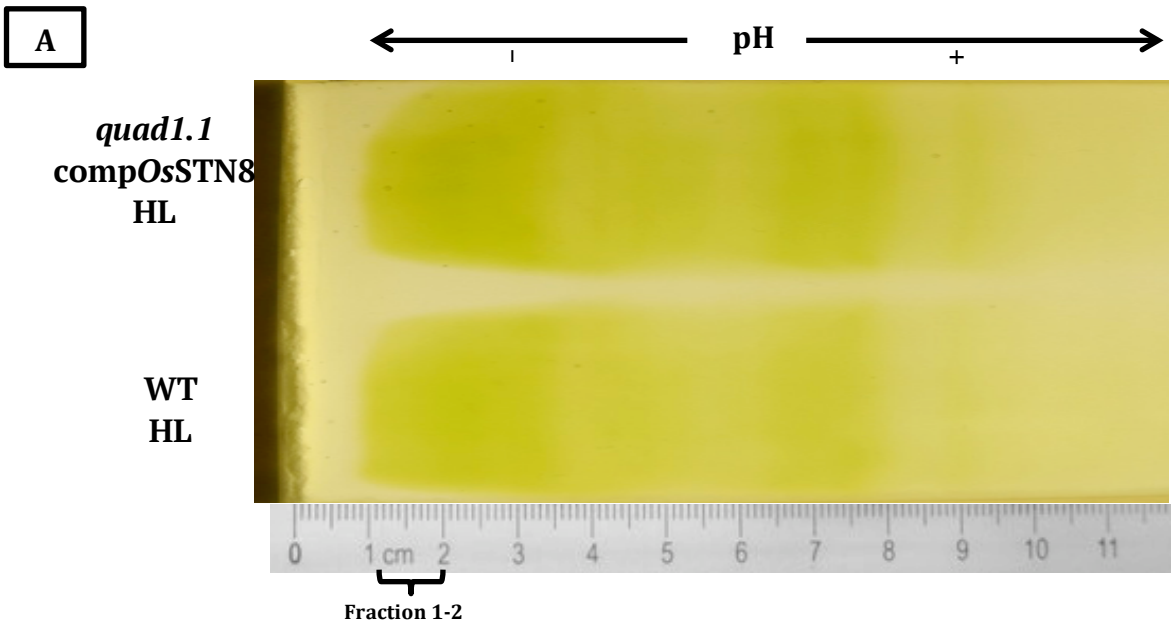


Fig. 3: Thylakoid and mLHC fraction phosphorylation pattern of double and quadruple mutants complemented with *Os*STN8. A Western blot analysis using an anti-phosphopthreonine antibody on thylakoid samples isolated from *A. thaliana* leaves of: *stn8* mutant complemented with *Os*STN8 (*stn8* comp*Os*STN8), double mutant *stn8pbcp* complemented with either *Os*STN8 (*stn8pbcp* comp*Os*STN8) or *At*STN8 (*stn8pbcp* comp*At*STN8), and quadruple mutant *stn8pbcpstn7pph1.1* (*quad1.1* comp*Os*STN8). D: dark treatment for 1-hour; HL: high light treatment for 1hr at 1300µmol photons m^{-2} s^{-1}; Rec: recovery of 30min after 1hr high light treatment. Loaded: 1,5µg of chlorophylls. **B** Native spectra of the two samples loaded in A **C** Tris-glycine SDS-PAGE (left) and western blot analysis (right) using an anti-phosphothreonine antibody on mLHC fractions from *A. thaliana* wild type and *quad1.1* comp*Os*STN8 lines. Loaded: 0,7µg of chlorophylls.

IEF of *quad1.1* comp*Os*STN8 mLHC fractions for P-CP29 isolation

In order to purify the phosphorylated form of *At*CP29, isoelectrofocusing was performed on the mLHC fraction obtained from *quad1.1*comp*Os*STN8 line (Fig. 4A). Together with the complemented line the mLHC fraction from wild type *A. thaliana* was loaded, where P-CP29 is not present, in order to discriminate the phosphorylated form of CP29. Phosphorylation should induce a shift in PI and therefore modify migration of CP29, as observed for *Zm*CP29 by Croce et al. (1996). However, no visible difference was detected between the two samples In figure 4A. The IEF gel was fractionated into 1cm sections and the proteins were eluted separately from the acrylamide, which were denominated according to their position (example fig. 4A: fraction 1-2 is the fraction delimitated by cm 1 and 2). Fractions were then eluted from the acrylamide and further separated through sucrose gradient ultracentrifugation to remove acrylamide, ampholytes and free pigments. As can be seen in figure 4B, no clear band was detectable after ultracentrifugation of the sucrose gradient in either of the two genotypes analyzed. Native absorption spectra of the initial IEF fractions harvested were measured, which were very similar to one another, with a peak in the red at 656nm circa (Fig. 4C). The various IEF fractions were further loaded on an SDS-PAGE colored with Coomassie, as shown in figure 4D, with the initial mLHC fraction of each corresponding genotype loaded as a reference.



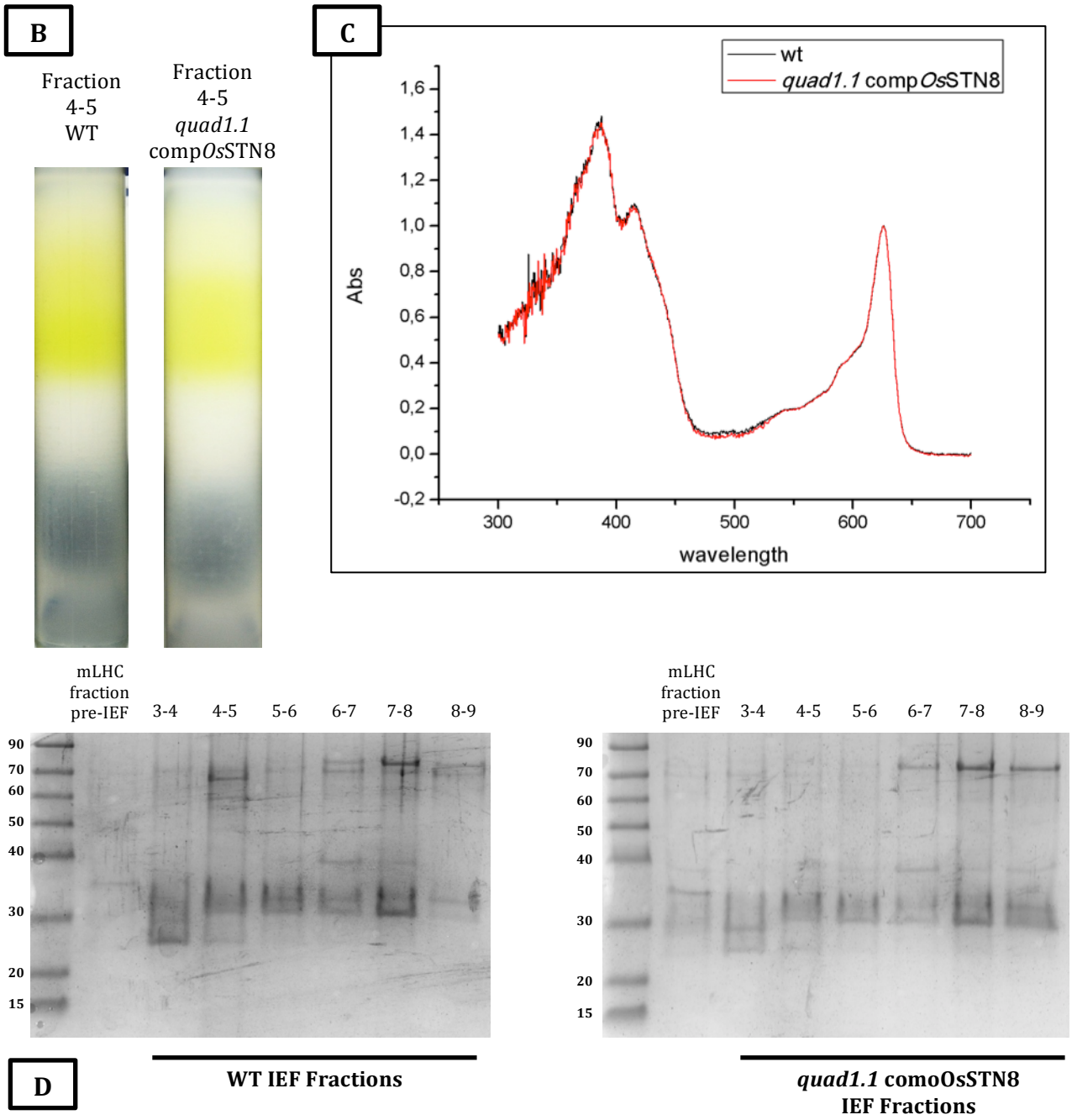


Fig. 4: IEF of the mLHC fractions from wild type and *quad1.1* comp*Os*STN8. A IEF analysis of wild type and quadruple mutant *stn8pbcpstn7pph1.1* complemented with *Os*STN8 (*quad1.1* comp*Os*STN8) mLHC fractions. Numbers on the bottom are used to indicate the various fractions recovered; HL: high light treatment for 1hr at 1300µmol photons m^{-2} s^{-1}; Loaded: 400µg of chlorophylls. **B** Example of the sucrose gradient fractionation obtained loading IEF recovered fractions. WT: wild type; *quad1.1* comp*Os*STN8: quadruple mutant complemented with *Os*STN8. Sucrose gradient was performed as described in chapter 2 **C** Example of native spectra, specifically fractions 4-5 recovered from IEF; maximum in the red was determined at 676nm **D** SDS-PAGE analysis of the fractions recovered from IEF of wild type (left) and *quad1.1* comp*Os*STN8 (right). Initial mLHC fraction was loaded as a reference (mLHC fraction pre-IEF). For each fraction, 0,5 µg of chlorophylls were loaded; for mLHC fractions pre-IEF, 0,7 µg of chlorophylls were loaded.

Discussion

Purification of phosphorylated CP29 from monocots has been previously reported. Croce and co-workers (1995) purified for the first time P-CP29 from *Zea mays*, and observed that there were differences between the phosphorylated and dephosphorylated form with respect to absorption spectra, fluorescence emission spectra and circular dichroism, suggesting changes on chromophore organization rather than composition. Further analysis of Chl fluorescence lifetime in detergent solution, Crimi et al. (2001) showed no change in fluorescence lifetime between P-CP29 and CP29. *In vivo* characterization of CP29 phosphorylation in various species, on the other hand, has led to various hypotheses on the effect of this high light-induced process (Bergantino et al., 1995, 1998; Pursiheimo et al., 2001; Betterle et al., 2015; discussed in Chapter 2). Generation of *A. thaliana* lines co-expressing *Os*STN8 and *At*CP29, in variants that either allow phosphorylation or not or even mimic constitutive phosphorylation is aimed to two major targets: first, we needed to discriminate between the activity of P-CP29 from that of P-PSII core proteins. The choice to utilize *A. thaliana* as an expression platform for CP29 and STN8 arose from the limitations of mutant libraries for monocots, while in the case of Arabidopsis coverage is satisfactory. Besides, genetic manipulation is very feasible, as opposed to rice or corn which require long and tiresome procedures for transformation. To this purpose, it is necessary to obtain a transgenic line expressing the rice kinase, which we determined to phosphorylate CP29 (Chapter 2), and CP29 in its WT and mutated form (Thr→Ala), where phosphorylation is suppressed (Fig. 2). A second strategy for obtaining a CP29 in its active form consisted in expressing *At*CP29 carrying a mutation at the phosphorylation site Thr→Glu, which mimics constitutive phosphorylation (Symington et al., 2007). If mimicking of constitutive phosphorylation could be obtained, co-expression with *Os*STN8 would no longer result necessary and this would allow determination of the individual contribution of CP29 to photoprotection, since PSII core phosphorylation would remain unaltered with respect to wild type. Furthermore, obtaining a transformed line where the totality of CP29 would result in a conformation similar to that induced by the

phosphorylated state, as opposed to only 50% as observed in rice (Chapter 1), would enhance its possible role on photoprotection, rendering its effect possibly more evident. Although, admittedly, I could not accomplish the programmed task within the time of my Ph.D. program, still I could obtain most of the genotypes planned and the preparation of the remaining genotypes is feasible, likely, within a relatively short period. Once the preparation of the genotypes will be accomplished, NPQ measurements and assessment of growth rate kinetics in different light and temperature conditions on the lines expressing *At*CP29CP, will allow verifying the hypothesis that reversible phosphorylation of CP29 is a method for regulating the sensitivity of the apparatus triggering excess energy dissipation, thus enhancing photoprotection, and that the mechanism can be transferred from monocots to dicots.

A second series of opportunities will spawn from the purification of CP29, from both *Oryza sativa* and *Arabidopsis thaliana,* which would allow for spectroscopic analysis of the purified protein for the identification of the physico-chemical mechanisms involved in quenching and their dependence on phosphorylation. Regarding *Os*CP29, the addition of a 6X Histag would facilitate purification of the phosphorylated form of the protein, once the transgenic lines co-expressing the rice kinase will be obtained, which is necessary for high light-induced phosphorylation (Chapter 2). In a similar manner, purification of P-*At*CP29 would allow to determine whether or not the phosphorylation of CP29 obtained in Arabidopsis induces conformational changes similar to those observed in the purified protein from monocots. To do so, mutant lines lacking the kinase and phosphatase of the PSII core were transformed with the coding sequence of *Os*STN8, to obtain phosphorylation of CP29 in a constitutive manner (Fig. 3A). Similarly, quadruple mutants for PSII core and LHCII kinase/phosphatase pairs were transformed with *Os*STN8. The absence of PBCP and PPH1 phosphatase should allow for accumulation of P-CP29 without possibility of cross-activity between the two phosphatases, as observed in the case of complemented *stn8pbcp* (Fig. 3A). Indeed, the quadruple mutant, when complemented with *Os*STN8, was the only line able to maintain a constitutive phosphorylated state of CP29. Preparative IEF was performed using the mLHC fractions obtained from *quad1.1* comp*Os*STN8 samples treated in high light, in order to purify P-CP29.

Unfortunately, the first attempt was unsuccessful due to the low amount of material available, which prompted us to perform IEF in small acrylamide slabs rather than in large preparative IEF resin (Bassi et al., 1999), which have far higher resolution and efficiency in recovery. Analysis of the fractions harvested showed that indeed purification of P-CP29 was not achieved, the most likely explanation for not observing differences in migration pattern between monomeric fractions from wild type and *quad1.1* comp*Os*STN8 lines as observed by Croce et al., (1996) in a successful separation.

Conclusions and future perspectives

The aim of this work was the determination of P-CP29 role in photoprotection with respect to that of P-PSII core, and at the same time purification of CP29 from rice and Arabidopsis with the purpose to characterize the conformational changes that phosphorylation induces. To do so, co-expression with the OsSTN8 kinase is necessary, as we have proven that it allows high light phosphorylation of the minor antenna protein (Chapter 2). As of today, these lines have yet to be obtained, but hopefully they will be in the near future, either through transformation of lines expressing the various forms of CP29, or through crossing with lines expressing the rice kinase. Alternatively, expression of AtCP29 mutated at Thr-83 to Glu, which mimics constitutive phosphorylation, could help complete this task without the need of co-expressing the kinase, and further elucidate P-CP29 role by obtaining the expression of the protein in a state where 100% phosphorylation is mimicked, as opposed to the 50% upper limit observed in rice (Chapter 1). Finally, expression of tagged versions of CP29 for improved purification will open the possibility of purifying P-CP29 and determine conformational changes imposed by phosphorylation and their effect on protein physic-chemical properties.

Materials and Methods

Plant material

A. thaliana Col-0 plants were all grown in control conditions for 3 weeks with a 16-h photoperiod at a photon flux density of circa 20µmol m^{-2} s^{-1}, at 22°C. double mutant lines *stn8pbcp* and quadruple mutants *stn8pbcpstn7pph1.1* and *stn8pbcpstn7pph1.3* were obtained from the laboratory of Prof. Goldschmidt-Clermont. Transformations and co-transformations with *Os*STN8 construct were performed according to Zhang et al., (2006).

*At*CP29 and *Os*CP29 constructs

*At*CP29 sequence was inserted in a Gateway vector pK7WG2, under the control of its native promoter and terminator. To do so, amplification was performed 1000bp ahead of the gene and 500bp after. *Os*CP29 construct was prepared inserting the cDNA sequence of the mature protein (LOC_Os07g37240.1) in the Gateway vector pK7WG2, carrying resistance for kanamycin. The sequence for expression for a 6X histidine tag was added to the reverse primer in order to link the tag to the C-terminus of the protein for purification through affinity chromatography. Mutagenesis was performed using QuickChange® site-directed mutagenesis kit (Strategene). Primers for mutation were designed using QuickChange Primer Design Program.

SDS-PAGE and western blot analysis

SDS-PAGE analyses were performed as described by Laemmli (1970) or with the Tris-Tricine buffer system (Schägger and von Jagow, 1987). For the separation of P-CP29 from P-D1, a Tris-Tricine gel with a 12%-18% gradient was utilised, and urea (2M) was also added into the running solution. Polypeptides, following SDS-PAGE, were transferred onto a nitrocellulose blotting membrane (Millipore) using a Mini Trans-Blot cell (Bio-Rad) and detected by use of homemade polyclonal antibodies, except for anti-phosphothreonine (Cell Signaling).

Isoelectrofocusing

mLHC fractions, isolated as described in chapter 2, were loaded on the flat bed IEF, which was prepared as according to Dainese et al., (1990), but with slight modifications, as according to Dall'Osto et al., (2005). Green bands were harvested and eluted from a small column with 10mM Hepes, pH 7.5, and 0.06% α-DM, and further fractionated on a 0.1 to 1M sucrose gradient containing 0.06% α-DM and 10mM Hepes, pH 7.5,

Thylakoids Isolation and Pigment Binding Complexes Purification

Thylakoids were purified from *A. thaliana* leaves as described in Rintamaki et al. (1996) with minor modifications. Leaves were harvested and macerated in extraction buffer (50mM HEPES-NaOH, pH 7.5, 300mM sucrose, 5mM $MgCl_2$, 1mM Na-EDTA, 10mM NaF, 1% (w/v) bovine serum albumin and 0.005M ε -aminocaproic acid, 0.001 phenyl-methylsulfonyl fluoride and 0.001 benzamidine as protease inhibitors). After filtration through Miracloth filters, samples were precipitated by centrifugation at 1500g for 5 min at 4°C and then resuspended in hypotonic buffer (10mM HEPES-NaOH, pH 7.5, 5mM sucrose, 5mM $MgCl_2$, 10mM NaF and protease inhibitors) and pelleted at 3000*g* for 3min. After centrifugation thylakoids were resuspended in a buffer containing 0.4M sorbitol, 0.015M NaCl, 0.005M $MgCl_2$, and 0.01M HEPES-KOH, pH 7.5. This procedure was performed in the dark at 4°C. Finally, thylakoids were either used directly or frozen in liquid nitrogen and stored at – 80°C until use.

For separation of pigment binding complexes 100 µg of thylakoid membranes were washed with 5mM EDTA and resuspended at a final concentration of 1 mg/ml in 10mM Hepes, pH 7,5. Samples were then solubilised at a final concentration of 0,5 mg/ml adding 1,2% α-DM and 10mM Hepes, pH 7,5 and vortexing for 1 min. After 10 min of ice incubation, thylakoids membranes were centrifuged at 15.000g for 10min to eliminate not solubilised materials. Fractionation occurred upon by ultracentrifugation on a 0,1 to 1 M sucrose gradient containing 0,03 α-DM and 10mM Hepes, pH 7,5 (SW60 Ti Rotor, 5h

30min at 60,000rpm, 4 °C). The green bands were harvested using a syringe needle.

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Conclusions

The aim of my work was the identification of the enzyme responsible for the high light-induced phosphorylation of CP29 in monocots and the attempt to transfer this mechanism to dicots. This was performed in order to both gain a deeper understanding on the role of CP29 phosphorylation and try to improve photoprotection in dicots, through the introduction of this high light response mechanism.

In the first part of my work I have focused on the identification and characterization of the kinase/phosphatase pair involved in the high light de/phosphorylation of CP29 in the model monocot *Oryza sativa*. This mechanism is present in many monocotyledonous plants, in response to environmental cues, such as cold stress, water stress or high light. Study of maize isogenic lines resistant to by cold-induced photo-inhibition, proved that these lines differed from sensitive ones in the presence of the phosphorylated form the minor antenna CP29. *In vivo* analysis on the purified phosphorylated protein indeed showed that this post-translational modification induced a conformational change that led to rearrangement of chromophores, leading to a more "quenched" conformation. Indeed, study of an *stn8* mutant of rice, where PSII core phosphorylation was suppressed when exposed to high light irradiancies, showed a much lower NPQ with respect to the wild type. Interestingly, in addition to the suppression of PSII core phosphorylation, that of CP29 was absent as well, given a first indication that this protein could be involved in photoprotection. More recently, it was demonstrated that this mechanism was not regulated by the STN7 kinase and PPH1 phosphatase, as opposed to what has been previously reported for LHC proteins, and that it enhanced NPQ and diminished ROS production.

A) Analysis of the *Osstn8* mutant allowed to determine that this kinase was indeed responsible for the high light induced phosphorylation of CP29. In high light conditions, *stn8* mutant is incapable of phosphorylating CP29 as opposed to wild type. This was previously unrecognized given the fact the phosphorylated CP29 migrates at the same level of P-D1 and P-D2 in SDS-PAGE gels. Furthermore, P-CP29/CP29 ratio was evaluated to be not higher than 50%, even

in the STN8 over-expressor. This was proposed to be associated to the distribution of the kinase within the grana membranes, which is absent from the center of grana disks. Regarding the phosphatase involved in CP29 dephosphorylation, previous reports demonstrated that PPH1, the phosphatase known to act on LHCs in Arabidopsis, was not responsible in rice for this activity. Demonstration that STN8 was indeed the kinase responsible for CP29 phosphorylation, suggested that PBCP, the PSII core phosphatase identified in Arabidopsis, could be the possible actor in CP29 dephosphorylation. Due to lack of appropriate rice mutants, we expressed *Os*PBCP coding sequence in *E.coli* and performed *in vitro* analyses using the recombinant protein. Although a *pbcp* mutant is not available at the moment for absolute proof, preferential dephosphorylation of PSII core proteins and CP29 with respect to LHCII on thylakoid membranes and isolated sucrose gradient fractions strongly supports this statement, in accordance also with what has been observed for PBCP in *A. thaliana.*

B) CP29 phosphorylation in high light is something of a unique mechanism to monocots. High light phosphorylation of the minor antenna has been reported before, but at very low levels and at sites different from the one characterized in monocots. The question arose on whether this mechanism was transferrable to dicots, and if this could improve photoprotection. If so, this could be a novel strategy for introducing characteristic advantageous traits of certain species to others. To do so, the rice kinase was expressed in *A. thaliana* mutants devoid of STN8, on the basis that while CP29 between the two species was highly conserved, STN8 showed a much longer N-terminal in monocots compared to dicots. Indeed, expression of the kinase led to restoration of PSII core phosphorylation in concomitance with that of CP29. This mechanism was proven to be high light dependent and reversible, as in rice, although phosphorylation did not induce difference in migration of the protein, as opposed to what has been observed in monocots. Slight NPQ increases were observed when complemented lines were exposed to high light irradiancies, indicating that indeed CP29 phosphorylation has an effect on photoprotection, although its true contribution has yet to be clarified.

C) The task of isolating the individual effect of P-CP29 on photoprotection is not simple. The strongest limitation lies in the scares availability of monocot mutants and the complexity of their genetic manipulation. To better understand CP29 phosphorylation and its effects, *A. thaliana* was employed, for which many more mutants are available and biochemical techniques have been optimized during the years. Lines expressing CP29 from both rice and *Arabidopsis* have been generated, carrying mutations at the known threonine residue, site of phosphorylation in monocots. Mutations were performed in order to suppress phosphorylation in one case, and mimic constitutive phosphorylation in the other. This will allow determination of the true effects of the minor antenna when co-expressed with *Os*STN8 to obtain high light phosphorylation, as described previously. Furthermore, the attempt to purify CP29 and P-CP29 of both species, will allow a deeper understanding of the conformational changes these proteins undergo upon phosphorylation. To this aim, different strategies are being carried out: i) in the case of *Os*CP29, we have tagged the protein with a 6X Histag, for chromatographic purification, and attempted co-expression with *Os*STN8 for high light induced phosphorylation. Initial co-transformation attempts have been negative, so now single lines expressing either CP29 or STN8 are being selected to perform crossing of the two lines to obtain a double transformant. ii) regarding *At*CP29, transformation of the quadruple mutant *stn8pbcpstn7pph1.1* has led to the generation of a line constitutively expressing P-CP29. Initial attempts of purifying the protein through IEF have failed, due to contamination of the fractions from the other monomeric antennae. Attempts utilizing different sucrose gradient fractions, such as the CP29-CP24-LHCII complex, could lead to more positive results in the near future.