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THYLAKOID PROTEIN PHOSPHORYLATION IN REGULATION OF PHOTOSYNTHESIS

by

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ABSTRACT

Once the seed has germinated, the plant is forced to face all the environmental changes in its habitat. In order to survive, plants have evolved a number of different acclimation systems. The primary reaction behind plant growth and development is photosynthesis. Photosynthesis captures solar energy and converts it into chemical form. Photosynthesis in turn functions under the control of environmental cues, but is also affected by the growth, development, and metabolic state of a plant. The availability of solar energy fluctuates continuously, requiring non-stop adjustment of photosynthetic efficiency in order to maintain the balance between photosynthesis and the requirements and restrictions of plant metabolism. Tight regulation is required, not only to provide sufficient energy supply but also to prevent the damage caused by excess energy. The very first reaction of photosynthesis is splitting of water into the form of oxygen, hydrogen, and electrons. This most fundamental reaction of life is run by photosystem II (PSII), and the energy required for the reaction is collected by the light harvesting complex II (LHCII). Several proteins of the PSII-LHCII complex are reversibly phosphorylated according to the energy balance between photosynthesis and metabolism. Thylakoid protein phosphorylation has been under extensive investigation for over 30 years, yet the physiological role of phosphorylation remains elusive. Recently, the kinases behind the phosphorylation of PSII-LHCII proteins (STN7 and STN8) were identified and the knockout mutants of these kinases became available, providing powerful tools to elucidate the physiological role of PSII-LHCII phosphorylation. In my work I have used the *stn7* and *stn8* mutants in order to clarify the role of PSII-LHCII phosphorylation in regulation and protection of the photosynthetic machinery according to environmental cues. I show that STN7-dependent PSII-LHCII protein phosphorylation is required to balance the excitation energy distribution between PSII and PSI especially under low light intensities when the excitation energy transfer from LHC to PSII and PSI is efficient. This mechanism differs from traditional light quality-induced “state 1” – “state 2” transition and ensures fluent electron transfer from PSII to PSI under low light, yet having highest physiological relevance under fluctuating light intensity. STN8-dependent phosphorylation of PSII proteins, in turn, is required for fluent turn-over of photodamaged PSII complexes and has the highest importance upon prolonged exposure of the photosynthetic apparatus to excess light.

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ABBREVIATIONS

77 K	77 degrees Kelvin = –196 degrees Celsius
CET	Cyclic electron transfer around PSI
Chl	Chlorophyll
ETC	Electron transfer chain between PSII and PSI
$F_{m'}$	Maximal fluorescence of light treated leaf
$F_{s'}$	Steady-state fluorescence under light
HL	High light intensity
LHCII	Light harvesting complex II
LL	Low light intensity
ML	Moderate light intensity
NPQ	Non-photochemical quenching of excitation energy
npq1	Mutant with no conversion of violaxanthin to zeaxanthin (low qE)
npq4	Mutant with no PsbS protein, no feedback de-excitation (qE)
pgr5	Mutant with no or impaired ferredoxin plastoquinone oxidoreductase-(FQR-) dependent cyclic electron transfer around PSI (CET)
P-LHCII	LHCII trimer with phosphorylated Lhcb 1 and/or Lhcb2
PSI	Photosystem I
PSII	Photosystem II
qE	Energy-dependent quenching
stn7	Mutant with no LHCII protein phosphorylation
stn7 npq4	Mutant with no LHCII phosphorylation, no qE
stn7 stn8	Mutant with no PSII-LHCII protein phosphorylation
stn8	Mutant with low PSII core protein phosphorylation
WT	Wild type

LIST OF ORGINAL PUBLICATIONS

This thesis is based on the following publications, which will be referred by their number in the text.

1. **Tikkanen M, Nurmi M, Suorsa M, Danielsson R, Mamedov F, Styring S, Aro EM.** (2008) Phosphorylation-dependent regulation of excitation energy distribution between the two photosystems in higher plants. Biochim Biophys Acta 1777: 425-432.

2. **Tikkanen M, Grieco M, Kangasjärvi S and Aro EM** (2009) Thylakoid protein phosphorylation in higher plants optimises electron transfer under fluctuating light. Manuscript.

3. **Tikkanen M, Piippo M, Suorsa M, Sirpiö S, Mulo P, Vainonen J, Vener AV, Allahverdiyeva Y, Aro EM.** (2006) State transitions revisited – a buffering system for dynamic low light acclimation of Arabidopsis. Plant Mol Biol. 62: 779-793.

4. **Tikkanen M, Nurmi M, Kangasjärvi S, Aro EM.** (2008) Core protein phosphorylation facilitates the repair of photodamaged photosystem II at high light. Biochim Biophys Acta. 1777:1432-1437.

Other publications related to the topic of the thesis:

Tikkanen M, Suorsa M and Aro EM (2009) The flow of solar energy to biofuel feedstock via photosynthesis. International Sugar Journal 11: 156-163.

Vainonen JP, Sakuragi Y, Stael S, Tikkanen M, Allahverdiyeva Y, Paakkarinen V, Aro E, Suorsa M, Scheller HV, Vener AV, Aro EM. (2008) Light regulation of CaS, a novel phosphoprotein in the thylakoid membrane of Arabidopsis thaliana. FEBS J. 275: 1767-1777.

1. INTRODUCTION

1.1. Challenges of photosynthesis research

Food, fossil fuels, and all the organic energy and matter in general are based on the conversion of solar energy into chemical form by photosynthesis. The human population is growing fast, and an increasing proportion of the growing population is achieving higher standards of living. This means that every day, more resources are needed to support the life of the human population. The stocks of unsustainable resources are shrinking and our only possibility of preserving peace and welfare is to strongly reduce the consumption of limited resources and to enhance the production of sustainable resources. Photosynthetic organisms are the primary producers behind all the organic resources we use to support our culture and economy. In order to provide security for future generations, we have to maximize the capacity to utilize photosynthetic production in a sustainable manner for the good of the human population as a whole.

Due to the fact that we cannot continue to increase the use of our shrinking resources, we have to find new strategies to provide resources that support of our lifestyle. One of the main goals must be the development of more efficient and more tolerant photosynthetic organisms that can utilize solar energy in a sustainable way with maximum yield and with the minimum of negative effects on society and on the environment. A deep understanding of the structure, function, and regulation of the photosynthetic machinery is a very important target on our way toward a sustainable future. Yet, our understanding of photosynthesis as an independent reaction is not enough. The photosynthetic machinery is the solar-powered energy plant of photosynthetic organisms, and it always functions under the control of the entire organism. We have to understand how the total output of a plant is regulated and what the factors that limit plant productivity are.

Downstream metabolism regulates the efficiency of photosynthetic primary production. If this metabolism cannot accept energy from photosynthesis, the excess energy is dissipated as heat. Indeed, the development of an unlimited sink for photosynthetic production would enhance the photosynthetic performance. Today, the raw materials (so-called feedstocks) of biofuels are hydrocarbons, which are far removed from the primary reactions of photosynthesis. With every biochemical step after the initial photochemical events, part of the energy harvested by photosynthesis is lost. Figure 1 illustrates the different solar energy conversion efficiencies and possibilities to enhance the efficiency of different fuels and feedstocks by using what we know about photosynthesis.

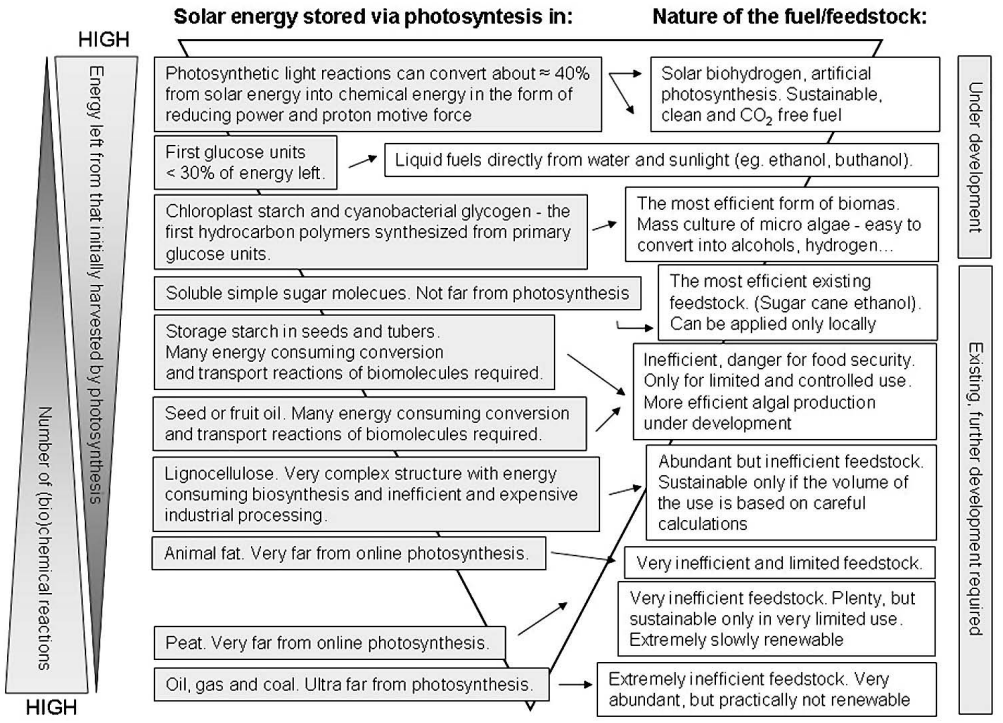

Figure 1. Solar conversion efficiency of different fuel feedstocks. *Bio- and fossil fuels originate from solar energy converted into chemical energy by photosynthesis. The highest amount of energy harvested by photosynthesis is stored in molecules that are the very first products of photosynthesis. After photosynthesis, the amount of energy stored in organic molecules gradually becomes reduced at each successive biochemical reaction. The usability and scalability of various feedstocks are a result of many different economical, political, technological, geographical, and biological factors. However, in order to develop biofuels that can truly compete with fossil fuels, the fundamental requirement is to enhance the solar conversion efficiencies to fuel feedstocks, which maximize the output and minimize the land area required and the negative effects on food production and biodiversity. The most efficient and promising possibilities to exploit photosynthesis sustainably for large-scale production of energy are shown at the top of the diagram. At the bottom are the traditional and most inefficient—as well as unsustainable—ways to use solar energy as a fuel. It is easy to recognize that an understanding of photosynthetic processes is very important when developing more efficient feedstocks for biofuels. From* Tikkanen, et al. (2009).

1.2. The balance of energy

There has to be a balance between photosynthetic production of energy and the use of energy in plant metabolism. This means that the photosynthesis is continuously regulated according to the physiological state of the plant and the availability of solar energy. Phosphorylation of PSII-LHCII proteins, the subject of my thesis work, is dynamically regulated according to the availability of light energy and the capability of the downstream metabolism to make use of the energy harvested (Aro and Ohad,

2003). Indeed, the phosphorylation status of PSII-LHCII dynamically reflects the energy balance of the photosynthetic machinery and is most probably one of the key factors in regulation of plant productivity. Yet, due to the complexity of plant metabolism, the exact physiological role of PSII-LHCII phosphorylation in regulation of plant efficiency has remained elusive.

1.3. The structure and function of the photosynthetic machinery in plants

1.3.1. The basic structure of the chloroplast membrane system

All green plant cells have chloroplasts, cell organelles that are the actual energy producing units of green cells. Figure 2 shows the basic structure of the chloroplast membrane system. Chloroplasts are surrounded by two layers of membranes called the envelope. Inside the chloroplast is the thylakoid membrane system, which is the structure carrying the protein complexes responsible for harvesting sunlight and converting the solar energy into chemical form. The thylakoid membrane network is surrounded by a soluble compartment called the stroma, where the carbon fixation reactions take place. The thylakoid system itself holds another soluble compartment, the thylakoid lumen. Such a system of two separate soluble compartments allows the storage of energy in chemical and electrical gradients. Thylakoid membranes are, in turn, divided into highly stacked grana membranes and unstacked stroma membranes, which connect the stacked grana

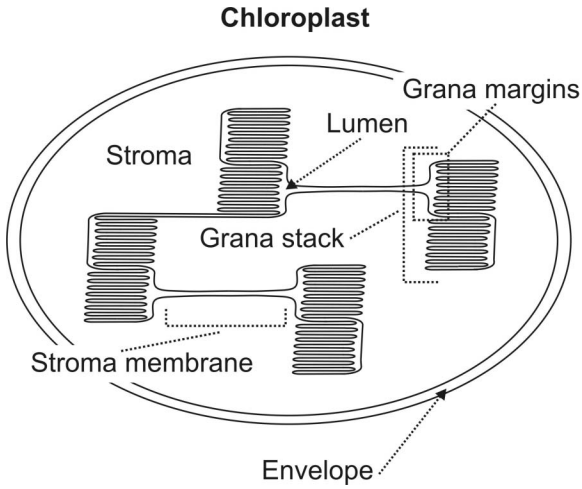

Figure 2. *The thylakoid membrane is composed of two laterally segregated compartments, highly stacked grana membranes and unstacked stroma membranes. The soluble part of the chloroplast is also distributed in two different compartments separated by the thylakoid membrane, that is, the thylakoid membrane holds the luminal compartment and is itself surrounded by the stroma. The grana stacks are enriched with PSII-LHCII complexes and grana margins and stroma membranes are enriched with PSI-LHCI complexes. ATP synthase is located in stroma membranes and cyt* b_6f *complex is more or less equally distributed between the grana and stroma membranes. Carbon fixation reactions take place in the soluble stroma.*

areas. The grana-stroma structure of the thylakoid membrane is dynamically regulated according to environmental cues. The photosynthetic light reactions occur in the thylakoid membrane and are run by four multiprotein complexes: photosystem II (PSII), cytochrome b6f (Cyt b6f), photosystem I (PSI), and the ATP synthase. The dynamic nature of the chloroplast membrane system is closely connected to the functionality of the photosynthetic protein complexes. Indeed, the proper functioning of photosynthetic pigment protein complexes requires tight regulation of the structure of the thylakoid membrane (Figure 2).

1.3.2. Production of chemical energy from sunlight by photosynthetic protein complexes

Figure 3 illustrates the structural and functional basis of photosynthetic light reactions. Light energy is absorbed by the light harvesting antenna system and the excitation energy is directed to the reaction centers in order to run the actual photosynthetic reactions. Harvested light energy excites the PSII reaction center chlorophyll molecule P680, from which the electron is transferred to a plastoquinone molecule. After receiving two electrons, the plastoquinol is released from the PSII complex and in the next step it becomes oxidized by the Cyt b_6f complex. Electrons are further transferred from Cyt b_6f to the PSI complex, which is again excited by light, and the electrons released from P700 are finally transferred to ferredoxin (Fd). Ferredoxin-$NADP^+$ oxidoreductase (FNR) oxidases ferredoxin and concomitantly generates NADPH from $NADP^+$.

Oxidized $P680^+$ in PSII is an extremely strong oxidant and it withdraws electrons from the manganese cluster of the oxygen evolving complex of PSII. The manganese cluster, in turn, finally withdraws the electrons from water and releases oxygen as a by-product. Water-splitting reactions and oxidation of plastoquinol create a proton gradient across the thylakoid membrane, which is released via the ATP synthase to produce ATP. Instead of directing electrons to carbon assimilation or to other reducing reactions in the stroma, PSI can cycle part of the electrons back to plastoquinol and by this mechanism it can enhance the production of ATP at the expense of NADPH production, and thus uncouple the regulation of the proton gradient from the water-splitting activity of PSII. Part of the electrons can also be directed to oxygen ($O_2 \rightarrow H_2O$) via chlororespiration (Peltier and Cournac, 2002) and the Mehler reaction (Badger et al. 2000).

1.3.3. Structure of the thylakoid membrane

Photosynthetic reactions require a highly organized and flexibly regulated structural environment to occur (Figure 2). Photosynthetic pigment protein complexes are embedded in the thylakoid membrane of plant chloroplasts (Figure 3). These membranes are structurally complex, highly organized, and dynamically regulated structures (Albertsson, 2000; Albertsson, 2001; Mustardy and Garab, 2003; Mustardy et al. 2008). Depending

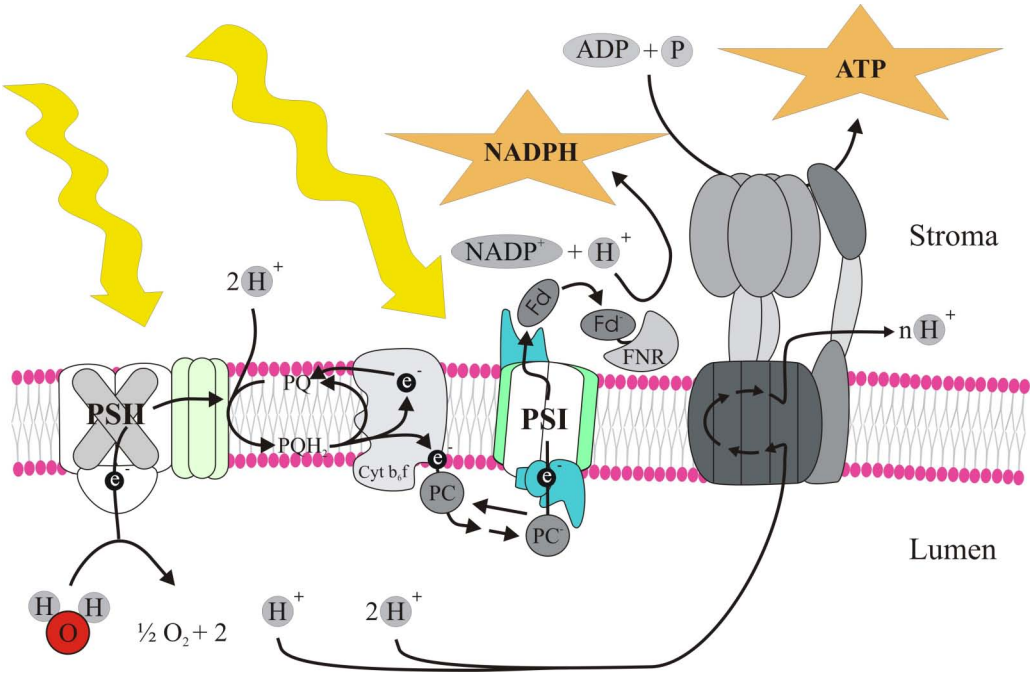

Figure 3. *Simplified scheme showing the structure and function of the photosynthetic light reactions and electron transfer routes in the thylakoid membrane. PSII = photosystem II; PQ = plastoquinone; Cyt b_6f = cytochrome b_6f complex; PC = plastocyanine; PSI = photosystem I; Fd = ferredoxin; FNR = ferredoxin-NADP$^+$ oxidoreductase; NADP = nicotinamide adenine; NADPH = the reduced form of NADP; ADT = adenosine diphosphate; P = phosphate; ATP = adenosine triphosphate.*

on the availability of solar energy (Rozak et al. 2002) and metabolic balance (Hausler et al. 2009), plants favor different structural organization of the thylakoid membrane (Figure 4). Shade and sun plants differ in the ultrastructure of their chloroplasts. As an adaptation to low light conditions, shade plants have evolved extensive granal stacking (Anderson et al. 1973). It is conceivable that in this way they maximize the thylakoid membrane area hosting the protein complexes involved in capturing solar energy. Conversely, the sun plants, living under excess light, have less appressed thylakoid membranes (Boardman, 1977), which could be an adaptation to maximize the space for the enzymes that are required for carbon fixation reactions. Moreover, the organization of the thylakoid membranes rapidly and reversibly responds to changes in light intensity and quality (Bjorkman and Holmgren, 1963; Anderson et al. 1973; Rozak et al. 2002; Chuartzman et al. 2008), which most probably occurs in the chloroplasts of both shade plants and sun plants.

The photosynthetic protein complexes are heterogeneously distributed in the thylakoid membrane (Figure 4): PSI-LHCI and ATP synthase are located in non-appressed stroma regions whereas PSII-LHCII is predominantly located in the stacked grana core

(Andersson and Anderson, 1980; Danielsson et al. 2004). In the grana margins—at the interface between PSII-LHCII-rich grana and PSI-LHCI-rich stroma membranes—the density of both reaction center complexes is high (Kaftan et al. 2002). Dynamic regulation of the thylakoid organization affects the interaction between the photosynthetic protein complexes, especially in the grana margin regions where both reaction centers are in close proximity to each other.

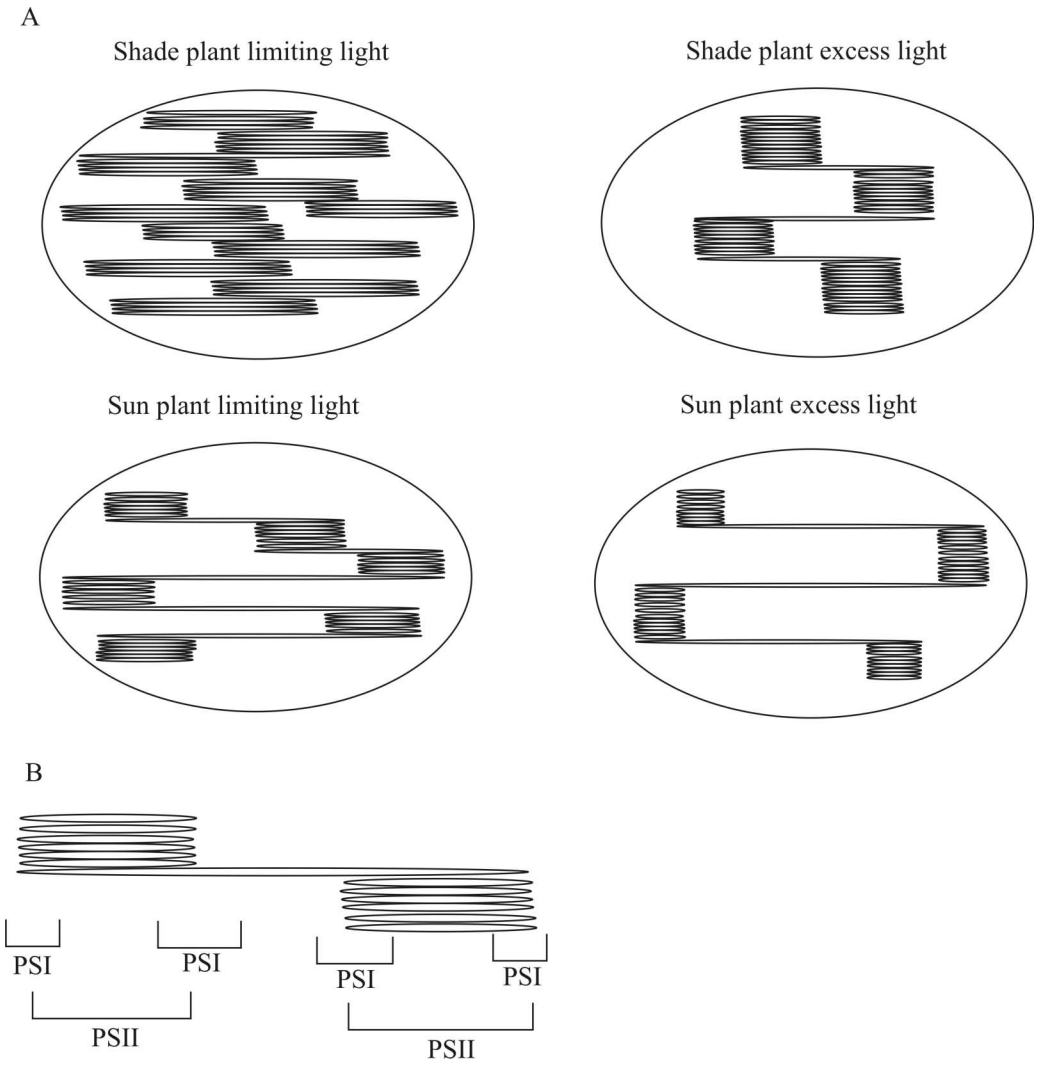

Figure 4. *Thylakoid ultrastructure and the lateral distribution of the PSII-LHCII and PSI-LHCI complexes in the thylakoid membrane is affected by adaptation and acclimation of plants to varying light intensity. A. Shade plants have more appressed thylakoid structure than sun plants. Saturating light intensity induces extensive segregation of the grana stacks and the stroma lamellae, and low light intensity relaxes the structure. B. PSII-LHCII complexes are located in the grana stacks and PSI-LHCI complexes are located mainly at the interface between grana stacks and stroma lamellae (grana margins).*

The dynamics in the lateral distribution of the photosynthetic protein complexes in the thylakoid membrane most probably have a vital role in regulation of the functions, maintenance, and efficiency of the photosynthetic apparatus. Yet, the exact roles of the structural flexibility in such processes are not fully understood. More is known about the role of lateral distribution of the pigment-protein complexes in relation to the synthesis and turnover of the photosynthetic protein complexes (Danielsson et al. 2006).

The water-splitting cofactors of the PSII complex are strong oxidants. High oxidizing capacity, which is required for the splitting of water, is also dangerous to the photosynthetic machinery itself (Barber and Andersson, 1992; Asada, 1999). The strongest oxidative pressure is targeted to the PSII reaction center proteins, closest to the water-splitting reaction. Indeed, PSII inevitably becomes damaged when light energy is present and the photosynthetic reactions take place. In order to maintain the photosynthetic activity, the damaged PSII complexes must be replaced with newly synthesized functional ones (Aro et al. 1993). For this turnover of PSII, a complex machinery of enzymes and assisting proteins runs a process whereby the damaged complexes migrate from grana to stroma membranes, where the non-functional complexes are disassembled, damaged subunits are replaced with newly synthesized ones, and after reassembly the PSII complex finally migrates back to grana membranes (Mulo et al. 2008).

1.3.4. Structure of the PSII-LHCII complex

Light energy is absorbed by the light harvesting chlorophyll (chl) a/b antenna complexes of PSII, which transfer the excitation energy to the PSII reaction center in order to drive the photosynthetic water-splitting reactions. The antenna system has to have high flexibility to respond to ever-changing light intensity. In order to have the flexibility required, the antenna system is rather complex and is built up of many different chlorophyll binding proteins. The function of the antenna system depends on which pigments and proteins are present in the antenna, and also on how the antenna complexes are interacting with each other and with the reaction center complexes in the three-dimensional structure of the thylakoid membrane. The structure of the PSII antenna system is quite well known, and consists of at least six different chl-binding proteins (Ganeteg et al. 2004). LHCII, the major antenna complex of PSII, is formed from trimers of three proteins—Lhcb1, Lhcb2, and Lhcb3—in different combinations (Jansson, 1994).

The monomeric PSII complex (Figure 5 A) is composed of more than 20 subunits (Zouni et al. 2001; Ferreira et al. 2004; Mulo et al. 2008). The PSII supercomplex (Figure 5 B) is composed of a dimeric core complex of PSII, which is associated with two copies of each minor light-harvesting protein, two strongly bound LHCII trimers, and one or more less tightly bound trimer(s) (Boekema et al. 2000; Yakushevska et al. 2001; Dekker and Boekema, 2005).

PSII-LHCII supercomplex is the functional form of PSII, and these supercomplexes are organized in arrays (Figure 5 C) and are mainly located in appressed grana membranes (Boekema and Braun, 2007). However, the assembly of newly synthesized PSII-LHCII subunits and the repair of photodamaged PSII core occur through the monomeric form of PSII. Dynamic and reversible oligomerization, monomerization, and reoligomerization of PSII are, indeed, required for the synthesis of PSII and for the maintenance of PSII activity. It is important to note that the monomerization process of the PSII-LHCII supercomplex requires lateral migration of different oligomerization states of PSII-LHCII (Figure 5) along the thylakoid membrane system (Figure 4). PSII-LHCII complexes form highly organized arrays of supercomplexes in the grana core, and the structure is gradually monomerized via the dimeric state of PSII towards the stroma lamellae (Danielsson et al. 2006).

During the past ten years, great progress has been made towards solving the three-dimensional structure of the PSII-LHCII and PSI-LHCI complex at high resolution. Several X-ray crystallographic structures have become available for cyanobacterial PSII (Zouni et al. 2001; Jordan et al. 2001; Ferreira et al. 2004; Kamiya and Shen, 2003; Guskov et al. 2009;) and the detailed structure of LHCII has been resolved (Liu et al. 2004; Standfuss et al. 2005; Yan et al. 2007). Yet, the complete structure of PSII from higher plants is still missing. The combination of structural information about the cyanobacterial PSII core and higher plant LHCII with experimental biochemical

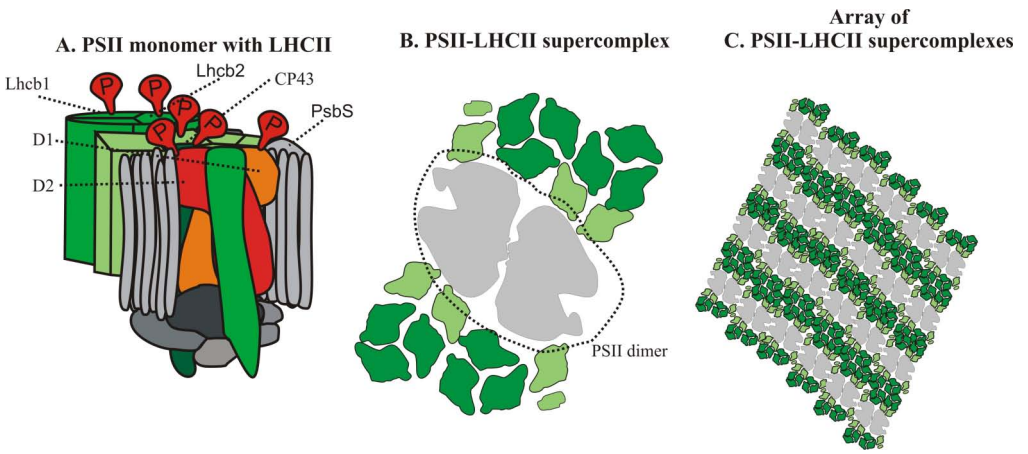

Figure 5. *Organization of the PSII-LHCII complexes in the thylakoid membrane. A. Illustration of the structure of the PSII-LHCII monomer. Only the major phosphoproteins are named and marked with P in the structure. PsbS is not a phosphoprotein, but a prerequisite in dynamic regulation of light energy transfer between the light harvesting pigment protein complexes (green) and the PSII reaction center (other colors). B. Functional PSII is composed of PSII-LHCII supercomplexes. Inner antennae CP26, 24, and 29 (light green), close to reaction centers, are composed of monomeric chl a/b, carotenoid binding proteins, and the outer ones (Lhcb1, Lhcb2 and Lhcb3) exist as trimers (dark green). C. In the grana, the PSII-LHCII supercomplexes form large highly organized arrays of protein complexes. The illustration has been drawn according to* Boekema and Braun (2007).

data has made it possible to model the structure of the PSII-LHCII complex of higher plants (Nield and Barber, 2006). The crystallographic structure of higher plant PSI has recently been resolved (Amunts et al. 2007). In general, solving of the structure of PSII at the highest resolution possible has become one of the most important goals of modern biology. Knowing the exact structure of the water-splitting apparatus of PSII would pave the way to development of photolytic bio-mimicking devices to produce hydrogen or electricity from water and sunlight.

1.3.5. Phosphorylation of PSII-LHCII proteins

Several proteins of the PSII core complex and LHCII are phosphorylated according to environmental and metabolic cues. The major phosphoproteins of the PSII core complex are D1, D2, and CP43, but the phosphorylation-specific threonine residues of PsbH and TSP9 proteins are also reversibly phosphorylated (Vener, 2007). The N-terminal threonine residues of the Lhcb1 and Lhcb2 proteins of the LHCII trimer are reversibly phosphorylated (Bennett, 1979). Three minor chl-binding proteins—Lhcb4 (CP29), Lhcb5 (CP26), and Lhcb6 (CP24)—are monomeric, and of these proteins Lhcb4 is the only phosphoprotein in higher plants (Bergantino et al. 1995; Bergantino et al. 1998). The phosphorylation of PSII-LHCII proteins is regulated by the redox state of the electron transfer chain and that of the chloroplast stroma (Aro and Ohad, 2003) via the STN7 and STN8 kinase-dependent phosphorylation pathways (Bonardi et al. 2005; Bellafiore et al. 2005). The phosphorylation of PSII core proteins is mainly under the control of STN8 kinase, but STN7 kinase also has a minor role (Vainonen et al. 2005). The phosphorylation kinetics of different phosphoproteins of the PSII core complex are individual, indicating that there is protein-dependent specificity of the STN7 and STN8 kineses in phosphorylation of the PSII core proteins. Even so, the reason for such specificity remains totally unknown. In contrast, LHCII proteins are phosphorylated solely by STN7 kinase (Bonardi et al. 2005).

Interestingly, the STN8 kinase not only phosphorylates the phosphoproteins of the PSII core. Recently, I was involved in a study demonstrating that STN8 kinase is responsible for phosphorylation of the 40-kDa thylakoid protein CaS (calcium-sensing protein) (Vainonen et al. 2008). About the same time, it was shown that the CAS protein is involved in regulation of stomata opening (Nomura et al. 2008). Together, these results suggest that STN8 kinase plays a role in inter-regulation of light reactions, carbon metabolism, and gas exchange.

1.4. Long- and short-term regulation of the PSII antenna system

Light acclimation is a result of many processes that modulate the function of the photosynthetic machinery to respond to the requirements and restrictions of the prevailing

environmental conditions and the metabolic state of the plant. Changes in environmental conditions take place on many different time scales. Under the canopy, fluctuations in shade and bright light occur in a fraction of a second, when wind is moving the leaves. The movement of clouds also causes fairly rapid changes in light intensity. The availability of light energy is very different on cloudy days and sunny days, which causes day-to-day variation in the availability of light. Moreover, the diurnal and seasonal availability of light energy peaks in the middle of the day and in the middle of summer, respectively. However, it is not only the availability of light but also the metabolic state of the plants that sets the requirements for the photosynthetic machinery. When growth is active and water and nutrients are abundantly available, a higher efficiency of photosynthesis is required as compared to the state when water, nutrients, temperature, or physiological state of the plant limit the need for energy. The photosynthetic machinery must be flexible enough to enhance the solar energy conversion efficiency when the photosyntesis limits the metabolism. Likewise, the photosynthetic machinery must have mechanisms to downregulate the efficiency of light harvesting when excess energy is available. It is also well known that the defense reactions caused by pathogen attach lead to transient metabolic reprogramming of plant growth and slowdown of photosynthesis (Bolton, 2009).

1.4.1. Long-term acclimation of photosynthetic light reactions

Depending on the average intensity and quality of light, plants synthesize different amounts of various protein complexes in the photosynthetic machinery. It is well known that the stoichiometry between PSII-LHCII and PSI-LHCI complexes, and especially the amount of LHCII, is variable and depends on the light conditions (Kim et al. 1993; Wagner et al. 2008). Plants grown under high light have more PSII reaction centers relative to PSI and less antenna complexes than plants grown under low light conditions (Bailey et al. 2001). The Lhcb1 protein accounts for about 70% of LHCII (Jansson, 1994), and it responds dynamically to the intensity of growth light (Bailey et al. 2001).

1.4.2. Short-term regulation of the photosynthetic machinery

As described above, plants have to have mechanisms for flexible adjustment of photosynthetic efficiency. Sometimes, relatively slow responses are enough to set the balance between photosynthesis and metabolism—but very rapid reactions are also required. The most important mechanisms in regulation of the efficiency of photosynthesis are related to the function of LHCII. Traditionally, short-term regulation of light harvesting has been divided into two distinct mechanisms: the LHCII phosphorylation-dependent state transitions (Allen et al. 1981; Allen and Forsberg, 2001) and the xanthophyll- and PsbS-dependent non-photochemical energy dissipation (NPQ) (Li et al. 2004).

1.4.2.1. State transitions

"State transitions" were discovered 40 years ago (Murata and Sugahara, 1969; Murata, 2009). This is a moderately short-term acclimation process that was believed to happen in the timescale of minutes. The conventional concept of state transition involved the rearrangement of thylakoid structure, and about 10 years later the phosphorylation of LHCII proteins was integrated into the concept (Bennett et al. 1980; Horton and Black, 1981), yet the relationship between the thylakoid rearrangements and protein phosphorylation has remained controversial. According to the later and generally accepted dogma, the non-phosphorylated LHCII mainly functions as an antenna for PSII, but migrates into contact with PSI as a result of light quality-dependent phosphorylation of Lhcb1 and Lhcb2. The STN7 LHCII kinase is needed for catalysis of Lhcb1 and Lhcb2 protein phosphorylation (Bellafiore et al. 2005), the activation of the kinase being regulated by the redox state of the photosynthetic electron transfer chain (Aro and Ohad, 2003; Zito et al. 1999; Finazzi et al. 2001). STN7 kinase is activated by binding of a reduced plastoquinone molecule to the Qo site of the cytochrome b_6f complex (Vener et al. 1997; Lemeille et al. 2009). Release of plastoquinone from the Qo site in turn deactivates the LHCII kinase. On top of this regulation mechanism, an increase in stromal reductants exerts an inhibitory effect on LHCII kinase, which occurs gradually—for example, with increasing irradiance (Rintamaki et al. 2000). Accordingly, the LHCII kinase is deactivated or inhibited upon transfer of plants to darkness or suddenly to high light, whereas the kinase is active under low and/or moderate light regimes (Rintamaki et al. 2000). The LHCII kinase is not only regulated at the functional level, but also the actual amount of enzyme expressed matches the quantity required to phosphorylate LHCII proteins (Lemeille et al. 2009).

1.4.2.2. Thermal dissipation of excess excitation energy

Of the mechanisms described in the literature that regulate the efficiency of the light harvesting machinery, the non-photochemical quenching of excitation energy (NPQ) is the most profound. NPQ, the event initially found as energy-dependent quenching of fluorescence (Murata and Sugahara, 1969), is composed of at least two components that function in different timescales. The faster one is called feedback de-excitation qE (energy-dependent quenching), and is very rapid, occurring in the time scale of seconds. The slower component is called qI (photoinhibitory quenching), and functions in minutes or tens of minutes. It is well documented that qE is fully dependent on the presence of the PsbS protein (Li et al. 2000), which functions as a sensor of luminal pH through luminally-exposed protonable residues (Li et al. 2000; Li et al. 2002; Li et al. 2004). However, despite the PsbS protein is obligatory for the induction of qE, this alone is not enough to induce NPQ to its full extent. In order to reach full NPQ, the violaxanthin carotenoid is converted to the form of another carotenoid, zeaxanthin (Demmig-Adams et al. 1990; Niyogi et al. 1997). The nature of qI remains elusive, but it

appears to be independent of the PsbS protein and is probably related to photoinhibition or accumulation of non-functional but undamaged PSII reaction centers.

Recently, there has been a great improvement in our understanding of the mechanistic basis of NPQ (qE). It is now evident that NPQ is based on PsbS protein-dependent reversible reorganization of PSII-LHCII protein domains in the thylakoid membrane, according to the light intensity (Kiss et al. 2008; Betterle et al. 2009). However, despite the biochemical breakthrough in our understanding of the mechanical basis of NPQ, the physical mechanisms that would explain how and by what pigment molecules the excess light energy is thermally dissipated remain a topic of heavy debate.

1.5. *Stt* and *stn* kinase mutants, the new tools to investigate the biological roles of thylakoid protein phosphorylation

Recently, a genetic approach led to the identification of the LHCII kinase Stt7 in *Chlamydomonas* (Depege et al. 2003) and its ortholog STN7 in *Arabidopsis thaliana* (Bellafiore et al. 2005). Studies with the stt7 and the *stn7* mutants have shown that LHCII phosphorylation is indeed a prerequisite for state transition to occur (Depege et al. 2003; Bellafiore et al. 2005) and it is also needed for long-term light quality acclimation when plants are grown under the light qualities that selectively excite PSII or PSI (Bonardi et al. 2005; Wagner et al. 2008). These findings demonstrate that the phosphorylation of LHCII proteins is crucial for regulation of the relative excitation of PSII and PSI on time scales that range from minutes to the whole lifetime of the plant.

Compared to wild-type (WT) plants, the *stn7* mutant plants have a lower rate of carbon assimilation when they have been grown under low light conditions (LL) (Bellafiore et al. 2005). Nevertheless, the rate of carbon assimilation in the *stn7* mutant grown at higher light intensity was no different from that of WT (Bellafiore et al. 2005). Despite its reduced carbon assimilation under LL, the *stn7* mutant has hardly any visual phenotype (Bellafiore et al. 2005). Compared to WT, the LL-grown *stn7* mutants have distinctly higher excitation pressure of PSII, whereas under higher growth light intensities this difference between WT and the *stn7* mutant diminishes (Bellafiore et al. 2005). In a study by Bonardi et al. (2005), several photosynthetic genes were found to be differentially expressed in the *stn7* mutant compared to the WT. In various studies, STN7 kinase has been suggested to function in different regulatory processes. Bellafiore et al. (2005) suggested that the kinase is part of the complex redox regulation in chloroplasts, while Bonardi et al. (2005) postulated that STN7 kinase functions as a transcriptional regulator. The function of PSII core protein phosphorylation by STN8 kinase in light acclimation of the photosynthetic machinery has remained controversial (Bonardi et al. 2005).

2. AIM OF AND MOTIVATION FOR THIS STUDY

The aim of my work was to investigate the role of PSII-LHCII protein phosphorylation in regulation of photosynthesis according to environmental cues. Because the phosphorylation of thylakoid proteins follows the energetic balance of the thylakoid membrane, it is highly likely that phosphorylation plays a central role in regulation of solar energy conversion efficiency in photosynthesis. In order to provided food and energy for the future generations, we have to break the records of the green revolution. To reach this goal, we have to understand how photosynthetic efficiency is controlled and which the actual limiting factors of plant productivity are.

Specific aims of my study were

1) To elucidate the role of LHCII protein phosphorylation in regulation of light harvesting and electron transfer upon short- and long-term acclimation;

2) To clarify the role of PSII core protein phosphorylation in regulation of PSII turnover;

3) To clarify the co-operation of STN7 and STN8 kinases in regulation of photosynthesis;

4) In general, to better understand the role of PSII-LHCII protein phosphorylation in the network of regulatory events in response to light absorption, quenching of excitation energy, and electron transfer.

3. METHODOLOGY

3.1. Growth of plants and light treatments

WT *Arabidopsis thaliana* (L.) ecotype Columbia (Col-0) and the *stn7* (Tikkanen et al. 2008), *stn8* (Vainonen et al. 2005), *stn7 stn8* (Bonardi et al. 2005), *npq4 stn7* (Frenkel et al. 2007), *npq4* (Li et al. 2000), *npq 2*-1 (Niyogi et al. 1998), and *pgr5* (Munekage et al. 2002) mutant plants were grown in a phytotron under 120 µmol photons m^{-2} s^{-1}, an 8-h photoperiod, and a relative humidity of 70%. Mature rosette leaves from 4 to 6-week-old plants were used for experiments. OSRAM PowerStar HQIT 400/D metal halide lamps served as a light source, both for plant growth and for high light (HL) and LL treatments. For HL and LL treatments, leaves were placed in a temperature-controlled chamber at 23°C and light was passed through a heat filter.

For the growth of plants under fluctuating light, an electronically controlled shading system was used. Five min of moderate low light (50 µmol photons m^{-2} s^{-1}) and 1 min of high light (500 µmol photons m^{-2} s^{-1}) were continuously following each other during the light period of 12 h followed by 12 h dark period.

Table 1. Knockout mutant lines used in this thesis work

Name	Description of the mutant
stn7	No LHCII protein phosphorylation
stn8	Low PSII core protein phosphorylation
stn7 stn8	No PSII-LHCII protein phosphorylation
npq4	No PsbS protein, no feedback de-excitation (qE)
npq1	No conversion of violaxanthin to zeaxanthin (low qE)
stn7 npq4	No LHCII phosphorylation, no qE
pgr5	No or impaired ferredoxin plastoquinone oxidoreductase- (FQR-) dependent cyclic electron transfer around PSI (CET)

WT plants were exposed to light favoring the excitation of PSII ("state-2" light or red light) and that of PSI ("state-1" light or far-red light) to ensure the maximum amount of phosphorylation and dephosphorylation of thylakoid proteins, respectively. A fluorescent tube (GroLux F58W/GROT8; Sylvania) covered with an orange filter (Lee 105 filter; Lee Filters) served as state-2 light and state-1 light was obtained from halogen lamps (500 W) covered with an orange filter (Lee 105) and a "Median blue" filter (Roscolux #83; Rosco Europe). The temperature was maintained at 23°C by a water-cooled glass chamber between the fluorescence tube and the plants.

3.2. SDS-PAGE, immunoblotting, and phosphoprotein staining

The amounts of protein corresponding to the linear response of each antibody were loaded in the gels. The polypeptides were transferred to an Immobilon-P membrane (Millipore, Bedford, MA), and the membrane was blocked with 5% fatty acid-free bovine serum albumin (Sigma-Aldrich). Western blotting was performed with standard techniques using P-thr antibody (New England Biolabs). Proteins were immunodetected using a Phototope-Star Chemiluminescence Kit (New England Biolabs).

In addition to western blotting, thylakoid proteins and the phosphorylation levels of thylakoid phosphoproteins were quantified from gels by using ProQ Diamond Phosphoprotein Gel Stain and SYPRO® Ruby Protein Gel Stain (Molecular Probes). Stains were used according to the manufacturer's instructions.

3.3. Chlorophyll fluorescence measurements

3.3.1. 77 K chl a fluorescence measurements

Thylakoid membranes and different thylakoid subfractions were diluted in buffer containing 50 mM HEPES/KOH, pH 7.5, 100 mM sorbitol, 10 mM $MgCl_2$, and 10 mM NaF to a chl concentration of 10 µg/mL. Then samples were immediately frozen in liquid nitrogen and 77 K fluorescence emission spectra were recorded with a diode array spectrophotometer (S2000; Ocean Optics, Dunedin, FL) equipped with a reflectance probe. To record the 77 K fluorescence emission curves, the samples were excited with white light below 500 nm, defined by LS500S and LS700S filters (Corion Corp., Holliston, MA). To obtain the 77 K fluorescence excitation spectra, the samples were excited with wavelengths from 400 to 540 nm in 5-nm steps by using an f/3.4 Monochromator (Applied Photophysics, Surrey, U.K.). The emission between 600 and 800 nm was recorded.

3.3.2. Fluorescence measurements at room temperature

PSII excitation pressure (1-qP) was measured with a PAM fluorometer (PAM 101/103; Heinz Walz) as described (Piippo et al. 2006).

PSII efficiency was determined as the ratio between variable fluorescence (Fv) and maximal fluorescence (Fm) measured from intact leaves with a Hansatech Plant Efficiency Analyser (Hansatech, King's Lynn, UK) after an incubation of 30 min in the dark.

Chl fluorescence parameters $F_{m'}$ and $F_{s'}$ were determined in intact leaves exposed to actinic light of 67, 590, 340, and 37 µmol photons m^{-2} s^{-1}, provided by green LEDs, using a JTS-10 spectrometer (Bio-Logic SAS, Claix, France). $F_{m'}$ was induced by a 0.25-

s saturating flash of green light (7,900 μmol·m^{-2}·s^{-1}). The first measuring points of F_m, and F_s, were given a value of 1 and all other values were normalized against this initial value.

3.4. Thylakoid isolation and fractionation

Thylakoid isolation and fractionation was performed according to Danielsson et al. (2004) and Danielsson et al. (2006). All thylakoid fractions were prepared without any detergent to keep the membrane as intact as possible. The membranes were broken mechanically by sonication and then submitted to an aqueous two-phase system to isolate the grana and stroma lamellae. The grana fraction was further purified to isolate the grana core fraction and the grana margin fraction (which does not contain end membranes). The Yeda press treatment of the thylakoid membrane followed by centrifugation steps resulted in the Y-100 fraction, which is considered to be the most purified stroma lamellae fraction (see Figure 6)

3.5. DNA microarray experiments

Arabidopsis cDNA microarray chips are based on the GEM1 clone set (from IncyteGenomics, Palo Alto, CA), which contains about 6,500 unique genes (Piippo et al. 2006). Each clone was spotted three times to an array to provide technical replicates.

4. OVERVIEW OF RESULTS

4.1. LHCII phosphorylation-dependent reorganization of PSII-LHCII complexes in the thylakoid membrane

In order to investigate the relationship between LHCII phosphorylation, lateral migration of LHCII proteins, and relative excitation of the reaction centers, I used five different fractions of thylakoid membrane (Figure 6) isolated both from thylakoids of dark-acclimated plants (D)—where LHCII is dephosphorylated—and from plants after 2 h of low light acclimation (LL) where LHCII is maximally phosphorylated (Paper 1).

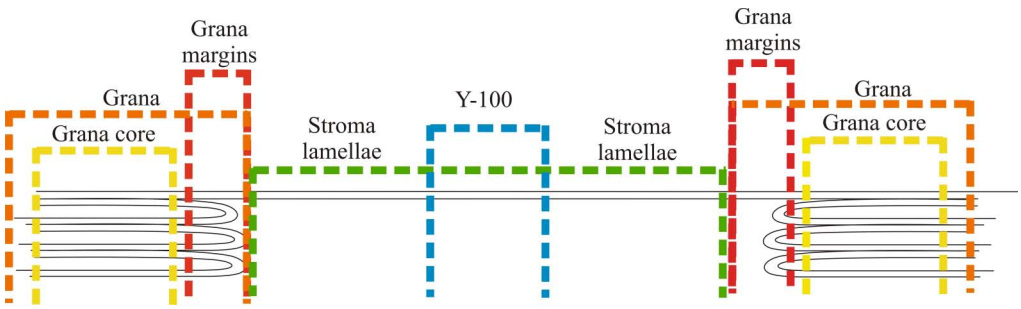

Figure 6. *Illustration of the five fractions isolated from dark- and low light-treated spinach plants. Thylakoid fractionation was performed using noninvasive mechanical membrane fractionation and a two-phase partition method* (Danielsson et al. 2004).

The fundamental question of the study in Paper 1 was to define the spatial origin of the change in relative excitations of PSII and PSI upon phosphorylation of LHCII. Figure 7 A shows that the increase in 77 K F733 nm/F685 nm (PSI/PSII) fluorescence ratio caused by h phosphorylation was localized mainly to the grana margin fraction of the thylakoid membrane. Indeed, this fraction showed the largest (almost 100%) increase in relative 77 K F733 nm/F685 nm fluorescence ratio; the entire grana showed a 20% increase and even in the grana core, 10% increase was recorded. On the other hand, the entire stroma lamellae showed only marginal (6%) increase in F733 nm/F685 nm ratio. In the Y-100 fraction, in fact, the change in the F733 nm/F685 nm ratio was negative, in practice meaning a 9% decrease in PSI excitation in the Y-100 fraction.

In order to define the mechanical basis of such LHCII phosphorylation-induced changes in relative excitation of the reaction centers, I analyzed the protein composition of the thylakoid fractions before and after the induction of LHCII phosphorylation. Migrations of LHCII proteins are known to serve the PSII and PSI reaction centers via state transitions. Thus, the prominent increase in the 77 K F733 nm/F685 nm ratio in the grana margins (Figure 7 A) was expected to result from an increased amount of P-LHCII in this

membrane fraction. However, no clear increase in the amounts of LHCII proteins could be detected in the grana margins (Figure 7 B). In sharp contrast to LHCII proteins, the amounts of the PSI-LHCI complexes and the ATP synthase clearly increased in the grana margins under LL (as compared to the dark). It was interesting to note that the amount of LHCII proteins increased in deep stroma (Y-100) membranes upon phosphorylation of LHCII (Figure 7 B), but no increase in relative excitation of PSI could be detected.

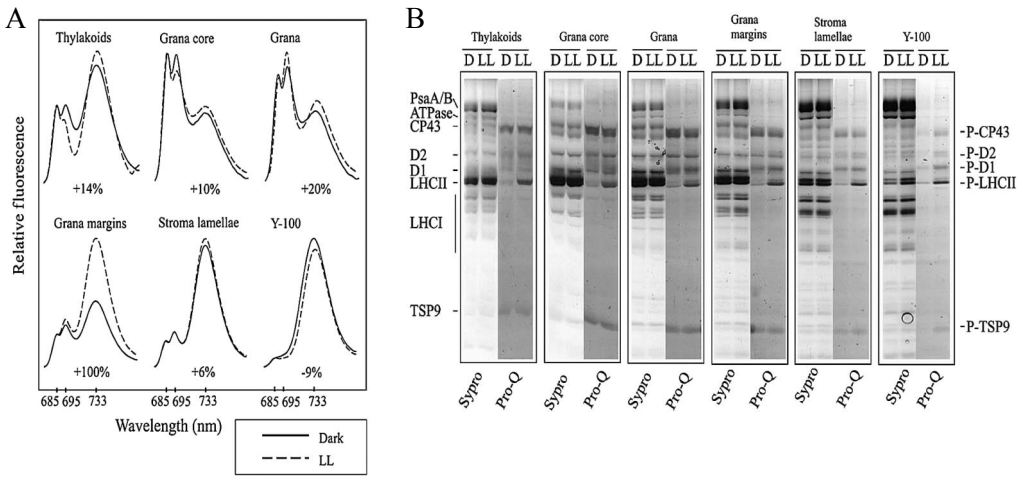

Figure 7. *Relationship between 77 K chl a fluorescence emission spectra and thylakoid protein composition. A. 77 K fluorescence spectra of intact thylakoid membrane and the membrane subfractions isolated from dark-treated and LL-treated spinach leaves. B. Quantitative protein and phosphoprotein staining of thylakoid membrane subfractions with SYPRO® Ruby Protein Gel Stain and Pro-Q® Diamond Phosphoprotein Gel Stain.*

4.2. STN7 and STN8 kinases in phosphorylation of the PSII-LHCII phosphoproteins

The phosphorylation patterns of the PSII core and the LHCII proteins are dynamically regulated by the STN7 and STN8 kinases according to light intensity (Paper 2). In Figure 8 A, WT and *stn* mutant plants were subjected to light treatments that changed in intensity from low to high every 30 min for a total duration of 180 min. Under low light, the level of phosphorylation of PSII core proteins, mainly controlled by the STN8 kinase, was low in relation to the level of LHCII phosphorylation controlled by the STN7 kinase. In contrast to the situation in LL, under HL the STN7 kinase-dependent phosphorylation of LHCII decreased and the STN8 kinase-dominated phosphorylation level of PSII core proteins increased.

Figure 8 B shows that the phosphorylation of PSII-LHCII protein can also be manipulated by using artificial conditions, but in that case the elegant co-regulation by STN7 and STN8 kinases becomes disturbed. Indeed, feeding of glucose to the leaves under darkness

activates both the STN7 and STN8 kinases, inducing a pattern of phosphorylation similar to that induced by the "state-2" light (Figure 8 C).

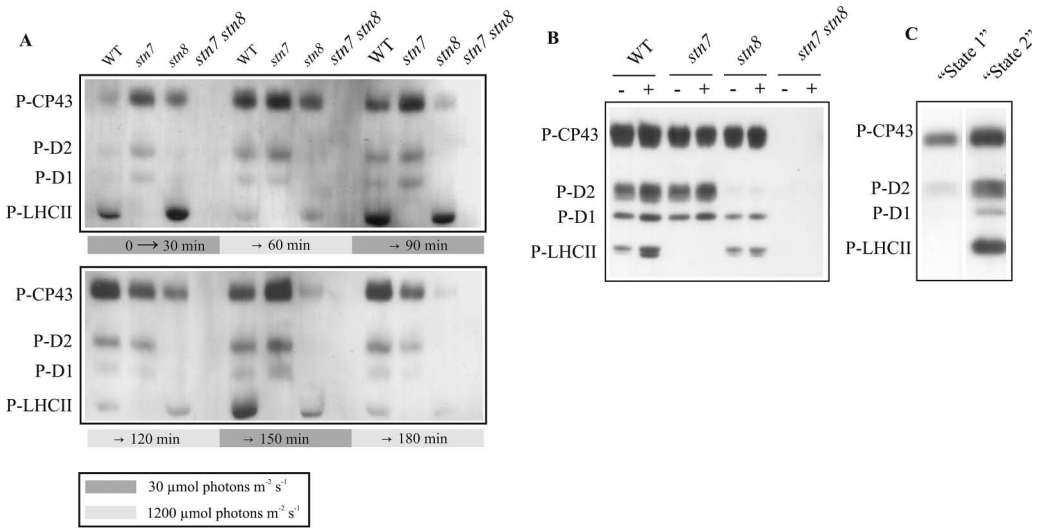

Figure 8. *Regulation of thylakoid protein phosphorylation in Arabidopsis by the STN7 and STN8 kinase pathways. A. Phosphorylation of thylakoid proteins in WT and in the stn7, stn8, and stn7 stn8 mutants exposed to high and low light at 30-min intervals. B. Phosphorylation of thylakoid proteins in WT, stn7, stn8, and stn7 stn8 mutants after 16 h of dark incubation in the absence (-) and presence (+) of external sugar. C. Phosphorylation of thylakoid proteins in WT plants after 1 h of treatment with "state-1" or "state-2" light (Paper 2).*

4.3. PSII-LHCII protein phosphorylation in the regulation of excitation energy distribution between the two photosystems

As described above, the modifications of PSII-LHCII phosphorylation by white light critically differ from those occurring upon the "state-1" to "state-2" transitions induced by artificial light conditions. Accordingly, I addressed the question of whether the white light intensity and "state-1" to "state-2"-induced modifications in the phosphorylation patterns of the PSII-LHCII proteins induce similar short-term changes in the functional properties of the PSI and PSII antenna systems, as reported for traditional state transitions (Paper 2). As expected, "state-2" light, which activates the phosphorylation of both the PSII core and LHCII proteins, strongly increased the relative excitation of PSI as compared to dark or "state-1" light (Figure 9 A). However, despite the dynamic regulation of PSII and LHCII phosphorylation, the effects of different intensities of white light on the relative excitation of PSII and PSI were only minor (Figure 9 B). WT plants kept the relative excitation of CP43, CP47, and PSI nearly unchanged, regardless of illumination with either low or high light, with very different effects on PSII-LHCII phosphorylation. However, the *stn7* mutant and the *stn7 stn8* double mutant showed

reduced relative excitation of PSI in dark-acclimated plants and after illumination with low light as compared to WT, but after exposure to high light, no differences to WT were recorded (Figures 9 C and E), indicating that STN7 kinase is required to reach the excitation balance between PSII and PSI specifically under low light.

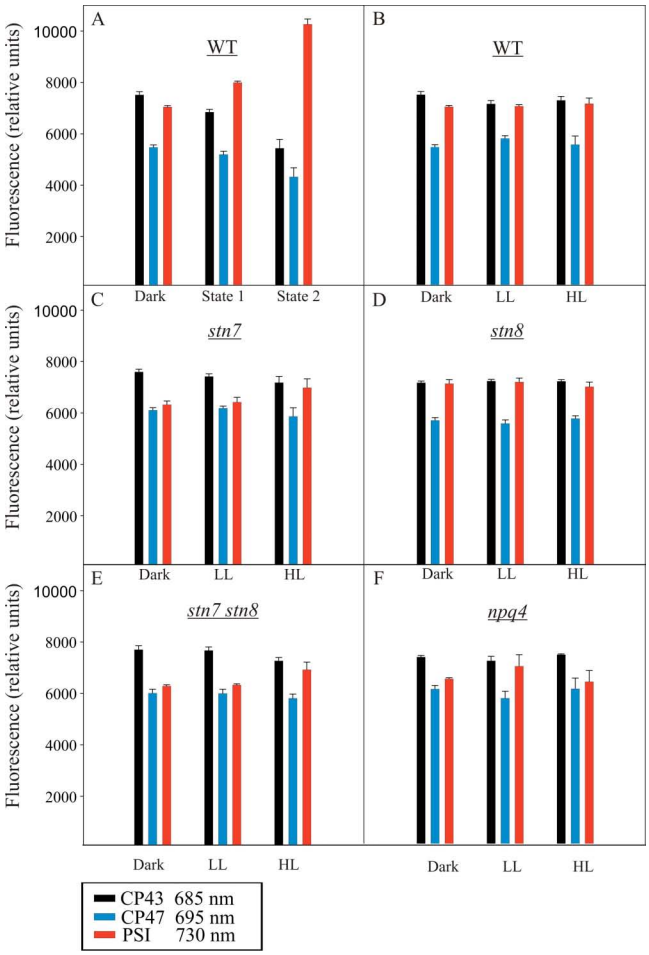

Figure 9. *77 K chl a fluorescence emitted from CP43, CP47, and PSI in WT, stn7, stn8, stn7 stn8, and npq4 plants after 16 h of incubation in the dark and a subsequent 1-h exposure to low and high light. WT plants were also exposed to "state-1" and "state-2" light for 1 h following the 16-h dark incubation.*

4.4. LHCII phosphorylation in regulation of the chlorophyll a/b ratio

It is well known that the chl a/b ratio is dependent on the relative amounts of pigment protein complexes, which change upon long-term light acclimation together with protein synthesis. In paper 3, I discovered that the chl a/b ratio not only responds to long-term light acclimation, but is also adjusted in relatively short time scales, as shown here in Figure 10. One very interesting observation was that such changes in the chl a/b ratio are linked to LHCII protein phosphorylation and they are absent from the *stn7* mutant.

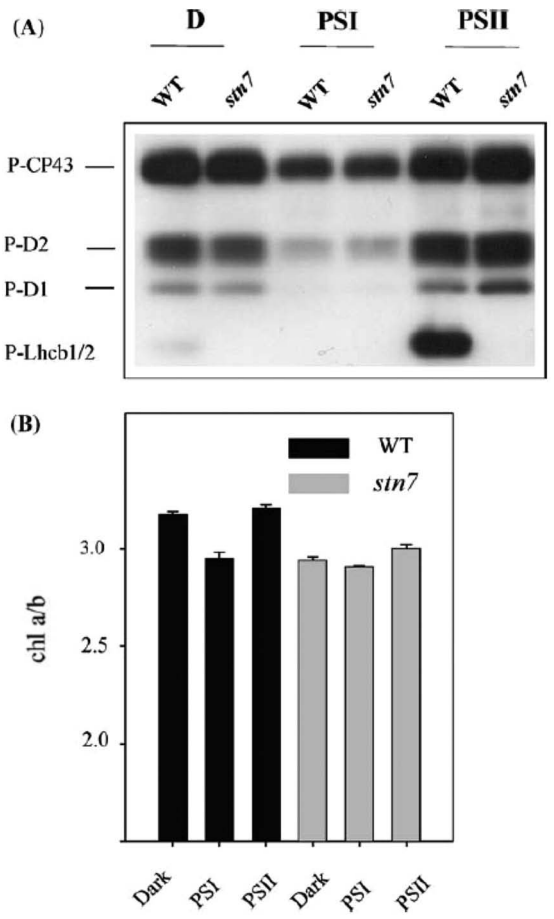

Figure 10. *Relationship between PSII-LHCII protein phosphorylation (A) and the chl a/b ratio (B) after a diurnal dark period (D) and after a subsequent 3-h treatment with "state-1" light (PSI) and "state-2" light (PSII), in WT plants and in stn7 mutants.*

4.5. Effects of the absence of PSII-LHCII phosphorylation on online regulation of photosynthetic electron transfer

In order to clarify the roles of LHCII and PSII core protein phosphorylation in dynamic light acclimation of the photosynthetic electron transfer reactions (Paper 2), I subjected WT and *stn* mutant leaves (Table I) to changing light intensities, which would be expected to require dynamic regulation of light harvesting in order to keep the electron transfer reactions in balance. All three *stn* mutants were capable of efficiently inducing NPQ when the light intensity suddenly increased (Figure 11). However, despite rapid induction of NPQ in all kinase mutants, the *stn7* mutant and the *stn7 stn8* double mutant had severe difficulties in maintaining NPQ after the rapid induction phase. Strong fluctuations between quenching states on changes in light intensity were strongly reflected in F_s, levels, indicating that there were difficulties in maintaining the redox balance under changing light intensities.

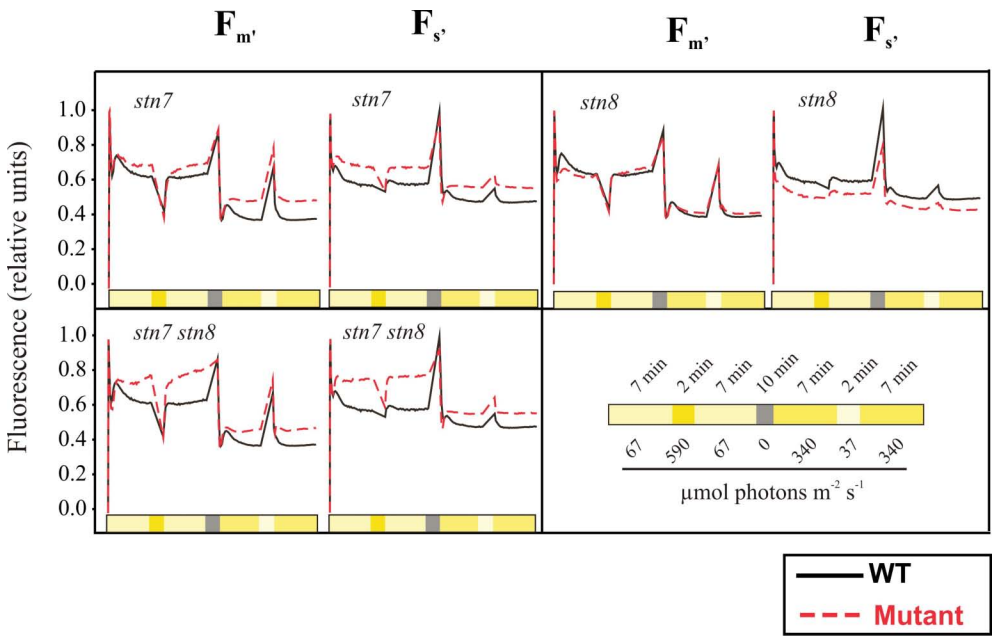

FIGURE 11. *Chlorophyll a fluorescence as a response to changes in light intensity and recorded from leaves of WT, stn7, stn8, and stn7 stn8 plants. $F_{s'}$ and $F_{m'}$ were monitored during illumination of leaves with programmed changes in light intensity as indicated in the figure. (See Paper 2 for details).*

4.6. The stn7 mutant compensates for the lack of LHCII phosphorylation by protein synthesis

As described above, the lack of LHCII phosphorylation leads to changed excitation balance of the two photosystems and to disturbed redox poise of electron transfer reactions, especially under low light intensity. Because of this, I investigated how the *stn7* mutant compensates for the lack of LHCII phosphorylation and changes the stoichiometry between photosynthetic protein complexes under different growth light intensities (Paper 3). The *stn7* kinase mutant showed a difference from WT in its thylakoid protein acclimation response, especially under low light (Figure 12). The a and b subunits of the chloroplast ATP synthase, the NdhI subunit of the chloroplast NDH-1 complex, and the PSI antenna proteins Lhca1 and Lhca2 were strongly upregulated under LL conditions in the mutant as compared to WT thylakoids. Also, the PsbS content of the *stn7* mutant was higher than that of WT, particularly in plants grown under low and moderate light intensities (LL and ML). All these changes in the thylakoid proteome of the *stn7* kinase mutant, as compared to WT, indicate problems in the mechanisms maintaining the excitation, redox, and protonation balance.

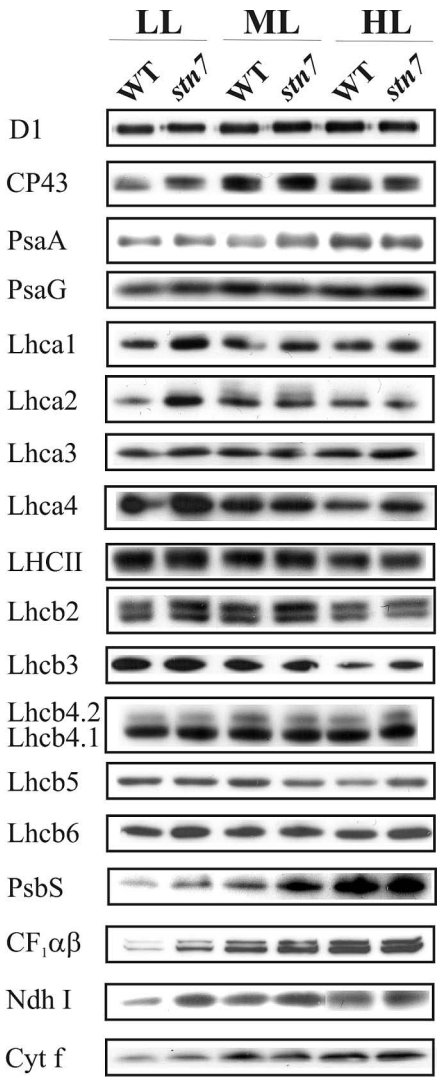

Figure 12. *Analysis of the thylakoid membrane proteins of the wild-type (WT) and the stn7 mutant plants by immunoblots of thylakoid membrane proteins from plants grown under LL (30 µmol photons m^{-2} s^{-1}), ML (100 µmol photons m^{-2} s^{-1}), and HL (500 µmol photons m^{-2} s^{-1}) (See Paper 3).*

4.7. PSII core protein phosphorylation and turnover of the PSII D1 protein

Phosphorylation of the PSII core protein has been tightly connected to the regulation of PSII turnover. It has been postulated that the light-induced phosphorylation of PSII core proteins, followed by controlled dephosphorylation, is a prerequisite for efficient turnover of damaged PSII (Aro et al. 1992; Koivuniemi et al. 1995; Kettunen et al. 1997). Recently, however, this model was challenged (Bonardi et al. 2005). In order to explain the role of PSII-LHCII phosphorylation in regulation of PSII turnover, I subjected WT and *stn* mutant plants to moderate high light stress, which in WT plants induces a maximal turnover rate of D1 protein but does not strongly exceed this

capacity, and so does not lead to any general damage and unspecific degradation of PSII (Paper 4). As shown in Figure 13 A, in the presence of the protein synthesis inhibitor lincomycin, the degradation of the D1 protein was retarded in the *stn8* and *stn7 stn8* mutants as compared to the WT and the *stn7* mutant. In order to find the mechanism behind the delayed degradation of D1 in the *stn8* and *stn7 stn8* mutants, I addressed the dynamics of the monomerization of PSII complex under photoinhibitory illumination. For this purpose, I compared the dynamics of thylakoid protein complexes in the WT and the *stn7 stn8* double mutant during high light illumination. As shown in Figure 13 B, it was clear that the unpacking of damaged PSII supercomplexes is severely delayed because the phosphorylation of PSII core protein is missing from the *stn7 stn8* double mutant.

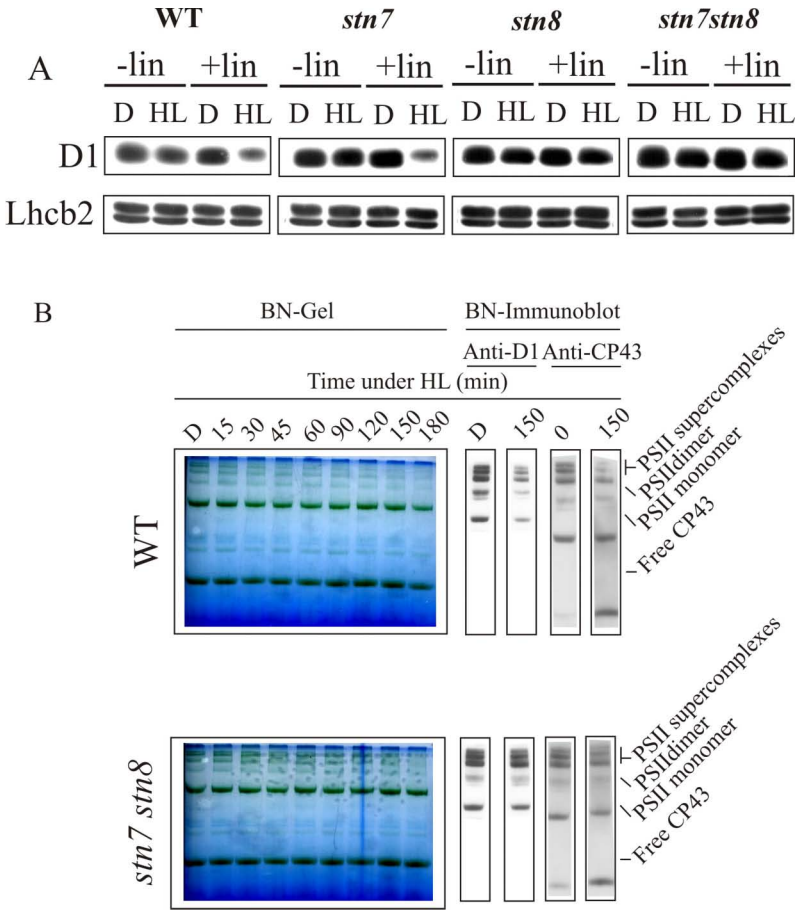

Figure 13. *Capability of the WT and the stn7, stn8, and stn7stn8 mutant plants to degrade damaged PSII upon illumination with HL (600–800 µmol photons m^{-2} s^{-1}). Leaves were exposed to high light in the absence (-Lin) and presence of the protein synthesis inhibitor lincomycin (+Lin). A .Immunoblots of the D1 and Lhcb2 proteins from the WT and the stn mutants after 2 h of HL treatment. B. Unpacking of PSII complexes upon high light stress in the WT and in the stn7 stn8 double mutant. (For details, see Paper 4).*

5. DISCUSSION

5.1. The role of PSII-LHCII phosphorylation in natural environments

The water-splitting photosystem II (PSII) and its light harvesting antenna (LHCII) are the basic units of life. LHCII absorbs sunlight and funnels the energy to PSII, which by splitting water releases oxygen and chemical energy that are the basis of the rest of life on Earth. Moreover, the proteins of the photosynthetic machinery are not only very important, but they are also the most abundant proteins on the planet. Since the late 1970s (Bennett, 1979), it has been known that several subunits of the PSII and LHCII complexes are reversibly phosphorylated. Indeed, in all green eukaryotic cells PSII-LHCII proteins are phosphorylated and dephosphorylated according to the energy balance of the plant or alga (Aro and Ohad, 2003). Despite the fact that the PSII-LHCII supercomplex is probably the most important and most abundant protein complex on Earth, the biological significance of the phosphorylation of PSII-LHCII proteins remains quite elusive.

Balancing of the light harvesting capacity between PSII and PSI through state transitions has long been recognized to be a regulatory mechanism to optimize the photosynthetic capacity (reviews: Allen and Forsberg, 2001; Haldrup et al. 2001; Rochaix, 2007; Kargul and Barber, 2008). Although the phenomenon of state transition has been a matter of extensive research over the past few decades, its mechanistic basis and biological significance under different natural light conditions have remained controversial. Here, I will focus first on the mechanistic basis of LHCII phosphorylation-dependent regulation of photosynthetic light reactions, and then on the role of such a regulatory process in the physiological performance of plants. I will also take a look at the physiological role of phosphorylation of the PSII core protein and try to address the phenomenon of cooperation between the STN7 and STN8 kinase pathways in regulation of photosynthesis.

5.2. Revision of the mechanistic model for LHCII phosphorylation-dependent regulation of the relative excitation of PSII and PSI reaction centers

According to the classical state transition theory (see Figure 7 in Paper 1), the phosphorylated LHCII migrates from PSII in the appressed membranes to serve as a light harvesting antenna for PSI in the stroma-exposed membranes, as a response to a change in light quality or quantity. Conversely, when LHCII becomes dephosphorylated, the excitation of PSII is again favored by the movement of LHCII back to PSII (Larsson et al. 1983; Kyle et al. 1984; Allen and Forsberg, 2001). It is well known that LHCII

phosphorylation is a prerequisite for dynamic regulation of relative PSI/PSII excitation under artifically induced state transition (Depege et al. 2003; Bellafiore et al. 2005; Bonardi et al. 2005) (Paper 3). However, there is no unambiguous evidence to support the movement of LHCII to stroma-exposed membranes as the mechanism behind increased PSI excitation when LHCII is phosphorylated. Moreover, such theories are based on unnatural conditions that cannot be found outside the laboratory. Because of this discrepancy between physiological and experimental conditions, I have preferred to use the conditions that correspond to real biological environments and thus the results can be expected to give more direct information about biological processes. Instead of using classical "state-1" and "state-2" light to manipulate LHCII phosphorylation and to induce state transitions, I used different intensities of white light that efficiently phosphorylate and dephosphorylate LHCII proteins (Figures 7 and 8).

Despite the strong phosphorylation of LHCII proteins in LL, I could measure only a minor transition to "state 2" from the 77 K chl fluorescence emission spectra (Figure 7 A). However, when analyzing the thylakoid subfractions in dephosphorylated state and in phosphorylated state, it became evident that the grana margins behave according to the traditional view of LHCII phosphorylation-induced state transition (Figure 7). It is important to emphasize that no "state-1" to "state-2" transition could be detected in the stroma lamellae, as was previously postulated (Larsson et al. 1983; Kyle et al. 1984; Allen and Forsberg, 2001). In fact, instead a "state-2" to "state-1" transition was detected upon phosphorylation in the stroma lamellae. This challenged the role of movement of P-LHCII to stroma membranes as a sole mechanism for dynamic redistribution of excitation energy between the two reaction centers, despite the fact that LHCII phosphorylation is obligatory for this mechanism to occur (Bellafiore et al. 2005; Depege et al. 2003). Likewise, the assumption that the induction of LHCII phosphorylation changes the balance of excitation energy between PSII and PSI might be misleading. Indeed, it is difficult to find any physiological relevance for such unbalancing of the redox poise upon change in light intensity, which changes the energy supply to the entire photosynthetic machinery but does not, as such, change the need for relative excitation of PSII and PSI. Thus, it is more logical to assume that changes in light intensity cause structural changes in the thylakoid membrane and that this, in turn, affects the energy distribution between the reaction centers. The prerequisite for fluent electron flow, however, is the balance between excitation of PSII and PSI. It seems highly probable that LHCII phosphorylation happens in order to maintain the excitation balance between PSII and PSI when changes occur in the organization of the thylakoid membrane. To date, it is not fully known why the ultrastructure of the thylakoid membrane dynamically changes according to the light intensity (Trissl and Wilhelm, 1993; Mustardy and Garab, 2003). On the other hand, it is well known that *in vitro* the modulations of thylakoid ultrastructure strongly affect the energy distribution between the reaction centers (Murata, 1969; Murata, 1971). In nature,

changes in light intensity dynamically regulate the thylakoid ultrastructure (Rozak et al. 2002), yet the energy distribution remains quite stable (Papers 1 and 2). Hence, it is conceivable that phosphorylation functions in order to maintain the excitation balance after light-induced changes in the thylakoid ultrastructure.

When considering the biological role of PSII-LHCII protein phosphorylation, it is important to differentiate between land plants and the unicellular aquatic microalgae. This is because the photosynthetic machinery functions under totally different environmental conditions in unicellular aquatic algae as compared to land plants with highly specialized cells. Depending on the environmental conditions, microalgae can swing between autotrophic, heterotrophic, aerobic, and anaerobic metabolism (Funes et al. 2007; Melis et al. 2007; Melis, 2007), and furthermore, they have a mechanism for carbon concentration under conditions of low inorganic carbon (Yamano and Fukuzawa, 2009). Due to them having such a high metabolic flexibility within one photosynthetic cell, the microalgae require an efficient mechanism to regulate the relative production of NADPH and ATP by photosynthesis. Through regulation of the relative energy supply between PSII and PSI, it is possible to switch between the water-splitting ATP- and NADPH-producing linear electron (LET) flow and the ATP-producing cyclic (CET) electron flow and chlororespiration (Nixon, 2000). Indeed, the model organism of the unicellular eukaryotic green algae, *Chlamydomonas reinhertii,* flexibly regulates the relative excitation between PSII and PSI (Wollman and Delepelaire, 1984), and through this mechanism, the ratio between LET and CET (Finazzi et al. 2002). Such regulation occurs according to the metabolic state of the cell (Cardol et al. 2003; Forti et al. 2003; Kruse et al. 2005) and depends on the availability of light energy, organic and inorganic carbon, O_2, and also nutrients (Wollman and Delepelaire, 1984; Palmqvist et al. 1990; Wykoff et al. 1998; Antal et al. 2006).

In contrast to the algae, the photosynthesis of land plants takes place in chloroplasts inside leaf cells, which have evolved just for photosynthesis, thus ensuring a highly buffered environment for photosynthesis to occur in. Due to this, shifts between different metabolic strategies are not required in plant cells specialized for photosynthesis—as they are in the cells of unicellular algae which are capable of hosting several different metabolic functions. However, with only minor differences, the phosphorylation of PSII-LHCII proteins and the respective kinases are conserved in the algae and higher plants (Depege et al. 2003; Bellafiore et al. 2005; Bonardi et al. 2005). Even so, the granal stacking is a much more extensive and dynamic process in higher plants than in algae (Doltchinkova and Lambreva, 2002), and probably evolved after the appearance of PSII-LHCII phosphorylation. Indeed, the extensive granal stacking in the chloroplasts of higher plants may have evolved to cooperate with the PSII-LHCII protein phosphorylation-based regulation in order to maximize the linear electron flow and the production of reducing power. Due to the long co-evolution of PSII-LHCII phosphorylation and

thylakoid stacking, today these two mechanisms function together very smoothly in order to maintain the balance between photosynthesis and downstream metabolism.

Figure 9 shows that the relative amount of excitation of PSII and PSI in higher plant chloroplasts remains stable upon changes in light intensity despite the distinct modulations in PSII-LHCII phosphorylation. It is also important to note that the *stn7* and *stn7 stn8* mutants have impaired capacity to establish WT-type excitation balance under low light, but not under high light, which inhibits the phosphorylation of LHCII in intact systems.

I suggest that LHCII phosphorylation in higher plant chloroplasts is required to create attractive forces between PSII-LHCII and PSI-LHCI complexes, thus enabling more efficient migration of PSII-LHCII-PSI-LHCI subcomplexes upon the light-induced changes in thylakoid membrane ultrastructure. Indeed, the phosphorylation of LHCII by STN7 kinase is required to maintain the functional balance between PSII and PSI when the light intensity has been changed (Figures 8 and 9). It is likely that the modulations in granal stacking disturb the excitation balance between the reaction centers, and result in loss of the redox poise. However, due to redox-regulated phosphorylation and subsequent tuning of the electrochemical properties of PSII-LHCII, the photosynthetic protein complexes can dynamically redistribute along the thylakoid membrane and maintain the redox balance between the two reaction centers.

Grana margins form the interface between the grana and stroma membranes; the protein complexes located in this regon are therefore prone to changes in grana-stroma structure. Indeed, I measured a 100% increase in relative PSI excitation in grana margins in the phosphorylated state. In this thylakoid region, PSII-LHCII and PSI-LHCI are widely present (Figure 5 and Figure 6 in Paper 1). These results strongly suggest that in the grana margins, the light-induced modulation of the thylakoid membrane strongly changes the energy distribution between the two reaction centers. Upon phosphorylation of LHCII, however, there are protein migrations in two directions: movement of PSI-LHCI towards the grana, and the movement of P-LHCII-PSII towards the stroma lamellae. Such PSII-LHCII phosphorylation-dependent lateral movements of protein complexes (Figure 7 B) are likely to balance the energy distribution of the thylakoid membrane, stabilizing the general distribution of excitation energy between PSII and PSI (Figure 9).

5.3. The physiological role of thylakoid protein phosphorylation

Traditionally, the physiological role of LHCII protein phosphorylation has been studied by shifting plants or microorganisms from the quality of light that specifically excites PSI to the light quality that specifically excites PSII—the light conditions that concomitantly strongly dephosphorylate and phosphorylate both the PSII core and the LHCII proteins. A fundamental problem with this experimental setup is that such sharp changes in light

quality, and the concomitant strong dephosphorylation and phosphorylation of both the PSII core and the LHCII proteins, do not occur in nature. Indeed, the "state-1" to "state-2" transitions are likely to be a laboratory artifact caused by an unphysiological pattern of phosphorylation of the PSII-LHCII proteins. Although far-red light, the PSI light, is enriched under the canopy, the "state-2" light (blue or red) required for transition to "state 2" (Figure 9) is not known to exist in natural environments.

5.4. PSII-LHCII proteins are phosphorylated through dynamic cooperation of the STN7 and STN8 kinases

PSII-LHCII proteins in the thylakoid membrane are not only phosphorylated according to the changes in light environment, but also according to the metabolic state of the plant or alga (Wollman and Delepelaire, 1984; Hou et al. 2002) (Figure 8). Under low light, the phosphorylation level of the PSII core proteins is low in relation to LHCII phosphorylation. In contrast to the situation in LL, under HL the phosphorylation of LHCII decreases and the phosphorylation level of the PSII core proteins increases. In the *stn7* mutant, which only expresses a functional STN8, LHCII proteins are not phosphorylated but PSII core proteins are phosphorylated more strongly than in the WT, especially under LL. This suggests that under LL, the *stn7* mutant has a more reduced electron transfer chain than the WT.

In the *stn8* mutant, the STN7 kinase phosphorylates LHCII strongly, and also the PSII core proteins relatively well, especially the CP43 protein (Figure 8). The STN7 and STN8 kinases are both activated when light is switched on, and the electron transfer chain between the reaction centers becomes reduced. However, when the light intensity increases and the whole system becomes more reduced, the STN7 kinase gradually loses its activity. In contrast, the activity of the STN8 kinase increases with increasing light intensity.

The reduction state of the thylakoid membrane is not only dependent on the availability of light energy but also on the metabolic state of the chloroplast (Wollman and Delepelaire, 1984; Hou et al. 2002). The metabolic state is determined by the ratio between incoming solar energy and the extent of the metabolic sink that is capable of utilizing the available energy. The redox state of the thylakoid membrane can be manipulated in the absence of light by chemical reducing power being fed to the system. In order to demonstrate the light-independent regulation of the STN kinases, I incubated WT and *stn* mutant leaves in darkness in the presence and absence of glucose and measured the effect of sugar feeding on the activities of the STN kinases. The feeding of glucose to the leaves in darkness activates both STN7 kinase and STN8 kinase. Such metabolic manipulation of the kinase activity in darkness results in a phosphorylation pattern similar to that induced by "state-2" light (Figure 8), which

strongly reduces the electron transfer chain (ETC) but does not induce the feedback downregulation of the STN7 kinase by "over"-reduction of the stroma (Rintamaki et al. 2000). This experiment emphases the fact that thylakoid protein phosphorylation is not only regulated by the intensity and quality of light, but also by the general metabolic status of the chloroplast. Indeed, under natural conditions the phosphorylation status of the thylakoid proteins is set by the prevailing light conditions in relation to the metabolic status of the chloroplast, and evidently the phosphorylation is a key regulatory mechanism to balance the photosynthetic efficiency in responding to the needs and restrictions of downstream metabolism.

5.5. STN7 kinase is a prerequisite for successful acclimation to low but fluctuating light

Just before I started my thesis work, the LHCII kinase was identified from a unicellular alga, *Chlamydomonas reinhardtii* (Stt7) (Depege et al. 2003), and two homologous kinase genes (STN7 and STN8) were shown to exist in the *Arabidopsis* genome. Knockout mutants from such kinases soon became available, providing an efficient tool to study the role of PSII-LHCII phosphorylation in regulation of photosynthesis. Before the availability of any mutants, it was well documented that phosphorylation of PSII-LHCII proteins does not directly influence the primary electron transfer reactions of PSII (Mamedov et al. 2002). Indeed, one of the main targets of my thesis work was to use the *stn7*, *stn8*, and *stn7 stn8* kinase mutants in order to explain the physiological roles of PSII-LHCII protein phosphorylation in online regulation of photosyntesis.

One of the key questions was whether the thylakoid protein phosphorylation is related to acclimation to excess light or to limiting light. One possibility for answering this question was to grow WT and mutant plants under different light intensities and to investigate how WT plants acclimate to such light conditions—and also to compare the differences between WT and mutant acclimation. In order to investigate the role of the STN7 kinase and LHCII phosphorylation in light acclimation, the WT and the *stn7* kinase mutant were grown under three different light intensities (Paper 3).

Because the *stn7* mutant could not enhance the excitation of PSI by a LHCII phosphorylation-dependent mechanism (Figure 4, Paper 3), it seemed clear that it has an impaired capacity to acclimate to low light, and this was reflected in high PSII excitation pressure relative to WT (Table 2, Paper 3). Interestingly, both the Lhca1 and Lhca2 light harvesting proteins of PSI were upregulated under LL growth conditions in *stn7* as compared to WT in order to compensate for the lack of enhancement of PSI excitation by LHCII. This is apparently a compensatory mechanism to increase the relative absorption cross section of PSI, which occurs via phosphorylated Lhcb proteins in WT thylakoids. The compensatory events were not limited only to the

light harvesting apparatus; a drastic upregulation of the CF1 α and β subunits of the ATP synthase and also of the NdhI subunit of the NDH-1 complex were also detected under LL and ML growth conditions in *stn7* (Figure 12). These findings suggest that, through a disturbed excitation balance between PSII and PSI, the lack of STN7 kinase also hampers the redox balance—and the proton gradient thus induces a wide set of compensatory events in the thylakoid membrane.

Despite the clear changes in the thylakoid proteome in the *stn7* mutant as compared to WT, it is clear from our DNA microarray experiments that the STN7 kinase activity as such does not maintain any differential gene expression at the level of transcription (Figure 8A, Paper 3). This suggests that the compensation for the missing phosphorylation occurs gradually upon the growth of the plant and strong permanent changes in gene expression are not required. It is conceivable that via long-term compensation mechanisms, the *stn7* mutant can maintain the cellular homeostasis in an almost similar manner to that in WT under stable and moderate light intensity. However, when the plants were shifted from stable growth light to excess light, the *stn7* mutant showed much stronger induction of stress-related genes as compared to WT (Figure 8 B, Paper 3). Indeed upon shift from growth light to excess light, the WT plants inhibit the STN7 kinase (Figure 1, Paper 2) and via this mechanism they can probably reduce the PSI excitation by LHCII. As shown in Figure 12, the *stn7* mutant compensates for the lack of P-LHCII excitation of PSI by increasing the amount of LHCI antenna. Indeed, the upregulation of LHCI can compensate for the lack of LHCII phosphorylation only under stable growth conditions, where the LHCII phosphorylation remains constant in WT. However, when transient downregulation of PSI light harvesting by dephosphorylation of LHCII would have been required, the *stn7* kinase mutant suffered from more severe oxidative stress than the WT.

5.6. LHCII phosphorylation-dependent regulation of the chlorophyll pool

It is well known that the long-term process of acclimation to varying light intensities strongly relies on differential synthesis of the light harvesting protein complexes, which is reflected in the chl a/b ratio. However, plants appear to regulate a certain part of the chl pool faster than changes can occur in the protein pool (Figure 10). One very interesting finding was that the *stn7* mutant is incapable of adjusting the chl a/b ratio upon short-term light acclimation (Figure 10). It seems that a certain pool of chl changes its form between chl a and chl b according the phosphorylation of LHCII, or due to LHCII phosphorylation-induced changes in the redox state of the photosynthetic machinery. The mechanism and biological role of such protein synthesis-independent and LHCII phosphorylation-related rapid regulation of the chl a/b ratio remains very elusive and requires further investigation.

5.7. A question of balance: distribution of tasks between NPQ, cyclic electron flow and PSII-LHCII phosphorylation

As described above, the phosphorylation of LHCII proteins is apparently required to maintain the excitation balance of the thylakoid membrane upon changes in light intensity. The next question concerned the possible role of the PSII core protein phosphorylation in such regulation of light harvesting, and the nature of the functional relationship between the PSII-LHCII phosphorylation, NPQ, and cyclic electron flow around PSI. In order to answer these questions, I employed knockout mutants of such reactions (Table 1). WT and mutant plants were subjected to fluctuating light and the dynamic acclimation process was followed by monitoring chl fluorescence. The $F_{m'}$ parameter was used to follow the yield of thermal dissipation of excitation energy, and $F_{s'}$ was measured in order to estimate the ability of plants to maintain fluent electron flow from PSII to PSI and to the downsteam electron acceptors when there are changes in light intensity.

First of all, it was clear that the PsbS protein-dependent NPQ is the main element in the dynamic regulation of PSII activity (Niyogi, 2000; Horton and Ruban, 2005; Kiss et al. 2007; Mozzo et al. 2008; Li et al. 2009). Indeed, the *npq4* mutant could not regulate the $F_{m'}$ and had high $F_{s'}$ under high light, indicating a strongly reduced electron transfer chain (Figure 4, Paper 2). This raises the question of the nature of the role left for the phosphorylation-dependent regulation. I demonstrated that, depending on the phosphorylation status of the PSII-LHCII proteins, the WT plants adjust NPQ at different levels (Figure 11). Under natural fluctuating light environments, the efficiency of light reactions requires non-stop regulation in order to keep the efficiency of light reactions in balance with the needs and restrictions of downstream metabolism. PsbS protein-dependent NPQ provides the means to regulate the primary reaction of photosynthesis. However, in order to set the thermal loss of excitation energy to respond to the needs of the prevailing light intensity, the NPQ machinery requires constant control according to the requirements of the downstream metabolism. Indeed, the balance between incoming light energy and metabolic consumption of the energy defines the redox state of the electron transfer chain and the stroma—both of which are known to control the phosphorylation of PSII-LHCII (Rintamaki et al. 2000). Phosphorylation, in turn, can change the functional stoichiometry between the photosynthetic complexes, which determines the relationship between the incoming energy and luminal protonation, which in turn regulates NPQ. Indeed, regulation of electron transfer, NPQ, and PSII-LHCII phosphorylation are functionally coupled, and change in one factor is most probably reflected to the function of other mechanisms.

It is clear that in the *stn7* mutant, where there is strong phosphorylation of the PSII core but no phosphorylation of LHCII, there is a strongly reduced electron transfer chain (Bellafiore et al. 2005; Tikkanen et al. 2006) and the WT level of NPQ cannot be

maintained under constant light intensity (Figure 11). Indeed, such a high light-mimicking phosphorylation pattern of PSII-LHCII in *stn7* seems to lead to a situation whereby the excitation of PSII is favored over that of PSI, which is analogous to the high PSII-to-PSI ratio present in high light acclimated plants (Vasilikiotis and Melis, 1994; Bailey et al. 2001;). In contrast to the *stn7* mutant, the *stn8* mutant strongly phosphorylates LHCII and weakly phosphorylates the PSII core proteins (Figure 8), and it has a slightly less reduced electron transfer chain ($F_{s'}$) compared to WT (Figure 11). Despite the opposing behavior of the single mutants, the *stn7 stn8* double mutant behaved like the *stn7* mutant, but the inability to maintain redox balance was even more obvious, stressing the importance of both kinase pathways in the regulation of electron transfer reactions.

It was interesting to note that similar to the *stn7* mutant and the *stn7 stn8* double mutant, the *pgr5* mutant (Munekage et al. 2002; DalCorso et al. 2008) with impaired cyclic electron flow could not maintain the WT level of NPQ. However, besides having low NPQ, the *pgr5* mutant also had very low $F_{s'}$, indicating a low reduction state of the electron transfer chain. In contrast to this, *stn7* and *stn7 stn8* had very high $F_{s'}$, indicating a strongly reduced electron transfer chain. These data provide evidence that contrary to the *stt7* mutant of *Chlamydomonas* (Finazzi et al. 2002), the *stn* kinase mutants of higher plants are not impaired in cyclic electron flow, and therefore phosphorylation-dependent modulation of NPQ must occur by the regulation of LET or by some other mechanism, which is discussed in Paper 2. It was also interesting to note that under limiting light, the *pgr5* mutant keeps the electron transfer chain (ETC) more oxidized than does the WT, but under high light the ETC becomes over-reduced compared to WT. This indicates that FQR-dependent CET is important under limiting light to facilitate ETC reduction, but under excess light it promotes downregulation of PSII by NPQ through participation in luminal protonation.

5.8. Phosphorylation of PSII core proteins facilitates the unpacking of damaged PSII complex and degradation of the D1 protein

It is not only LHCII but also the very important PSII core proteins D1, D2, and CP43 that undergo a strong and dynamic redox-regulated phosphorylation cycle (Aro and Ohad, 2003; Vener, 2007; Rochaix, 2007). As described above, LHCII is maximally phosphorylated under limiting light and it is needed to balance the excitation of PSII and PSI under such conditions. Moreover, the inhibition of LHCII phosphorylation appears to be required for acclimation upon transfer of plants from growth light to excess light. In contrast to LHCII phosphorylation, the PSII core proteins are maximally phosphorylated under excess light. This behavior of the PSII core protein pathway suggests that the PSII core protein phosphorylation is required for successful acclimation to high light. However, from experiments evaluating the capacity for dynamic light regulation, I could

not find any compromise in high light acclimation of the *stn8* or *stn7 stn8* mutants. Because of this, it is likely that PSII core protein phosphorylation and the STN8 kinase have more important roles on prolonged exposure to excess light.

Indeed, the light-induced phosphorylation of the PSII core proteins, especially that of the D1 protein, has been connected to the regulation of PSII protein turnover upon photodamage, and the repair of the damaged proteins (Aro et al. 1992). The D1 protein is always prone to photodamage when the photosynthetic light reactions are running. Rapid dynamic degradation of the damaged D1 protein and *de novo* synthesis and insertion of the new D1 protein into PSII are prerequisites for survival of the water-splitting photosynthetic organisms in a wide range of light intensities (for a review, see Aro et al. (2005)).

Initially, it was hypothesized that the damaged D1 is marked by phosphorylation, which then functions as a signal for migration of the damaged PSII from grana to stroma lamellae, where D1 is degraded and the newly synthesized D1 is inserted into PSII (Aro et al. 1993). Due to lack of kinase mutants at that time, such theories were based on indirect experimental evidence. When the kinase mutants became available, direct evidence was produced for the role of thylakoid protein phosphorylation in regulation of PSII turnover. Based on the *stn8* and *stn7 stn8* mutants, it was shown that PSII core protein phosphorylation does not significantly affect the photoinhibition of PSII or turnover of the D1 protein (Bonardi et al. 2005), challenging the earlier results assigning the function of PSII core protein phosphorylation to regulation of PSII turnover.

Due to the discrepancy between the old model and the new *stn8* mutant-based results concerning the role of phosphorylation in regulation of PSII turnover, I attempted to investigate the effect of PSII-LHCII protein phosphorylation on D1 protein degradation by using the *stn7, stn8,* and *stn7 stn8* mutants (Paper 4). The idea behind my work was that in the study by Bonardi et al. (2005), the plants were probably exposed to light that was too high (2,000 μmol photons m^{-2} s^{-1}), strongly exceeding the capacity of D1 turnover—and due to this, an uncontrolled degradation of PSII took place, masking the effect of PSII core protein phosphorylation on the control of D1 turnover. Indeed, in order to prevent uncontrolled damage, I exposed the plants to the high light treatments at about two times lower light intensity (Paper 4) as compared to the original study. I demonstrated that during the course of illumination, the degradation of the damaged D1 protein is retarded in the *stn8* mutant and in the *stn7 stn8* double mutant as compared to the WT or the *stn7* mutant (Figure 13 A). Further investigation revealed that the lack of PSII core protein phosphorylation disturbs the disassembly of the damaged PSII supercomplexes (Figure 13 B). It was also interesting to note that under prolonged high light stress, the *stn7 stn8* mutant accumulated more damaged PSII proteins and generated more oxidative stress than the WT (Figure 6, Paper 4). The mechanism facilitating the repair of PSII in the

WT as compared to the *stn7 stn8* mutant was found to reside in reversible dynamics of PSII oligomerization, enhanced by PSII core protein phosphorylation, thus allowing fluent migration of photodamaged PSII to stroma membranes for repair and detachment of the damaged D1 from the core complex (for a model, see Figure 7 in Paper 4). It is thus absolutely clear that PSII core protein phosphorylation has a role in regulation of PSII turnover, although the role is less than was expected. Based on a congress abstract (Nath et al. 2007) and a personal communication (Choon-Hwan Lee and Krishna Nath), it is worth mentioning that the *stn8* mutation in rice causes very severe problems in the turnover of damaged PSII and increases the susceptibility of PSII to high light stress even more strongly than in *Arabidopsis*.

5.9. The importance of PSII-LHCII protein phosphorylation-dependent regulation is highlighted under moderate light intensities

Under excess light, LHCII proteins become dephosphorylated (Figure 8 A) and the phosphorylation is not required any more to control the excitation balance between the two photosystems (Figure 9). This may be due to the fact that under excess light, LHCII dissociates from PSII and efficiently dissipate all the absorbed energy as heat (Kiss et al. 2008; Betterle et al. 2009;) and no energy to be distributed via phosphorylation-dependent mechanism any more exists. When comparing the results in Paper 4 with the results of Bonardi et al. (2005), it seems evident that even the PSII core protein phosphorylation loses its importance in regulation of efficient PSII D1 protein turnover under very strong light. Under such conditions, the granal stacking relaxes (Rozak et al. 2002), possibly due to a release of oxygen-evolving proteins from damaged PSII (Baena-Gonzalez and Aro, 2002). These proteins are required for efficient granal stacking (Yi et al. 2009). Due to the dissociation of LHCII from PSII and the relaxation of granal stacking, the highly organized structure of the thylakoid membrane becomes more randomized. It seems that the PSII-LHCII phosphorylation has the highest importance under low, moderate, and moderate high light intensities when the thylakoid ultrastucture is highly organized, energy flow from LHCII to PSII is efficient, and a relatively low proportion of the PSII centers are photodamaged.

6. SUMMARY

It is well known that LHCII phosphorylation changes the relative excitations of PSII and PSI in a process called state transitions. Phosphorylation of the LHCII and PSII proteins is dynamically regulated according to the light intensity. However, the underlying reason for such phosphorylation-dependent regulation under white light has remained elusive. I have shown that white light-induced changes in protein phosphorylation are strong, but that in intact leaves they have only a minor effect on the relative excitation of PSII and PSI. However, the lack of LHCII phosphorylation strongly affects the redox balance and the regulation of thermal dissipation of excitation energy, which is in turn dependent on proton concentration in the thylakoid lumen. Based on the data presented here, it is evident that the phosphorylation of LHCII proteins functions in order to maintain the functional stoichiometry between the PSII, PSI, and ATP synthase, through adjustment of the excitation balance between the reaction centers. PSII core protein phosphorylation possibly participates in the same regulatory system, but to a much lesser extent. Even so, PSII core protein phosphorylation plays a crucial role in plant acclimation to prolonged high light stress. It is required for fluent unpacking of PSII supercomplexes and dimers under high light stress, thereby facilitating the turnover of the D1 protein. Based on my thesis work, it appears that the dynamic and reversible phosphorylation of PSII-LHCII proteins functions in assisting the movements of PSII-LHCII-PSI-LHCI complexes among each other along the thylakoid membrane. This is a prerequisite for balanced function of PSII and PSI under ever-changing light intensity, and it is also required for fluent trafficking of the damaged PSII complexes into contact with the repair machinery in stroma thylakoids.

A schematic illustration of various roles of PSII-LHCII protein phosphorylation in higher plant chloroplasts under different light intensities is given in Figure 14. LHCII protein phosphorylation-based regulation of excitation energy distribution to PSII and PSI is of greatest importance under moderately low light intensities (A1). Under such conditions, thermal dissipation of excitation energy in LHCII is low and the deprotonated PsbS protein allows efficient energy transfer from LHCII to PSII (B, C1, and C2). Due to strict lateral heterogeneity of the thylakoid membrane (A, C1, and C2), the energy flow from PSII-LHCII to PSI through spillover is efficiently prevented (C2). In order to maintain sufficient excitation of PSI under such a “high PSII excitation, high lateral heterogeneity” state, an enhancement of PSI excitation occurs via LHCII protein phosphorylation-dependent rearrangement of PSII-LHCII-PSI-LHCI protein complexes (C3).

Moderate increase in light intensity still enhances the lateral heterogeneity, further reducing the energy spillover from PSII-LHCII to PSI (A2). However, concomitant protonation of the PsbS protein detaches the LHCII from PSII (C4 and C5), preventing the excitation energy flow from LHCII to PSII. Also, the light harvesting machinery switches to a quenched state, favoring thermal dissipation of absorbed light energy over the light harvesting, which reduces the efficiency of PSII light harvesting (C 5). All this occurs together with dephosphorylation of LHCII and phosphorylation of the PSII core proteins (C 6), the former preventing energy flow from LHCII to PSI and the latter helping to keep LHCII apart from PSII and facilitating the movement of damaged PSII from grana to stroma membrane for repair.

Under strong light (A3), the capacities of both the structural regulation of light absorption and the molecular regulation of excitation energy distribution are overrun (B). In this excess light state, the lateral heterogeneity of the thylakoid membrane is low, the light harvesting system is in maximally quenched state, and the organization of PSII-LHCII-PSI-LHCII complexes becomes more random. Due to the strong quenching and the randomization process of the thylakoid membrane protein system, the PSII-LHCII phosphorylation-dependent regulation and the PsbS protein-dependent regulation are no longer required to control the excitation energy transfer from LHCII to the two photosystems, as is the case in the highly organized state of the thylakoid system under lower light intensities. Now, also the damaged PSII centers have easier access to contact with the enzymes of the repair machinery, and for this reason the PSII core protein phosphorylation becomes of less importance in facilitation of PSII turnover.

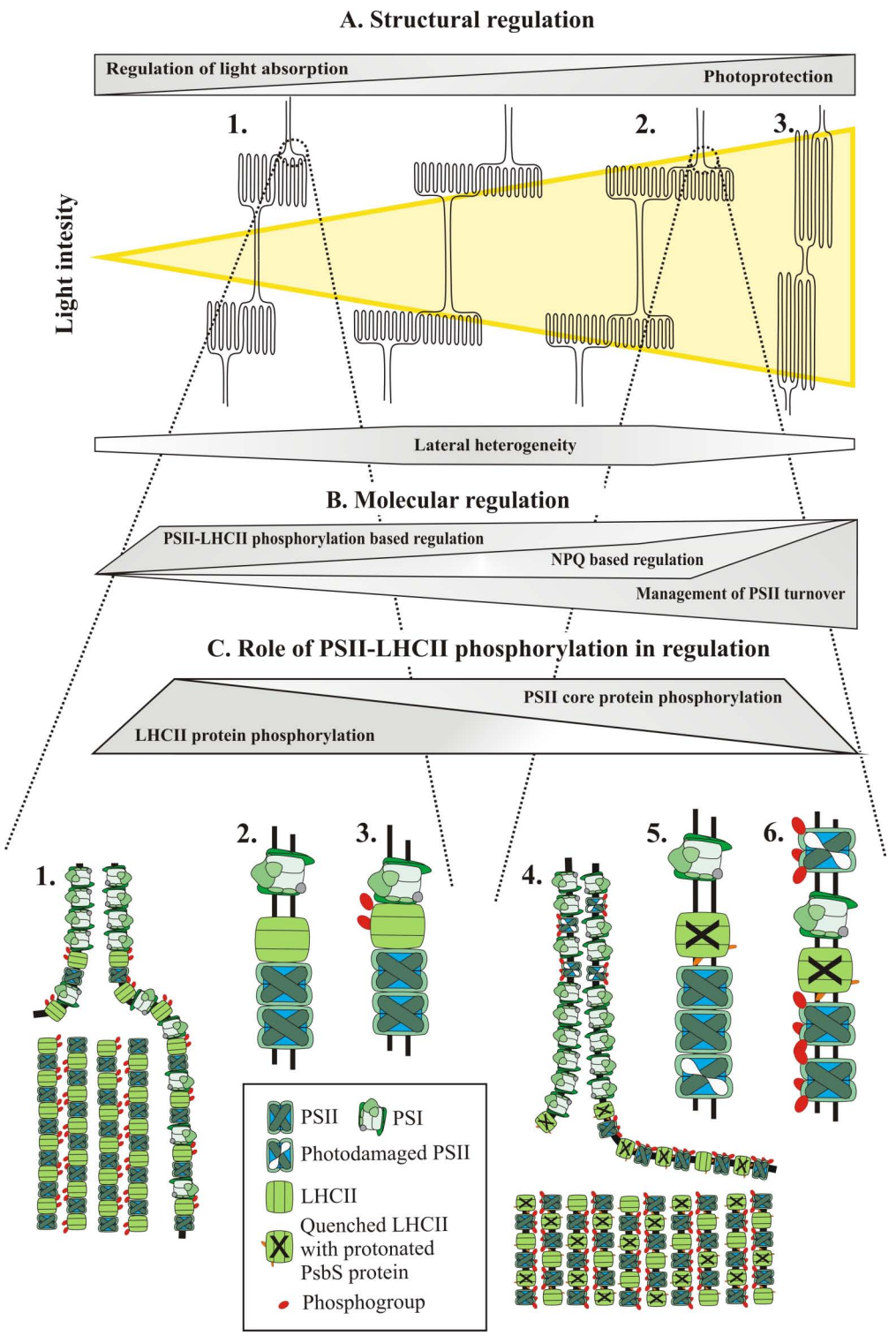

Figure 14. *Schematic model proposing the roles for PSII-LHCII protein phosphorylation in the regulatory network of photosynthetic light reactions. See the text for detailed information.*

7. FUTURE PERSPECTIVES

Despite the great progress that has been made during the past few years in our understanding of the role of thylakoid protein phosphorylation in regulation of the photosynthetic process, it is evident that the story of thylakoid protein phosphorylation as a part of the entire story of regulation of the primary energy metabolism is still in its infancy. We urgently need a better understanding of the relationship between PSII-LHCII protein phosphorylation and the ultrastructure of thylakoid membrane. We also have to know how the phosphorylation-based regulation is connected with other regulatory events of light reactions, and how this regulation communicates with downstream events such as carbon fixation reactions, starch synthesis, control of gas exchange, and hormonal regulation of growth and development. Such studies will pave the way toward the ultimate goal of plant science, producing plants that enable us to use solar energy in a sustainable way with maximal efficiency and with the minimum of negative effects on the environment.

8. ACKNOWLEDGEMENTS

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