Molecular-genetic characterization of thylakoid protein phosphorylation in *Arabidopsis thaliana*

Dissertation

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TABLE OF CONTENTS

“You can never write the word end.
As soon as you stop it is time to start again.”
(Pablo Ricasso)

Ai miei genitori

ABBREVIATIONS

ATP	adenosine triphosphate
°C	degree Celsius
BN	blue native
cDNA	complementary deoxyribonucleic acid
Chl	chlorophyll
Ci	Curie
Cyt b_6f	cytochrome b_6f
D	dark
Da	Dalton
DM	dodecylmaltoside
DNA	deoxyribonucleic acid
EST	expressed sequence tag
F	fluorescence
g	gram
h	hour
HL	high-light
HPLC	high performance liquid chromatography
l	liter
LB	left border
LHC	light-harvesting complex
LL	low-light
Lu	luthein
m	meter
M	molarity
min	minute
mol	mole
NPQ	non-photochemical quenching
Nx	neoaxanthin
PAGE	polyacrilamide gel electrophoresis
PAM	pulse amplitude modulation
PCR	polymerase chain reaction
PFD	photons flux density
PQ	plastoquinone

PS	photosystem
qN	non-photochemical quenching
qP	photochemical quenching
qT	state transition
RB	right border
RFP	red fluorescent protein
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcriptase-polymerase chain reaction
s	second
SD	standard deviation
SDS	sodium dodecyl sulphate
t	time
TCA	trichloroaceticacid
T-DNA	transfer-DNA
TGT	TARGET VALUE
v/v	volume per volume
VAZ	xanthophyll cycle pigments
w/v	weight per volume
WT	wild-type
β-Car	β-carotene
Φ_{II}	effective quantum yield of photosystem II

SUMMARY

Plants respond to changes in illumination conditions by modifying the thylakoid proteins post-translationally and by reorganizing the photosynthetic machinery. However, the mechanisms that characterize the short-term and the long-term responses are different. In the first case, the organism reacts to rapid illumination changes *via* phosphorylation of photosystem II (PSII) and light-harvesting (LHCII) proteins. Phosphorylation of PSII is thought to be relevant for PSII turnover, whereas LHCII phosphorylation is required for state transitions, which ensure the redistribution of the excitation energy between the two photosystems. Long-term imbalances in the energy distribution elicit changes in the composition and stoichiometry of the photosynthetic apparatus (photosynthetic acclimation). Two types of thylakoid protein kinases have been previously associated with LHCII phosphorylation, the TAK (thylakoid-associated kinase) proteins in *Arabidopsis thaliana* and Stt7 in *Chlamydomonas reinhardtii*. This work shows that the TAK proteins (TAK1, TAK2, and TAK3) are neither involved in LHCII phosphorylation nor in state transitions. In addition, evidences are provided that exclude any role of TAK2 and TAK3 in the photosynthetic electron flow. In *Arabidopsis*, two Stt7-like proteins exist, STN7 and STN8. Loss of STN7 blocks both LHCII phosphorylation and state transitions, indicating that this protein is a genuine Stt7 homolog. In contrast, STN8 is required for the quantitative phosphorylation of PSII core proteins. PSII activity under high-intensity light is affected only slightly in *stn8* mutants, and D1 turnover is indistinguishable from the wild-type (WT), implying that reversible protein phosphorylation is not essential for PSII repair. Functional characterization of *stn7* mutants showed that STN7 is not only associated with the short term response, but it is also required for the adaptation to long-term illumination changes including light-quality-induced changes in the mRNA expression of nuclear and plastid

genes for photosynthetic proteins. This indicates that short-term and long-term photosynthetic adaptations are coupled and that phosphorylation of LHCII, or of an unknown substrate of STN7, is crucial for the control of photosynthetic gene expression and readjustment of photosystem stoichiometry.

ZUSAMMENFASSUNG

Pflanzen reagieren auf veränderte Lichtbedingungen durch posttranslationale Modifikation von Thylakoidproteinen und durch Umbau des Photosyntheseapparates. Verschiedene Mechanismen liegen solchen kurz- oder langfristigen Adaptationen zugrunde. Kurzfristige Mechanismen sind bei raschen Änderungen der Lichtbedingungen aktiv und beinhalten die Phosphorylierung von Photosystem II (PSII) und Lichtsammel-Komplex II (LHCII) Proteinen. Dabei wird angenommen, dass die Phosphorylierung von PSII eine wichtige Rolle bei dem *Turnover* von PSII spielt, während die LHCII Phosphorylierung für die Umverteilung der Anregungsenergie zwischen den beiden Photosystemen (*State Transitions*) notwendig ist. Bei längerfristigen Änderungen der Lichtbedingungen kommt es zu Veränderungen in der Zusammensetzung und Stöchiometrie der Photosysteme (photosynthetische Akklimation). Zwei unterschiedliche Arten von Thylakoidprotein-Kinasen wurden in der Vergangenheit mit der LHCII Phosphorylierung in Verbindung gebracht: die TAK (thylakoid-associated kinase) Proteine bei *Arabidopsis thaliana* und Stt7 bei *Chlamydomonas reinhardtii*. In der vorliegenden Arbeit wurde gezeigt, dass die TAK Proteine (TAK1, TAK2, and TAK3) für die Phosphorylierung von LHCII Proteinen und somit für *State Transitions* nicht notwendig sind. Darüber hinaus konnte für TAK2 and TAK3 generell ein Einfluss auf den photosynthetischen Elektronenfluss ausgeschlossen werden. Bei *Arabidopsis* existieren zwei Stt7-ähnliche Proteine, STN7 und STN8. Die Inaktivierung von STN7 bewirkt, dass sowohl LHCII Phosphorylierung als auch *State Transitions* unterdrückt sind. Dieses zeigt, dass STN7 ein funktionelles Homolog des Stt7 Proteins darstellt. Das STN8 Protein ist notwendig für die quantitative Phosphorylierung von den PSII Kernproteinen. Die PSII Aktivität im Hochlichtstress ist in der *stn8* Mutante jedoch nur marginal beeinträchtigt

und der *Turnover* des D1 Proteins ist wie bei Wildtyp-Pflanzen. Daraus folgt, dass die reversible Phosphorylierung von Proteinen für die Reparatur von PSII nicht essentiell ist. Die funktionelle Charakterisierung von *stn7* Mutanten zeigte, dass STN7 nicht nur für die kurzfristige Anpassung, sondern auch für die Adaption des photosynthetischen Organismus an langfristige Veränderungen in der Lichtqualität -einschließlich Veränderungen in der Transkription von photosynthetischen Genen- verantwortlich ist. Somit ist die Phosphorylierung von LHCII oder eines unbekannten Substrates durch STN7 wichtig für die Kontrolle der photosynthetischen Genexpression und die Voraussetzung für die Anpassung der Photosystemstöcihiometrie an sich ändernde Lichtbedingungen.

1. INTRODUCTION

In oxygen-evolving photosynthesis, the conversion from light energy into chemical energy relies on the presence of two photosystems working in series: photosystem I (PSI) and photosystem II (PSII; Allen and Forsberg, 2001). These major components of the photosynthetic machinery are integrated into the thylakoid membranes of a highly specialized minifactory, the chloroplast.

PSI and PSII are connected to their antennae and wired electrically; thereby the rate of the electrons flowing through one photosystem must be equal to the one of those flowing through the other (Hill and Bendall, 1960; Duysens and Amesz, 1962). However, the antennae, which function as light-harvesting complexes (LHC), have a different pigment composition and, therefore, different light absorption properties. In fact, in chlorophyll *b* containing plants and algae, chlorophyll *a* is found in both photosystems, while chlorophyll *b* is mostly confined to PSII (Anderson et al., 1973). Thereby, in all green plants and in most eukaryotic algae, PS I uses preferentially far-red light (~700 nm), whereas PSII absorbs in the red region (~650 nm; Wollman, 2001).

In natural environments, both the intensity and the quality of light fluctuate with time, thus resulting in unbalanced excitation of the two photosystems and an overall decrease in the photosynthetic efficiency. Photosynthetic organisms have evolved various mechanisms to respond to negative environmental variation and to avoid the potentially damaging effects of light. This involves, in the short term, phosphorylation of PSII and LHCII proteins. The first is thought to be required for an efficient repair of PSII, following high-light damages and involving a rapid degradation of the D1 protein and its replacement with a newly synthesized one (Baena-González et al., 1999), whereas the second is associated with the relocation of LHCII and the redistribution of excitation energy (state transitions) between the two photosys-

tems (Wollman, 2001; Allen and Forsberg, 2001). Additionaly, imbalances in energy distribution between photosystems are counteracted, in the long term, by adjusting photosystem stoichiometry (Fujita, 1997; Melis, 1991).

1.1 State Transitions

Oxygenic photosynthetic organisms are able to respond rapidly to changes in light quality and quantity by altering the relative sizes of their PSI and PSII antennae, a process known as state transitions (Allen, 1992). State transitions were discovered almost 30 years ago in unicellular photosynthetic organisms (Bonaventura and Myers, 1969; Murata, 1969), and described as “a control mechanism of excitation transfer between the two photosystems” (Murata, 1970).

This adaptation mechanism requires the lateral migration of the mobile pool of LHCII, from PSII to PSI and *vice versa*, so that the two photosystems are excited at the same extent, thereby increasing the photosynthetic yield. During transition from state I to state II, which is induced by either preferential excitation of PSII or by low-light exposure, the mobile fraction of LHCII shifts from PSII-enriched grana regions to the PSI-enriched stroma lamellae. Conversely, under light favoring PSI or under high-light irradiances, the reverse migration of LHCII from PSI to PSII occurs (Figure 1.1). The kinetics of state transitions correlates with the phosphorylation status of LHCII: LHCII is phosphorylated under state II conditions, and dephosphorylated under state I (Allen, 1992; Bennett, 1977). Evidences of this were first reported in the early ´80s, when Bennet and Allen showed that reduction of the plastoquinone (PQ) pool, after overexcitation of PSII relative to PSI, triggers the activation of a protein kinase that specifically phosphorylates LHCII.

However, mutants of *Chlamydomonas reinhardtii* (Lemaire et al., 1987; Wollman and Lemaire, 1988) and *Arabidopsis thaliana* (Coughlan et al., 1988; Gal et al., 1988) lacking the cytochrome b_6f complex (Cyt b_6f) are

blocked in state I and unable to undergo transitions from state I to state II, even though the PQ pool is fully reduced.

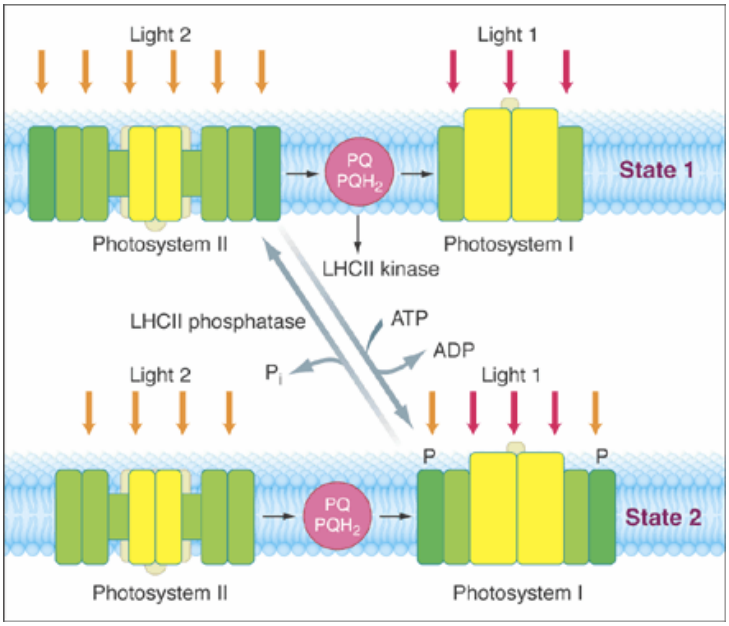

Figure 1.1 State transitions. PSI and PSII are composed of the inner reaction center domains (yellow) that drive the electron transfer into and out the PQ pool, and light-harvesting protein pigment complexes (light green), which collect and distribute light energy to the reaction centers. In addition, there is a mobile light-harvesting complex, LHCII (dark green), that collects light for PSI, when in the phosphorylated form, and for PSII, when in the dephosphorylated form. Under PSII light, the PQ pool becomes readily reduced and the LHCII kinase is activated. The mobile LHCII, once in the phosphorylated form, migrates to PSI and the imbalance in energy distribution is therefore corrected and PQ restored. Conversely, under light favoring PSI, the PQ pool becomes oxidized and the kinase switches off. LHCII is dephosphorylated by an LHCII phosphatase, and excitation balance is restored in state I as LHCII returns to PSII (from Allen, 2003).

Later on, Zito and coworkers (1999) showed that it is not the redox state of the PQ pool *per se* being critical in activating the LHCII kinase, but the occupancy of the reduced PQ pool to the Q_O site on the lumenal side of the Cyt b_6f. Further insights into how the kinase is activated by the Cyt b_6f came from the resolution of the 3D structure of the mitochondrial cytochrome *bc* complex (Iwata et al., 1998; Kim et al., 1998; Zhang et al., 1998; Hunte et al., 2001), which is highly homologous to the chloroplast Cyt b_6f. Zhang and coworkers (1998) showed that the position of the Rieske protein, one of the key subunit of the complex, changes depending on the presence or absence of stigmatellin – a Q_O site inhibitor. Thus, the reorganization of the trans-

membrane portion of the Cyt b_6f complex has been suggested to play a primary role in the activation of the LHCII kinase (Zito et al., 1999; Wollman, 2001).

During state transitions the mobile phosphorylated pool of LHCII migrates from the appressed region of PSII to the non-appressed region of PSI, but how this is achieved remains still unclear. Among the several possible explanations proposed, the most convincing one argues in favor of an electrostatic repulsion between the phosphorylated LHCII and the negative charges on the stromal grana surface (Allen, 1992). It has been shown that in *Arabidopsis* mutants deficient in the PSI subunit PsaH, LHCII – although phosphorylated – remains attached to PSII, indicating that PsaH is part of a high affinity binding site for the phospho-LHCII (Lunde et al., 2000). Recent papers attempted to investigate the composition of the LHCII pool migrating along the thylakoid membranes during state transitions, both in *Chlamydomonas* and in *Arabidopsis*. Kouřil and coworkers (2005) resolved the PSI-LHCII supercomplex from plant cells locked in state II using electron microscopy, and assigned unambiguously the density of a novel pear-shaped PSI-LHCII particle with an association of PSI and the trimeric LHCII. However, this is partially contradictory with new insights coming from studies in *Chlamydomonas*. It was, in fact, observed that CP29 (Kargul et al., 2005; Takahashi et al., 2006), CP26 and a previously unreported major LHCII protein type II, or LhcbM5 (Takahashi et al., 2006) might act as docking sites for the trimeric LHCII proteins in both PSI and PSII.

Redox control of the LHCII kinase activity seems to act at different levels. The maximal level of LHCII phosphorylation *in vivo* occurs at low light intensities, whereas, under higher irradiances, a strong down-regulation of LHCII phosphorylation takes place (Rintamäki et al., 1997; Pursiheimo et al., 1998). It has been proposed that light activates the LHCII kinase via reduction of the PQ pool and its subsequent binding to the regulatory site of the Cyt b_6f. This activation might induce a conformational change of the kinase (Vener et al., 1998) and a burial of a target disulfide bond (Rintamäki et al.,

2000). This state of LHCII kinase prevails at low-light irradiances, which induces maximal phosphorylation of LHCII proteins. On the contrary, higher light intensities induce an additional regulatory state of the LHCII kinase, in which the disulfide bond becomes exposed on the stromal side of the thylakoid membranes and is made accessible to reduction by a thioredoxin, which inactivates the kinase (Rintamäki et al., 2000; Fig. 1.2).

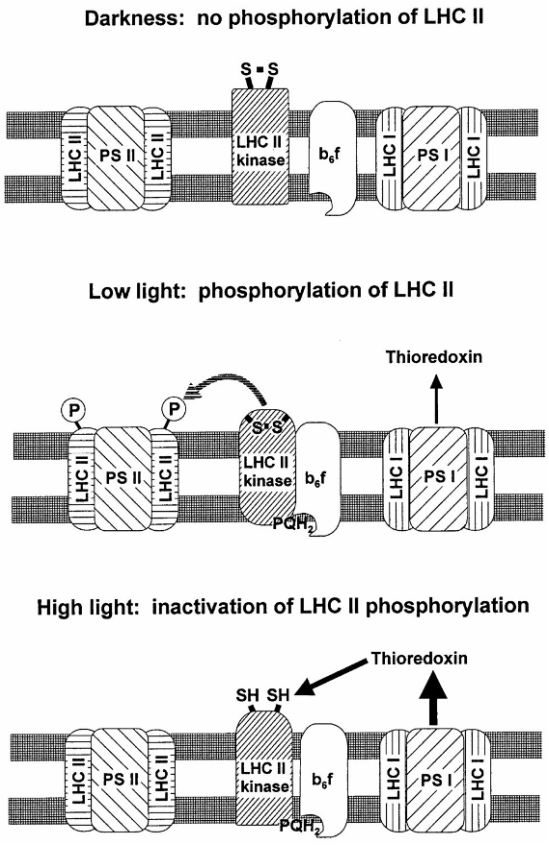

Figure 1.2 Regulation of LHCII phosphorylation *in vivo*. In the dark, the LHCII kinase is inactive because of the oxidized state of the Q_O site in the Cyt b_6f. Light inititates the electron transport in the thylakoid membranes, leading to the activation of the kinase via reduction of the PQ pool and its binding to the regulatory pocket in the Cyt b_6f. Under low-light intensities the LHCII kinase is fully active and undergoes a conformational change that protects its regulatory disulfide bond. Under high-light, the kinase exposes the disulfide bond on the stromal side, which is then reduced via the ferredoxin-thioredoxin system, resulting in stable inactivation of the LHCII kinase (from Rintamäki et al., 2000).

The hunting for the LHCII kinase has been elusive for years, although several attempts have been performed. Significant progress was offered by the approach of Snyders and Kohorn (1999), who screened for proteins interact-

ing with the N-terminal of the light-harvesting proteins, which is known to contain the target threonine site phosphorylated during state transitions. Nonetheless, this led to the identification of a kinase that was associated with the cell wall rather than with the thylakoid membranes. The antiserum against this kinase was then used to isolate a thylakoid-associated kinase (TAK), which is part of a small family of kinases – TAK1, TAK2, and TAK3 – that displays some homology with the human TGFβ1 receptor. This observation suggested that the TAK proteins may be part of a kinase cascade that could initiate with a redox signal, triggering the phosphorylation of LHCII and resulting in state transitions (Snyders and Kohorn, 2001). TAK1 was shown to be associated with both PSII and the Cyt b_6f complex. Moreover, antisense TAK1 lines were light sensitive, deficient in state transition and in LHCII phosphorylation. However, no TAK homolog was found in the genome of the green alga *Chlamydomonas reinhardtii*, which exhibits high levels of state transitions (Depège et al., 2003).

Chlamydomonas is, in fact, a model system in the study of photosynthetic state transition, since the lateral displacement of LHCII from PSII to PSI results in the transfer of about 80% of the excitation energy absorbed by LHCII (Delosme, et al., 1996), whereas in vascular plants only 15 to 20% of LHCII is mobile (Allen, 1992). Screens for mutants deficient in state transitions were perfomed in this green alga (Fleischmann et al., 1999; Kruse et al., 1999) and, among the ones isolated, two were found defective in a novel serine-threonine protein kinase, called Stt7 (Depège et al., 2003). This kinase is nucleus-encoded, associated with the thylakoid membranes, and required for LHCII phosphorylation. Interestingly, Stt7 is also conserved in *Arabidopsis*, whose genome contains two genes – *STN7* and *STN8* - that show significant homology to the *Chlamydomonas* gene.

1.2 Acclimation by adjustment of photosystem stoichiometry

Illumination conditions that preferentially excite PSI or PSII generate an imbalance in the excitation energy distribution between the two photosystems, which results in a decrease in the photosynthetic efficiency (Fujita et al., 1987; Kim et al., 1993). Photosynthetic organisms are able to respond to changes in the environmental light quality with an initial short-term response, the so-called state transitions, and a following long-term response that triggers the readjustment of photosystem stoichiometry and requires changes in photosynthetic gene expression (Allen, 1995). Both processes are regulated by the redox state of the PQ pool, which reflects the balance of light between both photosystems (Fujita et al., 1987; Taylor, 1989; Goldschmidt-Clermont, 1998). However, while state transitions rely on a pure post-translational mechanism, the adjustment of photosystem stoichiometry involves changes in the expression of PSI and PSII genes, and in the accumulation of chlorophyll *a* and *b* (Pfannschmidt et al., 2001). Acclimation ensures the photosynthetic organism to alter the proportion of incident light which is directed towards each photosystem, thus optimizing the electron transport and increasing the photosynthetic efficiency.

Recent studies highlighted the direct impact of photosynthetic processes on the expression of genes encoding photosynthetic components, in both the chloroplast (Pfannschmidt et al., 1999a; Pfannschmidt et al., 1999b) and the nucleus (Barkan and Goldschmidt-Clermont, 2000; Pfannschmidt et al., 2001). Thus, the chloroplast does not only provide energy but also represents a sensor for environmental information, and chloroplast redox signals help to acclimate the organism to changes in environmental factors (Anderson, 1995; Huner, 1998; Pfannschmidt, 2001). Various initial signals have been identified: changes in the redox state of the components of the electron transport chain, or of other soluble gears, like thioredoxin or glutathion, and reactive oxygen species (ROS) that are unavoidable by-products of photosynthesis (Pfannschmidt et al., 2003). This regulation is believed to provide a feedback loop that links photosynthesis to the expression of its own con-

stituents. In this scenario, redox signals allow the photosynthetic organism to acclimate its machinery to environmental light changes that otherwise would affect negatively the photosynthetic process. Such changes in the redox state seem to initiate signaling cascades, whose transduction is still largely unknown ultimately, affecting gene expression (Figure 1.3). Similar to state transitions, photosynthetic acclimation might be triggered by the activation of a sensor kinase, which translates the redox signal into a phosphorylation signal (Allen and Forsberg, 2001; Aro and Ohad, 2003; Haldrup et al., 2001).

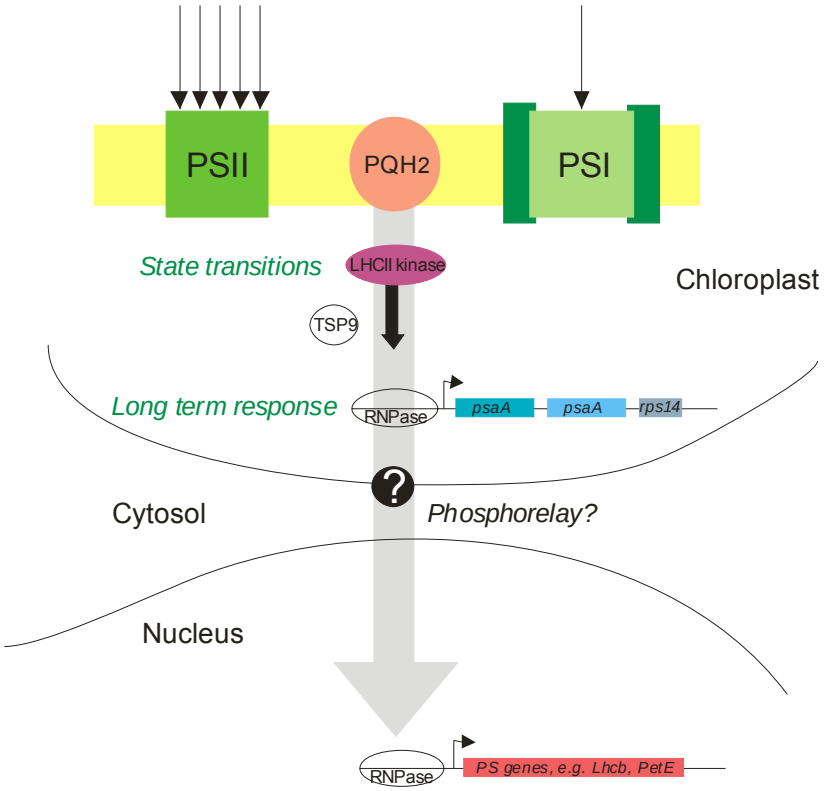

Figure 1.3 Proposed link between state transitions and control of gene expression. Under light favoring PSII, the PQ pool becomes reduced and therefore activates the LHCII kinase which, in turn, phosphorylates the mobile pool of LHCII (dark green). This migrates to PSI, enlarging its antenna cross-section (state transitions). The same signal activates the transcription of the *psaAB* operon through the plastid RNA polymerase (RNPase) controlling photosystem stoichiometry adjustment (long term response). The signal is also likely to be transported over the chloroplast double membrane by an unknown mechanism, perhaps involving TSP9, and transferred through the cytosol into the nucleus where it affects the expression of *Lhcb* and other nuclear-encoded photosynthetic proteins (adapted from Fey et al., 2005).

Regarding the time scale of short- and long-term response, the simple expectation that changes in gene transcription are slower than state transitions is certainly incorrect (Allen and Pfannschmidt, 2000). It has, in fact, been shown that post-translational and transcriptional responses to changes in the redox state of the PQ pool occur simultanuosly, with half-times measured in minutes (Pfannschmidt, 1999a). Thus, the two processes are likely to represent functionally coupled processes (Allen and Pfannschmidt, 2000; Pursiheimo et al., 2001), but it is still unclear how they relate to each other. Are they independent mechanisms controlled by branched cascades originating from a common signaling component – the reduction of the PQ pool –, or rather two subsequent processes regulated by a single consecutive signaling pathway?

A small 9-kDa thylakoid-bound protein of PSII, TSP9, has been proposed to be a a signal transducer candidate towards transcription. This protein is, in fact, partially released from PSII upon PQ reduction in spinach and, in addition, it carries a DNA binding domain (Carlberg et al., 2003). Homologs of the gene encoding TSP9 are present in *Arabidopsis* and rice, but not in cyanobacteria and green algae. In prokaryotes, a role for the PQ redox state in controlling photosystem stoichiometry through effects on biosynthesis of chlorophyll *a* has been suggested for cyanobacteria (Fujita et al., 1987). Since these organisms are the ancestors of chloroplasts, they might be a useful model to elucidate the development and molecular structure of chloroplast redox-signaling pathways.

1.3 Turnover of PSII units

Light is a prerequisite for photosynthesis, but it also can be harmful to the photosynthetic machinery. Exposure of photosynthetic organisms to high irradiances results in unbalances between excitation of the photosynthetic pigments and the ability of a plant metabolism to use the excitation energy. This may lead to a severe inactivation of PSII, referred to as PSII photoin-

hibition (Aro et al., 1993; Andersson and Aro, 2001), and to the production of reactive oxygen species (ROS) or other radicals, which are destructive to proteins and pigments, especially within PSII (Barber and Andersson, 1992; Niyogi, 1999). Photoinhibition of PSII is associated with both the induction of photoprotecting mechanisms, as well as light-mediated PSII inactivation followed by oxidative damage to the D1 protein (Krause and Weis, 1991; Prasil et al., 1992; Aro et al., 1993), and occurs at all light intensities. Under normal light conditions (Tyystjärvi and Aro, 1996), photosynthetic organisms are able to overcome photodamage by a rapid and efficient repair of PSII (Aro et al., 1993; Andersson and Aro, 2001). However, when plants are exposed to stronger light, the rate of photodamage exceeds the rate of repair, resulting in the photoinhibition of PSII.

Functional PSII centers are mainly located in the appressed grana membranes, where also photoinhibition and primary photodamage to PSII are taking place (Cleland et al., 1986; Mäenpää et al., 1987; Adir et al., 1990), whereas repair of damaged PSII occurs only in the stroma-exposed thylakoid regions (Guenther and Melis, 1990; Neale and Melis, 1991; Figure 1.4). The inactive complex migrates, in fact, to the non-appressed thylakoid regions, where the coordinated removal of the damaged protein and the insertion of a new copy into the PSII centers take place (Mattoo and Edelman, 1987; Adir et al., 1990; Kettunen et al., 1991; Barbato et al., 1992; Elich et al., 1992).

The PSII repair cycle is thought to be regulated by the phosphorylation state of the PSII core proteins, and primarily of D1, whose degradation is proposed to occur only after its prior dephosphorylation (Koivuniemi et al., 1995; Rintamäki et al., 1996). Phosphorylation of PSII core proteins in the grana regions has been suggested to prevent a premature disassembly of photodamaged PSII complexes and also to avoid D1 protein degradation under conditions in which the synthesis of a new copy is impossible. Co-immunoprecipitation experiments were interpreted in a way that D1, D2, CP47, usually present in the phosphorylated form when associated to intact

PSII centers, are also part of the PSII centers undergoing repair cycle, but only in the dephosphorylated form (Baena-González et al., 1999). Interestingly, CP43 was not detectable in the complexes to be repaired. Dephosphorylation of CP43 is believed to be necessary for detaching from the PSII monomer, and therefore for the access of the phosphatase to D2 and D1. Dephosphorylation of D2, on the other hand, might provide a further opening of the centers after detachment of CP43 to allow the replacement of the damaged D1. In this scenario, phosphorylation of PSII core proteins would be a protection mechanism to ensure a safe transport of the damaged PSII monomers to the stroma membranes. Dephosphorylation, conversely, would allow the coordination of protein degradation, synthesis, and insertion.

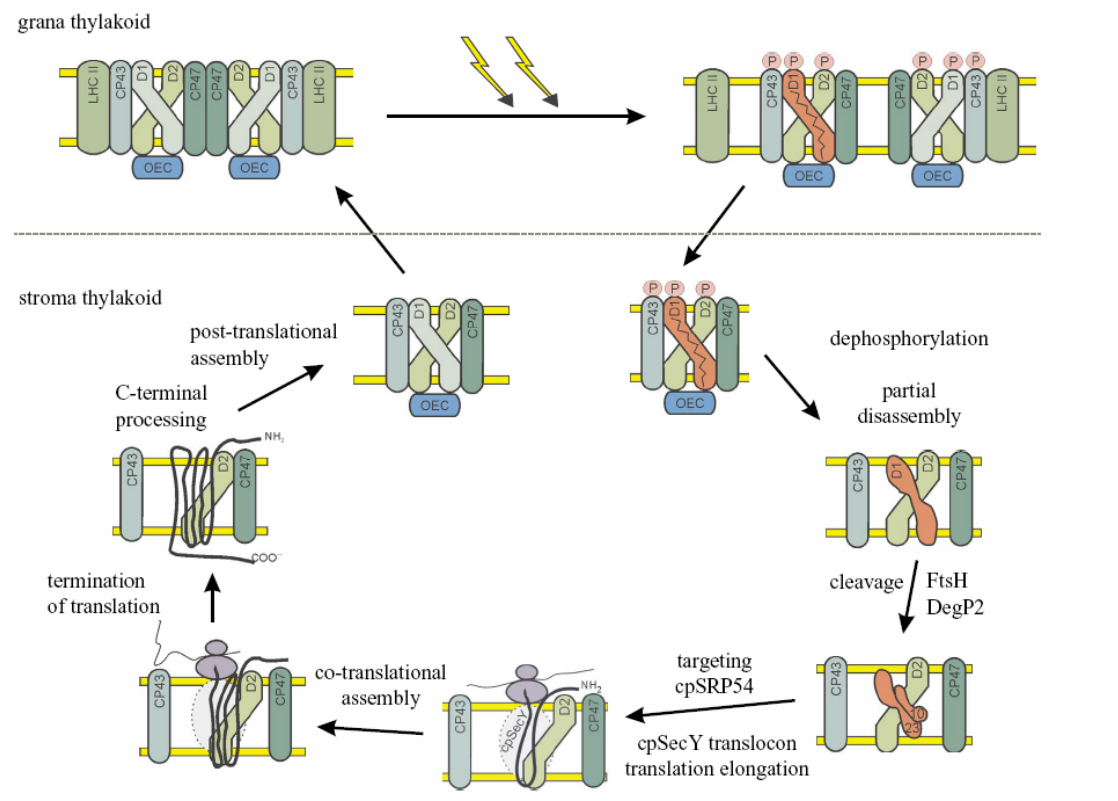

Figure 1.4 Model of the PSII centers repair *via* turnover of the D1 protein. Upon illumination, a pool of PSII centers in the grana undergoes phosphorylation. Prolonged light exposure can cause damage to D1, and when this occurs, it might induce monomerization of the PSII dimers. PSII monomers migrate to the stroma lamellae for repair, where dephosphorylation of the PSII core proteins takes place. D1, in the dephosphorylated form, is then degraded by one or more proteases and immediately replaced by a newly synthesized D1 copy that is inserted into existing PSII after depletion of the damaged D1. The repaired complex migrates back to the grana thylakoids (from Baena-González and Aro, 2002).

A recent work (Turkina et al., 2006) highlighted the occurrence of reversible D1 phosphorylation in *Chlamydomonas*, although it has been considered, so far, restricted to higher plant (Andersson and Aro, 2001; Baena-González et al., 1999). The authors were able to map, for the first time, the *in-vivo* phosphorylation sites in the PSII subunits D1, D2, CP43, CP29 and PsbR of the green alga after light-exposure. These modifications were proposed to be required for an efficient PSII repair cycle in lower plants, despite the lack of a well defined grana stack structure.

2. MATERIALS AND METHODS

2.1 Database analysis, Digital Northern, and prediction of subcellular targeting

For the analysis of gene models and their coverage by full-length mRNAs or ESTs, the NCBI (http://www.ncbi.nlm.nih.gov/), MIPS (http://mips.gsf.de/proj/thal/db/index.html) and AtGDB (http://www.plantgdb.org/AtGDB/prj/ZSB03PP/) databases were used.

Digital Northerns were performed by using the Genevestigator site (https://www.genevestigator.ethz.ch/; Zimmermann et al., 2004). The signal intensity values retrieved from this database are arbitrary units which depend on the choice of the target value (TGT). The TGT is the 2-98% truncated average signal intensity value of a chip processed during normalization, and in Genevestigator all TGT values are scaled to 1000.

Sequence data were analysed with the Wisconsin Package Version 10.0 GCG (Genetics Computer Group, Madison, Wisconsin; Devereux et al., 1984) and amino acid sequences were aligned using the CLUSTAL-W program (http://www.ebi.ac.uk/clustalw/; Chenna et al., 2003). Import sequence predictions were analyzed using the programs TargetP (version 1.1; http://www.cbs.dtu.dk/services/TargetP/; Emanuelsson et al., 2000), ChloroP (version 1.1; http://www.cbs.dtu.dk/services/ChloroP/; Emanuelsson et al., 1999), MitoProt (http://ihg.gsf.de/ihg/mitoprot.html; Claros et al., 1996), iPSORT (http://hc.ims.u-tokyo.ac.jp/iPSORT/; Bannai et al., 2002), PCLR (http://andrewschein.com/pclr/; Schein et al., 2001), Predotar (version 1.03; http://urgi.infobiogen.fr/predotar/predotar.html; Small et al., 2004) and PSORT (http://psort.nibb.ac.jp/ form.html; Nakai et al., 1999).

Segmentally duplicated genes were identified in the genome of *Arabidopsis thaliana* by employing the Paralogon database (http://wolfe.gen.tcd.ie/athal/dup; Blanc et al., 2003).

2.2 Plant lines and propagation

Arabidopsis seeds were sown in plastic trays with "Minitray" soil (Gebr. Patzer GmbH & Co. KG, Sinntal-Jossa, Germany) and incubated for 3 days at 2-5°C in the dark to break dormancy. Plants were grown in a growth chamber in a 16-h-light (20°C)/8-h-dark (18°C) cycle with a PFD of 80 µmol photons m^{-2} s^{-1}. Fertilization with "Osmocote Plus" (15% N, 11% P_2O_5, 13% K_2O, 2% MgO; Scotts Deutschland GmbH, Nordhorn, Germany) was performed according to manufacturer's instructions.

All insertion mutant lines, except *tak3-1*, were identified in the SALK collection (http://signal.salk.edu/; Alonso et al., 2003) which is made up of flank-tagged ROK2 T-DNA lines (ecotype Columbia-0) by searching the insertion flanking database SIGNAL (http://signal.salk.edu/cgi-bin/tdna express). The *tak3-1* mutant derives from the Versailles-INRA collection, which is made up of a flank-tagged GKB5 T-DNA lines (ecotype Wassilevskija), and was identified by searching the Flagdb database (http://urgv.evry.inra.fr/FLAGdb, Samson et al., 2004).

2.3 Nucleic acid analysis

Arabidopsis DNA was isolated as described (Liu et al., 1995) and insertion junction sites were sequenced after PCR-amplifications using a combination of gene- and insertion-specific primers. T-DNA primers specific for ROK2 were LBb1 (5´-GCGTGGACCGCTTGCTGCAACTC-3´) and RBb1 (5´-TCAGTGACAACGTCGAGCAC-3´); for pGKB5, RB1 (5´-CCAGACGTTGC CCGCATAA-3´), RB2 (5´-GTTAAAACTGCCTGGCAC-3´), LB1 (5´-CTATT GGTAATAGGACACTGG-3´) and LB2 (5´-CTCAACTGAAACGGGCCGG-3´). Primers specific for TAK1 were tak1-1153s (5´-ACGAATCGAGATGC GGAAGG-3´) and tak1-1464as (5´-CTCCAAAGCTAATTGGTCATTG-3´); for TAK2, tak2-215s (5´-AGGAGATTAAAGAGATCGTTC-3´) and tak2-1690as (5´-GAAAGTTCCCATCACACGAG-3´); for TAK3, tak3-1045s (5´-GATGCTC GTGTATGACTACG-3´), tak3-1566as (5´-CTGTCAACATTCCGGTGCAA-

3´); for STN7, stn7-1131s (5´-GATGGTCTTCACTCAAC TGG-3´) and stn7-1755as (5´-GCATTGGCCTCATCTTCCTT-3´); and for STN8, stn8-71s (5´-GATTCTCCACTCCCACTCAC-3´) and stn8-493as (5´-CCCTAACGTCATCA ACAAGAG-3´).

For determination of the kinases gene expression, total leaf RNA was extracted from fresh tissue using the method adapted from Chomczynski and Sacchi (1987) and TRIzol reagent (Invitrogen, Karlsruhe, Germany). First-strand cDNA was synthesized using the SuperScript Preamplification System (Invitrogen, Karlsruhe, Germany), and reverse transcriptase–mediated PCR (RT-PCR) was performed with primers specific for the genes *TAK1* (sense primer, 5'-TGGCGGATTCTCACTCAGTC-3'; antisense primer 5'-CGATGTGAATTTTGTTATCGTC-3'), *TAK2* (sense primer, 5'-AGGAGATTA AAGAGATCGTTC-3'; antisense primer 5'-TAACCACCTTCTCCGATTAC-3'), *TAK3* (sense primer, 5'-TCAAAGGAGATTAAAGAGATCGTTC-3'; antisense primer, 5'-TAACCACCTTCTCCGATTAC-3'), *STN7* (sense primer 5'-GGAG GAGCTTACATCGGAAC-3'; antisense primer 5'-AGGTCGAAACAATCTC TCAAC-3') and *STN8* (sense primer 5'-GATTCTCCACTCCCACTCAC-3'; antisense primer 5'-CCCTAACGTCATCAACAAGAG-3'). Oligos specific for *ACTIN1* (sense primer, 5'-TGCGACAATGGAACTGGAATG-3'; antisense primer, 5'-GGATAGCATGTGGAAGTGCATACC-3') were used as control.

2.4 Pigment analysis

Pigments were analyzed by reverse-phase HPLC as described previously by Färber et al. (1997). For pigment extraction, leaf discs were frozen in liquid nitrogen and disrupted in a mortar in the presence of acetone. After a short centrifugation, pigment extracts were filtered through a 0.2 µm membrane filter and either used directly for HPLC analysis or stored for up to 2 days at -20°C (the pigment analysis was performed in collaboration with Peter Jahns, Düsseldorf, Germany).

2.5 Immunoblot analysis

For quantification of Lhcb1 and 2 proteins, leaves from 4-week-old plants were harvested after overnight dark adaption or after following exposure to levels of illumination that favor phosphorylation (low light: 80 µmol photons m^{-2} s^{-1}, 2 h) or dephosphorylation (high light: 800 µmol photons m^{-2} s^{-1}, 2 h). Thylakoids were prepared from mesophyll chloroplasts as described (Bassi et al., 1985). Identical amounts of proteins equivalent to 2 µg of chlorophylls were loaded, separated by SDS-PAGE (12% acrylamide) as described by Schägger and von Jagow (1987) and transferred to Immobilon-P poly(vinylidene difluoride) membranes (Millipore, Eschborn, Germany). Replica filters were incubated with antibodies specific for Lhcb1 or Lhcb2 (Agrisera, Vännäs, Sweden). For the identification of phosphorylated threonine residues, thylakoid proteins were prepared as described (Haldrup et al., 1999) in the presence of 10 mM NaF. Protein amounts equivalent to 2 µg of chlorophylls were loaded for each genotype and each condition and fractionated by SDS-PA gradient gel (8 to 25% acrylamide). Filters were immunolabeled with a phosphothreonine-specific antibody raised in rabbits (Cell Signaling, Beverly, MA). Signals were detected using the Enhanced Chemiluminescence Western Blotting Kit (Amersham Biosciences, Sunnyvale, CA).

2.6 *In-vitro* protein import in purified chloroplasts

For *in-vitro* import in purified chloroplasts, [^{35}S]-labeled proteins were synthesized by coupled in vitro transcription and translation using the TNT-Kit (Promega, Madison, WI). Import experiments were performed as described (Becker et al., 2004). For sub-fractionation of chloroplasts after import, ten import assays were performed as outlined above. Subsequently, intact chloroplasts of all assays were pooled by re-suspension in 0.65M sucrose, 10mM sodium phosphate (pH 7.9). After two cycles of freeze and thawing, chloroplasts were ruptured by 30 strokes in a Dounce homogenizer and di-

luted 1:1 in 10mM sodium phosphate (pH 7.9). Chloroplast fractions were separated by centrifugation through sucrose step gradient composed of a 0.465M and 0.996M sucrose layer. Stromal proteins on the 0.465M sucrose layer were collected and TCA precipitated before gel loading. Mixed envelope vesicles collected on the 0.996M sucrose layer and pelleted thylakoids were taken without further treatment for SDS-PAGE analysis. After separation on a SDS-PAGE radio-labeled proteins were detected by exposure to a Biomax-film (Kodak, New York, NY; the *in-vitro* import assay was performed in collaboration with Thomas Becker and Enrico Schleiff, München, Germany).

2.7 Intracellular localization of dsRED fusions

The red fluorescent protein from the reef coral *Discosoma* (dsRED) (Jach et al., 2001) was used as a reporter to determine the intracellular localization of the kinases in transient gene expression assays. Mesophyll protoplasts were isolated from 6-week-old tobacco plants (*Nicotiana tabacum* cv. Petit Havana SR1) and transfected with 20 µg of pGJ1425 DNA (Jach et al., 2001), carrying the proteins cDNAs fused 5' to the *dsRED* gene, by polyethylene glycol–mediated DNA uptake as described (Pesaresi et al., 2003). The transfected protoplasts were cultured for 2 days at 26 °C in the dark in the presence of auxin (5 µM) and cytokinin (1 µM) and then analyzed using the Zeiss Axiophot fluorescence microscope (Jena, Germany) equipped with a filter set purchased from AF Analysentechnik (Tübingen, Germany). Photographs were taken using a video imaging system mounted on the microscope that consisted of a Hitachi charge-coupled device video camera (Tokyo, Japan) operated by the DISKUS software package (Technisches Büro Hilgers, Königswinter, Germany).

For intracellular localization of the dsRED fusion *in-planta*, a complementary DNA fragment encoding for the first 134 amino-acid residues of STN8 and the first 135 of STN7 were fused 5' to dsRED and inserted into the vector

pLEELA (kindly provided by M. Jakobi, Max-Planck-Institute for Plant Breeding Research, Germany). The vector carries the cauliflower mosaic virus 35S promoter, a kanamycin and a carbenicillin resistance gene for selection in *E. coli* and *Agrobacterium*, and a *pat* gene (phosphoinocitrin-N-acetyltransferase), as a plant selectable marker, conferring resistance to BASTA herbicide. The *Agrobacterium* strain GV3101 was used for *Arabidopsis* transformation. The strain has a C58C1 chromosomal background marked by a rifampicin resistance mutation, and carries pMP90RK as helper plasmid, encoding virulence functions for T-DNA transfer from *Agrobacterium* to plant cells (Koncz et al., 1990). Mutant plants were transformed as reported (Clough and Bent, 1998). Flowering plants were dipped for 20 s in an *Agrobacterium* suspension containing 5% (w/v) sucrose and 0,0005% (v/v) of the surfactant Silwet L-77. After dipping, plants were transferred to a growth chamber with high humidity conditions for two days and then to the greenhouse. Seeds were collected from transformed mutant plants and independent transgenic plants were selected. Confocal images were collected by laser scanning microscopy (TCS SP2; Leica, Wetzlar, Germany). Fluorescence was excited with a 461 nm HeNe laser and images were collected in the ranges 565-620 nm (dsRED fluorescence) and 670-750 nm (chlorophyll autofluorescence).

2.8 Chlorophyll fluorescence measurements

In-vivo chlorophyll *a* fluorescence of single leaves was measured using a pulse amplitude modulation 101/103 (PAM 101/103) fluorometer (Walz, Effeltrich, Germany) as described before by (Varotto et al. 2000a, b). Pulses (0.8 s) of white light (6000 µmol photons m^{-2} s^{-1}) were used to determine the maximum fluorescence (F_M) and the ratio (F_M - F_0)/F_M = F_V/F_M. A 15-min illumination with actinic light (80 µmol photons m^{-2} s^{-1}) was supplied to drive electron transport between PSII and PSI before measuring Φ_{II}, qP (photochemical quenching = (F_M' – F_S)/(F_M' – F_0)) and qN (non-photochemical

quenching = 1 – (F_M' – F_0')/(F_M – F_0)). State transitions (qT) of two leaves each from 5 wild-type and 5 mutant plants were measured with the PAM 101/103. After 30 min of dark incubation, F_M was measured by using a saturating white light pulse (0.8 s, 6000 µmol photons m^{-2} s^{-1}). Leaves were subsequently illuminated for 20 min with blue light (80 µmol photons m^{-2} s^{-1}) from a KL-1500 lamp (Schott) equipped with a BG39 filter (Walz, Effeltrich, Germany). The maximum fluorescence (F_M2), in state 2, was then measured. State 1 was induced by switching to far-red light (Walz 102-FR), and F_M1 was recorded 20 min later. qT was calculated according to the equation: qT= (F_M1-F_M2)/F_M2 (Jensen et al., 2000). The relative fluorescence changes of PSII (F_r) were measured as reported by (Lunde et al., 2000).

2.9 LHCII phosphorylation analysis

The LHCII phosphorylation level was analyzed both *in-vivo* and *in-vitro*. To determine the degree of LHCII phosphorylation *in-vivo*, dark-adapted leaves from 4-week-old plants were incubated in the presence of [^{33}P]-orthophosphate for 1 h and subsequently exposed to levels of illumination, as previously described. Identical amounts of thylakoid proteins (equivalent to 200 mg of fresh leaf) were prepared as described (Haldrup et al., 1999) in the presence of the phosphatase inhibitor NaF (10 mM), fractionated by SDS-PAGE (14% acrylamide), and labeled proteins were detected by phosphoimaging (Storm 860, Molecular Dynamics). For the *in-vitro* assay, thylakoids isolated from dark-adapted leaves (30 mg of fresh leaf) were incubated with [γ-^{33}P] ATP under reducing conditions in the dark (Pesaresi et al., 2002). Separation and detection of thylakoid proteins were performed as described for the *in-vivo* assay.

2.10 Immunoblot analysis of D1 protein upon exposure to high-intensity light

For experiments on the effects of high light exposure (PFD of 2000 µmol photons m^{-2} s^{-1}) to the accumulation of the D1 protein, leaf discs were vacuum-infiltrated either with water as control, or with lincomycin or streptomycin to inhibit protein synthesis in the plastids, before measurements (Bailey et al., 2002). Thylakoid proteins were extracted, fractionated on a SDS-PA gradient gel and transferred to Immobilon-P poly(vinylidene difluoride) membranes (Graßes et al., 2002). Filters were then probed with an antibody specific for D1 (kindly provided by D. Godde, Bochum, Germany). After stripping, the same filters were immuno-labelled with an Lhcb2-specific antibody (Agrisera, Vännäs, Sweden) to control the loading. Signals were detected by chemiluminescence (Amersham Biosciences, Sunnyvale, CA).

2.11 Pulse-chase measurement of D1 turnover

The pulse-chase procedure for the analysis of D1 turnover in pea (Aro et al., 1993) was modified for *Arabidopsis*. For radioactive labeling of thylakoid proteins, leaf discs of 3-week-old Arabidopsis plants were pressed extremely gently against coarse sand paper and then vacuum-infiltrated in a syringe containing 1 mCi of [^{35}S]-L-methionine in 10 ml of 1 mM KH_2PO_4 (pH 6.3)-0.1% Tween-20. Directly after infiltration, three leaf discs were frozen in nitrogen (t_0). Remaining leaves were transferred to high light (2000 µmol photons m^{-2} s^{-1}) and for each time point (t_{pulse}=15, 30, 60 min) three leaf discs were collected. Immediately after the pulse period, the remaining leaf discs were washed, incubated with 10 mM unlabeled L-methionine in the same buffer as before, and further exposed to high light for up to 8 h (t_{chase}= 60, 120, 180, 240, 300, 360, 420, 480 min). The three leaf discs for each time point were combined and thylakoid proteins were prepared, separated and detected as described for the LHCII phosphorylation analysis.

2.12 Measurement of acclimation to changes in light quality

Light conditions favoring either PSI or PSII (PSI or PSII light, respectively), as well as growth and acclimation conditions for *Arabidopsis*, have been described (Fey et al. 2005). Plants were initially grown for 10 days under white light followed by a 6-day acclimation period. Seedlings were acclimated either to PSI or PSII light for 6 days or to PSI light for 2 days followed by 4 days under PSII light or *vice versa*. For measurement of chlorophyll fluorescence to long-term changes in light quality, *in-vivo* chlorophyll *a* fluorescence was recorded by video imaging (Fluorcam 700MF, Photon System Instruments, Brno, Czech Republic; Wagner et al., 2005). Immediately after dark acclimation (15 min) the measuring light was turned on, and minimal fluorescence (F_0) was determined. Then leaves were exposed to a 1600-ms flash of saturating white light (3000 µmol photons m^{-2} s^{-1}) to determine maximal fluorescence (F_M). Subsequently, leaves were illuminated with 90 µmol photons m^{-2} s^{-1} of actinic red light of 620 nm for 10 min. Fluorescence was recorded until a stable level (F_T) was reached. Actinic light was then switched off to determine minimal fluorescence F_0' in the light-acclimated state. The steady-state fluorescence F_S was calculated as $F_T - F_0' = F_S$. A normal acclimation response to PSI or PSII light is characterized by a significant change in the F_S/F_M value as shown earlier (Pfannschmidt et al., 2001). For the measurement of chlorophyll content to changes in light quality, total chlorophyll was determined spectroscopically after grinding leaves in liquid nitrogen and extracting chlorophylls with 80% (v/v) buffered acetone. Concentrations of chlorophylls *a* and *b* were calculated using the extinction coefficients from previous studies (Porra et al., 1989). All values were calculated as the means of 50 individuals in at least three independent experiments, and the significance of differences between samples was tested using Student's *t*-test (the acclimation assays were performed in collaboration with Raik Wagner and Thomas Pfannschmidt, Jena, Germany).

2.13 mRNA expression profiling

Greenhouse-grown mutant and wild-type plants were analyzed. Generation and use of a 3292- GST nylon array enriched for nuclear genes for chloroplast proteins have been described (Richly et al., 2003). At least three experiments with cDNA probes from independent plant pools were carried out for each condition. cDNA synthesis was primed with an oligonucleotide mixture matching the 3292 genes in antisense orientation, and hybridized to the array as described (Richly et al., 2003, Kurth et al., 2002). Hybridisation images were read with a phosphorimager (Storm 860, Amersham Biosciences, Sunnyvale, CA), data were imported into *ArrayVision* (version 6.0; Amersham Biosciences, Sunnyvale, CA), and statistically evaluated using *ArrayStat* (version 1.0 Rev. 2.0; Amersham Biosciences, Sunnyvale, CA) as described (Richly et al., 2003; Pesaresi et al., 2003). Data were normalised with reference to all spots on the array (Kurth et al., 2002) and average expression ratios were analysed. Complete data sets were deposited at GEO (http://www.ncbi.nlm.nih.gov/geo/) under Accession Nos. GSE2620-GSE2622 (the transcription profiling was performed by Alexander Biehl, Köln, Germany).

2.14 2D BN/SDS-PAGE analysis of thylakoid proteins

Leaves from 4-week-old plants were harvested after overnight dark adaption or after following exposure to levels of illumination that favor phosphorylation (low light: 120 µmol photons m^{-2} s^{-1}; 2 h) or dephosphorylation (high light: 1200 µmol photons m^{-2} s^{-1}; 2 h). Thylakoids were then prepared as already described (Bassi et al., 1985). For 1D BN-PAGE analysis, protein amounts equivalent to 100 µg of chlorophylls for each genotype were washed with 10 mM Tris-HCl pH6.8, 10 mM $MgCl_2$ and 20 mM KCl, and subsequently solubilized in 750 mM ε-aminocaproic acid, 50 mM Bis-Tris pH 7.0, 5mM EDTA pH 7.0, 50 mM NaCl and 1.5% (w/v) digitonin. Solubilized samples were then incubated for 1 h at 4°C and afterwards centrifuged for 1 h at 19.000g at

4°C. Supernatants were supplemented with 5% (w/v) Coomassie-blue in 750 mM aminocaproic acid, and directly loaded onto BN gels (4-12% acrylamide). One–dimensional BN-PAGE and 2D BN/SDS-PAGE were carried out as described by Schägger and von Jagow (1991).

3. RESULTS

3.1 Protein sequence, gene structure and mRNA expression of the five kinase genes (*STN7*, *STN8*, *TAK1*, *TAK2*, and *TAK3*)

The *Arabidopsis thaliana* genome contains two genes, STN7 (At1g68830) and STN8 (At5g01920), that display significant sequence identity with the *Chlamydomonas* gene encoding the chloroplast Stt7 protein Ser-Thr kinase (Depège et al., 2003).

Of the two *Arabidopsis* proteins, STN7 is more similar (46%/55% identity/similarity) to *Chlamydomonas* Stt7 than STN8 (45%/54%), while the protein sequences of the two *Arabidopsis* proteins are 48%/59% identical/similar (Figure 3.1A). Database searches identified a rice protein sequence (GI:50932631) which is most closely related to STN7 (79%/84%). The two cystein residues in Stt7, separated by four residues and representing the tentative thioredoxin target site (Depège et al., 2003), are conserved in STN7 and in the rice protein, but not in STN8. In addition, STN8 is more related to the *Chlamydomonas* Stt7-like protein Stl1 than Stt7 itself. The *Stl1* sequence encodes a protein kinase of unknown function and with 29% sequence identity and 43% sequence similarity to Stt7 (Figure 3.1B). The computational prediction of subcellular targeting indicated for both STN7 and STN8 the presence of a classical chloroplast transit peptide (Table 3.1).

A family of three protein kinases, thylakoid-associated kinases (TAKs), has been previously described in *Arabidopsis* (Snyders and Kohorn, 1999). The three TAKs are 90% identical, and TAK2 (At1g01540) and TAK3 (At4g01330) are shorter than TAK1 (At4g02630; Figure 3.2). *In-vitro* translated TAK1 was shown to be imported into isolated chloroplasts, but import was not associated with chloroplast transit peptide cleavage (Snyders and Kohorn, 1999). Employing the Paralogon database (http://wolfe.gen.tcd.ie/athal/dup; Blanc et al., 2003), it was found that TAK2 and 3 are located in duplicated segments of the *Arabidopsis* genome, explaining their high level of homology (92%/94% identity/similarity). For none of the three TAK pro-

teins a chloroplast transit peptide was predicted with high reliability (Table 3.1), indicating that a putative chloroplast location of TAKs might be due to the action of either a non-canonical chloroplast transit peptide or of an internal chloroplast targeting signal.

The gene structures of the three TAK genes, as well as of STN7 and STN8, are shown in Figure 3.4A. The exon-intron boundaries were confirmed by both reverse transcriptase–mediated PCR (RT-PCR) and by analysis of full-length mRNA sequences available in public databases (MIPS, http://mips.gsf.de/proj/thal/db/index.html; and AtGDB, http://www.plantgdb.org/AtGDB/prj/ZSB03PP/). Only for TAK3, no full-length mRNA sequence was available; however, RT-PCR confirmed the predicted exon-intron structure for this gene. For TAK2, according to the AtGDB database two alternative types of transcripts exist due to alternative splicing; in leaves, however, only the 7-exon version which is shown in Figure 3.4A was detected by RT-PCR.

To investigate the mRNA expression of the five kinase genes during plant development and in different tissues, as well as under conditions relevant for photosynthetic state transitions, public available microarray data were analyzed (https://www.genevestigator.ethz.ch/; Zimmermann et al., 2004; Figure 3.3). The highest level of TAK1 mRNA was detected in cauline leaves. TAK2 and TAK3 were relatively constitutively expressed under the conditions listed in Figure 3.3. Transcripts of the STN7 and STN8 genes were most abundant in leaves, whereas in roots the two genes exhibited their lowest expression, as a typical trait of photosynthetic gene.

With respect to conditions differing in the quantity or spectral composition of light, none of the kinases showed a marked difference in transcript accumulation (Figure 3.3). Assuming that the LHCII kinase is represented by one or more of the five kinases, this indicates that the redox state dependency of LHCII-kinase activity is not associated with changes in transcript accumulation.

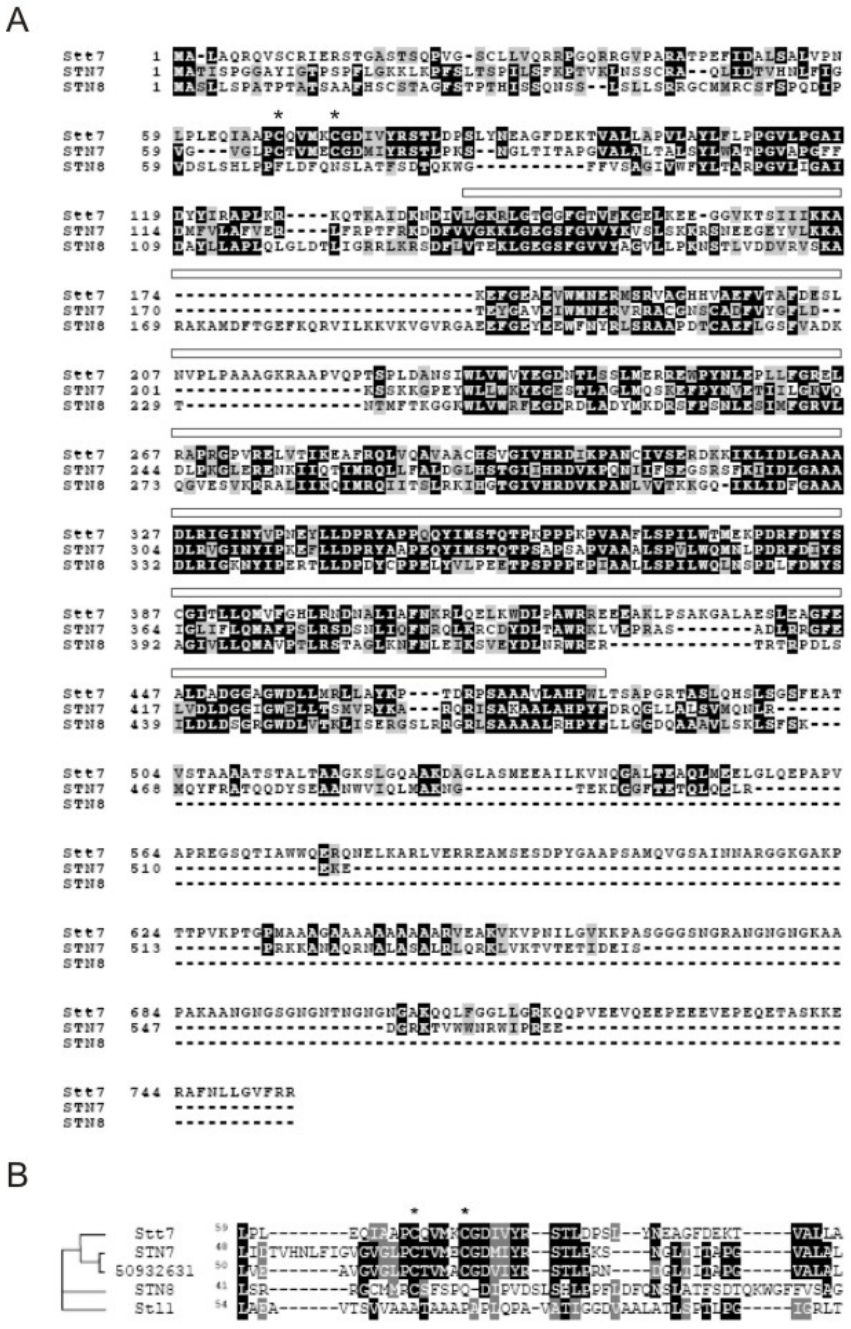

Figure 3.1 Sequence comparison of the *Chlamydomonas* Stt7 kinase and its homologs in *Arabidopsis*. The protein sequences were aligned with the Clustal W (Thompson et al., 1994) and Boxshade programs. Strictly conserved amino acids are highlighted by black boxes, whereas grey ones indicate closely related amino acids. (**A**) The kinase catalytic domain is indicated by an open box above the amino acid sequence while the asterisks indicate the Cys residues that are the potential targets for thioredoxin. (**B**) The portion of the aligned amino acid sequences of Stt7 and Stt7-like sequences from *Chlamydomonas* (Stl1), *Arabidopsis* (STN7 and STN8) and rice (GI:50932631) which contains the tentative target site of thioredoxin (indicated by asterisks) is shown. The phylogenetic tree at the left indicates the relatedness of the complete protein sequences.

Predictor	TAK1	TAK2	TAK3	STN7	STN8
ChloroP	-	-	-	C (0.555)	C (0.579)
TargetP	M (0.455)	-	-	C (0.740)	C (0.972)
Predotar	-	-	-	C (0.994)	C (0.976)
Mitoprot	M (0.0422)	M (0.0082)	M (0.0202)	M (0.2562)	M (0.6845)
iPSORT	M	C	-	C	C
PCLR	-	-	-	C (0.620)	C (0.976)
PSORT	C (0.681)	M (0.800)	ER (0.600)	P (0.378)	C (0.888)

Table 3.1 *In-silico* prediction of subcellular targeting of the protein kinases. The potential of the five kinases to be targeted to a distinct subcellular compartment was examined by the prediction algorithms ChloroP, TargetP, Predotar, Mitoprot, iPSORT PSORT and PCLR. Note that only output scores (listed in parentheses) in the same row are comparable. M, mitochondrion; C, chloroplast; ER, endoplasmic reticulum; P, peroxisome.

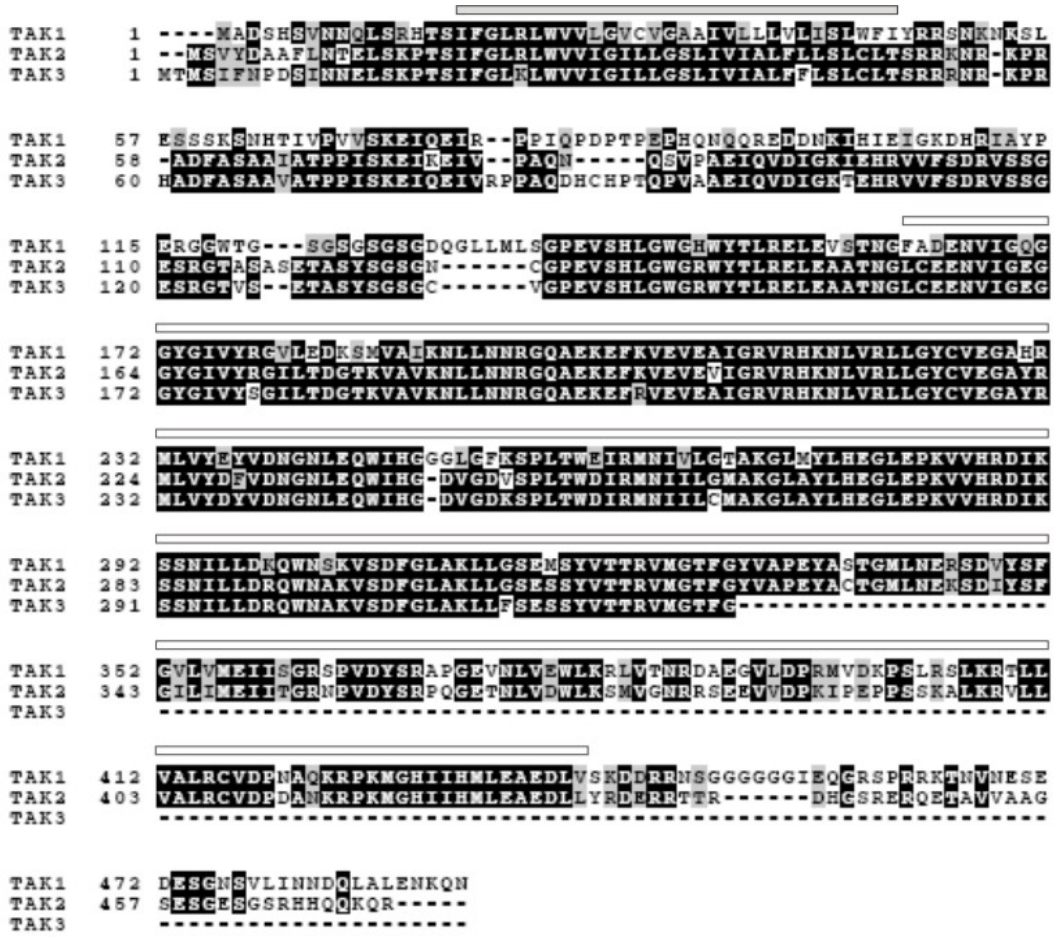

Figure 3.2 Sequence comparison of TAK proteins. The kinase catalytic domain is indicated by an open box above the amino acid sequence while the putative hydrophobic domain is marked by a grey box above the amino acid sequence.

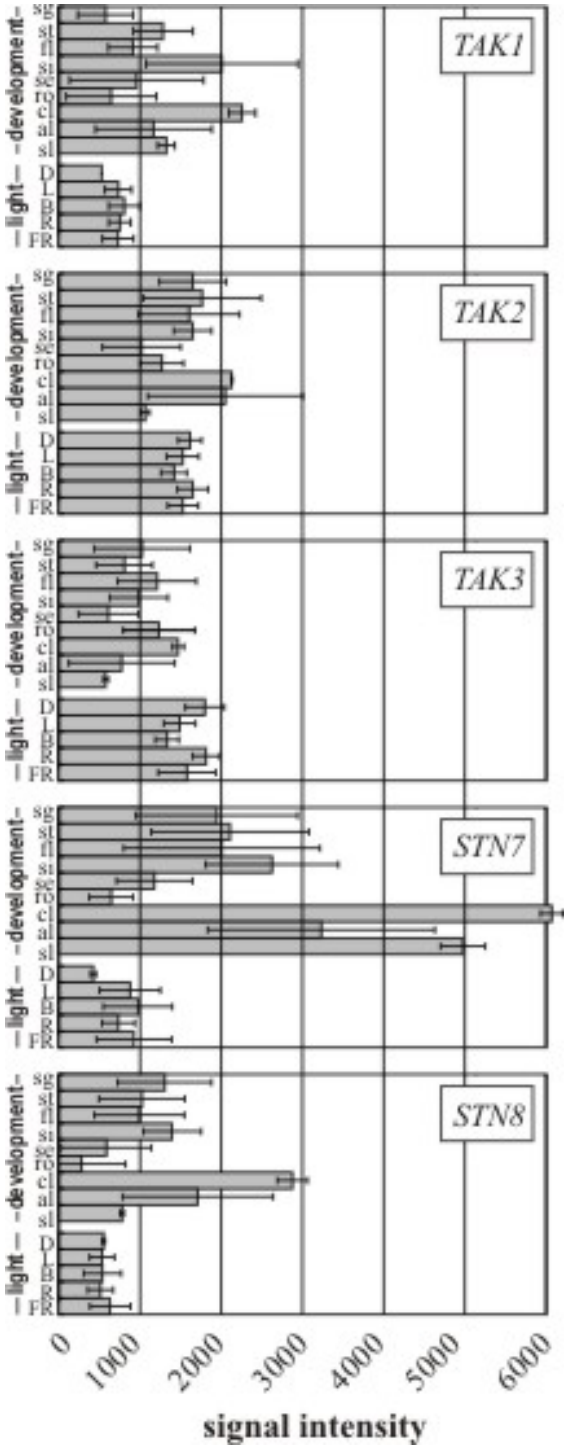

Figure 3.3 Digital Northern analysis of kinase genes. Data used to create the Digital Northerns were obtained from the *Genevestigator* site (http://www.genevestigator.ethz. ch/). Signal intensities for different developmental stages (sg, seedling; st, stem; fl, flower; si, siliques, se, seeds; ro, roots; cl, cauline leaf; al, adult leaf; sl, senescent leaf), as well as different light quantities (D, dark; L, light) or qualities (B, blue light; R, red light; FR, far-red light), are listed. Note that only signal intensities stemming from the same series of experiments are comparable, i.e. either from the different developmental stages ("GeneAtlas"), or from the experiments with different light conditions.

3.2 Knock-out mutations of the protein kinases and their effects on growth

Insertion mutants for each of the three TAK genes, as well as for *STN7* and *STN8*, were identified by searching T-DNA insertion flanking databases. In the obtained lines, the insertion sites were confirmed by PCR, employing gene- and T-DNA border-specific primers, followed by sequencing of the PCR products. For each of the genes, except TAK1, two independent alleles carrying insertions either in exons or introns were obtained (Figure 3.4A). To determine whether the gene disruptions resulted in suppression of the concerned mRNAs, mutant leaves were analyzed by RT-PCR. In none of the mutant lines, the transcript of the respective gene was detectable (Figure 3.8B). The *stn7 stn8* and *tak2 tak3* double mutants were generated by crossing *stn7-1* and *stn8-1*, and *tak2-1* and *tak3-2* single knock-outs respectively, and screening F_2 generation by PCR for homozygous double mutants. Given their high homology, only mutants lacking TAK2 and TAK3 were crossed among the *tak* plants.

All mutant lines could grow photoautotrophically in a greenhouse. Germination of seeds was not affected in the mutants and visual inspection did not reveal any change in phenotype compared to WT plants (data not shown). However, when plants were grown in a growth chamber, *stn7* mutants exhibited a retarded growth in comparison to WT or *stn8* plants (Figure 3.5). All genotypes lacking STN7 showed a decrease in rosette diameter and leaf number (Table 3.2). There was, however, no difference between WT and *stn8*. Moreover, it was proved that it is not the intensity of light to be responsible for this phenotype, since *stn7* mutants respond similarly under both high and high-light (1500 µmol photons m^{-2} s^{-1}; Figure 3.9B) and low light conditions (130 µmol photons m^{-2} s^{-1}; Figure 3.9A). Day-length was also shown not to influence mutants' growth; indeed, growth of *stn7* mutant plants was impaired under both short-day and long-day conditions (data not shown). In addition, recent observations (Pfannschmidt, unpublished data) indicate that also light quality (PSI- or PSII-light) cannot be considered as an

influent factor in reducing *stn7* growth in comparison to WT or *stn8* plants. Other factors that might lead to this phenotype could be humidity or temperature, but these are only speculations and have still to be proved.

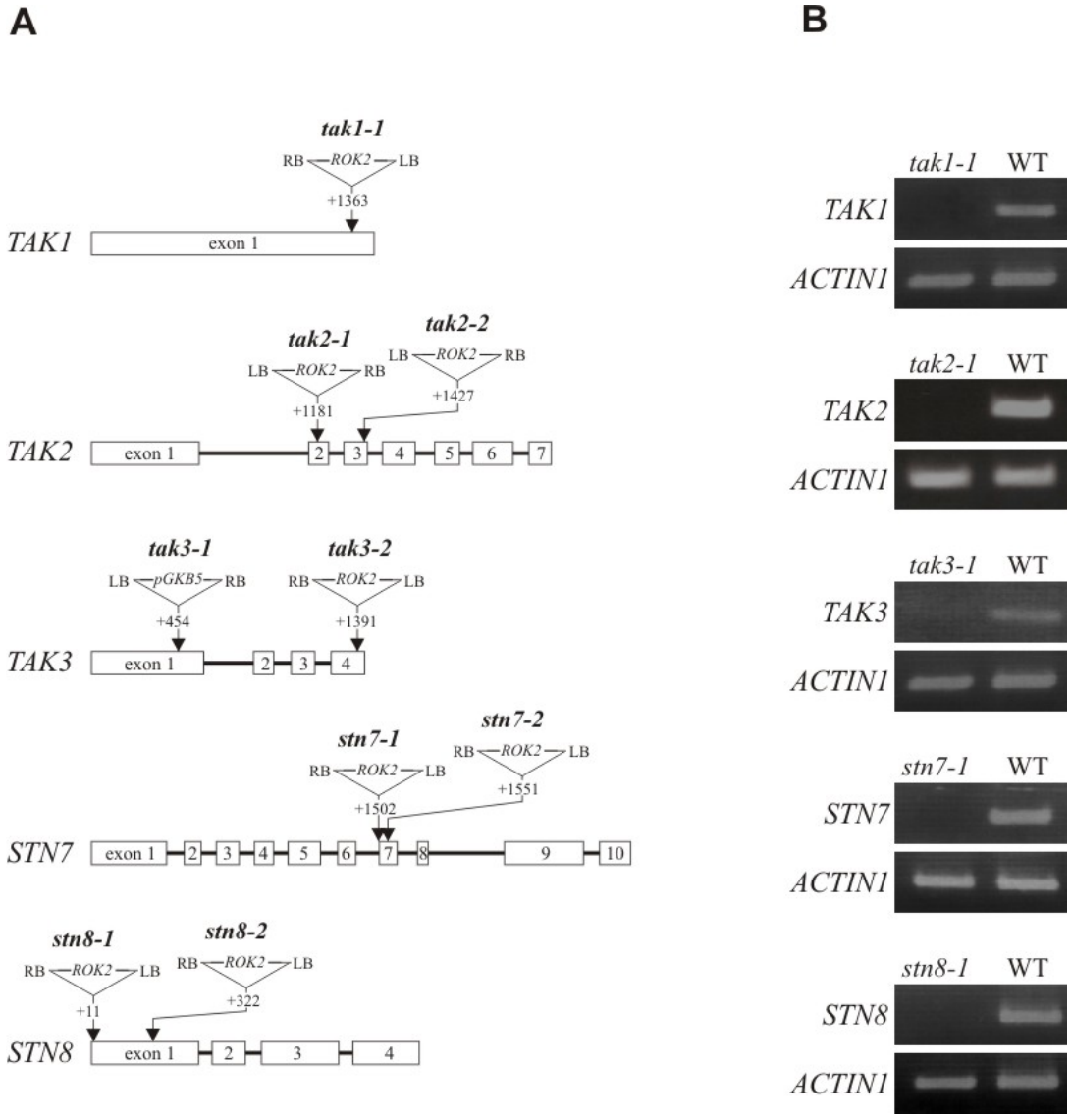

Figure 3.4 Gene models, mutations and their effects on transcript accumulation. (**A**) The translated parts of exon, as well as intron sequences, are indicated. For each T-DNA mutant used in this study, the insertion site and the orientation of the T-DNA is provided. In all mutants, except *tak3*-1, the concerned genes are disrupted by insertions of the ROK2 T-DNA. The corresponding lines were identified in the SALK T-DNA collection: *tak1-1*, SALK_097876; *tak2-1*, SALK_127328; *tak2-2*, SALK_008369; *tak3-2*, SALK_107777; *stn7-1*, SALK_073254; *stn7-2*, SALK_134469; *stn8-1*, SALK_060869; *stn8-2*, SALK_064913. In *tak3-1* (FST_455D12), the *TAK3* gene carries an insertion of the pGKB5 T-DNA. The T-DNA insertions are not drawn to scale. (**B**) RT-PCR analysis was performed on the transcripts of both WT and mutant plants with primers specific for the genes and for actin as a positive control.

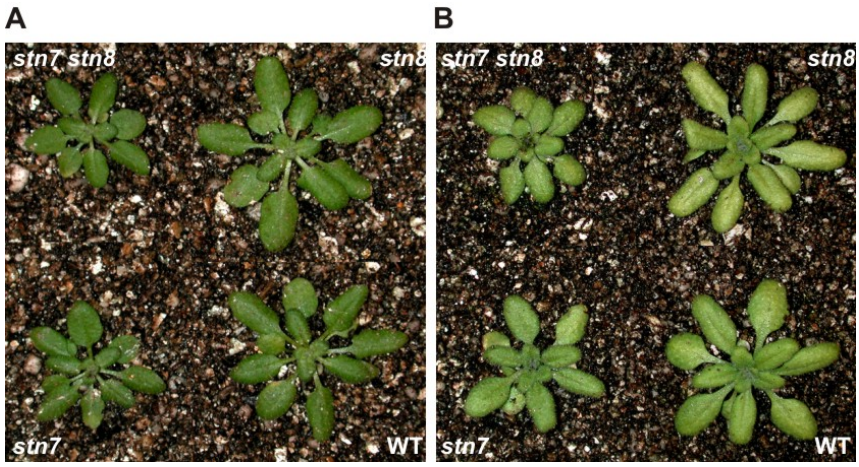

Figure 3.5 Growth of the *stn7* mutants is impaired in the growth chamber. WT and mutant plants were grown in a growth chamber under an 8 h/16 h light/dark regime, both under low-light (130 µmol photons m^{-2} s^{-1};**A**) and high-light (1500 µmol photons m^{-2} s^{-1}; **B**). Only plants lacking STN7 exhibited a retarded growth, regardless of the light intensity they grew under.

Parameter	WT	*stn7*	*stn8*	*stn7 stn8*
Rosette diameter (cm) in LL	6.2 ± 0.5	5.1 ± 0.4	6.0 ± 0.3	4.8 ± 0.1
Rosette diameter (cm) in HL	3.1 ± 0.7	2.8 ± 0.4	3.1 ± 0.6	2.6 ± 0.8
No. Of leaves in LL	14 ± 0.8	11.7 ± 1.5	14.25 ± 0.5	12.5 ± 0.6
No. of leaves in HL	11.5 ± 1.8	10.7 ± 1.2	11.7 ± 3.5	9.3 ± 1.6

Table 3.2 Rosette diameter and leaf number of low- and high-light grown plants. Data shown are the means ± standard deviation.

3.3 Leaf pigment composition and LHCII abundance remain unaltered in mutants

Effects of the mutations on leaf pigment composition were studied by HPLC (Table 3.3). In general, no dramatic alteration in thylakoid pigments was found; however, all mutants exhibited a slight decrease in the level of lutein, ß-carotene and total chlorophyll (Chl *a* + *b*) compared to WT plants. Only in *stn7* and *stn7 stn8* mutants, further decreases in the level of neoxanthin were detected (Table 3.3).

To determine whether the lack of one the kinases was affecting the accumulation of LHCII, the abundance of the most abundant antenna proteins (Lhcb1 and Lhcb2) was studied. Because photosynthetic function is depend-

ent on chlorophylls, and because the difference in chlorophyll content among the genotypes was not significant, protein levels were normalized based on chlorophyll content. No major differences were found between the genotypes for Lhcb1 and Lhcb2 proteins (Figure 3.10).

Leaf pigments	*tak1*	*tak2*	*tak3*	*tak2 tak3*	*stn7*	*stn8*	*stn7 stn8*	WT
Nx	40 ± 4	45 ± 2	44 ± 2	40 ± 2	39 ± 2	43 ± 5	35 ± 3	47 ± 8
VAZ	34 ± 3	38 ± 2.1	36 ± 2	39 ± 4	37 ± 2	38 ± 5	39 ± 4	39 ± 7
Lu	150 ± 15	156 ± 14	159 ± 8	145 ± 5	140 ± 5	149 ± 24	126 ± 15	169 ± 28
ß-Car	203 ± 18	190 ± 25	211 ± 9	203 ± 6	193 ± 11	186 ± 33	191 ± 19	223 ± 27
Chl *a*/*b*	3.09 ± 0.03	2.80 ± 0.24	3.01 ± 0.06	3.41 ± 0.07	2.91 ± 0.10	2.87 ± 0.14	3.42 ± 0.07	2.99 ± 0.10
Chl *a* + *b*	1281 ± 108	1332 ± 120	1388 ± 56	1400 ± 47	1237 ± 47	1267 ± 191	1267 ± 123	1435 ± 219

Table 3.3 Pigment composition of leaves from mutants compared to WT. Pigment content was determined by HPLC of four plants for each genotype. The pigment content is given as nmol per g FW. Mean values ±SD are shown. Nx, neoxanthin; VAZ, xanthophyll cycle pigments (violaxanthin + antheraxanthin + zeaxanthin) ; Lu, lutein ; ß-Car, ß-carotene.

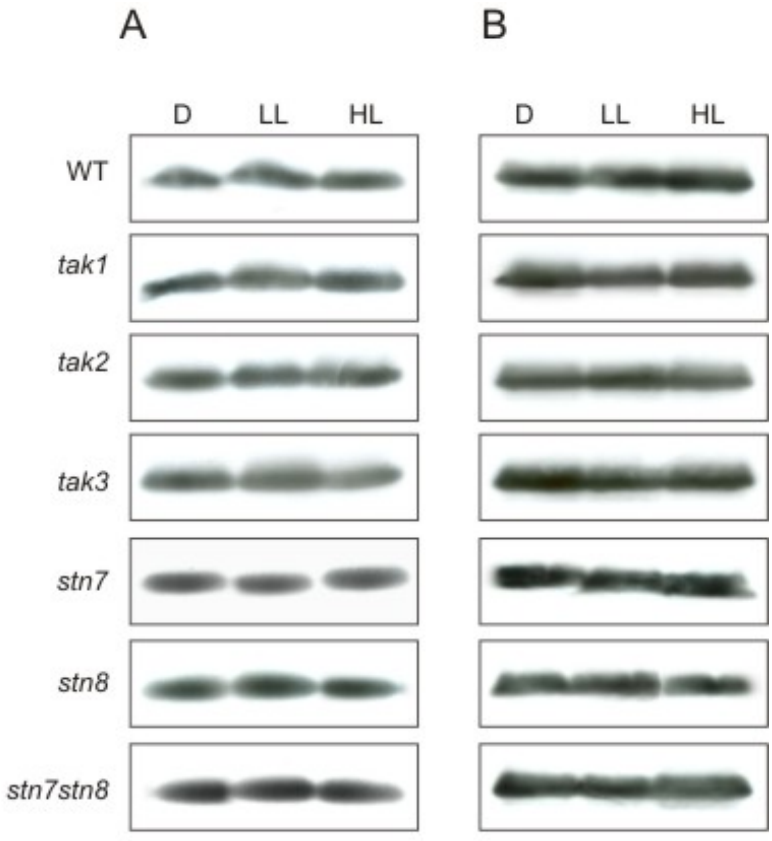

Figure 3.6 Accumulation of LHCII in WT and mutant plants. Immunoblot analysis of Lhcb1 (**A**) and Lhcb2 (**B**) proteins were performed on thylakoids from dark-adapted plants (D), subsequently exposed to low light (LL), and then to high light (HL).

3.4 STN7 and STN8 but not TAK2 and TAK3 are targeted to the chloroplasts

To further analyze the subcellular localization of the protein kinases, *in-vitro* and *in-vivo* targeting experiments were performed. *In-vitro* translated [^{35}S]-labeled proteins were incubated with isolated chloroplasts (Figure 3.7A).

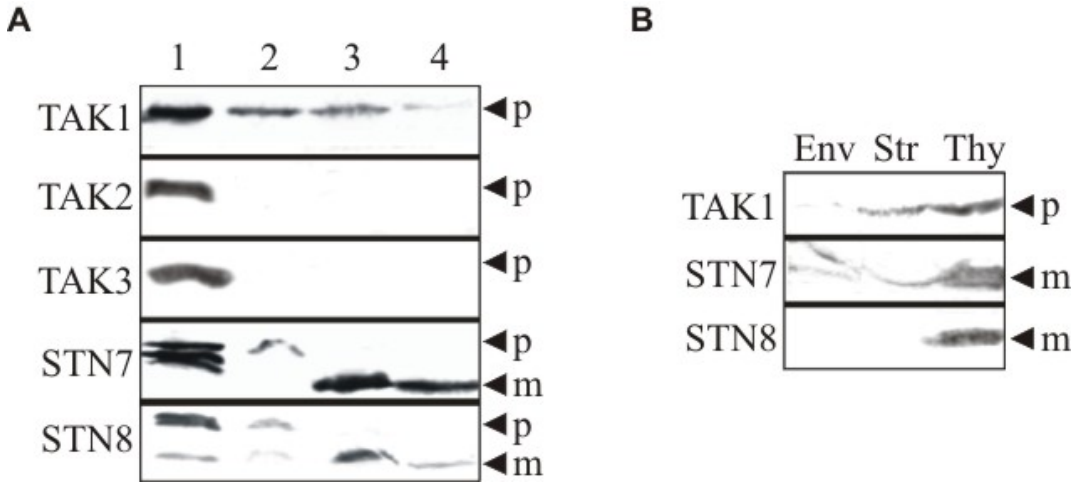

Figure 3.7 *In-vitro* import in pea chloroplasts**.** (**A**) *In-vitro* translated [^{35}S]-methionine-labeled proteins (10% translation product is shown for comparison in lane 1) were incubated with chloroplasts (20 µg chlorophyll, lane 2-4) for 20 min at 4°C (lane 2) or 25°C (lane 3, 4) followed by re-isolation through 40% Percoll. Chloroplasts were incubated with thermolysin (20 µg, lane 4), re-isolated and subjected to SDS-PAGE followed by visualization by autoradiography. "p" stands for precursor and "m" for mature protein. (**B**) Chloroplasts were sub-fractionated after import of *in-vitro* translated [^{35}S]-methionine-labeled proteins. Env, envelope; Str, stroma; Thy, thylakoids.

Only for TAK1, STN7 and STN8, but not for TAK2 and 3, an association to the chloroplast was detected. Post-treatment of the plastids with thermolysin resulted in the removal of the precursor only for STN7 and STN8 (Figure 3.7A, lane 4). Import of TAK1 was also observed in this assay, but it appeared to be less efficient than the one of the Stt7 homologues and it was not associated with the removal of a transit peptide (Figure 3.7A), in agreement with previous data obtained by *in-vitro* assays with isolated Arabidopsis chloroplasts (Snyders and Kohorn, 1999). Sub-fractionation of chloroplasts after import of *in-vitro* translated [^{35}S]-methionine labeled proteins revealed that TAK1, STN7 and STN8 are associated with the thylakoids (Figure 3.7B).

For both TAK1 and STN7, a weak signal was also obtained in the stroma fraction.

To investigate *in-vivo* the subcellular localization of the protein kinases, a transient expression assay in tobacco protoplasts was first employed (Figure 3.8).

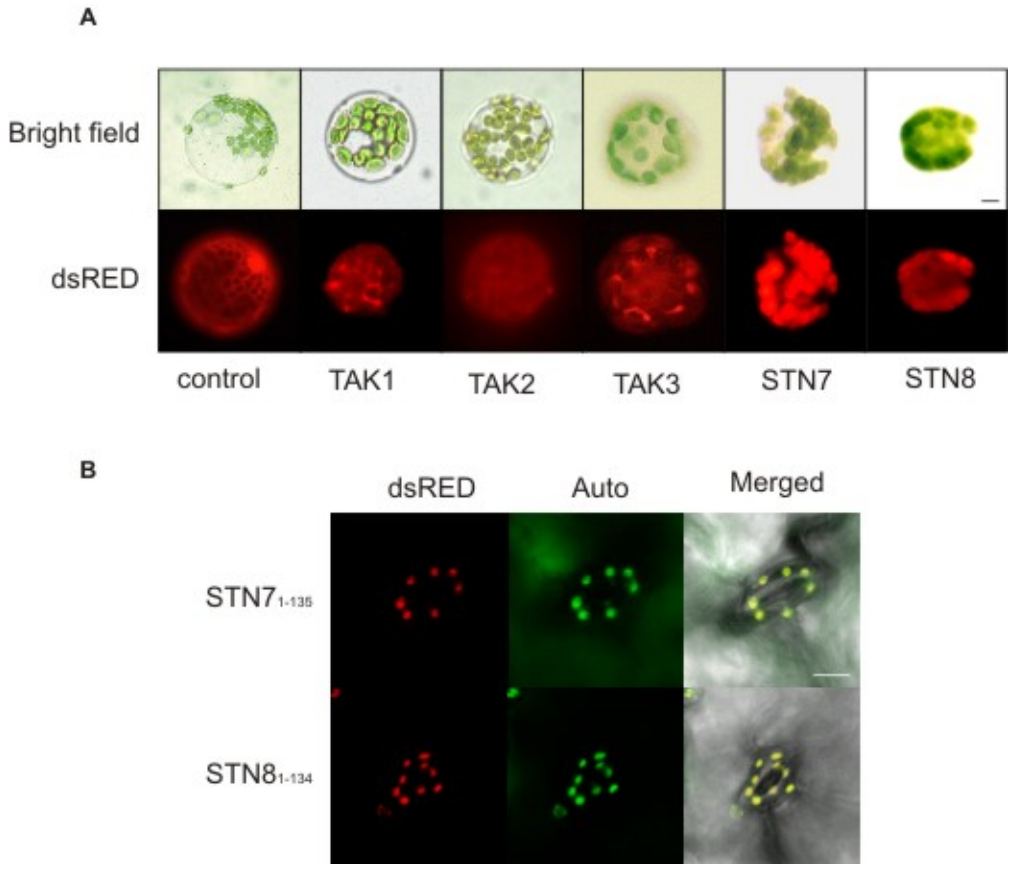

Figure 3.8 *In-vivo* localization of the protein kinases. (**A**) Tobacco protoplasts were transiently transfected by polyethylene glycol-mediated DNA uptake with the fusion constructs of the full kinase coding sequence with dsRED, or a construct encoding the dsRED protein without fusion ("control"), and analyzed using fluorescence microscopy. In the lower panels, the red fluorescence of the fusion proteins is shown; in the upper ones the bright field view. Scale bar, 10 µm. (**B**) The $STN7_{1-135}$- and $STN8_{1-134}$-dsRED fusion were stably introduced into *stn7* and *stn8* plants respectively. Guard cells were analyzed by confocal laser microscopy. In the left panels, dsRED fluorescence identifying the fusion protein is displayed; in the middle ones, the chloroplasts are revealed by chlorophyll fluorescence (in false color); in the right ones, the merged images are shown. Scale bar, 50 µm.

The full-length coding sequence of each kinase was fused upstream to the *dsRED* reporter gene (Jach et al., 2001) and used to transfect *Nicotiana benthamiana* protoplasts. Again, chloroplast localization was readily de-

tected for the STN7 and STN8 (Figure 3.8A). None of the three *TAK-dsRED* fusions, however, was imported into chloroplasts. As a control, tobacco protoplasts were transfected with *dsRED* and the reporter protein could be localized in the cytosol and, because of its small size, also in the nucleus.

In addition, *Arabidopsis* plants were stably transformed with a construct harboring the amino-terminal region of STN7 and STN8 fused to the dsRED protein under the transcriptional control of the cauliflower mosaic virus 35S promoter. *stn7* plants were transformed with the $STN7_{1-135}$-dsRED fusion construct, whereas *stn8* mutants were transformed with the $STN8_{1-134}$-dsRED construct (Figure 3.8B). *In-vivo* subcellular localization showed that the amino-terminal regions of both STN7 and STN8 are sufficient to trigger the import of the dsRED protein in *Arabidopsis*.

3.5 Only in *stn7* state transitions are suppressed

Photosynthetic electron flow and state transitions were characterized by measuring parameters of chlorophyll fluorescence (Table 3.4). In all mutants, the maximum quantum yield (F_V/F_M) and the effective quantum yield (Φ_{II}) of PSII were similar to the WT. Also, 1-qP, the fraction of Q_A (the primary electron receptor of PSII) present in the reduced state, and qN, the non-photochemical quenching, were not altered in any of the mutants. This indicates that loss of function of any of the five kinases does not result into a marked perturbation of photosynthetic electron flow.

State transitions in WT and mutant plants were followed by measuring maximum PSII fluorescence signals in state 1 (F_M1) and state 2 (F_M2), after irradiating plants at wavelengths that target PSII and PSI, respectively, and normalizing these values to the maximum PSII fluorescence of dark-adapted leaves (F_M). In the WT, F_M1/F_M and F_M2/F_M differed significantly (0.86 ± 0.01 versus 0.93 ± 0.01), while of the mutants only *stn7* exhibited a different behavior. Here, both values were essentially the same (0.89 ± 0.01 versus 0.90 ± 0.01). This corresponds to a reduction of more than 90% in qT in the mu-

tant (0.11 ± 0.02 in the WT, and 0.01 ± 0.00 in *stn7*), indicating a severe impairment in the redistribution of excitation energy between the photosystems in this genotype.

Parameter	*tak1*	*tak2*	*tak3*	*tak2tak3*	*stn7*	*stn8*	*stn7stn8*	WT
F_V/F_M	0.81 ± 0.01	0.80 ± 0.01	0.82 ± 0.01	0.84 ± 0.01	0.82 ± 0.07	0.83 ± 0.00	0.84 ± 0.01	0.81 ± 0.02
Φ_{II}	0.78 ± 0.01	0.77 ± 0.02	0.76 ± 0.03	0.77 ± 0.01	0.76 ± 0.01	0.77 ± 0.01	0.76 ± 0.01	0.77 ± 0.01
1-qP	0.02 ± 0.01	0.02 ± 0.02	0.04 ± 0.01	0.04 ± 0.00	0.04 ± 0.02	0.02 ± 0.01	0.05 ± 0.01	0.03 ± 0.02
F_M1/F_M	0.96 ± 0.01	0.95 ± 0.00	0.98 ± 0.01	0.93 ± 0.01	0.89 ± 0.01	0.94 ± 0.02	0.88 ± 0.03	0.86 ± 0.01
F_M2/F_M	0.88 ± 0.01	0.86 ± 0.01	0.89 ± 0.01	0.83 ± 0.02	0.90 ± 0.01	0.87 ± 0.01	0.88 ± 0.03	0.93 ± 0.01
$(F_M1-F_M2)/F_M2$	0.09 ± 0.01	0.11 ± 0.02	0.12 ± 0.01	0.12 ± 0.03	0.01 ± 0.00	0.10 ± 0.01	0.00 ± 0.01	0.11 ± 0.02

Table 3.4 Spectroscopic data for mutants and WT leaves. Mean values for five plants (± SD) are shown. For the mutants, only the data for one of the two independent knock-out alleles are listed; in both cases the second allele behaved similarly. Note that the differences in 1-qP between genotypes lacking STN7 (*stn7* and *stn7 stn8*) and WT plants are not statistically significant.

3.6 PSII core protein phosphorylation requires STN7 and STN8

Reversible LHCII phosphorylation, which is associated with state transitions, was studied *in-vivo* in dark-adapted WT and mutant plants incubated with [^{33}P]-orthophosphate, and then exposed to different light conditions (Figure 3.9A). In WT and mutant leaves, but not in *stn7*, low-light treatment caused a marked increase in the phosphorylation of LHCII, whereas subsequent exposure to high-intensity light reduced the amount of phosphorylated LHCII. Only when STN7 was missing, however, reversible LHCII phosphorylation was not detectable in any of the illumination conditions tested. When phosphorylated LHCII was monitored using a phosphothreonine-specific antibody, only very weak and constant signals were found in *stn7* plants (Figure 3.9B), while the other mutants behaved like WT. Moreover, determination of LHCII phosphorylation with an *in vitro* assay (performed only on *stt7*-like mutants) in conditions under which the LHCII kinase should be maximally active (Figure 3.9C), showed that only the loss of STN7 function suppresses the phosphorylation of LHCII. These results together with the spectroscopic data, indicate not only that STN7 kinase is specifically involved in LHCII phosphorylation and its loss blocks state transition (confirming other results;

Bellafiore et al., 2005), but also that STN7 and STN8 do not act in series in this function.

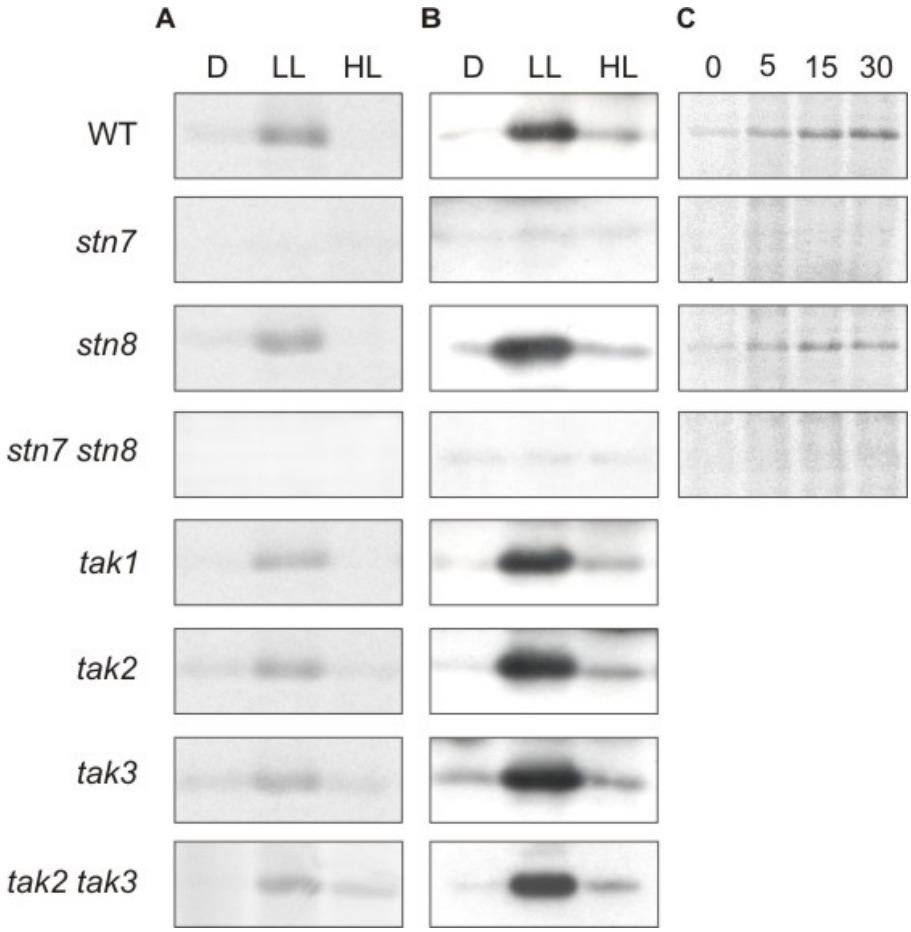

Figure 3.9 Phosphorylation of LHCII. (**A**) *In vivo* assay. Leaves from WT and mutants were incubated with [^{33}P]-orthophosphate and thylakoid membrane proteins were extracted in the dark (D), after exposure to low light (LL), and after subsequent exposure to high light (HL). Proteins were then fractionated by SDS-PAGE and autoradiographed. (**B**) Immunoblot analysis. Leaves were treated and proteins fractioned as in **A** before being immunobotted with the phosphothreonine-specific antibody. (**C**) *In vitro* assay. Thylakoids from dark-adapted leaves were incubated with [γ-^{33}P]ATP under reducing conditions for 0, 5, 15 and 30 min in the dark. Proteins were fractionated as in **A**.

In addition, when phosphorylation of the PSII core proteins was monitored with a phosphothreonine-specific antibody (Figure 3.10), *stn7* plants exhibited the same phosphorylation pattern shown in the WT, showing high levels of phosphorylated PSII core proteins under all light regimes tested, whereas LHCII phosphorylation was greatly decreased. However, plants lacking STN8 showed a marked decrease in the total amount of PSII core phospho-

proteins, in particular under high-light, whereas LHCII phosphorylation was like in the WT.

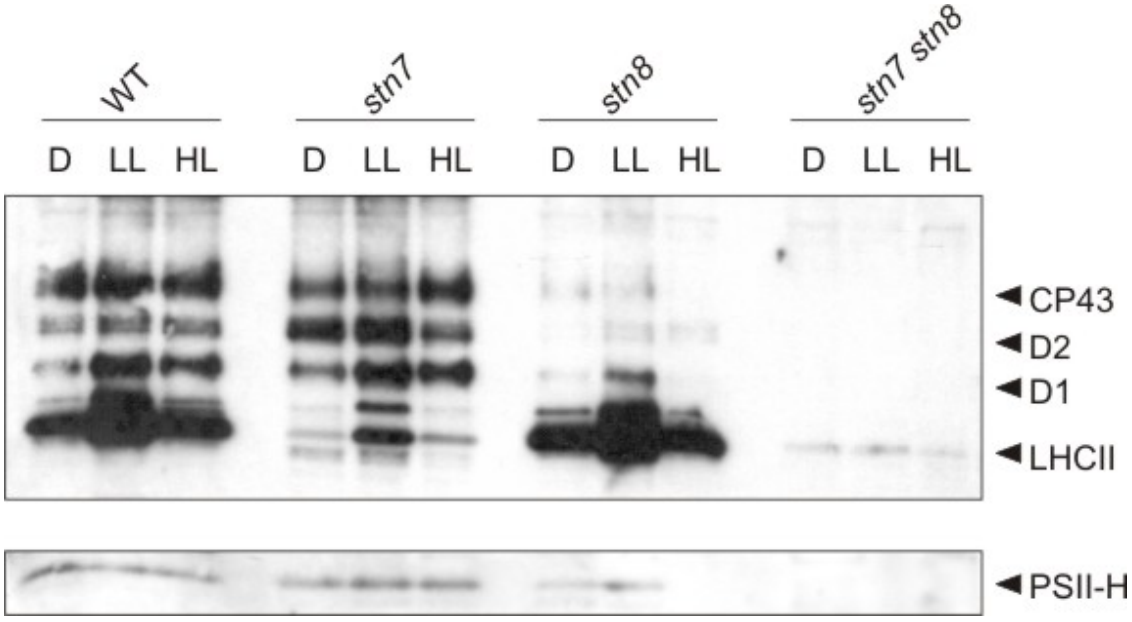

Figure 3.10 Phosphorylation of thylakoid membrane proteins. Plants were dark-adapted (D), subsequently exposed to low light (LL), and then to high light (HL). Thylakoid membrane proteins were isolated and fractionated by SDS-PAGE. PSII and LHCII phosphoproteins were detected by immunoblot analysis with an anti-phosphothreonine antibody.

In *stn7 stn8* the LHCII and PSII core protein phosphorylation was completely absent under all light conditions examined, indicating that the two kinases must have some degree of overlap in their substrates specificities. On the other hand, the existence of two kinases, one for LHCII and another one for PSII core proteins with distinct redox regulation systems, is supported by several observations made both *in-vivo* and *in-vitro*. LHCII phosphorylation, but not PSII core protein phosphorylation, was shown to be inhibited in Cyt b_6/f deficient mutants (Bennett et al., 1988; Vener et al., 1995). In addition, PSII and LHCII protein phosphorylation was found differentially regulated by ambient light conditions *in-vivo* (Ebbert and Godde, 1994; Rintamäki et al., 1997). The results shown here are the first evidence that, indeed, only two kinases are cooperatively phosphorylating all the PSII proteins, where STN7 phosphorylates preferentially LHCII, while STN8 is acting more specifically on PSII core proteins.

The complete absence of PSII core protein phosphorylation in *stn8* mutants under high light conditions supports the idea that STN7 and STN8 have different modes of regulation. Under high light, in fact, STN7 is subjected to redox inactivation by a thioredoxin (Rintamäki et al, 2000) and therefore cannot phosphorylate PSII proteins in *stn8* background. However, a residual LHCII phosphorylation by STN7 is observed also under high-light irradiances, indicating that its regulation might be more complex.

Moreover, differences in the phosphorylation level were not attributable to a decrease in PSII core protein content, as revealed by polyacrilamide-gel electrophoresis (PAGE) analysis (Figure 3.11).

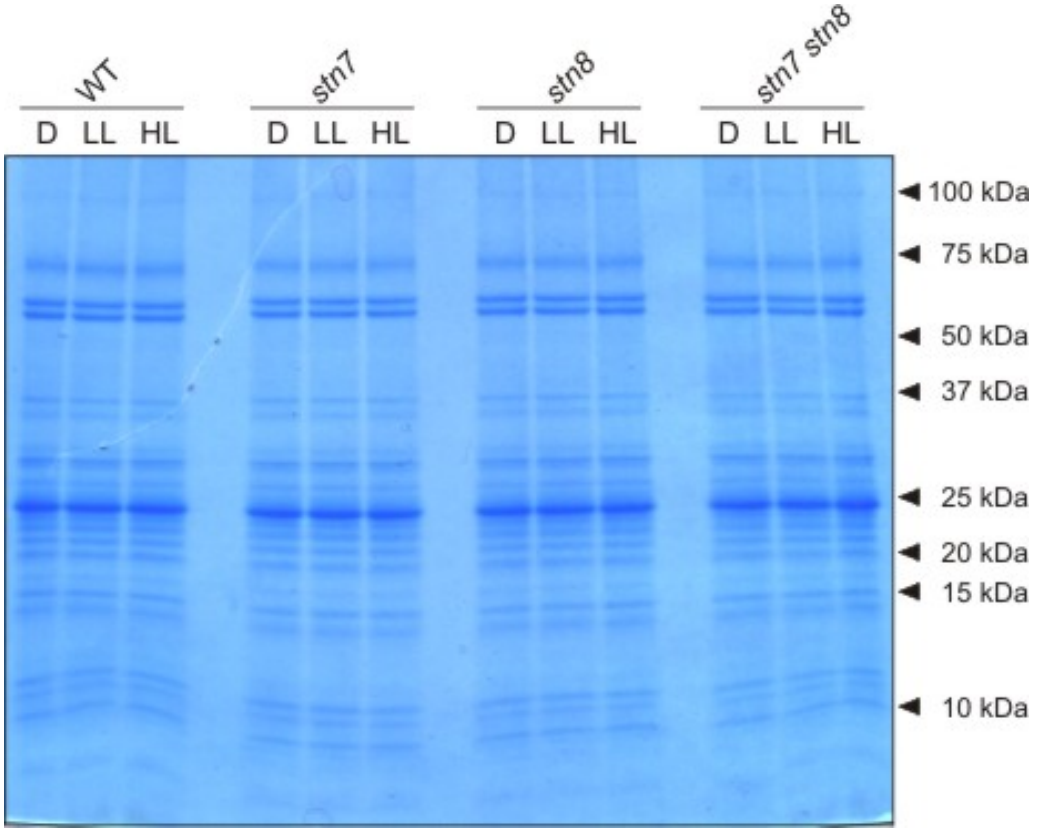

Figure 3.11 Coomassie-staining of a representative protein gel, from which immunoblot displayed in Figure 3.10 was derived.

3.7 PSII activity during high light stress is unaffected in *stn7* and *stn8* mutants

Reversible phosphorylation of the PSII core proteins has been proposed to have a regulatory function during photoinhibition of PSII (Baena-González et al., 1999). In particular, prolonged light exposure induces phosphorylation of, and causes damage to, PSII core proteins, although the phosphorylated form of damaged D1 is resistant to proteolysis. Phosphorylated PSII polypeptides are supposed to act in the translocation of the damaged complex from the grana thylakoids to the stroma lamellae. To test if indeed suppression of PSII core phosphorylation is affecting PSII repair by changing the rate of D1 turnover, the inactivation of PSII under high light exposure, the recovery of PSII activity and the degradation of D1 were investigated. The maximum efficiency of PSII photochemistry, measured as the fluorescence parameter F_v/F_M, was monitored during the photoinhibitory treatment (Figure 3.12). Illumination of leaf discs at high light intensity (2000 µmol photons m^{-2} s^{-1}) led to a slightly stronger inactivation of PSII in *stn8* and *stn7 stn8* plants than in *stn7* and WT plants. Recovery of PSII efficiency in a subsequent low light phase (20 µmol photons m^{-2} s^{-1}) was also somewhat retarded in *stn8* and *stn7 stn8* after prolonged high light treatment for 4 h, when compared to *stn7* and WT plants (Figure 3.12A).

In presence of streptomycin, an inhibitor of plastid protein synthesis and, by that, of the PSII repair cycle, PSII was completely inactivated after 5 h of high light exposure in all the genotypes (Figure 3.12B). Moreover, no recovery of PSII activity occurred during subsequent low light treatment for 16 h (data not shown). The removal of differences in PSII activity among the genotypes after treatment with streptomycin indicates that D1 turnover could be, indeed, responsible of the slight differential sensitivity of *stn8* and *stn7 stn8* in comparison to WT and *stn7* plants. To test this, the degradation rate of the D1 protein was determined by immunoblot analysis.

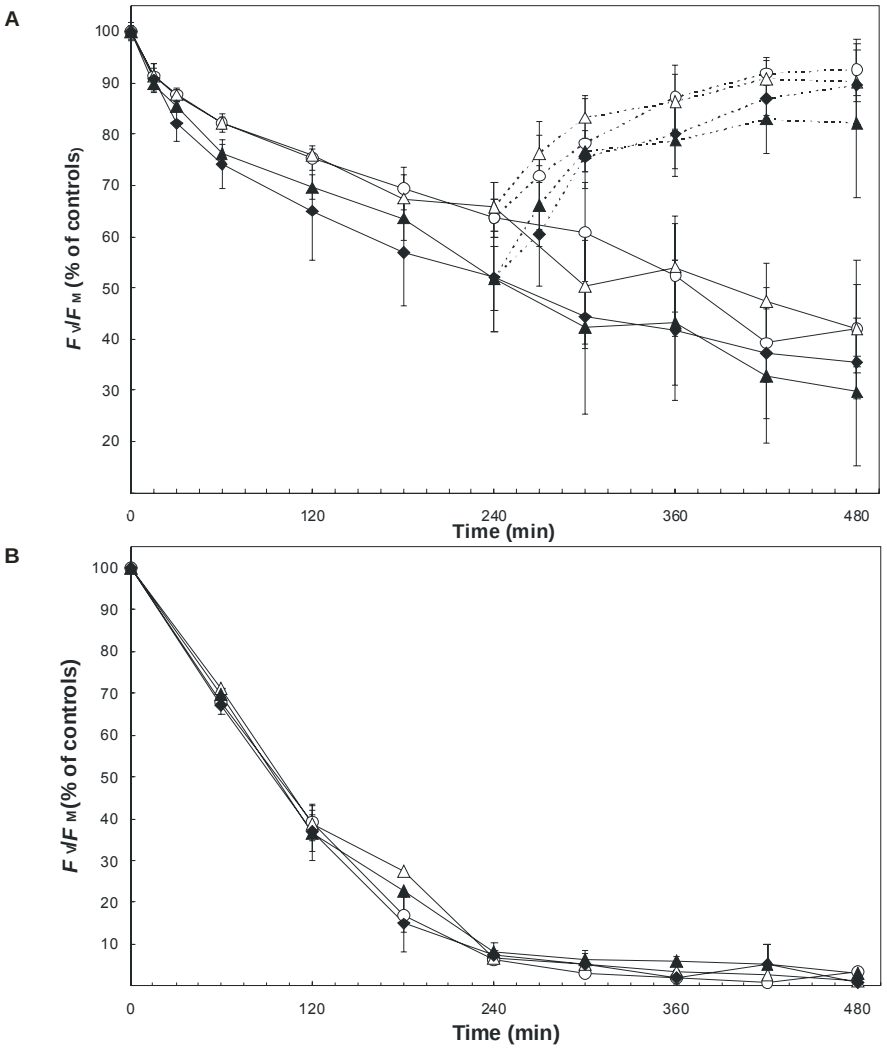

Figure 3.12 PSII activity under high intensity light in WT and mutant leaves. (**A**) Time course of high light induced PSII inactivation (solid lines) and recovery under low light (dashed lines). Leaf discs were floated on water (20°C) and illuminated at a PFD of 2000 µmol photons m^{-2} s^{-1}. For the recovery experiment, leaves were transferred to low light (20 µmol photons m^{-2} s^{-1}) after 4 h of high light treatment. (**B**) Time course of high light induced photoinactivation in presence of streptomycin. Leaf discs were floated on a solution containing 3 mM streptomycin. Prior to high ligh exposure at a PFD of 2000 µmol photons m^{-2} s^{-1}, the uptake of streptomycin was performed during 30 min of illumination at low light, followed by 60 min dark incubation. Error bars indicate standard deviation. Open triangles, *stn7*; filled triangles, *stn8*; diamonds, *stn7 stn8*; circles, wild type.

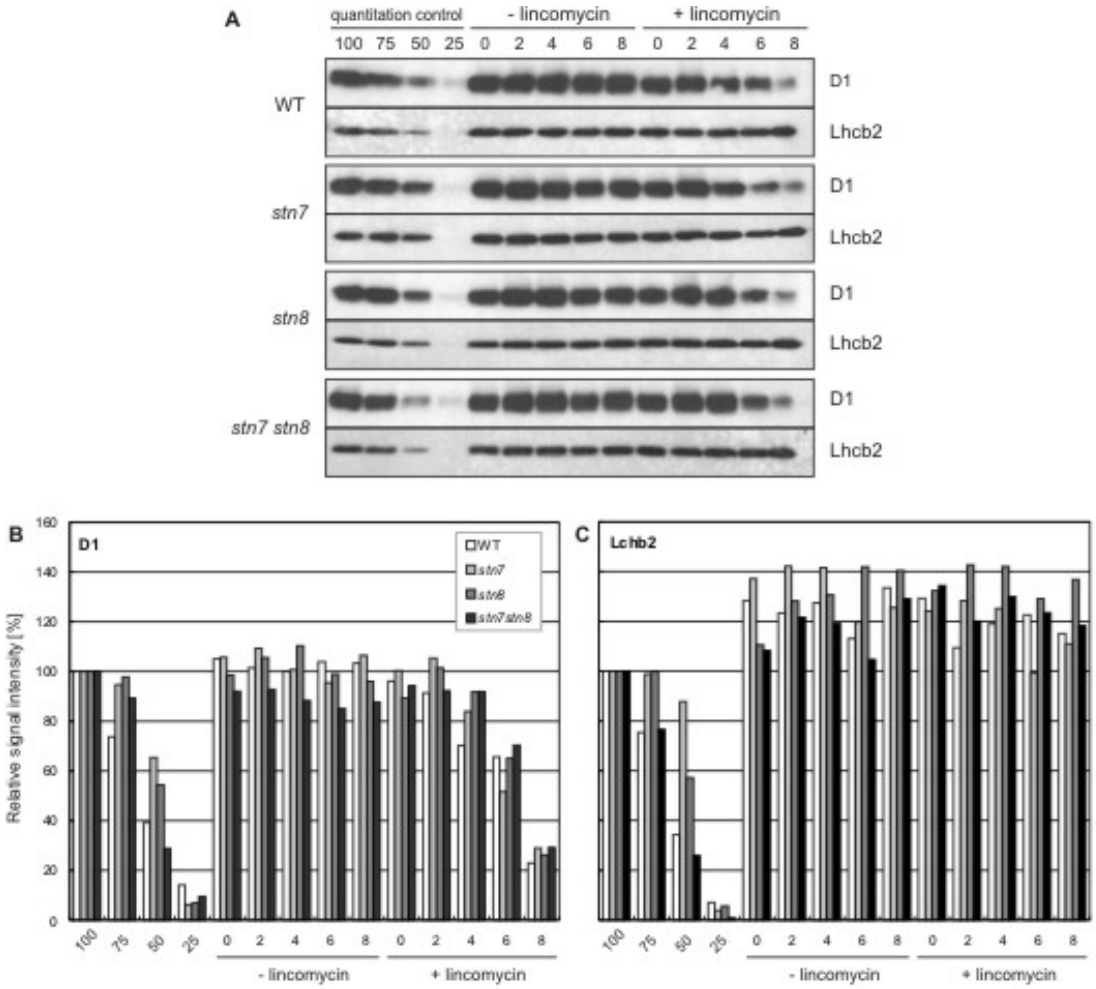

Figure 3.13 Immunoblot analysis of D1 protein upon exposure to high-intensity light. (**A**) Leaf discs of WT, *stn7*, *stn8* and *stn7 stn8* plants were incubated for 90 min in the absence or presence of 1 mM lincomycin to inhibit chloroplast protein synthesis. During the first 30 min of incubation, leaf discs were illuminated with 20 µmol photons m^{-2} s^{-1} and then kept in the dark for 1 h. Immediately after illumination with high light (2000 µmol photons m^{-2} s^{-1}) for the indicated time (0, 2, 4, 6 and 8 h), thylakoid proteins (equivalent to 100 mg fresh leaf) were extracted, loaded in each lane and probed with antibodies raised against the D1 protein or Lhcb2 (as control). Decreasing amounts of thylakoid proteins (corresponding to 1, 0.75, 0.5 and 0.25 µg of chlorophyll) were loaded in the first four lanes (100, 75, 50 and 25), allowing to control that the signals being measured were within the linear range of detection. (**B**-**C**) Signals were quantified (LumiAnalyst, Boehringer) and the results of quantitation are displayed. Signals obtained for samples corresponding to 1 µg of chlorophyll (lane "100") were set as 100% relative signal intensity.

In agreement with earlier studies (Kettunen et al., 1991), in absence of a protein synthesis inhibitor only minor degradation of the D1 protein was found (Figure 3.13A). In contrast, after incubation of leaf discs with lincomycin, a chloroplast-encoded protein synthesis inhibitor, and illumination for 8 h at

high-light intensity (2000 µmol photons m^{-2} s^{-1}), D1 was nearly completely degraded. However no significant differences were observed among the mutant plants in comparison to the WT, neither in the presence nor in the absence of lincomycin (Figure 3.13B-C). These results together indicate that the increased photosensitivity of PSII in *stn8* and *stn7 stn8* mutants was not reflected in changes in the rate of light induced D1 degradation.

To exclude that a third PSII core protein kinase is active during photoinhibition and subsequent recovery, the phosphorylation state of PSII core and LHCII was monitored under these conditions (Figure 3.14). In both cases no PSII phosphoproteins were detected in *stn7 stn8* plants.

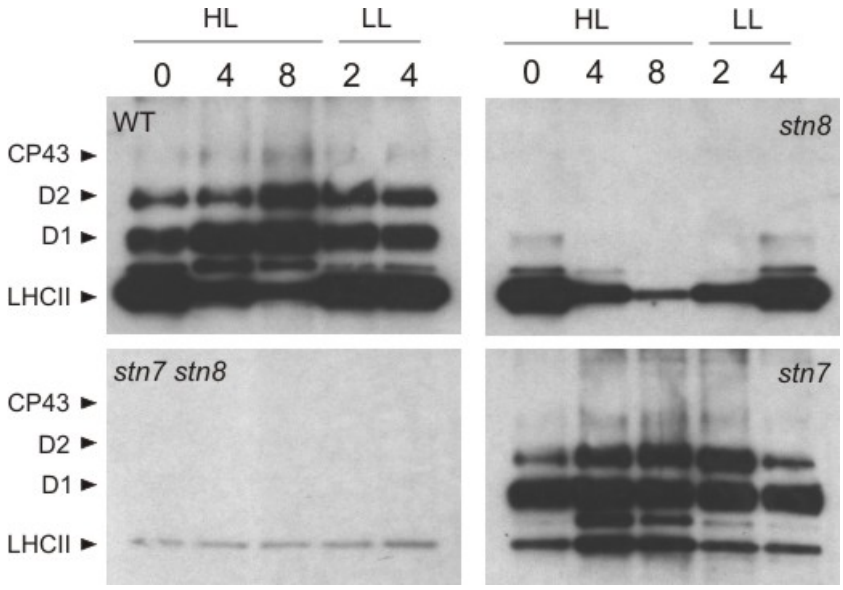

Figure 3.14 Phosphorylation of thylakoid proteins during photoinhibition and subsequent recovery. For each time point, three leaf discs were exposed to high light (HL; 2000 µmol photons m^{-2} s^{-1}) for up to 8 h. For the measurement of thylakoid phosphorylation during recovery from photoinhibition, leaf discs were exposed for up to 4 h to low light (LL; 20 µmol photons m^{-2} s^{-1}) after 4 h of high light. Phosphorylation was detected by immunoblot analysis with a phosphothreonine-specific antibody as described in Figure 3.10. Note that *stn7 stn8* film was over-exposed.

To learn more about the rates of D1 synthesis and degradation during photoinhibition, pulse-chase experiments were performed at high light. Leaf discs from all genotypes were pulse-labeled with [^{35}S]-methionine under photoinhibitory conditions for up to 60 min, and subsequently subjected to chase experiment for additional 480 min (Figure 3.15A). No differences in

the rate of D1 synthesis were observed during the pulse period in comparison to WT plants in which, after 60 min, D1 protein was heavily labeled. Similarly, decreases in the radioactivity of pulse-labeled D1 protein during the chase time were identical in all the tested genotypes (Figure 3.15B).

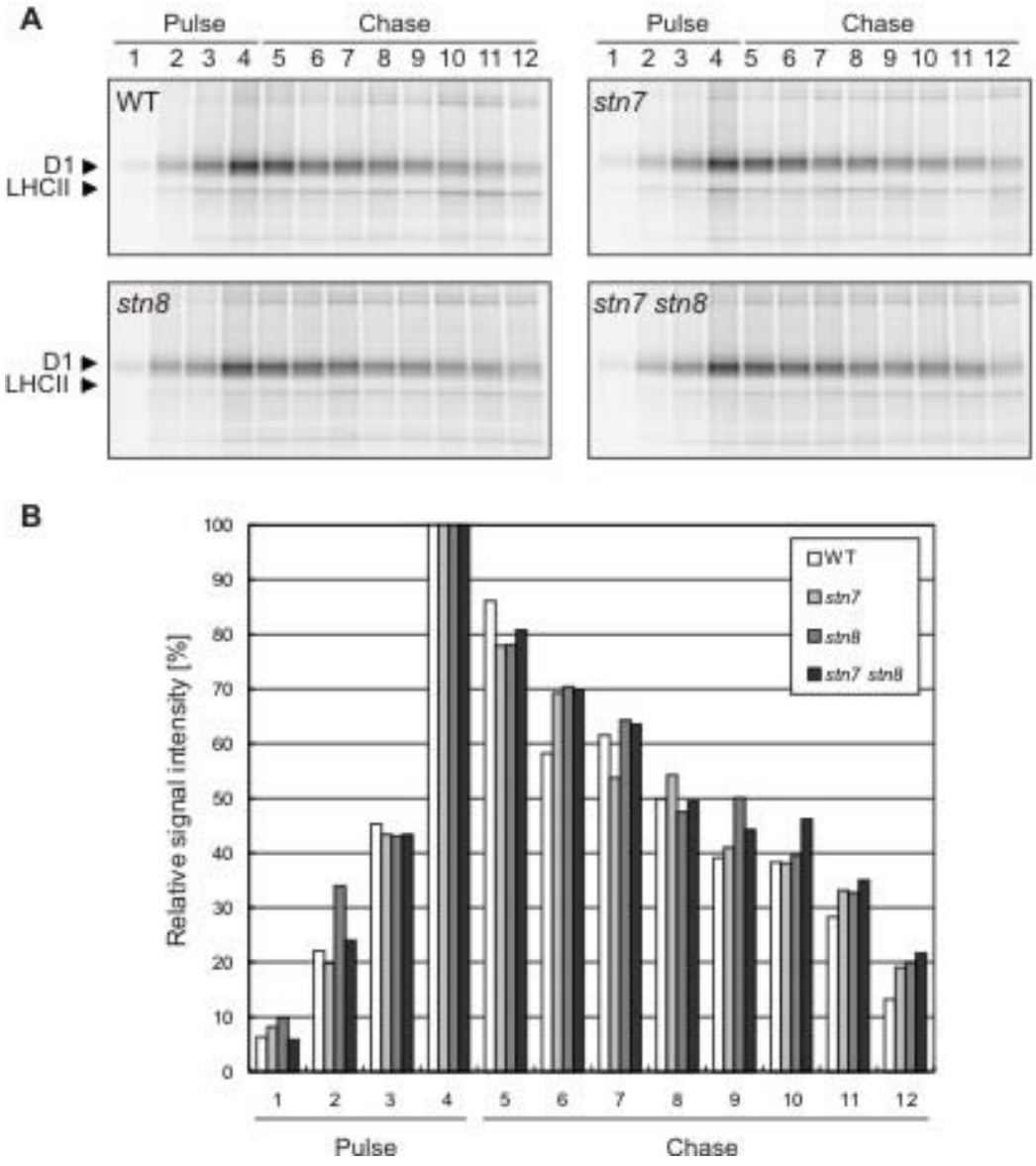

Figure 3.15 D1 turnover under high intensity light. (**A**) Autoradiogram of thylakoid membrane proteins resolved by SDS-PAGE after pulse-labeling with [^{35}S]-methionine for 0, 15, 30 and 60 min (lanes 1-4) and subsequent chase in unlabeled medium for 60, 120, 180, 240, 300, 360, 420 and 480 min (lanes 5-12), both under high light (2000 µmol photons m^{-2} s^{-1}). (**B**) Signals were quantified as in Figure 12. Because signals obtained after 60 min of pulse (lane 4) were almost identical in all genotypes tested, these signal intensities were set as 100% relative signal intensity.

3.8 STN7 is required for acclimation to long term changes in light quality

Photosynthetic organisms acclimate to long term changes in the environmental light quality by an adjustment of their photosystem stoichiometry to maintain photosynthetic efficiency (Pfannschmidt et al., 2001). This, in turn, requires a signaling network that coordinates photosynthetic gene expression in plastids and nucleus (Pfannschmidt, 2003). Moreover, recent studies suggested that state transitions and photosystem stoichiometry adjustment may operate coordinately, and may both be triggered by changes of redox state of interphotosystem electron carriers (Allen and Pfannschmidt, 2000).

To test if lack of STN7 or STN8 was affecting the acclimation ability to changes in light quality, and therefore the readjustment of antenna structure and photosystem stoichiometry, changes in chlorophyll *a*/*b* ratios and *in vivo* chlorophyll *a* fluorescence (indicated as Chl *a*/*b* and the parameter F_S/F_M, respectively) were monitored. In higher plants, in fact, chlorophyll *a* is found in both photosystems, whereas chlorophyll *b* is mostly confined to PSII. An increase in the stoichiometry of PSII to PSI hence produces a decrease in the ratio of chlorophyll *a*/*b*. Seedlings of WT and mutant plants were grown for 6 days under each photosystem-selective light regime (PSI or PSII light), or acclimated for 2 days to PSI light followed by 4 days under PSII light, or *vice versa*. In WT plants acclimation to PSII light, as well as transfer from PSI to PSII light, caused an increase in Chl *a*/*b* ratio as expected for a decrease in PSII to PSI ratio, whereas acclimation to PSI light, together with transfer from PSII light to PSI light, caused a decrease in Chl *a*/*b* ratio, consistent with an increase in PSII to PSI (Figure 3.16B). On the other hand, WT plants acclimated to PSI light (or transferred from PSII to PSI light) showed a higher level in F_S/F_M when compared with PSII light acclimation (or after transfer from PSI to PSII light), confirming differences in the photosynthetic electron transport efficiency, which result from the different acclimation (Figure 3.16A). The *stn8* mutant responded to the light treatments like the WT, indicating that the photosynthetic acclimation was not impaired. However, in

the *stn7* and *stn7 stn8* mutants both F_S/F_M and Chl *a*/*b* ratio were typical of plants acclimated to PSI light under all light regimes tested, suggesting that the mutants lost their ability to acclimate to changes in light quality. This implies that STN7 coordinates the short and long term response to changes in light conditions.

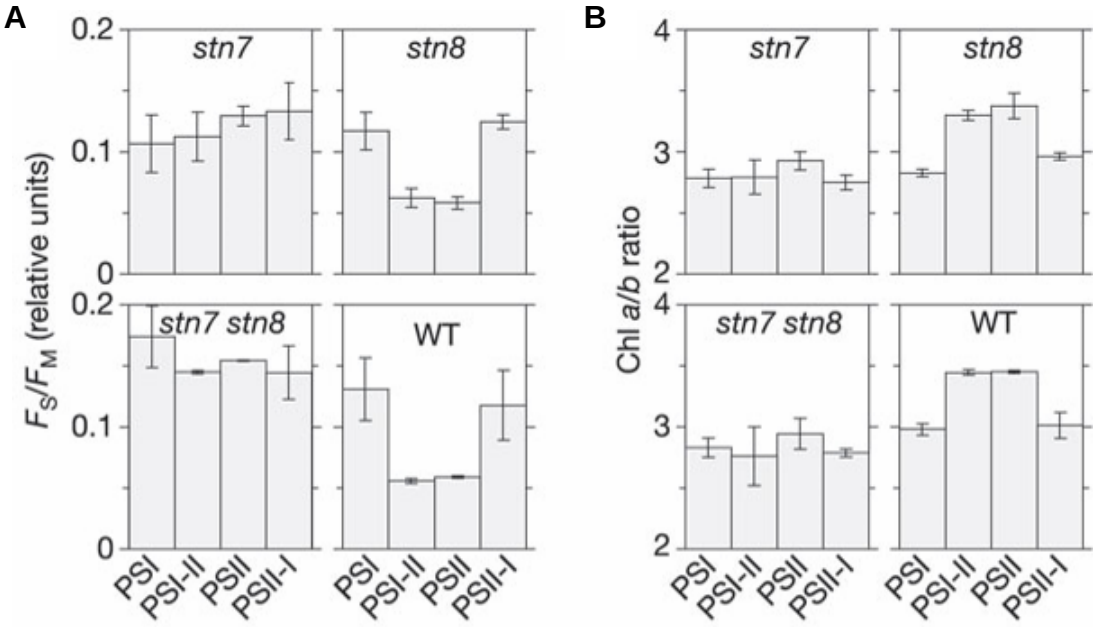

Figure 3.16 Photosynthetic acclimation to PSI and PSII light sources. Seedlings were acclimated for 6 days to PSI or PSII light (indicated as PSI and PSII respectively), or grown for 2 days under PSI light followed by 4 days under PSII light (PSI-II), or *vice versa* (PSII-I). F_S/F_M values (**A**) and Chl *a*/*b* ratios (**B**) were monitored. Error bars indicate standard deviation.

Recent reports suggested that expression of nuclear (Pfannschmidt et al., 2001) and chloroplast genes (Pfannschmidt et al., 1999a) is functionally linked to changes in photosynthetic efficiency during adjustment in photosystem stoichiometry. To test whether lack of STN7 impairs this signaling pathway, blocking the acclimation response to changes in light quality, the transcription of photosynthetic genes was studied. Greenhouse-grown *stn7* and *stn7 stn8* plants exhibited differential expression of only relatively few photosynthetic genes, in comparison to the WT, in which a large set of photosynthetic genes is markedly down-regulated (Figure 3.17A). A list of photosynthetic genes whose expression pattern is significantly different in the absence of STN7 with respect to the *stn8* mutant is provided in Supplementary

Table I and characteristic expression profiles of two nuclear genes and of two plastid genes are displayed in Figure 3.17B.

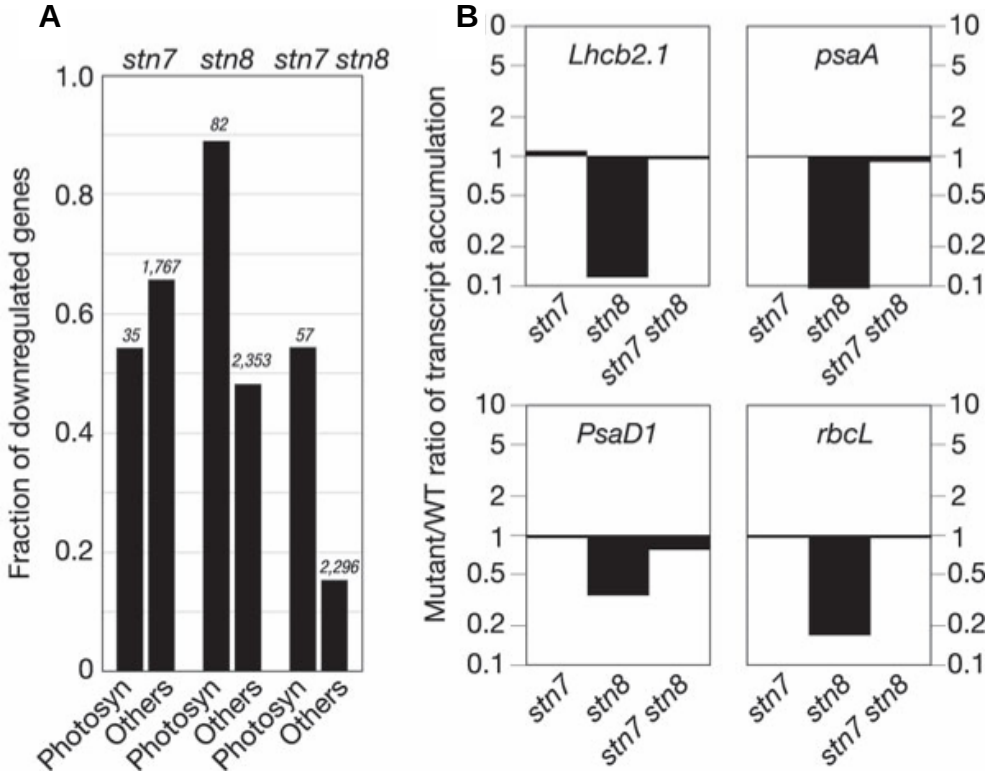

Figure 3.17 mRNA expression profiling. (**A**) Nuclear transcript accumulation in greenhouse-grown plants is shown. Numbers above bars indicate the total amount of genes that are significantly differentially expressed in mutants with respect to WT plants. Bar lengths indicate the fraction of differentially regulated genes that were downregulated. Photosyn, photosynthetic genes; others, other genes encoding non-photosynthetic proteins. Exemplary expression profiles of two nuclear (*Lhcb2.1* and *PsaD1*) and two plastid genes (*psaA* and *rbcL*) are displayed as mutant *versus* WT ratios of transcript levels (logarithmic scale) in **B**.

3.9 STN7 is required for the association of LHCII with PSI

State transitions involve the reorganization of LHCII within the thylakoid membranes to guarantee that both photosystems are excited at the same extent (reviewed by Allen, 1992). This process correlates with the phosphorylation state of LHCII and, thus, with the LHCII kinase activation (Allen and Nilsson, 1997). In low-light conditions the active state of STN7 prevails (Figure 3.10), inducing maximal phosphorylation of LHCII proteins *in vivo*. Higher irradiances result in a stable inactivation of the kinase that becomes

available for reduction by thioredoxin (Rintamäki et al., 2000). To test the ability of the mobile antenna complex to associate to PSI when STN7 or STN8 are missing, the supramolecular structure of photosystems was investigated by 2D BN/SDS-PAGE. The analysis was performed in dark-adapted plants, subsequently exposed to low-light irradiances (120 µmol photons m^{-2} s^{-1}; 2h), and then to high-light (1200 µmol photons m^{-2} s^{-1}, 2h). Thylakoids were solubilized in the presence of 1.5% digitonin, which proved to be a very suitable detergent for the solubilization and stabilization of supercomplexes of *Arabidopsis* mitochondria (Eubel et al., 2003, 2004). Thylakoid protein complexes were identified by comparison to previous separations of digitonin solubilized chloroplast protein complexes from *Arabidopsis* on the basis of 1D BN-PAGE (Heinemeyer et al., 2004). Under all the light regimes tested, LHCII failed to associate with PSI when STN7 was missing (Figure 3.18), leading to an accumulation of PSI-LHCI complex that, nevertheless, was not accompanied by a correspondent increase in the amount of trimeric LHCII. In contrast, *stn8* plants accumulated PSI-LHCII complex at the same extent as the WT plants. Even under prolonged higher irradiances (1200 µmol photons m^{-2} s^{-1}), when STN7 should be inactive and LHCII dephosphorylated, a small fraction of PSI was still associated with LHCII in *stn8* and WT plants. However, in dark conditions, LHCII failed to migrate to PSI in all the genotypes tested.

The complexes and the supercomplexes resolved on 1D BN-PAGE after solubilization of the thylakoid proteins by digitonin considerably differed in comparison to those resolved employing dodecylmaltoside (DM). In the latter case, in fact, PSI-LHCII complex was not detectable (data not shown), indicating that the association between LHCII and PSI is indeed weak and can be disrupted during solubilization with DM.

Moreover, blue-native gels were combined with SDS-PAGE to elucidate identities of the resolved complexes on the basis of subunit compositions (Figure 3.19). Only in *stn7* and *stn7 stn8*, no signals indicative for subunits of PSI-LHCI-LHCII complex could be observed (data are shown only for *stn7*).

This confirms that LHCII associates to PSI-LHCI complex, only when it is in the phosphorylated form.

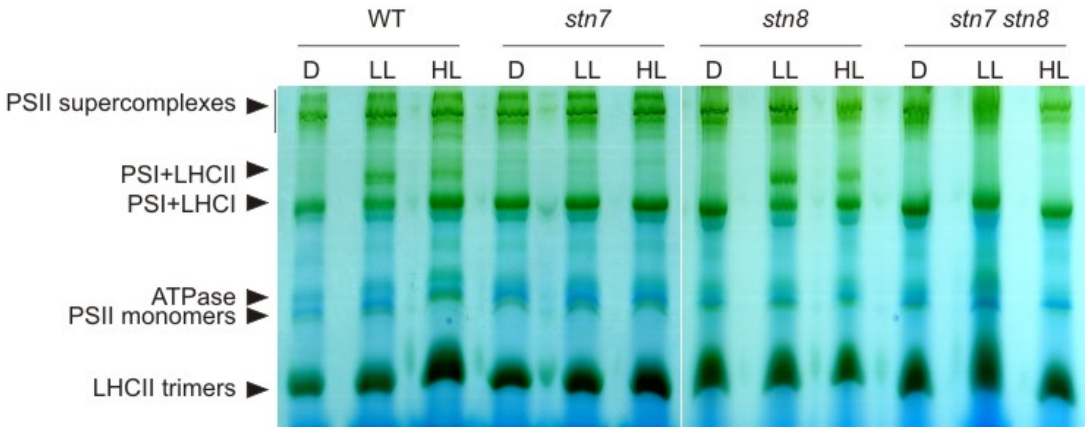

Figure 3.18 Separation of thylakoid protein complexes and supercomplexes by 1D BN-PAGE. Thylakoids were extracted from dark-adapted plants (D), after exposure to low-light (LL; 120 µmol photons m^{-2} s^{-1}; 2h), and after subsequent exposure to high-light (1200 µmol photons m^{-2} s^{-1}; 2 h). Proteins were then solubilized by digitonin at a final concentration of 1.5% (w/v) and run into a BN-PAGE.

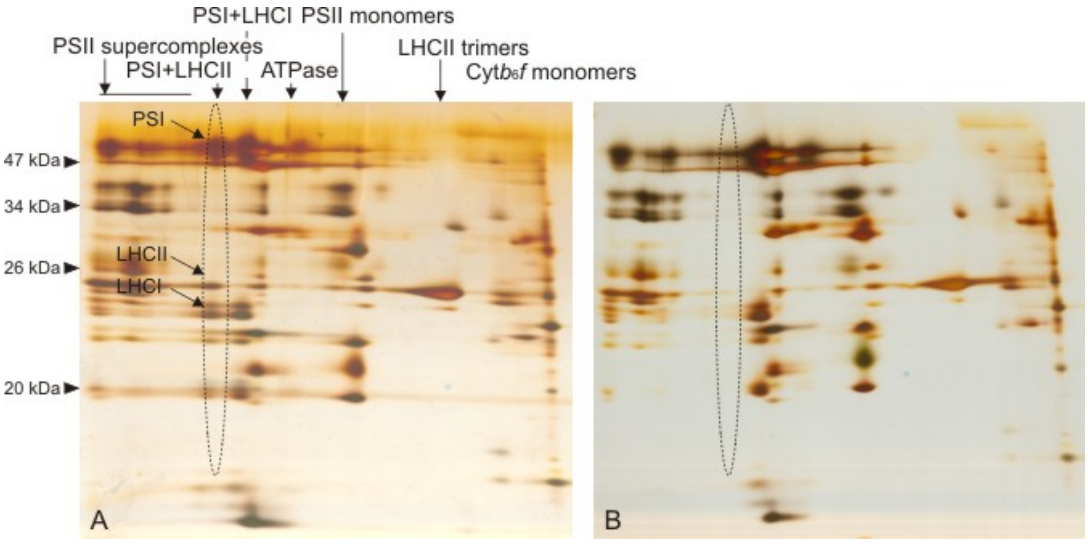

Figure 3.19 Two-dimensional resolution of thylakoid protein complexes and supercomplexes by BN/SDS-PAGE. After the separation of the protein complexes on Blue-Native gel, the lanes corresponding to WT (low-light; **A**), and *stn7* (low-light; **B**) were excised, laid on the top of SDS-PAGE (12.5% acrylamide, 4M urea), and the proteins of each macromolecular complex were separated. The gels were subsequently stained with silver. Identification of the macromolecular protein complexes of thylakoid membranes is given on the top of the first gel (A). Black circles indicate PSI-LHCI-LHCII complex. Note that the analysis is not quantitative.

4. DISCUSSION

Although LHCII phosphorylation is known since almost thirty years (Bennett et al., 1977; 1979), the search for the kinase involved in this process has been so far elusive. The TAK proteins, in particular TAK1, have been the prime candidates of the last years for the LHCII kinase in vascular plants (Snyders and Kohorn, 1999; 2001). *In-vitro* translated TAK1 was shown to be imported into isolated chloroplasts, but import was not associated with cleavage of a transit peptide (Snyders and Kohorn, 1999). TAK1, 2 and 3 were thought to be associated whith each other and with the reaction center of PSII and Cyt b_6/f (Snyders and Kohorn, 1999, 2001). Moreover, a chloroplast thylakoid associated serine-threonine protein kinase, named Stt7, was found to be required for the phosphorylation of the major-light harvesting protein complex and for state transitions in *Chlamydomonas reinhardtii* (Depège et al., 2003). The *Arabidopsis* genome contains two genes, STN7 and STN8, which show homology to the *Chlamydomonas* gene Stt7 (Depège et al., 2003; Bellafiore et al., 2005). Both of them encode, like their orthologous, serine-threonine protein kinases that are targeted to the chloroplasts and associated to the thylakoid membranes.

Our data strongly argue against a chloroplast location of TAK2 and 3, because neither computational prediction, nor *in vitro* or *in vivo* import assays could demonstrate chloroplast localization of the two proteins. In the *in vitro* assay, import of TAK1 into chloroplasts was detected, but it appeared to be less efficient than the one of the Stt7 homologs. Cleavage of the presequence of the imported TAK1 protein was not found, an observation in line with previous data (Snyders and Kohorn, 1999)This indicates that, if TAK1 is indeed imported in the chloroplast, it is not imported in the conventional way; moreover, it would explain the partially contradictory results obtained from *in-vivo* and *in-vitro* assay. The lack of an efficient import of TAK1 in tobacco protoplasts might be due to the modified structure of TAK1, after its fusion to

the RFP sequence. The burial of an internal or C-terminal sequence necessary for the targeting might, in fact, prevent the import.

Antisense TAK1 expression led to increased photosensitivity and influenced the phosphorylation of multiple thylakoid proteins; in particular loss of LHCII phosphorylation and a reduction in state transitions were observed (Snyders and Kohorn, 2001). The genetic analyses described here, employing an insertional mutant of the *TAK1* gene, however, argue against an essential role of TAK1 in LHCII phosphorylation and state transitions. In the *tak1* mutant, neither a drop in LHCII phosphorylation nor a change in state transitions were observed, indicating that the effects observed in the antisense experiment (Snyders and Kohorn, 2001) cannot be attributed to the specific suppression of TAK1 function. Because antisense experiments are prone to pleiotropic effects due to the suppressed expression of multiple genes, the effects on state transitions and LHCII phosphorylation in TAK1 antisense lines might be due to such additive effects. The presence of at least 10 *TAK*-like genes in *Arabidopsis* genome, none of which, however, features a predicted chloroplast transit peptide (data not shown), supports this idea. Since both TAK2 and TAK3 are not targeted to chloroplasts, they cannot be considered as candidates for LHCII kinases anymore. Although TAK1 could be targeted to the chloroplasts, the data here reported clearly suggest that it is certainly not the LHCII kinase.

Among the knock-out mutants described, only the inactivation of the *STN7* gene suppresses state transitions, as indicated by spectroscopic data. In *stn7* and *stn7 stn8*, LHCII phosphorylation is permanently reduced, as indicated by immunoblot, *in-vivo* labeling and *in-vitro* phosphorylation assays, confirming previous results (Bellafiore et al., 2005), although the antenna size is indistinguishable in respect to the WT. Absence of STN7 activity, however, had no consequences on overall photosynthetic electron flow (F_V/F_M and Φ_{II}), photochemical (qP) or non-photochemical (qN) quenching. Snyders and Kohorn (1999) raised the possibility that a cascade of kinases associated with the thylakoid membranes could be part of the signal trans-

duction chain of state transitions. However, taken together, the results argue against an involvement of STN8 kinase or TAK proteins in this putative cascade, indicating that STN7 does not act in series in phosphorylating LHCII neither with STN8, nor with any of the TAK proteins. In the absence of biochemical data, it remains to be shown whether STN7 is capable to directly phosphorylate LHCII or whether it is indeed a component of a protein kinase cascade which ultimately leads to LHCII phosphorylation.

In agreement, again, with previous results, growth of *stn7* mutant plants was impaired relative to the WT (Bellafiore et al., 2005), and the retarded growth rate was even more pronounced in the *stn7 stn8* mutants. Bellafiore et al. (2005) attributed this phenotype to the changing light conditions, assuming that STN7 and state transitions function in response to environmental changes. The data shown here, however, indicate that it is not the fluctuating light itself which retards the growth of plants defective in state transitions. Indeed, the growth of mutants lacking STN7 was retarded when plants were grown under both low- and high-light, as well as both short- and long-day regimes, suggesting that also the light intensity and the day length are not responsible of this phenotype. In addition, recent observations suggested that also the light quality (PSI- and PSII-light) does not influence the growth rate of *stn7* mutants, since plants grown under PSI-light exhibited a similar phenotype to plants grown under PSII-light (Pfannschmidt, unpublished data). Moreover, growth rate of *stn7* plants was indistinguishable from the WT in a greenhouse. It can only be assumed that other factors like temperature, soil composition or humidity could retard the growth of plants lacking STN7, but what and how have still to be clarified.

The growth retardation observed in *stn7* and *stn7 stn8* plants is nonetheless in disagreement with previous results obtained with *Arabidopsis* plants lacking PsaH and deficient in state transitions (Lunde et al., 2000), where mutant plants were unaffected in growth under optimal conditions. However, as suggested before (Bellafiore et al., 2005), this difference could be due to the particular growth conditions used. In fact, *stt7* mutants of *Chlamydomonas*

grow at a slower rate than the WT (Fleischmann et al., 1999). The absence of an obvious effect on plant fitness under environmental conditions, together with the significantly smaller pool of mobile LHCII in vascular plants compared to green algae, raises questions on the biological relevance of state transitions in higher plants. The availability of the *stn7* mutant which is specifically impaired in the phosphorylation of LHCII allows now to design experiments to analyze the physiological impact of the absence of state transitions due to impaired LHCII phosphorylation. Taken together, the present results support the view of Gutman and Niyogi (2004) that the integration of data resulting from the genetic analysis of state transitions in *Chlamydomonas* and *Arabidopsis* is a powerful tool to dissect conserved photosynthetic mechanisms.

Both, STN7 and its *Chlamydomonas* orthologue Stt7, but not STN8, contain a tentative thioredoxin target site (Figure 3.1), which is necessary for the down-regulation of LHCII phosphorylation under high light intensities (Rintamaki et al., 1997; Carlberg et al., 1999; Rintamaki et al., 2000). Strikingly, no TAK homolog exists in the current draft release of the *Chlamydomonas reinhardtii* genome sequence (http://genome.jgi-psf.org/Chlre3/Chlre3.home.html), indicating that, at least in this species, Stt7 might represent both the sensor of the thylakoid redox state and the kinase that phosphorylates LHCII.

Furthermore, the relatively low sequence similarity between STN7 and STN8 argues against a recent duplication and functional conservation of the two genes, suggesting that STN8, which might be indeed considered as the ortholog of *Chlamydomonas* Stl1, might act specifically on substrates other than LHCII. Various experimental data support the existence of two different kinases for PSII phosphoproteins, one for LHCII and another for PSII core proteins with distinct redox regulation systems (Bennett et al., 1988; Gal et al., 1997; Rintamäki et al., 1997; Carlberg et al., 1999). The significant decrease in the total amount of PSII core phosphoproteins, but not in LHCII phosphorylation, in *stn8* mutants indicates, in fact, that the main substrates

of STN7 and STN8 are different, in accordance with a recent study (Vainonen et al., 2005). Whereas LHCII phosphorylation is mostly dependent on STN7, the phosphorylation of PSII core proteins requires almost exclusively STN8. However, only in *stn7 stn8* plants the phosphorylated forms of LHCII and the PSII core proteins are completely absent under all light regimes tested, indicating that STN7 and STN8 must show some degree of overlap in their substrate specificities. The clear distinction between the phosphorylation phenotypes of the two single mutants argues in favor of a parallel action of STN7 and STN8. This supports the view that the two proteins could be directly responsible for phosphorylating the LHCII and PSII core proteins, respectively.

Reversible phosphorylation of PSII proteins is considered to function in the repair of PSII via turnover of the D1 protein (Baena-González et al., 1999). Light induces inactivation of PSII and damage to D1 reaction center protein (Ohad et al., 1984) which undergoes very fast turnover (Mattoo and Edelman, 1987) regulated by the phosphorylation status of D1 (Baena-González et al., 1999). Phosphorylation of PSII core proteins during high-light exposure is thought to control the stability of the photosystem, whereas dephosphorylation of CP43, D2 and D1 proteins would allow the coordination of protein degradation, synthesis and insertion (Baena-González et al., 1999). Here we show that absence of STN8, and consequently of D1 phosphorylation, results only in a slight increase in photosensitivity of PSII that, however, is not reflected in changes in the rate of light-induced D1 turnover. Neither synthesis nor degradation of D1 was affected in the absence of PSII core protein phosphorylation, as demonstrated by immunoblot analysis on the D1 protein in the presence of a plastid protein inhibitor. The turnover of the D1 protein is believed to occur at all light intensities, being by far the fastest of all the PSII subunits. Such a high turnover rate results in a preferential labeling of D1 in pulse-chase experiments, which are considered as an efficient tool for monitoring the different assembly steps of the PSII complex (Bena-González and Aro, 2002). Nevertheless, no clear difference in the rate of D1

synthesis or degradation was observed in mutants impaired in the phosphorylation of PSII core polypeptides. In addition, *stn8* plants showed a very similar rate growth and leaf pigment composition when grown under high light intensities. Taken together, the data indicate that STN8-mediated phosphorylation of D1 is not crucial for D1 turnover and PSII repair. If, indeed, PSII core protein phosphorylation is essential for D1 turnover, a third kinase that might take part during photoinhibition, and subsequent recovery, should exist. However, the action of an additional PSII core kinase must be excluded since no PSII core phosphorylation was detected under these conditions.

Changes in light conditions are thought to result, in the long term, in the adjustment of photosystem stoichiometry, which requires a signaling network that coordinates photosynthetic gene expression in plastids and nucleus (Fujita, 1997; Melis, 1991; Pfannshmidt, 2003). A functional relationship between LHCII phosphorylation (and state transitions) and readjustment in photosysthem stoichiometry (and long-term light quality imbalance) has been recently suggested (Allen and Pfannschmidt, 2000; Pursiheimo et al., 2001; Pfannschmidt, 2003). Both processes, in fact, are thought to be regulated by the redox state of the plastoquinone (PQ) pool, which reflects the balance of light use between both photosystems (Pfannschmidt, 2003). Defects in the function of STN7, indeed, not only affect the capacity to perform state transitions, but also the long term response to changes in light quality. Recent studies proposed the existence of a signaling pathway that links changes in photosynthetic stoichiometry to the level of gene expression in plastids (Pfannschmidt, 1999a) and the nucleus (Pfannschmidt et al., 2001; Fey et al., 2005). For nuclear genes encoding chloroplast proteins two modes of transcriptional regulation are known: a master switch that acts on most genes (Richly et al., 2003) and an additional mechanism specific for photosynthetic genes (Biehl et al., 2005). Lack of STN7 in greenhouse-grown *stn7* and *stn7 stn8* plants results in the differential expression of only relatively few photosynthetic genes, in contrast to *stn8* plants in which a large set of

photosynthetic genes is markedly down-regulated. The impairment of the transcriptional regulation of certain *stn8*-responsive genes in the absence of STN7, together with the inability to long-term respond to changes in light quality, argues in favor of a function for STN7 in the regulation of nuclear and plastid gene expression. We can only speculate how STN7 triggers changes in photosynthetic gene expression but, in principle, three hypothesis are available: first, the phosphorylation state of LHCII directly provides information for signaling; second, state transitions and the associated conformational changes of thylakoids stimulate signaling; and third, an unknown protein is phosphorylated by STN7 and participates in signaling (Wollman, 2001; Vallon et al., 1991). In favor of the last hypothesis, recent studies suggested the possible existence of a protein factor that could be released from thylakoids after light-induced phosphorylation and that could link the gene expression to the redox state of the plastoquinone pool (Escoubas et al., 1995). Carlberg and coworkers (2003) identified the plant-specific protein TSP9 (Thylakoid Soluble Phosphoprotein of 9 kDa) as the first candidate for such a signaling factor. Future work will be required to clarify whether, indeed, TSP9 is an additional substrate of the STN7 kinase and whether defects in TSP9 interfere with the signaling pathway that is responsible of the plastid-to-nucleus communication.

Why state transitions and photosystem stoichiometry depend upon a common protein kinase is an intriguing question. Certainly, LHCII phosphorylation and regulation of transcription both respond to the same redox signal, and within a time scale of a few minutes. A recent review focuses on how the two processes might be linked (Allen, 2005) and suggests that it is likely that other protein kinases and phosphoprotein phosphatases, as yet unreported, will fit into a network of redox signal transduction within chloroplasts, connecting their photosynthetic performance with expression of chloroplast genes (Figure 4.1).

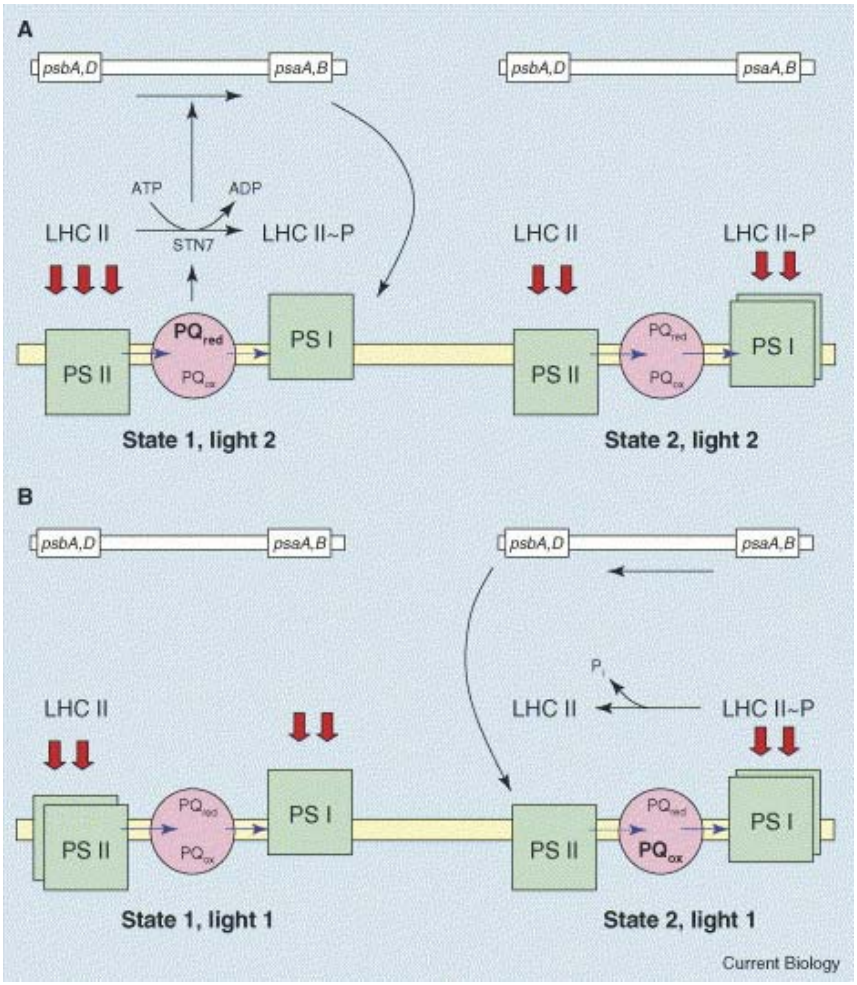

Figure 4.1 State transitions and control of gene expression in chloroplasts. STN7, besides having a primary role in state transitions, required for the adjustment of photosystem stoichiometry, by means of transcriptional control of PSI reaction center genes (e.g. *psaA,B*) and PSII reaction center genes (*psbA,D*). (Model from Allen, 2005)

In green algae, the lateral displacement of the mobile pool of LHCII from the PSII-rich grana to the PSI-rich lamellar thylakoid regions results in transfer to PSI of about 80% of the excitation energy absorbed (Delosme et al., 1996), whereas in land plants only 15 to 20% of LHCII is mobile (Allen, 1992). Moreover the transition from state I to state II induces a switch from linear to cyclic electron flow in *Chlamydomonas reinhardtii* (Finazzi et al. 1999; Finazzi et al., 2002). This correlation between the redistribution of antenna complexes during state transitions and the onset of cyclic electron flow is however absent in vascular plants. It is, therefore, tempting to speculate that triggering of the long term response, rather than the short term response in

terms of state transitions, represents the major function of STN7 in flowering plants. In this respect, the slower growth of *stn7* plants in controlled light conditions might be due to disturbances of transcriptional regulation rather than being a physiological consequence of defects in state transitions. Future analysis must clarify how the short term and long term responses are coupled, and whether STN7 and STN8 are necessary and sufficient for the phosphorylation of thylakoid proteins.

Until now, PSI has been regarded to play a passive role in state transitions, but biochemical evidence for a physical association between LHCII and PSI is yet weak. Two reasons for this have been proposed: first, the binding of the LHCII proteins to the PSI core under state II is loose and lead to the detachment during isolation by standard methods; second, the amount of energy redistribution is too small in higher plants, making the LHCII proteins that migrate to PSI difficult to detect (Takahashi et al., 2006). Some reports suggested that even normally grown plants can have some LHCII associated to PSI (Bennet et al., 1988; Andreasson and Albertsson, 1993). In addition, an increase in energy transfer from LHCII to PSI has been observed in plants undergoing state I to state II transition (Bennet et al. 1988; Horton, 1999). Two recent papers show an attempt to identify the mobile pool of LHCII in *Arabidopsis* by reconstructing single-particle images of PSI-LHCI/LHCII supercomplexes (Kouřil et al., 2005; Kargul et al., 2005). These reports are, however, contradictory in the sizes, locations, and identities of the densities assigned as the mobile LHCII(s). Biochemical evidences on the association of LHCII with PSI were given in *Chlamydomonas* (Takahashi et al., 2006), where CP26, CP29, and LhcbM5 (a previously unreported major LHCII protein type II) were shown to shuttle between PSI and PSII during state transitions acting as docking sites for the trimeric LHCII proteins in both PSI and PSII. Here, the isolation of the PSI-LHCII supercomplex, employing 2D BN/SDS-PAGE, is described. This allowed confirming that LHCII phosphorylation is required for its migration from PSII to PSI. In absence of STN7, in fact, LHCII fails to associate to PSI, thereby increasing the amount

of free PSI-LHCI. In agreement with previous results (Kouřil et al., 2005; Kargul et al., 2005), this association is only detectable when a mild detergent is used. Digitonin proved to be a very suitable detergent in the solubilization and stabilization of supercomplexes of *Arabidopsis* mitochondria (Eubel et al., 2003, 2004) and chloroplasts (Heinemeyer et al., 2004). Haldrup and coworkers (2001) observed that LHCII phosphorylation *per se* is not sufficient to dissociate LHCII from PSII, instead LHCII coupling to PSI is a prerequisite for an efficient dissociation from PSII. In fact, mutants of *Arabidopsis* lacking a specific PSI subunit, PSI-H, are unable to perform state transitions because the phosphorylated LHCII remains attached to PSII. The authors suggested that the binding of LHCII to one of the two photosystem is a result of a binding equilibrium. In this scenario, our results support the idea that phosphorylation is the motor that changes the binding affinity of LHCII during state transitions, regardless of the light regime. Under higher irradiances, LHCII is still associated to PSI, although the complex is less abundant in comparison to low-light conditions. This indicates that the abundance of the PSI-LHCII complex is proportional to the phosphorylation state of LHCII. LHCII can only migrate to PSI when is in the phosphorylated state and PSI-LHCII is formed also under forms still present, although to a less extent, suggesting that the residual phosphorylation of LHCII under high-light in WT plants is promoting the migration of phospho-LHCII from PSI to PSII. Contrarily, *stn7* mutant plants are still impaired in shuttling LHCII to PSI. Under high-light conditions, indeed, LHCII is still partially phosphorylated in WT plants, although the regulatory disulfide bond of STN7 should become exposed and be made accessible to the reduction by a thioredoxin (Rintamäki et al., 2000). How a residual activity of STN7 can be observed under higher irradiances is not yet clear. However, one plausible explanation might be that, under these conditions, the kinase could be partially still active. If it is true that a conformational change in STN7 occurs after the reduction of the disulfide bond (Rintamäki et al., 2000), this does not induce a stable inactivation of the kinase.

It is known that state transitions, in the unicellular alga *Chlamydomonas reinhardtii*, are primarily aimed at switching the photosynthetic apparatus from the oxygenic type, to the bacterial one (Finazzi et al., 2002). Moreover, in the same species, state transitions seem to play an additional role under high-light irradiances in protecting from photoinhibition (Finazzi et al., 2001). This, nevertheless, does not apply to higher plants, where the function of state transitions is still unclear. Bellafiore and coworkers (2005) suggested that state transitions are important for adaptation and this explains the retarded growth of *stn7* mutants; however the data presented here suggest that this is not a consequence of the absence of state transitions, but rather a defect in photosynthetic acclimation. PSII core phosphorylation was, until now, considered as a mechanism specific to higher plants. Only recently it has been proposed that also in *Chlamydomonas* phosphorylation of D1, D2, CP43, CP29, PsbR and CP26 proteins take place under high-light exposure (Turkina et al., 2006), indicating that PSII core phosphorylation has likely conserved its function during evolution. However, the data shown here argue against a role in PSII repair cycle. The absence of an obvious phenotype of plants defective in PSII phosphorylation and, at the same time, the conservation of this mechanism among higher plants and their ancestors raise an intriguing question: what is PSII core phosphorylation required for? The availability of *stn7* and *stn8* single and double mutants, defective in phosphorylation, should enable to design experiments to unravel the physiological role of state transitions, on one hand, and of PSII core phosphorylation, on the other.

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6. Supplementary information

Accession No	Gene	*stn7*		*stn8*		*stn7 stn8*	
		Significance	Ratio	Significance	Ratio	Significance	Ratio
Lhcb genes							
At1g29920	*Lhcb1.1*	no	1.01	yes	0,12	no	1.08
At1g29910	*Lhcb1.2*	no	1.02	yes	0,17	no	1.04
At2g34430	*Lhcb1.4*	no	1.14	yes	0,19	no	0.96
At2g34420	*Lhcb1.5*	no	1.04	yes	0,16	no	1.07
At2g05100	*Lhcb2.1*	no	1.10	yes	0,12	no	0.95
At2g05070	*Lhcb2.2*	no	1.25	yes	0,20	no	0.98
At5g54270	*Lhcb3*	no	1.08	yes	0,13	yes	0.68
At3g08940	*Lhcb4.2*	no	1.16	yes	0,43	yes	1.24
At4g10340	*Lhcb5*	no	0.90	yes	0,20	yes	0.74
At1g15820	*Lhcb6*	no	1.12	yes	0,69	no	1.16
PSI genes							
psaA	*psaA*	no	0.98	yes	0,07	no	0.9
At4g02770	*PsaD1*	no	0.95	yes	0,34	yes	0.78
At1g03130	*PsaD2*	no	0.83	yes	0,25	yes	0.73
At4g28750	*PsaE1*	no	0.88	yes	0,26	no	0.95
At2g20260	*PsaE2*	no	0.87	yes	0,16	no	0.89
At1g55670	*PsaG*	no	1.04	yes	0,20	no	1.15

At3g16140	*PsaH1*	no	0.88	yes	0,26	no	0.98
At1g52230	*PsaH2*	no	0.83	yes	0,31	no	1.04
At1g30380	*PsaK*	yes	0.71	yes	0,15	yes	0.6
Others							
At1g06680	*PsbP1*	yes	1.30	yes	0,28	no	0.88
At2g30790	*PsbP2*	no	1.05	yes	0,35	no	0.86
At3g50820	*PsbO2*	no	0.99	yes	0,26	no	0.86
At1g79040	*PsbR*	no	1.17	yes	0,26	no	0.91
At3g21055	*PsbT1*	no	0.83	yes	0,37	yes	0.74
At5g23120	*HCF136*	no	0.87	yes	0,44	no	0.84
At4g37200	*HCF164*	no	1.17	yes	0,56	no	1.18
At1g76100	*PetE1*	no	0.86	yes	0,45	no	1.13
At1g20340	*PetE2*	no	0.84	yes	0,36	no	1.15
At4g17600	*Lil3*	no	0.84	yes	0,49	no	0.96
At3g22840	*ELIP.1 (Lil1.1)*	no	1.18	yes	0,20	no	1.04
At3g61470	*Lhca2*	no	0.84	yes	0,27	no	0.83
At4g15510	*PsbP-related*	no	0.98	yes	0,45	yes	1.23
At1g76450	*PsbP-related*	no	0.88	yes	7,19	no	1.21
rbcL	*rbcL*	no	0.95	yes	0,17	no	0.95

Supplementary Table I Photosynthetic genes with STN7-dependent differential expression in the *stn8* mutant. **"**Ratio" indicates differential expression ratios of genes in *stn7*, *stn8* and *stn7 stn8* mutants with respect to the WT. "Significance" refers to whether, after application of the false-positive error correction method used (FDR), the calculated differential expression values are considered statistically significant or not. Note that genes, which are not differentially expressed, usually result in expression values that are not statistically significant (Significance: "no").

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ORAL COMUNICATIONS

- Two kinases are required for LHCII and PSII core protein phosphorylation. PSICO meeting, Copenhagen, Denmark (2005)
- Functional characterization of thylakoid protein phosphorylation. Tagung, MPIZ, Köln, Germany (2004)
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Ehrenwörtliche Versicherung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständing und nur unter Verwendung der angegebenen Hilfsmittel und Quellen angefertigt habe. Die Autorin hat zuvor nicht versucht, anderweitig eine Dissertation einzureichen oder sich einer Doktorprüfung zu unterziehen. Die Dissertation wurde keiner weiteren Prüfungskommission weder in Teilen noch als Ganzes vorgelegt.

München, den 01.08.2006

Vera Bonardi