Characterisation and functional analysis of a lumenal proline isomerase from photosynthetic membranes of higher plants and cyanobacteria

Dissertation

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ABBREVIATIONS

A (Abs)	absorbance
amp	ampicillin
APC	allophycocyanin
APS	ammonium persulfate
Arabidopsis	*Arabidopsis thaliana*
ATP	adenosine 5'-triphosphate
BASTA	Glufosinate-ammonium
bp	base pairs
BSA	bovine serum albumin
cDNA	complementary DNA
Cfx	Cefotaxin-Na-salt
Cm	chloramphenicol
dCTP	deoxy cytosine 5'-triphosphate
DNase	deoxyribonuclease I
DNA	deoxyribonucleic acid
EDTA	ethylene diamintetracetic acid Fe(III)Na-Salt
EtBr	ethidium bromide
Gm	gentamycin
h	hours
HEPES	N-(2-hydroxyethyl)piperazine N'-(2-ethane sulfonic acid)
HL	high light conditions (450 $\mu Em^{-2}s^{-1}$, 30°C)
IgG	immunoglobulin G
IPTG	isopropyl ß-D-thiogalactoside
kb	kilobases
kDa	kilo Dalton
LHCII	Light harvesting complex II
LiCl	Lithium Cloride
µg	microgram
min	minutes
MOPS	4-morpholinopropansulfonacid
nm	nanometre
ORF	open reading frame
PAA	polyacrylamide

PAG	polyacrylamide gel
PAGE	polyacrylamide gel electrophoresis
PBS	phycobilisome
PC	phycocyanin
PCR	polymerase chain reaction
PSI	photosystem I
PSII	photosystem II
RC	reaction centre
rpm	rotations per minute
RNase	ribonuclease
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
sec	seconds
SDS	sodium dodecylsulfate
spinach	*Spinacia oleracea*
SSC	standard saline citrate
ST	standard conditions (50 $\mu Em^{-2}s^{-1}$, 30°C)
Synechocystis	*Synechocystis* sp. PCC 6803
TBE	Tris-borate-EDTA
TCA	trichloroacetic acid
TE	Tris-EDTA
TEMED	N, N, N', N'-tetramethylethylene diamine
TFE	Trifluoroethanol
Tris	Tris (hydroxymethyl) aminomethane
Tween 20	polyoxyethylensorbitanmonolaurat

Amino acids

A, Ala	Alanine	M, Met	Methionine
C, Cys	Cysteine	N, Asn	Asparagine
D, Asp	Aspartic acid	P, Pro	Proline
E, Glu	Glutamic acid	Q, Gln	Glutamine
F, Phe	Phenylalanine	R, Arg	Arginine
G, Gly	Glycine	S, Ser	Serine
H, His	Histidine	T, Thr	Threonine
I, Ile	Isoleucine	V, Val	Valine
K, Lys	Lysine	W, Trp	Tryptophan
L, Leu	Leucine	Y, Tyr	Tyrosine

DNA Bases

A	Adenine
C	Cytosine
G	Guanine
T	Thymine
U	Uracil

1. Introduction

1.1 Oxygenic photosynthesis

Oxygenic photosynthesis is the process which converts light energy into chemical energy according to general reaction

$$H_2O + CO_2 \xrightarrow{\text{Light}} (CH_2O) + O_2$$

By this reaction CO_2 (inorganic source of carbon) is ultimately converted into sugar (organic source of carbon). The process requires also water. An important consequence of the whole process is the release of molecular oxygen into environment. The photosynthetic process can be divided into two phases: a light phase in which light energy is stored in ATP and NADPH and a dark phase (i. e. that does not need light directly) in which ATP and NADPH are used to fix CO_2 into an organic compound (GAP, glyceraldehyde-3-phosphate). The light reaction of photosynthesis takes place in thylakoid membranes which are present in photosynthetic bacteria and in photosynthetic organelles of higher plants and algae.

1.2 Primary, secondary and tertiary endosymbiosis

The evolution of the oxygenic photosynthetic apparatus took place in cyanobacteria-like cells which lived on earth for at least 3.5 billion years ago (Schopf, 1993). A homologous apparatus was found in recent eukaryotic cells where it is compartmentalized in an organelle, known as chloroplast. It is widely accepted that chloroplasts evolved from the engulfment of a cyanobacterium by a protist. This event is known as primary endosymbiosis (Mereschkowsky, 1910; Douglas, 1998; Delwiche, 1999; Cavalier-Smith, 2000; see Fig. 1). Chloroplasts deriving from a primary endosymbiotic event possess two surrounding membranes of different evolutionary origin. Molecular data suggest that the inner membrane has a prokaryotic origin while the outer contains both prokaryotic and eukaryotic components (Bölter *et al*., 1998; Reumann *et al*., 1999). The consortium between

a cyanobacterium and a eukaryotic cell became obligate because of the gene transfer from the bacterium to the eukaryotic nucleus (Herrmann, 1997; Martin and Herrmann, 1998; Race *et al.*, 1999; Abdallah *et al*., 2000; Rujan and Martin, 2001). It is believed that 90% of the bacterial genome which comprised photosynthetic and non-photosynthetic genes was transferred to the host cell (Martin and Herrmann, 1998). At the time where prokaryotic genes were transferred into the host cell, the new cell had to elaborate a system to import proteins into chloroplasts. This was achieved by the evolution of signal peptides at the N-termini of proteins and of specific transporter (Martin and Herrmann, 1998; Blanchard *et al*., 2000). The primary endosymbiosis event gave rise to three different lineages: Chlorophytes and Streptophytes which comprise green algae and land plants, Rhodophytes which comprise red algae, and Glaucocystophytes (Martin *et al*., 1998; see Fig. 1).

Many algae possess chloroplasts which are surrounded by three or even four membranes instead of two which are found in primary chloroplasts. It was suggested that these chloroplasts derived from a secondary endosymbiosis event in which a protist engulfed an existing algae (Gibbs 1978; Whatley *et al*., 1979; Ludwig and Gibbs, 1987; Gibbs, 1993; McFadden and Gilson, 1995). This type of eukaryote-eukaryote endosymbiosis explains the vast majority of algae diversity. The three or four membranes which surround these chloroplasts are from the outermost to the inner membrane the remnants of the phagotropic vacuole of the host, the plasma membrane of the endosymbiont and the two membranes of the primary chloroplasts (Cavalier-Smith, 2000). The fourth membrane has been lost in some groups (Euglenophyta and most of the Dinoflagellates). Some groups (Cryptophytes and Chlorarachniophytes) still contain a reduced nucleus of the endosymbiont, the nucleomorph (Gilson *et al*., 1997), which is located between the second and the third membrane (the former cytoplasm of the algae endosymbiont). A final important marker of a secondary endosymbiosis event is the presence of an extended N-terminus in nuclear-encoded chloroplasts proteins encoding a bi- or tri-partite targeting sequence permitting the transport of chloroplast proteins across the three or four membranes.

Tertiary endosymbiosis is limited to some Dinoflagellates and refers to the uptake by a eukaryotic cell of a photosynthetic symbiont originating from secondary

endosymbiosis (Zhang *et al*., 1999; Yoon *et al*., 2002; Palmer, 2003). The photosynthetic symbiont derived originally from a red alga. The cellular organisation between these species is quite similar: a single membrane surrounds a subcellular compartment, the symbiont, containing a nucleus, mitochondria and chloroplasts (Tomas and Cox, 1973; Jeffrey and Vesk, 1976; see Fig. 1).

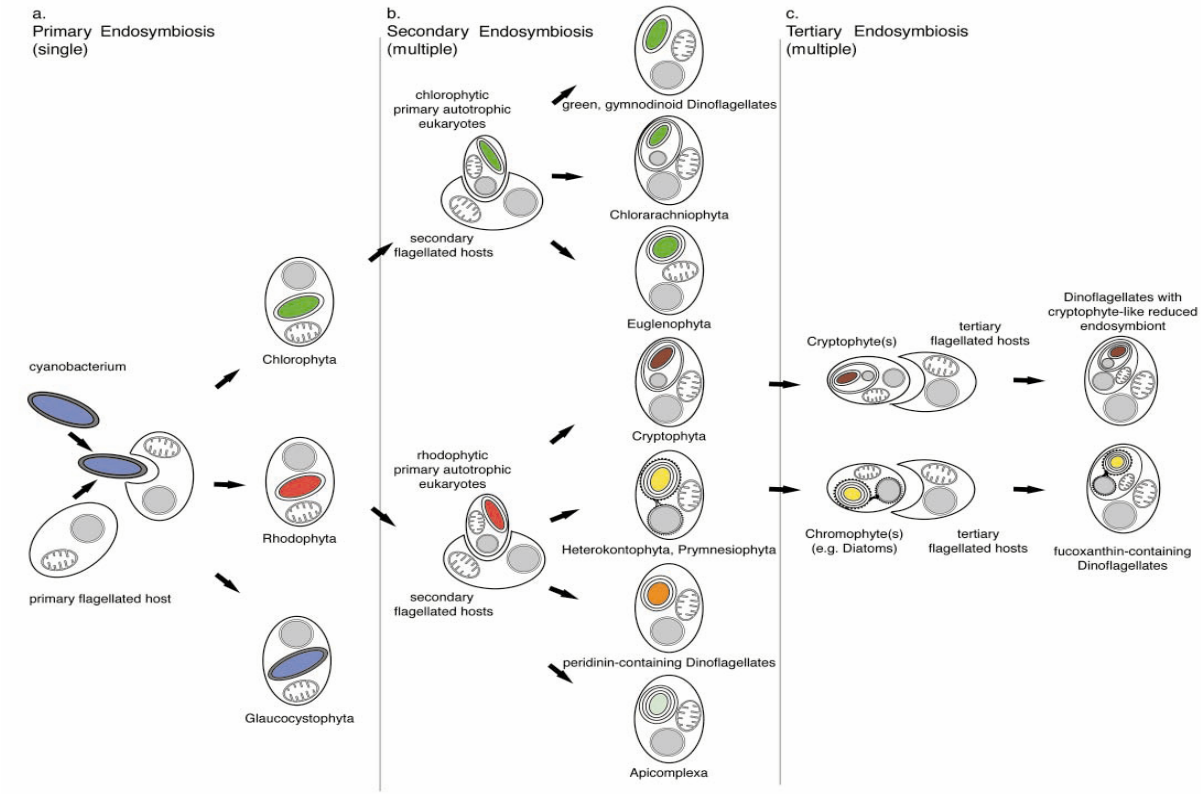


Fig. 1. Schematic presentation of primary, secondary and tertiary endosymbiosis (adapted from Stoebe and Maier, 2002).

1.2.1 Chloroplast in algae and higher plants

The chloroplast is the organelle of the photoautotrophic eukaryotic cell, in which photosynthesis takes place. In higher plants and algae it contains three compartments which are separated by different membranes. The chloroplast envelope is composed of two membranes which delimit an intermembrane space. The stroma is a compartment between the chloroplast envelopes and the inner membrane system. It contains the enzymes necessary for the dark phase of photosynthesis as well as plastid DNA and RNA. The lumen is delimited by the

thylakoid system and contains proteins involved in the evolution of oxygen and electron transport of photosynthesis. Thylakoids are organised into stacked region, which are called grana. Grana are connected between them by single thylakoid membranes, called stroma lamellae. Such a thylakoid organisation is not present in photoautotrophic prokaryotic cells. A scheme of a chloroplast is presented in Figure 2.

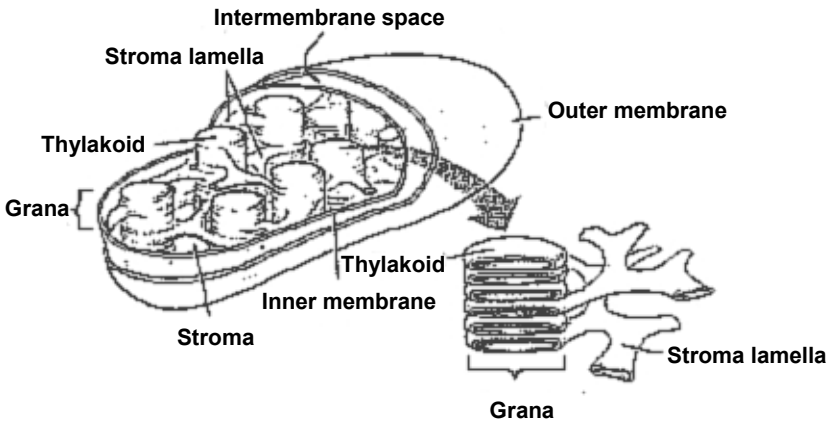


Fig. 2. Schematic presentation of a typical chloroplast of higher plants and algae.

1.2.2 *Synechocystis* as a model of eukaryotic photosynthetic process

Since the ancestor of chloroplasts was a cyanobacteria-like cell, cyanobacteria represent a convenient system to study functions and structure of the photosynthetic apparatus in the eukaryotic cell. One of the most used models is *Synechocystis* sp. PCC 6803 whose genome was completely sequenced (Kaneko *et al*., 1997). This bacterium is naturally competent and can be easily transformed (Williams, 1988) by homologous recombination that allows interruption of a specific part of the chromosome. Another important advantage is the possibility to grow *Synechocystis* photoheterotrophically that means that mutations which alter the photosynthetic processes can be recovered by addition of a carbon source in the medium (Williams, 1988).

1.3 Photosynthetic reaction centres in higher plants and in *Synechocystis*

Photosynthesis involves the cooperation of two reaction centres, known as photosystem I (PSI) and photosystem II (PSII), which operate according to the "Z-scheme" (Fig. 3) where electrons move from PSII to PSI to generate the strong reductant necessary for $NADP^+$ reduction. In chloroplasts, PSII is mainly found in appressed thylakoid membranes (grana lamellae), while PSI is distributed in non-appressed thylakoids (stroma lamellae) and peripheral regions of the grana. It is not quite clear why reaction centres of PSII and PSI are laterally separated between grana and stroma lamella in eukaryotic organisms. In prokaryotes thylakoid membranes do not form grana and photosystems II and I appear to be intermixed. The electron transport between two photosystems is bridged by the cytochrome b_6f complex, which is evenly distributed throughout thylakoid membranes, and two freely diffusing electron carries, plastoquinone (PQ) and plastocyanin (PC). Light-driven electron transfer through PSII and PSI reaction

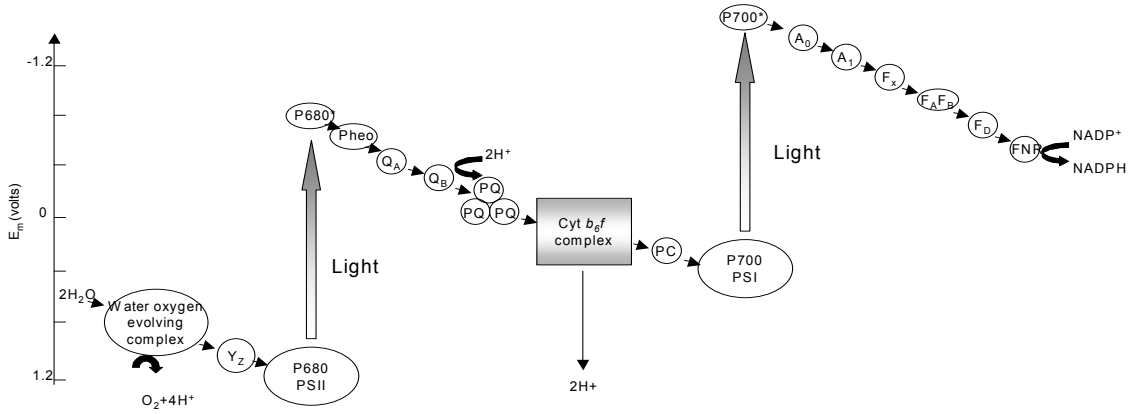


Fig. 3. The "Z-scheme" of oxygenic photosynthetic electron transport. The vertical scale shows the potential (E_m) of the components. Y_Z is the tyrosine 161 of the D1 protein; P680 is the reaction centre chlorophyll *a* of PSII; Pheo is pheophytin; Q_A is plastoquinone; Q_B is a plastoquinone which binds and dissociates from the PSII; PQ is a pool of mobile plastoquinone molecules; Cyt b_6f is the cytochrome b_6f complex; PC is plastocyanin; P700 is the reaction chlorophyll *a* centre of PSI; A_o is a special chlorophyll *a;* A_1 is the vitamin K; F_X, F_A, F_B, are iron sulphur centres; F_D is ferredoxin; FNR is ferredoxin-NADP reductase and $NADP^+$ is nicotinamide-adenine dinucleotide phosphate.

centres generates a proton electrochemical potential across thylakoid membranes which is released during ATP synthesis. A schematic presentation of the light-driven reactions of photosynthesis is shown in Figure 3.

1.3.1 Photosystem II

Oxygenic photosystem II (PSII) is the only protein complex which can oxidize water and release molecular oxygen. Studies on PSII have been stimulated by its close homology to the purple bacteria reaction centre although this ancient form of the photosystem can not evolve oxygen. Although PSII is found in cyanobacteria (e.g. *Synechocystis*), green algae (e.g. *Chlamydomonas reinhardtii*) and higher plants, the overall structure is similar in all these organisms. PSII consists of a central reaction centre core surrounded by a light-harvesting antenna system. The first chemical reaction of PSII is a charge separation within the reaction centre and it is driven by absorbed light energy. The chemical nature of the primary donor, known as P680, is likely a dimer of chlorophyll *a* but its exact nature has still not been resolved. Subsequent electron transfer steps prevent the primary charge separation from recombining by transferring the electron to pheophytin and then to Q_A which becomes Q_A^-. From Q_A^- the electron is transferred to another plastoquinone molecule, Q_B, which after two photochemical turnovers becomes completely reduced (PQH_2). While the electron removed from P680 is rapidly sent away, an electron from a tyrosine residue (Y_Z) in the reaction centre protein D1 reduces $P680^+$. Electrons for the reduction of Y_Z are extracted from water by the water-oxidizing complex. Since one absorbed photon drives the transfer of one electron, the overall photochemical reaction of PSII is

$$2H_2O + 2PQ + 4H^+ + (4hv) \rightarrow O_2 + 2PQH_2 + 4H^+$$

where hv is the energy of one single photon according to Einstein's law of photochemistry. In all oxygen-evolving organisms, the reaction centre of PSII contains membrane proteins D1, D2 as well as CP43 and CP47, and some other proteins which are required for oxygen evolution and protection.

1.3.1.1 D1 protein

The D1 protein, known also to carry the Q_B-binding site, is encoded in higher plants and algae by the chloroplast gene *psbA*. It is a membrane protein with five transmembrane α-helices and four connecting loops (Trebst, 1986). D1 can reduce the reaction centre P680* through tyrosine 161 and may provide the ligands to the manganese cluster of the water-oxidizing complex at the lumenal side of thylakoid membrane (Debus, 1992; Rutherford *et al*., 1992). Site-directed mutagenesis in *Synechocystis* generated several mutations of the charged residues that are predicted to be located in the lumen (reviewed by Debus, 1992). In general, these substitutions eliminated or impaired photoautotrophic growth, showing that some of these residues were important for stability, assembly or efficiency of PSII. In particular, an extensive mutational analysis on aspartate 170 (D170) suggested that the first manganese atom of the water-oxidizing complex is bound to PSII through this amino acid residue (Nixon and Diner, 1992).

1.3.1.2 D2 protein

The D2 protein is an intrinsic protein of PSII reaction centre. Its protein sequence, encoded by chloroplast gene *psbD*, is highly conserved between different species and quite homologous to D1 protein. It was proposed that D1 and D2 bind the primary reactants of PSII (Nanba and Satoh, 1987) because of their high homology to the L and M subunits of the purple bacterial reaction centre (Deisenhofer, 1985; Allen *et al*., 1988). This hypothesis was confirmed by the isolation and characterisation of a PSII reaction complex that contained only D1, D2 and some small polypeptides (Nanba and Satoh, 1987); such a complex could perform the primary charge separation but could not evolve oxygen. The mutational analysis of the D2 polypeptide followed the same strategy of D1 analysis where negatively charged residues as well as histidines, glutamines and asparagines were mutated to identify the residues which are involved in PSII stability and/or functionality. The only mutation which gave rise to a photoheterotrophic phenotype was glutamate 68 which was substituted to glutamine and valine (Vermaas *et al*., 1990; Yu and Vermaas, 1993). While the valine-substituted strain could not evolve oxygen at all, the glutamine-substituted

strain contained assembled and functional PSII although three times less than the wild-type (based of chlorophyll content). However, oxygen evolution was rapidly inhibited suggesting a faster rate of PSII photoinhibition. These data suggested that also glutamate 68 of D2 might be a ligand to the manganese cluster.

1.3.1.3 CP47 protein

The CP47 protein is encoded by the *psbB* gene. With CP43 it serves as the proximal antennae for PSII. In addition to this role, both proteins interact with the proteins associated with the site of water oxidation. It was hypothesized that CP47 possesses six transmembrane α-helices separated by five extrinsic loops (A - E) exposed alternatively to the lumenal or stromal site (Vermaas *et al*., 1987; Bricker, 1990). The large loop E on the lumenal side was target for mutational analysis. The biochemical studies on the mutated strains provided strong evidence that the extrinsic E loop of CP47 interacts with the manganese-stabilizing protein. In particular, the well-conserved arginine residues at positions 385 and 386 seem to form a binding site for the manganese stabilizing protein (Qian *et al*., 1997). When arginines at positions 385 and 386 were mutated into glutamate, the phenotype of the resulting strain was very similar to the *ΔpsbO* mutant which lacks the manganese-stabilizing 33 kDa protein. The interaction of CP47 with the manganese-stabilizing protein was also confirmed by limited proteolysis performed on extracted PSII in the presence or absence of the PsbO protein: CP47 was cleaved only in the absence of PsbO indicating that the manganese-stabilizing protein shields CP47 from proteolytic attack (Bricker and Frankel, 1997).

1.3.1.4 CP43 protein

The CP43 is encoded by the *psbC* gene and despite low homology with CP47 (less than 20%), hydropathy analysis predicted a similar structure as for CP47. Early mutagenesis studies on the E-loop of CP43 concluded that CP43 is required for a functional PSII (Kuhn and Vermaas, 1993); deletion of a stretch of well-conserved amino acid residues resulted in obligate photoheterotrophs, loss of oxygen evolution, and electron transfer to Q_A. In most of these mutants the reaction centre core proteins were detected immunologically, even in lower amount in comparison to wild-type, but it was unclear whether these proteins could

assemble into PSII. Proteolytic studies showed that tryptic cleavage in the large extrinsic E-loop of CP43 occurred in the absence of the manganese-stabilizing protein (Enami *et al*., 1997). Moreover, several studies indicated that under photoinactivation conditions, CP43 could become covalently crosslinked to D1 (Mori and Yamamoto, 1992). Considering all of these findings, Bricker and Frankel (2002) hypothesized that the interaction of the extrinsic E-loop of CP43 with lumenal-exposed domains of D1 are required for efficient assembly and stability of the oxygen-evolving complex.

1.3.1.5 Cytochrome b_{559} protein

Cytochrome b_{559} contains two small subunits, alpha and beta, which are encoded by the chloroplast *psbE* and *psbF* genes, respectively (Carrillo *et al.,* 1986). Both have a single transmembrane helix and it is well accepted that they form a heterodimer (Widger *et al*. 1985, Cramer *et al*, 1986). Since each subunit contains only one histidine residue, the heme group must cross-link the two subunits and be exposed to the stromal side. α- and β-subunits of the cytochrome b_{559} are components of the minimal PSII reaction centre complex which is still capable of charge separation. Deletion of the *psbE* and/or *psbF* genes prevented the formation of functional PSII suggesting that cytochrome b_{559} plays also a structural role in PSII assembly (Pakrasi *et al*., 1988, 1990).

1.3.1.6 The 33 kDa and other extrinsic proteins of PSII

Several extrinsic protein subunits are associated to PSII at the lumenal side of the thylakoid system. The 33 kDa PsbO protein is present in all oxygenic organisms. It influences the properties of the manganese catalytic site. However, the mechanism is not clear. Moreover, it has been reported that the 33 kDa protein is essential for oxygen evolution in green algae while cyanobacteria in which the *psbO* gene was deleted, were still able to evolve oxygen (Mayfield *et al*., 1987; Burnap *et al*. 1989). This indication suggested that the 33 kDa protein may play a different role in eukaryotes and cyanobacteria. This discrepancy can be partially explained by substantial differences in protein sequences of plant and cyanobacterial 33 kDa protein and by the presence of different extrinsic components in plants and cyanobacteria. The 23 kDa and 16 kDa proteins were

found in plants, green algae and cyanobacteria and are encoded by the nuclear genes *psbP* and *psbQ*, respectively. Their binding to PSII requires the presence of PsbO. Their absence decreases oxygen evolution but the wild-type phenotype can be restored with elevated levels of Ca^{2+} and Cl^- (Ghanotakis *et al*., 1984; Miyao and Murata, 1984). Therefore, these subunits regulate the access of Ca^{2+} and Cl^- which are cofactors of oxygen evolution. In addition, cyanobacterial PSII contains other extrinsic proteins which are the 15 kDa cytochrome c_{550} (*psbV*) and a 12 kDa protein encoded by the gene *psbU*. The role of these proteins is still unclear but it has been suggested that they are important in regulation of oxygen evolution (Shen and Inoue, 1993).

1.3.1.7 Low molecular mass proteins

There are several low molecular mass (less than 10 kDa) hydrophobic polypeptides associated with PSII. The PsbI protein was found in both, higher plants and cyanobacteria; it was predicted to have one membrane-spanning region and it is tightly bound to the PSII reaction centre. This protein is not essential for PSII biogenesis and function but plays a role in the optimisation of PSII activity (Ikeuchi *et al*., 1995). PsbH was found in eukaryotes and prokaryotes. It is a bitopic membrane protein and in *Synechocystis* seems to be involved in the optimization of electron transfer between Q_A and Q_B (Mayes *et al*., 1993) as well as in the binding of bicarbonate to PSII (Komenda *et al*., 1995). Also PsbL was found to be important for a functional PSII and was found in PSII core complex of higher plants, green algae and cyanobacteria (Ikeuchi *et al*., 1989; Zheleva *et al*., 1998; Swiatek *et al.,* 2001; Kashino *et al*., 2002; Suorsa *et al.,* 2004). Deletion mutant of PsbL in *Synechocystis* and tobacco could not grow photoautotrophically suggesting that PsbL is essential for the normal function of PSII (Anbudurai and Pakrasi, 1994; Iwata *et al*., 2001,). There are several other small subunits which are associated with PSII: PsbJ, PsbK, PsbM, PsbN, PsbR, $PsbT_C$, $PsbT_n$, PsbW, PsbX, PsbY (Gau *et al*., 1998) and PsbZ (Swiatek *et al*., 2001). All of them are chloroplast encoded with the exception of PsbW. PsbR, $PsbT_n$ and PsbW were not found in cyanobacteria. Deletion mutants in *Synechocystis* showed in general that these subunits are required for optimisation of PSII activity but their function is dispensable (reviewed by Shi and Schroeder, 2004).

1.3.1.8 Crystal structure of PSII

According to crystal structure at 3.5 Ångstrom resolution (Ferreira *et al*., 2004), PSII in cyanobacteria is a dimer composed of two almost identical monomers. The reaction centre of the monomer contains the proteins D1 and D2 which are flanked by CP43 and CP47 located on opposite sites of the heterodimer. Besides of the proteins of PSII core complex, 13 transmebrane helices were detected and were assigned to low molecular mass proteins according to previous studies (Zouni *et al*., 2001; Hankamer *et al*., 2001; Kamiya *et al*., 2003): PsbL, PsbM and PsbT were assigned to the interface of dimerisation as well as PsbI and PsbX. PsbZ, PsbK PsbJ, PsbE and PsbF were located on the opposite site. Three extrinsic proteins PsbO, PsbU and PsbV are located at the lumenal side and, together with the C-terminus of the D2 protein, form a cap covering the oxygen-evolving complex. Anyway the sites of ligands to the oxygen-evolving complex are provided by D1 (Ferreira *et al*., 2004).

1.3.2 Photosystem I

PSI is an integral membrane protein complex which reduces $NADP^+$. It is the strongest predicted reductans in any known biological system. The reaction centre is known as P700 which by absorption of light becomes P700*. The electron is then transferred to a series of bound low-potential electron acceptors. The dimeric structure of P700 was confirmed in 1993, where a structural analysis of PSI complex, identified a pair of chlorophylls near the lumenal surface of the membrane lying on the same axis of PSI first acceptor (Krauss *et al*., 1993). The first electron acceptors are a monomeric chlorophyll *a* molecule (A_0) and a phylloquinone (A_1). The electron is then transferred to a series of membrane bound Fe-S centres (F_X, F_A and F_B) until ferredoxin which is a soluble electron carrier. Ferredoxin-NADP reductase reduces then $NADP^+$ to NADPH. Despite the differences of PSI between species, all PSI complexes contain two subunits PsaA and PsaB, which form a heterodimer and bind P700, A_0, A_1 as well as F_X. The terminal acceptors in PSI, F_A and F_B, are bound to a low-molecular mass subunit, PsaC, which is tightly associated with the PsaA/PsaB heterodimer. Other subunits are present in the PSI complex, which are involved in PSI function and

stabilization. PsaE and PsaF are implicated in the interaction of PSI with the soluble electron carrier (plastocyanin and ferredoxin). The function of other subunits (Psa G, H, I, J, K, M and N) is not completely clear. In cyanobacteria, PSI exists as a trimer and each monomer is composed of twelve protein subunits, 96 chlorophyll *a* molecules, 22 carotenoids, three [4Fe4S] clusters, and two phylloquinones (Estreicher *et al*., 2003). Plant PSI is a monomer (Scheller *et al*., 2001; Ben-Sherm *et al*., 2003 a) containing fourteen subunits. Plant PSI lacks two subunits that are unique to cyanobacteria (PsaX and PsaM), and contains four subunits that are absent in cyanobacteria (PsaG, PsaH, PsaN and PsaO). Both, plant and cyanobacterial PSI can bind external antenna systems when extra light-harvesting capacity is required. A structure of plant PSI-LHCI supercomplex has been obtained by X-ray structure analysis at 4.4 Å resolution (Ben-Sherm *et al*., 2003 b) which showed that the four antenna Lhca assembled in two dimers (Lhca 1-4 and Lhca 2-3) around the reaction core complex.

1.3.3 Light harvesting antenna of higher plants and *Synechocystis*

Despite of the high similarity in the organisation and function of photosystems, the antenna system of higher plants and *Synechocystis* are quite different. The higher plant light-harvesting complex of PSII (LHCII) constitutes about one third of the total chloroplast protein content and binds half of the total chlorophyll. It is encoded by three nuclear genes, lhcb1, lhcb2 and lhcb3 (Jansson *et al*., 1992) whose products have an average length of 232, 229 and 223 amino acids after removal of the respective chloroplast import signals. It is located in the thylakoid membrane where it is present in a trimeric form (Peter and Thornber, 1991). The structure of LHCII was resolved at a near atomic level (Kühlbrandt *et al*., 1994) and showed that each single protein molecule forms three transmembrane α-helix domains which are connected by two hydrophilic loops located on opposite sides of the membrane. Crystallographic and biochemical data confirmed that LHCII binds 7 chlorophylls *a*, 5 chlorophylls *b* and 3 carotenoids (Bassi *et al*., 1993; Kühlbrandt *et al*., 1994). LHCII is mostly associated with PSII, but under highly reducing conditions in thylakoid membranes (i. e. when PSII is more active than PSI), it is phosphorylated and migrates to the stroma lamella where PSI is located.

This process is known as state transition (Allen *et al*., 1981, 1992; see below). CP29, CP26, CP24 apoproteins are encoded by the nuclear genes lhcb4, lhcb5 and lhcb6 and form a minor antenna complex associated with the core of PSII. The LHCI antenna (genes: lhca1-4) associate exclusively with PSI. It is believed that the common ancestor of the LHC proteins is a cyanobacterial high-light inducible protein with one membrane-spanning helix (Dolganov *et al*., 1995). After the endosymbiotic event the *lhc* gene progenitor was transferred to the nucleus, where subsequent duplication and deletion events took place, leading to the large *lhc* gene family that exists today (Green and Kühlbrandt, 1995; Herrmann, 1997; Jansson, 1999).

Phycobilisomes (PBS) are peripheral water-soluble complexes in cyanobacteria (and red algae) that harvest light energy and transfer it to the photosynthetic centres. The major PBS components are the chromophore-bearing biliproteins: phycocyanin (PC), allophycocyanin (APC) and phycoerythrin (PE). The last one is not present in *Synechocystis* strain 6803 (Glazer, 1985). The entire PBS structure is stabilised by non chromophorylated linker (L) proteins which are also involved in the regulation of PBS quaternary structure and optimization of the energy transfer (Tandeau de Marsac and Cohen-Bazire, 1977; MacColl, 1998). Phycobiliproteins are composed of α and β subunits which associate in heterodimers. Heterodimers aggregate in trimers $(\alpha\beta)_3$ and hexamers $(\alpha\beta)_6$. PBS are organised in two structural units: the APC-containing core and the PC- and PE-containing rods. The general structure of PBS is comprised of generally six rods, each composed of a stack of PC and PE (when present) radiating from the core. The core is constituted by APC trimers and contains a high molecular mass polypeptide (L_{CM}) which has homology both with phycobiliproteins and with linker proteins (Capuano *et al*., 1991). L_{CM} polypeptide binds the tetrapyrrole chromophore which serves as a PBS terminal energy acceptor. A schematic diagram of the PBS structure is presented in Figure 4.

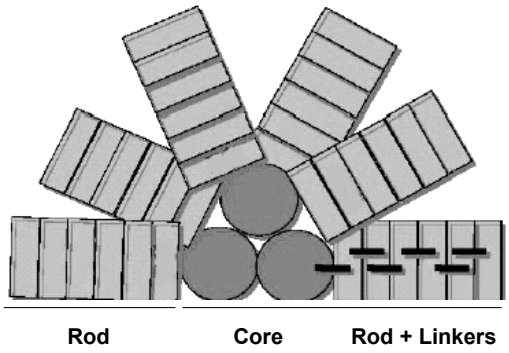


Fig. 4. Schematic presentation of PBS structure

The energy transfer from PBS to photosystems follows the pathway from PE to PC and APC and finally to L_{CM} (MacColl, 1998). Genes encoding α and β subunits of each phycobiliproteins are contiguous on the cyanobacterial genome and are cotranscribed. Often, polycistronic transcripts encode phycobiliproteins subunits and associate L polypeptides. It is still under debate whether PBS transfers energy only to PSII or also to PSI in analogy to LHCII in higher plants (Murata, 1969; Allen *et al*., 1985; Salehian and Bruce, 1992; Kuhl *et al*., 1999; see also below). In addition to the antenna function, PBS represent the major source of nitrogen for cyanobacterial cells, since they represent about 50% of the total protein content in the cell (see below).

1.4 Short-term and long-term adaptation of thylakoid membranes

The conversion of light energy into chemical energy in thylakoid membranes is achieved by the cooperative action of the two photoreaction centres, PSII and PSI. The activity of these photoreaction centres has always to be coordinated in order to maintain a high efficiency of energy conversion. In natural life the environmental conditions are never constant, so there are several situations which can lead to an imbalance of electron flow between the photosystems. For example, photosynthesis is very sensitive to light quality (which can favour either PSII or PSI) or to requirements of extra energy (which favour a cyclic electron flow around PSI). Thylakoid membranes and the photosynthetic apparatus should therefore be flexible to acclimate to the environmental changing. The adaptation processes can

differ in time ranges from seconds or minutes (short-term), to hours and days (long-term).

1.4.1 Short-term adaptation

The key process of short-term adaptation is known as state transition which is a regulatory mechanism controlling the distribution of energy between photosystems. In chloroplasts this is achieved by the distribution of antenna proteins (LHCII) which can transfer their collected energy either to PSII or to PSI. The reduction of the plastoquinone pool (Horton *et al*., 1981) and/or cytochrome b_6f complex (Gal *et al*. 1988) activates a thylakoid-bound kinase which phosphorylates LHCII antenna bound to PSII (state 1). This phosphorylation causes a change in protein recognition resulting in the migration of LHCII to PSI (state 2). The overall result is a redistribution of energy from PSII to PSI. Dephosphorylation of LHCII proteins seems to occur by a constitutive redox independent phosphatase. The state transition in vascular and green alga chloroplasts optimises the amount of energy reaching the photosystems by regulation of the energy transfer from light-harvesting antenna to the two photosystems. An important element of the proposed model is the spatial separation of two photosystems in thylakoid membranes. Red alga and cyanobacterial thylakoids are not stacked and thus lack the heterogeneous localisation of photosynthetic complexes. In addition they have different antenna systems which probably reflect differences in the state transition mechanisms. There are few models which try to explain the state transition in cyanobacteria and red alga (Fig. 5).

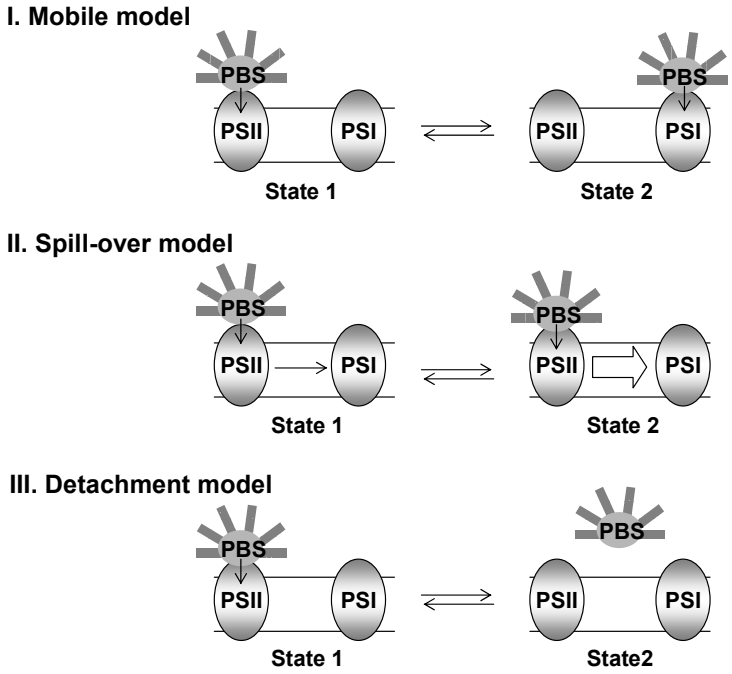


Fig. 5. Proposed models for state transition in red alga and cyanobacteria. For explanation, see text. (Figure adapted from Yoshihiko *et al*., 1994)

The first model, known as mobile model, is similar to that for chloroplasts and suggests a movement of antenna complexes (phycobilisomes, PBS) between the photosystems (Allen *et al*., 1985; Allen and Holmes, 1986; Sanders and Allen, 1987; Sanders *et al*., 1989). However, this model cannot explain why during state transition in cyanobacteria there is also a change in the contribution of chlorophyll *a*-absorbed excitation energy relative to PSII and PSI and not only in the contribution of PBS absorbed excitation energy. This observation supports the so-called spill-over model proposed originally by Murata (1969). This model suggests that PBS transfer their absorbed energy only to PSII and that PSI takes energy from PSII with a constant rate depending on conditions. This model suggests then that changes in the rate constant for excitation energy transfer between PSII chlorophyll *a* and PSI chlorophyll *a* are responsible for the observed changes in the distribution of both PBS- and chlorophyll a-absorbed energy between the two photosystems. However, this model is inconsistent with the observation that changes in energy transfer to PSII are always greater than those in the transfer to PSI (Salehian and Bruce, 1992). This discrepancy is explained by the third and last model, the detachment model, in which some PBSs detach from PSII without association to PSI during illumination with PBS-exciting light. Anyway, until now,

none of these models can adequately explain all the experimental observations so far reported. It has also still not been solved which signal drives the state transition. Satoh and Fork (1983) proposed that cyclic electron flow is related to the state transition by the induction of an electrochemical gradient around the photosystems: this gradient would change the relative affinity of photosynthetic complexes leading to the state transition. On the other hand, Mullineaux and Allen (1990) suggested that the state transition occurs in response to the redox state of the cytochrome b_6f complex. Recent studies in Spirulina showed that 20% of PBS s are bound to PSII, while 60% of PBSs transfer the energy to the PSI trimer and 20% are associated with PSI monomer (Rakhimberdieva *et al*., 2001).

1.4.2 Long-term adaptation

There are several phenomena which are referred to as long-term adaptation as for example the regulation of the stochiometry of PSI relative to PSII, the regulation of antenna size in cyanobacteria and the degradation of PBS under nitrogen starvation. All of these regulatory responses occur quickly, but the effect of the response depends on synthesis and/or degradation of new proteins. This change has a time scale from hours until days and for this reason is referred to as long-term. Experimental data showed that in cyanobacterial cells the PSI : PSII ratio increases (2- to 3-fold) when cells are grown under conditions which excite PSII (green-enriched light), while the ratio decreases (around half) when the light conditions excite PSI (red-enriched light) (Kawamura *et al*., 1979; Myers *et al*., 1980). In addition, the PSI : PSII ratio is regulated also in respect to the source of inorganic carbon (CO_2 versus HCO_3^-) (Eley, 1971; Manodori and Melis, 1984). The quality of light irradiating cells influences not only the ratio PSI : PSII but also the structure of PBS. This phenomenon, known as complementary chromatic adaptation (CCA), reflects a changing in the PE : PC ratio depending on light conditions (Bennet and Bogorad, 1973; Bryant and Cohen-Bazire, 1981). In particular in green-enriched light, PBS contains a single PC hexamer and up to three PE hexamers: if cells are moved to red-enriched light, they have almost no PE and up to three PC hexamers. CCA is the result of a coordinated activation/suppression of the genes encoding PE and PC (*cpe* operon and *cpc* operon, respectively) (Conley *et al*., 1985, 1986; Mazel *et al*., 1988; Federspiel

and Scott, 1992). Photosynthetic organisms are sensitive not only to the quality of light, but also to its intensity. Under light-saturating conditions cyanobacteria reduce their antenna size and the photosystem content which is accompanied with a marked decrease in chlorophyll *a* and phycobiliproteins (Hihara *et al*., 1998; He *et al*., 2001; Havaux *et al*., 2002). Phycobilisome sizes and photosystem contents are reduced to avoid absorption of excess light energy. In higher plants, high light induces the expression of a family of chlorophyll *a/b* binding protein (ELIPs) which are localised in the thylakoid membranes and are involved in photoprotection (Grimm and Kloppstech, 1987; Kolanus *et al*., 1987; Adamska and Kloppstech, 1991; Adamska *et al*., 1992). In *Synechococcus* sp. PCC 7942 a homologue to the ELIP family has been described (Dolganov *et al*., 1995). This protein, designated HliA, is located in the thylakoid membrane and induced at high light and UV light (Dolganov *et al*., 1995; He *et al*., 2001; Van Waasbergen *et al*., 2002). The expression of HliA, was found to be under the control of NblS, a sensor histidine kinase of a typical two-component regulatory system of bacteria. The absence of NblS influences also regulation of the *psbA* genes and *cpcBA* operon at high light (Van Waasbergen *et al*., 2002) and the degradation of PBSs during nitrogen and sulphur starvation (Collier and Grossman, 1992, 1994; Grossman *et al*., 2001). Adaptation of the cell to nutrient starvation is well-studied in cyanobacteria (Allen and Smith, 1969; Yamanaka and Glaser, 1980; Collier and Grossman, 1992; Baier *et al*., 2001; Grossman *et al*., 2001; van Waasbergen *et al*., 2002). When cyanobacterial cells are grown in nitrogen-depleted medium, their colour changes from a normal green-blue to chlorotic yellow or yellow-green. This phenomenon, referred to chlorosis, is the result of the PBS degradation which represents a source of nitrogen for the cells. Chlorosis is also induced in the absence of sulphur and phosphorous in *Synechococcus*, but not in *Synechocystis* (Collier and Grossman, 1992; Richaud *et al*., 2001). Degradation of PBS is triggered by the NblA protein, although the mechanism of its function is not yet clear. It has been suggested that NblA can tag PBS for degradation or activate a protease responsible of PBS degradation. It has also been suggested an interaction of NblA with NblS through NblR which could represent a response regulator for NblS (Schwarz and Grossman, 1998; Grossman *et al*., 2001).

1.5 Phosphorylation pathways in plants and cyanobacteria

Photosynthesis is a process which is strictly dependent on environmental conditions, in particular light and temperature. Photosynthetic organisms have developed the ability to adjust the photosynthetic processes regulating energy distribution between reaction centres and synthesis/degradation of photosynthetic protein components. The facility by which one or more phosphate groups can be added or removed from a protein makes phosphorylation an attractive way to regulate cellular responses in respect to the environment (Pawson, 1994). Bacteria usually use two-component systems for signal transduction (Stock *et al*., 1990; Alex *et al*., 1994) which, in the simplest form, includes two proteins: a histidine kinase and a response regulator. The histidine kinase autophosphorylates on a conserved histidine residue in response to a stimulus. The phosphate group is then transferred to an aspartate residue on a cognate response regulator. In *Synechocystis* more than 80 ORFs have been found to encode proteins of two-component systems (Mizuno *et al*., 1996). Eukaryotic organisms employ Ser/Thr and Tyr kinases and Ser/Thr and Tyr phosphatases as well as several accessory components for the same purpose. However, some prokaryotic-like two-component systems were identified as well. The first was identified in the ETR1 family in *Arabidopsis* and it is involved in the ethylene perception (reviewed in Kieber, 1997). Until now, about 14 genes encoding proteins similar in sequence to response regulators were identified in *Arabidopsis.* These proteins are induced by different plant hormones, but the function for most of them is still unclear (reviewed in D´Agostino and Kieber, 1999). Two-component systems in eukaryotes appear to be more complicated than those of in bacterial cells, since they include additional proteins containing phosphorylable aspartate or histidine residues between the sensor and the response regulator. In eukaryotes some signal transduction systems similar to the typical bacterial ones were described. Conversely, Ser/Thr kinases and phosphatases resembling the eukaryotic models were found in bacteria (reviewed in Zhang, 1996 and in Kennelly and Potts, 1996). Seven Ser/Thr kinases and seven Ser/Thr phosphatases were found in the *Synechocystis* genome (Zhang *et al*., 1998). Unlike their eukaryotic counterparts, these genes are clustered in one operon. In addition, in the same cluster genes which could encode regulatory functions and

genes for a two-component signal transduction were found. These findings raised the idea that the action of these eukaryotic-like transduction pathways can be coupled with the two-component system. This would be in analogy with the discovery of two-component systems in eukaryotes. In the sensing of ethylene in *Arabidopsis*, in fact, a two-component system acts upstream of a Ser/Thr kinase in the same signal transduction pathway (Chang *et al*., 1993).

1.5.1 Phosphorylation in higher plant thylakoid membranes

Phosphorylation/dephosphorylation of LHCII is an example of protein regulation by the addition/removing of a phosphate group in chloroplasts. It was shown that light-activated membrane protein kinases lead to phosphorylation of about 20 thylakoid membrane proteins (Bennet *et al*., 1980; Allen, 1992; Vener *et al*., 1995; Gal *et al*., 1997; Vener *et al*., 1998; Snyders and Kohorn, 1999). The major thylakoid phosphoproteins are LHCII and PSII proteins, the reaction centre proteins D1, D2, CP43 and PsbH. The kinase which phosphorylates specifically LHCII is the chloroplastic protein STN7 in *Arabidopsis* (Bellafiore *et al*., 2005). T-DNA insertion line of *Arabidopsis* for the gene *stn7* could not perform any state transition and LHCII was not phosphorylated. The phosphorylation pattern of D1, D2, CP43 and PsbH were unaffected indicating that this protein specifically phosphorylates LHCII (Bellafiore *et al*., 2005). The reaction core proteins are phosphorylated by the thylakoid membrane kinase that belongs to the TAK family (thylakoid associated kinase, Snyders and Kohorn, 1999). It was shown that this protein is activated by reducing conditions in chloroplasts and it is associated with the PSII reaction centre and the cytochrome b_6f complex (Snyders and Kohorn, 1999). Phosphorylation of LHCII and reaction centre proteins of PSII is a reversible process and several phosphatases were proposed to be involved in the process of the dephosphorylation of these subunits (Sun *et al*., 1989; Hammer *et al*., 1997; Vener *et al*., 1999). LHCII is likely to be dephosphorylated by a stroma phosphatase (Hammer *et al*., 1997) while D1, D2 and CP43 are dephosphorylated by an intrinsic protein phosphatase (Vener *et al*., 1999; Rokka *et al*., 2000). In particular, this phosphatase seems to be regulated by the cyclophilin-like protein TLP40 in spinach chloroplasts (Fulgosi *et al*., 1998; Vener *et al*., 1999; Rokka *et al*., 2000; see below).

Phosphorylation controls the stability, degradation and turnover of the reaction centre proteins and depends not only on light but also on all those environmental conditions which interfere with PSII functions (Andersson and Aro, 1997; Barber *et al*., 1997; Kruse *et al*, 1997; Baena-Gonzales *et al*., 1999, Rokka *et al*. 2000). The D1 reaction centre protein of PSII shows the highest turnover rate among all PSII proteins (Barber and Andersson, 1992). Restoration of PSII activity requires the proteolytic degradation of the damaged protein and the integration of a newly-synthesised one in the complex (Kyle *et al*., 1984; Ohad *et al*., 1984; Aro *et al*., 1993; Andersson and Aro, 2001). Under light stress D1 protein becomes phosphorylated. However, its degradation takes place only after its dephosphorylation (Koivuniemi *et al*., 1995; Rintamäki *et al*., 1996). The phosphorylation-dephosphorylation of D1 is proposed to be a key regulatory event to coordinate the degradation with the integration of a new protein into PSII complex (Andersson and Aro, 1997). While the mechanism of PSII repair is well-accepted, the hypothesis about the reason of PSII photoinactivation was recently challenged (Ohnishi *et al*., 2005; Hakala *et al*, 2005). The currently accepted hypothesis on the reason of PSII photoinactivation suggested that the absorption of an excess light by the PSII reaction centre causes the generation of highly oxidative species (ROS) which damage the D1 protein (Vass *et al*., 1992; Okada *et al*., 1996; Keren *et al*., 1997). However, recent studies on the mechanism of PSII inactivation suggest that phoinhibition of PSII occurs in two steps. The first step is the inactivation of the oxygen-evolving complex by UV and/or blue light. The second step is the inactivation of PSII by visible light (absorbed by chlorophylls and pigments) and occurs only after the oxygen-evolving complex is inactivated (Ohnishi *et al*., 2005). This hypothesis, suggested for *Synechocystis*, is valid also for plants (Hakala *et al*., 2005).

1.5.2 Phosphorylation in *Synechocystis* thylakoid membranes

A repair cycle that selectively replaces damaged D1 subunits within PSII operates also in *Synechocystis* (Goloubinoff *et al*., 1988; Komenda and Barber, 1995), but the regulation of D1 turnover is not dependent on D1 phosphorylation state. In *Synechocystis*, in fact, reaction centre proteins of PSII are not phosphorylated.

Phosphorylation of PBS protein was shown for β–PC subunit (Allen *et al*., 1985; Sanders and Allen, 1987; Sanders *et al*., 1989) although these data are still a matter of debate. This phosphorylation was light-dependent and was postulated to drive state transitions in cyanobacteria in analogy with LHCII in chloroplasts (Allen and Holmes, 1986; see also above). However, it was reported later that the conditions for light-induced phosphorylation described by Allen and colleagues (1985) were inadequate to induce state transition in cyanobacteria (Biggins and Bruce, 1989). Recently, it was reported that linker proteins of PBS are phosphorylated in *Synechocystis* (Piven *et al*., 2005). In particular, it was shown that the dephosphorylation of linkers coincides with PBS degradation. Degradation of PBS proteins, in particular during high light stress and nutrient-deprivation, is governed by the *nbl* gene family through a bacterial two-component system (Schwarz and Grossman, 1998; Grossman *et al*., 2001; Van Waasbergen *et al*., 2002). In particular, degradation of PBS requires the activity of the NblA and NblB proteins (Baier *et al*., 2001; Richaud *et al*., 2001; Grossman *et al*., 2001; Van Waasbergen *et al*., 2002). While NblB is clearly a lyase which removes the chromophore from phycobiliproteins before degradation, the function of the NblA protein is still not clear. NblA is likely not a protease itself. It was suggested that NblA can tag phycobiliproteins for degradation or induce some conformational changes which would give access for the activity of phosphatases and proteases and/or other enzymes involved in PBS degradation.

1.5.3 TLP40, a possible phosphatase regulator found in spinach chloroplasts

TLP40 (thylakoid lumen PPIase of 40 kDa) represents the first complex immunophilin protein identified from plants. In spinach, it is located in chloroplasts. It was found during the purification and characterization of thylakoid membrane phosphatases (Fulgosi *et al*., 1998; Vener *et al*., 1999). The sequence of the protein is shown schematically in Figure 6.

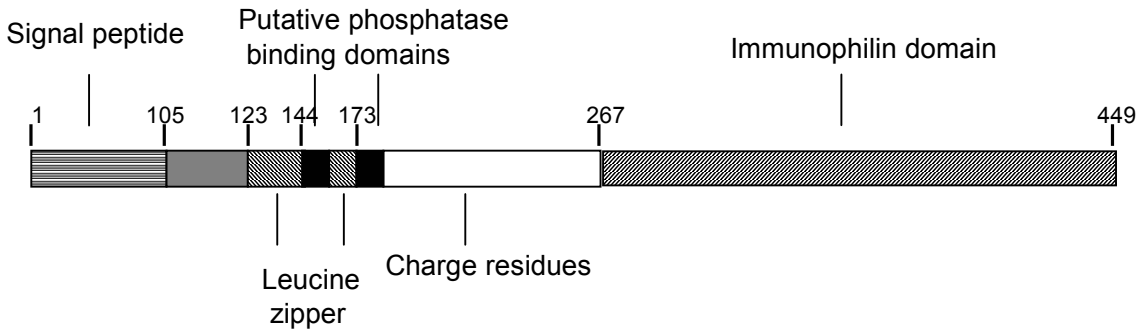


Fig. 6. Schematic presentation of structural domains of TLP40 protein from spinach (according to Fulgosi *et al*., 1998)

Sequence analysis and secondary structure predictions revealed a complex domain structure of TLP40. The most striking feature of the protein is the immunophilin domain located at the C-terminus (residues 267 – 449). Immunophilins comprise a superfamily of ubiquitous proteins which despite the lack of structural similarity catalyze the *cis-trans* isomerisation of proline peptide bonds (PPIase or rotamase activity, Fischer *et al*., 1989). Such isomerisation is one of the rate limiting steps during protein folding. For this reason, the main physiological role of these components is a chaperon function for newly synthesized or translocated proteins. However, immunophilins play also roles in cellular signalling (Schreiber *et al*., 1991) and in the regulation of other cellular processes. In *Arabidopsis* a total of 29 immunophilins were found. Within them, one is located in the chloroplast stroma and five are found in the lumen (Romano *et al*., 2004). Immunophilins that comprise cyclophilins and FKBPs (FK506/rapamycin-binding proteins) target the immunosuppressive drugs cyclosporin A (CsA) and FK506/rapamycin respectively (Schreiber, 1991). A third subfamily, designated parvulins, has rotamase activity but is insensible to any immunosuppressive drug (Rahfeld *et al*., 1994; Hani *et al*., 1995). Recently, a new subfamily has been described which contains both the cyclophilin and FBK domain (Adams *et al*., 2005). TLP40 belongs to the cyclophilin subfamily even if the homology with the human cyclophilin A, used as basic reference for this class of enzymes, is only 25% (Fulgosi *et al*., 1998). The N-terminal domain of TLP40 contains several structural features which might provide a site of interaction with other proteins. In particular, it is possible to distinguish two leucine zipper domains (residues 123 - 173) which can interact with other leucine zippers or with coiled-

coil-containing proteins and a cluster of charge residues (19 acidic and 13 basic, residues 179 - 266) which in other high molecular weight cyclophilins were shown to bind to other proteins. In addition, there are also two short stretches GLKALDSVERN[158] and AGLAESKKDRG[185] which possess 60% similarity to sites of the known-immunophilin FKBP12, which bind the phosphatase calcineurin (Aldape *et al*., 1992). These two short peptides could explain the association of TLP40 with the thylakoid phosphatase during its purification. The presence of a bipartite transit peptide, in the amino acid sequence and *in organello* import experiments showed that the protein is soluble and located in the thylakoid lumen as free protein and also associated to the membrane. Separation of thylakoids into grana and stroma lamellae and Western analysis using a specific polyclonal antibody raised against the full length TLP40, showed that the protein is enriched in the stroma lamellae (Fulgosi *et al*., 1998). This localisation corroborates the probable association of TLP40 with a thylakoid membrane phosphatase also located in stroma lamellae. This intrinsic phosphatase was shown to be involved in the specific dephosphorylation of the PSII core proteins CP43, D1 and D2 during PSII turnover (Vener *et al*., 1999). Structurally the phosphatase belongs to the family of the PPP phosphatases. Among these, some were shown to be regulated by immunophilins in mammalian and yeast cells (Schreiber *et al*., 1992; Cardenas *et al*., 1994). The association/dissociation of TLP40 to the phosphatase was suggested to be a regulatory mechanism of the membrane phosphatase. It was shown *in vitro* that the activation of the phosphatase coincided with the release of TLP40 into the thylakoid lumen (Rokka *et al*., 2000).

1.6 Aim of the work

Homologous proteins of TLP40 were found in *Arabidopsis thaliana* and in *Synechocystis* sp. PCC 6803. While the finding of TLP40 in *Arabidopsis* was not surprising, the identification of a TLP40 homolog in *Synechocystis* was intringuing, since the phosphorylation pattern as a way of regulation is completely different in cyanobacteria in comparison to higher plants. Most of this work is based on the characterisation of the insertional mutant for the respective gene of TLP40 in *Synechocystis* and on comparison of bacterial TLP40 with that of spinach. Initial work on selection on *Arabidopsis* T-DNA insertion line is also presented.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals and enzymes

All chemicals used in this study were of p. a. quality. They were purchased from the following companies: Difco Laboratories (Detroit, USA), Merck AG (Darmstadt, Germany), Serva Feinbiochemica (Heidelberg, Germany), Roche (Basel, Switzerland), Roth GmbH & Co. (Karlsruhe, Germany) and Sigma Chemical Company (Munich, Germany).

Radiochemicals (α^{32}P-dCTP, ^{35}S-Met, γ^{32}P-ATP) were from Amersham Pharmacia Biotech (Freiburg i. Br., Germany). Restriction endonucleases, DNA- and RNA-modifying enzymes, DNA-, RNA-polymerases were purchased from MBI Fermentas (St. Leon-Rot, Germany) and Roche (Grenzach-Wyhlen, Switzerland).

2.1.2 Molecular weight markers

RNA and DNA length molecular standards

The 0.24 - 9.5 kb RNA Ladder from Gibco/BRL (Karlsruhe, Germany) was used as a RNA length standard. The DNA λ EcoRI/HindIII and 1 kb-DNA-Extension-Ladder from Gibco/BRL (Karlsruhe, Germany) were used as DNA length standards.

Protein molecular weight standards

The apparent molecular weight of proteins in SDS polyacrylamide gel electrophoresis was determined according to SDS-7 molecular weight marker (14 - 66 kDa) from Sigma Chemical Company (Munich, Germany). PeqGOLD high molecular weight protein marker (14.4 – 116 kDa) and peqGOLD prestained

protein marker (20 - 122 kDa) were from PeqLab Biotechnologie GmbH (Erlangen, Germany).

2.1.3 Vectors, bacterial strains and plant material

Vector

pVZ321(Zinchenko *et al*., 1999)

Bacterial strains

E. coli BL21 (DE3) pLysS (Studier and Moffat, 1986)
E. coli C600 (Appleyard, 1954)
E. coli DH5α (Hanahan, 1985)
E . coli R751
Synechocystis sp. PCC 6803

Plant material

Arabidopsis thaliana, ecotype Columbia
Spinacia oleracea var. Monopa

2.1.4 Antibodies

Antisera against subunits of PSI (PsaA, PsaB; dilution 1 : 1.000), PSII (D1, D2, CP43, CP47; dilution 1 : 1.000), Cytochrome b_6f complex (anti-Rieske Fe/S protein; dilution 1 : 1.000) ATP synthase (α subunit; dilution 1 : 5.000) TLP40 and cTLP40 (dilution 1 : 1.000) were kindly provided by Prof. Dr. R. G. Herrmann.

2.1.5 Oligonucleotides

All specific oligonucleotides that are listed below were obtained from MWG-BIOTECH (Ebersberg).

Primers specific for *Arabidopsis thaliana*

FWDsynara	5’-TTA CGG AGG ACC GGA CTG GGC CAA AGC ACC CA-3’
TLP40FULEC	5´-TTA GGA TCC ACC GGC GAT TTT GTA ACT CG-3´
TLP40FULECII	5’-TTA GGA TCC TTA GCG AGG TTC TCT AAA CC-3’
TLP40FULEN	5´-ATT GAA TTC ATG GCG GCG GCG TTT GC-3´
TLP40IMMN2	5’-ATT GAA TTC CGA AAA AGA GAC GTT GC-3’
TLP40NXhoI	5’-ATT CTC GAG ATG GCG GCG GCG TTT GCC T-3’
TLP40CXhoI	5’-ATT CTC GAG TTAACC GGC GAT TTT GTA ACT C-3’
TLP40UR3106	5’-ATA CTT CCA AAG GCG GAG G-3’
TLP40121UF	5’-CCC AAC TAA CGA CCC ATC A-3’
TLP40578UR	5’-GGG GCT TTT TGG AGC AAG A-3’
TLP402000UF	5’-GGT ACA AAC GGG AGA TCC A-3’
TLP402581UF	5’-CCC GAG TTA CAA AAT CGC C-3’

Primers specific for T-DNA insertion

LBREVERSE	5’-ATT GAC GCT TAG ACA ACT TAA T-3’
LB3cut	5’-TCA TAA CCA ATC TCG ATA CAC-3
SAILLB1	5´-GAA ATG GAT AAA TAG CCT TGC TTC C-3´

Primers specific for *Synechocystis*

apcA_for	5’-ATG AGT ATC GTC ACG AAA TC-3’
apcA_rev	5’-CTA GCT CAT TTT TCC GAT AAC-3’
apcendfwd	5’-GGG CGT ACG GGA AAT GTA TC-3’
D17045F	5’-AAA ACG CCC TCT GTT TAC C-3’
D17252R	5’-CGC TGT TGG AGA GTC GTT-3’
D18149F	5’-AGC CAA GGC CGG GTA ATC G-3’
D18311R	5’-TTA ACC GTT GAC AGC AGG A-3’
D13523557F	5’-AAG GGA AGT TAA TCA ATG CC-3’
D13523747R	5’-CTG TAA TCC TAA TTG GGT AG-3’

REVarasyn 3’	5’-TTA CGG AGG ACC GGA CTG GGC CAA AGC ACC CA-
Sll0408Fover	5’-ATT CTC GAG ATG GGG GCG TTG AAG TGG-3’
SLL0408N	5’-CGG AAT TCG CTG ACT TCC ATC TCC TCT-3’
SLL0408ROVER	5’-TTA CTG CAG TCA ACT ATT GCC GTT AAC TAA-3’
SLL0409FWD	5’-GTA ACA GCC CAT GGC CAT T-3’
SLL0409REV	5’-TCT GTT ATG GGT TCC CTT AG-3’
slr0906endfwd	5’-ATC TGG CAT GGT TCC CGG A-3’
slr0906fwd	5´-TCT TAA GCT GTG GTT AAA AGC TGT-3´
16sendfwd	5’-AAT TCG TTC CCG GGC CTT GTA-3
16S_RNA fwd	5’-AAT GGA GAG TTT GAT CCT GGC T-3’
16S_RNArev	5’-AGG TGA TCC AGC CAC ACC TTC-3’

2.1.6 Synthetic peptides for the kinetic assay

Substituted peptides were purchased from Genscript Corporation, Pinscht, New Jersey

2.1.7 General buffers and solutions

TE buffer	10 mM Tris/HCl, pH 8.0 1 mM EDTA
STET buffer	8% sucrose 5% Triton X-100 50 mM EDTA 50 mM Tris/HCl, pH 8.0
10 x TBE	1 M Tris 0.5 M boric acid 20 mM EDTA
20 x SSC	3 M NaCl 0.3 M Na_3-citrate x 2 H_2O, pH 7.2

10 x Blotting buffer	1.5 M glycin
	0.2 M Tris/HCl, pH 8.0
10 x PBS	750 mM NaCl
	30 mM KCl
	45 mM Na_2HPO_4
	15 mM KH_2PO_4

Antibiotic stock solutions:

Ampicillin	100 mg/ml (in 50% ethanol)
Gentamycin	10 mg/ml (in water)
Chloramphenicol	25 mg/ml (in 50% ethanol)
Lincomycin	100 mg/ml (in 50% ethanol or water)

2.1.8 Media

2.1.8.1 *E.coli* growth medium

LB	10 g Bacto-Trypton
	5 g Yeast Extract
	10 g NaCl
	H_2O up to 1 l

For solid media 15 g/l of agar were added. After autoclaving, antibiotics (100 µg/ml ampicillin, 20 µg/ml chloramphenicol) were added from stock solutions.

2.1.8.2 BG11 medium for *Synechocystis*

Macro-elements (except $NaNO_3$) were prepared as a 1000 x stock solution and added to the medium before autoclaving. Micro-elements, calcium chloride ($CaCl_2$

x H_20) and magnesium sulphate ($MgSO_4$ x $7H_2O$) were added after autoclaving. For solid medium 1% Agar in BG11 (Difco, Detrit, USA) was added.

Macro-elements (1 x)	g/l
$NaNO_3$	7.5
K_2HPO_4 x 3 H_2O	0.051
$MgSO_4$ x 7 H_2O	0.075
$CaCl_2$ x 2 H_2O	0.036
citric acid	0.006
ferrum ammonium citrate	0.006
EDTA (disodium salt)	0.001
Na_2CO_3	0.02

Micro-elements (1000 x)	g/l
H_3BO_3	2.86
$MnCl_2$ x 4 H_2O	1.81
$ZnSO_4$ x 7 H_2O	0.222
$NaMoO_4$ x 2 H_2O	0.39
$CuSO_4$ x 5 H_2O	0.079
$Co(NO_3)_2$ x 6 H_2O	0.05

2.1.8.3 MS-medium for *Arabidopsis*

Micro-elements and vitamins were prepared as a 1000 x stock solutions and added to the medium before autoclaving.

Macro-elements (1 x)	g/l
NH_4NO_3	1.65
KNO_3	1.9
$CaCl_2$ x $2H_2O$	0.22
$MgSO_4$ x $7H_2O$	0.37
KH_2PO_4	0.17

Micro-elements (1000 x)	g/l
KI	0.83
H_3BO_4	6.2
$MnSO_4$ x H_2O	22.3
$ZnSO_4$ x 7 H_2O	8.6
Na_2MoO_4 x H_2O	0.25

$CuSO_4$ x 5 H_2O	0.025
$CoCl_2$ x 6 H_2O	0.025
EDTA	40

Vitamins and amino acids (1000 x)	g/l
inosit	100
pyridoxin HCl	0.5
thiamin HCl	0.1
glycin	2
nicotinic acid	0.5

Arabidopsis plates were prepared by adding 0.8% of purified agar and 1.5% of sucrose to ½ MS medium. The pH was adjusted to 5.8 with KOH. After autoclaving the medium was cooled down to 55°C and Cefotaxin (Cfx) was added to a final concentration of 200 µg/ml. When required BASTA was added to a final concentration of 30 µg/ml.

2.1.9 Blotting membranes

Hybond nitrocellulose membranes for Southern and Northern blots were purchased from Amersham (Braunschweig). PVDF and nitrocellulose PROTRAN membrane for Western blots were obtained from PALL (Portsmouth, England).

2.2 Methods

Standard molecular biology methods like DNA plasmid extraction from *E. coli,* DNA gel electrophoresis, DNA restriction analysis, DNA phenol/chloroform extraction, DNA ethanol/isopropanol precipitation, DNA dephosphorylation and ligation were performed according to Sambrook *et al*. (1989) or according to instruction manuals unless otherwise indicated. DNA fragments were purified from low melting agarose gels.

2.2.1 Sequence analysis

Genome sequences were obtained from *Synechocystis* and *Arabidopsis* databanks (*http://www.kazusa.or.jp/cyano/* and *http://www.mips.biochem.mpg.de/* respectively).

Analysis of protein and gene homologies was performed using BLAST search services of NCBI (*http://www.ncbi.nml.nih.gov/BLAST*). Hydropathy plots were performed according to Kyte and Doolittle (1982).

2.2.2 Strains and growth conditions for *Synechocystis*

Wild-type and mutant strains were cultivated in BG11 medium at a light intensity of 50 µmol photons m^{-2} s^{-1} (low light, LL) and 30°C (standard conditions, ST). For the analysis of the strains at high (HL) cultures were grown at 450 µmol photons m^{-2} s^{-1}. Mutant strains were grown in BG11 in presence of 0.24 µg/ml of gentamycin.

2.2.3 Growth conditions for *Arabidopsis thaliana* and *Spinacia oleracea*

Plants were grown either on soil or plates. In the first case plants were kept in the green house at a temperature of 25°C and a day/night cycle of about 14/10 h. Plants on plates were cultivated in growing chambers at a constant temperature of

22°C and a day / night cycle of 14/10h. In both cases the germination of the seeds was synchronized by incubation for 2 days at 4° C.

2.2.3.1 Sterilisation of *Arabidopsis* seeds

When *Arabidopsis* was grown on plates, it was necessary to sterilise the seeds to eliminate contaminations.

Sterilisation buffer 5% Dimamin
0.02% Triton X-100

Seeds were incubated in 300 µl of sterilisation buffer for 1 min, collected by centrifugation, washed 3 times with sterile water and finally distributed on the plates.

2.2.4 Conjugal transfer of plasmids into cyanobacterial cells

Conjugation is a general strategy for transfer of plasmid DNA from *E. coli* into cyanobacterial cells using the broad host range conjugal apparatus of an IncP plasmid (such as R751 plasmid). Conjugal transfer of pVZ321 plasmids into cyanobacterial cells was performed via triparental mating using three parents, *E. coli* bearing the conjugal plasmid, *E. coli* bearing the cargo plasmid plus helper(s) and the target cyanobacteria, as described by Zinchenko *et al*. (1999).

Cyanobacterial cells of wild-type or mutant *Synechocystis* strains were grown until late exponential growth phase (A_{750} of 1.0 – 1.5) at standard conditions. The cells were then collected by centrifugation and washed with 0.9% NaCl. Overnight cultures of *E. coli* were washed with LB to remove antibiotics. Then 1 ml of cyanobacterial cells was mixed with *E. coli* (1 ml), which contained plasmids pVZ321 and R751, washed twice with 0.9% NaCl and finally resuspended in 30 - 40 µl of BG11. All three strains were spotted onto a nitrocellulose filter resting on solid BG11 medium containing 5% of LB and incubated at 30°C and dim light until the appearance of colonies. Transformed colonies were selected on medium

containing increased concentrations of antibiotics (kanamycin 5 – 50 µg/ml; chloramphenicol 0.5 - 20 µg/ml or gentamycin 0.5 - 3 µg/ml).

2.2.5 DNA and RNA extraction

2.2.5.1 DNA isolation from *Synechocystis*

For isolation of chromosomal DNA *Synechocystis* cells from 3 – 5 ml of culture at the late exponential phase (A_{750} = 1.0) were collected, washed with 1 ml of TE buffer and resuspended in 270 µl STET buffer (Section 2.1.7). The cell suspension was mixed with 15 µl of chloroform and vigorously vortexed for 5 min. 30 µl of lysozyme (20 mg/ml) was added and the cell suspension was incubated for 30 min at 37°C. The lysozyme-treated cells were lyzed for 50 min at 65°C by addition of 100 µl of 10% (w/v) SDS and further incubated for 10 min after addition of 100 µl 5 M NaCl. Proteins were removed from the lysate by chloroform extraction. DNA was precipitated with isopropanol, washed with 70% ethanol and dissolved in sterile water.

2.2.5.2 RNA isolation from *Synechocystis*

Total RNA was isolated from mid log-phase liquid culture (A_{750} = 0.5) using TRIZOL reagent according to the manufacturer instructions (GibcoBRL Life technologies, Invitrogen GmbH, Karlsruhe). Cyanobacterial cells were harvested by centrifugation at 4°C for 15 min at 12.000 x g, quickly frozen in liquid nitrogen and homogenized with 1 ml of Trizol solution. The samples were incubated for 5 min at RT to insure complete dissociation of nucleoprotein complexes. An equal volume of phenol-chloroform-isoamyl alcohol mixture, pH 6.6 - 7.9, (Ambion) was added, and the lysate and incubated on ice for 5 min. The suspension was centrifuged at 12.000 x g for 5 min at 4°C. The aqueous phase containing RNA was mixed with 1/10 aqueous phase volume of 3 M sodium acetate and shaken for about 10 sec. Acid-phenol-chloroform (Ambion) was added and the mixture was incubated for 5 min at 4°C, followed by centrifugation at 12.000 x g for 5 min at 4°C. The RNA in the upper phase was precipitated overnight with isopropanol and collected by centrifugation at 12.000 x g for 30 min at 4°C. The RNA pellet

was washed once with 70% EtOH, dried at RT, dissolved in RNAse-free water and incubated at 55°- 60°C for 15 min. RNA concentrations were determined by measuring the absorbace at a wavelength of 260 nm.

2.2.5.3 DNA isolation from *Arabidopsis thaliana*

Buffer 1 (2 x)	600 mM NaCl
	100 mM Tris/HCl, pH 7.5
	4 mM EDTA
	4% N-lauryl sarcosine, sodium salt
	1% SDS

Extraction buffer (50 ml)	25 ml buffer 1 (2 x)
	20 ml 12 M urea
	2.5 ml phenol, pH 8.0
	2.5 ml deionised water

3 - 4 leaves of *Arabidopsis* were cut and frozen immediately in liquid nitrogen. Leaves were broken with a pestle and resuspended in 250 µl of freshly prepared extraction buffer. Leaf disruption was repeated once more and another 250 µl of extraction buffer was added. After mixing 400 µl of phenol/chloroform were added and the mixture was centrifuged at 16000 x g for 15 min at 20°C. Total DNA in the aqueous phase was precipitated with 0.8 volumes of isopropanol for 10 min at RT. DNA was collected by centrifugation at 16000 x g for 15 min at 20°C, washed once with ethanol 70%, dried and resuspended in 20 - 50 µl of sterile water. DNA concentrations were determined by measuring the absorbance at a wavelength of 260 nm.

2.2.6 Analysis of gene expression

2.2.6.1 Northern analysis

Hybridization solution	0.5 M sodium phosphate, pH 7.2
	7% SDS

1 mM EDTA

Sample loading buffer	1.2 ml deionized formamide
	0.4 ml formaldehyde
	0.24 ml 10 x MOPS
	bromophenol blue (trace)

RNA samples (15 µg of total RNA per lane) were mixed with 2.5 volumes of loading buffer, denatured for 7 min at 80°C and electrophoresed on 1.2% agarose gels in MOPS-buffer with 2.2 M formaldehyde. RNA marker was used for the estimation of molecular masses. RNA was transferred to nylon membranes (Hybon-N+, Amersham) with 10 x SSC as described in Sambrook *et al*. (1998) and crosslinked to the membrane for 1 hour at 80°C.

The hybridization probes were synthesized by the random priming method with α-[^{32}P]-dCTP and Klenow fragment of DNA polymerase (Feinberg and Vogelstein, 1983). RNA blots were prehybridized for at least 2 h at 42°C in hybridization solution. Hybridization was carried out overnight at 42°C. After hybridization, membranes were washed at least two times with decreasing concentrations of SSC solution containing 0.5% SDS. Dried membranes were exposed to Phosphoimaging plates (Fuji GmbH, Düsseldorf), which were read out with a TLA 3000 Phosphoimage Reader and AIDA software (Raytest Isotopenmessgeräte, Straubenhardt).

2.2.6.2 Methylene blue staining of RNA on nylon membranes

Methylene blue solution	0.03% methylene blue
	0.3M NaOAc, pH 5.2

The RNA on nylon membranes was stained before or after hybridization by soaking in methylene blue solution for one minute and washing several times with distilled water.

2.2.6.3 RT-PCR

Before RT-PCR, residual DNA in the preparation of RNA was removed by DNAse I treatment at 37°C for one hour. After denaturation of RNA for 15 min at 95°C (necessary for removing the secondary structure of RNA), cDNA synthesis was performed for 30 min at 55°C using 1 µg of total RNA in a final volume of 20 µl according to the manufacturer's instructions (Roche, Grenzach-Wyhlen). As a negative control for DNA presence, 1 µg of RNA was subjected to PCR amplification with specific primers for the 16S rRNA gene.

2.2.6.4 Real-time PCR

Real-time PCR was performed in a reaction volume of 20 µl according to the instruction manual (Qiagen). Measurements and analysis of results were done in a Roche Light Cycler 24 and supplied software (Roche, Grenzach-Wyhlen, Switzerland).

2.2.7 Determination of cell density in *Synechocystis*

The cell density of *Synechocystis* was estimated by measuring the absorbance of suspension at 750 nm (A_{750}) (spectrophotometer Uvikon, Kontron). For most of experiments cells were collected when they reached an A value between 0.3 - 0.5.

2.2.8 Measurement of pigment concentrations

2.2.8.1 Determination of chlorophyll concentrations in *Arabidopsis* and spinach

Pigments were extracted from chloroplasts or thylakoid membranes with 80% (v/v) acetone. After centrifugation at 10.000 x g for 10 min at 4°C, the chlorophyll content was determined from the absorbance of the acetone extract at 645 (chlorophyll b) and 665 nm (chlorophyll *a*) according to the equation:

$$\text{total chlorophyll (µg/ml)} = 20.2 \times A_{645} + 8.02 \times A_{665} \times \text{dilution}$$

2.2.8.2 Determination of chlorophyll concentration in *Synechocystis* cells

Pigments were extracted from cell suspensions and total membranes with 90% (v/v) methanol. After centrifugation at 10.000 x g for 10 min at 4°C, chlorophyll *a* contents were calculated from the absorbance of the methanol extract at 652 and 665 nm using the equation:

$$\text{chlorophyll } (\mu g/ml) = 16.82 \times A_{665} - 9.28 \times A_{652}.$$

2.2.8.3 Determination of carotenoid concentrations in *Synechocystis*

Carotenoid concentrations were determined by measuring the absorbance of the methanol extract (2.2.8.2) at 470 nm and the concentration of chlorophyll:

$$\text{carotenoids } (\mu g/ml) = (1000 \times A_{470} - 1.91 \times [\text{ Chl }]) / 225$$

2.2.8.4 Determination of phycobilin concentrations in *Synechocystis*

The phycobilin content was estimated according to Grossman *et al*. (1992). Cell samples were heated at 75°C for 10 min and the phycocyanin content was determined according to the following equation:

$$\text{phycocyanin } (\mu g/ml) = [A_{620} - A_{750} \text{ (unheated)}] - [A_{620} - A_{750} \text{ (heated)}]$$

2.2.9. Isolation of photosynthetic membranes

2.2.9.1 Isolation of photosynthetic membrane proteins from *Synechocystis*

Buffer A	50 mM HEPES-NaOH, pH 7.0
	500 mM sucrose
	15 mM NaCl
	5 mM $MgCl_2$

Buffer B	10 mM Tricine/HCl, pH 7.5
	6% sucrose
	30 mM DTT

Cells were harvested by centrifugation at 4°C, resuspended in 600 µl of buffer A and then broken by vortexing with glass beads (3 times for 2 min each and keeping the samples for 1 min on ice between cycles). Unbroken cells were removed by a brief centrifugation (3500 x g for 1 min at 4°C). Thylakoids were sedimented from supernatant by centrifugation at a maximum speed of 20.000 x g for 30 min at 4°C. Thylakoids were washed twice in buffer A and finally resuspended in buffer B.

2.2.9.2 Isolation of chloroplasts from *Arabidopsis* and spinach

Homogenization buffer	20 mM EDTA
(10 x)	10 mM $MgCl_2$
	10 mM $MnCl_2$
	500 mM HEPES/KOH, pH 7.6
	3.3 M sorbitol

This buffer was stored at -20°C. It was diluted tenfold with water before use and 5 mM of Na-ascorbate was added.

PBF-Percoll stock	3% (w/v) PEG 6000
	1% (w/v) BSA
	1% (w/v) Ficoll 400

Dissolve all components in Percoll

45% Percoll	330 mM sorbitol
	50 mM HEPES/KOH, pH 7.6
	2 mM EDTA
	1 mM $MgCl_2$
	45 % (v/v) PBF-Percoll stock

85% Percoll	330 mM sorbitol

50 mM HEPES/KOH, pH 7.6
2 mM EDTA
1 mM $MgCl_2$
85% (v/v) PBF-Percoll stock

The subsequent procedure refers to 120 g of leaves. Buffer volumes can be scaled up linearly for different amounts of starting material.

Leaves were divided into two portions and homogenized 3 times for 20 sec in 180 ml of homogenization buffer. After filtration, chloroplasts were precipitated in a JA-14 rotor (Beckman) at 5.000 rpm without brake. The pellet was resuspended gently in 2 ml of homogenization buffer and intact chloroplasts were separated from the broken ones by centrifugation on Percoll gradients. Percoll gradients were prepared in glass tubes with 10 ml of 85% Percoll solution at the bottom and 12 ml of 45% Percoll solution at the top. The gradients were centrifuged in a JS-13 rotor (Beckmann) at 10.000 rpm for 10 min. Intact chloroplasts were collected from the interface between the layers. Chloroplasts were washed carefully twice with 20 ml of homogenization buffer (5.000 rpm in JS-13 rotor without brake) to remove Percoll and finally resuspended in 2 ml of homogenization buffer.

2.2.9.3 Rupture of thylakoid membranes by sonication

Thylakoid membranes of *Synechocystis* or *Arabidopsis* were broken by three cycles of sonication at 70 Watt for 2 min. Between each sonication step thylakoids were kept on ice for 3 min. Membrane proteins were separated from soluble lumenal ones by centrifugation at 45000 x g at 4°C for 30 min. The lumenal fraction was then transferred to a new vessel, while membranes were washed few times with the actual buffer and finally resuspended to the desired chlorophylls concentration.

2.2.10. Isolation and separation of photosynthetic complexes

2.2.10.1 Isolation of photosynthetic complexes from *Synechocystis* by sucrose gradient ultracentrifugation

SMN buffer: 0.4 mM sucrose
10 mM NaCl
50 mM MOPS, pH 7.0

Cells were harvested at the late exponential growth phase and resuspended in SMN solution containing 0.2 mM PMSF and 5 mM benzamidine. Cells were broken by vortexing with glass beads and thylakoids were sedimented by centrifugation at 50.000 x g for 60 min. Thylakoid membranes (200 µg chlorophyll/ml) were incubated for 30 min at RT with 1 mM $CaCl_2$ in SMN buffer and then solubilized by addition of β-dodecyl-D-maltoside (Sigma, Taufkirchen) to a final concentration of 1.5% for 15 min on ice. Insoluble material was pelleted by centrifugation at 20.000 x g for 15 min at 4°C. Solubilized membranes were loaded onto a linear 12 - 28% sucrose gradient in 10 mM MOPS, pH 7.0, 0.05% β-dodecyl-D-maltoside and centrifuged at 160.000 x g for 16 h using 12-ml tubes of a SW40 rotor (Beckman).

2.2.10.2 Isolation of photosynthetic complexes from *Arabidopsis* and spinach by sucrose gradient ultracentrifugation

TMK 10 mM Tris/HCl, pH 6.8
10 mM $MgCl_2$
20 mM KCl

Intact chloroplasts were isolated as described in Section 2.2.9.2. Thylakoid membranes (400 µg chlorophyll) were isolated from chloroplasts by incubation in TMK buffer for 15 min on ice and centrifugation for 5 min at 3500 x g at 4° C. Thylakoid membranes were washed twice with 500 µl TMK and finally resuspended in 100 µl of TMK. Membranes were solubilized by addition of 300 µl of 2% β-dodecyl-D-maltoside (end concentration 1.5%) for 15 min on ice. Insoluble

material was pelleted by centrifugation at 20.000 x g for 15 min at 4°C. Solubilized membranes were loaded onto a linear 0.1 – 1.0 M sucrose gradient in TMK containing 0.06% β-dodecyl-D-maltoside and centrifuged at 160.000 x g for 16 h using 12-ml tubes of a SW40 rotor.

2.2.10.3 Separation of photosynthetic complexes by non-denaturing gel electrophoresis (Blue Native PAGE)

Cathode buffer	50 mM Tricine
	15 mM Bis-Tris/HCl, pH 7.0
	0.02% (w/v) Coomassie G250
Anode buffer	50 mM Bis-Tris/HCl, pH 7.0
Gel buffer	0.5 mM ε-Aminocaproic acid
	0.05 Bis- Tris/HCl pH 7.0
ACA buffer	750 mM ε-Aminocaproic acid
	50 mM Bis-Tris/HCl, pH 7.0
	0.5 mM EDTA-Na_2
Sample buffer	5% (w/v) Coomassie Brilliant Blue G250
	750 mM ε-Aminocaproic acid
Denaturation buffer	2% (w/v) SDS
	66 mM DTT
	66 mM Na_2CO_3

Thylakoid membranes of *Synechocystis* (12 µg of chlorophyll per lane), *Arabidopsis* or spinach (50 µg of chlorophyll per lane) were solubilized in ACA buffer containing 1% β-dodecyl-D-maltoside. Unsolubilized material was collected by ultracentrifugation at 45.000 x g for 10 min at 4°C. Solubilized thylakoids were mixed with sample buffer (5% v/v) and loaded onto a 4 - 12% acrylamide gradient gel (containing a gradient of glycerol from 0 to 20%). The gel was run overnight at 5 mM at 4°C. When the front had reached half of the gel length size, cathode

buffer containing Coomassie Blue G250 was replaced by the same buffer without staining reagent. The gel was denatured by incubation in denaturation buffer for 20 min at RT.

2.2.10.4 Denaturating gel electrophoresis according to Laemmli *et al*. (1970)

10 x Laemmli buffer	0.25 M Tris/HCl, pH 8.5 1.92 M glycine 1% SDS
4 x sample buffer	0.25 M Tris/HCl, pH 6.8 8% SDS 40% glycerol 20% ß-mercaptoethanol 0.016% Bromophenol Blue

Table 1. Scheme for PAA gels

Solution	Separating gel (end concentration)	Stacking gel (end concentration)
Tris/HCl, pH 8.8	0.375 M	-
Tris/HCl, pH 6.8	-	0.125 M
acrylamide (acrylamide/ bisacrylamide = 29:1)	depending on desired concentration	5%
SDS	0.1% (w/v)	0.1% (w/v)
sucrose (for gradient gels)	10 – 17.5% (w/v) (gradient)	-
APS	0.06% (v/v)	0.08% (v/v)
TEMED	0.006% (v/v)	0.008% (v/v)
H_2O	until desired volume	until desired volume

In some cases the resolution of the gel was increased by addition of 4 M urea in the gel.

2.2.11 Staining of PAA gels

2.2.11.1 Coomassie Brilliant Blue staining

Staining solution	45% ethanol
	9% acetic acid
	0.2% (w/v) Coomassie Brilliant Blue R 250
Destaining solution	45% ethanol
	9% acetic acid

The gel was stained for 30 min in staining solution (which could be previously warmed up to 50°C to accelerate the process). It was destained in destaining solution to clear the background.

2.2.11.2 Silver staining (Blum *et al.*, 1987)

Fixing solution	50% ethanol
	12 % acetic acid
	0.05% of 37% formaldehyde
Thiosulfate solution	0.02% (w/v) $Na_2S_2O_3$
Silver solution	0.2% (w/v) $AgNO_3$
	0.075% of 37% formaldehyde
Developing solution	6% (w/v) Na_2CO_3
	0.05% of 37% formaldehyde
	4 µg/ml $Na_2S_2O_3$
Stop solution	50% ethanol
	12% acetic acid

Silver staining was used for detection of low protein quantities in gels. The gel of 1 mm thickness was incubated for 1 h in fixing solution, washed three times for 20 min in 50% ethanol and soaked for 1 min in thiosulfate solution. Afterwards, the gel was washed three times with water for 30 sec and incubated in silver solution for 20 min in darkness with constant agitation. The gel was then washed with water again and incubated in developing solution for 5 to 30 min. The reaction was stopped by addition of stopping solution.

2.2.11.3 Imidazol staining

Imidazol buffer	0.2 M Imidazol
Zinc sulphate buffer	0.3 M $ZnSO_4$
Destaining solution	1 x blotting buffer

This method was used for detection of protein bands in a gel before Western transfer. The gel was shaken for 10 min in imidazol-containing buffer and then briefly incubated in zinc sulphate buffer until visualization of protein bands. The transparent protein bands were visible in a milk-white background of the gel. Staining was terminated by incubation with water. The gel was stored at 4°C until blotting.

2.2.12 Immunological detection of proteins

2.2.12.1 Western transfer by the semi-dry blotting system

Anode buffer I	0.3 M Tris (no adjustment of pH required)
Anode buffer II	0.025 M Tris (no adjustment of pH required)
Cathode buffer	40 mM ε-aminocaproic acid 0.01% (w/v) SDS

Proteins were transferred onto membranes using a semi-dry blotting system (PHASE GmbH, Lübeck). The transfer membranes and PAA gels were incubated for 10 min in anode buffer II prior to transfer. Three layers of Whatman paper were soaked in cathode buffer and placed onto the bottom (cathode part). Then the gel was placed on paper and covered with the transfer membrane. Gel and transfer membrane assembly was covered by two layers of Whatman paper soaked in anode buffer II and three layers of Whatman paper soaked in anode buffer I. The transfer was performed for 2 h at 0.8 mA/cm^2 of gel.

2.2.12.2 Western transfer by the wet-blotting system

Blotting buffer	0.02 M Tris/HCl, pH 8.0
	0.15 M glycin

Proteins were transferred onto membranes using a wet-blotting system (Renner GMBH, Dannstadt). Transfer membranes and PAA gels were incubated for 1 min in blotting buffer prior to transfer. Two layers of Whatman paper were equilibrated in the blotting buffer and placed onto the cathode side. The gel was then placed on top of these papers and covered with the transfer membrane. Two layers of Whatman paper soaked in blotting buffer covered the transfer membrane. The transfer was performed for 2 h for a PAA gel of 1 mm thickness. After protein transfer, the membrane was dried for 10 min at 80°C.

2.2.12.3 Staining blots with Ponceau S

Ponceau S solution	0.2% (w/v) Ponceau S
	1.0% acetic acid

For the detection of proteins after transfer, membranes were incubated in Ponceau S solution for 15 min at RT under constant agitation. Membranes were then rinsed in water and the position of molecular weight standards was marked.

2.2.12.4 Western analysis using horseradish peroxidase-conjugated antibodies

Blocking buffer	1 x PBS

	5% dry milk
	1% Tween 20
Washing buffer	1 x PBS
	1% Tween 20

Developing stocks

Solution 1	2.5 mM luminol (in DSMO)
	0.4 mM p-coumaric acid (in DMSO)
	100 mM Tris/HCl, pH 8.5
Solution 2	5.4 mM H_2O_2
	100 mMTris/HCl, pH 8.5

After electrophoretic transfer of proteins nitrocellulose membranes were incubated in blocking buffer for 1 h at RT. The antiserum diluted to the desired concentration in blocking buffer was incubated for 2 h at RT or overnight at 4°C. Primary antibody was removed by washing the membrane with blocking buffer four times for 10 min. Then anti-rabbit IgG peroxidase conjugate antibodies (Sigma) were diluted in blocking buffer and incubated with the membrane for 1 h. The membrane was then washed four times in washing buffer. The membrane was developed in a mixture containing solutions 1 and 2 in a 1 : 1 ratio for 1 min. Signals were detected by exposition of the developed membrane to X-ray films (Hyperfilm, Amersham Life Science, Freiburg).

2.2.12.5 Stripping of nitrocellulose membrane

Stripping buffer	62 mM Tris/HCl, pH 6.8
	2%SDS
	0.7% (v/v) β-mercaptoethanol

Nitrocellulose membrane was incubated in stripping buffer for 15 min at 65°C under constant agitation. Stripping buffer was removed by 2 times washing with 1 x PBS/1 x Tween 20 for 15 min at RT.

2.2.13 Radioactive pulse-labeling experiments

To analyze the kinetics of synthesis and degradation of cyanobacterial proteins, *Synechocystis* cultures were radioactively labelled *in vivo* with ^{35}S-methionine (> 1000 Ci/mmol, Amersham, Freiburg). Cells were grown in liquid BG11 medium to a final A of 0.3 to 0.5 and resuspended to a final chlorophyll concentration of 0.3 mg/ml. ^{35}S-methionine was added to a final concentration of 2.5 µCi/µg chlorophyll and cells were labelled at the required light conditions for 25 min. The reaction was stopped by freezing cells in liquid nitrogen. For chase-labelling radioactivity was removed by washing cells 3 times with BG11 buffer and cell cultures were incubated under desired conditions for different periods. Total membranes were isolated and separated by SDS-PAGE. Dried gels were exposed to Phosphoimaging plates (Fuji GmbH, Düsseldorf), which were read out and evaluated with a TLA 3000 Phosphoimage reader and the supplied AIDA software (Raytest Isotopenmessgeräte, Straubenhardt).

2.2.14 Measurements of oxygen evolution by a Clark-type electrode

Oxygen evolution of photosynthetic membranes of *Synechocystis* cells was measured with a Clark-type electrode (Hansatech Instruments Ltd, Reutlingen). *Synechocystis* cells (at a chlorophyll *a* concentration of 5 µg/ml) were adapted to darkness for 10 min, then continuously stirred at 30°C and illuminated with white light at 50 µE m^{-2} s^{-1} (LL) or at 1000 µE m^{-2} s^{-1} (HL). Lincomycin was added to a final concentration of 200 µg/ml before dark adaptation of cells, if required.

2.2.15 Low temperature fluorescence analysis (77K)

77K fluorescence was recorded using a SPEX Fluorolog-2 model F212 spectrofluorometer (Industries, Inc., USA). *Synechocystis* cells grown under different light intensities were resuspended at 2 µg of chlorophyll *a,* dark adapted for 10 min and rapidly frozen in liquid nitrogen. For investigation of energy distribution between phycobilinproteins, PSI and PSII emission spectra between 600 and 760 nm were recorded with excitation light of 435 nm or 570 nm.

2.2.16 Kinetic measurements of immunophilin activity

The concentrations of components in each reaction are listed below.

	Stock solution	End concentration (µM)
p-nitroaniline, control peptide	6 mM in 470 mM LiCl/TFE	60
synthetic peptides	6 mM in 470 mM LiCl/TFE	60
chymotrypsin	186 µM in 10 mM Tricine/NaOH, pH 7.8	0.93

Plant immunophilin was used at an estimated concentration of 0.01 µM. *Synechocystis* immunophilin was diluted until the rate of the reaction was comparable to that of plant immunophilin with the control peptide. The progress of the reaction was measured spectrophotometrically at RT at 390 nm. Components were diluted in 10 mM Tricine/NaOH pH 7.8. The total volume for each measurement was 1 ml. 1 µl of antiserum against *Synechocystis* TLP40 was added in order to block the immunophilin activity.

3. Results

3.1 Analysis of protein sequences of TLP40

Spinach cyclophilin TLP40 was described as a regulator of a protein phosphatase involved in the dephosphorylation of damaged PSII proteins (Fulgosi *et al*., 1998). The homologous components of TLP40 were identified in the *Arabidopsis thaliana* and *Synechocystis* sp. PCC 6803 genomes and designated cTLP40 (cyanobacterial TLP40) in the latter case.

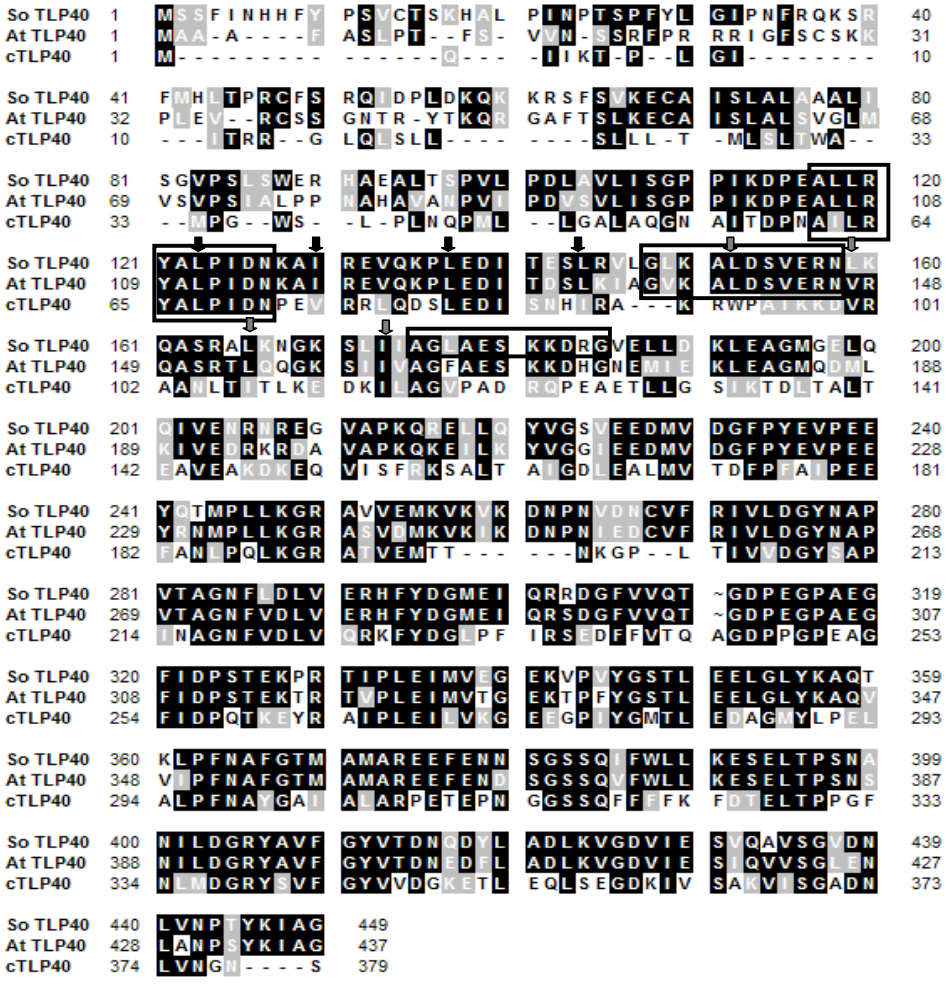


Fig. 7. Sequence comparison of TLP40 homologues from spinach (*Spinacea oleracea*, So), *Arabidopsis thaliana* (At), and *Synechocystis* (cyanobacterial TLP40, cTLP40). The alignment was performed with the Bioedit 7.0.1 software. Identical residues are shaded in black while similar residues are shaded in gray. Arrows point to the leucine/isoleucine amino acid residues of the deduced leucine zipper domains. Black boxes indicate the putative phosphatase binding domains.

The TLP40 homologue in *Arabidopsis* is a protein of 437 amino acid residues, encoded by the nuclear gene *At3g01480*. In *Synechocystis*, the TLP40 homologue is a protein of 402 amino acid residues and encoded by a single copy gene designated *sll0408*. Fig. 7 shows the alignment of protein sequences from spinach TLP40 and its *Arabidopsis* and *Synechocystis* homologues (cyanobacterial TLP40: cTLP40).

Sequence identity between TLP40 proteins from spinach and from *Arabidopsis* is 70% while it is 37% with cTLP40. The highest homology is found in the C-terminal part which includes the immunophilin domain. In this region spinach TLP40 is identical with the *Arabidopsis* homologue by nearly 90%, and 54% with cTLP40. The highest homology of the N-terminal domain is between residues 110 - 174 in spinach TLP40 and residues 55 - 114 in *Synechocystis* TLP40. In spinach these regions contain two leucine zipper domains constituted of a repetition of leucine (Leu) and isoleucine (Ile) residues at every seventh amino acid residue (indicated by arrows in Fig. 7). This domain can fold in an α–helical conformation forming a hydrophobic surface of Leu/Ile residues on one side of the helix and a hydrophilic region on the other one. The leucine zipper domains are generally believed to enable the interaction with other leucine zipper or coiled-coil-domain containing proteins (Lupas, 1996). In *Arabidopsis* TLP40 and cTLP40 the repetition of each Leu/Ile is interrupted by valine residues (Val; amino acid residue 147 in *Arabidopsis* TLP40 and residues 74 and 100 in cTLP40). Since Val is also a hydrophobic amino acid residue with a structure close to Ile and Leu, this domain can be considered as a structural alternative of a leucine zipper (Moitra *et al*., 1997). In addition to the leucine zipper domain, the N-terminal part of spinach TLP40 contains three putative phosphatase binding domains (residues 117 – 127, 148 – 158 and 175 – 185, respectively; Aldape *et al*., 1992), one of which could explain the association of spinach TLP40 to a thylakoid phosphatase during its purification (Fulgosi *et al*., 1998). Two of these domains are also present in *Arabidopsis* (residues 105 – 115 and 136 – 146) and only one in *Synechocystis* (residues 61 – 71). However, the substitution of an arginine residue, which was suggested to impair phosphatase-binding in human complex immunophilin (Aldape *et al*., 1992) to alanine in *Synechocystis* did not impair TLP40 function in the mutagenized strain (Irina Piven, personal communication).

3.2 Characterization and functional analysis of cTLP40

3.2.1 Cellular localization of cTLP40

Hydropathy analysis of the cTLP40 amino acid sequence according to Kyte and Doolittle (1982) predicted a hydrophilic protein with a highly hydrophobic N-terminus, which could represent a transmembrane domain (Fig. 8). However, lumenal targeting sequences are typically highly hydrophobic as well and often confused with a putative transmembrane domain (Peltier *et al*. 1997). In addition, a recent proteomic analysis of *Synechocystis* thylakoid membranes confirmed the localisation of cTLP40 in the lumen (Srivastava *et al*., 2005). A computer prediction analysis suggests that cTLP40 is imported into thylakoids by the Tat pathway and defines the cleavage site after the residue Ala[55]. Therefore, the hydrophobic N-terminus is most likely a targeting sequence for thylakoid lumen.

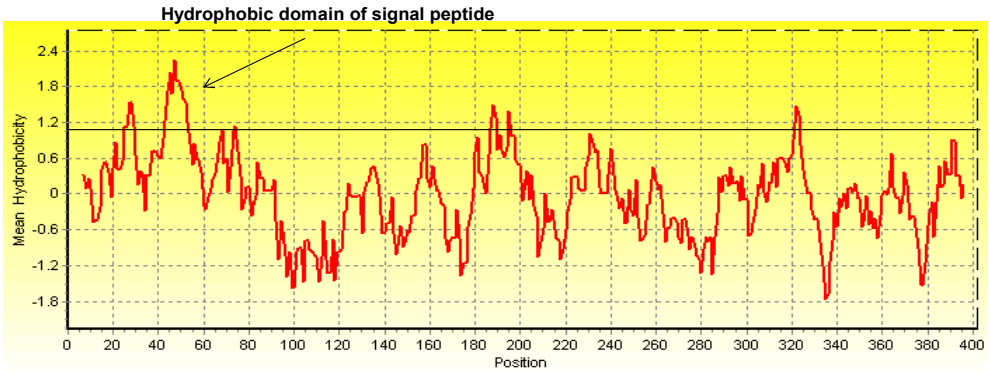


Fig. 8. Hydropathy plot according to Kyte and Doolittle (1982). The position of amino acid residues is indicated at the bottom of the graph. An arrow points to the putative transmembrane domain. The scan window size is 13.

In order to analyse the localisation of TLP40 *Synechocystis* cells were divided into cytoplasm and thylakoid membranes which were further separated into lumen and the membrane fraction. The presence of cTLP40 was checked immunologically using specific antisera raised against cTLP40 (Fig. 9). Western analysis clearly showed that cTLP40 is localised in thylakoid membranes (Fig. 9). Presence of the control proteins α subunit of the ATP synthase complex (AtpA) and 33 kDa protein of the water-evolving complex (PsbO) in the cytoplasm might be due to

contamination, since both components are readily release from thylakoid membranes. Within thylakoids cTLP40 could be detected in association with membranes but also released in the lumen (Fig. 9).

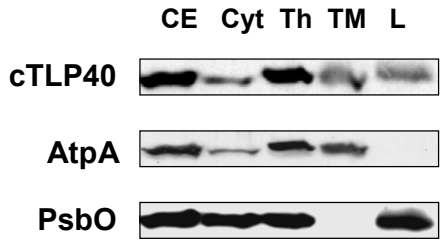


Fig. 9. Localisation of cTLP40 within cyanobacterial cells. *Synechocystis* cells grown at standard conditions were broken by vortexing with glass beads (CE) separated into cytoplasm (Cyt) and thylakoids (Th) by centrifugation. Thylakoid membranes were further fractionated by repeated cycles of sonication and separation into the soluble lumenal (L) and membrane fraction (TM) by centrifugation. The localization of cTLP40 was performed by Western analysis using specific antibodies against the full-length protein. Antisera against α-subunit of ATP-synthase complex (AtpA) and PsbO were used as controls for purification of thylakoid membranes and thylakoid lumen, respectively (this analysis was performed by Irina Piven).

3.2.2 Association of cTLP40 with thylakoid membranes

In order to define more precisely the localisation of cTLP40, a blue-native/SDS-PAGE analysis was performed to check whether cTLP40 binds to a specific photosynthetic complex in thylakoid membranes (Fig. 10). For the first dimension thylakoid membranes were mildly solubilized in order to keep the integrity of protein complexes and photosynthetic complexes were separated by 4 - 12% BN-PAGE (Schägger and von Jagow, 1991). The protein composition of complexes was identified by separation of the subunits by SDS-PAGE in the second dimension and visualized with silver staining (Fig. 10). PSII was found both, in the dimeric and monomeric forms by separation on BN-PAGE in the first dimension. PSII monomer represents an intermediate of PSII assembly (Komenda *et al*., 2004). PSI could be found in the monomeric and trimeric form (Herranen *et al*., 2004). In this case, the core proteins of PSI trimer were not detected by silver staining so it was not possible to localize exactly its position in the first-dimension

gel. Cytochrome b_6f and phycobilisomes are complexes with low molecular masses and migrate slightly slower than the fraction of free proteins (Fig. 10).

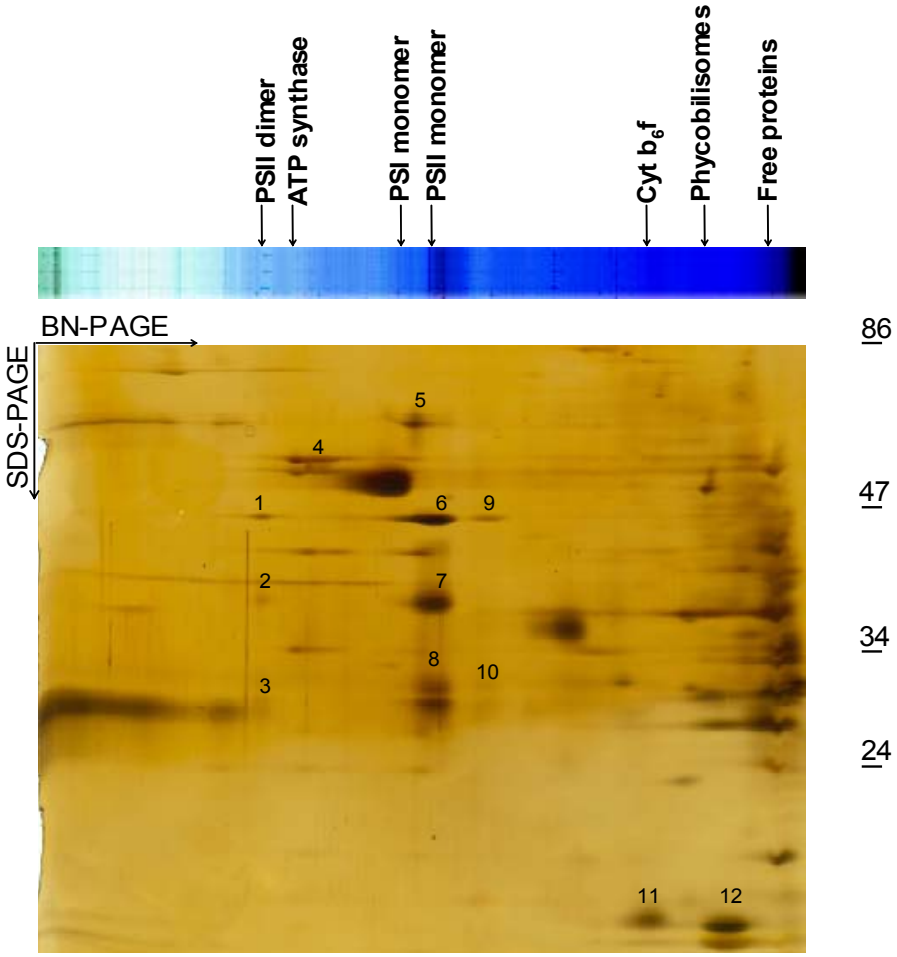


Fig. 10. Separation of photosynthetic complexes by blue-native PAGE (first dimension) and SDS-PAGE (second dimension). For the first dimension thylakoid membranes were solubilized with 1% β-dodecyl-maltoside as described in Materials and Methods and the lysate loaded on a 4 - 12% polyacrylamide gel. Subunits of the complexes were separated afterwards on a 10% SDS-PAGE containing 4 M urea and stained with silver nitrate. Proteins which were identified according to Herranen *et al*. (2004) are: 1 - CP47 in PSII dimer; 2 - CP43 in PSII dimer; 3 - D2 and D1 in PSII dimer; 4 - α– and β subunits of ATP-synthase complex; 5 - PsaA and PsaB of PSI monomer; 6 - CP47 in PSII monomer; 7 - CP43 in PSII monomer; 8 - D2 and D1 in PSII monomer; 9 - CP 47 in PSII intermediate monomer (RC47); 10 - D2 and D1 in PSII intermediate monomer; 11 - cytochrome b_6 in cytochrome b_6f complex; 12 - phycocyaniṇ β-subunits of phycobilisomes.

To detect cTLP40, Western analysis was performed after protein separation in a second dimension gel using specific antibodies against the protein. To define the localisation of cTLP40 more precisely, the same membrane was stripped and tested with specific antibodies raised against CP47, cytochrome *f* and the α subunit of ATP synthase (Fig. 11). Western analysis showed that cTLP40 is mainly detectable as a free soluble protein with a small amount of cTLP40 migrating at the size of cytochrome b_6f monomer (Fig. 11).

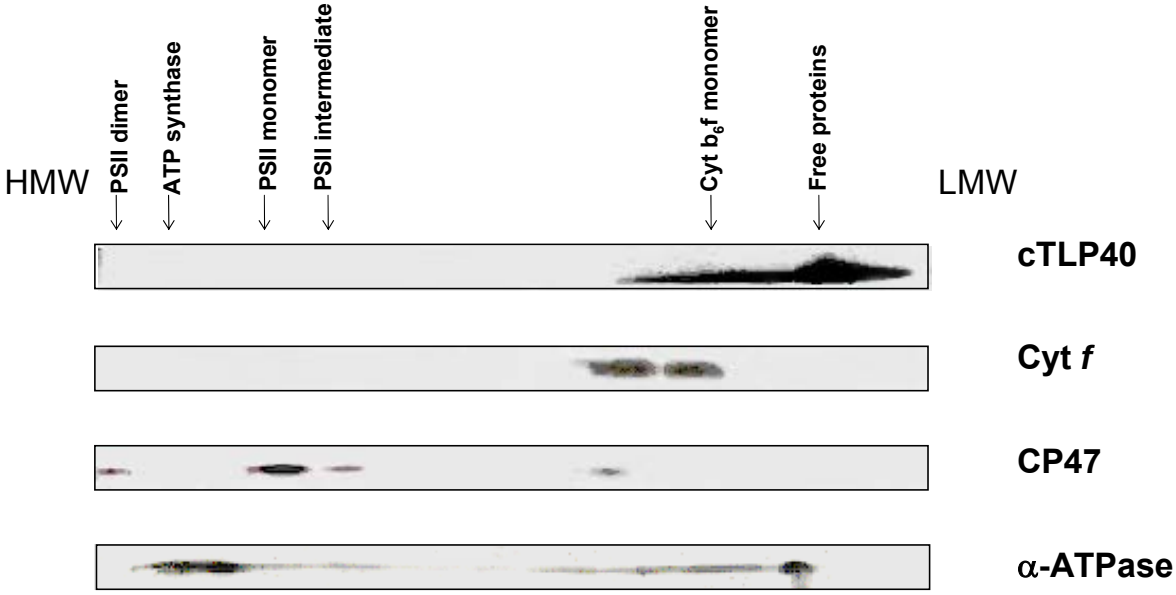


Fig. 11. Western analysis of thylakoid membrane lysates after separation by blue native/SDS-PAGE. The same membrane was tested with specific antibodies raised against cTLP40, cytochrome *f,* CP47 and β subunit of ATP synthase complex. The direction of separation is indicated by HMW, high molecular weight and LMW, low molecular weight proteins, respectively.

Even though most of the cTLP40 protein runs as free protein it is not possible to exclude an association with other proteins. It is possible that the used solubilization conditions could have modified the stability of a cTLP40 protein complex. Besides that, the integrity of the complex could have been destroyed by the electric field applied for blue native gel electrophoresis. To overcome the latter problem, the association of cTLP40 with some complexes was tested using linear sucrose gradient centrifugation in a first dimension. In this case, the separation of

complexes and proteins is driven by the centrifugal force applied during ultracentrifugation and depends mostly on density and molecular weight. Therefore, the separation conditions are different if compared to the blue-native analysis.

Thylakoid membranes of *Synechocystis* wild-type were solubilized with β-dodecyl-maltoside and loaded on a 12% - 25% linear sucrose gradient. After centrifugation the gradient was collected in fractions of 400 µl (40 in total). Samples of each fraction were subjected to SDS-PAG and, after electrophoresis, proteins were transferred to a nitrocellulose membrane. The membrane was incubated with antibodies raised against cTLP40, Cyt *f* and CP47 (Fig. 12).

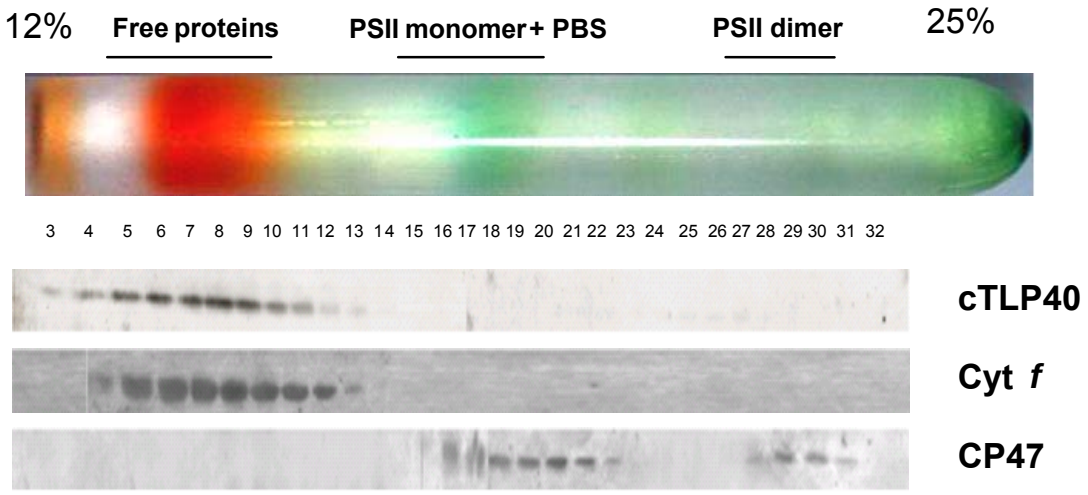


Fig. 12. Separation of photosynthetic complexes from *Synechocystis* wild-type by sucrose density gradient centrifugation. Thylakoid membrane were isolated from fresh cells and solubilized by β-dodecyl-maltoside. Solubilized thylakoid membranes were loaded onto a 12 - 25% sucrose linear gradient. Photosynthetic complexes were separated by ultracentrifugation for 16 h at 4°C. The gradient was collected in 400 µl fractions and loaded onto a 12.5% SDS-PAG. Proteins were transferred onto a nitrocellulose membrane which was tested with antibodies raised against cTLP40, Cyt *f* and CP47.

The results of this analysis were similar to those of blue-native gels. Despite some differences between these two techniques, cTLP40 was found in association or comigration with the cytocrome b_6f complex.

3.2.3 Expression of cTLP40 at different environmental conditions

Photosynthesis is highly dependent on environmental conditions such as light and temperature modifications. Thylakoid membranes react to external conditions by regulating the amount of several proteins in order to optimise the efficiency of photosynthesis. Since cTLP40 was found in thylakoid membranes, it was checked whether the amount of cTLP40 transcript changed with environmental conditions. *Synechocystis* wild-type was grown under normal conditions until A_{750} = 0.4. Cell cultures were divided into four flasks which were grown under normal conditions or adapted to high light (500 µE $m^{-2}s^{-1}$), high temperature (42°C) or low temperature (17°C) for 12 h. RNA was extracted from acclimated cells and cTLP40 transcripts were detected by a *sll0408* gene-specific probe.

Comparison of the RNA loaded detected by methylene staining of the membrane and the hybridisation with cTLP40 probe demonstrated that the gene was down regulated when cells were adapted to heat stress. When the cell culture was adapted to high light or low temperature the expression of the gene *sll0408* was increased relative to normal growth conditions.

It was checked then whether the decrease in gene expression at stress conditions reflected a change in protein amount as well. Thylakoid membranes were extracted from cells adapted to the above mentioned stress conditions and the amount of protein was detected by Western analysis (data not shown). The down regulation of the mRNA at high light and low temperature conditions did not reflect a significant decrease in protein amount: the cTLP40 amount did not change during light and cold stress in comparison to amount at standard growth conditions (Irina Piven, personal communication).

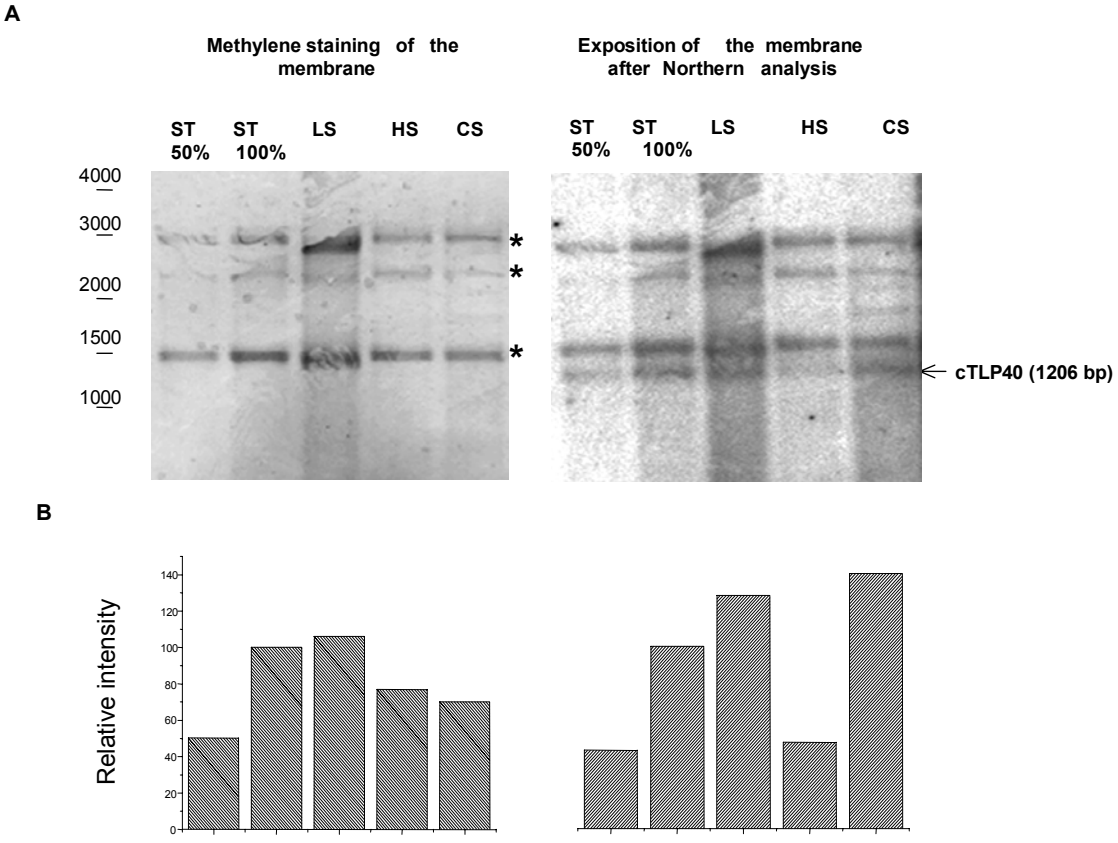


Fig. 13. Northern analysis of cTLP40-transcript in *Synechocystis* cells adapted to different environmental conditions. (A) Prior to hybridization the membrane with transferred RNA was stained with methylene blue for RNA-quantification. The components of the 70S ribosomal RNA, which were detectable by this staining, are marked by asterisks. The membrane was hybridized overnight at 42°C using the ^{32}P-radiolabelled *sll0408* gene sequence as a probe. The amount of loaded total RNA in each lane was 15 µg (100%) or 7.5 µg (50%), respectively. ST - standard conditions, LS - light stress (500 µE m^{-2} s^{-1}), HS - heat stress (42°C) and CS - cold stress (17°C). (B) The amount of total RNA loaded was evaluated with the software TINA 2.01 (left panel). The intensity of the cTLP40 signal was evaluated with the software TINA too and corrected relative to the amount loaded (right panel)

3.3 Characterization of a *Synechocystis* strain lacking cTLP40 protein

3.3.1 Construction of a cTLP40-deletion mutant (Δ*sll0408*)

The construction of the plasmid used for transformation of *Synechocystis* in order to generate a *sll0408* gene insertion mutant is shown in Figure 14 (Fulgosi, 1999). *Sll0408* gene was interrupted by the insertion of the gentamycin resistance gene cassette into the BamHI restriction site 386 base pairs after the ATG start codon. The transformation of *Synechocystis* wild-type with this construct was performed in the laboratory of Prof. S. Shestakov (Moscow, Russia). Transformants were selected on solid media containing increasing amounts of antibiotica till full segregation had been completed.

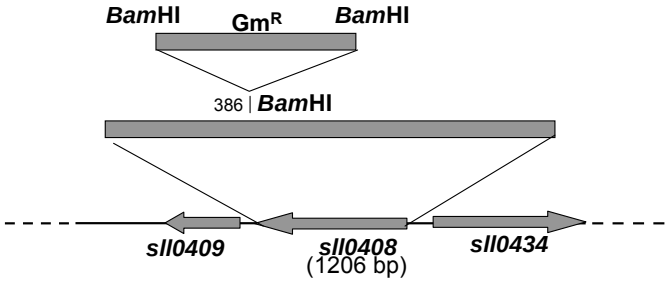


Fig. 14. Construction of the insertional mutagenesis cassette for the gene *sll0408* in *Synechocystis*. The gentamycin resistance cassette was inserted into the BamHI restriction site of the wild-type gene *sll0408* coding for cTLP40.

3.3.2 Construction of a vector for the complementation of *Δsll0408* mutant

The disruption of a distinct gene by insertional mutagenesis can influence the expression of closely located genes. In that case the phenotype of a mutant strain can be caused either by deletion of the gene of interest or by a side effect on expression of closely located genes. To exclude a pleiotropic effect of gene

disruption and to prove that the phenotype corresponds exclusively to the disrupted gene, a complementation analysis of the *Δsll0408* mutant strain was performed. Wild-type gene *sll0408* was amplified from genomic DNA using the primer pair sll0408N/sll0408Rover. The amplification product contained the entire coding sequence of *sll0408* with additional 380 bp of the 5´- untranslated region. This approach was chosen to ensure the presence of the promoter region of the gene in the amplified fragment. The PCR product was amplified using the Pfu polymerase which guarantees a high fidelity of transcription and does not add any adenine at the 3´end of the copied gene. The PCR product was cloned into the singular SmaI site of the self-replicating vector pVZ323 (Fig.15) and introduced in to the mutated strain *via* triparental mating as described in Materials and Methods (see 2.2.4). Transformants were selected on BG11 plates containing 25 µg/ml chloramphenicol.

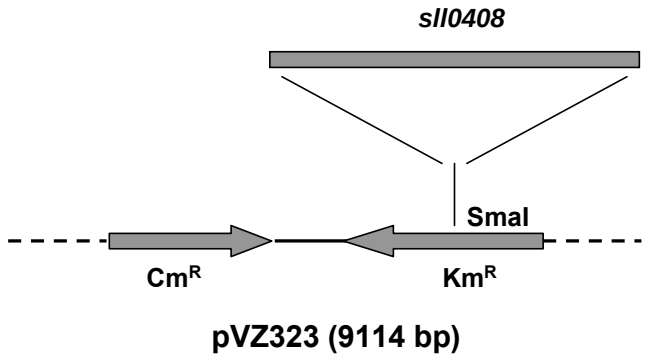


Fig. 15. Construction of complementation vector for mutant *Δsll0408*. Wild-type *sll0408* gene was amplified from genomic DNA and cloned into the unique SmaI site within a kanamycin resistance cassette of pVZ323 plasmid. Positive transformants where selected on chloramphenicol-containing plates.

The expression of cTLP40 in the complemented strain (pVZsll0408) was checked by Western analysis. Wild-type, *Δsll0408* mutant and the complemented strain pVZsll0408 were grown at normal conditions. Thylakoid membranes were extracted, separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was immunodecorated with specific antibodies raised against cTLP40 (Fig. 16).

Western analysis showed that cTLP40 was present in the wild-type and in the complemented strain where the protein was present even in higher amounts than in the wild-type.

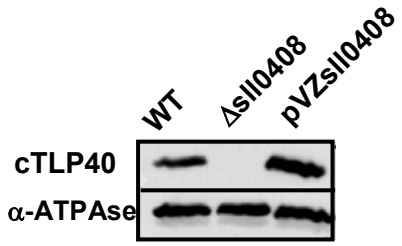


Fig. 16. Western analysis of thylakoid membranes of *Synechocystis* wild-type, mutant *Δsll0408* and complemented strain pVZsll0408. *Synechocystis* cells were grown at normal conditions to an A_{750} = 0.5 - 0.7. Thylakoid membranes were extracted, separated on a 12% SDS-PAGE and transferred onto a nitrocellulose membrane. cTLP40 was detected immunologically with specific antibodies. α-subunit of ATP synthase was used as loading control.

During transformation of the self-replicating plasmid the gene of interest can be independently expressed but it can be integrated by homologous recombination into genomic DNA as well, thereby promoting complementation of the mutant. A PCR approach excluded a possible homologous recombination between *Synechocystis* chromosomal DNA and the *sll0408* gene from the complementation plasmid (data not shown). Collectively, the outlined data excluded a pleiotropic effect in the deletion strain.

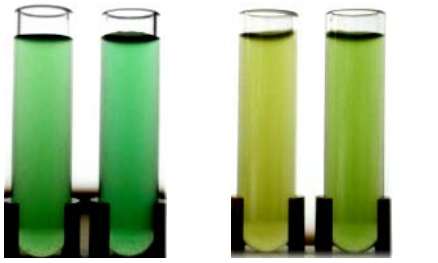
3.4 Characterization and functional analysis of the *Δsll0408* mutant

3.4.1 Phenotypic analysis of *Δsll0408*

Growth of wild-type and mutant strains was first analysed phenotypically under normal and high light conditions. In addition, doubling time and pigment

concentration of the cyanobacterial cell cultures were analyzed under the same light regimes (Fig. 17).

A



B

	Doubling time (h)	Chlorophyll (µg/cell)	Carotenoids (µg/cell)	Phycocyanin (µg/cell)
wild-type ST	12 ± 1.0	1.55 ± 0.07	0.93 ± 0.03	19.99 ± 0.5
wild- type HL	16 ±0.5	1.00 ± 0.11	1.47 ± 0.22	14 ± 2.3
Δsll0408 ST	14 ± 0.2	1.47 ± 0.05	0.85 ± 0.01	23.72 ± 1.3
Δsll0408 HL	36 ± 1.0	1.26 ± 0.04	0.88 ± 0.03	18.8 ± 0.4

Fig.17. Phenotypical and pigment characterization of wild-type and mutant strain. (A) Phenotype of *Synechocystis* wild-type and *Δsll0408* cell cultures grown under normal conditions (ST) or adapted to high light for 24 h (HL). (B) Doubling time and pigment concentration of wild-type and *Δsll0408* mutant grown under normal conditions (ST) or adapted to high light for 24 h (HL). This analysis was performed by Irina Piven.

No significant phenotypical differences were observed when cells were grown at normal conditions (Fig. 17A). Concentrations of chlorophyll *a* and carotenoids were comparable in cells of wild-type and *Δsll0408* mutant grown under standard regimes (Fig. 17B). Phycocyanin concentration was slightly higher in the mutant at standard conditions and this resulted in a more intensive blue colour of the mutant cell culture. Under high light cell cultures presented clear differences in their phenotypes reflected by differences in pigment concentrations and cell doubling times (Fig. 17). In both, wild-type and *Δsll0408* mutant, a decrease in the total chlorophyll amount was detectable reflecting a decrease in the amount of pigment-binding proteins of PSI and PSII. In addition, amounts of phycobilin proteins, which act as light-harvesting antenna in *Synechocystis*, were decreased to prevent an excess of light absorption at high light.

A significant difference between wild-type and mutant strains is presented in the amount of carotenoids after adaptation to high light. While carotenoid concentrations increased in wild-type cells grown under high light conditions

relative to normal conditions, the carotenoid content in *Δsll0408* cells remained nearly constant in both light intensities Carotenoids protect the photosynthetic apparatus from photo-oxidative damage by de-excitation of radical species (for example O_2^-) which form when PSII is overexcited (Sistrom *et al*., 1956; Niyogi *et al*., 1997; Havaux and Nyogi, 1999; Bassi and Caffarri, 2000). The two strains were also different in respect to their growth rate under high light: the doubling time of wild-type cells increased up to 30% upon acclimation from low to high light regimes while mutant cells showed an increase of nearly 300%.

3.4.2 Oxygen evolution at different light adaptation

To analyze whether cTLP40 influences the activity of photosynthetic membranes, the oxygen evolution of wild-type and *Δsll0408* strain under different light conditions was measured using a Clark-type electrode. Both strains were grown at normal conditions and acclimated to dark for 10 min before irradiation with low light (LL; 50 µE $m^{-2}s^{-1}$) or high light (HL; 1000 µE $m^{-2}s^{-1}$). Oxygen content decreased during first 10 min of dark adaptation because of a high respiration activity of cyanobacterial cells. Activation of photosynthesis under illumination resulted in the production of oxygen with an initial rate depending on light intensity.

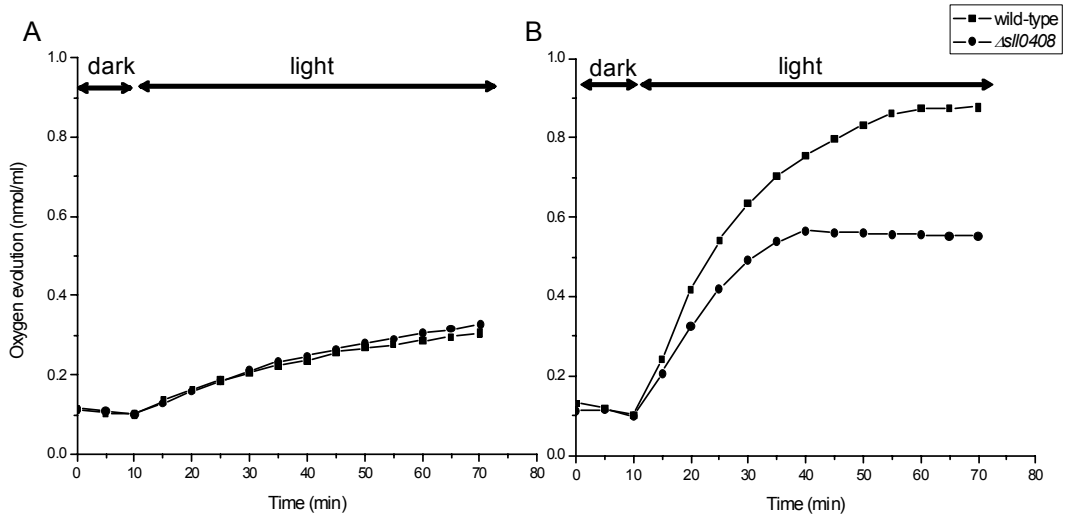


Fig. 18. Oxygen evolution of wild-type and mutant strain under low and high light. Cells were grown at normal conditions until an A_{750} of 0.3 - 0.5. Cells were collected by centrifugation and resuspended to a final chlorophyll concentration of 5 µg/ml. Respiration activity was measured for 10 min in the dark. Oxygen production was measured at a constant temperature of 30°C either under low light (50 µE $m^{-2}s^{-1}$, panel A) or light of high intensity (1000 µE $m^{-2}s^{-1}$, panel B).

Irradiation with low light resulted in similar rates of oxygen production in both strains (Fig. 18A). These results were expected since the two strains showed no significant differences in growth rate and pigment content under normal conditions (see Fig. 17). Oxygen evolution was then measured at high light intensity where the behaviour of the two strains revealed major differences. Oxygen production in the mutant was clearly lower than in the wild-type at high light irradiance. After 40 min of high light treatment the oxygen production rate of the mutant was nearly zero indicating that the oxygen production and its utilisation by respiration were in equilibrium.

The reaction centre of PSII is the most sensitive one of the supramolecular thylakoid complexes to high irradiance. Excess of light causes PSII photoinactivation which is mostly a result of damage and degradation of the PSII reaction center protein D1. This damage results in a drop of photosynthetic activity and, therefore, in a decrease of oxygen production. The restoration of PSII function (the so called “repair cycle”) comprises the removal and degradation of damaged D1 and integration of newly synthesised D1 subunits into PSII complexes. The reduction of oxygen evolution indicated a lower functionality of PSII in the mutant. Possible explanations are an inefficient repair of damaged PSII or a higher PSII damage rate in the mutant. To check these alternatives oxygen evolution of both strains was measured in the presence of lincomycin, an antibiotic blocking the reinitiating of protein translation. After addition of lincomycin damaged PSII can not be repaired any longer. Since no significant differences in oxygen evolution of the two strains at low light were noted, the measurement with lincomycin was performed only with high light (Fig. 19).

In the presence of lincomycin oxygen production of wild-type and mutant was similar. The decrease of PSII activity which reflects the reduction of oxygen evolution started after 40 min of high light treatment in both cell cultures (Fig. 19A). This was similar to oxygen production in the mutant irradiated with high light in the absence of lincomycin (Fig. 19B). These results support the first of the above mentioned hypotheses. The lower photosynthetic activity in the mutant at high light can be a consequence of an improper translation of D1 or other PSII proteins, assembly and or repair of PSII or lack of D1 degradation under high light.

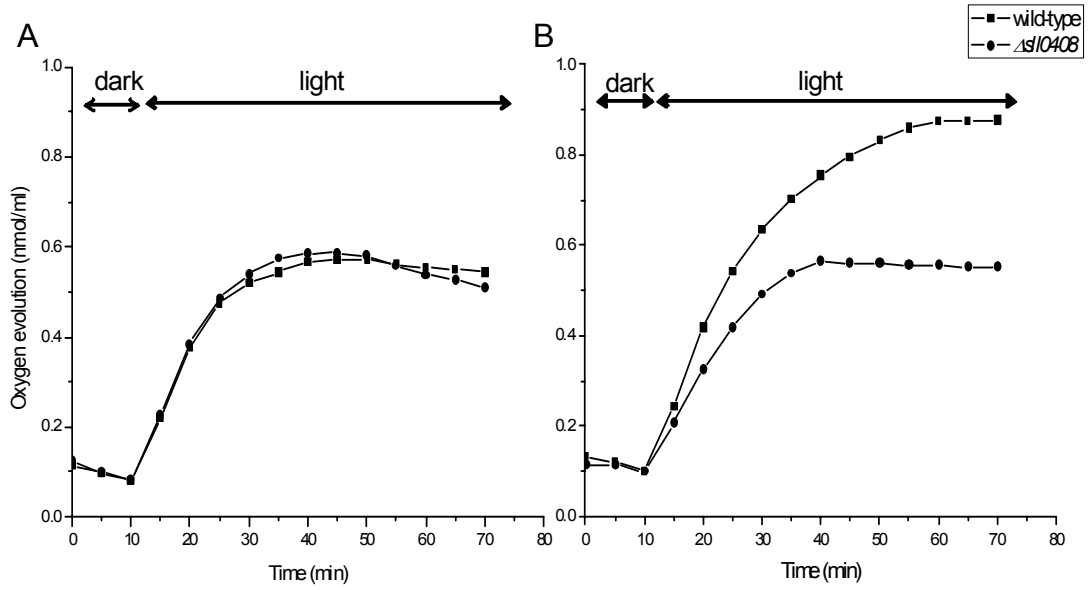


Fig. 19. Oxygen evolution of wild-type and mutant strain under high light in the presence of lincomycin. (A) Cells were grown at normal conditions until an A_{750} of 0.3 - 0.5, collected by centrifugation and resuspended to a final chlorophyll concentration of 5 µg/ml. Lincomycin was added to a final concentration of 200 µg/ml. Respiration activity was measured for 10 min in the dark. Oxygen production was measured under light of 1000 µE m^{-2} s^{-1} at a constant temperature of 30°C. (B) Oxygen evolution measurements under high light without lincomycin are shown for comparison (compare also Fig. 18).

3.4.3 Fluorescence emission spectra at 77K

The functionality of both photosystems in terms of energy transfer and organisation of the antenna system was analysed in wild-type and mutant strain by low temperature fluorescence. The excitation of *Synechocystis* cells frozen in liquid nitrogen at 440 nm (chlorophyll *a*) results in the emission of fluorescence yielding spectra with three typical peaks at 685 nm, 695 nm and 725 nm. These peaks derive from the emission of inner antenna of PSII (685 and 695 nm) and of PSI (725 nm; Nakatani *et al*., 1984; van Dorssen *et al*., 1987; Haag *et al*., 1993). The emission fluorescence spectra were recorded for wild-type and mutant grown at normal and high light conditions and were normalised to the emission of PSI at 725 nm.

The emission spectra of mutant cells showed a slightly higher ratio of PSI/PSII than in the wild-type when both were grown under normal conditions. This could argue either for a lower amount of PSII or a higher amount of PSI in the mutant (Fig. 20A).

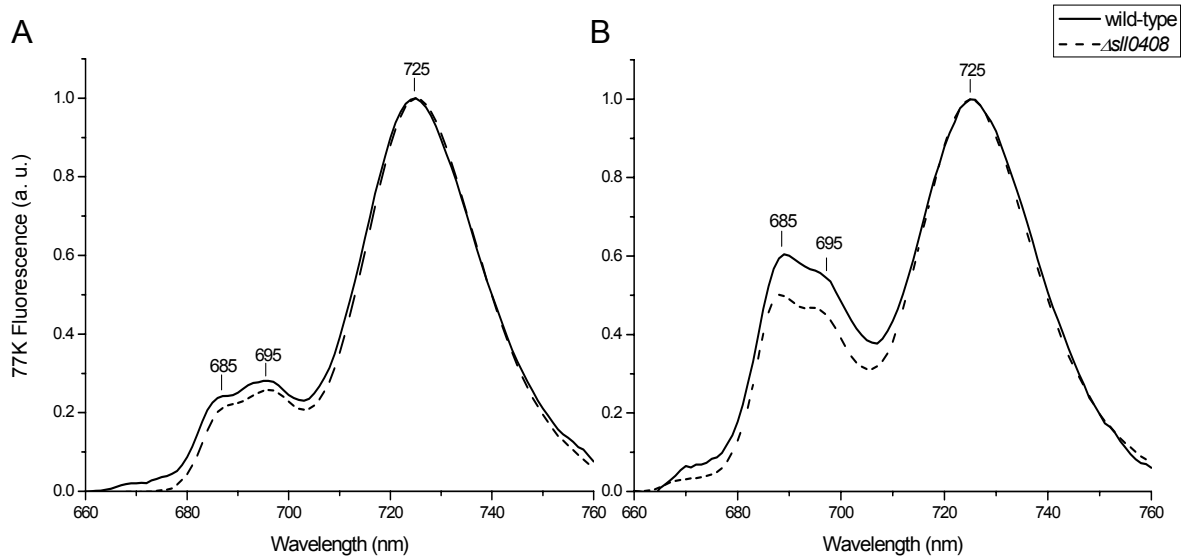


Fig. 20. Fluorescence emission spectra of wild-type and mutant cells by excitation at 440 nm (chlorophyll *a*). Cells were grown under normal conditions to an A_{750} = 0.3 – 0.5 (A) or adapted to high light (B), adjusted to a final chlorophyll concentration of 2 µg/ml and frozen in liquid nitrogen. Emission spectra were red upon excitation of chlorophyll *a* at 440 nm. The spectra were normalized to PSI emission.

For the analysis of fluorescence emission of photosynthetic complexes at high light cell cultures were adapted to high light for 24 h and emission spectra were recorded upon excitation of chlorophyll *a* at 440 nm (Fig. 20B). Both strains showed a higher PSII/PSI ratio of fluorescence signal in comparison to the spectra recorded with cultures grown at normal conditions (Fig. 20A). The relative increased PSII-fluorescence depends on a decrease of the PSI amount and reveals a new equilibrium in the contents of both photosystems as a consequence of high light adaptation (Kawamura *et al*., 1979; Muramaki and Fujita, 1991; Hihara *et al*., 2003; Tu *et al*., 2004). The increased relative fluorescence signal of PSII in the mutant after high light adaptation could argue for less PSII as well as a higher amount of PSI (Fig. 19B).

The analysis of pigment concentrations showed that the mutant contained a higher amount of phycocyanin than the wild-type (see Fig. 17). The distribution of phycobilisomes can be estimated by excitation of PC/APC at 570 nm. The emission spectrum resulted in three peaks: two peaks were derived from PSII and PSI emissions (as already described) and an additional peak between 650 – 670 nm derived from the emission of free phycobilisomes which were not associated with PSI or PSII. The resulting emission spectra showed that most of the light energy is emitted from phycobiliproteins not associated with the photosystems (between 650 – 670 nm Fig. 22A). Moreover, the transfer of energy from the antenna to PSII seemed to be slightly higher in the mutant if compared to wild-type (Fig. 21A). The same spectra were recorded with cultures which were adapted for 24 h to high light (Fig. 21B). The ratio of PSII to PSI fluorescence in both strains increased under high light (see also Fig. 20). The emission of PBS and PSII fluorescence was higher in the mutant when compared to the wild-type. This can result from a higher amount of phycobiliproteins (compare Fig. 17) or higher amount of PSII in the mutant. However, this last possibility was excluded since excitation at 440 nm did not show a higher amount of emitting PSII (Fig. 20B).

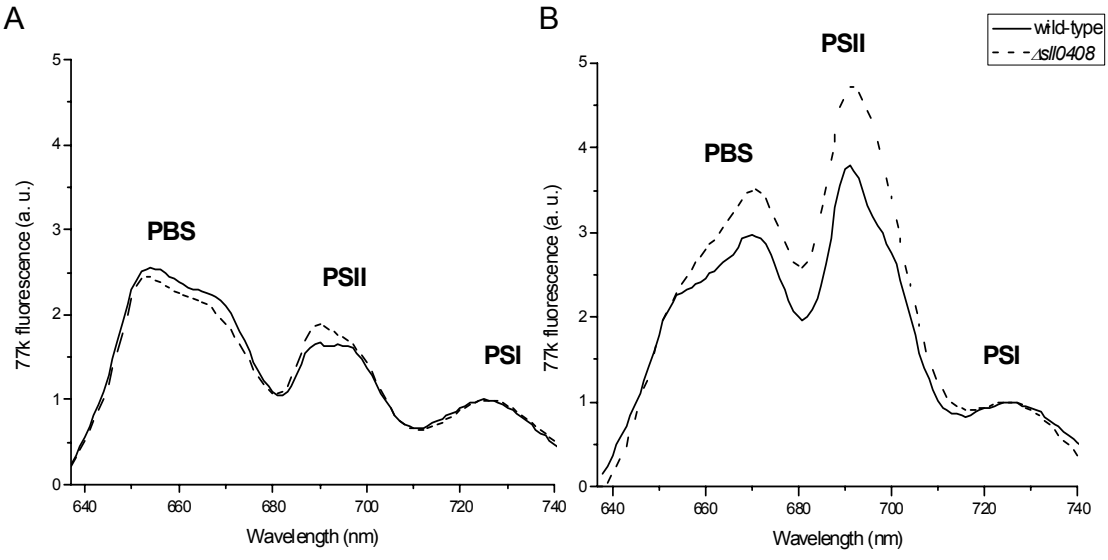


Fig. 21. Fluorescence emission spectra of wild-type and mutant cells by excitation at 570 nm. Cells were grown under normal conditions to an A_{750} = 0.3 – 0.5 (A) or adapted to high-light (B), adjusted to a final chlorophyll concentration of 2 µg/ml and frozen in liquid nitrogen. Emission spectra were taken upon excitation at 570 nm. The spectra were normalized to PSI emission.

3.4.4 Western analysis of PSII subunits in wild-type and *⊿sll0408*

Oxygen evolution measurements and 77K fluorescence emission spectra demonstrated that the mutant *⊿sll0408* differed from wild-type mainly under acclimation of cyanobacterial cells to high light. Under these conditions the mutant produced 40% less oxygen than the wild-type. In addition, the fluorescence emission of PSII by excitation at 440 nm in the mutant was lower than in wild-type under the same conditions. Taken together, these results suggested a decreased amount of functional PSII in the mutant. The amount of PSII subunits was therefore analysed immunologically using antisera raised against various PSII subunits. Thylakoid-enriched membranes were extracted from wild-type and mutant strains grown at ST or adapted to HL for 24 h and their lysates separated by 12.5% SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane and immunodecorated with antibodies elicited against PSII subunits (Fig. 22).

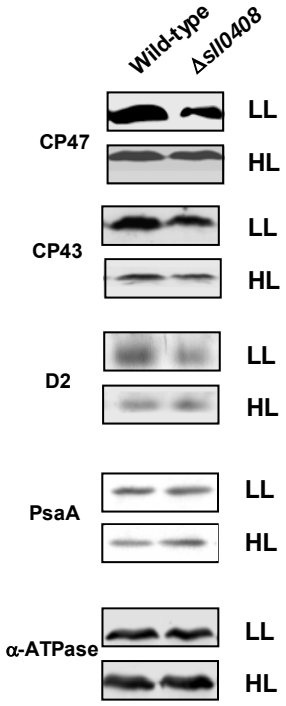


Fig. 22. Immunological analysis of PSII subunits from *Synechocystis* wild-type and mutant strains. *Synechocystis* cell cultures were grown at low light (LL) or adapted to high light (HL) for 24 h. Thylakoid-enriched membranes were extracted and thylakoid proteins were separated by 12.5% SDS-PAGE. Proteins were transferred onto a nitrocellulose filter and tested with polyclonal antibodies raised against the major PSII-subunits CP47, CP43 and D2 and PSI subunit PsaA The α subunit of the ATP synthase complex was used as a loading control. Western analysis was performed by Irina Piven.

The Western analysis showed that the mutant contains up to 30% less PSII core complex proteins under low light (Fig. 22). The amount of PSI core protein was similar to that of wild-type under the same conditions. When cells were adapted to high light a general decrease in protein amount was detectable both in wild-type and mutant. This decrease was a consequence of the adaptation of the two strains to high light. Under high light the amount of PSII core protein in the two strains was quite similar (Fig. 22). These immunological analyses showed the total amount of PSII proteins, but did not give any information on the functionality of the proteins. Nevertheless, these results together with the 77K fluorescence data described before (see 3.4.3, Fig. 20 and 21) suggest that the reduced PSII fluorescence in the mutant upon excitation of chlorophyll *a* could be attributed to the presence of a higher amount of non-functional PSII.

3.4.5 Analysis of the steady-state level of *psbA* mRNA coding for D1

Western analysis of the major photosynthetic proteins in wild-type and mutant strains showed that the mutant had less PSII subunits, in particular when cells were grown under standard conditions. The steady-state protein amount depends on mRNA-levels as well as on translation and degradation rates of proteins which change with growth conditions. Expression of all PSII subunits must be coordinated to guarantee a correct synthesis of the supramolecular complex which can support the cell at a given condition. D1 is encoded by the gene *psbA* which is present in three copies in the *Synechocystis* sp. PCC 6803 genome (Williams, 1988). It has the highest turnover rate among all PSII subunits and *psbA* gene expression is highly dependent on light intensity. While *psbA1* is not expressed in this strain, *psbA2* and *psbA3* encode identical gene product. Both are actively transcribed with the level of *psbA2* transcript being normally much higher than *psbA3* (Mohamed and Jansson, 1989; Mohamed *et al*., 1993). To investigate, whether the mutant *Δsll0408* shows altered mRNA levels coding for PSII subunits, the *psbA* mRNA amount was monitored by RT-PCR analysis as representative for mRNAs of subunits for the whole complex. For this analysis, total RNA was extracted from cyanobacterial cells, residual DNA was removed by a DNase treatment and cDNAs were synthesised using random hexanucleotide primers. Afterwards, the *psbA2* cDNA was amplified in a real time PCR using specific

primers for *psbA2*. The increase of the product was evaluated relative to the amplification cycle using the Light Cycler device. From these curves the amount of *psbA* mRNA was evaluated for wild-type and mutant strains grown under standard conditions with and without adaptation to high light for 24 h (Fig. 23). The amount of *psbA* transcripts increased in both strains after adaptation to high light. This is in agreement with a higher damage of D1 protein under high light which requires a higher synthesis of D1 to replace the damaged one into PSII complex. The level of *psbA* transcript in the mutant was higher than in the wild-type under both growth conditions (Fig. 23). The mRNA level was three times higher in the mutant than in wild-type under standard growth regimes while only about 40% higher under high light. However, this seems not to affect directly the protein levels (see Fig. 22). Since the amount of a protein is not only controlled by the mRNA level but also by translation and degradation rate, protein synthesis was then analysed by *in vivo* labelling experiments.

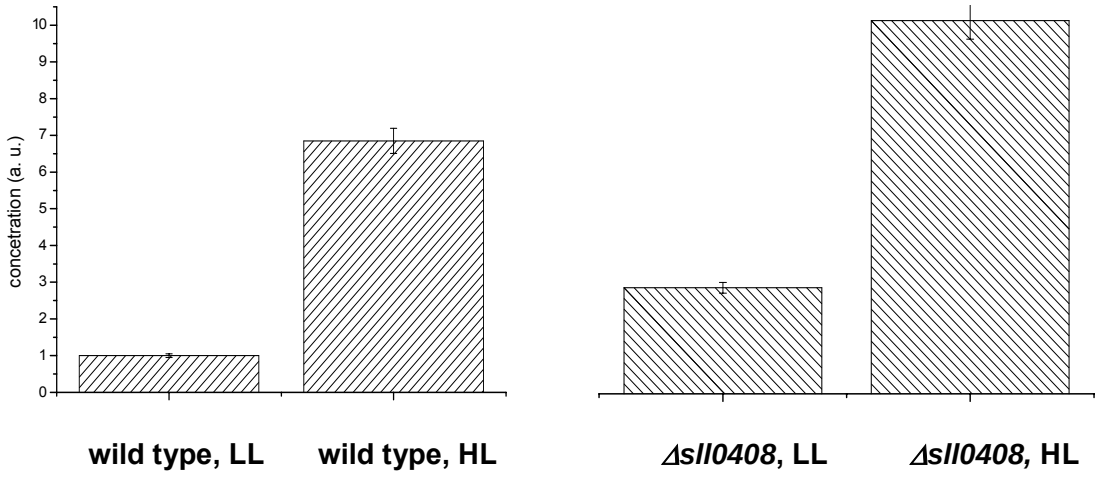


Fig. 23. Real time PCR of *psbA* transcript. Wild-type and mutant strain were grown under standard conditions (ST) or adapted for 24 h to high light (HL). Total RNA was extracted and used for cDNA synthesis with random hexanucletide primers. D1 RNA was amplified from the cDNA library using two specific primers for *psbA2* gene, D18149F and D18311R. The amount of wild-type grown at ST was adjusted to 1 and the amount of the other samples is shown relative to this.

3.5 Labelling of *Synechocystis* cells *in vivo*

3.5.1 Analysis of protein synthesis *in vivo*

Western analysis showed that the mutant *Δsll0408* possessed less photosystem II subunits in comparison to the wild-type when strains were grown under standard

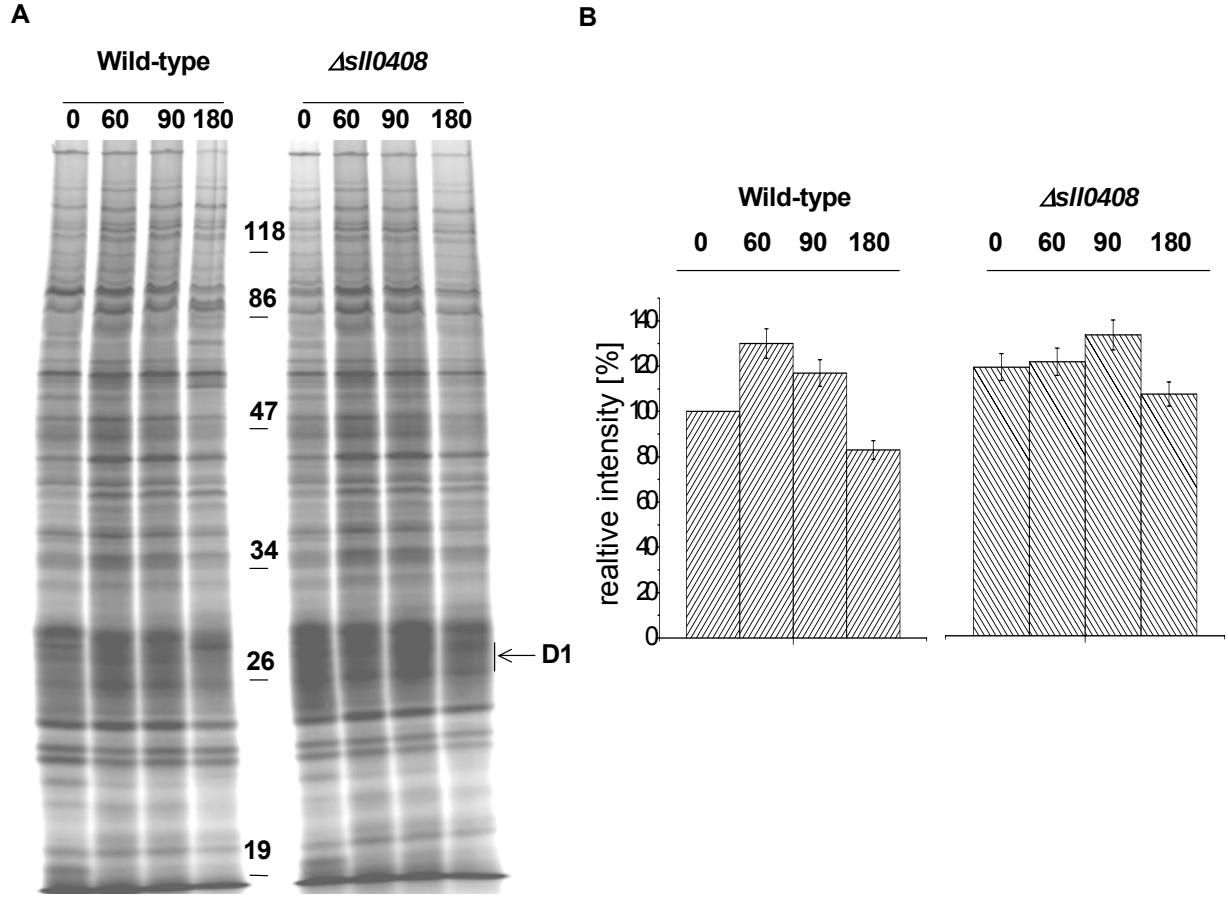


Fig. 24. Analysis of protein translation rates in wild-type and *Δsll0408* mutant by pulse-labelling with [^{35}S]-methionine. (A) *Synechocystis* cell cultures were grown at standard conditions to an A_{750} of 0.3 - 0.5. Cellular proteins were labelled by addition of [^{35}S]-methionine to the medium for 25 min at ST (0) and after adaptation to HL for 60, 90 and 180 min. Thylakoid membranes were extracted and separated on 10% SDS PAG containing 4 M urea. Evaluation of protein band intensities was done using the software TINA 2.01. (B) Relative intensities of incorporated radiolactivity in D1 protein in wild-type and the mutant. The intensity of labelled D1 protein was normalized to wild-type grown under standard conditions (0; 100%).

conditions. After adaptation to high light for 24 h, there was a general decrease of photosynthetic proteins both in wild-type and in the mutant. The decrease of photosystem II proteins was higher in wild-type than in the mutant so that at high light the content of PSII proteins between the two strains was similar. Analysis of *psbA* transcript levels showed that expression of *psbA* is higher in the mutant than in the wild-type independent of the growth conditions. Therefore, it was checked whether the mutant was impaired in protein synthesis. The protein synthesis rate was checked by *in vivo* labelling of *Synechocystis* cell cultures under normal light regime and after adaptation to high light for different times. Cellular proteins were labelled with [^{35}S]-methionine for 25 min as described in Materials and Methods (see section 2.2.13) and the reaction was stopped by freezing the cells in liquid nitrogen. Afterwards, thylakoid membranes were extracted and separated by 10% SDS-PAGE.

To maintain the functionality of photosystem II, damaged proteins need to be removed from the complexes and exchanged by newly synthesised and inserted ones. The damage rate and, therefore, the synthesis rate of proteins increases with light. For this reason the synthesis rate of photosynthetic proteins is expected to be higher at least at the beginning of high light adaptation. The rate of synthesis in wild-type and mutant strains was evaluated through analysis of D1 protein since it is the protein with the highest turnover of all photosystem II subunits (Mattoo *et al*., 1984; Ohad *et al*., 1985). Comparing the intensities of the D1 bands for wild-type and *⊿sll0408*, it was possible to note that accumulation of radioactivity in D1 (which indicated the synthesis of new protein) was mostly higher in the mutant relative to the wild-type, even when the cultures where grown and labelled at standard conditions (Fig. 24). This contradicted with results of Western analysis that showed a lower amount of photosystem II subunits in the mutant under these growth conditions. A similar rate of D1 protein was observed for cells acclimated to high light. In the wild-type D1 was higher synthesised within first 60 min of acclimation to high light. Afterwards the accumulation decreased and reached a level lower after 3 h of high light growth than under standard conditions. This could be caused by a lower content of PSII after 3 h of acclimation to high light.

3.5.2 Determination of protein degradation rate in *Synechocystis* cells

When wild-type and mutant strains were compared for their D1 synthesis rate, it was found that protein accumulation in the mutant was slightly higher then in wild-type. To explain the result of Western analysis which showed a relatively lower amount of PSII subunits in the mutant, we analyzed the rate of D1 protein degradation. Cell grown at standard conditions, were labelled for 25 min with [^{35}S]-methionine. Afterwards methionine was removed by washing cells several times with BG11 medium. Finally, cells were resuspended in BG11 and incubated under standard and high light conditions for different times. Thylakoid membranes were extracted from cells frozen in liquid nitrogen and proteins were loaded on a 10% polyacrylamide gel.

Degradation was estimated by the decrease of the radioactive signal. As expected, the degradation rate was higher under high light than under standard conditions, both in wild-type and mutant strain. After 3 h of growth under standard conditions, D1 protein decreased up to 30% due to the turnover of the radiolabelled protein in the wild-type. This value increased up to 50% after 3 h of high light adaptation. In the mutant D1 had a faster turnover rate, both at standard growth and high light conditions (50% and 60% of decrease of radioactive signal, respectively). This experiment showed that in the mutant, D1 protein had a faster turnover than in the wild-type. Moreover, the differences in the D1 degradation rate in the two cultures were higher when they were grown under standard conditions. These data support the findings of the Western analysis.

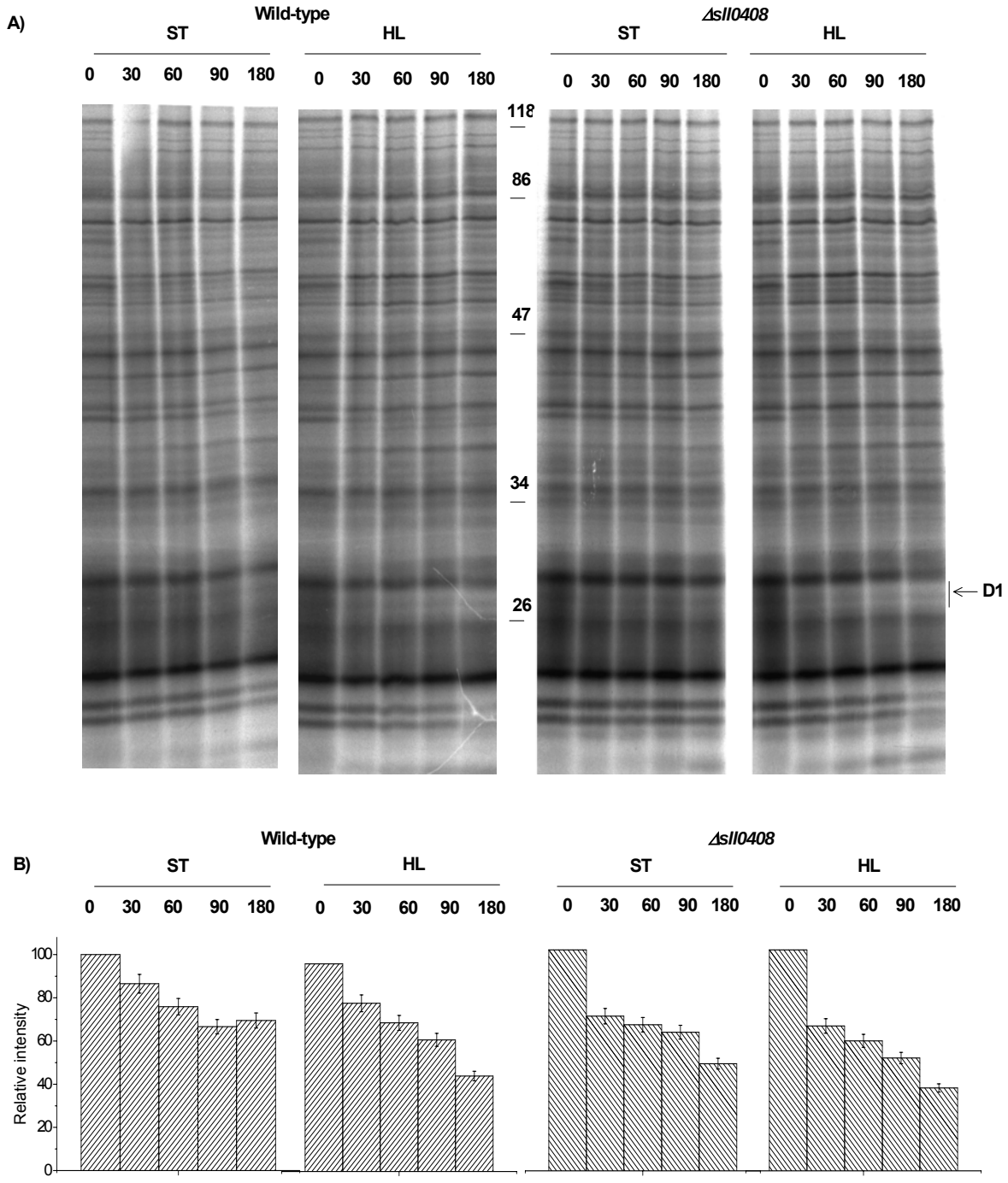


Fig. 25. Analysis of protein degradation in wild-type and *Δsll0408* mutant. (A) *Synechocystis* cells were grown under standard conditions and labelled with [^{35}S]-methionine (0). After removing [^{35}S]-methionine, degradation of proteins was followed at standard conditions and high light for 30, 60, 90 and 180 min. Thylakoid membranes were extracted and proteins were separated on a 10% SDS polyacrylamide gel containing 4 M urea. (B) The labelling of D1 protein was evaluated with TINA 2.01 software and normalized to the starting point which was taken as 100%.

3.5.3 Separation of photosynthetic complexes by 2D-PAGE

Previous analysis has shown that the *⊿sll0408* mutant contains less PSII subunits in comparison to the wild-type under standard growth conditions even though the mutant exhibited no visible phenotype. Under high light, the mutant showed a decreased growth rate and lower oxygen evolution. Under these conditions the amounts of major PSII subunits in the mutant strain were comparable with those in the wild-type. To clarify the reason for the different accumulation rates of PSII components in the mutant strain the distribution of the major PSII subunits between PSII complexes was analyzed immunologically. Thylakoid membranes of the wild-type and mutant strains were extracted from cells grown at standard conditions or adapted to high light for 6 h. Protein complexes were separated by blue-native PAGE in the first and by SDS-PAGE in the second dimension. Proteins transferred onto a nitrocellulose membrane were then analysed by a Western approach using specific antibodies raised against the major subunits of the PSII complex (Fig. 26).

The major subunits of PSII could be found in free form, in PSII intermediates (D1/D2/CP47 complex and PSII monomer) and in the PSII dimer. The high molecular weight complexes including PSII subunits represented some aggregates of the highly hydrophobic protein during sample denaturation or derived from non-completely solubilized thylakoid membranes. When cells were grown under normal conditions, the major subunits of PSII could be detected in free form and in PSII complexes in both, wild-type and mutant. After acclimation of cell cultures to high light the amount of PSII proteins decreased in both, wild-type and mutant strains compared to normal conditions. While in the wild-type, a PSII dimer complex was clearly detectable by antisera against D2 and CP47, PSII dimer was not detectable in the mutant with any of the antibodies used against PSII subunits. This indicated that the mutant contained less of PSII dimers under high light.

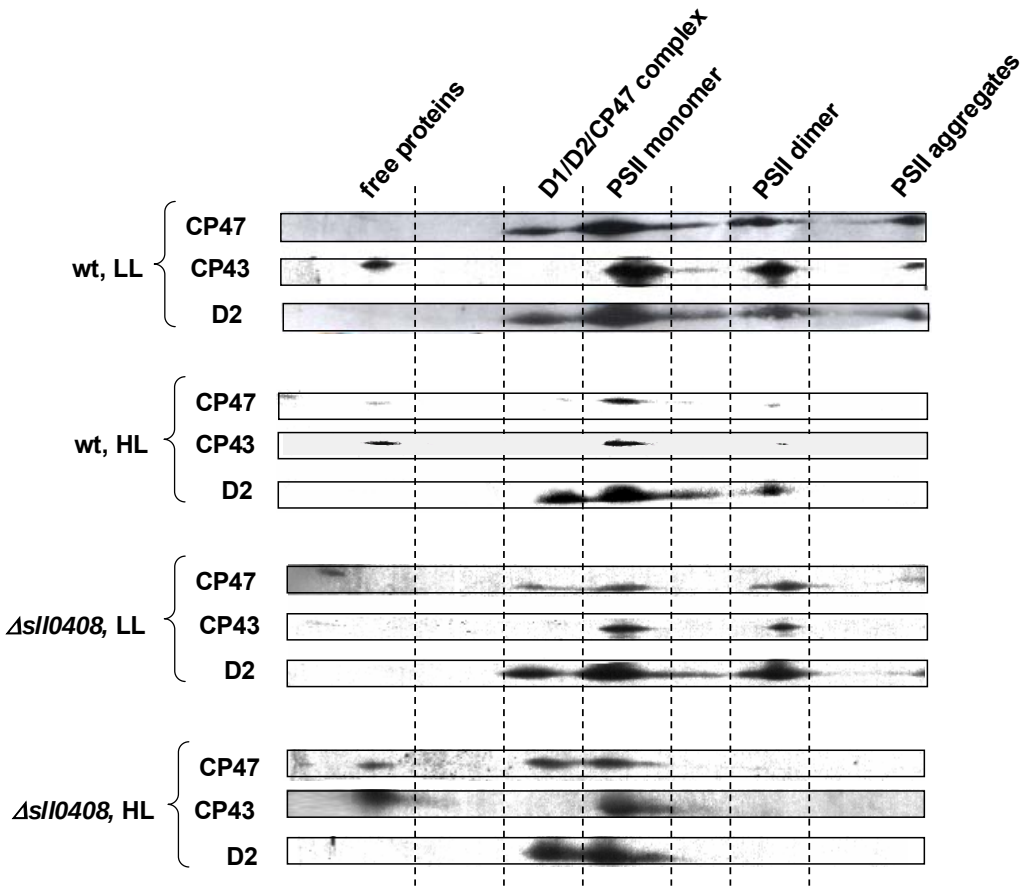


Fig. 26. Separation of PSII complexes from wild-type and mutant strain by 2D-blue-native/SDS-PAGE. Wild-type and mutant strain were grown at standard conditions (LL) or adapted to high light (HL) for 6 h. Total membranes were extracted and their partial lysates separated by 4 - 12% acrylamide blue-native PAGE in a first dimension and in a 10% SDS-PAG containing 4 M urea in the second dimension. Proteins were then transferred onto nitrocellulose membrane and tested with specific antibodies raised against the core proteins of PSII complex, CP47, CP43 and D2.

3.5.4 Separation of photosynthetic complexes by 2D-PAGE after labelling with [^{35}S]-methionine *in vivo*

Separation of PSII complexes by 2D-PAGE with subsequent Western analysis showed that PSII dimers could not be detected in the mutant after high light acclimation. These results indicated that *Δsll0408* could be affected in the assembly of PSII and mainly in formation of PSII dimer. To verify this idea the more sensitive procedure based on protein labelling with [^{35}S]-methionine was applied. Wild-type and mutant cell cultures were labelled with [^{35}S]-methionine for

25 min under standard conditions or after adaptation to high light. The reaction was stopped by freezing the cultures in liquid nitrogen. Thylakoid membranes were extracted and their photosynthetic complexes were separated by blue-native PAGE. Subunits of each complex were then separated by SDS-PAGE in a second dimension. A typical separation profile of radiolabelled proteins is shown in Figure 27.

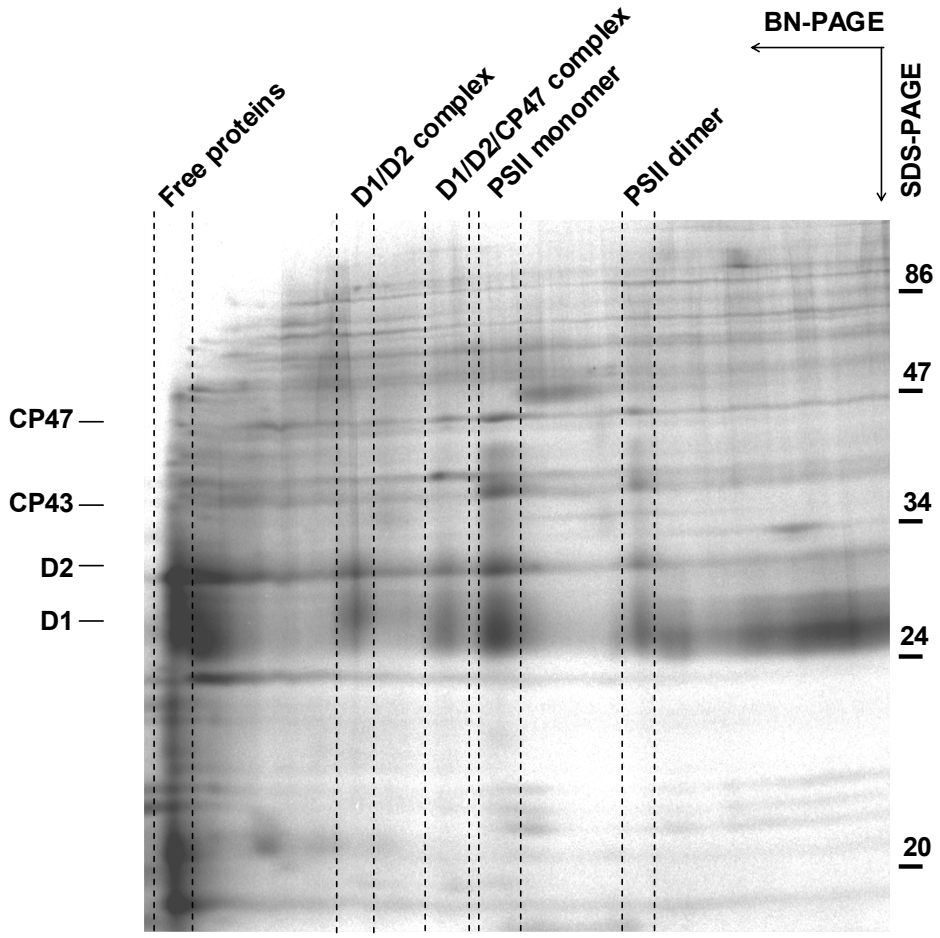


Fig. 27. 2D-BN/SDS-PAGE analysis of thylakoid-enriched membranes isolated from radiolabelled *Synechocystis* wild-type cells. Cultures were grown under standard conditions and labelled for 25 min with [^{35}S]-methionine. Membranes were extracted and photosynthetic complexes were separated by 4 - 12% blue-native PAGE. Protein subunits were subsequently separated in a 10% SDS gel containing 4 M urea in the second dimension. Localization of core intermediates and of PSII is indicated on the top of the figure.

By this 2D analysis PSII can be detected in its monomeric and dimeric forms. In addition, assembly of PSII intermediate which contain the core proteins except

CP43 (RC47) was also detectable. It is worth noting that D1 is the most highly labelled protein among PSII subunits due to its higher turnover (Komenda *et al*., 2004).

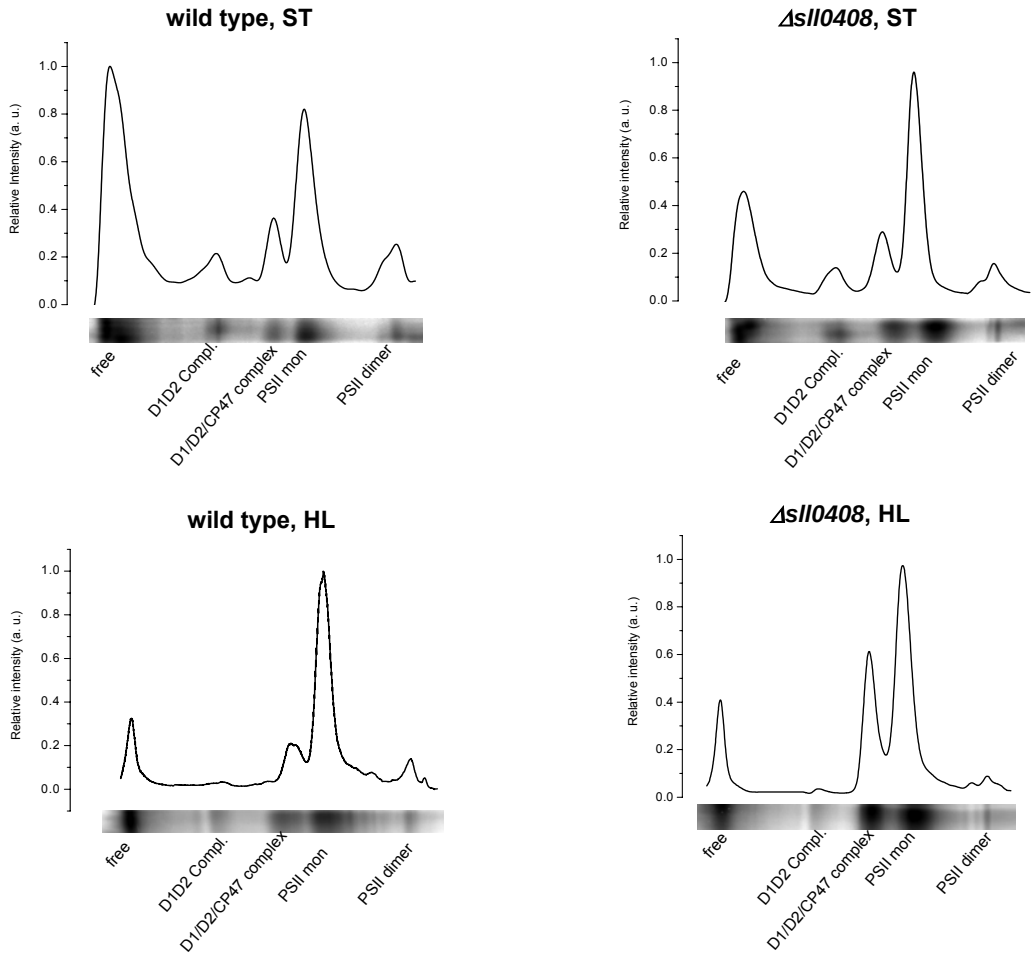


Fig. 28. Quantification of D1 protein in PSII assemblies of [^{35}S]-methionine labelled wild-type and *Δsll0408* strain. Wild-type and mutant cell cultures were labelled with [^{35}S]-methionine under standard conditions or after acclimation to high light for 6 h. Thylakoid membranes were extracted, solubilized and separated by 4 - 12% BN-PAGE and subsequently by 4 M urea containing 10% SDS-PAGE. Amounts of radiolabelled D1 protein were evaluated using TINA 2.01 software and normalized to the highest peak taken as 1.0. Compl.: complex; interm.: RC47 intermediate and mon.: monomer.

Accumulation of PSII subunits within complexes was evaluated in the wild-type and mutant through quantification of the radiolabelled D1 protein. As shown in Figure 28, D1 protein could be found as free protein, in PSII intermediates formed by D1/D2 and D1/D2/CP47, respectively, as well as in PSII monomer and dimer.

Amounts of D1 protein were evaluated at standard conditions and after 6 h of high light acclimation. In *Δsll0408* similar PSII assembly steps were detectable if compared to the wild-type (Fig. 28). Upon acclimation to high-light the relative amount of D1 protein as free protein and in the PSII dimer complex was decreased. When cultures were grown and labelled under standard growth conditions the amount of PSII dimer was slightly reduced in the mutant in comparison to wild-type. After acclimation to high light, this difference was more pronounced and most of the D1 protein in the mutant was mainly found in the PSII intermediates and PSII monomer. This experiment suggested that PSII dimer is assembled slower and/or is faster damaged and degraded in *Δsll0408* than in the wild-type. Both explanations account for an accumulation of D1 in PSII intermediates. From this experiment it was not possible to distinguish between these two alternatives.

3.5.5 Kinetic analysis of D1 protein synthesis in *Synechocystis*

A combined 2D analysis of *Synechocystis* PSII proteins by immunological visualisation and protein labelling showed that under high light the mutant possessed less PSII dimer form. This could depend on a slower assembly rate or a higher degradation rate of the PSII dimer. To distinguish these two processes, the assembly of PSII was analysed by labelling proteins with [^{35}S]-methionine for different times in cells adapted to high light for 6 h. After extraction of thylakoid membranes and separation of proteins by 2D-BN/SDS-PAGE, incorporation of [^{35}S]-methionine was checked for D1 protein at different times (Fig. 29).

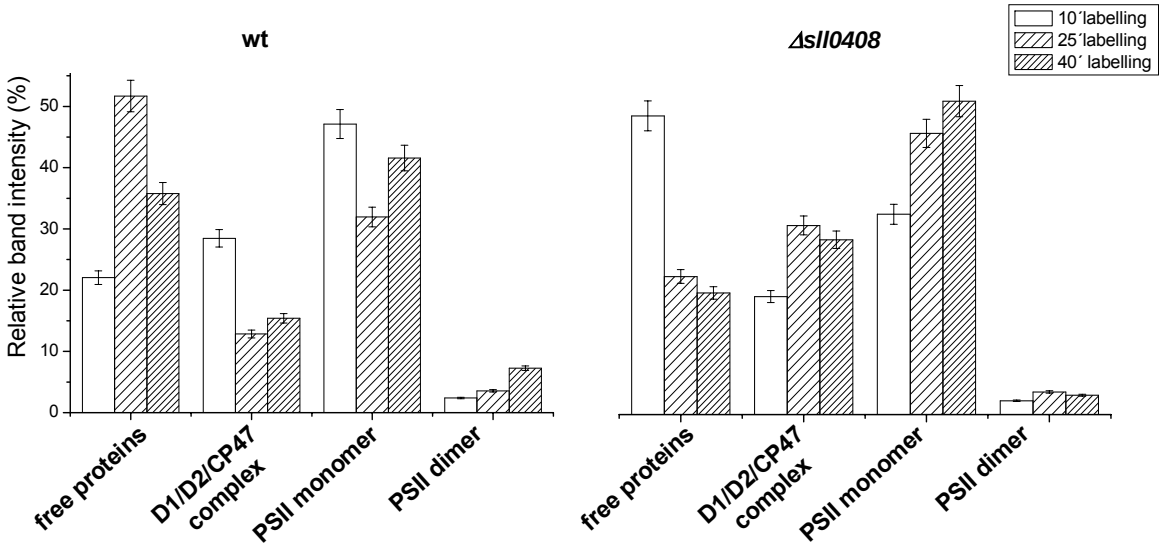


Fig. 29. Kinetic analysis of protein synthesis in wild-type and *ΔsII0408* cells. Wild-type and mutant strains were adapted to high light for 6 h and labelled for 10, 25 and 40 min with [^{35}S]-methionine. Reactions were stopped by freezing cells in liquid nitrogen. Thylakoid membranes were extracted and proteins from partial lysates were separated by 4 – 12% BN-PAGE in a first dimension. The composition of photosynthetic complexes was analyzed on a 10% SDS-PAG containing 4 M urea. Incorporated radioactivity was estimated using the software TINA 2.01. For each experiment, the total radioactivity of D1 protein was taken as 100%.

The rate of PSII assembly was quantified from the radiolabelled gel using TINA software. In wild-type the amounts of PSII dimer were proportional to the labelling periods. After 40 min of labelling the dimer contained about 7% of the total amount of radiolabelled D1 protein. In addition, with increasing labelling time, the amount of the intermediate complexes decreased in favour of PSII dimer (see D1/D2/CP47 complex and in minor part PSII monomer, Fig. 29). In the mutant strain the amount of PSII detectable after 40 min of labelling represent only 3% of the radiolabelled D1 protein. Moreover, the amount of PSII intermediates was increased with labelling time (Fig. 29). This did not discriminate between retardation in PSII assembly in the mutant or higher degradation rate.

To analyse the stability of PSII dimer at high light, wild-type and mutant proteins were labelled with [^{35}S]-methionine for 25 min. After removal of [^{35}S]-methionine by several washing steps with fresh BG11-medium, adaptation of cells to high light

was prolonged for further 40 min. Samples of the wild-type and mutant cells were taken after different times and frozen in liquid nitrogen. Thylakoid membranes were extracted and the proteins were separated by 2D BN/SDS-PAGE. The distribution of D1 protein between the PSII forms was analysed and the results are shown in Figure 30.

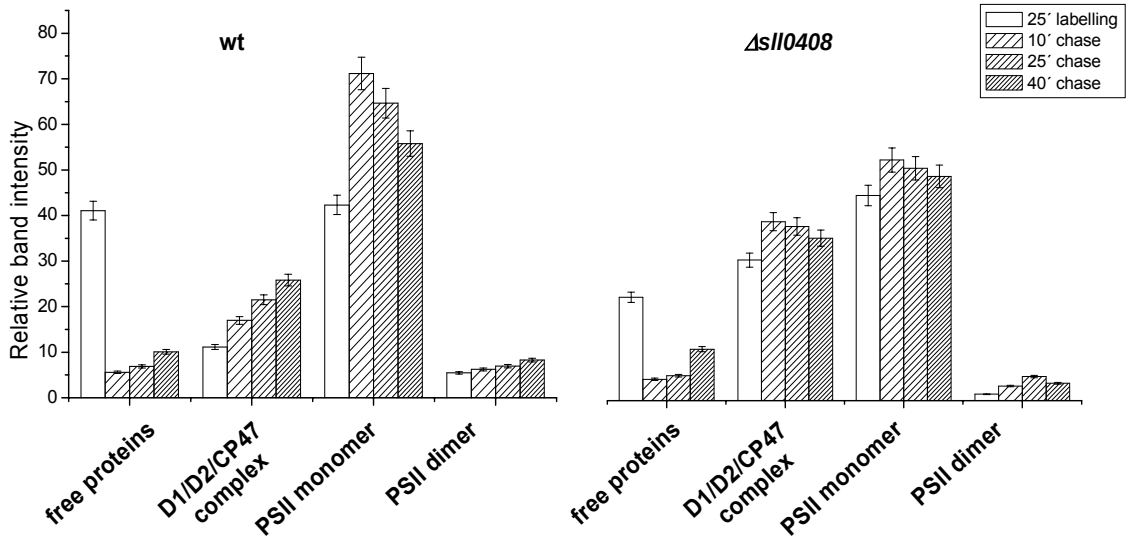


Fig. 30. Analysis of D1 protein degradation in wild-type and *ΔsII0408* cells under high light. Wild-type and mutant strains were adapted to high light for 6 h and labelled for 25 min with [^{35}S]-methionine. Radioactivity was then removed by washing cells with fresh BG11 medium three times. Cells were finally resuspended in BG11 and high light adaptation was prolonged for 10, 25 and 40 min. Reactions were stopped by freezing cells in liquid nitrogen. Thylakoid membranes were extracted and separated by 4 – 12% BN-PAGE in the first dimension and by 4 M urea containing 10% SDS-PAGE in the second dimension. Incorporated radioactivity was estimated using the software TINA 2.01. For each experiment, the total radioactivity of D1 protein was taken as 100%.

This experiment showed that the mutant assembled less PSII dimer in comparison to the wild-type. Moreover, the highest signal in the wild-type was concentrated always in the PSII monomeric form. In the mutant the difference between D1 in D1/D2/CP47 complex and PSII monomer was less pronounced than in the wild-type. In addition, in wild-type there is an increase in the amount of PSII dimer with the chase time. This indicated not only that PSII dimer was actively assembled in the wild-type but also that the dimeric form was stable. Increase of PSII dimer was limited in the mutant to the first 25 min. Afterwards, the signal of the PSII dimer decreased and this could suggest a reduced stability of the complex.

3.6 Rotamase activity of cTLP40

The C-terminal domain of cTLP40 protein shows high homology to peptidyl-proline-isomerases that can catalyze the isomerisation of the proline bonds from the *cis* to the *trans* conformation. PPIase or rotamase activities is involved *in vivo* in several biological processes (see Introduction). The PPIase activity can be tested *in vitro* using a colorimetric reaction which comprises a chromogenic substrate and the proteolytic enzyme chymotrypsin (Fischer *et al*., 1989). In this conventional PPIase assay, the peptide substrate N-succ-Ala-Ala-Pro-Phe-p-nitroanilide can be recognised by PPIase and hydrolysed by chymotrypsin only in the trans conformation. The hydrolysis releases p-nitroanilide which leads to an absorbance increase at 390 nm. The *cis* and *trans* forms of each nitroaniline-containing peptide are naturally present in solution, in equilibrium which depends on the concentration of the peptide and on the type of solution. In aqueous solution, for example, the equilibrium population of the *cis* relative to the *trans* conformation does not exceed 10%. To increase the absorbance value the substrate was dissolved in LiCl containing buffer which increases the quantity of the *cis* conformation up to 50 - 70% (Kofron *et al*., 1991). This assay was performed with *in vitro* overexpressed cTLP40 to check whether this enzyme can indeed catalyze the isomerisation of the proline bonds. cTLP40 was overexpressed by a rapid translation system from Roche based on expression of proteins in a bacterial cell lysate. The full length cTLP40 was overexpressed with Histidine-tag at its N-terminus and purified by Irina Piven.

Before starting the PPIase colorimetric assay, it was checked whether cTLP40 can be proteolyzed by chymotrypsin. cTLP40 protein was incubated with chymotrypsin at 4°C and room temperature for different times. The reaction was stopped and proteins were loaded onto a 10% SDS-PAGE. The degradation of cTLP40 was analyzed by Western blot with cTLP40 antisera (Fig. 31).

Fig. 31. Proteolytic activity of chymotrypsin against overexpressed cTLP40. cTLP40 (lane 1) was incubated with chymotrypsin either on ice for 30 sec or 5 min (lane 2 and 3) or at RT for 30 sec or 5 min (lane 4 and 5). Reaction was stopped by addition of PMSF (1 mM) and proteins were denatured by addition of Laemmli loading buffer. Proteins were separated by a 10% SDS-PAGE, transferred onto a nitrocellulose membrane and tested with polyclonal antibodies raised against cTLP40.

The data showed that cTLP40 was degraded completely within 30 sec, both on ice and at room temperature. To prevent rapid degradation of cTLP40 by chymotrypsin peptide N-succ-Ala-Ala-Pro-Phe-p-nitroanilide (control peptide, pepC) was first incubated with cTLP40 on ice for 5 min. Chymotrypsin was then added afterwards and the absorbance was recorded. Addition of cTLP40 increased the activity of chymotrypsin (measured as an increase of absorbance at 390 nm) of about 50%. This experiment confirmed that addition of cTLP40 increased the *trans* population of the peptide (Fig. 32).

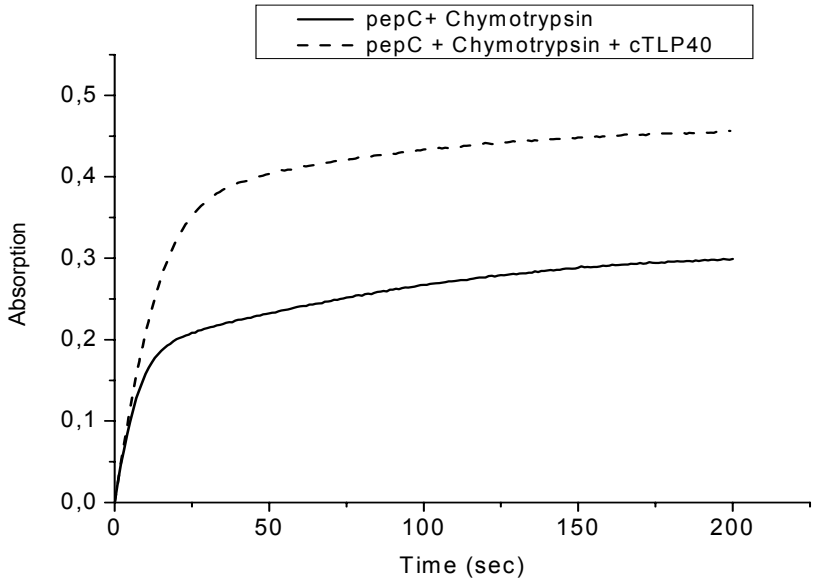


Fig. 32. Activity of cTLP40 against pepC substrate. cTLP40 was diluted with 10 mM Tricine/NaOH, pH 7.8, up to a final concentration of 0.01 µM. Afterwards, pepC (60 µM end concentration) was added to cTLP40 and incubated on ice for 5 min. Chymotrypsin (0.93 µM end concentration) was added immediately before recording the absorbance at 390 nm. Total volume of each reaction was 1 ml.

cTLP40 was overexpressed using an *in vitro* system which contains *E. coli* cellular proteins. It was previously reported that *E. coli* contains few highly active rotamases (Hottenrot *et al*., 1997). To check whether rotamase activity was specific for cTLP40 or could be caused by other PPIase activities, measurements were repeated in the presence of specific antibodies raised against cTLP40. cTLP40 and antisera against cTLP40 were mixed and incubated on ice for 5 min. After addition of the substrate and repeated incubation on ice for 5 min chymotrypsin was added and absorbances were recorded (Fig. 33).

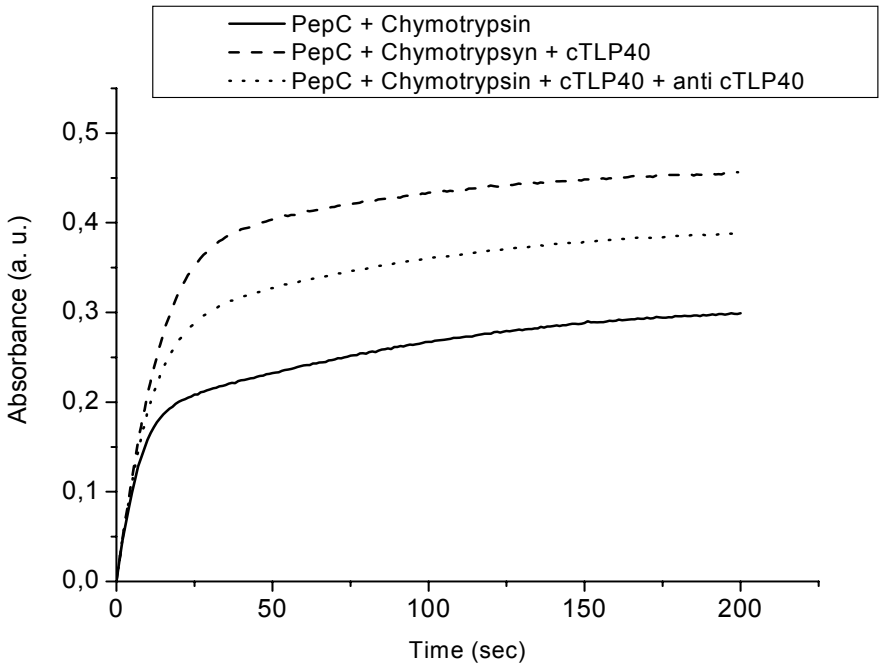


Fig. 33. Inhibition of cTLP40 PPIase activity by addition of antisera. cTLP40 and antisera were mixed and incubated on ice for 5 min. After pepC addition the reaction was incubated on ice for 5 min. Chymotrypsin was added and absorbance was recorded at 390 nm for 200 sec at RT.

Addition of anti cTLP40 inhibited the proteolytic activity of chymotrypsin by about 50%. This experiment showed that despite the purification of overexpressed cTLP40, some bacterial rotamase(s) were still present and contributed to isomerisation of pepC. However, it was not possible to exclude that cTLP40 antisera could also be a substrate for chymotrypsin and that it could compete with pepC as a substrate.

3.6.1 Enzymatic activity of TLP40 on synthetic peptides derived from CP43 and PsbI

It was previously shown that cTLP40 possesses PPIase activity, i. e. it can catalyze the *in vitro* isomerisation of the peptide N-succ-Ala-Ala-Pro-Phe-p-nitroanilide in a conventional PPIase assay (Fischer *et al*., 1989; Kofron *et al*., 1991). Functional analysis of *Synechocystis* strain *Δsll0408* showed that cTLP40 seems to be involved in the assembly/stabilisation of PSII dimer. Several PSII subunits have a critical role in assembly and/or stabilisation of PSII dimer and we suggested that several proteins could be a target of cTLP40. Since cTLP40 is located at the lumenal side of thylakoid membranes we suggested that cTLP40 could be involved in the isomerisation of the lumenal loop of CP43 and PsbI which are involved in the dimerisation of PSII (Ikeuchi *et al*., 1995; Anderson *et al*., 2002). It was suggested that proline 351 and 355 in CP43 could be important for the proper orientation of tryptophan 351, a target of posttranslational oxidation. The role of this posttranslational modification is not known but it was suggested to be important in CP43 turnover (Anderson *et al*., 2002). *Synechocystis* mutant lacking PsbI protein has a decreased PSII activity (about 25 - 30%) and so it was suggested that PsbI could play a role in the optimisation of the PSII structure (Ikeuchi *et al*., 1995). Two peptides containing proline in the lumenal E loops of CP43 and one peptide of the lumenal part of PsbI were synthesized for the colorimetric PPIase assay (Table 2).

Table 2. List of substrates used in the PPIase assay

Pep1	CP43-1	N-Succ-^{349}RGP^{351}F-p-nitroaniline
Pep2	CP43-2	N-Succ-^{353}LEP^{355}F-p-nitroaniline
Pep3	PsbI	N-Succ-^{26}SDP^{28}F-p-nitroaniline

At the beginning it was checked whether chymotrypsin can hydrolyze the *trans* form of each peptide. Peptide substrates were diluted in 1 ml of 10 mM Tricine pH 7.8 and reactions were performed in the presence of chymotrypsin. The hydrolysis of the substrate was recorded by an increase of absorbance at 390 nm (Fig. 34).

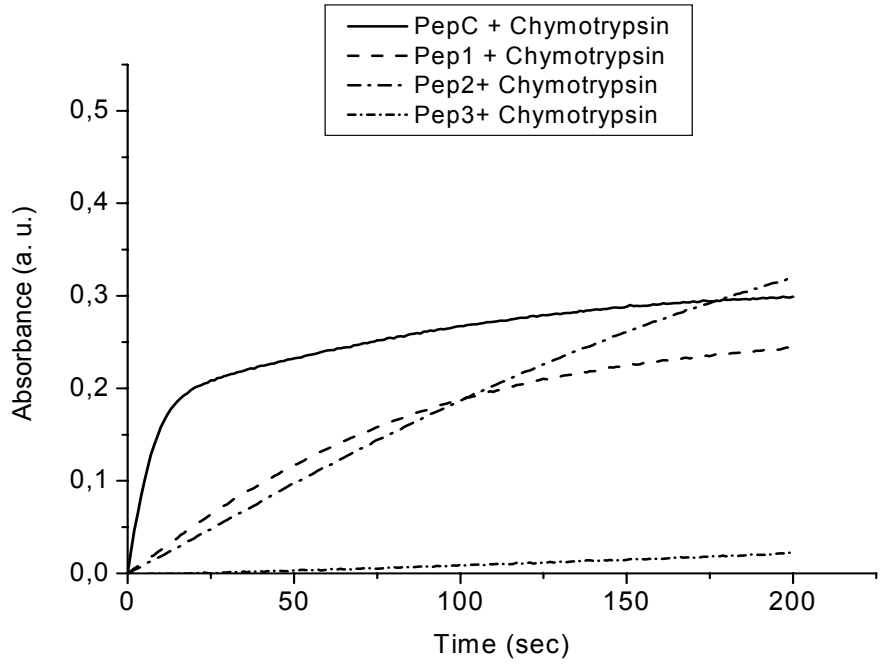
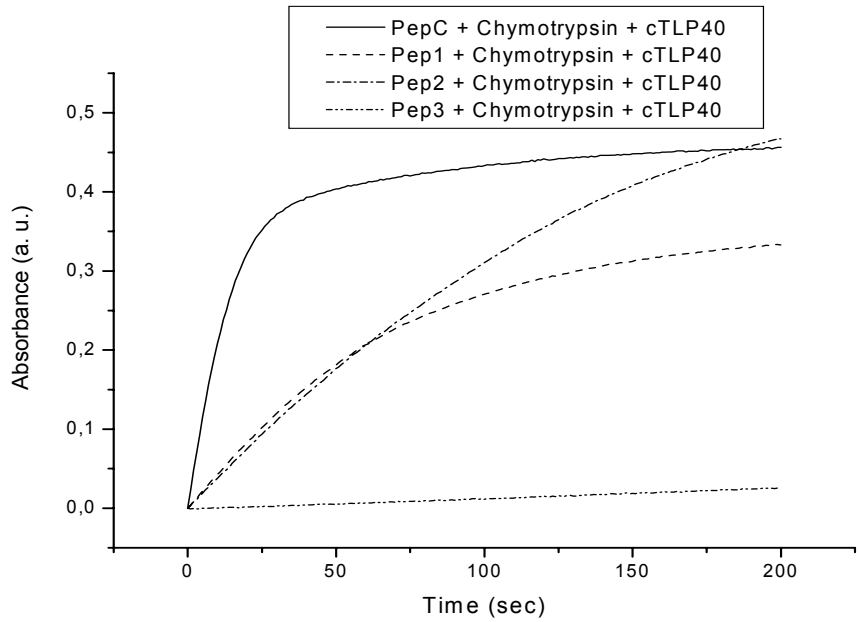


Fig. 34. Measurement of chymotrypsin activity on different peptide substrates. Chymotrypsin was diluted in 10 mM Tricine/NaOH, pH 7.8, to a final concentration of 0.93 µM. Absorbance measurement at 390 nm was started immediately after addition of the peptides (60 µM end concentration). The absorbance was recorded for 200 sec at RT.

Figure 34 shows that the efficiency of the hydrolysis was different between the peptides. This difference can depend on the relative percentage of *cis* and *trans* conformations. In particular Pep3 seems to be completely in *cis* conformation and no significant increase of absorbance could be observed during the measurement. It is also worth noting that the absorbance increase for peptide 2 was linear and did not reach the maximum value during the time of measurement (as in case of PepC and Pep1). The same measurement was repeated in the presence of the overexpressed rotamase (Fig. 35). Substrates were incubated first with cTLP40 on ice for 5 min. After addition of chymotrypsin, the absorbance increase was measured at 390 nm for 200 sec.

A



B

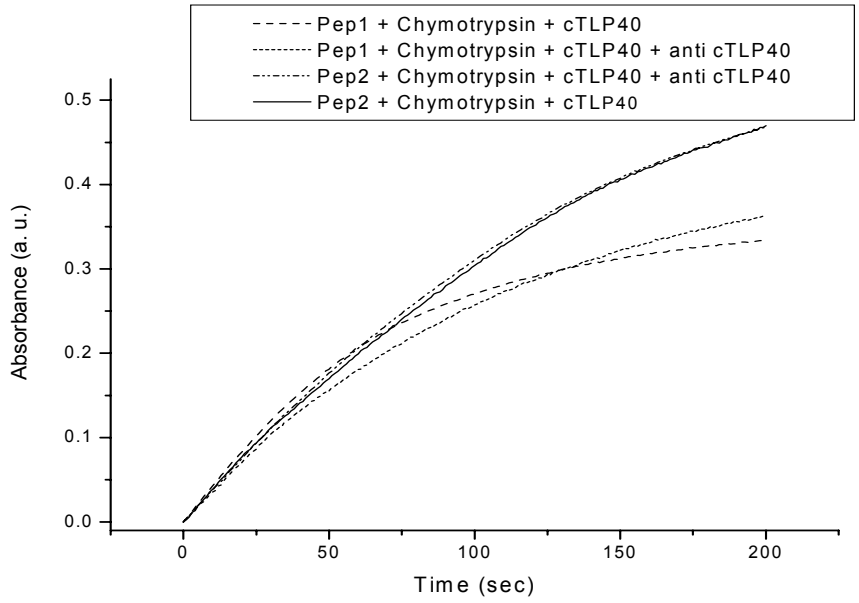


Fig. 35. (A). Activity of cTLP40 against different peptides. A) cTLP40 was diluted in 10 mM Tricine/NaOH, pH 7.8, at a final concentration of 0.01 µM. Peptides (60 µM end concentration) were added to cTLP40 and incubated for 5 min on ice. Chymotrypsin (0.93 µM end concentration) was added immediately before the recording of the absorbance at 390 nm. The total volume of the reaction was 1 ml. The absorbance was measured for 200 sec at RT. (B) Inhibition of cTLP40 activity by addition of antisera against cTLP40. cTLP40 and anti-cTLP40 were mixed and incubated on ice for 5 min. After the peptides were added, the reaction was incubated for an additional 5 min. Chymotrypsin was the added and the 390 nm absorbance was recorded for 200 sec.

The addition of cTLP40 mixture increased the population of the *trans* form when Pep1 and Pep2 were used as substrates. The addition of cTLP40 increased the activity of chymotrypsin for about 35% for Pep1 and for 47% for Pep2. Anyway the rate of isomerisation for both peptides was slower than the rate of isomerisation of PepC. For this peptide the maximum value of the absorbance was reached in 50 s while the maximum value for Pep1 was reached at the end of the reaction. For Pep2 the maximum was not reached during the measuring time. When Pep3 was used as substrate, there was no difference in the activity of chymotrypsin in the absence or in the presence of cTLP40. It was then analyzed whether *E. coli* isomerases were present in the overexpressed mixture and could also be involved in the isomerisation process of the peptide substrates. The activity of cTLP40 was blocked by the addition of anti-cTLP40. Then, the peptides were added and the mixture was incubated for other 5 min. Chymotrypsin was added and the 390 nm absorbance was immediately recorded for 200 sec (Fig. 35, Panel B). The result of the last experiment was not clear. The addition of anti cTLP40 seems not to interfere with the proceeding of the reaction. A possible explanation of that is that anti-cTLP40 did not inhibit the activity of cTLP40: this is possible since anti-cTLP40 were raised against the denatured protein, while in this experiment cTLP40 was used in its native form. However, it is not possible to exclude the presence of other rotamases which catalyze the isomerisation of the synthetic peptides and their activity is not impaired by the addition of anti-cTLP40. This experiment excludes the possibility that anti-cTLP40 is a substrate of chymotrypsin since there is no decrease in the protease activity.

3.7 Analysis of TLP40 in higher plants

3.7.1 Localisation of TLP40 within thylakoid membranes

Analysis of the protein sequence and import assay *in vitro* showed that TLP40 is indeed located in thylakoid lumen and can be either associated with the thylakoid membrane or exist in free form in the lumen (Fulgosi *et al*., 1998). In order to define more precisely the localisation of TLP40, a blue-native/SDS-PAGE analysis was performed to check whether TLP40 associated with some complex of thylakoid membranes. For the first dimension thylakoid membranes of spinach were solubilized with the detergent β-dodecyl-maltoside. The complexes were then separated by a 4 - 12% blue-native PAGE. Proteins constituting the complexes were separated in the second dimension by SDS-PAGE and transferred to a nitrocellulose membrane. The presence of TLP40 within thylakoid membrane and its association with photosynthetic complexes was tested immunologically with specific antibodies raised against TLP40, CP47 and cytochrome *f.*

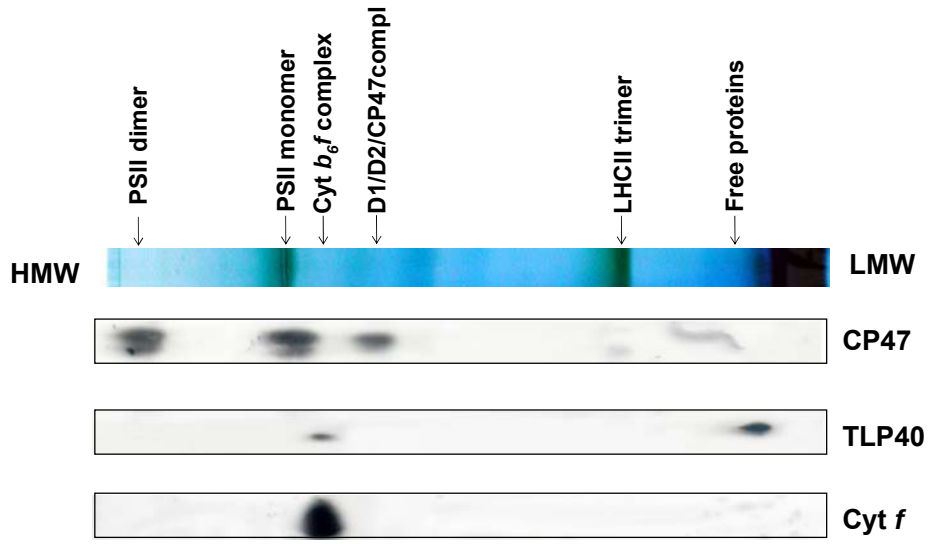


Fig. 36. Western analysis of partially lysed spinach thylakoid membranes separated by 2D gel electrophoresis. Proteins transferred into nitrocellulose membrane were identified with antibodies raised against TLP40, cytochrome *f* and CP47. The direction of blue-native separation is indicated by the shortcut HMW, high molecular weight, and LMW, low molecular weight proteins. The major photosynthetic complexes are indicated on the first dimension according to Ossenbühl *et al*. (2004).

Western analysis of TLP40 after separation by 2D gel-electrophoresis showed two signals in the region of free proteins and in the region of the cytochrome *b₆f* complex. The two TLP40 signals differed in molecular weight and one could argue for possible TLP40 modifications (for example phosphorylation or other amino acid modifications) which alter the electrophoretic mobility of the protein.

3.7.2 Association of TLP40 with the cytochrome *b₆f* complex in tobacco

It was previously shown that TLP40 from spinach thylakoid membrane lysates separated by sucrose gradient partially comigrated with the cytochrome *b₆f* complex (Weber, 2001). Similar results were obtained when thylakoid membranes were separated by blue-native and SDS gel electrophoresis (see Fig. 36). Comigration of two proteins in a sucrose gradient or in a blue-native PAGE does not necessarily imply that proteins belong to the same complex. To check, whether TLP40 is associated with cytochrome *b₆f* complex, the localisation of TLP40 was analysed in tobacco plants deleted in *petG* (encoding subunit V) which resulted in a deficiency of the cytochrome *b₆f* complex. Homoplastomic lines were able to grow heterotrophically and demonstrated a pale green phenotype and a retarded growth rate under standard growth light (100 µE $m^{-2}s^{-1}$; Legen, 2003). Thylakoid membrane lysate of tobacco wild-type and mutant *ΔpetG* were separated on a 0.1 – 1 M sucrose gradient. The gradient fractions were collected and loaded onto a SDS-PAG. Proteins were transferred onto a nitrocellulose membrane and tested with antibodies raised against cytochrome *f* and TLP40 (Fig.37).

As shown in Figure 37, TLP40 migrated mostly as a free protein and was not associated with the cytochrome *b₆f* complex. This finding contrasts with what was previously reported (Weber, 2001) A similar pattern was found when thylakoid membranes of mutant *ΔpetG* were tested for the localisation of TLP40. Evidence that the absence of cytochrome *b₆f* complex does not influence the migration pattern of TLP40 indicated that TLP40 is not associated with cytochrome *b₆f* complex.

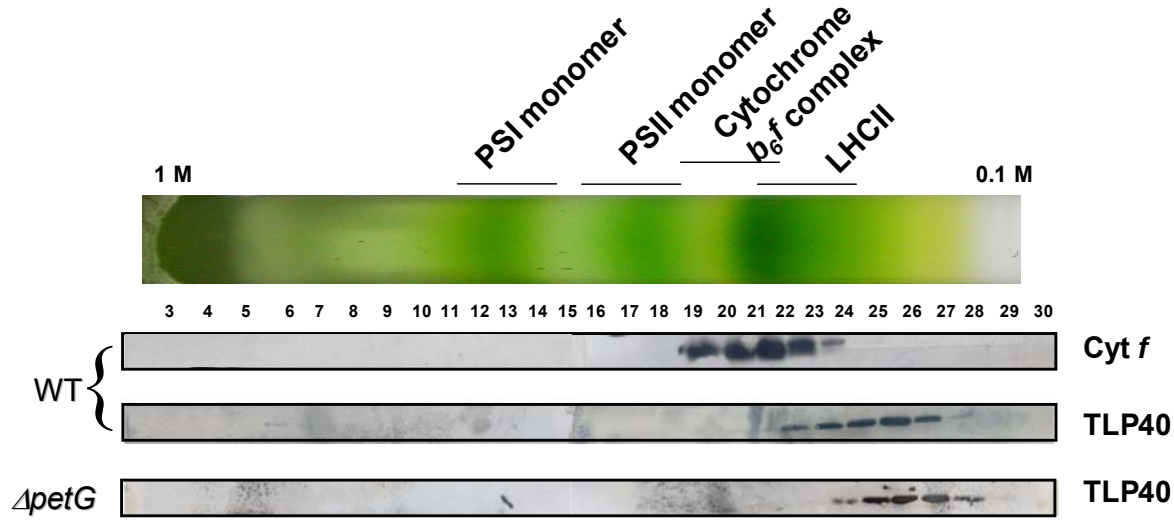


Fig. 37. Separation of thylakoid membranes from tobacco wild-type and *ΔpetG* by sucrose gradient centrifugation. Thylakoid membranes were isolated from fresh harvested leaves and solubilized with β–dodecyl-maltoside. Solubilized membranes were loaded onto a linear 0.1 – 1 M sucrose gradient. Photosynthetic complexes were separated by ultracentrifugation for 16 h at 4°C. The gradient was collected and protein fractions were loaded on a 12.5% SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane and tested with antibodies raised against TLP40 and cytochrome *f*. The fractions of tobacco wild-type and *ΔpetG* were kindly provided by Dr. Julia Legen.

3.7.3 Association of TLP40 with thylakoid membranes *in vivo*

It was suggested that TLP40 in chloroplasts could be involved in the regulation of the phosphatase that dephosphorylates PSII subunits (Fulgosi *et al*., 1998; Vener *et al*., 1999; Rokka *et al*., 2000). In particular, it was proposed for TLP40 to be a negative regulator of protein phosphatase: upon binding of TLP40 to thylakoid membranes and thus to phosphatase, its activity could be inhibited. The activation of phosphatase coincides with the release of TLP40 into the lumen (Vener *et al*., 1999; Rokka *et al*., 2000). This hypothesis was tested *in vitro* by detecting the association/dissociation of TLP40 with thylakoid membranes under different temperature regimes. It was observed that the rise of temperature coincided with an increase of the dephosphorylation activity and with a release of TLP40 into the lumen (Rokka *et al*., 2000). The association/dissociation of TLP40 to the lumen was checked *in vivo*. Spinach was grown at green-house conditions for five weeks.

Afterwards, the plants were adapted to different light and temperature regimes for different periods. Thylakoids were extracted from acclimated plants and separated into lumenal and thylakoid membrane proteins. Fractions were loaded onto SDS-PAGE, transferred to a nitrocellulose membrane and tested with polyclonal antibodies raised against TLP40 (Fig. 38).

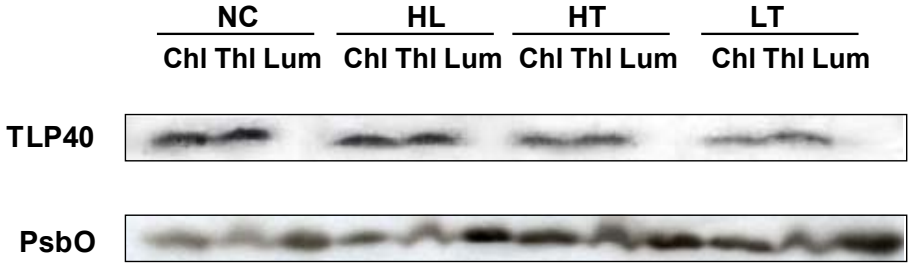


Fig. 38. Association of TLP40 with thylakoid membranes. Spinach plants were grown for 5 weeks in a green house (normal conditions, NC, 25°C). Later on, plants were adapted to HL (750 µE $m^{-2}s^{-1}$, 25°C), high temperature (HT, 42°C, 50 µE m^{-2} s^{-1}) for 2 h and to low temperature (LT, 4°C, 50 µE m^2 s^{-1}) for 24 h. Thylakoid membranes were extracted and separated into lumenal and thylakoid membrane proteins. For each experiment chloroplast (chl), thylakoid (thl) and lumenal (lum) fractions were separated on a 12.5% SDS-PAGE, transferred to a nitrocellulose membrane and tested with antibodies raised against TLP40. PsbO protein was used as a control for lumenal proteins.

Figure 38 shows that the adaptation of spinach plants to high temperature did not increase the release of TLP40 into the lumenal compartment as it was suggested previously from experiments *in vitro* (Rokka *et al*., 2000). In addition, it was checked whether other conditions, could cause the dissociation of TLP40 from thylakoid membrane. Spinach plants were acclimated to low temperature and to high light, conditions that normally impair the function of photosynthetic complexes. Similar to high light TLP40 was mostly found attached to the membrane. These results are controversial under our chosen conditions with those reported by Rokka *et al*., (2000).

3.8 T-DNA insertion lines of *Arabidopsis thaliana*

Seeds of T-DNA insertion lines of the gene encoding TLP40 were obtained from Syngenta Biotechnology (Inc. Research Triangle Park, North Carolina 27709). Insertion lines were generated in the Columbia ecotype of *Arabidopsis thaliana*. The sequence analysis of the flanking regions of T-DNA insertion was made using a specific primer for left border (LB). This sequence analysis allowed to define an orientation of T-DNA insertion which could be either in plus/plus (reading frame of T-DNA corresponds to the sense direction of targetted gene) or plus/minus in the opposite case. Few lines with different T-DNA localisation were selected for further analysis (Table 3).

Table 3. *Arabidopsis* T-DNA insertion lines of the gene *At3g01480* coding for TLP40 protein.

Line	TDNA vector	Selectable marker	Strand orientation
96_C04	pCSA110	BASTA	plus/minus
125_E05	pCSA110	BASTA	plus/plus
611_H10	pDAP101	BASTA	plus/minus
714_A07	pDAP101	BASTA	plus/minus
862_G07	pDAP101	BASTA	plus/plus
1296_H02	pDAP101	BASTA	plus/plus

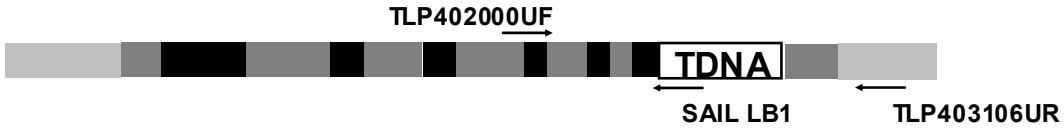
Four lines, 125_E05, 862_G07, 1296_H02 and 714_A07 contained T-DNA insertions in their 5´ and 3´ non-coding regions (Fig. 39). Two lines, 96_C04 and 611_H02 contained T-DNA insertion in the 5^{th} intron and in the 7^{th} exon, respectively. Since the T-DNA insertion for lines 125_E05, line 714_A07 and 862_G07 were quite distant from the gene coding region, these three lines were not considered for analysis.

Fig. 39. Localisation of T-DNA insertions in *Arabidopsis* lines for the gene *At3g01480* (TLP40). Non-coding regions, exons and introns of TLP40 are coloured in light grey, grey and dark grey, respectively. The length of the T-DNA (white) is not in scale to the length of TLP40 gene.

3.8.1 PCR analysis of T-DNA insertion lines

Total DNA was extracted from *Arabidopsis* leaves of the mutant lines and a PCR analysis was performed to define the exact localisation of the insertion and to check the homozygous state. PCR of T-DNA insertion lines was performed on

Line 611_H10 and line 96_C04 (orientation plus/minus)



Line 1296_H02 (orientation plus/plus)

Fig. 40. Schematic presentation of T-DNA insertions in *Arabidopsis* lines. Primers used for each PCR reaction are also shown (arrows). Non-coding regions, exons and introns of TLP40 are coloured in light grey, grey and dark grey, respectively. The length of T-DNA (white) is not in scale to the length of TLP40 gene.

genomic DNA with three different primers, two gene-specific and one T-DNA-specific, in order to check, with a single reaction, the presence/absence of the T-DNA insertion and of the wild-type gene (Fig. 40).

Because of high molecular fragment of T-DNA insertion (7451 or 4763 bp for pCSA110 and pDAP101 T-DNA vectors, respectively), no PCR products could be obtained with TLP40 specific reverse and forward primers on genomic DNA containing the T-DNA fragment. The expected lengths of PCR products are shown in Table 4.

Table 4. Length of amplified PCR producs in T-DNA lines and wild-type gene

Line	With TDNA	Without TDNA (wild-type gene)
611_H10	ca 650 bp	1106 bp
96_C04	ca 400 bp	1106 bp
1296_H02	ca 500 bp	450 bp

Typical PCR results of four different second generation plants for each line are shown in Figure 41.

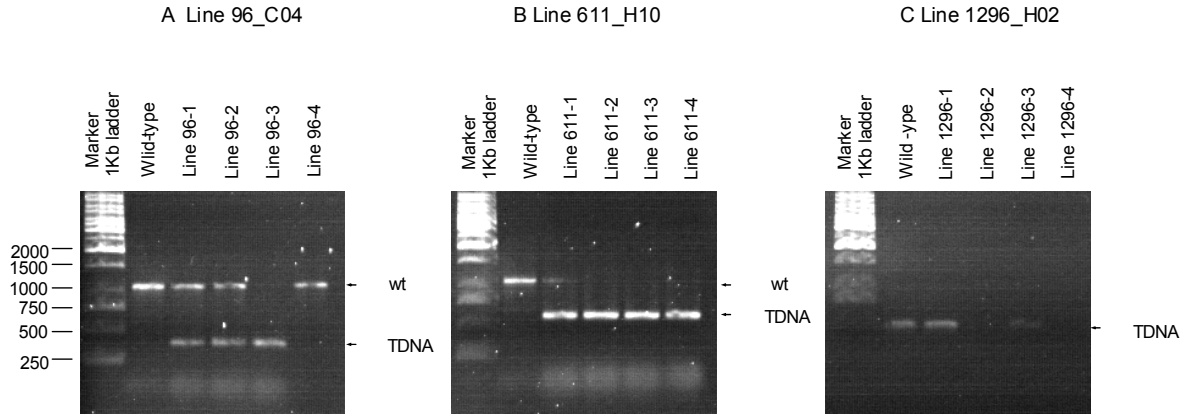


Fig. 41. PCR analysis of T-DNA insertion lines in the gene encoding TLP40 protein. Arrows indicate the fragment coming from amplification of the wild-type gene (wt) or T-DNA disrupted gene. The molecular weight marker is indicated at the left of panel A.

PCR analysis demonstrated that lines 96-1 and -2 were heterozygous while line 96-3 was homozygous. Line 96-4 contained no insertion in the TLP40 gene. Line 611-1 was heterozygous while lines 611-2, 3 and 4 were homozygous for the T-DNA insertion. In the case of line 1296, three lines were homozygous for the insertion.

To localise the position of the T-DNA inside the gene, the PCR products obtained with T-DNA specific primers, were sequenced using the primer SAIL LB1. Sequence analysis showed that insertion was located 22 bp from the beginning of the last exon (exon 7) in line 611_H10, 4 bp before start of exon 6 (intron 5) in line 96_C04, and -195 bp of the 5´ untranslated region in line 1296_H02 (Fig. 42).

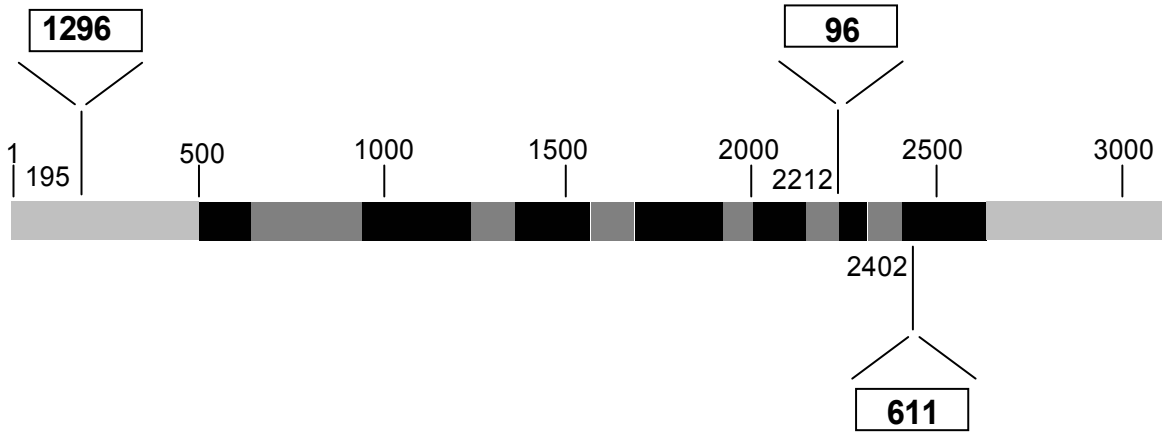


Fig 42. Localisation of the T-DNA insertion in lines 1296_H02, 96_C04 and 611_H10. Non-coding regions, exons and introns of TLP40 are coloured in light grey, grey and dark grey, respectively. The length of the T-DNA is not in scale to the length of the TLP40 gene. The annotation of the DNA sequence is according to the MIPS database.

3.8.2 T-DNA sequence

Sequences of T-DNA lines with SAIL LB1 primer revealed part of the sequence which corresponded to the T-DNA sequence (Fig. 43).

5´- ATATTGTGGTGTAAACAAATTGACGCTTAGACAACTTAATAACACATTGCG
GCCGTTTTTAATGTACTGNATTAACGCCGAATTGAATTCGATTTGGTGTATCG
AGATTGGTTATGAAA - 3´

Fig. 43. Part of the T-DNA sequence in lines 611_H10 and 1296_H02 (containing pDAP101 T-DNA vector). The T-DNA fragment was amplified with primer SAIL LB1 (or LB3cut) and LBreverse. The final lengths of the PCR product were 150 - 200 bp.

Line 611_H10 was interrupted with T-DNA in the seventh exon of the coding region. The resulting protein was 64 amino acid residues shorter than the wild-type protein. These 64 amino acid residues seemed not to impair the PPIase activity of the C terminal part. When the protein sequence was submitted to the NCBI server, a PPIase domain was recognized at the C-terminus.

4. Discussion

4.1 Sequence analysis of TLP40 from higher plants and *Synechocystis*

TLP40 (thylakoid lumen PPIase of 40 kDa) was the first complex immunophilin described in spinach chloroplasts (Fulgosi *et al*., 1998). The main characteristic of the protein is the presence of an immunophilin domain located at the C-terminus. Such a domain catalyzes the *cis-trans* isomerisation of a proline imidic peptide bond (rotamase or PPIase activity) (Fischer *et al*., 1989) which is thought to be a rate-limiting step of protein folding. TLP40 belongs to the cyclophilin subfamily even if the homology with the human cyclophilin A, which is usually used as a basic reference for this class of enzymes, is only of 25% (Fulgosi *et al*., 1998). In addition, TLP40 misses the tryptophan at position 332 which binds the immunosuppressive drug cyclosporin A in the human cyclophilin A (Zydowsky *et al*., 1992). This critical tryptophan residue is substituted into an isoleucine in spinach TLP40 and cTLP40 and into a valine in *Arabidopsis* TLP40. Another feature of the TLP40 immunophilin domain is the substitutions F334E and H406Y which are required for PPIase activity in the human cyclophilin A (Zydowsky *et al*., 1992). The same substitutions are present in *Arabidopsis* TLP40 (F322E and H394Y) and in cTLP40 (F268E and H340Y). However, TLP40 retained the PPIase activity as it was shown *in vitro* (Fulgosi *et al*., 1998). The rotamase activity of cTLP40 was confirmed also *in vitro* (this work, see Section 3.6) while the rotamase activity of *Arabidopsis* TLP40 still remains to be proven. However, since the C-terminus of *Arabidopsis* possesses 90% homology with the rotamase domain of TLP40, it is likely that the C-terminal domain of *Arabidopsis* TLP40 possesses PPIase activity as well.

In addition to the C-terminal rotamase domain, the N-terminus of TLP40 possesses two leucine zipper and three putative phosphatase-binding domains. Leucine zipper domains are characterised by the regular repetition of leucine and/or isoleucine at every seventh position. This domain folds in an α–helical conformation forming a hydrophobic surface with leucine/isoleucine residues on

one side of the helix and a hydrophilic region on the other. This domain can interact with other leucine zipper-containing or coiled-coil proteins (Lupas, 1996). Two leucine zipper domains are present both in *Arabidopsis* TLP40 and cTLP40. The first leucine zipper domain in *Arabidopsis* TLP40 is identical to the one of TLP40 (disregarding the conservative substitution E130D in *Arabidopsis*). The second domain contains one valine instead of a leucine in position 147. In cTLP40 the amino acid valine substituted isoleucine and leucine in both helixes (I76V and L100V in *Synechocystis*). In addition, the second leucine zipper domain is shorter in cTLP40 than one of spinach TLP40 due to the lack of the first heptade.

Amino acid positions in each heptade of a leucine zipper domains are conventionally identified using the nomenclature $(a, b, c, d, e, f, g)_n$ (McLachlan and Stewart, 1975), where the position d is normally occupied by a leucine o isoleucine residues. It was shown that leucine is the most stabilising amino acid in the d position upon dimerisation of a leucine zipper structure (Moitra *et al*., 1997). However, other amino acid residues can be present at this position. Valine belongs to the group of unpolar branched amino acid residues as leucine and isoleucine and can also provide a hydrophobic side for protein interaction in the leucine zipper domain. When valine is located in position d, the stability of the leucine zipper domain decreases about 30% (Moitra *et al*., 1997). It is possible to conclude that the presence of valine in position d can interfere with the stability of the interaction of the leucine zipper with another leucine zipper or with a coiled-coil structure. The specifity of interaction depends on the amino acid residue in position a (Vinson *et al*., 2002). While in *Arabidopsis* the amino acid residue in position a is always conserved in respect to TLP40, in cTLP40 this amino acid is not conserved particularly in the second leucine zipper domain. In addition, this last domain of cTLP40 is shorter that that of TLP40 and the amino acid sequence is not well conserved. This could indicate that the second leucine zipper domain specifies an interaction which is typical for two different types of organism (e. g. eukaryotic and prokaryotic organisms).

The N-terminal domain of TLP40 contains also three putative phosphatase-binding domains (Fulgosi *et al*., 1998). The presence of such domains in TLP40 support the hypothesis that TLP40 is involved in the regulation of PSII turnover by binding

to the phosphatase which dephosphorylates major subunits of PSII (Fulgosi *et al*.,1998; Vener *et al*., 1999; Rokka *et al*., 2000). Two phosphatase-binding domains are present in *Arabidopsis* TLP40 and only one in *Synechocystis* (Fig. 7). These domains share homology between 30 - 50% with the phosphatase-binding domain of the immunophilin FKBP12 (Aldape *et al*., 1992). A point mutation analysis of the phosphatase-binding domain of FKB12 showed that the binding to the calcineurin phosphatase was highly affected when the arginine was mutated into alanine (Aldape *et al*., 1992). The same approach was performed in cTLP40, but the corresponding R64A mutation did not lead to any phenotypical modification of the resulting strain (Irina Piven, personal communication).Different from higher plants, PSII major subunits are not phosphorylated in *Synechocystis* and so the involvement of cTLP40 in PSII turnover, if any, follows a different way of regulation involving dephosphorylation. On the other side, the hypothesis of TLP40 involvement in PSII turnover by the regulation of a thylakoid protein phosphatase, despite one evidence (Fulgosi *et al*., 1998; Vener *et al*., 1999; Rokka *et al*., 2000) still remains to be tested *in vivo* and then it is possible that TLP40 and cTLP40 have a similar function in thylakoids but they use another mechanism than protein phosphorylation.

4.2 Characterization and functional analysis of TLP40 and cTLP40

4.2.1 Localisation of cTLP40

The isolation of TLP40 was done during work aimed at purification of thylakoid membrane phosphatases (Fulgosi *et al*, 1998, Vener *et al*., 1999). The thylakoid localisation of TLP40 was subsequently confirmed by an import experiment *in vitro* (Fulgosi *et al*., 1998). The N-terminus of TLP40 contains a hydrophobic region and a possible protease recognition site, DLA, at position of 104 amino acids, and thus, can be proposed to be a lumenal-targetting presequence (von Heijne *et al*., 1989; Fulgosi *et al*., 1998). The homologue of TLP40 in *Arabidopsis* was found in the lumen in an experimental proteome analysis aimed to identify lumenal and peripheral thylakoid proteins (Peltier *et al*., 2002). The localisation prediction of *Arabidopsis* TLP40 protein with softwares TargetP (Emanuelsson *et al*., 1999) and

SignalP (Nielsen *et al*., 1997, 1999) identified the possible cleavage site at position ^{82}AHA-VA (Peltier *et al*., 2002). If the cTLP40 sequence is analysed with the same localisation program, the prediction is a cytoplasmatic protein. Western analysis of fractionated *Synechocystis* proteins showed that the protein is located in thylakoid membranes (Fig. 9). Prokaryotic lumenal or periplasmatic signal peptides carry a N-terminal hydrophobic region enriched in leucine and alanine, and a polar region immediately before the cleavage site. At position –3 and –1 in respect to the cutting site are normally found small amino acids, most likely alanine residue (Von Heijne 1983, 1985; Nielsen *et al*.,1997). The presequence of cTLP40 contains all these features. A charged region (amino acids 5 – 14) is followed by a hydrophobic one, enriched in leucine (amino acids 15 – 28), and by a polar one (amino acids. 29 – 48). According to this analysis is possible to predict the cleavage site at position 51 after the sequence ALA. However, a recent proteomic analysis defines the cleavage site after the residue Ala^{55} (Srivastava *et al*., 2005).

4.2.2 Association of TLP40 and cTLP40 with thylakoid membrane

Hydropathy analysis of cTLP40 according to the algorithm proposed by Kyte and Dolittle (1992) suggested a hydrophilic protein with a membrane domain located at the N-terminus of the protein (Fig. 8). Western analysis of thylakoid membrane of *Synechocystis* showed that cTLP40 can be found inside thylakoids associated with the membrane and free in the lumen. Differences between *in silico* analysis and immunological procedures can be easily explained considering the fact that lumenal targeting sequences are often misinterpreted with membrane domains because of the presence of the hydrophobic region (Peltier *et al*. 1997). However, at least 18 amino acid residues are required to span the lipid bilayer of a membrane in an α–helical conformation.

The same localisation as with cTLP40 was found for TLP40 in spinach. In addition, it was shown *in vitro* that the association with thylakoid membranes depended on temperature regimes (Rokka *et al*., 2000). In the present work it was analysed whether temperature could influence the association/dissociation of TLP40 within thylakoid membranes *in vivo*. For this analysis whole plants were adapted to

different stress conditions and thylakoid membranes were extracted immediately after the treatment. TLP40 was found associated to the membrane under all tested conditions (Fig. 38). These results are controversial with data obtained from *in vitro* experiments reported by Rokka *et al*. (2000). A possible explanation is that temperature conditions or method used in *in vitro* experiment were too harsh and caused a release of TLP40 into the lumen.

The association of TLP40 to the thylakoid membrane was analysed by 2D gel-electrophoresis with separation of protein complexes on a blue-native gel or on a sucrose gradient in the first dimension. Both techniques attempt to isolate integral membrane protein complexes. They differ in the way of separation; in blue- native PAGE proteins are separated by their molecular weight and charge, while on sucrose gradients the separation of proteins depends on density and molecular weight. In both approaches, TLP40 was found partially as a free protein and partially comigrating in a complex close to cytochrome b_6f complex. In particular, when the complexes were separated by a blue native PAGE in the first dimension, the signals of TLP40 differed in the molecular weight. The presence of a double signal on Western analysis was already noted in separation including SDS and argued for a possible post-traslational modification of TLP40 (Fulgosi *et al*., 1998). To exclude that TLP40 is bound to the cytochrome b_6f complex the distribution of TLP40 in tobacco mutant plants deficient in cytochrome b_6f complex was analysed. The distribution of TLP40 in this mutant was not significantly affected. This suggests that TLP40 is not associated with the cytochrome b_6f complex. The distribution of cTLP40 in thylakoid membranes of *Synechocystis* resembled that of spinach: with the same approach used for the analysis of association of TLP40 to the membranes, cTLP40 was found to migrate close to the cytochrome b_6f complex and as free protein. The association of TLP40 and cTLP40 in the membrane could then depend on the interaction of TLP40 with a minor integral complex/protein and could be clarified by an immunoprecipitation experiment.

4.2.3 Phenotypical analysis of a *Synechocystis* mutant lacking cTLP40

Mutant *Δsll0408* was generated by insertional mutagenesis in the gene coding for cTLP40 (Canino *et al*., 2005). Growth rate and pigments were analyzed from cells

grown under low light and after adaptation to high light. Photosynthesis is strictly dependent on environmental conditions so it was checked whether various light conditions could raise phenotypic differences between the strains. The major differences were indeed found after adaptation to high light. Strain *Δsll0408* had a longer doubling time (2.3 slower than the wild-type) and differed in the amount of pigments in particular of carotenoids and phycocyanins.

Carotenoids are ubiquitous among microrganisms, animals and plants where they protect cells from oxygen radicals. In this sense they are especially important in oxygen-evolving organisms since many environmental conditions cause the formation of oxygen radicals (Codgell and Frank; 1987; Yamamoto and Bassi, 1996). In addition to photoprotective action, carotenoids are involved in light harvesting (Codgell and Frank; 1987; Yamamoto and Bassi, 1996) and in stabilisation and/or assembly of photosynthetic protein complexes (Bassi *et al*, 1993; Masamoto *et al*., 2004; Wenk *et al*, 2005). *Synechocystis* possesses zeaxanthin, echinenone, beta-carotene and myxoxanthophylls. Between these, zeaxanthin showed to be the most effective in oxygen radical scavenging (Schafer *et al*., 2005). Oxygen radicals are produced at any light intensity, but it is especially at high light that the role of carotenoids is fundamental for PSII protection. The amount of carotenoids increased by about 60% in wild-type cells when they were adapted to high light conditions. In the mutant, the total amount of carotenoids did not change upon adaptation to high light (Fig. 17). It is not known whether the reduced amount of carotenoids in the mutant depends directly on the absence of cTLP40 or is a secondary effect. PBS content (measured in terms of phycocyanin amount) was higher in the mutant *Δsll0408* compared to wild-type at both light conditions (Fig. 17). Fluorescence emission spectra at 77K confirmed this result (Fig. 21) and showed that PBS were mostly attached to PSII at low and high light regimes.

4.2.4 Oxygen evolution and 77K fluorescence emission spectra of wild-type and *Δsll0408* mutant strain

Localisation of cTLP40 inside the thylakoid membrane and the catalytic PPIase activity, suggested that cTLP40 could be involved in some process related to

photosynthesis. To measure the photosynthetic activity of thylakoid membrane oxygen production was checked in wild-type and mutant irradiated with low light (50 μE m^{-2} s^{-1}) or high light (1000 μE m^{-2} s^{-1}). The production of molecular oxygen did not show any significant differences at low light irradiation. The major differences werefound when cells were irradiated with high light. After 40 min of high light treatment total oxygen production in the mutant was zero, indicating that all the molecular oxygen produced was used for cell respiration. The decline of oxygen production in wild-type started slowly after 70 min of high light treatment. The minimum PSII complex which can evolve oxygen comprises the reaction centre proteins of PSII (CP43, CP47, D1 and D2), PsbO and cytochrome b_{559} (Rögner *et al*., 1987). According to this, both PSII monomer and dimer can evolve oxygen (Rögner *et al*., 1987). Decrease of oxygen production at high light conditions in the mutant was a result of a lower amount of PSII monomer and dimer. It is well known that high light causes the photodamage of PSII in particular, of the reaction core protein D1 (Mattoo *et al*., 1984; Ohad *et al*., 1985; Aro *et al*., 1993). The repair of PSII comprises removal of damaged D1 and insertion of a newly synthesized molecule into the complex (Kyle *et al*., 1984; Ohad *et al*, 1984; Andersson and Aro, 2001). The result of oxygen measurements at high light conditions suggested then that the mutant was unable to repair efficiently PSII in high light conditions. To check this idea the same measurement was repeated after addition of the antibiotic lincomycin which inhibits protein synthesis *de novo*. Since removal of damaged D1 from PSII complex and insertion of a new one is a process coordinated strictly, the inhibition of protein synthesis results in the blockage of PSII repair. When lincomycin was added, wild-type oxygen evolution decreased after 40 min of high light treatment as a result of the inability to substitute damaged D1. The addition of lincomycin did not change significantly oxygen evolution in the mutant and this was taken as evidence that the mutant could not repair efficiently PSII under high light conditions. A high damage rate of PSII in the mutant was confirmed also during the analysis of PSII protein distribution into PSII subcomplexes after adaptation of the culture to high light conditions (Fig. 28). In all these experiments accumulation of D1/D2/CP47 complex, which is both, an intermediate of synthesis and repairing (Komenda *et al*., 2004), indicated a higher amount of damage PSII. Possible reasons of that will be discussed in Section 4.3.

Functionality of both photosystems in terms of energy transfer and organisation of the antenna system was analyzed by low temperature fluorescence emission spectra (Figs 20 and 21). The emission spectra of the mutant, despite differences in the relative peak intensity, were similar to the wild-type indicating that the overall organisation of photosystems and antenna is not modified. Wild-type and mutant emission spectra were normalized relative to PSI fluorescence and differed in the relative peak intensity of PSII and of free phycobilisomes in particular after high light treatment and excitation of phycobiliproteins. Western analysis of proteins under these conditions showed that the PSII and PSI protein amounts were similar in wild-type and mutant. At low light the total amount of PSII subunits is reduced to about 30% compared to wild-type. *In vivo* labelling and Western analysis of proteins extracted from cells grown at normal conditions showed that the PSII is mostly assembled in the mutant. Assembled PSII contains phycobiliprotein antenna which transfer the absorbed energy to PSII reaction centre. When cells are irradiated at 440 nm (which excites mostly chlorophyll *a*) a nearly identical fluorescence emission had been observed between wild-type and mutant strain at low light. At high light, the amounts of PSII subunit are similar in both strains. However, pulse-labelling experiments and Western analysis showed that there is a lower amount of assembled PSII in the mutant under this light regime. The excitation of chlorophyll *a* in the cells acclimated to high light resulted in a higher ratio of PSI versus PSII emission in the mutant when compared to the wild-type. Considering that both analysed strains contain equal amounts of PSI one could conclude that either mutant cells contained less amount of PSII proteins or less functionally active PSII molecules. The comparison of the fluorescence data with biochemical analysis let us to conclude that PSII of the mutant strain acclimated to high light emitted less than the wild-type due to lower amounts of assembled PSII dimer.

4.3 Role of cTLP40 in the biogenesis of PSII

Analysis of oxygen evolution in *Δsll0408* showed that PSII functions were impaired when cells were adapted to high light. However, Western analysis showed that under these conditions the amount of the major PSII subunits was similar in wild-

type and mutant. The activity and functionality of PSII depends on the amount of functional complex which is present in thylakoid membranes. It was investigated then whether wild-type and mutant, despite a similar amount of PSII subunits at high light, contained different amounts of functional PSII. The biogenesis of PSII complex can be operationally divided into several steps (Fig. 44):

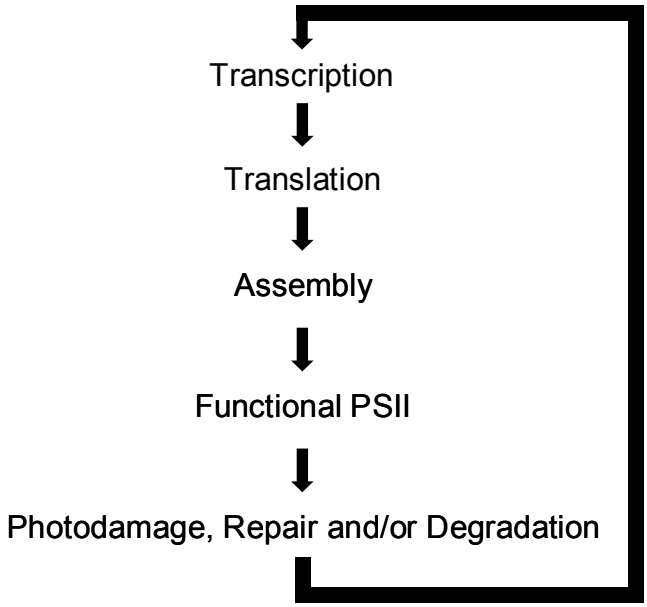


Fig. 44. Scheme of PSII biogenesis and maintenance

Since D1 protein has the higher turnover rate than any other thylakoid proteins in light (Mattoo *et al*., 1984; Ohad *et al*., 1985), a lot of studies on PSII focused on D1 synthesis, assembly and turnover, both in cyanobacteria and in chloroplasts (Herrin and Michaels, 1985; Kim *et al*., 1991; Golden, 1994; Tyystjärvi *et al*., 2001). Following the same idea, the major steps of PSII biogenesis in cTLP40-deficient strain were monitored *via* the D1 protein.

4.3.1 Analysis of transcription, translation and degradation rate in wild-type and *Δsll0408* strain

It was shown that the amount of *psbA* mRNA was relatively constant when chloroplasts were adapted to different external conditions (Danon and Mayfield, 1991; Staub and Maliga, 1993; Hirose and Sogiura, 1996). On the other side, the

amount of *psbA* transcript in *Synechocystis* was highly dependent on environmental conditions (Mohamed and Jansson, 1989; Tyystjärvi *et al*., 1996, 2001) and increased several times when cells were acclimated to high light (Tyystjärvi *et al*., 2001). The reason for this difference in *psbA* mRNA expression between chloroplasts and *Synechocystis* resides in the fact that in chloroplasts D1 protein amounts are regulated at the level of transcription initiation, while in *Synechocystis* the regulation is at the level of translational initiation that means that the increase of D1 protein amount requires an increase of the correspondent mRNA transcript (reviewed in Baena-Gonzalez and Aro, 2001). First, we checked whether the amount of *psbA* transcript in mutant *Δsll0408* could explain the lower amount of PSII subunits. In *Synechocystis*, there are three *psbA* genes (Williams, 1988), but only *psbA*2 and *psbA3*, that encode identical gene products are transcribed (Mohamed and Jansson, 1989; Mohamed *et al*., 1993; Komenda *et al*., 2000). The amount of D1 transcript in wild-type and mutant at standard growth conditions and after adaptation to high light were compared with real time PCR (Fig.23). When the cultures were adapted to high light, the amount of D1 transcript increased both in wild-type and mutant as it was previously reported (Tyystjärvi *et al*., 2001). Relative to the conditions used, *psbA* transcript increased about seven times in wild-type and three times in the *Δsll0408* mutant. However, the amount of *psbA* mRNA was always higher in the mutant under both light conditions. Since the regulation of D1 transcript it is at the level of trascription (reviewed in Baena-Gonzalez and Aro, 2001), a higher amount of transcript suggested a higher requirement of D1 protein in the mutant. This hypothesis was also supported by the analysis of D1 synthesis in the wild-type and mutant strain at standard growth conditions and after short adaptation to high light. Under both light conditions, the synthesis of D1 protein was higher in the mutant than in wild-type according to a higher amount of *psbA* transcript in the mutant (Fig. 24). Pulse-chase experiments showed that newly synthesised D1 protein is inserted into PSII only when it is required to substitute a damaged one into the complex (Adir *et al*., 1990; Tyystjäri *et al*., 2001). The higher amount of D1 transcript as well as a faster synthesis rate in the mutant under the growth conditions analysed supported the idea of a higher damage rate of PSII in the mutant. This conclusion was supported by measurements of oxygen evolution at high light (Section 4.3.4). In addition, analysis of degradation rates in wild-type and mutant showed that D1 is faster

exchanged in the mutant than in the wild-type. When cells were grown at standard conditions, about 30% of D1 in the wild-type and 50% in the mutant was exchanged within three hours (Fig. 25). If it is considered that the amount of all subunits of PSII is strictly coordinated and regulated, a higher degradation rate of D1 protein in the mutant explained the Western results which showed then, despite a general higher synthesis rate, PSII subunits were reduced in the mutant. When cells were adapted to high light, the degradation rate increased in both strains according to a higher damage rate of D1. During high light adaptation the degradation of D1 was again faster in the mutant even if the difference with the degradation rate in the wild-type was not so pronounced as for standard conditions (55% in wild-type and 65% in the mutant after 3 h of high light adaptation). In this case is not possible to compare this result with that of the Western analysis since the adaptation time to high light was different between the two experiments.

4.3.2 Photosystem II assembly in wild-type and mutant *Δsll0408*

The distribution of the major PSII subunits in thylakoid membranes was analysed by blue-native and subsequent SDS-PAGE. By this separation, PSII proteins can be found both, in free or assembled forms (Komenda *et al*., 2004; Herranen *et al*., 2004). In particular, by short pulse labelling of *Synechocystis* proteins followed by blue-native/SDS-PAGE separation of thylakoid membrane proteins, it was possible to identify several PSII intermediate in addition to the dimeric form (Komenda *et al*., 2004). These intermediates are thought to represent steps of PSII assembly or repair and were combined in a model for PSII turnover (Fig. 46) (Komenda *et al*., 2004). According to this model, the first step of PSII biogenesis comprises the association of the cytochrome b_{559} subunits, *psbE* and *psbF*, with D2 protein. Then D1 (as pD1, D1 precursor) associates to the complex and after a first D1 processing, CP47 can associate as well. The formation of the D1/D2/CP47 complex (RC47) and the association of CP43 protein, leads to the PSII monomer and then to the dimer (Komenda *et al*., 2004; Ossenbühl *et al*., 2004). Before addition of CP43 to the D1/D2/CP47 complex, a second processing step of D1 takes place (Zhang *et al*., 2001).

The repair cycle of the PSII complex includes the removal of damaged D1 from the D1/D2/CP47 (RC47) complex, and the insertion of a newly synthesised one. It is important to note that according to this model, D1/D2/CP47 complex is an intermediate of both newly assembled and repaired PSII (Komenda *et al*., 2004).

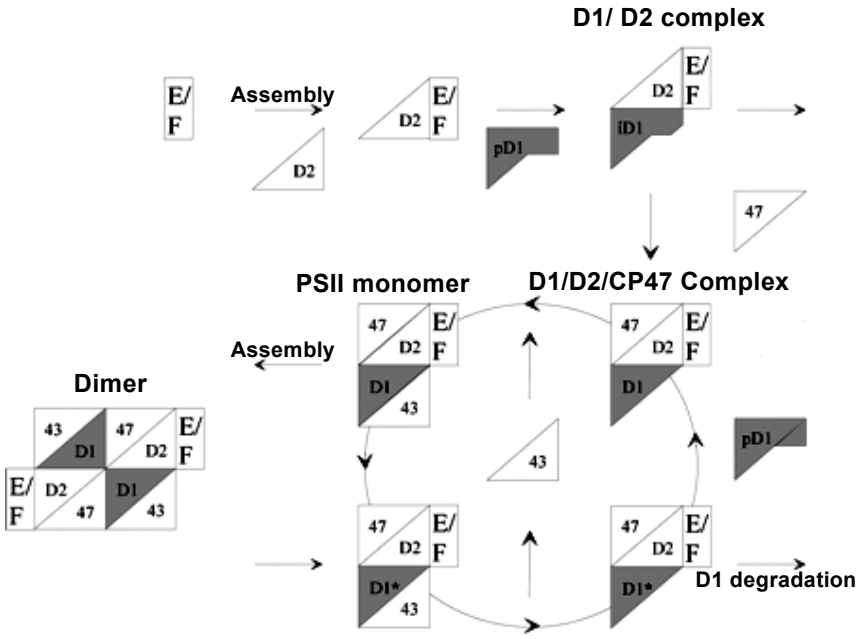


Fig. 45. Model for PSII assembly in *Synechocystis*. The scheme is adapted from Komenda *et al*., (2004). Names of intermediate PSII complexes were modified according to this work. For explanations see the text.

The composition of PSII complexes in the wild-type and mutant strain was first analysed by Western analysis which shows a steady-state amount of PSII proteins in each complex. When cells were cultivated at normal conditions, it was possible to detect proteins in their free or assembled form. The D1/D2 complex was not detected in both strains, indicating that this complex is rapidly converted into the D1/D2/CP47 complex. At high light conditions PSII dimer form was not detectable in the mutant indicating that the amount present was below detection of ECL-based procedure. In addition, the relation between D1/D2/CP47 complex and PSII monomer was higher in the mutant than in the wild-type. Since the D1/D2/CP47 complex represents an assembly and a repair intermediate of PSII, these data suggested a high damage rate of PSII or a slower assembly of the complex in the

mutant. After acclimation to high light, increased amounts of free PSII proteins were found in the mutant, indicating an elevated requirement of PSII proteins high light. This was already concluded from *psbA* mRNA analysis and D1 synthesis rate of in the mutant. Radioactive labelling of *Synechocystis* cells provided new information on the dynamic of PSII assembly. During 25 min labelling under standard growth conditions most of radioactivity was incorporated in two PSII intermediates, D1/D2/CP47 and PSII monomer, in both strains. After high light adaptation, the highest amount of radioactivity was incorporated in PSII monomer in the wild-type, while in the mutant the D1/D2/CP47 complex and PSII monomer were equally synthesised. This finding suggested that while the two strains can assemble PSII until the monomer form with a comparable rate when grown under standard conditions, there is retardation in the assembly between D1/D2/CP47 intermediate and PSII monomer in the mutant after adaptation to high light. These results were confirmed by chase-labelling analysis where, after removal of radioactive methionine, the amount of D1/D2/CP47 complex relative to PSII monomer was higher in the mutant than in the wild-type. A lower amount of PSII dimer in the mutant at high light can depend on a retardation of PSII assembly although instability of PSII dimer (which would increase the amount of D1/D2/CP47 complex because of faster photoinhibition) could not be excluded.

4.4 Different mechanisms of regulation of PSII biogenesis in higher plants and cyanobacteria

TLP40 was first described from spinach where it was purified because of its association to a thylakoid protein phosphatase (Fugolsi *et al*., 1999). Subsequent evidence suggested a role for TLP40 as a negative regulator of such phosphatase (Fulgosi *et al*., 1998; Vener *et al*., 2000; Rokka *et al*., 2000). Phosphorylation is a common signal mechanism in both, prokaryotic and eukaryotic organisms (see Introduction, Section 1.5). In chloroplasts there are several examples of protein phosphorylation and it has been claimed that phosphorylation/dephosphorylation of proteins provide signals for stabilisation, degradation and turnover for PSII reaction proteins (Andersson and Aro, 1997; Barber *et al*., 1997; Kruse *et al*, 1997; Baena-Gonzales *et al*., 1999, Rokka *et al*. 2000). In *Synechocystis*, proteins of

PSII are not phosphorylated. If the function of TLP40 as a regulator of PSII protein dephosphorylation is accepted, a difference in mechanism for cTLP40 must be the consequence.

4.4.1 Rotamase (PPIAse) activity of cTLP40

Functional analysis of *⊿sll0408* showed that the absence of cTLP40 decreased the performance of the strain in particular after adaptation to high light. Under these conditions the mutant possesses a longer duplication time, a different set of pigments and a lower oxygen evolution rate. Studies on the assembly and stability of PSII by immunological and *in vivo* labelling experiments showed that mutant had a lower amount of PSII dimer. In addition, the mutant had a higher synthesis rate of PSII proteins (evaluated through the D1 content) but higher degradation rate which resulted in a similar steady state level of PSII proteins. We tried to analyze a possible reason for the lower amount of PSII dimer focusing on two possible mechanisms: inefficient repair of PSII or instability of the complex.

The repair of PSII complex (the so-called repair cycle of PSII) comprises the removal of damaged protein (D1) from PSII and the insertion of a newly synthesised one into the complex. Damaged D1 is degraded after its removal. Damage of D1 occurs after photoinhibition of PSII which is caused by high light, extreme temperature regimes or nutrient deficiency. It is well known that the photoinhibition of PSII is caused by formation of reactive oxygen species which are generated in PSII during oxygen evolution (Vass *et al*., 1992; Okada *et al*., 1996; Kerne *et al*., 1997). However, recent studies challenged this idea and showed that oxidative stress inhibited the repair of PSII by blocking the translation of D1 protein but do not inactivate PSII directly (Nishiyama *et al*., 2004). In addition it has been suggested that the photoinhibition of PSII occurs only after the complete inactivation of the oxygen-evolving complex (Ohnishi *et al*., 2005).

Considering the enzymatic activity of cTLP40, we suggested that cTLP40 could be involved in the isomerisation of proline residues of PSII subunits. The isomerisation of the proline bond can significantly change the structure of a protein exposing or hiding possible protein-protein interaction site. The absence of

cTLP40 could cause the presence of structurally modified proteins interfering with the normal PSII cycle. The effect of absence of cTLP40 is stronger at high light conditions, where, due to higher photoinhibition rate, PSII should be faster repaired and assembled. Since cTLP40 is located in the thylakoid lumen, our candidate/s should have one or more proline residues facing the lumenal side of thylakoids. In addition, we considered that during PSII assembly studies most of D1 was distributed between D1/D2/CP47 complex and PSII monomer. This raised the idea that the incorporation of CP43 into D1/D2/CP47 complex could be impaired in mutant *Δsll0408*. CP43 is a six-helix transmembrane protein with three connecting loops located on the lumenal side (see also Section 1.3.1.4). In particular, the last connecting loop was intensively studied because of its large lumenal extension (reviewed in Bricker and Frankel, 2002; Anderson *et al*., 2002). One of these studies showed that CP43 in *Synechocystis* can have a posttraslational modification (different level of oxidation) on the tryptophan 352 (Anderson *et al*., 2002). This tryptophan residue is located in the E-loop in the region A^{350}PWELPLRGPN^{360} which was shown to be highly conserved in different organisms. Considering that mutation analysis in this region impaired severely the assembly and functionality of PSII (Kuhn and Vermaas, 1993; Anderson *et al*., 2002), the authors suggested a possible role of tryptophan modification as a signal in PSII turnover. Computer modelling analysis of this region, suggested an important role of proline 351 and 355 for the orientation of that tryptophan residue (Anderson *et al*., 2002). Since PPIase activity can be tested in an *in vitro* assay (Fischer *et al*., 1989; Kofron *et al*., 1991), we chose two peptides containing proline 351 and proline 355 which could be important for tryptophan orientation (Fig. 35). Full-length cTLP40 containing a His-tag was overexpressed in *E. coli* and purified by metal affinity chromatography. One possible contaminant in this procedure could be the bacterial protein SlyD which is a rotamase (Mukherjee *et al*., 2003). We used a modifed version of Ni^{2+}-metal affinity chromatography which excludes copurification of SlyD (Mcmurry and Macnab, 2004). However, our experiment showed a possible residual rotamase activity in the bacterial lysate which could depend from another minor rotamase activity present in *E. coli* (for example CypA and CypB, Hayano *et al*., 1991). This rotamase can efficiently isomerise the two CP43-derivedpeptides. Thus, the result then of our experiment

was not conclusive and should be repeated employing a new purification procedure for cTLP40 to remove all contaminating rotamase activities.

To test whether the absence of cTLP40 can interfere with the dimerisation of PSII, we analyzed small subunits of PSII which are involved in the dimerisation and stabilisation of the complex (reviewed in Shi and Schröder, 2004). The best candidate was the PsbI protein which has one transmembrane domain and contains a lumenal extension with a single proline residue. In addition, PSII crystallographic analysis localised PsbI at the interface of the dimerisation site, suggesting a role in the stabilisation of PSII dimer (Ferreira *et al*., 2003). The *Synechocystis* strain deficient in PsbI was previously described (Ikeuchi *et al*., 1995). The mutant could still grow autotrophically and was more sensible to light than the wild-type. In addition, analysis of the mutant revealed that the absence of PsbI protein resulted in a loss of PSII activity of 25 - 30%. Since this phenotype was not very strong and resembled the phenotype of *Δsll0408*, it was checked whether PsbI could be a substrate for cTLP40. However, the data showed that the PsbI-peptide-containing proline could not be isomerased from cTLP40.

4.4.2 Possible involvement of cTLP40 in gene expression

The fact that the mutant has less carotenoids and a higher PBS content at high light (Fig.17) could be a reason of the reduced amount of PSII: carotenoids scavenge oxygen radicals which are abundant under high light and damage PSII. A reduced amount of PBSs at high light limits the absorption of light and decreases photodamage of PSII. The expression of genes encoding PBS components showed that they are under control of the histidine kinase DspA homologue of NblS in *Synechocystis* (Tu *et al*., 2004; Hsiao *et al*., 2004). It was shown that cyanobacterial cells lacking DspA could not degrade PBS during nitrogen starvation (He *et al*., 2001). In addition, absence of DspA blocked cells in the high-light-phenotype even when they were grown at low light (Tu *et al*., 2004). Transcription of genes coding for PSI proteins was decreased while transcription of the genes of PSII subunits was elevated in the mutant at low light (Tu *et al*., 2004). Mutant *Δsll0408* resembled partially this phenotype. Analysis of *psbA* transcript level showed that the amount of D1 mRNA was higher in the mutant

than in the wild-type even under standard light condition. A possible model depicting the role of DspA in controlling the response of cells to environmental conditions was proposed for the homolog in *Synechococcus* (NblS Fig. 46).

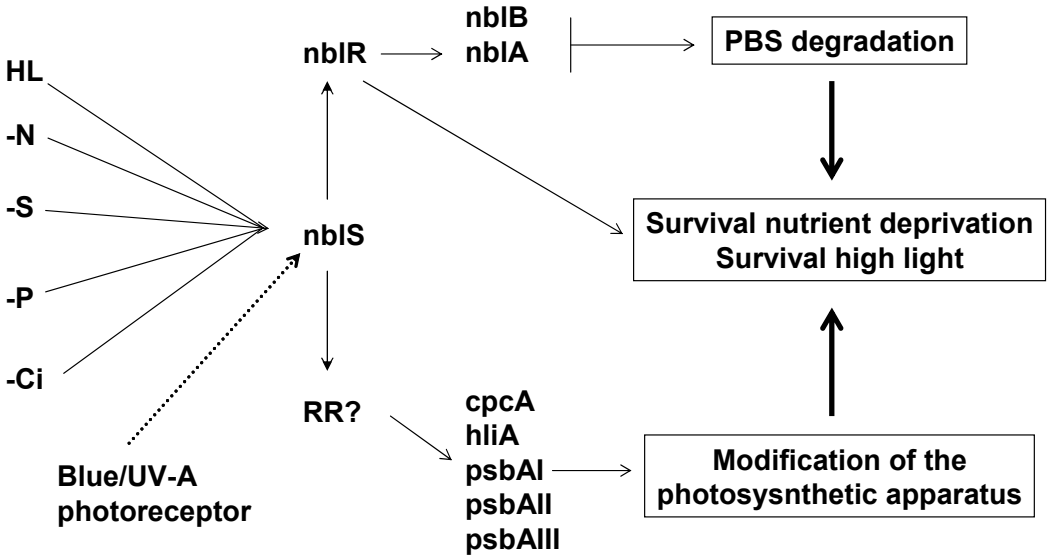


Fig. 46. Possible role of NblS. HL, -N, -S, -P and -Ci stand for high light and absence of nitrogen, sulphur, phosphorous and inorganic carbon. RR stands for response regulator. For explanation, see text (adapted from Grossmann *et al*., 2001)

According to this model, *nblS* senses different external conditions of light and nutrient availability. It controls PBS degradation through *nblA*, *nblB*, *psbA*, *hliA* (high light induced protein) and *cpcA* (phycocyanin α-subunits) expression through an unknown response regulator (Grossman *et al*., 2001). The analysis of the NblS sequence suggests that it is a histidine kinase spanning twice the membrane. Since cTLP40 was found to be associated with the thylakoid membrane (see Fig. 11 and 12), this suggests that it could interact with DspA modulating the expression of *cpcA* and *psbA*. The association of cTLP40 to the membrane was checked in the *Δhik33* mutant (kindly provided by N. Murata and described in Suzuki *et al*., 2000) but no difference was found in comparison to the wild-type (data not shown). However, it was not possible to exclude completely the interaction of cTLP40 with DspA since *Δhik33*, despite showing a phenotype, was not completely segregated. The same experiment should be repeated with the null DspA mutant (described in Tu *et al*., 2004 and Hsiao *et al*., 2004).

5. Summary

TLP40 is the first complex immunophilin which was described from plant chloroplasts (Fulgosi *et al*., 1998). For this protein a role in regulation of PSII protein phosphorylation has been suggested (Fulgosi *et al*., 1998; Vener *et al*., 1999; Rokka *et al*., 2000). Homologous proteins were found in *Arabidopsis thaliana* and in *Synechocystis*. In *Synechocystis,* different from higher plants, the relevant PSII proteins are not phosphorylated. The main topic of this work was therefore to characterise the homologous protein of TLP40 in *Synechocystis* (named cTLP40, cyanobacterial TLP40). The investigation shows:

- cTLP40 contains the major structural domains of TLP40 both at the N- and at the C-termini. A higher homology is found in the immunophilin domain located at the C-terminal end.
- As in chloroplasts, cTLP40 is also located in the thylakoid lumen where it is present in free form or associated to the membrane.
- A *Synechocystis* strain lacking cTLP40 (*Δsll0408*) could grow photoautotrophycally under normal grown conditions in a way comparable to wild-type. However, after adaptation to strong light the mutant strain showed higher photosensibility. Under these conditions, there was a decrease in oxygen evolution.
- The total amount of PSII dimer was reduced in the mutant under high light. The lower amount of PSII can be attributed to a slower assembly rate of the complex and/or to higher degradation rate. Protein synthesis was not impaired under any of the tested conditions.
- The PPIAse activity of cTLP40 was tested *in vitro* on synthetic proline-containing peptides of PSII proteins which are exposed to the lumenal face in thylakoids. The *in vitro* assays showed that cTLP40 possesses PPIAse activity on control peptides but can not efficiently isomerise the specific synthetic peptides.

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Curriculum Vitae

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Career

Since July 2005 Employment as a research associate at the Department for Molecular Botany at the University of Ulm (Germany) in the laboratory of Prof. Dr A. Brennicke.

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Studies

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Diploma thesis „Siti di legame dei carotenoidi nelle proteine CP26 e CP29 delle piante superiori“ (Carotenoid binding sites of the proteins CP26 and CP29 from higher plants) supervised by Prof. Dr. R. Bassi, Department for Biochemistry of the University of Verona.

September 1989-

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Publications

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Eidesstattliche Erklärung:

Hiermit versichere ich, daß ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel und Quellen angefertigt habe.

23.01.2006

Giusy Canino