Characterisation of mechanisms and components of protein phosphorylation in photosynthetic membranes of *Synechocystis* sp. PCC 6803

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Abbreviations

A	absorbance
APC	allophycocyanin
APS	ammonium persulfate
bp	base pair
BSA	bovine serum albumin
CS	cold stress
D	dark
DNA	deoxyribonucleic acid
DM	dim light
DMSO	dimethyl sulfoxide
DCMU	3-(3,4-dichlorphenyl)-1,1-dimethylurea
DTT	dithiotreitol
DS	double stress
EDTA	ethylenediaminetetraacetic acid
FNR	ferredoxin $NADP^+$ oxidoreductase
Gm	gentamycin
h	hour(s)
HEPES	N-(2-hydroxyethyl)piperazine N'-2(2-ethanesulfonic acid)
HL	high light
HS	heat stress
IgG	immunoglobulin G
IPTG	isopropyl β-D-thiogalactoside
Km	kanamycin
kBp	kilobases pair
kDa	kilodalton
LB	Luria-Bertani medium
LL	low light
LHCII	chlorophyll-binding PSII light-harvesting complex
min	minute
MOPS	3-(N-Morpholino)propanesulfonic acid
nm	nanometer
ORF	open reading frame

PAA	polyacrylamide
PAGE	polyacrylamide gel electrophoresis
PAM	pulse amplitude-modulated fluorometer
PBS	phosphate-buffered saline
PBS	phycobilisome
PC	phycocyanin
PCR	polymerase chain reaction
PE	phycoerithrin
PK	protein kinases
PMSF	phenylmethylsulfonyl fluoride
PP	protein phosphatases
PPIases	peptidyl-prolyl *cis/trans* isomerases
PSI	photosystem I
PSII	photosystem II
PVDF	polyvinylidene difluoride
qP	photochemical chlorophyll *a* fluorescence quenching
rpm	rotations per minute
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SSC	standard saline citrate
TBE	Tris-borate-EDTA buffer
TCA	trichloroacetic acid
TE	Tris-EDTA
TEMED	N, N, N', N'-tetramethylethylene diamine
Tris	Tris-(hydroxymethyl)-aminomethane
Tween	polyoxyethylenesorbitane monolaurate
v/v	volume per volume
w/v	weight per volume

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

Bases

A:	Adenine
C:	Cytosine
G:	Guanine
T:	Thymine
U:	Uracil

1. INTRODUCTION

1.1 Phosphorylation in higher plants

Reversible protein phosphorylation is an important regulatory mechanism controlling cellular events in prokaryotic and eukaryotic cells. Various thylakoid proteins of higher plants undergo reversible light-dependent phosphorylation (Bennett, 1977, 1980; Silverstein *et al*., 1993). Phosphoproteins in chloroplast membranes were first found by Bennett (Bennett, 1977, 1980). Later, reversible phosphorylation of proteins has been shown to occur also in the soluble stroma and envelope membranes (Bhalla and Bennett, 1987; Soll and Bennett, 1988; Tiller and Link, 1993; Danon and Mayfield, 1994). Phosphorylation of thylakoid proteins is regulated by light *via* the redox state of electron transfer components in thylakoid membranes (Bennett, 1977; Allen *et al*., 1981). Dephosphorylation of thylakoid phosphoproteins occurs in darkness (Bennett, 1991), but both, light-dependent and light-stimulated dephosphorylation of thylakoid proteins, have also been reported (Elich *et al*., 1993; Ebbert and Godde, 1994; Rintamäki *et al*., 1996, 1997; Pursiheimo *et al*., 1998). So far, only membrane-bound kinases have been reported to phosphorylate thylakoid proteins, while both soluble and membrane-bound protein phosphatases are active in the dephosphorylation processes (Bhalla and Bennett, 1987; Sol *et al*., 1988; Sun *et al*., 1989; Hammer *et al*., 1995; Hast and Follmann, 1996; Snyders and Kohorn, 1999; Vener *et al*., 1999; Fulgosi and Soll, 2001).

The major proteins that can be phosphorylated in thylakoid membranes of higher plants belong mainly to photosystem II (PSII) and antenna complexes (LHCII). Four proteins of the multisubunit PSII complex of higher plants, the reaction centre proteins D1, D2, 43 kDa chlorophyll *a*-binding (CP43) and PsbH proteins undergo reversible phosphorylation (Ikeuchi *et al*., 1987; Michel *et al*., 1988). Exposure of phosphoprotein phosphorylation site(-s) to protein kinases is regulated by light-induced conformational changes. Reversible phosphorylation of core subunits of PSII, including two reaction centre proteins D1 and D2, controls stability, degradation and turnover of this complex (Ohad *et al*., 1984; Elich *et al*., 1993; Koivuniemi *et al*., 1995; Andersson and Aro, 1997; Kruse *et al*., 1997). Three proteins of the light-harvesting antenna, Lhcb1 and Lhcb2 (designated as LHCII), as well as Lhcb4 also undergo light-dependent phosphorylation (Bennett, 1977, 1991). Lhcb1 and Lhcb2 proteins form, together with Lhcb3, the major trimeric light-harvesting complexes (LHCII) (Jansson, 1994; Simpson and Knoetzel, 1996; Boekema, 1999). The inner trimeric complexes are tightly

associated with PSII, while the outer trimeric complexes can be reversibly detached from the PSII antenna upon phosphorylation of the Lhcb1 and Lhcb2 proteins (Larsson *et al*., 1987). Reversible phosphorylation of LHCII is involved in the redistribution of light excitation energy between PSII and PSI in a process designated as state transitions (Bonaventura and Myers, 1969; Murata, 1969). Lhcb4 (also named CP29), which belongs to the minor chlorophyll *a/b* antenna proteins, was shown to be phosphorylated in a light-dependent manner in thylakoid membranes of maize leaves (Bergantino, 1995). The protein kinase which can phosphorylate LHCII is located in thylakoid membranes and its activation is controlled by redox state of the plastoquinone pool and the cytochrome b_6f complex, and is modulated by the thiol reduction state (Allen, 1992; Vener *et al*., 1995; Gal *et al*., 1997; Vener *et al*., 1997, 1998). The LHCII is maximally phosphorylated at low light and almost completely dephosphorylated in darkness and at high light irradiances. The inactivation of the LHCII kinase at high light intensities is a general feature for chlorophyll *b*-containing photosynthetic organisms (Schuster *et al*., 1986; Rintamäki *et al*., 1997).

New phosphoproteins are still detected in thylakoid membranes. A most recently identified thylakoid phosphoprotein is a 15.2 kDa polypeptide of the cytochrome b_6f complex, also designated subunit V (Hamel *et al*., 2000). Another phosphopeptide, which was identified in thylakoid membranes, is a soluble phosphoprotein of 9 kDa (TSP9) (Carlberg *et al*., 2003). TSP9 is a membrane-associated plant-specific protein which is partially released from the thylakoids into the chloroplast stroma in its phosphorylated form during light-induced phosphorylation (Carlberg *et al*., 2003). TSP9 possesses three phosphorylation sites. It was proposed that the protein could be a plant-specific transcription or translation regulatory factor that is released from the thylakoid membranes in response to reduction of the plastoquinone pool (Carlberg *et al*., 2003).

The phosphorylation of PSI proteins was less discussed than that for the PSII. First evidence for protein phosphorylation in PSI was reported by Hauska *et al*. (1970) but no phosphoproteins could be identified. The first identified phosphoprotein of PSI was PsaD that assists in the electron transfer from PSI to ferredoxin, and the second one was TMP14 (thylakoid membrane phosphoprotein of 14 kDa), which belongs to a nuclear-encoded protein with a chloroplast-targeting transit peptide. Both phosphoproteins are novel subunits of plant PSI and were designated as PSI-D and PSI-P, respectively (Hansson and Vener, 2003; Khrouchtchova *et al*., 2005). It was demonstrated that about 25% of the PsaD protein could be

phosphorylated. However, observations under conditions activating the LHCII kinase (with light intensity for state 2) showed no change in the phosphorylation level for both, PsaD and PsaP proteins (Khrouchtchova *et al*., 2005). It was hypothesized that kinases specific for PSI are activated at different conditions. It was also suggested that the phosphorylation of PSI subunits is involved in post-translational modifications rather than the light-regulated processes (Khrouchtchova *et al*., 2005).

Recent studies suggested the existence of five protein kinases associated with photosynthetic thylakoid membranes and required for phosphorylation of proteins of chloroplast thylakoid membranes: the family of three kinases, thylakoid-associated kinases (TAKs), and two kinases STN7 and STN8, that are responsible for phosphorylation of LHCII and PSII core proteins. TAKs were able to activate phosphorylation of LHCII *in vitro* (Snyders and Kohorn, 1999). It was found that TAKs interact with cytochrome b_6f and LHCII complexes *in vivo* and their association with the cytochrome b_6f complex could activate the phosphorylation of LHCII (Snyders and Kohorn, 2001). It was also suggested that TAKs could be involved in other phosphorylation processes than state transitions (Snyders and Kohorn, 2001).

The other two kinases STN7 and STN8 have significant sequence identity to chloroplast Ser/Thr protein kinase Stt7 from the green alga *Chlamydomonas reinhardtii* (Depege *et al*., 2003). One of these kinases, STN7, is required for phosphorylation of light-harvesting complex II and is involved in the regulation of the state transition process (Bellafiore *et al*., 2005; Bonardi *et al*., 2005). Although it is evident that STN7 phosphorylates LHCII, it is still not known whether this kinase phosphorylates LHCII directly or whether other kinases could be involved in this process (Bellafiore *et al*., 2005). It was also proposed that STN7 may regulate the expression of nuclear and plastid genes through phosphorylation of LHCII (Bonardi *et al*., 2005). The other one, STN8, is required for phosphorylation of PSII proteins (Bonardi *et al*., 2005; Vainonen *et al*., 2005). STN8 kinase does not phosphorylate LHCII proteins and is not required for state transitions. Rather, it was found that phosphorylation of PSII core proteins almost exclusively depends on STN8 (Bonardi *et al*., 2005; Vainonen *et al*., 2005). Vainonen and co-workers showed that STN8 specifically phosphorylates D1, D2, and CP43 proteins at their N-terminal threonines, and PsbH at Thr-4. The Thr-2 residue of PsbH should be controlled by another kinase (Vainonen *et al*., 2005). Moreover, it was indicated that STN8-mediated phosphorylation of D1 is not crucial for D1 turnover and PSII repair (Bonardi *et al*., 2005).

All PSII and Lhc*b* phosphoproteins in higher plants identified so far are phosphorylated at Thr residues at the N-termini exposed to the stromal surface of the thylakoid membrane (Mullet, 1983; Michel and Bennett, 1987; Michel *et al*., 1988; Testi, 1996). Phosphorylation of serine (Vescovi and Lucero, 1990) and tyrosine residues (Tullberg *et al*., 1998) of thylakoid membrane proteins has also been reported. However, these phosphoproteins are only poorly characterized and the kinases involved are unknown.

1.2 Protein phosphorylation in various photosynthetic organisms

Reversible phosphorylation of PSII proteins in lower photosynthetic organisms is less characterized. Organisms performing oxygenic photosynthesis can be divided into three groups with respect to protein phosphorylation of thylakoid proteins (Pursiheimo *et al*., 1998). In the first group, which is composed by phycobilisome-containing cyanobacteria and red algae, proteins of PSII and light-harvesting complex are not phosphorylated at all. Although phosphorylation of both, thylakoid and cytoplasma proteins, has previously been reported from cyanobacteria (Sanders and Allen, 1987; Harrison *et al*., 1991; Allen, 1992; Pursiheimo *et al*., 1998), none of these phosphorylated proteins was recognized with specific antibodies against D1, D2 or CP43 proteins. Lower plants (green algae, mosses and liverworts) represent the second group in respect to phosphorylation of photosynthetic proteins. Notably, all species in group 2 exhibit reversible phosphorylation of Lhcb1 and Lhcb2 proteins, as well as of D2 and CP43. The phosphorylation of the D1 protein has never been detected in these species (Wollman and Delepelaire, 1984; de Vitry *et al*., 1991; Andronis *et al*., 1998). Reversible phosphorylation of all three PSII core proteins, D1, D2 and CP43, occurs only in the higher plants, which comprise the third group of photosynthetic organisms. Reversible D1 protein phosphorylation of higher plants has been reported to play an important role in the regulation of D1 protein turnover, and hence, the repair of PSII centres under photoinhibitory conditions (Aro *et al*., 1992; Rintamäki *et al*., 1996).

There are three major physiological functions of thylakoid protein phosphorylation/dephosphorylation in photosynthetic membranes of higher plants. The first one, reversible phosphorylation of LHCII, has been recognized as the molecular basis for state transitions, an important mechanism of *short-term* adaptation. State transitions reflect a regulatory mechanism that controls the distribution of excitation energy from light-harvesting

antenna to photosystems I and II (Allen *et al*., 1981; Allen, 1992; Allen and Forsberg, 2001; Wollman, 2001).

The second process, which involves reversible phosphorylation/dephosphorylation of PSII proteins, affects the stability, degradation and turnover of damaged PSII proteins. Reversible phosphorylation of PSII proteins (particulary of D1) appears to be a crucial regulatory process for PSII functionality (Aro *et al*., 1992, 1993; Rintamäki *et al*., 1996; Andersson and Aro, 1997; Kruse *et al*., 1997).

As the third essential role, protein phosphorylation was inferred to be involved in the regulation of the gene expression. This process involves both, nucleus- and chloroplast-encoded photosynthetic proteins (Allen *et al*., 1995; Allen and Pfannschmidt, 2000; Pursiheimo *et al*., 2001; Bellafiore *et al*., 2005). The redox state of plastoquinone, a component of the photosynthetic electron transport chain that connects PSI and PSII control the rate of transcription of photosynthetic genes. Redox control of chloroplast transcription initiates *long-term* adjustments that compensate the imbalance in energy distribution and adapt the whole plant to altered light environments (Pfannschmidt *et al*., 1999; Allen and Pfannschmidt, 2000). Changes in the redox state of the chloroplast controls a signalling cascade that modulates the expression of both chloroplast and nuclear genes (Pursiheimo *et al*., 2001). The redox signal should be transmitted from the chloroplast to the nucleus and the pathway involves protein kinases and phosphatases (Allen *et al*., 1995). The redox-activated kinase(-s) triggers a cascade leading to regulation of gene expression (Pfannschmidt *et al*., 1999). Previous evidence for control of expression of photosynthetic genes at the level of plastoquinone has been obtained for the nuclear-encoded LHCII of green algae (Escoubas *et al*., 1995). Escoubas *et al*. (1995) suggested that increased reduction of the plastoquinone pool in chloroplasts is coupled with repression of *lhcb* gene transcription in the nucleus, leading to a decrease in the light-harvesting antenna of PSII. Most direct evidence has been obtained from chloroplast gene transcription (Pfannschmidt *et al*., 1999). It was observed that *psaAB* transcription (reaction-centre apoproteins of PSI) is induced when plastoquinone is reduced (PQH_2), and repressed when it is oxidized (PQ). Expression of *psbA* that encodes the D1 reaction centre protein of PSII, is induced when plastoquinone is oxidized and repressed when it is reduced (Pfannschmidt *et al*., 1999; Allen and Pfannschmidt, 2000). As a result the stoichiometry of PSI to PSII can be modified. This mechanism is able to sense the changes in the redox state of the chloroplast and initiates a signalling cascade that modulates the expression of both, the

chloroplast and nuclear genes. It has been hypothesized that LHCII kinase, rather than the plastoquinone pool (Escoubas *et al*., 1995), acts as a primary redox sensor initiating the signalling cascade from chloroplast to the nucleus (Pursiheimo *et al*., 2001). Activity of the LHCII kinase, that is strictly controlled by environmental cues, would regulate the amount and/or the activity of such regulatory proteins and thereby the transcription of *lhcb* genes, possibly *via* a cascade of phosphorylation/dephosphorylation reactions (Pursiheimo *et al*., 2001). It was observed that phosphorylation of LHCII is crucial for the control of photosynthetic gene expression (Bonardi *et al*., 2005). The LHCII kinase, STN7, plays an important role in coordinating *long-term* and *short-term* responses to changes in light conditions and was suggested to be involved in the regulation of nuclear and plastid gene expression as well (Bonardi *et al*., 2005).

1.3 Energy distribution between photosystems I and II through state transitions mechanisms

The phenomenon of light-induced fluorescence changes that later was termed „state transitions" was discovered independently by Murata (1969) in the phycobilisome-containing red alga *Porphyridium cruentum*, and by Bonaventura and Myers (1969) in the green alga *Chlorella pyrenoidosa*. State 1/state 2 transitions reflect a rapid physiological adaptation mechanism that regulates the way in which absorbed light energy is distributed between PSI and PSII. State transitions occur in both, green plants and cyanobacteria, although the light-harvesting complexes involved are very different.

1.3.1 State transitions in chloroplasts of higher plants

The state transitions in the chloroplasts of higher plants regulate the energy from the light-harvesting antenna to the photosystems I and II. This *short-term* regulation is based on the reversible phosphorylation of LHCII by a thylakoid-bound kinase (Allen *et al*., 1981; Pursiheimo *et al*., 1998; Carlberg, 1999; Bellafiore *et al*., 2005). An important feature of the photosynthetic membranes in higher plants is the lateral heterogeneity of the two photosystems: PSII complexes are localised mostly in grana stacks, while PSI complexes were found in non-stacked stromal thylakoids. When the plastoquinone pool is oxidized, LHCII is associated with PSII and this is known as state 1. When a change in light intensity or quality leads to reduction of the plastoquinone pool, the LHCII kinase is activated, and LHCII becomes phosphorylated

and the mobile pool of LHCII (Lhcb1 and Lhcb2) moves to PSI (state 2) (Vener *et al*., 1997; Allen and Forsberg, 2001; Haldrup *et al*., 2001; Wollman, 2001; Khrouchtchova *et al*., 2005). The plastoquinone becomes oxidized and the LHCII kinase is inactivated. The LHCII phosphatase which is continuously active (Silverstein *et al*., 1993), catalyses the dephosphorylation of LHCII, thereby returning excitation energy to PSII. The correlation between plastoquinone redox level, state transitions and phosphorylation of LHCII has been demonstrated in numerous studies. Nevertheless, a demonstration that a reduced plastoquinine pool or highly LHCII phosphorylation causes or is required for state transitions has not been presented (Haldrup *et al*., 2001; Scheller *et al*., 2001) and the exact function of state transitions is still not clear. Although the phosphorylation of LHCII could be involved in regulation of state transitions, it could also be a stabilizing factor that prevents the degradation of LHCII that later becomes exposed in the stroma lamellae (Haldrup *et al*., 2001). It was proposed that state transitions more likely could be involved in optimizing the photosynthetic yield and thus, growth under low light conditions. Bellafiore *et al*. (2005) proposed that state transitions are important for adaptation of plants to natural environments where light quality and quantity change frequently.

1.3.2 State transitions in cyanobacterial cells

Cyanobacteria, similar to higher plants, also use state 1/state 2 transitions as the major mechanism for energy transfer. In cyanobacteria this process was discovered by Fork and Satoh (1983). The regulatory mechanisms involved are not as well characterized as those in higher plants. Different from higher plants, in cyanobacteria the main light-harvesting antennas are extrinsically associated with thylakoid membranes and represent supramolecular structures known as phycobilisomes (PBSs). PBSs are primarily composed of bilin-containing phycobiliproteins (PBP). The PBSs have been known to act preferentially as a light-harvesting antenna for PSII, although some of the energy trapped by the PBS is also transferred to PSI (Glazer, 1989). The PBS-covered thylakoids of cyanobacteria are not stacked, and thus some difference from higher plants may occur in the mechanism for state transitions in this organism. The mechanisms of state transitions in PBS- and chlorophyll *ab*-containing organisms are presently controversery discussed and several models have been proposed. The PBS antenna are able to uncouple from and couple to PSII and PSI, respectively, during such transition (the „mobile antenna model”), or state transitions may occur due to a spill-over of excitation energy from PSII to PSI (the „spill-over model”). Accordingly to the third model (the „detachment

model"), some PBSs become detached from PSII without association with PSI during illumination with PBS-absorbed light (shift to state 2). However, a contribution of PBSs can also be recognised in the action spectrum of PSI, and state transitions phenomena can affect the coupling between the PBSs and PSI, as well as with PSII. A mutant of *Synechocystis* sp. PCC 6803 with no detectable PSII chlorophyll-binding proteins showed quite efficient energy transfer from PBSs to PSI (Mullineaux, 1994). It was found that in cyanobacteria PSII is immobile, but PBSs diffuse rapidly on the membrane surface. The association of PBSs with reaction centres is highly dynamic; there are no stable phycobilisome-reaction centre complexes *in vivo* (Sarcina *et al*., 2001; Joshua and Mullineaux, 2004). It was also suggested that PBS mobility is necessary for state transitions in cyanobacteria (Joshua and Mullineaux, 2004; Mullineaux and Emlyn-Jones, 2005). The physiological role of state transitions is still a matter of debate. It was found that state transitions are physiologically important only at very low light intensities; they play no role in protection from photoinhibition. Thus, state transitions are a way to maximize the efficiency of light-harvesting at low light intensities (Mullineaux and Emlyn-Jones, 2005). Recently, the gene required for the state transitions has been identified in the cyanobacterium *Synechocystis* sp. PCC 6803. This gene (*sll1926*) encodes a putative integral membrane protein of 16 kDa, which was designated as RpaC (regulator of PBS association C) (Emlyn-Jones *et al*., 1999; Mullineaux and Emlyn-Jones, 2005).

1.4 Phosphorylation in cyanobacterial cells

Contrary to higher plants, no phosphorylation of major antenna proteins has been found in cyanobacteria (Pursiheimo *et al*., 1998) (Table 1). However, several observations on phosphorylation of phycobilisome-associated proteins has been reported (Allen *et al*., 1985; Harrison, 1990; Emlyn-Jones *et al*., 1999). For example, it has been found that the phosphorylation state of a 15 kDa thylakoid protein and a 18.5 kDa soluble protein in *Synechococcus* sp. PCC 6301 is light-dependent (Allen *et al*., 1985; Harrison *et al*., 1991). The 18.5 kDa protein copurified with PBSs (Sanders and Allen, 1987) and was later identified as a ß subunit of phycocyanin (ß-PC) (Harrison, 1990; Mann and Newman, 1997). It was suggested (Allen, 1992) that state 2 is induced by reduction of the plastoquinone pool, leading to phosphorylation of β–PC plus a PSII-associated 15 kDa membrane protein and to the dissociation of the PBSs from PSII. The 18.5 kDa protein was proposed as a PBS component, the 15 kDa protein as a component of PSII.

Table 1. Phosphoproteins in photosynthetic membranes of *Synechocystis* and higher plants

Substrates	Protein		Phosphorylation	
	Synechocystis	Chloroplast (higher plants)	*Synechocystis*	Chloroplast (higher plants)
D1 protein of PSII	+	+	-	+
D2 protein of PSII	+	+	-	+
CP43 protein of PSII	+	+	-	+
10 kDa phosphoprotein	+	+	-	+
LHCII	-	+	-	+
Polyphenoloxidase	-	+	-	+
Subunit V of cytochrome b_6f complex	-	+	-	+
TSP9	-	+	-	+
PsaD	+	+	unknown	+
PsaP (TMP14)	-	+	-	+

However, a mutant of *Synechocystis* sp. PCC 6803 lacking phycocyanin (PC) is able to perform state transitions (Vernotte *et al*., 1990). Therefore, reorganisation of thylakoid components during state transitions does not necessarily require phosphorylation of antenna proteins (van Thor *et al*., 1998). There is strong evidence that the state transitions are regulated by the redox status of an intermediate electron carrier between PSII and PSI (Mullineaux and Allen, 1986, 1990; Dominy and Williams, 1987; Schreiber *et al*., 1995). The reduction of this electron carrier induces a shift toward state 2, whereas the oxidation results in a state 1 transition. Plastoquinone (PQ) or some other closely associated components could serve as an electron carrier (Mullineaux and Allen, 1990). Moreover, it was shown that the redox state of the PQ pool regulates the state transitions *via* the cytochrome b_6f complex in cyanobacteria (Mao *et al*., 2002). The detailed mechanisms of state transitions in cyanobacteria are not completely understood. One of the suggestions was that PBSs could diffuse rapidly along the surface of thylakoid membranes and this lateral diffusion could be involved in the state transition mechanism (Mullineaux *et al*., 1997). Furthermore, Bald and co-workers (1996) proposed that processes of dynamic coupling and uncoupling of PBSs to PSI and PSII were analogous to the mechanisms, which regulate the LHCII association with photosystems I and II in the chloroplast.

1.5 Characterisation of protein kinases and phosphatases

It appears that protein phosphorylation is a key mechanism for intracellular signal transduction in both, prokaryotic and eukaryotic cells. The key enzymes of the protein phosphorylation are protein kinases and phosphatases. There are three distinct families of protein kinases: serine/threonine, histidine- and tyrosine kinases, where the latter one were predominantly found in eukaryotes (Jansson, 1994; Hunter, 1995). Histidine kinases are involved in bacterial two-component signalling pathways (Stock *et al*., 1989; Parkinson and Kofoid, 1992; Andronis *et al*., 1998). In eukaryotes, protein kinases often form cascades or networks of protein phosphorylation in order to co-ordinate cellular responses to a variety of signals (Jansson, 1994; Hunter, 1995).

Bacteria use so called two-component regulatory systems that consist of a sensor, a histidine kinase, and a response regulator. Histidine kinases are usually transmembrane proteins that sense a specific environmental signal(-s) and transfer this information to the cytoplasmic response regulator protein through phosphorylation reactions (Raivio and Silhavy, 2001). Histidine kinases can be autophosphorylated on conserved histidine residues, and then transfer the phosphate group to a conserved aspartate (Asp) residue of a response regulator. Phosphorylation of the response regulator generally activates transcription of a specific set of target genes (Stock *et al*., 1990; Andronis *et al*., 1998; Vener *et al*., 1998; Raivio and Silhavy, 2001). Thus, adaptive changes in the structure, behaviour and physiology of the bacterial cell in response to a given cue can be initiated through signal induced alterations in a simple, conserved set of phosphotransfer reactions between the histidine kinase and response regulator. The mechanism of signal transduction through two-component systems is highly conserved and the physiological roles of this process are well established (Andronis *et al*., 1998). More than 80 open reading frames (ORFs), representing 2.5% of the total genome capacity were found to encode proteins of two-component systems in the cyanobacterium *Synechocystis* sp. strain PCC 6803 after its genome sequence became available (http://www.kazusa.or.jp/cyano/cyano.html) (Kaneko *et al*., 1996; Mizuno *et al*., 1996).

It became evident during the past years that, in addition to this signalling mode, protein O-phosphorylation by serine/threonine kinases, which were previously thought to be only important in eukaryotic signal transduction, occurs in various groups of Gram-negative and Gram-positive bacteria (Kennelly and Potts, 1996; Keren *et al*., 1997). They are particularly

widespread in cyanobacteria (Kruse *et al*., 1997). Surveys of the genome of *Synechocystis* sp. PCC 6803 revealed more than 20 putative serine/threonine and also tyrosine-specific protein kinases (PK) and protein phosphatases (PP) (Keren *et al*., 1997; Kruse *et al*., 1997). Eukaryotic-type protein kinases can be divided into two classes based on their substrate specificity: serine/threonine and tyrosine kinases.

The dephosphorylation of thylakoid phosphoproteins has been shown to be catalysed predominantly by enzymes integral to the thylakoid membrane (Bennett, 1980). Serine/threonine phosphatases are classified according to their substrate specificity, requirement for divalent cations and susceptibility to inhibitors (Pierre *et al*., 1997; Elanskaya *et al*., 1998; Mao *et al*., 2002). Using these criteria two major categories of phosphatases have been defined: the Mg^{2+}-dependent PPM family (including phosphatase PP2C) and Mg^{2+}-independent PPP family (which can be divided into PP1, PP2A and PP2B subfamilies). Many of these phosphatases consist of a catalytic, regulatory and targeting subunits (Fruman *et al*., 1994; Barford, 1996). The third family of protein phosphatases, the PTP family, includes low-molecular weight PTPs, and PTPs with dual specificity because of their ability to dephosphorylate both, phospho-serine/threonine and phospho-tyrosine residues (Fauman and Saper, 1996).

Although the existence of serine/threonine and/or tyrosine kinases in prokaryotes has been established, knowledge of the molecular networks of bacterial signal transduction systems involving protein O-phosphorylation and their function in cell regulation is still very preliminary (Bakal and Davies, 2000). For example, a putative protein kinase is involved in motility of *Synechocystis* cells (Kamei *et al*., 2001) and in the filamentous cyanobacterium *Anabaena* sp. PCC 7120. PK and PP activities play a role in the differentiation of nitrogen fixating heterocysts (Zhang *et al*., 1998a). In most cases it is still unclear, how eukaryotic-type protein kinases and phosphatases are involved in bacterial signal transductions. One possibility is that they participate in signal transduction through a cascade of serine/threonine and tyrosine phosphorylation/dephosphorylation processes, in a way similar to that which takes place in eukaryotes (Jansson, 1994; Tullberg *et al*., 1998). Another possibility would be the coupling of some serine/threonine kinases and phosphatases to two-component systems, with sensors of two-component systems acting as the membrane receptors (Phalip *et al*., 2001). It is interesting to note, that several genes encoding serine/threonine kinases or phosphatases in *Synechocystis* sp. PCC 6803 are found in the same cluster as those encoding members of two-component

systems or, as in the case of *slr1983*, the same protein contains both, a response regulator domain and a protein serine/threonine phosphatase domain (Keren *et al*., 1997). Thus, the post-translational modification of proteins by phosphorylation is a universal molecular mechanism of adaptation that enables cells to sense and respond to changes in their environments. The phosphorylation or dephosphorylation of serine/threonine, tyrosine or histidine residues generally triggers conformational changes in target proteins and alters their biological properties (Weber *et al*., 2001).

1.6 Structure, degradation and role of PBSs in cyanobacteria

Phycobilisomes (PBSs) are multimeric highly organised protein complexes that may constitute 50% of the soluble proteins of the cyanobacterial cell. Contrary to light harvesting antennae, LHCII, of higher plants which are located within the thylakoid membrane, the PBS antenna complexes of cyanobacteria are peripherally associated with the outer cytoplasmic surface of the photosynthetic membranes (Glazer *et al*., 1983, 1985; Bryant, 1986) (Fig. 1). PBSs are not only antenna complexes for light harvesting, but can also be used as storage materials for carbon and nitrogen (Bryant, 1987).

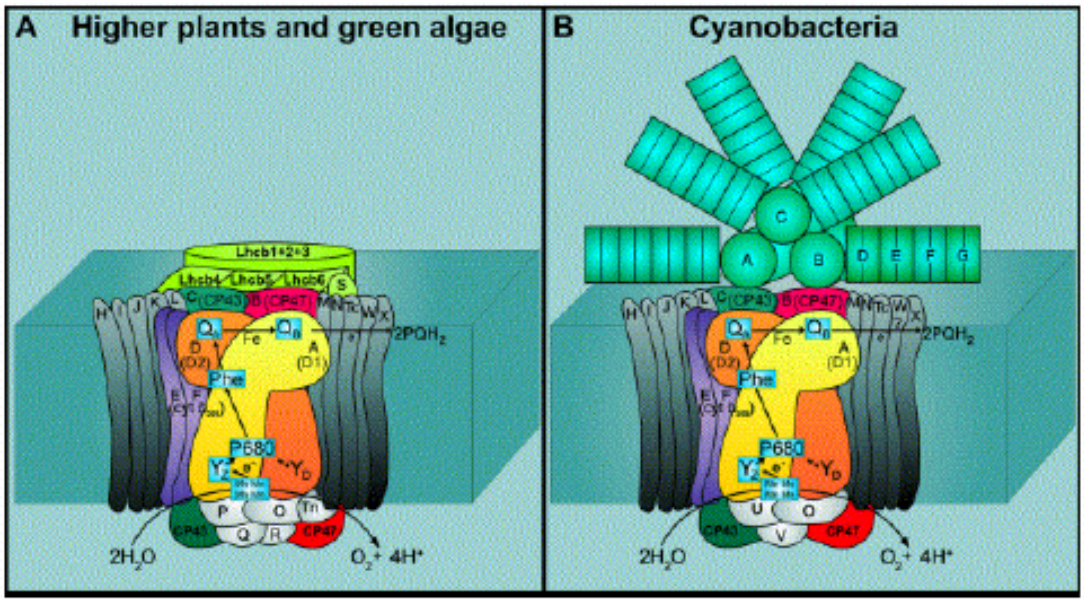


Figure 1. Comparison of photosystem II of cyanobacteria and higher plants. Adapted from Hankamer *et al*. (2001).

The polypeptides composing the PBSs have been divided into three groups: major bilin-containing proteins, phycocyanin (PC), allophycoyanin (APC) and phycoerithrin (PE) (Glazer, 1987, 1988), colorless linker polypeptides, and PBS-associated proteins, like ferredoxin $NADP^+$ oxidoreductase (FNR), phycobilin lyases, etc. The phycobiliproteins comprise 85% of the PBSs. Each type of phycobiliproten is composed of two subunits, α and β, that are organized into $(\alpha\beta)_6$ hexamers. The excitation energy absorbed by PE is transferred to PC, AP and then to the chlorophyll molecules associated with the reaction centres of photosystems II and I. In *Synechocystis* sp. PCC 6803 PBSs are comprised of bilin-containing phycobiliproteins, allophycocyanin (APC) and phycocyanin (PC) (Fig. 2).

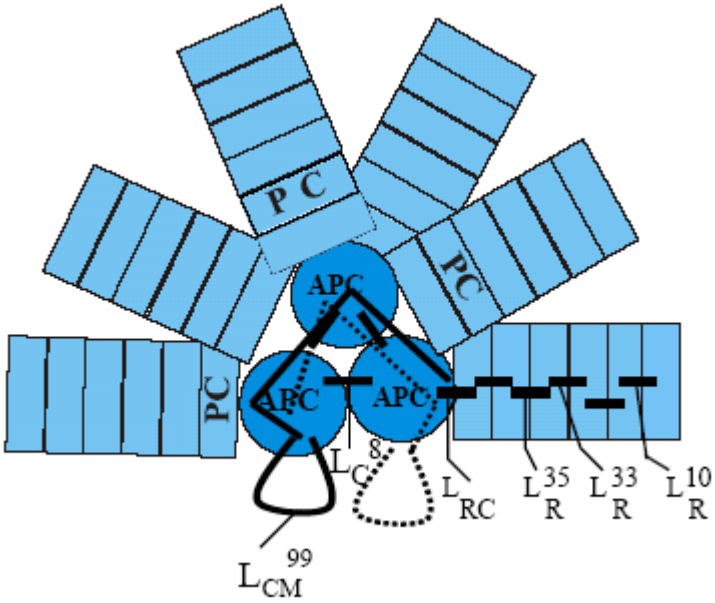


Figure 2. The schematic presentation of the structure of PBSs of the *Synechocystis* sp. PCC 6803. PC – phycocyanin, APC – allophycocyanin, L_{CM} – core-membrane linker, L_C – core linker, L_{RC} – rod core linker, L_R – rod linker.

The non-pigmented linker proteins are integrated into the PBS structure (Fig. 2). These polypeptides with molecular masses from 8 kDa to 120 kDa serve several functions in the PBSs. They help stabilizing the PBS structure, determine the positions of specific phycobiliproteins in the complex, facilitate assembly of phycobiliprotein-containing substructures, modulate the absorption characteristics of the phycobiliproteins to promote unidirectional transfer of energy within the PBS and from PBS to the chlorophylls of the photosynthetic reaction centres, and physically link the entire complex to the photosynthetic membranes (Glazer *et al*., 1983, 1985, 1988, 1989; Bryant, 1991; Grossman *et al*., 1993b). Linker proteins can be divided into four groups: rod core linkers (L_{RC}) that attach the peripheral rods to the PBS core, rod linkers (L_R^{10}, L_R^{33}, L_R^{35}) that associate PC substructures into rod segments, the small core linker (L_C^8) that are associated with trimeric allophycocyanin at the

peripheries of the core cylinders, and the core-membrane linker (L_{CM}^{99}), that acts in the organisation of the PBS core, in the PBS attachment to the membrane and also as the major terminal energy emitter to PSII (Glazer, 1989; Capuano *et al*., 1991; Sidler, 1994). The latter one was suggested to couple PBSs to the membrane *via* a domain called the PB-loop, but deletion mutagenesis of this domain that has been proposed to be a transmembrane domain, did not show any changes in the association of PBSs with thylakoid membranes in the mutant strain (Ajlani and Vernotte, 1998b).

FNR may be attached to PBSs at the core-distal phycocyanin hexamer by its N-terminal domain (which was shown also to be similar to the small phycocyanin rod linker L_R) at a position topologically equivalent to the L_R binding site (de Lorimier *et al*., 1990; Schluchter and Bryant, 1992; Zhao *et al*., 1992). The two C-terminal domains serve for the enzymatic activity of FNR (Schluchter and Bryant, 1992; Fillat *et al*., 1993; van Thor *et al*., 1998). However, recent studies have shown that in *Synechocystis* sp. PCC 6803 FNR is tightly bound to the PBSs at a different site from that predicted on the sequence similarity of the binding domain with sequences of linker polypeptides. It was found that FNR binds to PBSs not at the core-distal but at the core-proximal PC hexamer *via* its N-terminal domain, with approximately two copies per complex (van Thor *et al*., 1999). Also, it was concluded by fluorescence measurements that the presence of FNR in the PBS complexes does not influence the distribution of excitation energy absorbed by the PBSs between PSII and PSI, and also does not affect state transitions (van Thor *et al*., 1999).

In cyanobacteria the level, structure and polypeptide composition of PBSs varies widely among various cyanobacterial strains. Moreover, different environmental parameters can significantly alter the composition or abundance of PBSs for a single strain. It is controlled by changes in the light intensity, light quality, temperature and nutrient availability (Allen and Smith, 1969; Tandeau de Marsac, 1977; Bryant and Cohen-Bazire, 1981; Anderson *et al*., 1983; Collier and Grossman, 1992; Grossman *et al*., 1993b). Cyanobacteria have evolved several molecular mechanisms for acclimation of the PBS antennae that operate at the transcriptional (Belknap and Haselkorn, 1987; de Lorimier *et al*., 1991), translational and post-translational levels (Grossman *et al*., 1993a; Pojidaeva *et al*., 2004). Low light intensities may stimulate the synthesis of PBSs and cause an increase in size of the rod structure (Öquist, 1974a, 1974b; Lönneborg *et al*., 1985). Acclimation to higher light intensity occurs primarily through changes in gene expression that result in a decreased number of PBSs per cell and in a shortening of

PBS rods (Raps *et al*., 1985; Lomax *et al*., 1987; Kalla *et al*., 1993). Light quality can also cause a dramatic change in PBS composition (Bogorad, 1975; de Marsac *et al*., 1980; Bryant and Cohen-Bazire, 1981; Anderson and Grossman, 1990; Federspiel and Grossman, 1990; Sobczyk *et al*., 1993).

High rates of PBS degradation were observed for various cyanobacterial strains also upon nutrient deprivation (Duke *et al*., 1989; Collier and Grossman, 1992; Grossman *et al*., 1993a). Starvation for nitrogen (N) or sulfur (S) triggers the rapid and complete degradation of the PBSs (Allen and Smith, 1969; Yamanaka and Glazer, 1980; Schmidt *et al*., 1982; Jensen and Rachlin, 1984; Wanner *et al*., 1986; Collier and Grossman, 1992, 1994; Grossman *et al*., 1995). The PBS degradation also occurs in response to phosphorus (P) (Ihlenfeldt and Gibson, 1975), carbon (C) (Miller and Holt, 1977) or iron (Fe) starvation (Sherman and Sherman, 1983). Degradation of PBSs could provide amino acids or carbon skeletons for production of other cellular constituents required during nutrient deprivation (Allen and Smith, 1969) and reduce absorption of excitation energy, making cells less susceptible to photodamage (Schwarz and Grossman, 1998). Screening of *Synechococcus* sp. PCC 7942 mutants that were unable to degrade their PBSs during sulfur or nitrogen limitation growth led to the identification of several genes that control PBS degradation in cyanobacteria (Collier and Grossman, 1994; Schwarz and Grossman, 1998; Dolganov and Grossman, 1999). The *nbl* gene family encodes a major group of proteins that is involved in stress signalling and controls the PBS degradation. Two signalling components, a response-regulator and a histidine kinase, are encoded by the *nblR* and *nblS* genes, respectively (Schwarz and Grossman, 1998; van Waasbergen *et al*., 2002), while two other genes code for NblA and NblB that are required for coordination of PBS degradation under nutrient deprivations (Collier and Grossman, 1994; Dolganov and Grossman, 1999; Baier *et al*., 2001, 2004). Two tandem copies of *nblA* are present in the genome of *Synechocystis* sp. PCC 6803. In this strain PBS degradation has been studied only under nitrogen starvation (Elmorjani and Herdman, 1987). More recently, it was demonstrated that nitrogen deprivation leads to induction of *nblA*, followed by PBS degradation in *Synechocystis* 6803, while S starvation does not (Richaud *et al*., 2001). PBS degradation in N- and S-deprived *Synechococcus* strains occurs in an ordered manner (Yamanaka and Glazer, 1980; Collier and Grossman, 1992). Degradation begins at the periphery of the complex with the elimination of the terminal PC-hexamer and their associated linker polypeptides. This is followed by degradation of the next PC-hexamer and linker polypeptides. The degradation of PBS polypeptides results in a decrease of the PBS size and reduction in the ratio of PC to APC.

After these processes the PBS structure is completely destroyed in a process called „core degradation". Hence, cellular phycobiliprotein content is controlled at the level of both, PBS size and number. Both, rod trimming and PBS core degradation, occurs in other cyanobacteria as well (Yamanaka and Glazer, 1980; Duke *et al*., 1989; Grossman *et al*., 1993b). The cleavage of linker proteins has been suggested to be a prerequisite for complete degradation of phycobiliproteins (Glazer, 1988; Gottschalk *et al*., 1991; Pojidaeva *et al*., 2004). Since PBSs represent tightly organised structures it is not clear yet which protein determinants can trigger the initiation of the disassembly process and how such structural components as linker proteins, which are embedded in rod discs (Yu and Glazer, 1982; Reuter *et al*., 1999; Stec *et al*., 1999), can become accessible to regulatory proteins and proteolytic enzymes. It has been suggested that chaperones may be essential in the first steps of PBS assembly when the degradation of biliproteins competes with protein biosynthesis (Anderson and Toole, 1998).

1.7 Possible roles of peptidyl-prolyl *cis/trans* isomerases

Various auxiliary enzymes are essential for the biogenesis, maintenance and acclimation of the thylakoid system. Thylakoids form a continuous membrane network enclosing an inner space designated the thylakoid lumen (Kieselbach *et al*., 1998). This cellular compartment has become increasingly interesting with the advances in understanding regulation and stress protection of the photosynthetic process. Until recently the thylakoid lumen was considered to contain just a few soluble proteins facilitating electron transfer and oxidation of water, but recent proteomic studies have revealed at least 80 different proteins to be present in the lumen space (Kieselbach *et al*., 1998; Peltier *et al*., 2000; Peltier *et al*., 2002; Schubert *et al*., 2002). Unexpectedly, the largest group of these proteins was classified as peptidyl-prolyl *cis/trans* isomerases (PPIases) or rotamases (Peltier *et al*., 2002; Schubert *et al*., 2002). PPIases is a widely distributed group of proteins that is involved in the isomerisation of peptidyl-prolyl imide bonds in peptides and thus might be involved in protein folding (Bang and Fischer, 1991; Gething and Sambrook, 1992; Matouschek *et al*., 1995; Fischer *et al*., 1998). PPIases can also perform chaperone functions or cooperate with other chaperone proteins (Freskgard *et al*., 1992; Freeman *et al*., 1996; Pratt *et al*., 2001). In diseased plants they can function as chaperon-like molecules in order to decrease the opportunity for proteolytic degradation (Marivet *et al*., 1994). PPIases can regulate diverse cellular processes including cell signalling and biogenesis, and can activate different cellular receptors (Luban *et al*., 1993; Lu *et al*., 1996; Harrar *et al*., 2001; Schiene-Fischer and Yu, 2001). They participate in important processes in

plant development. It has been shown that PPIase-like proteins are essential for normal cell division and differentiation in *Arabidopsis thaliana* (Vittorioso *et al*., 1998). One of these components was a critical regulator of normal development of leaf size and shape in *Arabidopsis thaliana* (Berardini *et al*., 2001). The peptidyl *cis-trans* isomerase activity can be required for acceleration of the processing of native polypeptides. Members of PPIases include also stress-related proteins. Under stress conditions their higher amounts might also be needed to accelerate folding steps and, therefore, the maturation processes of newly synthesized proteins induced during stress, and are believed to play protective functions (Ho and Sachs, 1989; Bowles, 1990; Linthorst, 1991). Despite these possible multiple roles, the physiological functions of most of immunophilins are not established yet (Kay, 1996).

PPIases are divided into three families: cyclophilins, FK506-binding proteins (FKBPs) and parvulins (Vener, 2001). The cyclophilins and FKBPs are also collectively designated immunophilins because of their possibility to bind the immunosuppressive drugs cyclosporin A (CsA) and FK506, respectively, which inhibit their PPIase activity (Schreiber, 1991).

Although PPIases have been identified in all plant tissues (Gasser *et al*., 1990; Breiman *et al*., 1992; Luan *et al*., 1996; Chou and Gasser, 1997; Saito *et al*., 1999), the understanding of their cellular functions and roles is incomplete. PPIases are present in all cellular compartments of plant cells such as the cytosol (Breiman *et al*., 1992; Lippuner *et al*., 1994), mitochondria, chloroplast (Brieman *et al*., 1992; Mattoo, 1998), and endoplasmic reticulum (Sheldon and Venis, 1996; Saito *et al*; 1999). In chloroplasts, the major PPIase activities were found in the stroma compartment (Brieman *et al*., 1992). Moreover, Lippuner and co-workers (Lippuner *et al*., 1994) had found the PPIase activity only in the chloroplast stroma, and not in thylakoid membranes or lumen. However, the recent proteomic studies of Schubert and colleagues revealed three putative cyclophilins and five FKBPs in the thylakoid lumen of *Arabidopsis thaliana* (Schubert *et al*., 2002). The study by Peltier and co-workers identified five and predicted at least four additional putative PPIases in the same compartment (Peltier *et al*., 2002). However, only one active PPIase from the thylakoid lumen has been experimentally characterized up to now (Edvardsson *et al*., 2003). This first characterized PPIase is a cyclophilin-like protein TLP40 (thylakoid lumen PPIase of 40 kDa) of the complex type, located in the lumen of plant photosynthetic membranes and has been co-isolated with thylakoid protein phosphatases of PP2A-type from thylakoid membranes of spinach (Fulgosi *et al*., 1998). It was proposed that TLP40 is responsible for PPIase activity and protein folding in

the lumen in addition to its regulatory function (Fulgosi *et al*., 1998; Vener *et al*., 1999). TLP40 possesses a cyclophylin-like C-terminal catalytic domain and an N-terminal domain with a number of protein-binding modules (Fulgosi *et al*., 1998; Vener *et al*., 1998, 1999; Vener, 2001). It has been shown that reversible binding of TLP40 to the inner thylakoid membrane surface regulates the activity of a membrane-bound phosphatase of the PP2A type at the outer surface. It has been proposed that binding of TLP40 to the protein phosphatase inhibits dephosphorylation of the PSII phosphoproteins, the process required for protein turnover during repair of damaged PSII reaction centres (Fulgosi *et al*., 1998; Vener *et al*., 1999; Rokka *et al*., 2000).

More recently, a novel enzyme TLP20 (thylakoid lumen PPIase of 20 kDa) from the thylakoid lumen of spinach chloroplasts has been found (Edvardsson *et al*., 2003). It was demonstrated that TLP20 was inhibited by cyclosporin A and thus belongs to the cyclophylin family of PPIases. It was suggested that TLP20 is responsible for common protein folding catalysis in the lumen of chloroplast thylakoids, while TLP40 has a specialized regulatory function(-s) (Edvardsson *et al*., 2003).

The homolog of the gene for the chloroplast lumenal immunophilin protein (TLP40) was found in the genome of *Synechocystis* sp. PCC 6803 (Kaneko *et al*., 1996). Since chloroplast TLP40 is involved in the control of dephosphorylation of PSII subunits, the process which is absent in cyanobacterial thylakoid membranes, the role of *Synechocystis* TLP40 homologue in the cyanobacterium was not clear. Lack of protein phosphorylation of photosystem II subunits suggested different mechanisms or regulatory functions of cyanobacterial TLP40 homologue.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and enzymes

The chemicals used in this work were of analytical grade or of best available quality. They were obtained from Biomol (Hamburg), Difco Laboratories (Detroit, USA), Fluka (New-Ulm), Merck AG (Darmstadt), Roth GmbH & Co (Karlsruhe), Serva Feinbiochemica (Heidelberg), Sigma Chemical Company (Munich), Difco Laboratories (Detroit, USA) and Roche (Basel, Switzerland).

Molecular biology enzymes were purchased from Boehringer (Mannheim), New England Biolabs (Schwalbach) and MBI Fermentas (Vilnius, Lithuania). Alkaline phosphatase and protease inhibitors were obtained from Sigma, radiolabeled nucleotides from Amersham Pharmacia Biotech (Freiburg).

2.1.2 Molecular weight markers

DNA and RNA molecular weight standards

As DNA length standards were used λ DNA digested by *Eco*RI/*Hind*III and the 1 kBp ladder obtained from Gibco/BRL (Karlsruhe).

Protein molecular weight standards

Protein molecular mass standards for SDS protein gel electrophoresis, SDS-7 (14 – 66 kDa), peqGOLD protein marker (14.4 – 116 kDa) and prestained protein marker (20 – 122 kDa), were obtained from Sigma Chemical Company (Munich) and PeqLab (Biotechnologie GmbH), respectively.

2.1.3 Bacterial strains

E. coli DH5α (Hanahan, 1985)
E. coli BL21 (DE3) pLysS (Studier and Moffat, 1986)

E. coli C600 (Maniatis et al., 1984)

E. coli XL10-Gold (Stratagene's QuikChange® XL site-directed mutagenesis kit)

E. coli R751 (Prof. V. Zinchenko, Moscow State University)

Wild-type *Synechocystis* sp. PCC 6803 (Prof. S. Shestakov, Moscow State University)

PAL *Synechocystis* sp. PCC 6803 strain (Ajlani and Vernotte, 1998)

CK *Synechocystis* sp. PCC 6803 strain (Ajlani and Vernotte, personal communications)

2.1.4 Vectors

pBluescript II KS (Stratagene, San Diego)

pIVEX 2.4b (Roche Diagnostics GmbH, Basel, Switzerland)

pVZ323 (Prof. V. Zinchenko, Moscow State University)

pUC4K (Taylor and Rose, 1988)

R751 (Meyer and Shapiro, 1980)

pSL762 (Schweirer, 1993)

2.1.5 Oligonucleotides

Gene-specific oligonucleotides were obtained from MWG-BIOTECH (Ebersberg, Germany).

***Synechocystis* gene-specific primers**

sll0408Fover	5'-ATTCTCGAGATGGGGGCGTTGAAGTGG-3'
sll0408Rover	5'-TTACTGCAGTCAACTATTGCCGTTAACTAA-3'
sll0408N	5'-CGGAATTCGCTGACTTCCATCTCCTCT-3'
sll0408C	5'-GCTCTAGAGTTGACAGTAACTGAGAGAG-3'
sll0408Mfor	5'-GCAATAGTTGATAAATCAGTCGACCATTGTCAGAAACT-3'
sll0408Mrev	5'-AGTTTCTGACAATGGTCGACTGATTTATCAACTATTGC-3'
sll0408Fmut	5'-GATCCCAATGCCATTTTGGCGTACGCTCTGCCCATC-3'
sll0408Rmut	5'-GATGGGCAGAGCGTACGCCAAAATGGCATTGGGATC-3'
sll0408p1	5'-CCGGGATGGAGCTTGCCTTTG-3'
sll0408p2f	5'-GCCGGCCAACGCTTAGCCCG-3'
sll0408Nterfor	5'-GCTTAGCCCGGGCACTTTTGAGCTTGGTCCG-3'
sll0408Nterrev	5'-GGCTACAAGCTTTCACACCACAATGGTCAGGGGG – 3'

sll0408L6for	5’-CCTTTGAACCAGCCGATGGCGTTGGGTGCTTTGGCCCAGG-3’
sll0408L6rev	5’-CCTGGGCCAAAGCACCCAACGCCATCGGCTGGTTCAAAGG-3’
sll0408Ctermfor	5’-CCTCAACTCAAGGGCCGG-3’
sll0408Ctermrev	5’-GGTACTAAGCTTTCAACTATTGCCGTTAACTAA-3’
sll0408NCfusionrev	5’-GCCCTTGAGTTGAGGTTGCATCATAATGATTGTGG-3’
CtermRpmF	5’-GACGGTTTACCTTTTATCCTTTCGGAAGATTTCTTTGTGAC-3’
CtermRpmR	5’-GTCACAAAGAAATCTTCCGAAAGGATAAAAGGTAAACC GTC-3’
CtermQpmF	5’-GATTTCTTTGTGACTCTAGCGGGGGATCCCCCAG-3’
CtermQpmR	5’-CTGGGGGATCCCCCGCTAGAGTCACAAAGAAATC-3’

2.1.6 Media for bacterial growth

E. coli growth medium

LB-medium	10.0 g	Bacto-Trypton
	5.0 g	Yeast extract
	10.0 g	NaCl
	H_2O	add till 1 l

For solid media 15 g/l of agar was added (end concentration 1.5%). For selection of antibiotica resistant clones antibiotica were used in the following concentrations: kanamycin – 50 µg/ml, ampicillin – 100 µg/ml and chloramphenicol – 25 µg/ml. The recombinant clones were selected by blue-white selection on LB-medium containing X-gal (50 µg/ml) and IPTG (0.5 mM).

Synechocystis sp. PCC 6803 growth medium

Cyanobacterial strains were cultivated on BG11 medium (Rippka, 1988). Macro- and micro-elements were used as 1000 x stock solutions. For solid medium 1% agar (Difco, Detroit, USA) was added to BG11 medium. For photoheterotrophic growth the BG11 medium was supplemented with sterile 5 mM glucose. For selection of mutant strains gentamycin (Gm; 1 – 3 µg/ml), chloramphenicol (Cm; 15 – 25 µg/ml) and kanamycin (Km; 40 – 100 µg/ml) were added to solid media. In liquid medium the concentration of antibiotics was decreased twofold.

Macro-elements (1 x stock)	g/l
$NaNO_3$	1.5
K_2HPO_4	0.04
Citric acid	0.006
Ferric ammonium citrate	0.006
EDTA (disodium salt)	0.001
Na_2CO_3	0.02
$MgSO_4$ x 7 H_2O	0.075
$CaCl_2$ x 2 H_2O	0.036

Micro-elements (1000 x stock)	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	0.0494

2.1.7 General buffers and solutions

TE buffer	10.0 mM	Tris-HCl, pH 8.0
	1.0 mM	EDTA
10 x TBE	1.0 M	Tris-HCl, pH 8.0
	0.5 M	boric acid
	20.0 mM	EDTA
STET-buffer	8%	sucrose
	5%	Triton X-100
	50 mM	EDTA
	50 mM	Tris-HCl, pH 8.0
10 x PBS	750 mM	NaCl

	30	mM	KCl
	45	mM	Na_2HPO_4
	15	mM	KH_2PO_4
10 x Transfer buffer	1.5	M	glycin
	0.2	M	Tris-HCl, pH 8.0
20 x SSC	3.0	M	NaCl
	0.3	M	tri-sodium citrate x 2 H_2O, pH 7.2
10 x MOPS	0.05	M	Na-acetate, pH 7.0
	0.1	M	MOPS
	0.1	M	EDTA

2.1.8 Antibodies

Polyclonal goat anti-rabbit IgG peroxidase conjugate, goat anti-mouse IgG peroxidase conjugate and rabbit anti-chicken IgG peroxidase conjugate antibodies were obtained from Sigma Chemical Company (Munich). Rabbit polyclonal anti-phosphothreonine/-serine antibodies were obtained from Zymed Laboratories (San Francisco, USA). Antisera against phycobiliproteins (phycocyanin, allophycocyanin and linker proteins) were kindly provided by Prof. A. Grossman (Stanford University, USA).

2.1.9 Transfer membranes

Hybond™ PVDF (polyvinylidene difluoride) membrane for nucleic acid transfer was purchased from Amersham (Braunschweig, Germany). Nitrocellulose PROTRAN® transfer membrane was obtained from Schleicher and Schuell (Dassel, Germany).

2.2 Methods

2.2.1 Standard methods

Basic molecular procedures, such as electrophoresis of DNA on agarose gel, restriction of DNA, dephosphorylation with alkaline phosphatase, DNA precipitation, ligation with T4 DNA ligase have been performed according to Sambrook *et al*. (1989). Plasmid DNAs were isolated by the alkaline lysis procedure (Birmboim and Doly, 1979) or by the boiling lysate method (Holmes and Quigley, 1981). Protein concentrations were determined according to a modified Lowry procedure (Markwell *et al*., 1978).

2.2.2 Sequence analysis

Sequence data were obtained from the *Synechocystis* genome bank (http://www.kazusa.or.jp/cyano/cyano.html). Hydropathy plots were done according algorithms developed by Kyte and Doolittle (1982) and the SOSUI program (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html). Analysis of protein and gene homologies was performed using BLAST search on NCBI (http://www.ncbi.nlm.nih.gov/BLAST/).

2.2.3 Growth conditions for *Synechocystis*

Wild-type and mutants were cultivated in standard BG11 media under low light (LL; 40 μE m^{-2} s^{-1}). For acclimation to stress conditions cells were grown at LL to exponential growth phase (A_{750} = 0.6 – 0.8) and then transferred to high light (HL; 400 μE m^{-2} s^{-1}), cold stress (CS; 17°C; 40 μE m^{-2} s^{-1}), double stress (DS; 17°C; 400 μE m^{-2} s^{-1}), or heat shock (HS; 42°C; 40 μE m^{-2} s^{-1}). Photoheterotrophic cell growth was obtained in the medium supplemented with 5 mM glucose under dim light (approximately 5 μE m^{-2} s^{-1}).

Medium lacking nitrogen was prepared by replacing ferric ammonium citrate, $NaNO_3$ and $Co(NO_3)_2$ by $FeCl_3$, NaCl and $CoCl_2$. The cyanobacterial cultures were grown at LL regime in standard BG11 medium and harvested by centrifugation at 2.500 x g for 15 min at a cell density of A_{750} = 0.6 – 0.8. Cells were washed once in a medium lacking nitrogen, resuspended in 50 ml of medium depleted in nitrogen and transferred to LL for 1 to 10 days.

2.2.4 Construction of *Synechocystis* mutants

To obtain knock-out and complementation mutants (Table 2), parts of the coding regions of the desired genes were amplified from *Synechocystis* genomic DNA using specific primers (Section 2.1.5). PCR products were cloned into pBluescript II KS or pVZ323 vectors. To generate the mutants, a 0.8 kBp gentamicin (Gm^R) resistance gene from pSL762 or a 1.2 kBp kanamycin (Km^R) resistance gene from pUC4K vectors were used. The plasmids containing the interrupted gene of interest were used for transformation (Section 2.2.4.1) or conjugal transfer (Section 2.2.4.2) into *Synechocystis* cells.

Table 2. Strains that were obtained for analysis of *sll0408* gene

Name of mutant strains	Type of mutation	Size (kBp) of cloning fragment	Vector	Antibiotic resistance
Δsll0408 (*Δctlp40*)	insertion mutant	2.050	pBluescript II KS	Gm
Δsll0408pVZ323	complementation strain	1.209	pVZ323	Gm, Cm
Δsll0408Ala	(R) CGC → (A) GCG	1.864	pBluescript II KS	Km
Δsll0408^K	insertion mutant	1.864	pBluescript II KS	Km
ΔN-termsll0408	N-terminal deletion	0.867	pVZ323	Gm, Cm
ΔC-termsll0408	C-terminal deletion	0.843	pVZ323	Gm, Cm
Δsll0408pmL2	(L2) CTG → (A) GCG	1.209	pVZ323	Gm, Cm, Km
Δsll0408pmR	(R) CGT → (L) CTT	1.209	pVZ323	Gm, Cm, Km
Δsll0408pmQ	(Q) CAA → (L) CTA	1.209	pVZ323	Gm, Cm, Km
Δsll0408pmR/Q	(R) CGT / (Q) CAA → (L) CTT / (L) CTA	1.209	pVZ323	Gm, Cm, Km

2.2.4.1 Transformation of *Synechocystis*

Transformation of *Synechocystis* cells was carried out as described by Grigorieva and Shestakov (1982). For integration of genetic material into cyanobacterial chromosome, the *E. coli* plasmid pBluescript II KS containing a fragment of the *Synechocystis* chromosome was

used. Cyanobacterial cells were grown for three days until an A_{750} = 0.6 – 0.8. Then 1 ml of *Synechocystis* cells was mixed with 200 µl of a donor *E. coli* cell culture, centrifuged, and resuspended in 50 – 100 µl of fresh BG11 medium. After overnight incubation at 20 – 30 µE m^{-2} s^{-1} cells were plated on selective solid BG11 medium containing low concentration of antibiotica. Segregation of transformants, which appeared within 7 – 12 days, was carried out with several passages on an increased concentration of antibiotics such as gentamycin (0.5 – 3 µg/ml), kanamycin (20 – 100 µg/ml), chloramphenicol (10 – 25 µg/ml). The complete segregation of mutations was confirmed by PCR analysis of chromosomal DNA isolated from transformed strains with gene-specific primers.

2.2.4.2 Conjugal transfer of plasmids into cyanobacterial cells

Conjugal transfer of pVZ323 plasmid was performed *via* triparental crossing on membrane filters (13 mm diameter, 0.22 µm pore size; Millipore GVWP). Mobilization of the non-conjugative autonomously replicating plasmid pVZ323 with the gene of interest was performed with the *E. coli* conjugative plasmid R751 of the IncP group as described by Zinchenko *et al*. (1999). Cyanobacterial cells were grown for three days until a cell density A_{750} = 0.7. Cyanobacterial cell cultures (1 – 2 ml) were mixed with 0.2 ml of overnight grown *E. coli* cells, one of them contained the pVZ323 plasmid with a cloned fragment and the another one plasmid R751. After centrifugation, the mixture was resuspended in 100 µl of BG11 and applied on a filter placed on the surface of the BG11 medium supplemented with 5% LB in a Petri dish. Dishes were incubated under dim light (10 µE m^{-2} s^{-1}) for one day. Afterwards cells were resuspended in fresh BG11 medium and plated onto selective media containing various concentrations of antibiotics.

2.2.5 DNA analysis

2.2.5.1 DNA isolation from *Synechocystis*

Synechocystis cells were grown for 5 – 7 days and cells were harvested by centrifugation (from 3 ml), washed with 1 ml of buffer, containing 50 mM Tris-HCl, pH 8.0, 50 mM EDTA, and then resuspended in 270 µl of the STET buffer (Section 2.1.7). The cell suspension was mixed with 15 µl of chloroform and vigorously stirred for 5 min. Then 30 µl of 20 mg/ml lysozyme was added and the mixture was incubated for 30 min at 37°C. After that 100 µl of 10% (w/v)

SDS and 100 µl of 5 M NaCl were added and the mixture incubated at 65°C for 60 min. The lysate was deproteinized twice with chloroform. DNA was precipitated with an equal volume of isopropanol, washed twice with 70% ethanol and dissolved in TE buffer (Section 2.1.7).

2.2.5.2 Isolation of plasmid DNA from *E. coli* by the „boiling lysate" method

The pVZ323 plasmid DNA from *E coli* was isolated by the boiling lysate method according to Holmes and Quigley (1981). Overnight grown *E. coli* cells (1.5 – 3 ml) were collected by centrifugation at 10.000 x g for 3 – 5 min at room temperature. The pellet was resuspended in 350 µl STET buffer (Section 2.1.7). The mixture was incubated for 5 min at room temperature after addition of 25 µl lysozyme (10 mg/ml) and proteins were denatured by incubation for 40 sec at 95°C. The lysate was centrifuged at 10.000 x g for 10 min at room temperature and the sedimented pellet was removed. The supernatant containing plasmid DNA was precipitated with 0.5 volume of 7.5 M NH_4-acetate and 0.6 V of isopropanol for 20 min at room temperature. After centrifugation at 10.000 x g for 20 min the DNA pellet was washed twice with 70% ethanol, dried, resuspended in 50 µl of TE buffer and stored at –20°C.

2.2.6 Site-directed mutagenesis *in vitro*

The QuikChange® XL site-directed mutagenesis kit (Stratagene) was used for the construction of point mutations in the *sll0408* gene. Mutagenesis was performed in pBluescript II KS vector containing the *sll0408* gene. Two specific synthetic oligonucleotide primers containing the desired mutation, each complementary to opposite strands of the vector and *PfuTurbo*® DNA polymerase were used in a thermal temperature cycler. The final product was treated with *Dpn*I to digest the methylated, non-mutated parental DNA template and to select for mutation-containing synthesized DNA. This DNA was transformed into XL10-Gold® ultracompetent cells. Plasmid DNA was extracted and modified sequences were proven by DNA sequence analysis.

2.2.7 Protein overexpression and generation of antisera

2.2.7.1 Protein overexpression in *E. coli* cell lysates

For overexpression of cTLP40 protein, ecoded by the *sll0408* gene the Rapid Translation System based on *E. coli* cell lysates (Roche Diagnostics GmbH, Basel, Switzerland) was used. The full-length *sll0408* gene was amplified with specific primers sll0408Fover and sll0408Rover and cloned into the overexpression vector pIVEX2.4b. The resulting plasmid was used for protein overexpression according to manual instructions.

2.2.7.2 Preparation of the probes for rabbit immunization

E. coli cell lysates containing overexpressed cTLP40 were loaded onto a 12.5% PAA gel. The electrophoretically separated proteins were stained with imidazol and the band corresponding to overexpressed cTLP40 protein was excised from the gel. The gel slice was ground between two glasses in 1 x PBS solution. The gel suspension was vortexed for 2 min and kept at 4^{o}C. The supernatant that was obtained after centrifugation at 15.000 x g and 4^{o}C for 15 min was used for injection into rabbits. For the first injection the protein extract was diluted in a 1 : 1 ratio with adjuvant TiterMax^{TM} (the maximum volume was 300 µl). Freund's adjuvant was used in the following injections instead TiterMax^{TM}.

2.2.7.3 Injection of rabbits and antiserum preparation

A preimmune serum was analysed for crossreaction with cyanobacterial proteins before protein injection. Antigen-TiterMax^{TM} suspension was injected generally at three places of the rabbit's back. The injection with Freund's adjuvant was repeated in four week intervals. Serum samples were taken in two-week intervals after the second boost and blood was allowed to clot for 30 – 60 min at 37°C. The clot was placed at 4°C overnight. The serum was separated from the clot by centrifugation at 10.000 x g for 10 min at 4°C. Na-azide (0.01%) was added to the supernatant which was aliquoted and stored at –20°C.

2.2.8 Pigment analysis of *Synechocystis* cells

The cell density of *Synechocystis* was estimated by measuring the absorbance of the culture at 750 nm (A_{750}) with the spectrophotometers 922 Uvikon (Bio-Tek, Kontron) or Ultrospec^{R}3000 (Pharmacia Biotech).

2.2.8.1 Determination of chlorophyll *a* concentrations

The concentration of chlorophyll *a* was estimated spectrophotometrically by methanol extraction as described by Lichtenthaler (1987). A cell culture aliquot of 1 ml was centrifuged at 10.000 x g for 10 – 15 min at 4^{o}C and chlorophyll was extracted from the cell pellet with 1 ml of 90% (v/v) methanol for 1 h at 4^{o}C. The chlorophyll *a* content was determined after centrifugation at 10.000 x g and 4^{o}C for 10 min by measuring the absorbance of the methanol extract at 652 and 665 nm. The concentration of chlorophyll *a* was calculated by the following equation:

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

2.2.8.2 Determination of carotenoid concentrations

Carotenoid concentrations were determined from the methanol extract as described in Section 2.2.8.1 by measuring the absorbance at 470 nm. Carotenoid concentrations resulted from equation:

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

[Chl] = concentration of chlorophyll *a* measured previously.

2.2.8.3 Determination of phycocyanin concentrations

The concentration of phycocyanins was estimated spectrophotometrically from the following equation:

$$\text{phycocyanin } (\mu g/ml) = 138.79 \times (A_{620} - A_{730}) - 35.58 \times (A_{678} - A_{730})$$

2.2.9 Protein analysis

2.2.9.1 Protein gel electrophoresis

2.2.9.1.1 SDS-denaturing PAA gels

Pipetting scheme of PAA gels

Solution	Separating gel (end concentration)	Stacking gel (end concentration)
2 M Tris-HCl, pH 8.8	0.375 M	-
1 M Tris-HCl, pH 6.8	-	0.125 M
40% acrylamide (acrylamide : bis-acrylamide – 29 :1)	depending on desired concentration	5%
10% SDS	0.1%	0.1%
80% sucrose (for gradient gels)	10 - 17.5% (gradient)	
10% APS	0.06%	0.08%
TEMED	0.006%	0.008%
H_2O	until desired volume	until desired volume

2.2.9.1.2 Urea-containing SDS-denaturing PAA gels

Urea-containing denaturing gels were prepared as described in Section 2.2.9.1.1 with the addition of 4 or 6 M urea.

2.2.9.1.3 Laemmli gel system (Laemmli, 1970)

10 x Laemmli buffer 0.25 M Tris-HCl, pH 8.5
1.92 M glycine
1% SDS

4 x Laemmli sample buffer 0.25 M Tris-HCl, pH 6.5

8%	SDS
40%	glycerol
20%	β-mercaptoethanol
0.016%	Bromphenol Blue

2.2.9.2 Staining of PAA gels

2.2.9.2.1 Coomassie Brilliant Blue staining of PAA gels

Staining solution	40%	ethanol
	5%	acetic acid
	0.2% (w/v)	Coomassie Brilliant Blue R-250 (Serva, Heidelberg)
Destaining solution	30%	ethanol
	7%	acetic acid

Gels were incubated in staining solution for 30 min under constant shaking and destained in destaining solution until the protein bands were well visible. Gels were scanned or stored for some days in destaining buffer.

2.2.9.2.2 Imidazol staining

Buffer 1	0.2 M	imidazol
Buffer 2	0.3 M	zink sulfate

Imidazol staining, which does not fix proteins irreversibly, was used for detection of proteins on the gels used for protein transfer onto the membrane. The gel was incubated in buffer 1 under constant shaking for 10 min. Proteins were visualised as transparent bands on a white background after exchanging buffer 1 with buffer 2. The staining was stopped by washing the gel with water. For protein transfer onto the membrane the gel was destained in blotting buffer.

2.2.9.2.3 Silver staining of PAA gels

Fixation solution	50%	ethanol
	12%	acetic acid
	0.05%	37% formaldehyde
Thiosulfate solution	0.02% (w/v)	$Na_2S_2O_3$
Silver solution	0.2% (w/v)	$AgNO_3$
	0.075%	37% formaldehyde
Developing solution	6% (w/v)	Na_2CO_3
	0.05%	37% formaldehyde
	0.0004%	$Na_2S_2O_3$

Gels were incubated at least for 1 h in fixation solution, washed three times for 20 min in 50% ethanol and soaked for 1 min in thiosulfate solution. Gels were washed three times for 20 – 30 sec with water and incubated in silver solution for 20 min in darkness. The washing steps (twice for 20 – 30 sec) were repeated and gels were incubated in the developing solution until the bands reached the desired intensity. The reaction was stopped by addition of fixation solution. The gels were stored for some days in fixation buffer or in 50% ethanol.

2.2.9.2.4 Luminescent staining

For protein visualisation gels were stained with the luminescent dye SYPRO® Ruby (Molecular Probes, Leiden, Netherlands). Phosphoproteins were detected by staining gels with luminescent dye Pro-Q™ Diamond (Molecular Probes, Leiden, Netherlands). The SYPRO® Ruby and Pro-Q™ Diamond stains were detected by a fluorescent image analyser FLA-300 (Fujifilm). Gels were scanned with the orange filter O580 with excitation/emission wavelengths at 475/580 nm. For detection of PBS bilin-containing proteins by self-fluorescence, non-stained gels were scanned by the red filter R675 with excitation and emission at 633/675 nm.

2.2.9.3 Analysis of proteins on the membranes

2.2.9.3.1 Transfer of SDS-denatured proteins onto nitrocellulose and PVDF membranes

10 x blotting buffer	1.5 M	glycine
	0.2 M	Tris-HCl, pH 8.0

Proteins separated on a PAA gel were transferred onto membranes using the wet-blot system. Transfer membranes and PAA gels were incubated for 10 min in a blotting buffer prior to transfer. PVDF membranes were pre-wetted in 100% methanol. Two sheets of Whatman paper of the gel size were soaked in blotting buffer and placed onto the plastic plate marked „minus". The gel was placed on top of the paper and covered with nitrocellulose or PVDF membrane. The membrane was covered with two layers of Whatman paper soaked in blotting buffer and a plastic plate. Air bubbles were removed with a rolling glass tube. The transfer was performed for 2 – 3 h at 45 V. Thereafter, the membrane was backed for 10 min at 80^{o}C.

2.2.9.3.2 Membrane staining with Ponceau S

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

Ponceau S (3-hydroxy-4-[2-sulfo-4-(sulfo-phenylazo) phenylazo]-2,7-naphtalene disulfonic acid) staining can be used to monitor transfer and to locate the molecular weight markers. Membranes were incubated in Ponceau S solution for 15 min at room temperature under constant agitation. They were washed in water and the positions of the molecular weight standards were marked. The membranes were destained by washing few times with water.

2.2.9.4 Immunological detection of proteins

2.2.9.4.1 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

Chemiluminescent detection

Stock solutions	0.25 M	Luminol (in DMSO)
	0.09 M	p-Coumaric acid (in DMSO)
Solution 1	2.5 mM	luminol
	0.4 mM	p-coumaric acid
	0.1 M	Tris-HCl, pH 8.5
Solution 2	5.4 mM	H_2O_2
	0.1 M	Tris-HCl, pH 8.5

After electrophoretic transfer of proteins from PAA gels onto nitrocellulose membrane, the gels were incubated in a blocking buffer for 1 h at room temperature. The antisera raised against the protein of interest were diluted to the desired concentration (1 : 500 – 1 : 2000) in blocking buffer and incubated with the membrane for 2 h at room temperature or overnight at 4^{o}C. The primary antisera were removed by washing the membranes in blocking buffer four times for 10 min. Anti-rabbit IgG peroxidase conjugate (Sigma) antisera were diluted in blocking buffer and incubated with the membranes for 1 h at room temperature. The membranes were again washed four times for 10 min in a washing buffer. They were developed in a mixture of solutions 1 and 2 (1 : 1) by incubation for 1 min. Chemiluminescence reaction was detected by exposure of membrane to X-ray film (Hyperfilm; Amersham Life Science, England) for variable periods.

2.2.9.4.2 Western analysis with phospho-specific antisera

Antisera raised against phosphorylated threonine or serine amino acid residues (pThr and pSer) were used for immunological detection of phosphoproteins. Western analysis was performed in the same way as described before (Section 2.2.9.4.1) with minor modifications. Proteins were transferred onto PVDF (polyvinylidene difluoride) membranes, which were pre-wetted in methanol before blotting. For immunological analysis the following solutions were used:

Blocking solution	3%	BSA
	10 mM	Tris-HCl, pH 7.5
	50 mM	NaCl
	0.1%	Tween 20
Washing solution	1 x	PBS
	0.1%	Tween 20

2.2.10 Isolation and fractionation of thylakoid membranes

2.2.10.1 Isolation of total cellular and membrane proteins from *Synechocystis*

Thylakoid membrane and cell extract proteins were isolated as described in Shukla *et al*. (1992).

Buffer A	0.5 M	sucrose
	0.05 mM	HEPES-NaOH, pH 7.0
	0.015 mM	NaCl
	0.005 mM	$MgCl_2$
	0.001 volume	of a proteinase inhibitor cocktail (Sigma)
Buffer B	0.01 mM	Tricine-HCl, pH 7.5
	6%	sucrose
	0.03 mM	DTT
	0.001 volume	of a proteinase inhibitor cocktail (Sigma)

Cells were grown in liquid BG11 medium and harvested by centrifugation. They were resuspended in buffer A and broken by vortexing (three times for 2 min with intervals on ice at least for 1 min) with glass beads (0.25 – 0.50 µm, Roth, Karlsruhe). Unbroken cells were removed by centrifugation for 10 min at 1.600 x g, and the supernatant was used as a total cell extract. Thylakoids were sedimented by ultracentrifugation at 45.000 x g for 30 min at 4°C. The pellet was washed in buffer A, resuspended in buffer B and immediately frozen.

2.2.10.2 Isolation of lumenal proteins

Thylakoid membranes were isolated as described previously (Section 2.2.10.1). Thylakoids were resuspended in buffer A. Then, they were sonicated using a Sonifier^{R}B-12 (Branson Sonic Power Company). The suspension was separated by ultracentrifugation for 30 min at 45.000 x g at 4^{o}C into lumenal (supernatant) and thylakoid membrane proteins (pellet). The pellet was washed once in buffer A and the final pellet was dissolved in buffer B for analysing thylakoid membranes (Section 2.2.10.1).

2.2.10.3 Isolation of peripheral membrane proteins

In order to characterize the nature of protein interactions with the membrane, thylakoid membranes were incubated with chaotropic salts and alkaline solution. Thylakoids (0.1 mg/ml chlorophyll) were resuspended in HEPES/sucrose buffer (10 mM HEPES-NaOH, pH 8.0, 300 mM sucrose) containing 2 M NaBr, 2 M NaSCN, or 0.1 M NaOH, or without any addition. After incubation on ice for 30 min, thylakoids were diluted 1 : 2 with a buffer lacking salts and sedimented by centrifugation for 10 min at 30.000 x g. Thylakoid membranes were resuspended in 2 x Laemmli sample buffer (LSB) and denatured for 10 min at 80^{o}C. Extracted polypeptides were collected by precipitation in 100% ice-cold acetone (2 h at –20^{o}C), dried and resuspended in 2 x LSB.

2.2.10.4 Preparation of phycobilisomes

Phosphate buffer

K_3PO_4	0.9 M, pH 7.0

Phycobilisomes (PBSs) were isolated according to Glazer (1988) with some modifications. Cells were grown in liquid BG11 medium and harvested by centrifugation. Cells were suspended in 0.9 M phosphate buffer (mixture of K_2HPO_4 and KH_2PO_4) and broken by vortexing with glass beads (0.25 – 0.5 mm, Roth). Cell extracts were solubilized with 2% Triton X-100 during 20 min at room temperature under constant agitation. Unbroken cells were removed by centrifugation at 10.000 x g for 30 min at 15^{o}C and the blue supernatant was loaded on a sucrose gradient (0.25 M – 0.79 M sucrose in phosphate buffer). After

ultracentrifugation at 130.000 x g for 16 – 18 h at 15°C the fraction of intact PBS that formed the lower band in the gradient was diluted in 0.9 M phosphate buffer and centrifuged at 80.000 x g for 4 h at 4°C. The PBS pellet was dissolved in H_2O and frozen.

2.2.10.5 Isolation of photosynthetic complexes from *Synechocystis* cells by sucrose gradient

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

Isolation of photosynthetic complexes was performed according to Sun *et al*. (1998). Cells were harvested at the late exponential stage of growth and suspended in SMN solution with protease inhibitors, PMSF (0.2 mM) and benzamidine (5 mM). Cells were broken by vortexing with glass beads and thylakoids were isolated by centrifugation at 50.000 x g for 60 min. Membranes (200 µg chlorophyll per ml) were incubated for 30 min at room temperature with 1 mM $CaCl_2$ in SMN buffer. The solubilization was performed by addition of 1.5% (final concentration) dodecyl-β-D-maltoside and incubated for 15 min on ice. Insoluble material was pelleted by centrifugation at 20.000 x g for 15 min and the supernatant was loaded on a 12 – 28% sucrose linear gradient in 10 mM MOPS, pH 7.0 and 0.05% dodecyl-β-D-maltoside. Ultracentrifugation at 160.000 x g for 16 h resolved the pigmented complexes of the photosynthetic membranes into distinct bands.

2.2.11 Phosphorylation of cyanobacterial proteins *in vivo*

Phosphorylation of *Synechocystis* cellular proteins *in vivo* was performed according to Forchhammer and Tandeau de Marsac (1994). Cells were grown in BG11 medium under LL regime (40 µE m^{-2} s^{-1}; 30°C) to an A_{750} of 0.6. Cells were harvested by centrifugation and resuspended in BG11 medium lacking phosphate and buffered with 10 mM HEPES, pH 8.0, to an A_{750} of about 3.0. For each sample 1 ml of cell culture was transferred to a small Petri dish to provide equal illumination. The phosphorylation was performed by addition of 30 µCi of carrier-free [$^{32}P_i$] with subsequent incubation under LL (40 µE m^{-2} s^{-1}) for 1.5 h. After incubation cyanobacterial proteins were precipitated with 5% (w/v) trichloracetic acid (TCA),

washed with 100% acetone and denatured in 4 x Laemmli sample buffer and heating at 80°C for 10 min.

2.2.12 Dephosphorylation of proteins by alkaline phosphatase *in vitro*

Dephosphorylation of thylakoid and phycobilisome (PBS) proteins was performed according to de Vitry *et al*. (1991). Thylakoid membrane and PBSs were incubated with bovine alkaline phosphatase (Sigma, Schnelldorf) in a buffer containing 0.1 M glycine, pH 10.4, 1 mM $MgCl_2$ and 1 mM $ZnCl_2$ for 30 min at room temperature. The reaction was stopped by additional of 20 mM EDTA.

2.2.13 Chlorophyll fluorescence measurements using a PAM fluorometer

Fluorescence measurements of *Synechocystis* cultures were performed with a pulse amplitude modulated fluorometer (PAM 2000; Walz, Germany). The dark-adapted cells were treated with high light (1.000 µE m^{-2} s^{-1}) to determine the maximum fluorescence (F_M) and the ratio F_V/F_M = $(F_M-F_0)/F_M$. Actinic light of 40 µE m^{-2} s^{-1} was used to transfer electrons through photosynthetic complexes without full reduction of PSII. The fluorescence quenching parameter qP (photochemical quenching) = $(F_M-F_S)/(F_M-F_0)$ was determined according Schreiber *et al*. (1986).

2.2.14 Low temperature (77K) fluorescence analysis

77K fluorescence was recorded using SPEX Fluorolog-2 model F212 spectrofluorometer (Industries, Inc., USA). *Synechocystis* wild-type and mutant cells were grown under LL (40 µE m^{-2} s^{-1}) and HL (400 µE m^{-2} s^{-1}) until an A_{750} = 0.5 – 0.6. Cells were normalized to 2 µg chlorophyll and adapted to the dark for 10 min. Afterwards cells were rapidly frozen in liquid nitrogen. For study of energy distribution between phycobiliproteins, PSI and PSII emission spectrum at excitation of phycobiliproteins at 570 nm was recorded. To monitor changes in pigment composition of PSII the excitation spectrum was recorded at an emission of 680 nm for phycobiliproteins.

3. RESULTS

3.1. Protein phosphorylation in thylakoids of *Synechocystis* sp. PCC 6803 wild-type

3.1.1 Detection of protein phosphorylation in *Synechocystis* thylakoids

Phosphorylation of photosynthetic proteins in *Synechocystis* was analysed in two assays: by immunological detection with antisera raised against phosphorylated amino acid residues (phospho-threonine, pThr, and phospho-serine, pSer; Zymed, San Francisco) or by *in vivo* phosphorylation with carrier-free [^{32}Pi]. For immunological analysis, thylakoid-enriched protein fractions were extracted from wild-type *Synechocystis* cells grown under standard conditions (40 µE m^{-2} s^{-1}, 30°C). Proteins were separated by 12.5% SDS-PAGE and visualised by silver-staining (Fig. 3, panel A, lane „silver") or transferred onto PVDF membranes for immunological detection of phosphorylated proteins (Fig. 3, panel A, lane „pThr"). The application of phospho-threonine and -serine antisera resulted in similar phosphoprotein profiles (Fig. 3, panel B).

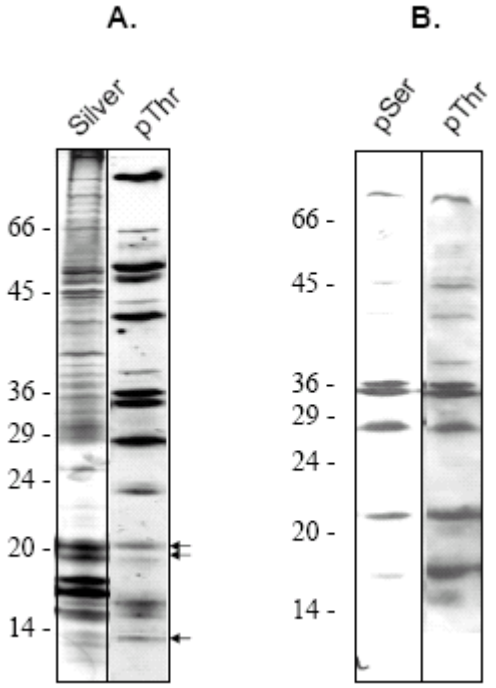


Figure 3. Detection of phosphosubstrates in isolated thylakoids of *Synechocystis* wild-type strain. Thylakoid-enriched protein fractions were extracted from wild-type cells of *Synechocystis* grown under low light (LL ~ 40 µE m^{-2} s^{-1}, 30°C) and separated by 12.5% SDS-PAGE. Protein profiles were visualised by silver-staining (panel **A**) or immunologically with antisera against phospho-threonine and -serine amino acid residues (panels **A** and **B**). Protein molecular weight markers are indicated at the left side.

To check whether similar phosphoprotein patterns resulted from serological cross-reactivity of phospho-threonine and -serine residues or from the presence of both phospho-residues, inhibitors blocking phospho-threonine or -serine antisera were applied during the

immunological assay. For blocking unspecific cross-reactivity of phospho-threonine antisera, the inhibitors masking phospho-serine residues (Zymed, San Francisco) were mixed in one immunological reaction. As a result only the signals from phospho-threonine amino acid residues should be detected. The electrophoretically separated, thylakoid proteins transferred onto PVDF membranes were treated with a mixtutre of pThr-antisera/anti-phospho-serine inhibitor or pSer-antisera/anti-phospho-threonine inhibitor. Thylakoid phosphoproteins detected with pThr or pSer antisera were used as a control (Fig. 4). Since these treatments did not change significantly the profile of phosphorylated proteins (Fig. 4) it was assumed that the phosphorylation occurred at both, threonine and serine residues. In subsequent studies we restricted analysis to the use of phospho-threonine antisera.

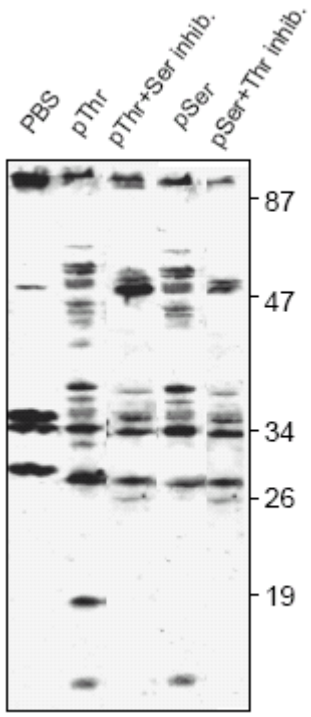


Figure 4. Cross-reactivity of antisera against phospho-threonine and -serine amino acid residues. Thylakoid proteins from wild-type strain grown under standard regime were separated by 12.5% SDS-PAGE and transferred onto a PVDF membrane. For immunological analysis antisera against phospho-threonine or -serine residues were treated with specific inhibitors for phospho-serine or -threonine residues, respectively. The immunological analysis of thylakoid phosphoproteins with antisera without inhibitor treatment was used as a control.

Between 10 and 15 phosphoproteins were detected in thylakoid preparations with antisera elicited against phospho-threonine residues (Fig. 3). The strongest phosphorylation signals were obtained for proteins with molecular masses of 16, 20, 23, 29, 33, 35, 40, 50 and 90 kDa. Obviously these proteins represent a set of phosphoproteins that are stable enough to be detected by an immunological approach. In order to detect phosphoproteins that undergo rapid phosphate exchanges an *in vivo* labelling approach with [^{32}Pi] was applied. The cell culture of wild-type *Synechocystis* was grown under normal conditions (40 µE m^{-2} s^{-1}; 30°C) until an A_{750} = 0.6 – 0.7. The cells were harvested by centrifugation and resuspended in BG11 medium without phosphate and carrier-free [^{32}Pi] (30 µCi) was added. The radiolabelled cell

suspensions were incubated in the dark (D) or under low light (LL) for 1.5 h and 3 h. The reactions were stopped by precipitation of proteins with TCA (5% w/v). Proteins were separated by 12.5% SDS-PAGE followed by blotting on to nitrocellulose membrane. The protein labelling was visualised by autoradiography (Fig. 5). The data demonstrated that the phosphorylation of some thylakoid proteins was light-dependend. The strongest phosphorylation was revealed after 3 h illumination under low light (40 µE m^{-2} s^{-1}). With the help of this strategy we have identified another set of phosphoproteins in thylakoid membranes of cyanobacteria with molecular weights in the range of 20 – 22, 27 – 36 kDa and a highly phosphorylated protein band of 66 kDa (Fig. 5).

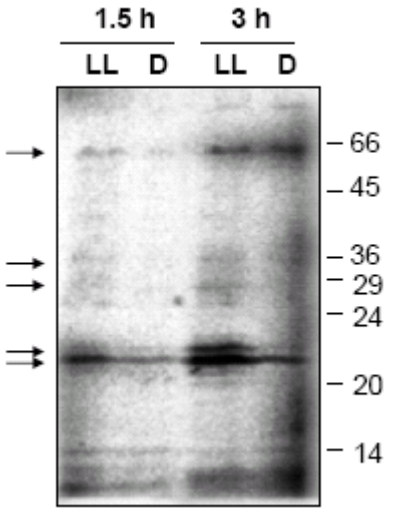


Figure 5. Labelling of thylakoid membrane proteins of wild-type *Synechocystis* with [^{32}Pi] *in vivo*. Radiolabelling of wild-type cells of *Synechocystis* was performed in BG11 medium depleted in phosphate by addition of carrier-free [^{32}Pi] (30 µCi end concentration). The radiolabelled cell suspensions were incubated in the dark (D) or low light (LL ~ 40 µE m^{-2} s^{-1}) for 1.5 and 3 h. The proteins were separated by 12.5 % SDS-PAGE and transferred onto nitrocellulose membrane. Afterwards the blot was autoradiographed and protein phosphorylation was analysed. The arrows indicate the bands which were stronger phosphorylated under LL.

3.1.2 Phosphorylation of PBS antenna proteins in the wild-type *Synechocystis* sp. PCC 6803

Comparison of silver-stained thylakoid membrane polypeptides with those of the immunological analysis suggested that the major phosphorylated proteins migrated in an electrophoretic position of phycobilisome subunits. Therefore, PBSs were isolated from wild-type *Synechocystis* cells grown under standard condition. PBS proteins were purified on sucrose gradients, separated by SDS-PAGE and proteins were visualised by silver-staining (Fig. 6, panel A, lane „silver"). The preparation of PBSs contained predominantly two groups of proteins, heavily stained phycobilin-containing proteins in the molecular weight range of 16 – 22 kDa, and less abundant proteins corresponding to the FNR protein (ferredoxin $NADP^+$ reductase) and non-bilin-containing linker peptides, rod linkers L_R^{33} and L_R^{35}, rod-core linkers L_{RC}, as well as two, mature and truncated, forms of membrane linker, L_{CM}^{99} and L_{CM}^{78},

respectively (Glazer, 1988). Immunodetection for phosphorylation with PBS preparation revealed five heavily phosphorylated bands that corresponded to linker proteins (L_{CM}^{99}, L_R^{35}, L_R^{33} and L_{RC}) and FNR which were also markedly labelled in thylakoid membrane preparations. The rod core linkers with 26 and 27 kDa were detected as one band probably due to the very close apparent molecular masses of the proteins. Although two highly phosphorylated bands in the molecular weight range between 16 and 22 kDa were detected in thylakoid membrane preparations (Figs. 2 and 3), no signals in the same region could be visualised in PBSs at least with the phospho-threonine antisera (Fig. 6, panel A, lane „pThr"). This showed that no protein phosphorylation of phycocyanin and allophycocyanin, the major proteins of cyanobacterial antenna that migrate in this size range could be detected at least by an immunological strategy.

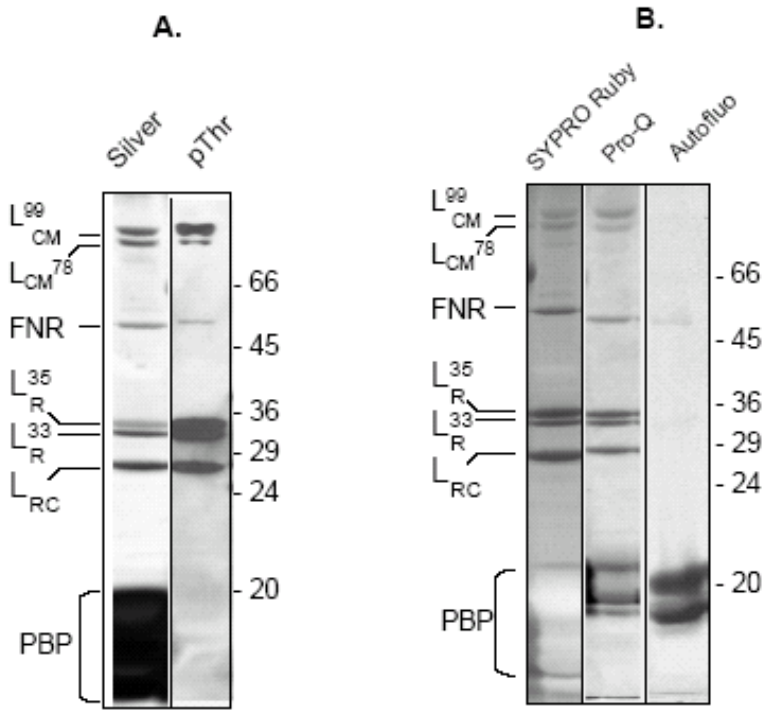


Figure 6. Phosphorylation of purified PBS antenna in *Synechocystis* wild-type strain. PBSs were isolated from wild-type *Synechocystis* cells grown under standard light regime and separated by 12.5% SDS-PAGE. The phosphorylated proteins were detected either with anti-phospho-threonine sera (panel **A**) or with fluorescent dye Pro-Q Diamond (panel **B**). Protein profiles were visualised by silver-staining (panel **A**) or with fluorescence dye SYPRO Ruby (panel **B**). The fluorescence of bilin-containing proteins (PBP), APC and PC, was detected by scanning the non-stained gels with a red laser (panel **B**, lane „Autofluo"). Protein molecular weight markers are indicated at the right; PBS proteins are marked at the left (L_{CM}^{99} and L_{CM}^{78} – core membrane linkers; $L_R^{33,\ 35}$ - rod linkers, L_{RC} - rod core linkers, FNR – ferredoxin $NADP^+$ reductase).

Another approach for analysis of protein phosphorylation in PBS antenna was based on the utilisation of protein staining with fluorescence dyes SYPRO Ruby and Pro-Q Diamond (Fig. 6, panel B). SYPRO Ruby, which is a quantitative and highly sensitive dye for protein detection stained saturating amounts of phycobiliproteins (APC and PC) negatively. Phosphorylated proteins were visualised with fluorescence dye Pro-Q Diamond that reacts with phosphorylated residues (Fig. 6, panel B). The results that were obtained with the fluorescence Pro-Q Diamond dye, were similar to those with phosphospecific serum, although the dye displayed additional signals in the range of PC/APC. To understand the nature of the signals originating in PC/APC proteins the non-stained gel loaded with PBS proteins was scanned with a red laser set at an excitation/emission wavelength suitable for detection bilin-containing proteins. (Fig. 6, panel B, lane „Autofluo"). It was observed a very weak signal comigrating with FNR and intense signals from the PC and APC bands. This showed that the PC/APC signals obtained from Pro-Q staining corresponded to self-fluorescence of bilin proteins and not to phosphorylation of PC/APC. A limited shift in position of the FNR between lanes „Pro-Q" and „Autofluo" versus lanes „Silver" and „SYPRO Ruby" (Fig. 6, panel B) occured due to changes in gel sizes during various staining and blotting procedures.

3.1.3 Phosphorylation of thylakoid proteins in PBS-deficient mutants

In order to assess whether the phosphorylation signals actually originated from the PBS linkers rather than from minor comigrating contaminating polypeptides, the protein phosphorylation strategy was tested in the mutant strains, PAL and CK, deficient in various PBS proteins. The PAL mutant (PC^-; *ΔapcAB/apcE*) bears a deletion in genes encoding the L_{CM}^{99} (*apcE*) and allophycocyanin proteins (*apcAB*) in a phycocyanin deficient background (Ajlani and Vernotte, 1998a). The CK mutant was deleted in the entire *cpc*-cluster including *cpcB* (β-PC), *cpcA* (α-PC), *cpcC* (L_R^{33} and L_R^{35}), *cpcD* (L_R^{10}) genes (Ughy and Ajlani, unpublished). These mutations resulted in a decrease of PBS sizes because of a lack of rods (CK mutant) or in a complete lack of PBS antenna (PAL mutant). Staining of the PBS proteins after SDS-PAGE with Coomassie Brilliant Blue showed that PBSs were still present in CK and were smaller in size but contained only APC (Fig. 7, panel A). Since not all genes encoding PBS-constitutive proteins were deleted from the cyanobacterial genome, both strains were analysed for the presence of all linker proteins by immunodetection with antisera raised against the various linker and bilin-containing polypeptides (Fig. 7, panel B). According to immunological analysis none of the PBS proteins was present in the PAL strain. In the CK strain the absence of phycocyanin rods

and rod linkers $L_R^{33, 35}$ prevented the accumulation of newly synthesised L_{RC} that connect the APC-containing core and the PC rods of PBS (Fig. 7, panel B).

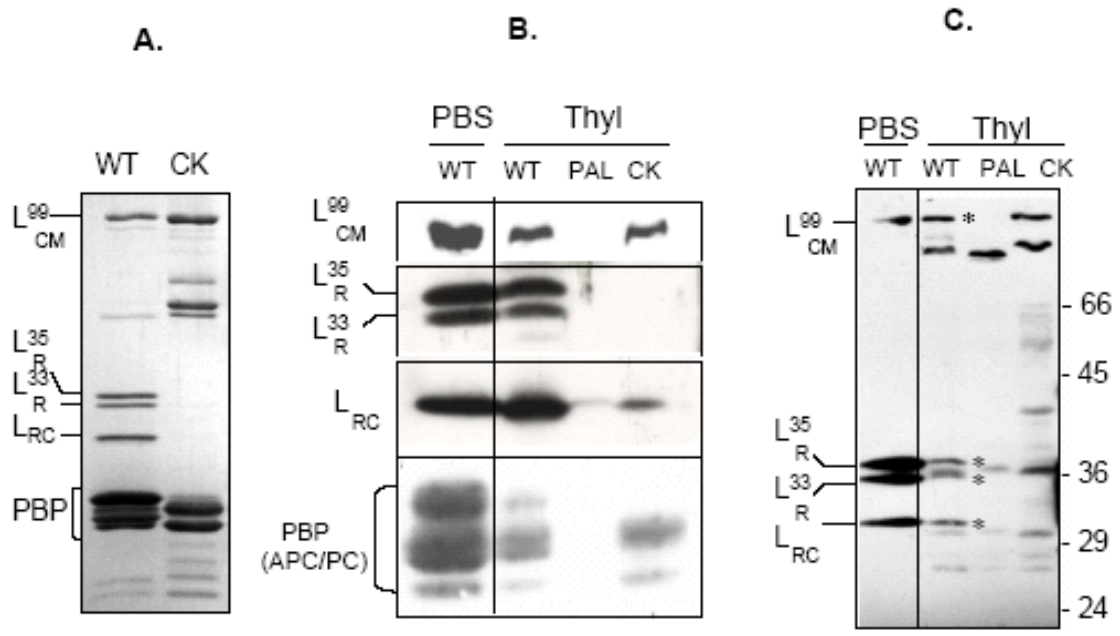


Figure 7. Analysis of phosphoproteins in the wild-type and mutant strains deficient in PBS proteins. PBSs were extracted from the wild-type and linker-deficient CK strains. PBS antenna proteins were separated by 12.5% SDS-PAGE and stained with Coomassie Brilliant Blue (panel **A**). Thylakoid proteins extracted from the wild-type, CK and PAL mutants were detected immunologically using antisera raised against core membrane (L_{CM}^{99}), rod ($L_R^{33, 35}$), rod-core (L_{RC}) linkers, and APC/PC proteins (panel **B**). The extracted pure PBSs from the wild-type were used as a control. Phosphorylated proteins from thylakoid membranes of the wild-type, CK and PAL mutants were identified by immunodetection with phospho-threonine antisera (panel **C**). PBSs extracted from wild-type cells were used as a control to identify linker proteins among other phosphorylated thylakoid proteins. PBS proteins are marked at the left side, protein molecular weight markers are indicated at the right side. Stars indicate the phosphorylated linker proteins in the wild-type thylakoid preparation.

These rod core linkers were still detectable in the CK strain but to a much lower extent than in the wild-type. The CK strain still contained allophycocyanin in PBS core and L_{CM}^{99} linker in a similar amount as in the wild-type. The phosphorylation patterns of thylakoid membranes of wild-type, PAL and CK mutant strains were also probed with phospho-threonine antisera (Fig. 7 panel C). PBSs extracted from the wild-type were loaded as a control for migration of linker proteins. High resolution of the 29 – 36 kDa region revealed three phosphorylated bands in the range of rod linkers with the central one showing no correspondence to PBS subunits. No phosphorylated bands were detected at any linker position in the PAL mutant (Fig. 7, panel C). The core-membrane linker (L_{CM}^{99}) remained detectable as a phosphoprotein in the CK mutant

at the same rate as in the wild-type, while no phosphorylated band remained at the positions of the other linkers. Since the absence of linker polypeptides in the mutants resulted in the loss of the corresponding phospho-threonine signals, it was concluded that the rod linkers were phosphorylated in the PBSs of the wild-type.

3.1.4 Dephosphorylation of PBS linker proteins *in vitro*

As an additional proof of linker phosphorylation *in vivo*, PBSs were dephosphorylated *in vitro* by incubation with alkaline phosphatase (Sigma, Schnelldorf) that is specific for O-dephosphorylation of threonine or serine residues.

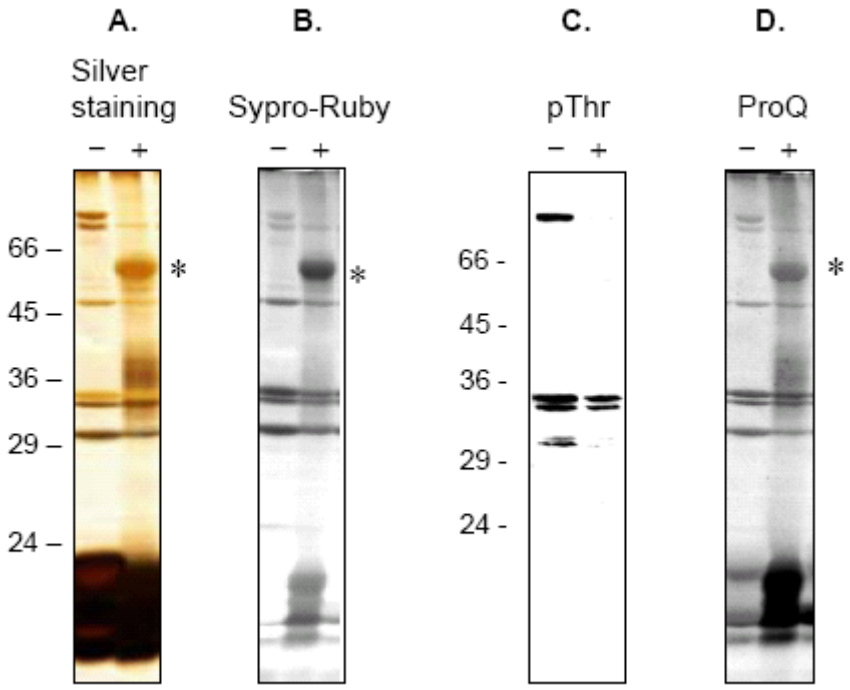


Figure 8. Dephosphorylation of the PBS linker proteins by alkaline phosphatase *in vitro*. The PBSs were isolated from wild-type *Synechocystis* and treated (+) with alkaline phosphatase (AP). Non-treated (-) PBSs were used as a control. Phycobilisome proteins were separated by 10% SDS-PAGE. The protein profiles were visualised by staining with silver nitrate (panel **A**) or fluorescence dye SYPRO Ruby (panel **B**). Protein phosphorylation was immunologically detected with phospho-threonine antiserum (panel **C**) or by staining with fluorescent dye Pro-Q Diamond (panel **D**). Star indicates the alkaline phosphatase protein band.

The PBSs extracted from wild-type *Synechocystis* were treated with alkaline phosphatase according to de Vitry *et al*. (1991). Non-treated PBSs were used as a control. The proteins separated by 10% SDS-PAGE were visualised by staining with silver nitrate or fluorescence

dye SYPRO Ruby (Fig. 8, panels A and B). For an analysis of the dephosphorylation rate proteins were transferred onto PVDF membrane and immunodetected with phospho-treonine antiserum (Fig. 8, panel C) or the gel was stained with fluorescence dye Pro-Q Diamond (Fig. 8, panel D). Only partial dephosphorylation of the linker proteins was observed. The membrane linker L_{CM}^{99} and rod core linkers L_{RC} have demonstrated a higher rate of dephosphorylation than rod linkers $L_R^{33, 35}$.

3.1.5 Dephosphorylation of PBS linkers is involved in the protease recognition

One of the major roles of protein phosphorylation is the signalling for protein degradation. Since linker proteins can be degraded under exposure of cyanobacterial cells to higher light intensity or nitrogen deprivation we raised the question, whether phosphorylation/dephosphorylation of linker proteins is involved in their degradation and/or can influence the stability and assembly of PBSs. To check this hypothesis, thylakoid proteins were dephosphorylated *in vitro* by incubation with alkaline phosphatase and non-treated and dephosphorylated samples were subjected to protein degradation *in vitro*.

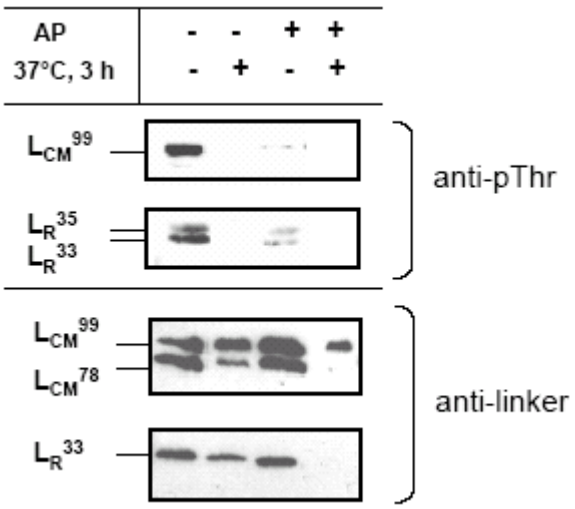


Figure 9. Degradation of linker proteins *in vitro*. Thylakoid-enriched proteins were isolated from wild-type *Synechocystis* cells grown under low light (LL ~ 40 µE m^{-2} s^{-1}, 30°C). Isolated thylakoid proteins were incubated in the absence (-) or presence (+) of alkaline phosphatase (AP). Treated and non-treated with alkaline phosphatase thylakoid fractions were incubated at 37°C (+) or at 20°C (-) for 3 h. The phosphorylation grade was immunologically detected with anti-phospho-threonine serum. The level of linker protein degradation was immunologically detected with antisera raised against various linker proteins.

Non-treated and dephosphorylated thylakoid proteins were incubated in a water bath for 3 h at 37°C for activation of proteolytic processes involving thylakoid associated or membrane-integrated proteases. The dephosphorylated state of linker proteins was tested with anti-phospho-threonine serum. At least 70 – 80% of the linker protein preparations could be

dephosphorylated by alkaline phosphatase (Fig. 9, lane „+/-"). The rate of dephosphorylation of linker proteins with alkaline phosphatase was higher than observed before (Fig. 8, panel C) due to using thylakoid membranes that were frozen and thawn afterwards. Dephosphorylation of linker proteins also occurred in the absence of alkaline phosphatase during incubation for 3 h at 37^{o}C (Fig. 9, lane „-/+"). Western analysis with linker proteins showed that they were partially degraded in the latter case. When PBSs were dephosphorylated prior to incubation at 37°C, their degradation was almost complete (Fig. 9, lane „+/+"). These data indicated that the dephosphorylation of linker proteins could enhance their degradation rate.

3.1.6 Dephosphorylation occurs only in partially disassembled phycobilisomes

The linker proteins are usually highly embedded in PC hexamers and different enzymes cannot have an easy access without partial disassembly of the PBS structure. To check, whether only partially disassembled or intact PBSs can be dephosphorylated, intact PBSs were resuspended in water or in phosphate buffer of very low concentration, conditions which lead to partially disorganization of PBS structure (Rusckowski and Zilinskas, 1982), and treated with alkaline phosphatase. PBSs pre-treated or non-treated with alkaline phosphatase were separated on sucrose gradients. As shown by the silver-staining pattern of the 16 – 22 kDa region containing the PC/APC proteins, intact PBSs were found at the bottom of the gradient (Fig. 10, fraction 1), while partially or fully disassembled PBSs were found at the top of the gradient (Fig. 10, fractions 2 to 20). Phosphorylated linker proteins in the region of 24 – 37 kDa were detected immunologically with an anti-phospho-threonine serum and are presented in the lower panels of Fig. 10. Without alkaline phosphatase treatment, linker proteins were heavily phosphorylated in fraction 1 but less efficiently in fractions 9 – 16 where most PBS proteins were detected by silver nitrate staining. After dephosphorylation by alkaline phosphatase, the PBS preparation resolved phosphorylated linkers only in intact PBSs (fraction 1), but no other phosphorylation was observed all over the gradient fractions (lower panel, + AP). At the same time the phycobiliproteins were all shifted to the upper part of the gradient indicating that they underwent further disassembly. This experiment demonstrated that linker proteins can be subjected to dephosphorylation only in partially disassembled PBS structures and that their dephosphorylation enhances disassembly of the PBS antenna.

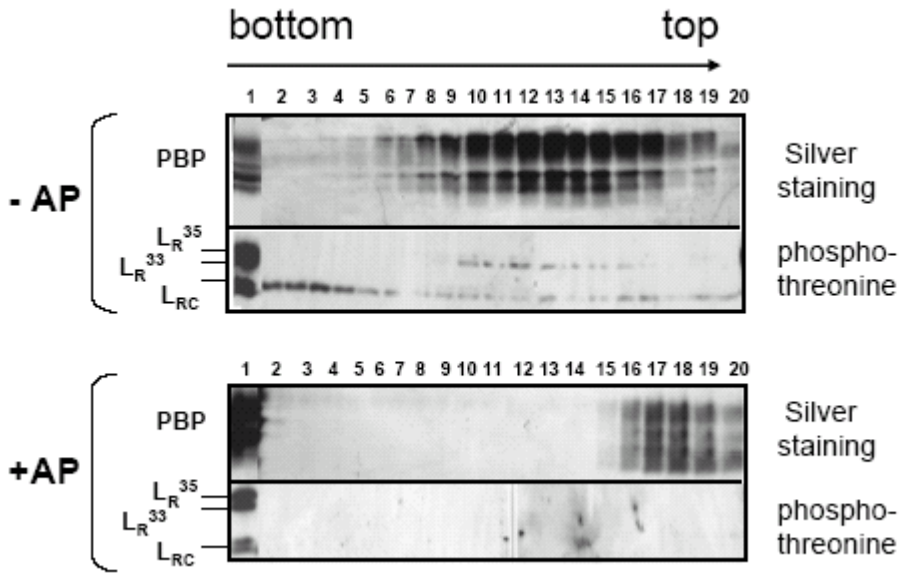


Figure 10. Dephosphorylation of the PBS linker proteins by alkaline phosphatase and their separation in the sucrose gradients. PBS fractions treated and non-treated with alkaline phosphatase (+ AP and – AP) were separated on 0.25 – 0.79 M sucrose gradients. Fractions of 0.2 ml were collected from the bottom of the gradient and separated by 12.5% SDS-PAGE. Proteins were detected by silver-staining (the upper panels show experiments with and without AP) and by immunological analysis with phospho-threonine antiserum (lower panels). From silver-staining, only the 16 – 22 kDa region with APC/PC proteins (PBP; phycobiliproteins) is presented. For immunological analysis, the part of the gels of 24 – 38 kDa with rod and rod-core linkers is shown.

3.1.7 Level of phosphorylation of linker proteins during state transition

Light is one of the major environmental factors that are involved in the modification of light-harvesting antenna in higher plants and cyanobacteria. Phosphorylation/dephosphorylation of light-harvesting antennae in higher plant chloroplasts, which is involved in state transitions and antenna degradation, is controlled by light intensity and quality.

In higher plants in the state 1 the plastoquinone pool becomes oxidized and the mobile pool of LHCII antenna proteins is dephosphorylated and move to PSII. State 2 is caused by reduction of the plastoquinone pool and the mobile pool of LHCII antenna proteins is phosphorylated and move to PSI (Haldrup *et al*., 2001; Wollman, 2001; Takahashi *et al*., 2006). In cyanobacteria the mechanisms involved in regulation of light induced state transitions are not well characterized. It was suggested that state transitions are not controlled by phosphorylation of antenna proteins. To check whether the phosphorylation status of PBS linker proteins is

modified upon state transitions, the wild-type *Synechocystis* cells were placed in state 1 and state 2 and phosphorylation of proteins was analysed immunologically. To obtain state 2 cyanobacteria cells were grown under LL regime until an A_{750} = 0.6 – 0.8 and afterwards cells were incubated in the dark for 30 min. Alternatively, for state 2 cells were transferred from dark to HL (400 μE m^{-2} s^{-1}) for 30 min. The adaptation of cyanobacterial cells in the dark leads to respiratory electron flow and generally poises the electron transport chain toward a reduced state. To block cells in state 1, cells were grown under LL and the inhibitor 3-(3,4-dichlorphenyl)-1,1-dimethylurea (DCMU) (10 μM), that induces the closure of all PSII centres and inhibits the reduction of the PQ pool, was added immediately. Afterwards, the cells were rapidly frozen in liquid nitrogen, thylakoid membranes were isolated and probed with anti-phospho-threonine antibody. The phosphorylation/dephosphorylation rate of linker peptides was not changed during state transitions (Fig. 11).

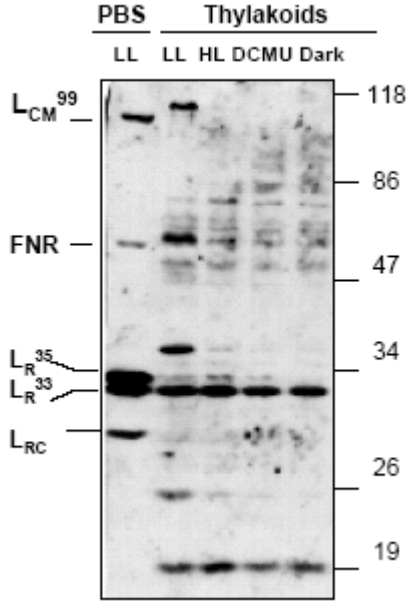


Fig. 11. Phosphorylation of linker proteins during state transitions. *Synechocystis* wild-type cells were grown under LL. The cell cultures were then transferred to state 2 or state 1. For state 2 cells were acclimated to the dark (D) or were transferred to HL for 30 min. For state 1 cells were acclimated to the LL with addition of DCMU (10 μM). To check the level of phosphorylation of linker proteins during short-term acclimation, the *Synechocystis* cells were rapidly frozen in liquid nitrogen, thylakoid membranes were isolated and the phosphorylation grade was immunologically detected with phospho-threonine antisera. As a control, thylakoid membrane and PBSs were used.

The acclimation of cyanobacterial cells to HL for 30 min led to dephosphorylation of L_{CM} linker, FNR and few proteins with molecular weights of about 36 and 25 kDa (Fig. 11). The state transition in cyanobacteria is a very rapid process that takes place in time periods from second to several minutes (Joshua and Mullineaux, 2004). The acclimation of cells to HL for 30 min that induces the reduction of plastoquinone also obviously activates some other mechanisms distinct from state transitions.

3.1.8 Dephosphorylation of PBS linker proteins is enhanced under high light or under nitrogen limiting conditions

The cyanobacterial PBS antenna are highly rearranged and in part degraded upon increasing light intensities as well as under nitrogen limiting conditions (Grossman *et al*., 1993a). The effects of high light and nitrogen deprivation on the *in vivo* phosphorylation of linker proteins was analysed in wild-type *Synechocystis* cells that were acclimated to these conditions for 24 h. To better distinguish all phospholinker polypeptides from the other phosphoproteins, the whole cell protein extracts were separated into membrane (thylakoid-enriched) and soluble (cytoplasm) fractions. After 10% SDS-PAGE the protein profiles were visualised by staining with Coomassie Brilliant Blue (Fig. 12, panel A).

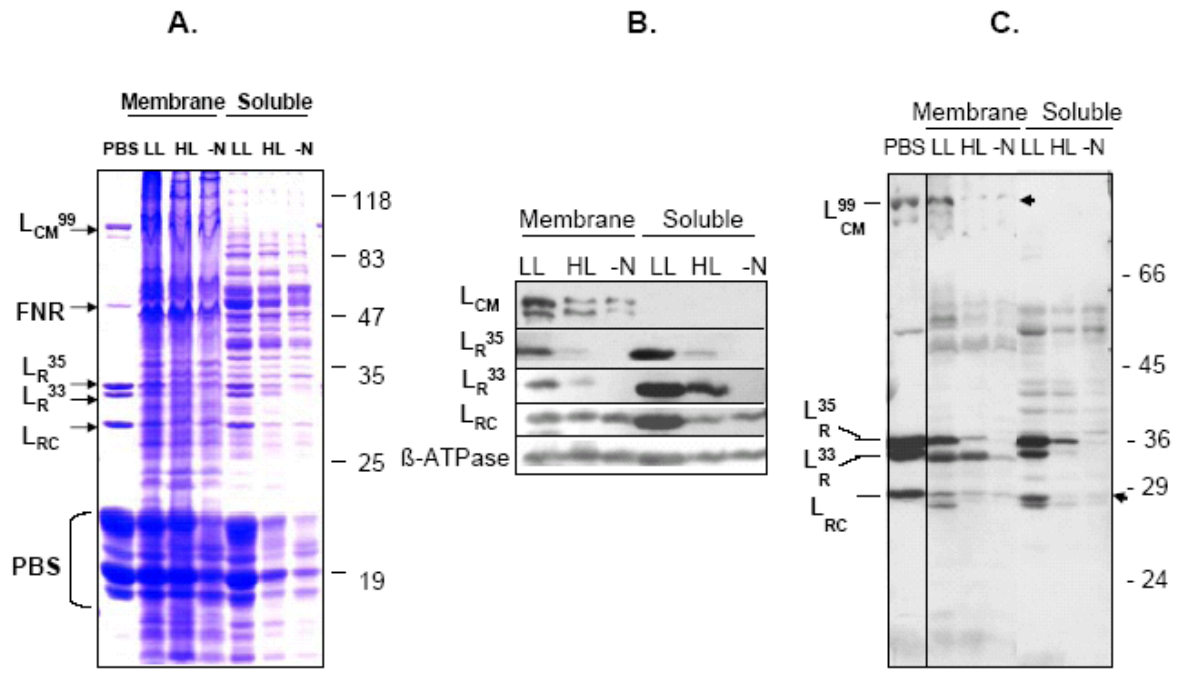


Figure 12. Phosphorylation levels of PBS linker proteins under stress conditions. *Synechocystis* wild-type cells were grown under LL and then acclimated to HL and nitrogen deprivation (- N) for 24 h. Cell cultures were normalised to the same optical dencity and proteins were separated into membrane (thylakoid-enriched) and soluble (cytoplasm) fractions. Proteins were separated by 10% SDS-PAGE and stained with Coomassie Brilliant Blue (panel **A**). PBSs were used as a control for identification of linker proteins among other thylakoid proteins. The amount of linker proteins was controlled by antisera elicited against linker proteins (panel **B**). The ß subunit of ATP synthase complex was used as a loading control. The phosphorylation rate was detected by immunological analysis with phospho-threonine antisera (panel **C**). Arrows indicate the strongly dephosphorylated L_{CM} and L_{RC} linkers.

In order to assess the rates of phosphorylation of each linker relative to its actual content under each growth condition the thylakoid-enriched and soluble fractions were probed with antibodies raised against linker proteins (Fig. 12, panel B) and phospho-threonine amino acid residues (Fig. 12, panel C). Immunological analysis revealed PBS proteins in both, the membrane and soluble fractions, with exception of the L_{CM}^{99} linker that was not detected in the cytoplasm (Fig. 12, panel B). The absence of L_{CM}^{99} could be due to rapid degradation upon release of PBSs from the thylakoid membranes. Under HL all linkers were detected in the membrane fraction but the amount of the distal L_R^{33} and L_R^{35} linkers decreased significantly.

Immunological analysis with phospho-threonine amino acid residues showed that HL and nitrogen deprivation caused a marked decrease in the phosphorylation rate of the L_{RC} and L_{CM}^{99} linkers relative to their quantity in the soluble and membrane fraction. The drop in the phosphorylated state was respectively two and four times higher than the drop in the total content. The dephosphorylation rates of L_R^{33} and L_R^{35} were not higher than their degradation rates which argues for a rapid degradation of the dephosphorylated forms. Thus, the environmental changes that cause an increased disassembly and degradation of PBS are accompanied by a dephosphorylation of several PBS linkers that include the membrane and rod-core linkers.

3.1.9 Characterisation of thylakoid protein phosphorylation in *Synechocystis* kinase and phosphatase mutants

3.1.9.1 Dephosphorylation of linker proteins in the mutants deficient in serine/threonine phosphatases

The *Synechocystis* sp. PCC 6803 genome encodes eight genes for members of the PP2C (PPM superfamily) and one gene for a putative PPP-type phosphatase families (Keren *et al*., 1997; Kruse *et al*., 1997). It has been reported that the members of phosphatase PPM superfamily participate in modulating responses to environmental stresses, such as anoxia, heat, osmotic shock, etc. (Kruse *et al*., 1997). In order to find the phosphatase(-s) responsible for the dephosphorylation of PBS linker proteins the phosphorylation/dephosphorylation levels of corresponding proteins were analysed in the phosphatase-deficient mutants. The two mutants, *Δsll1033* and *Δsll1771* (*ΔpphA*), inactivated in the genes encoding the PP2C-type phosphatases (PPM superfamily) were kindly provided by K. Forchhammer (Germany, Gießen). The Pph

phosphatase is the product of the *sll1771* gene and is the first protein phosphatase in *Synechocystis* 6803 for which the role has been identified recently (Irmler and Forchhammer, 2001). This phosphatase is essential for the dephosphorylation of the PII signalling protein which represents the central signal transducer of nitrogen control in proteobacteria. The mutants deleted in *sll1771* and *sll1033* genes were checked for their ability to acclimate to nitrogen deprivation. Since the acclimation of wild-type *Synechocystis* cells to high light or to nitrogen starvation for at least 24 h led to a marked decrease of the phosphorylation grade of the L_{CM}^{99} and L_{RC} linkers (Section 3.1.8), the *Δsll1033* and *Δsll1771* (*ΔpphA*) mutants were analysed for dephosphorylation of linker proteins after acclimation of cell cultures to one of these stress conditions.

The wild-type and *Δsll1033* and *Δsll1771* mutant strains were grown under LL regime. Cell cultures were normalized to an A_{750} = 0.5 and grown for the next 24 h in nitrogen-deprived BG11 medium. The mutant strains did not exhibit any phenotypical differences to wild-type under LL in the full BG11 medium (Fig. 13 panel A, „+ N"). The absorption spectra of the cyanobacterial cultures also did not show any changes between strains under this regime (Fig. 13, panel B, „+ N"). Under nitrogen deprivation wild-type cells bleached slightly more rapidly than the mutant strains (Fig. 13, panel A, „– N"). In wild-type cells the loss of PBSs could be visualised by a decrease of the absorption peak at 620 nm which corresponds to PC and APC (Fig. 13, panel B, „– N"). In both mutant strains the loss of PC/APC absorbance peak was also observed (Fig. 13, panel B, „– N"). However, the decreases of PC absorption in cells grown in medium sufficient in nitrogen relative to nitrogen-defficient medium was less pronounced than in the wild-type. The ratio of absorbance peaks of PC at nitrogen supplied versus PC at nitrogen deficient media was ~ 1.4 in the wild-type and ~ 1.21 in *Δsll1033* and *Δsll1771* mutant strains.

The phosphorylation state of linker proteins was determined by separation of cellular proteins by denaturing gel electrophoresis followed by immunological analysis. The wild-type, *Δsll1033* and *Δsll1771* mutants were grown under LL (40 μE m^{-2} s^{-1}) until an A_{750} = 0.5 and depleted in nitrogen for 24 h. Equal volumes of cell cultures were normalized to the same optical density and used for isolation of cyanobacterial cellular proteins. Total proteins were separated into membrane (thylakoid-enriched) and soluble (cytoplasm) fractions. The thylakoid proteins were separated by electrophoresis on a 10% SDS-PAG and phospho-proteins were immunodetected with antibodies against phospho-threonine residues (Fig. 14, panel A).

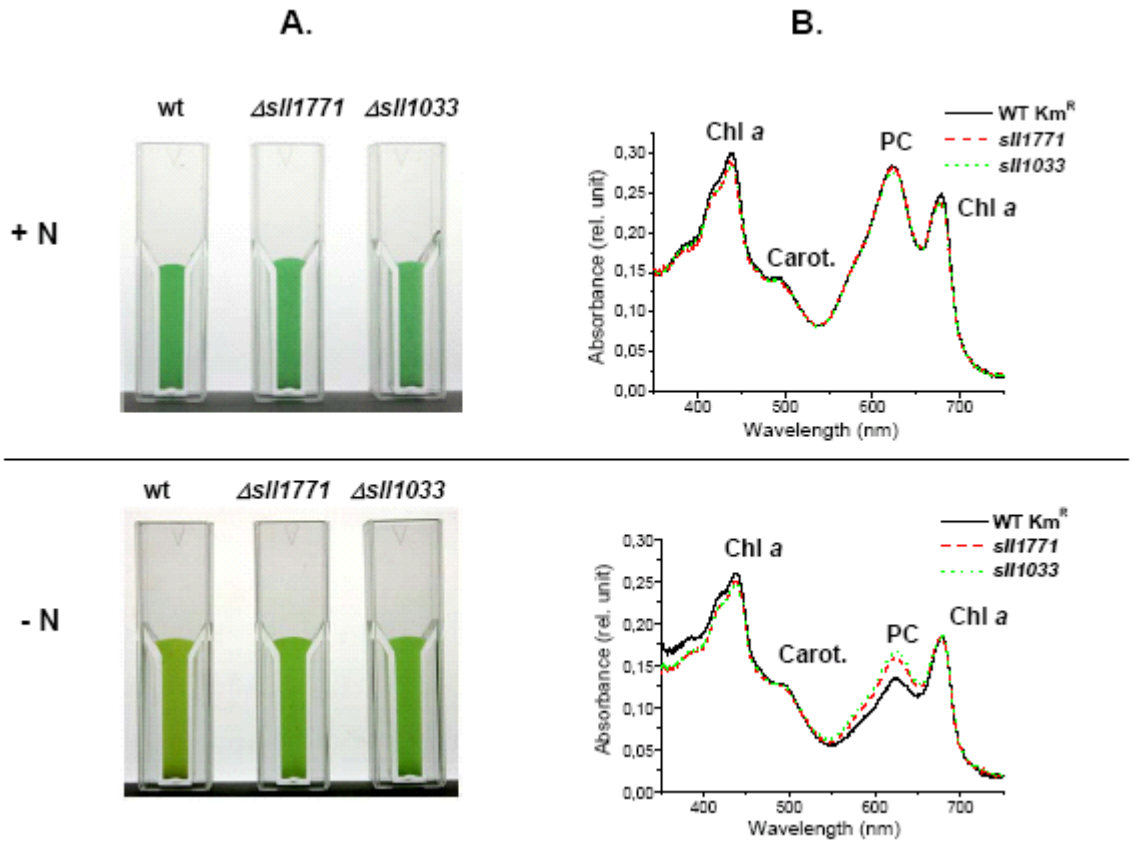


Figure 13. Phenotypical and spectroscopic analysis of wild-type and mutant cells deleted in serine/threonine phosphatases under nitrogen-sufficient and –deficient conditions. Phenotypical modifications of wild-type, *Δsll1771* (*ΔpphA*) and *Δsll1033* mutant strains in full (+ N) and nitrogen-depleted (- N) BG11 media for 24 h (panel **A**). For measurements of the cell absorbance at A_{750} cell suspensions were normalised to 0.4 – 0.5. Absorption spectra were taken for cells grown in full and nitrogen-depleted BG11 media (panel **B**).

The amount of PBS linker proteins was controlled by visualisation with antibodies raised against L_{CM}, L_R[33, 35] and L_{RC} polypeptides (Fig. 14, panel B). Western analysis demonstrated that the phosphorylation rate of L_{CM}^{99} and L_R^{33} proteins under nitrogen deprivation was unaffected in *Δsll1033* strain when compared to the wild-type (Fig. 14, panel A). Interestingly, the phosphorylation level of L_{CM}^{99} linker protein in *Δsll1771* (*ΔpphA*) mutant was increased even under LL conditions in comparison with the wild-type and *Δsll1033* mutant (Fig. 14, panel A), while the rate of degradation of L_{CM}^{99} under nitrogen deprivation in mutant *ΔpphA* was similar to that of the wild-type (Fig. 14, panel B). Higher stability of PBS linker proteins L_{CM}^{99} and L_R[35, 33] was observed in the mutant deleted in the *sll1033* gene (Fig. 14, panel B). Comparative analysis of phosphoproteins also demonstrated some changes in the degree of dephosphorylation of several thylakoid proteins including core-membrane (L_{CM}^{99}) and rod

linker (L_R^{35}) proteins in the *Δsll1033* mutant, which were more stably phosphorylated (Fig. 14, panel A). Thus, the dephosphorylation of membrane linker proteins under nitrogen starvation was inhibited in the mutant deficient in Sll1033 serine/threonine phosphatase.

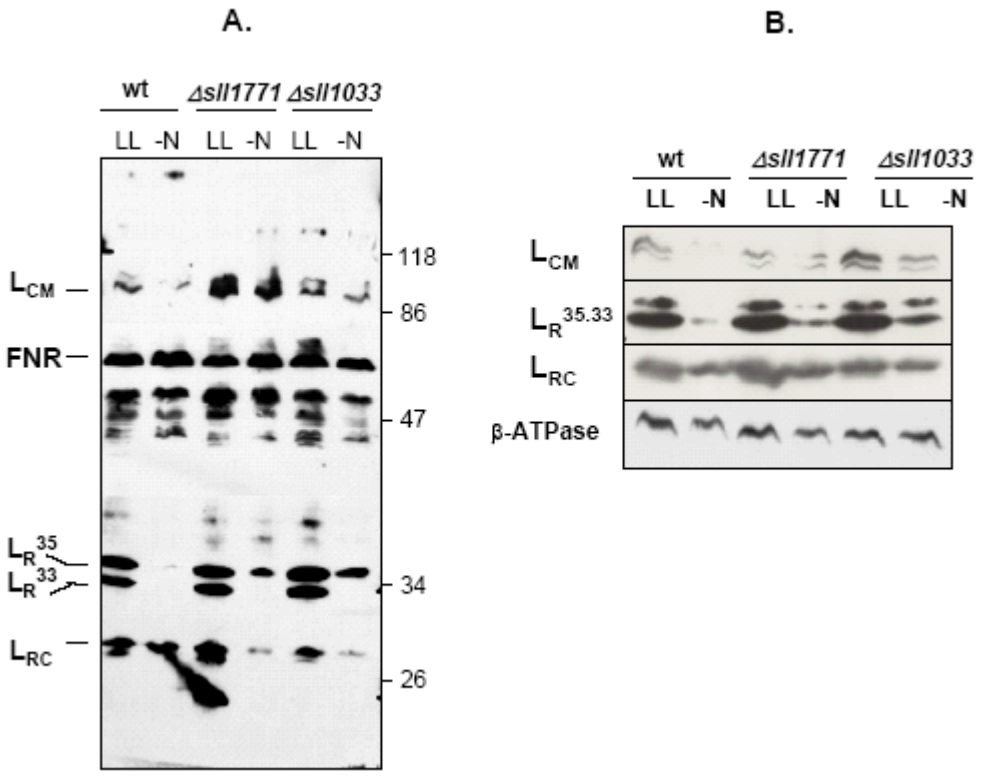


Figure 14. Phosphorylation levels of thylakoid proteins in mutants deficient in serine/threonine phosphatases. *Synechocystis* wild-type and the phosphatase deficient mutants *Δsll1771* and *Δsll1033* were grown under LL in BG11 medium. Cell cultures were normalised to an A_{750} = 0.5, resuspended in BG11 medium deficient in nitrogen and grown for further 24 h (- N). Thylakoid-enriched proteins were separated by 10% SDS-PAGE. The phosphorylation degree was detected by immunological analysis with phospho-threonine antisera (anti-pThr) (panel **A**). Amounts of linker proteins were controlled by antisera raised against linker proteins (panel **B**).

3.1.9.2 Phosphorylation state of linker proteins in the kinase mutants

For identification the kinase(-s) that could be involved in the phosphorylation of linker proteins, three kinase-deficient mutants, *Δsll0776, Δslr0559* and *Δslr0152* (kindly provided by Dr. S. Bedu; Marceille, CNRS), were analysed in the same way as the phosphatase-deficient mutants (Section 3.1.9.1).

The analysis of phenotypical modifications and absorption spectra did not revealed any visual changes between wild-type and mutants grown under low light regime in BG11 medium (Fig. 15, panels A and B, „+ N"). Under nitrogen deprivation for 24 h all mutant strains tested exhibited a phenotype similar to that of the wild-type. However, the *Δslr0559* mutant bleached slightly less then the other strains (Fig. 15, panel A, „– N"). Although all strains demonstrated a decrease of the PC/APC absorption peak at 620 nm, mutant *Δslr0559* showed a higher PC/APC versus chlorophyll *a* peak (Fig. 15, panel B, „– N").

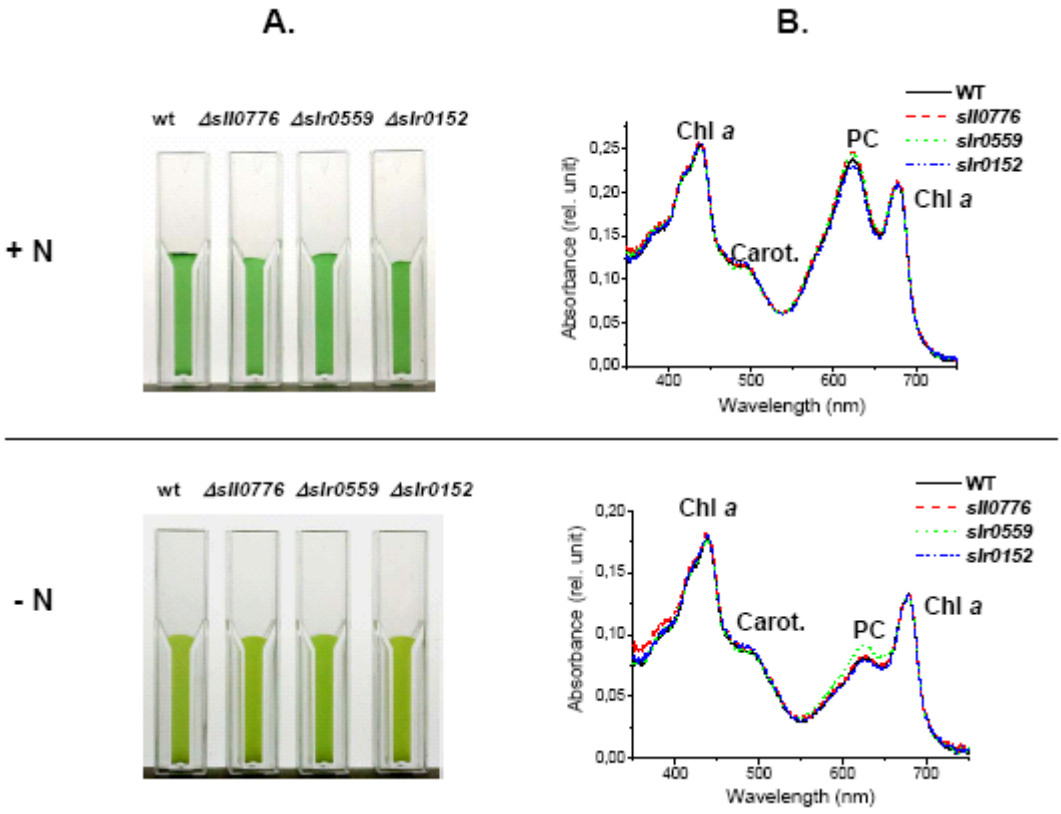


Figure 15. Phenotypical characterisation and absorption spectra of wild-type and kinase-deficient strains under nitrogen deprivation. Cyanobacterial wild-type and mutant cells were normalized until an A_{750} = 0.4 – 0.5 and absorption spectra were measured on the cells grown under nitrogen supplied (+ N) and deficient (- N) conditions.

The effect of nitrogen deprivation on the phosphorylation/dephosphorylation degree of linker proteins in the kinase-deficient mutants was detected immunologically with an anti-phospho-threonine serum (Fig. 16, panel A). In order to assess the actual content of these proteins they were probed with antibodies raised against linker proteins (Fig. 16, panel B). The ß subunit of ATP synthase complex was used as a loading control. Western analysis demonstrated that in wild-type, *Δsll0776* and *Δslr0152* mutants the amounts of L_{CM}^{99} strongly decreased under

nitrogen deprivation. Under the same conditions the degradation of L_{CM}^{99} linker retained in *Δslr0559* mutant. A comparative analysis of phosphoproteins demonstrated some changes in the phosphorylation rate of a few proteins in the *Δslr0559* mutant (L_{CM}, L_R^{35} and L_{RC} linkers). Surprisingly, the level of L_{CM}, L_R^{35} and L_{RC} phosphorylation was higher in *Δslr0559* mutant.

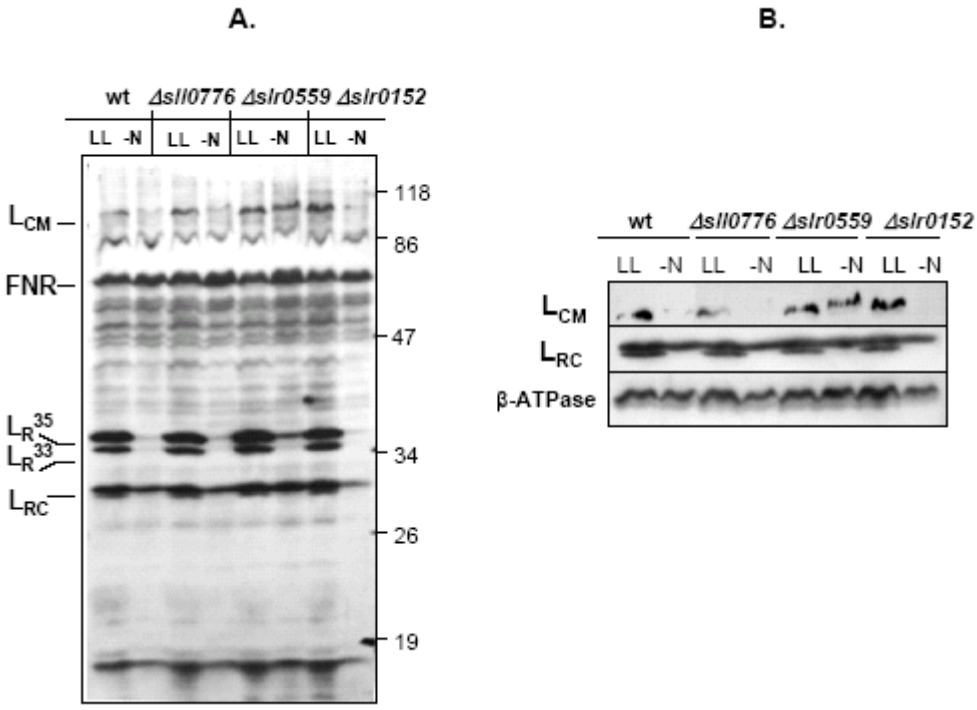


Figure 16. Phosphorylation levels of thylakoid proteins in the kinase mutants. Cell cultures of the wild-type and kinase deficient mutants (*Δsll0776, Δslr0559* and *Δslr1052)* were grown in the BG11 medium. Cells were normalised to an A_{750} = 0.5, sedimented and incubated in the nitrogen-depleted (- N) medium. Cells were grown for further 24 h. Thylakoid-enriched proteins were separated by 10% SDS-PAGE and transferred onto PVDF membranes. The phosphorylation rate was detected by immunological analysis with phospho-threonine antisera (anti-pThr) (panel **A**). Amounts of linker proteins were controlled by antisera against linker proteins (panel **B**). The immunoreaction with antisera raised against β subunit of ATP synthase was used as a loading control.

3.2 Functional analysis of the cTLP40 PPIase

3.2.1 Analysis of the cTLP40 protein sequence

TLP40 (thylakoid lumen PPIase) was the first identified complex immunophillin-like protein associated with the photosynthetic membranes in spinach chloroplasts. This protein, which is a soluble protein of 40 kDa in the thylakoid lumen, exhibits multifunctional properties including peptidyl prolyl *cis-trans* isomerase (PPIase) activity that is involved in protein folding. It was suggested that TLP40 is essential for the regulation of the protein phosphatase, which dephosphorylates the reaction centre subunits of photosystem II (PSII; Fulgosi *et al*., 1998). The genome of the cyanobacterium *Synechocystis* sp. PCC 6803 (Kaneko *et al*., 1996; http://www.kazusa.or.jp/cyano/cyano.html) encodes a homologous protein named cTLP40 (cyanobacterial TLP40). cTLP40 is a protein of 42 kDa that displayed high protein homology (about 47%) and a similar domain structure to the plant component. Hydropathy analysis of the deduced cTLP40 amino acid sequence suggested a predominantly hydrophilic protein similarly to TLP40 (Fig. 17).

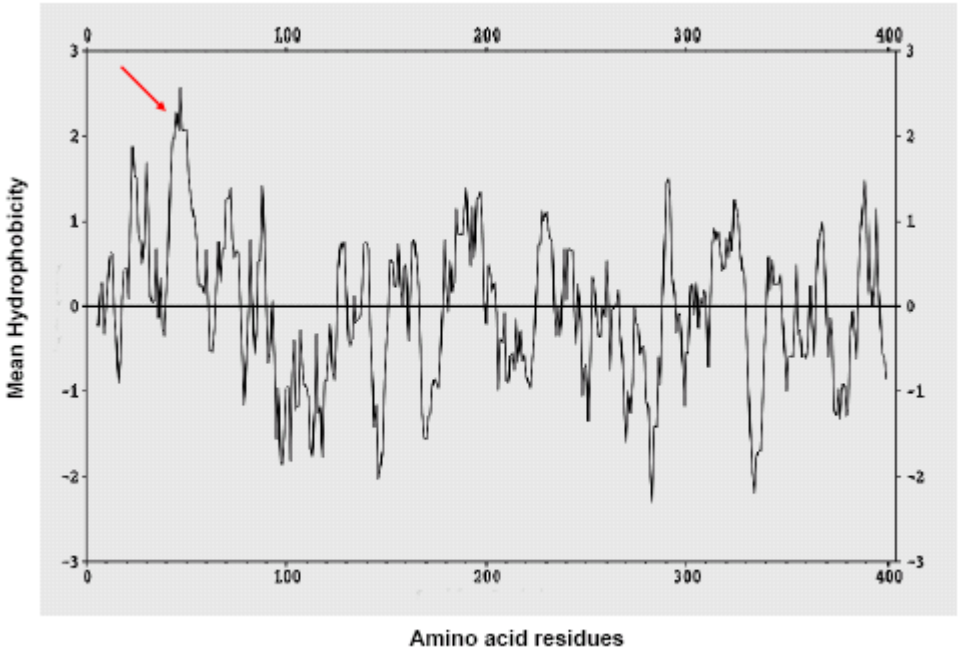
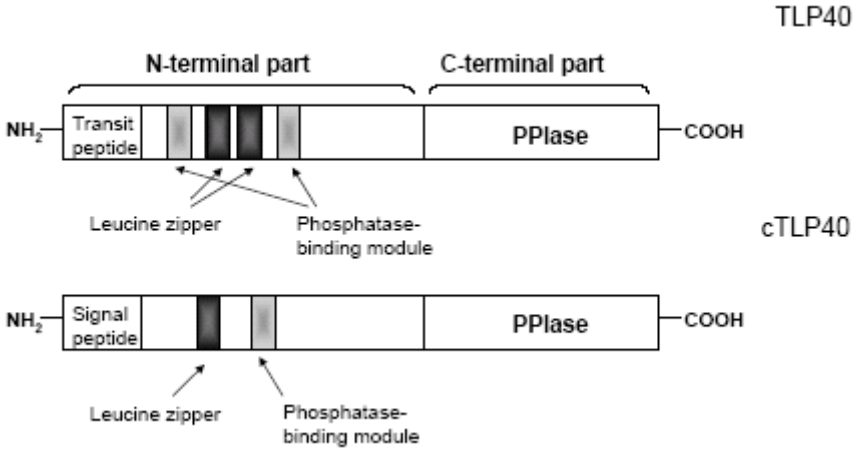


Figure 17. Hydropathy profile of the *Synechocystis* cTLP40 protein according to Kyte and Doolittle (1982). Amino acid positions are indicated at the bottom of the graph. Hydrophobic amino acid residues are placed above the zero line. An arrow indicates the hydrophobic domain of the signal peptide.

A.



B.

```
TLP40so   1   MSSFINHHFYPSVCTSKHALPINPTSPFYLGIPNFRQKSRFMHLTPRCFSR-QIDPLDKQ 59
cTLP40    1   ------------------------------------------------------MQII  4

                                                                  I
TLP40so  60   KKRSFSVKECAISLALAAALISGVPSLSWERHA-EA-LTSPVLPDLAVLISGPPIKDPEA117
cTLP40    5   KTPLGIITRRGLQLSLLSLLLT-MLSLTWAMPGWSLPLNQPML--LGALAQGNAITDPNA 61
                                           *     *     *

                *     *     *     *     *     *     *     *
TLP40so  118  LLRYALPIDNKAIREVQKPLEDITESLRVLGLKALDSVERNLKQASRALKNGKSLIIAGL177
cTLP40    62  ILRYALPIDNPEVRRLQDSLEDISNHIR--A-KRWPAIKKDVRAANLTITLKEDKILAGV118
              *↑
                  II
TLP40so  178  AESKKDRGVELLDKLEAGMGELQQIVENRNREGVAPKQRELLQYVGSVEEDMVDGFPYEV237
cTLP40   119  PADRQPEAETLLGSIKTDLTALTEAVEAKDKEQVISFRKSALTAIGDLEALMVTDFPFAI178

TLP40so  238  PEEYQTMPLLKGRAVVEMKVKVKDNPNVDNCVFRIVLDGYNAPVTAGNFLDLVERHFYDG297
cTLP40   179  PEEFANLPQLKGRATVEM------TTNKGP--LTIVVDGYSAPINAGNFVDLVQRKFYDG230

TLP40so  298  ME-IQRRDGFVVQTGDPEGPAEGFIDPSTEKPRTIPLEIMVEGEKVPVYGSTLEELGLYK356
cTLP40   231  LPFIRSEDFFVTQAGDPPGPEAGFIDPQTKEYRAIPLEILVKGEEGPIYGMTLEDACMYL290

TLP40so  357  AQTKLPFNAFGTMAMAREEFENNSGSSQIFWLLKESELTPSNANILDGRYAVFGYVTDNQ416
cTLP40   291  PELALPFNAYGAIALARPETEPNGGSSQFFFKFDTELTPPGFNLMDGRYSVFGYVVDGK350

TLP40so  417  DYLADLKVGDVIESVQAVSGVDNLVNPTYKIAG* 450
cTLP40   351  ETLEQLSEGDKIVSAKVISGADNLVNGNS*---- 380
```

Figure 18. Schematic presentation of the spinach and cyanobacterial TLP40 domain structures (A) and comparison of the protein sequences (B) of TLP40 homologues. TLP40so – spinach PPIase, cTLP40 – *Synechocystis* homologous TLP40. Arrowheads indicate the processing site of the precursor proteins. The leucine and isoleucine residues of the potential leucine-zipper domains are marked by asterisks and underlined. The arrow indicates the proposed major amino acid residue responsible for the phosphatase-binding in cTLP40. Phosphatase-binding domains are framed.

Recent proteomic studies of the thylakoid membrane proteins of *Synechocystis* sp. PCC 6803 (Srivastava *et al*., 2005) revealed that cTLP40 has an N-terminal signal peptide with 33 amino acid residues. This signal peptide contains a typical *Tat* epitope (Fulda *et al*., 2000) with the recognition cleavage site for the processing peptidase (Fig. 18, panels A and B). This indicated that the cTLP40 protein should be located in the thylakoid lumen (Srivastava *et al*., 2005). The C-terminal part of the protein (203 – 380 amino acid residues) contains a PPIase (cyclophilin) domain that could be responsible for proline isomerase activity. The N-terminal domain of cyanobacterial cTLP40 demonstrates less sequence homology to TLP40 of higher plants but possesses similar structural elements. Comparably to plant TLP40, the cyanobacterial protein includes a leucine-zipper motif, which starts from L39 to L63 (Fig. 18) and contains repeated leucine or isoleucine residues at every seventh position. Leucine zipper motifs have structural and functional importance and could be involved in protein-protein interactions for oligomerization and/or interaction of the TLP40 with the thylakoid inner membrane surface (Fulgosi *et al*., 1998, 2002). Two stretches which flank the leucine zipper motifs in the chloroplast TLP40 protein (amino acid residues 114 – 121 and 175 – 185) have been identified as potential phosphatase binding sites (Fulgosi *et al*., 1998). Sequence analysis of the cyanobacterial homologue revealed just one short region (58 – 67 amino acids residues) that may include one possible site for phosphatase binding (Fig. 18, panel B).

3.2.2 Localisation of the cTLP40 protein within the cyanobacterial cell

3.2.2.1 Overexpression of the cTLP40 protein in *E. coli* lysates

To obtain an antiserum against the cTLP40 protein, the coding region of the *sll0408* gene (1.143 kBp) was amplified on the chromosomal DNA of *Synechocystis* sp. PCC 6803 wild-type by a PCR approach. Two gene-specific oligonucleotide primers (sll0408Fover and sll0408Rover), which were specific for the 5' and 3' ends of the *sll0408* gene and contained introduced restriction sites for *Xho*I and *Pst*I endonucleases, respectively, were chosen for PCR analysis. The resulting amplification product of 1.242 kBp was inserted in-frame into the *Xho*I/*Pst*I sites of the pIVEX2.4b expression vector and the correctness of its insertion was confirmed by sequence analysis. The pIVEX2.4b vector has been developed and optimised for the application with the Rapid Translation System provided by Roche (Roche Diagnostic GmbH, Basel, Switzerland) and designed for *in vitro* protein expression in *E. coli* cell lysates. The gene expression starts with the T7 promoter of the pIVEX2.4b vector and protein is

translated with a N-terminally located hexa-His-tag fusion that allows an easy detection and purification of the expressed protein (Fig. 19).

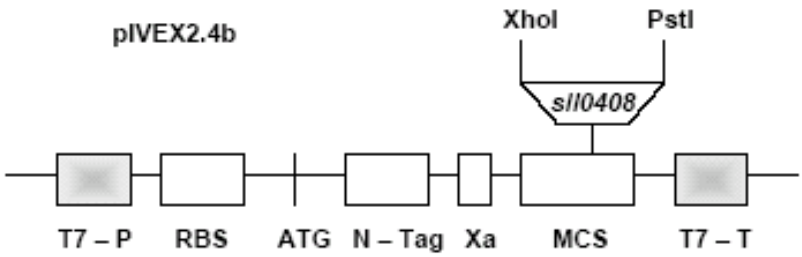


Figure 19. Schematic presentation of the cTLP40 overexpression construct. The full-length *sll0408* gene (1.143 kBp) was amplified with sll0408Fover and sll0408Rover primers, which contained *Xho*I and *Pst*I restriction sites, respectively, and the resulting fragment was cloned into pIVEX 2.4b vector in the frame with the ATG start codon of the plasmid. T7-P and T7-T – phage T7 polymerase promoter and terminator sequences, respectively; RBS – ribosomal binding site; ATG – plasmid provided start codon; N-Tag – His-tag sequence for purification; Xa – factor Xa cleavage site; MCS – multiple cloning site.

Overexpression of the full-length *sll0408* gene was performed with Roche translation system at 30^{o}C for 24 h (Fig. 20, panel A). Analysis of *E. coli* cell lysates demonstrated that the overexpressed protein migrated as a protein with molecular mass of approximately 42 kDa on a 12.5% SDS-PAG. *E. coli* cell lysates with (+) and without (-) an overexpressed protein were used for immunological analysis with anti-His serum (Fig. 20, panel B). Overexpressed protein was then used for immunization of rabbits and production of a protein-specific antiserum. The specificity of the antiserum was tested immunologically. Thylakoid membranes were isolated from the wild-type and *sll0408*-deficient strains. Proteins were separated by electrophoresis, electroblotted onto nitrocellulose membrane and reacted with cTLP40 antiserum (Fig. 20, panel C). Immunological analysis confirmed the presence of cTLP40 protein in wild-type cells and absence of this protein in mutant cells.

3.2.2.2 Intracellular localisation of the cTLP40 protein

The localisation of cTLP40 within cyanobacterial cells was analysed immunologically using the antisera raised against full-length cTLP40 protein. For this analysis thylakoids from wild-type cells were isolated and fractionated into soluble cytoplasma and membrane proteins, which were fractionated afterwards into lumenal and thylakoid membrane-enriched fractions.

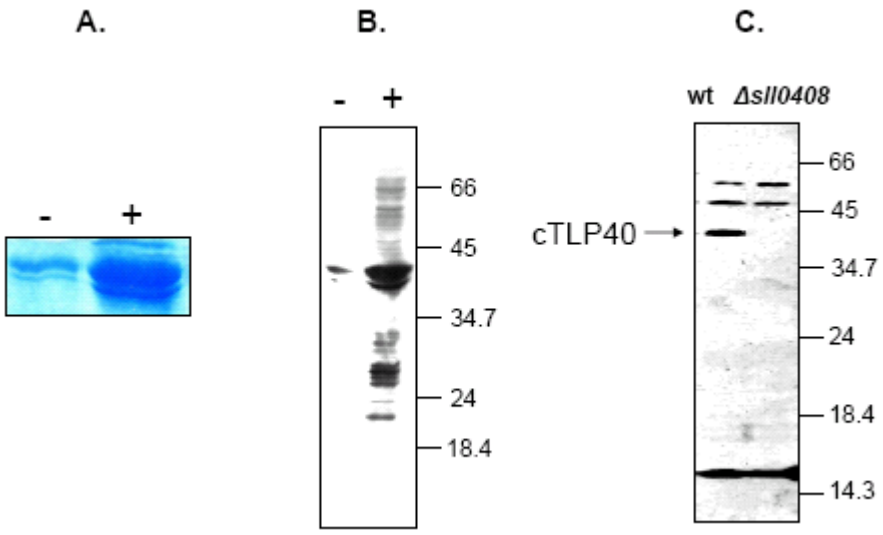


Figure 20. Overexpression of cTLP40. Full-length cTLP40 protein was amplified in *E. coli* cell lysates using the overexpression Rapid Translation System from Roche. Protein was synthesised in a Rapid Translation machine at 30°C for 24 h. (**A**) Overexpressed protein (+) was monitored after separation on a 12.5% SDS-PAGE by Coomassie Blue staining. (**B**) Immunological analysis of overexpressed protein (+) with anti-His serum. As a control the *E. coli* cell lysates without overexpressed protein (-) were loaded. (**C**) Western analysis of thylakoid-enriched proteins of the wild-type and *ctlp40*-deficient *Synechocystis* strain with antiserum against cTLP40 protein. Arrows indicate the overexpressed cTLP40 with a molecular weight of 42 kDa. The molecular weight marker is shown at the right side.

Polypeptides were analysed by SDS-PAGE followed by Western analysis with antisera raised against cTLP40 protein. Serological analysis demonstrated that cTLP40 was enriched in the whole protein extract and in the thylakoid membrane fraction (Fig. 21, lanes „CE" and „TM"). The sonication of thylakoids with subsequent ultracentrifugation resulted in the separation of lumenal and thylakoid membrane-enriched proteins. Despite prediction of cTLP40 localisation in the soluble cell compartment, it was found in two forms, associated with thylakoid membranes and soluble in the thylakoid lumen (Fig. 21, lanes „Th" and „L"). The β subunit of ATP synthase complex and the 33 kDa protein of oxygen-evolving complex were used as controls for thylakoid membrane and lumenal proteins, respectively. The 33 kDa protein was exclusively found in the thylakoid lumen, while the ATP synthase β subunit due to its peripheral association with thylakoid membranes was partially found in thylakoid membrane and lumenal fractions (Fig. 21).

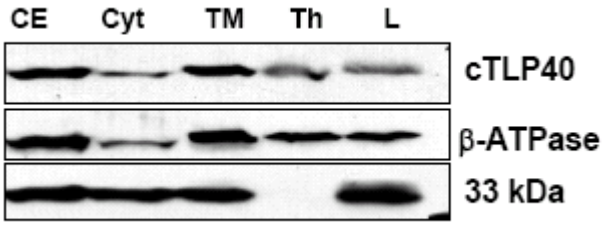


Figure 21. Intracellular localisation of TLP40 in cyanobacterial cells. Whole protein cell extracts (CE) were isolated from the *Synechocystis* wild-type cells and fractionated into soluble cytoplasma (Cyt), thylakoid-enriched (Th), thylakoid membrane (TM) and lumenal (L) proteins. After separation of proteins by 12.5% SDS-PAGE and their transfer onto nitrocellulose membrane cTLP40 was immunodedected with corresponding antisera. β subunit of ATP synthase and 33 kDa protein of oxygen-evolving complex were used as controls for the purity of protein fractions.

3.2.2.3 Association of the cTLP40 with thylakoid membranes

To estimate the nature of association of the cTLP40 with thylakoid membranes and to prove that the *sll0408* gene product is a soluble protein and not a membrane-integral component, thylakoid fractions were treated with chaotropic salts (2 M NaBr or 2 M NaSCN) or with alkaline solution (0.1 M NaOH). In these assays thylakoids isolates from wild-type cells, resuspended at a chlorophyll concentration of 0.1 mg/ml in HEPES/sucrose buffer containing NaBr, NaSCN or NaOH or without any addition (as a control), were incubated on ice for 30 min. After centrifugation the polypeptide composition of the supernatant and membrane pellet was immunologically analysed with cTLP40 antiserum. It appeared that NaBr and NaSCN were not able to release the protein from the membranes (Fig. 22). Partial release of cTLP40 from the membrane was observed after treatment with the strong chaotropic reagent NaSCN that disturbs lipids interactions within the membrane and makes them leaky. The treatment with harsh alkaline solution, NaOH, which is able to destabilize most electrostatic interactions between molecules and removes peripheral and even intrinsic polypeptides led to the complete removal of the protein from the membrane. Therefore, these data suggested that cTLP40 is a soluble in lumen protein but can be strongly attached with but not integrated in the thylakoid membrane.

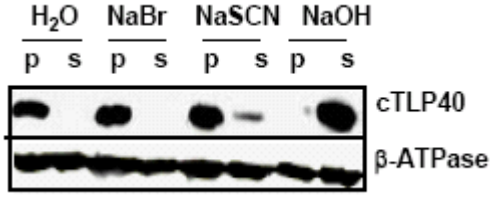


Figure 22. Association of cTLP40 with the thylakoid membranes. The association of TLP40 with thylakoid membranes was analysed by treatment with chaotropic salts (2 M NaBr or 2 M NaSCN) and alkaline solution (0.1 N NaOH); incubation buffer was used as a control. After centrifugation thylakoid proteins were separated into pelleted membrane (p) and soluble (s) proteins and immunodetected with antisera against cTLP40 and β subunit of ATP synthase.

3.2.2.4 Localisation of the cTLP40 within photosynthetic membranes

The immunological analysis had shown that some amount of cTLP40 could be associated with thylakoid membranes. To identify possible association of cTLP40 with some of photosynthetic proteins and/or multimeric complexes, membranes from wild-type cells were extracted, solubilized and separated on sucrose gradients. After fractionation of sucrose gradient, the protein composition was assayed by SDS-PAGE and immunological analysis. Aliquots of each

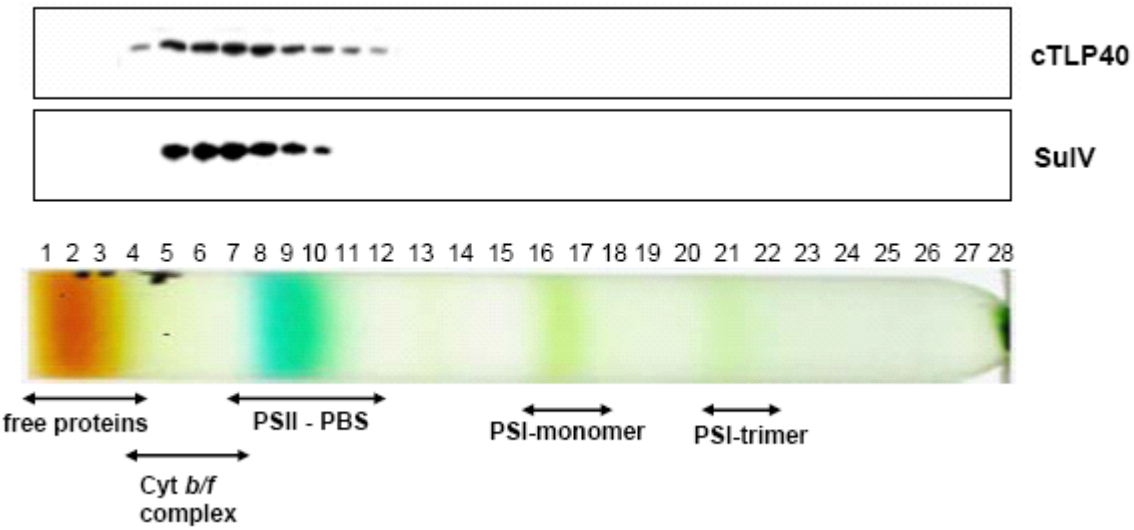


Figure 23. Localisation of cTLP40 within thylakoid membranes. Solubilized by ß-dodecyl-maltoside *Synechocystis* thylakoid membranes were separated on a 12 to 24% sucrose gradient. The fraction aliquots of 0.3 ml were analysed on 12.5% SDS-PAG and immunodecorated with antisera against cTLP40 protein and subunit IV (SuIV) of the cytochrome b_6f complex.

fraction (1 – 28) were analysed using antibodies directed against cTLP40 and major proteins of photosynthetic complexes. The analysis revealed that the membrane-associated cTLP40

comigrated with the cytochrome b_6f complex (Fig. 23). These data confirmed previous studies on chloroplast TLP40, which was also found to be associated with the cytochrome b_6f complex (Weber, 2001; Weber *et al*., 2001).

3.2.3 Functional analysis of the cTLP40 by interposon mutagenesis in cyanobacterial cells *in vivo*

3.2.3.1 Construction of the *Δsll0408* knock-out and complementation strains

An open reading frame *sll0408* encodes a protein with 381 amino acid residues. To study molecular functions of the cTLP40 protein the corresponding gene was inactivated by insertion of the gentamycin resistance cassette in *Synechocystis* wild-type cells (Fulgosi, 1999; Fig. 24 panel A). A chimeric construct was transformed into wild-type *Synechocystis* cells and colonies resistant to gentamycin were selected and segregated in the presence of increasing amounts of the antibioticum. Since the *Synechocystis* genome contains up to 12 chromosome copies per cell the gene of interest should be replaced in all DNA copies of chromosome by homologous recombination. PCR analysis with the gene-specific primers sll0408Fover and sll0408Rover indicated that *sll0408* contained the gentamycin insertion in all chromosome copies and that the mutant was homozygous (Fig. 24, panel B).

Four additional genes are located on the complementary strand downstream of *sll0408* and transcribed in the same direction as *sll0408*. The mutagenesis of *sll0408* gene could influence the expression of the closely located genes. The promoter region of the closely located to *sll0408* gene *sll0409* (104 bp downstream of *sll0408)* could overlap with *sll0408* reading frame. The interruption of *sll0408* could therefore influence the expression of *sll0409* as well. To check the correctness and specificity of the *sll0408* gene disruption, a complementation analysis of the mutant strain was performed. A wild-type copy of *sll0408* gene was inserted into the autonomously replicating vector pVZ323 containing the replicon of the RSF1010 plasmid. This construct has been obtained by G. Canino (Canino, 2006). Conjugal transfer of the pVZ323 plasmid carrying the wild-type *sll0408* gene into *Δctlp40* cyanobacterial mutant has been done *via* triparental crossing. The construct for the complementation analysis, plasmid pVZsll0408, was obtained by insertion of the full-length gene into the unique *Sma*I restriction site in the kanamycin resistance gene of the pVZ323 plasmid. Mobilization of the non-conjugative plasmid pVZsll0408 in *Δctlp40* mutant cells was performed with the helper

plasmid R751. Transconjugants *pVZsll0408* were selected on the plates, containing BG11 medium which was supplemented with 15 µg ml^{-1} chloramphenicol and 3 µg ml^{-1} gentamycin

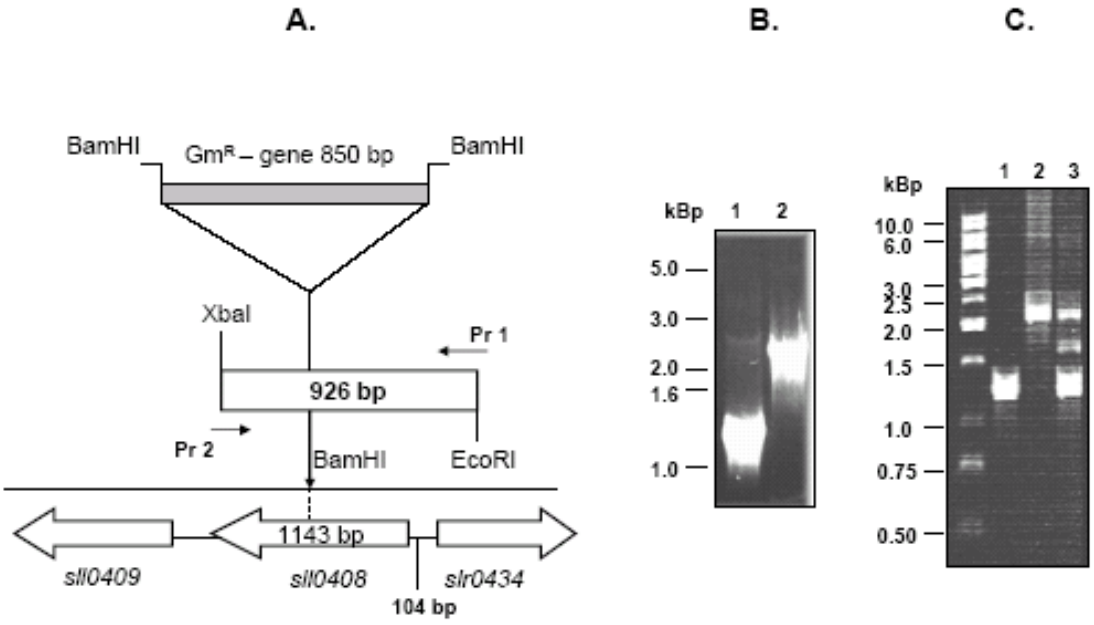


Figure 24. Construction of the knock-out mutant strains for the *sll0408* gene from *Synechocystis* sp. PCC 6803 and molecular-biological analysis of the *Δsll0408* and complementation strains. (**A**) The *sll0408* gene was interrupted by insertion of the gentamycin resistance gene into the *Bam*HI restriction site of the *sll0408* reading frame (Fulgosi, 1999). (**B**) The chromosomal DNA from the wild-type (lane 1) and knock-out mutant *Δsll0408* (lane 2) were tested by PCR with the *sll0408* gene specific primers, sll0408Fover and sll0408Rover. The amplification of the wild-type *sll0408* gene resulted in a 1.25 kBp fragment, whereas the *sll0408* gene interrupted with *Gm* resistance cassette (0.85 kBp) was 2.1 kBp long. The molecular mass markers (λ phage DNA digested with *Eco*RI/*Hind*III) are indicated at the left. (**C**) The chromosomal DNA from the wild-type (lane 1), knock-out *Δsll0408* (lane 2) and complementation *pVZsll0408* (lane 3) mutants were tested by PCR with the *sll0408* gene specific primers, sll0408Fover and sll0408Rover. The amplification resulted in two fragments of 1.25 and 2.1 kBp. One fragment (1.25 kBp) resulted from the amplification of the pVZ323 plasmid. Another one (2.1 kBp) resulted from the wild-type copy of *sll0408* interrupted with *Gm* resistance cassette (0.85 kBp) which was present on the chromosomal DNA of the *Δsll0408* mutant. An additional band of 1.6 kBp in *pVZsll0408* complementation strain appeared due to unspecific reaction of the primers. The 1 kb DNA ladder molecular mass marker is indicated at the left.

and analysed. Since during insertion of the self-replicating plasmid the *sll0408* gene from the complementation pVZ323 plasmid can be independently expressed from the vector and it can be integrated into genomic DNA of *Synechocystis* by reverse recombination as well, a PCR approach was performed. The DNA from wild-type, *Δctlp40* and *pVZsll0408* strains was used

for PCR analysis with the primers sll0408Fover and sll0408Rover corresponding to 69 bp upstream of the 5' end and specific to 3' end of *sll0408* gene. The resulting fragments were about 1.25 kBp for wild-type and 2.1 kBp of the *Δctlp40* mutant due to the 0.85 kBp *Gm*-resistance gene. The *pVZsll0408* strain possesses two fragments: one from the plasmid which corresponded to 1.25 kBp of the wild-type copy and another one from the chromosomal DNA of the *pVZsll0408* strain. The latter has the same size of 2.1 kBp as the product of the *Δctlp40* mutant strain (Fig. 24, panel C), since the 0.85 kBp *Gm*-resistance gene is present on chromosomal DNA of both, the *Δctlp40* and *pVZsll0408* strains. PCR analysis performed with DNA from the *pVZsll0408* complemented strain revealed one additional band at about 1.6 kBp which appeared as an unspecific signal.

The deficiency of the *sll0408* gene product in the mutant strain was tested by immunological analysis with antiserum raised against the cTLP40 protein. cTLP40 protein was detected in the wild-type and complementation strain but not in the *ctlp40*-depleted strain (Fig. 25). These molecular-biological and biochemical studies confirmed the correctness of the gene inactivation in the *Δctlp40* strain.

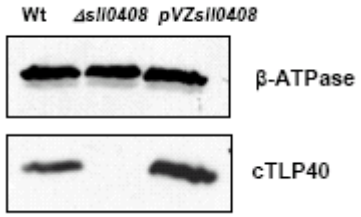


Figure 25. Detection of cTLP40 in the wild-type, *Δsll0408* and complementation strains. Thylakoid-enriched proteins were isolated from the wild-type, *Δsll0408* and complementation strains. Proteins were transferred onto nitrocellulose membrane and immunologically detected with antisera against cTLP40 and β subunit of ATP synthase as a control.

3.2.3.2 Physiological characterisation of the *Δctlp40* mutant under standard and stress conditions of growth

3.2.3.2.1 Phenotypical analysis of the *Δctlp40* mutant

The *Δctlp40* mutant was capable to photoautotrophic growth as the wild-type. Phenotypical analysis under standard growth conditions (40 μE m^{-2} s^{-1}; 30°C) did not result in strong differences compared to wild-type, although the *Δctlp40* strain showed a slightly enhanced cell pigmentation when cells of the wild-type and mutant were normalised to the same cell density (Fig. 26, panel A). The growth rate of *Δctlp40* was slightly slower than that of the wild-type (Fig. 26, panel B).

The possible functional role of plant TLP40 is regulation of protein phosphatase which dephosphorylates the PSII reaction centre proteins (Vener *et al*., 1999; Rokka *et al*., 2000). The phosphorylation/dephosphorylation of thylakoid proteins is a universal molecular mechanism that is involved in acclimation and probably signal transduction and can be highly dependend on light, temperature and other stress conditions.

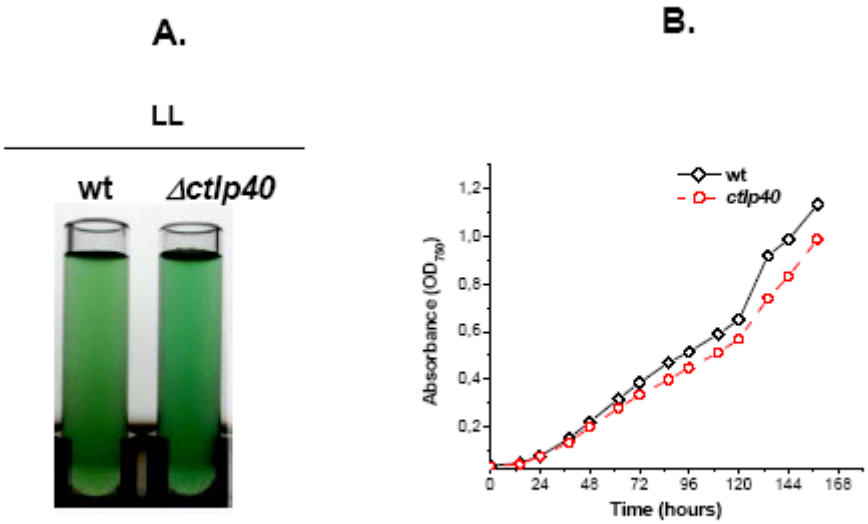


Figure 26. Characterisation of the wild-type and *Δctlp40* mutant strains under LL regime (40 µE m^{-2} s^{-1}, 30°C). Phenotypical modification (**A**) and growth rate (**B**) of the wild-type and mutant cells.

Since cyanobacteria lack the phosphorylation of PSII proteins, the role of cTLP40 could be different than that of the plant one. To analyse the possible functional and regulatory role of cTLP40 in signal transduction, the cyanobacterial mutant strain was acclimated to different stress conditions. The wild-type and mutant cell cultures were grown at LL to the exponential phase, diluted to an A_{750} of 0.5 – 0.8 and transferred to different regimes like high light (HL; 400 µE m^{-2} s^{-1}; 30°C), cold (CS; 40 µE m^{-2} s^{-1}; 17°C), heat (HS; 40 µE m^{-2} s^{-1}; 42°C) or double (DS; 400 µE m^{-2} s^{-1}; 17°C) stresses. The phenotypical modifications were analysed 24 – 32 h after exposure to the stresses. The phenotypical modifications of the mutant strain, which were not apparent under LL started to be more evident when the strain was acclimated to various stress conditions. The phenotypical analysis showed that *Δctlp40* cells were stronger pigmented (dark green) than the wild-type under HL, CS and HS (Fig. 27, panel A). The strongest differences between wild-type and mutant strains were revealed under double stress. This regime led to a complete bleaching of the *Δctlp40* mutant within 26 – 32 h. As a result the

mutant cells became completely depigmented (Fig. 27, panel B). However, several hours before bleaching the mutant strain was intensively blue that indicated that the chlorophyll *a* and carotenoids declined probably first, and only afterwards bilin-pigments were degraded as well (Fig. 27, panel C). At the same time the wild-type showed also some bleached phenotype but chlorophyll *a* and billin pigments probably declined simultaneously.

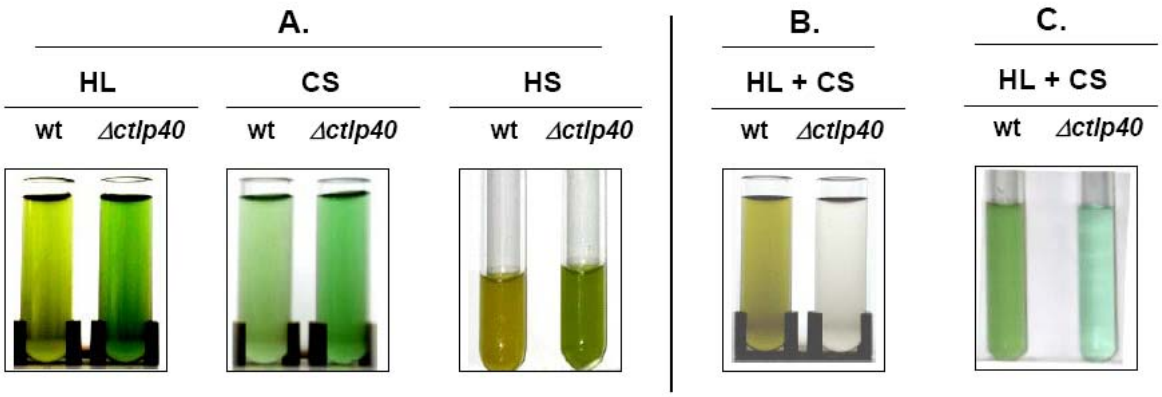


Figure 27. Phenotypical modifications of the wild-type and *Δctlp40* strains upon acclimation to various light and temperature conditions. Wild-type and *Δctlp40* mutant were grown under LL regime and afterwards transferred to HL (400 µE m^{-2} s^{-1}; 30°C), CS (40 µE m^{-2} s^{-1}; 17°C), HS (40 µE m^{-2} s^{-1}; 42°C) for 24 h (panel **A**) or DS (400 µE m^{-2} s^{-1}; 17°C) (panels **B** and **C**).

Acclimation responses to stress conditions include changes in the cell morphology, metabolism and pigment content. The pigment content can be analysed by absorption spectra of whole cells. The absorption spectra of the wild-type and *Δctlp40* cells grown under standard and stress regimes were taken in order to compare the pigment contents of both strains. The spectra of the whole cyanobacterial cells visualised four absorption peaks with wavelengths around 430 and 680 nm corresponding to chlorophyll *a*, 500 nm to carotenoids and 620 nm to phycocyanin/allophycocyanin (PC/APC). Despite that the *Δctlp40* mutant did not differ phenotypically from wild-type grown under LL condition, the absorption peak at 620 nm corresponding to PC/APC was slightly higher in mutant cells compared to wild-type, suggesting an increased content of bilin-pigments (Fig. 28, panel LL). The absorption spectra of the wild-type and *Δctlp40* cells grown under stress conditions (for instance, HL, CS or DS before full bleaching of the culture) showed that during acclimation to these stresses at least for 20 – 28 h the respective PC/APC and chlorophyll *a* absorption peaks at 620 nm and 430 nm remained higher in the mutant strain. (Fig. 28, panels HL, CS and DS/24 h). The absorption

spectra of the wild-type and *Δctlp40* mutant cells after adaptation to double stress for 30 h displayed a strong decrease, probably due to complete degradation, of all photosynthetic pigments (Fig. 28, panel DS/30 h).

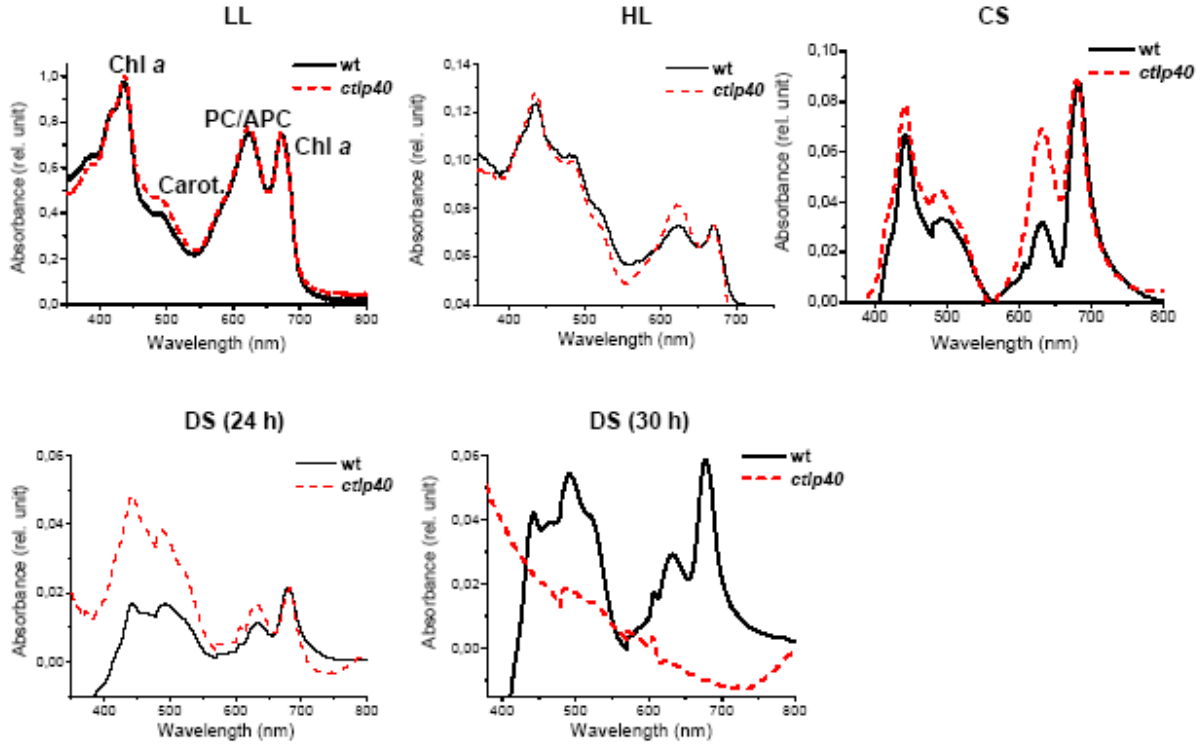


Figure 28. Characterisation of the wild-type and *Δctlp40* mutant under LL and various stress regimes. Absorption spectra of the whole cyanobacterial cells of the wild-type (solid line) and *Δctlp40* mutant (dashed line) were measured after acclimation to LL (panel LL) and stress conditions (panel HL, CS and DS) by normalizing an A_{750} of cells to 0.4 – 0.5. Absorption spectra under HL, CS and DS were taken after cell transfer (A_{750} = 0.5) from LL (40 μE m^{-2} s^{-1}) to these stresses for 24 h and after acclimation to DS for 30 h (panel DS, 30 h).

Since, PBSs are the major light-harvesting complexes involved in cell acclimation, the degradation of the PBSs can reflect one of the protection mechanisms for controlling photosynthetic activity during stress conditions. The reduction of the PBSs can lead to a decrease of absorbed energy, thus preventing cells from photodamage. The adaptation of the PBSs to stresses occurs through a decrease of the rod size, the number of rods and the number of PBSs that are controlled at the transcriptional and post-translational levels. The PBSs are mobile structures on the surface of the thylakoid membrane. The association of PBSs with photosystems II and I is transient and PBSs can be found in a membrane-bound and non-associated form with PSI/PSII. PBSs efficiently harvest light energy and transfer it to the chlorophyll *a* of PSII or PSI when they are attached to these complexes. Thereby, the ratio of

the membrane-bound and free PBSs (soluble in the cytoplasm) may provide information on energy absorption and transfer through PBSs to the reaction centres of the two photosystems. To determine the ratio between membrane-bound and free PBSs thylakoid and cytoplasmic protein fractions were isolated from cell cultures of wild-type and mutant, which were grown under LL and acclimated to various stresses (HL, CS and DS) for 24 h. The absorption of PBSs in the cytoplasm and thylakoid membranes was then measured at 620 nm.

Expectedly, HL and CS treatments led to the degradation of PBSs in wild-type cells causing an insignificant reduction of PBSs associated with membranes under HL and CS conditions, while under the double stress regime (HL + CS) the ratio of the membrane associated PBSs to free ones was reduced 2.5 times compared to that at LL (Table 3). At the same time, the *Δctlp40* strain contained an increased amount of membrane-bound PBSs already under normal conditions in comparison to wild-type. Interestingly, under stress conditions, such as a HL and CS, the amount of PBSs associated with the membrane was slightly increased in the *Δctlp40* strain, an effect that was opposite in wild-type cells. The measurement also showed that double stress in mutant cells as in wild-type led to a decline of the content of membrane-bound phycobiliproteins but to a much lesser extent. In *Δctlp40* cells the ratio of membrane associated to free phycobiliproteins was reduced only to 1.5 times under this strong stress comparable to that under normal conditions (Table 3). Therefore, mutant cells are probably affected in the regulatory mechanisms that control the association and disassociation of the PBSs with photosynthetic membranes.

Table 3. Ratio of the membrane-associated and non-associated phycobilisomes.

	LL ($40\ \mu Em^{-2}\ s^{-1}$; 30^oC)	HL ($400\ \mu Em^{-2}\ s^{-1}$; 30^oC)	CS ($40\ \mu Em^{-2}\ s^{-1}$; 17^oC)	HL+CS ($400\ \mu Em^{-2}\ s^{-1}$; 17^oC)
wild-type	16.75	14.02	14.64	6.7
Δctlp40	23.95	29.37	25.29	15.4

3.2.3.2.2 Pigment analysis under standard and high light conditions

Since the mutant strain showed different to the wild-type phenotypical modifications under all tested conditions, we decided to perform a detailed functional and biochemical analysis of the

Δctlp40 strain under acclimation to HL first, a stress condition under which *Δctlp40* exhibited the non-bleaching phenotype (Fig. 27, HL). For an analysis of growth rates and pigment contents cells of the wild-type and mutant were grown at LL until log-phase, diluted to the same optical density (A_{750} = 0.5) and transferred to HL for 3 days. The concentration of major pigments (chlorophyll *a*, PC and carotenoids) was measured every 12 h within 3 days of HL acclimation. It was observed that the growth rate was slower in *Δctlp40* than in wild-type under LL regime. Under HL the duplication time decreased 2.57 in mutant cells and only 1.33 in the wild-type (Table 4).

Table 4. Growth rate of the wild-type and *Δctlp40* strains under LL (40 µE m^{-2} s^{-1}) and HL (400 µE m^{-2} s^{-1}) regimes.

	Doubling time (h)	Cell concentration per OD = 0.1	Cell concentration per ml at T_{24}	Cell concentration per ml at T_{48}
WT LL	12 ± 1	1.33 ± 0.07	15.7 ± 0.5	19.9 ± 0.13
WT HL	16 ± 0.5	1.03 ± 0.04	16.8 ± 1.5	26.12 ± 4
Δsll0408 LL	14 ± 2	1.4 ± 0.06	16.2 ± 1.7	21.6 ± 2.2
Δsll0408 HL	36 ± 1	1.6 ± 0.1	21.44 ± 1.7	30.77 ± 0.8

Microscopic observations revealed that the amount of mutant and wild-type cells per ml of culture and their size were the same under LL regime. However, during acclimation to HL the amount of mutant cells per ml increased 1.5 times and cells were smaller than those of the wild-type cells. Thus, the analysis of pigments was performed on a cell basis due to differences in their size. Determination of pigment content of cells grown under LL showed that the mutant contained higher amount of phycocyanin, a lower quantity of chlorophyll *a* and similar amounts of carotenoids (Fig. 29 panels A, B and C, respectively). The analysis of pigments in wild-type cells grown under LL and then acclimated for 24 and 48 h to HL demonstrated that the content of chlorophyll *a* and phycocyanin decreased upon acclimation to HL to about 35 and 30%, respectively, while the amounts of carotenoids increased to 58%. The acclimation of the mutant strain to HL led to a decrease of all pigments including carotenoids, but to a much lesser extent of chlorophyll *a* and phycocyanin than in the wild-type (15 and 21%, respectively). A comparative analysis of pigment concentrations per cell is presented in Table 3. These data confirmed the measurements of absorption spectra and showed that pigment degradation occurred more slowly in the mutant strain than in the wild-type.

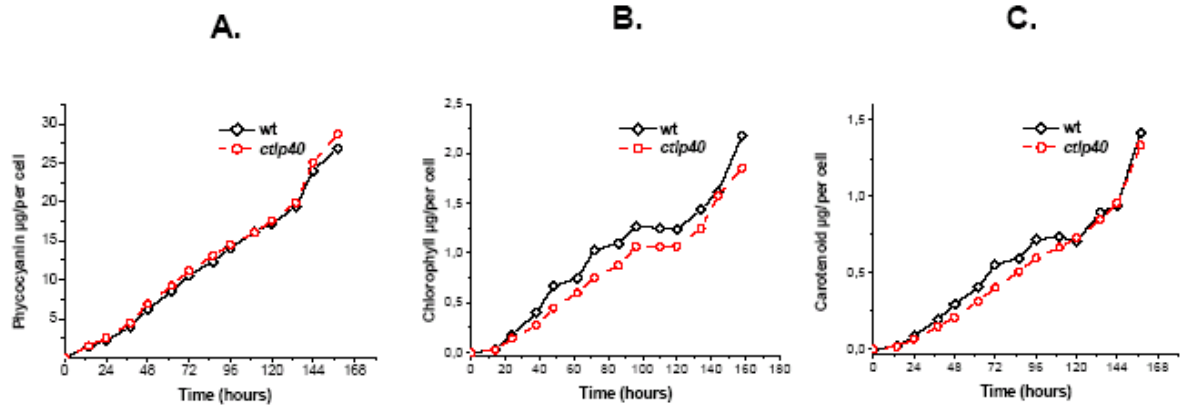


Figure 29. Pigment characterisation of the wild-type and *Δctlp40* strains under LL regime. Phycocyanin (panel **A**), chlorophyll *a* (panel **B**) and carotenoid (panel **C**) concentrations were measured and calculated per cell culture volume. The measurements were performed every 12 h during 7 days.

Table 5. Characterisation of cell pigments in wild-type and strain *Δctlp40* acclimated to LL and HL

	Chlorophyll (µg/cell)			Carotenoids (µg/cell)			Phycocyanin (µg/cell)		
	LL	**HL (24h)**	**HL (48h)**	**LL**	**HL (24h)**	**HL (48h)**	**LL**	**HL (24h)**	**HL (48h)**
wt	1.55 ±0.07	1.3±0.02	1.04±0.11	0.93±0.026	1.37±0.14	1.47±0.22	19.9±0.5	19.12±1.2	14±2.3
Δctlp40	1.47±0.05	1.27±0.08	1.26±0.04	0.92±0.01	0.9±0.02	0.88±0.03	23.72±1.3	19±0.8	19±0.4

Another strong phenotypical effect was observed during long time incubation of *Δsll0408* cells under LL. Upon long-term growth of cyanobacterial cells in BG11 medium cells start to starve because of lack of fresh nutrients and degradation of photosynthetic pigments (PC/APC and chlorophyll *a*) that lead to cell de-pigmentation. Cell cultures were grown under standard photoautotrophic growth conditions for two weeks and were maintained for a prolonged growth period under LL at 30°C without additional aeration. The wild-type cells bleached after 15 – 18 days of growth, while mutant *Δctlp40* stayed green within 30 – 45 days of growth under the same conditions (Fig. 30, panel A). Non-bleaching of the mutant cell culture could result from the stability of cell pigments (chlorophyll *a* or/and PC/APC). The absorption spectra of whole cyanobacterial cells also showed that the PC/APC and chlorophyll *a* absorption peaks at 620 nm and 430 nm, respectively, remained higher in the mutant strain (Fig. 30, panel A).

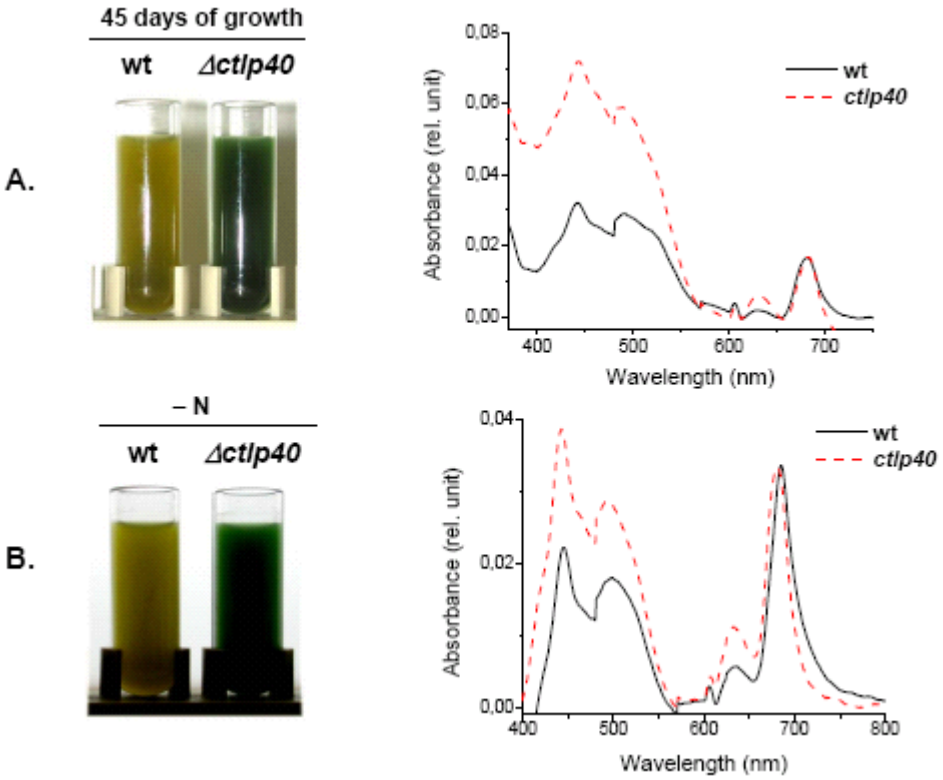


Figure 30. Growth phenotype of wild-type and *Δctlp40* mutant during long-term growth (panel A) and nitrogen starvation (panel B). Cell cultures were grown for 45 days without shaking (panel **A**). Cells were grown at LL until an A_{750} of 0.6 – 0.7, then washed with BG11 medium depleted in nitrogen and transferred to the same medium for 3 days (panel **B**).

The highest degradation of cell pigments, specially of bilin-containing PBSs has been observed upon nitrogen limitation (Duke *et al*., 1989; Collier and Grossman, 1992; Grossman *et al*., 1993a). Degradation of phycobiliproteins is part of an acclimation process in which growing cells differentiate into non-pigmented cells able to endure long periods of starvation (Gorl *et al*., 1998). To check the influence of nitrogen starvation on the behaviour of wild-type and mutant strains, cells of both strains were harvested in the exponential growth phase by centrifugation, washed and resuspended in a medium lacking nitrogen. As expected, wild-type cells showed a bleaching phenotype after three-days of growth under nitrogen-depletion due to a rapid decline of phycobiliproteins, PC and APC. The cTLP40-deficient mutant remained blue-green and did not exhibit visual changes in pigmentation (Fig. 30, panel B). The measurement of absorption of the whole wild-type and mutant cells indicated an increased content of bili-pigments (PC/APC) and chlorophyll *a* (absorption peaks at 620 nm and 430 nm, respectively) in *Δctlp40* mutant cells (Fig. 30, panel B). However, a longer starvation period (about 10 – 12 days) also led to cell bleaching. These observations suggested that mutant

Δctlp40 is affected in sensing nitrogen limitation that could lead to PBS degradation and cTLP40 might be involved in the regulation of degradation of phycobiliproteins and/or another pigments.

3.2.4 Chlorophyll fluorescence analysis of photosynthetic complexes in the *Δctlp40* mutant with the PAM device

The phenotypical differences of the mutant strain under HL suggested that deletion of *sll0408* could affect the photosynthetic apparatus. Chlorophyll fluorescence analysis could give more information about the state of major photosynthetic complexes and acclimation capacity of cells to increased light intensities. Usually, the chlorophyll fluorescence from PSII at room temperature predominates and changes if the redox-state of PSII modifies. Thus, the level of fluorescence can be changed by the state of the primary acceptor Q_A. When Q_A is oxidized, a minimal level of fluorescence can be observed, and a maximum level, when Q_A is fully reduced (Bissati *et al*., 2000). Fig. 31 shows the fluorescence analysis of the wild-type and *Δctlp40* mutant cells.

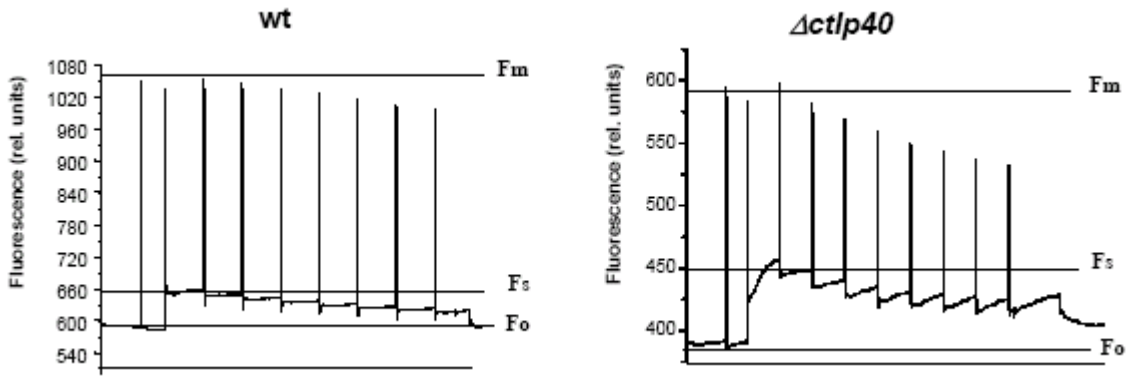


Figure 31. PSII fluorescence at room temperature. Wild-type and *Δctlp40* strains were adapted in the dark for 10 min before measurements. The minimal fluorescence level, $F_{0,}$ was determined by illuminating cyanobacterial cells with a red-modulated light of low intensity (0.14 µE m^{-2} s^{-1}). The actinic light for measuring the steady-state fluorescence level, $F_{S,}$ was 40 µE m^{-2} s^{-1}. The maximal fluorescence level, $F_{M,}$ was determined by illuminating the dark-adapted cells with HL (1.000 µE m^{-2} s^{-1}). The light induction was measured by illuminating cells with 1 s flashes with intervals of 2 min.

The wild-type and mutant cell cultures were grown under standard LL regime until an A_{750} = 0.5 – 0.6 and cyanobacterial cell cultures were normalized to chlorophyll *a* (3 µg ml^{-1}). Afterwards, cells were adapted to dark for 10 min. The minimal fluorescence level, F_0 (all PSII

reaction centres open), was determined by illuminating the dark-adapted cells (end chlorophyll concentration 3 μg ml^{-1}) with a low-intensity red-modulated beam (0.14 μE m^{-2} s^{-1}). The steady-state fluorescence, F_S, was measured by cell treatment with actinic light (40 μE m^{-2} s^{-1}) and the maximal fluorescence, F_M (all PSII centres are closed), was determined by illuminating cells for 1 sec HL (1.000 μE m^{-2} s^{-1}). The light induction curve of PSII fluorescence was recorded by applying about ten saturation light pulses (1.000 μE m^{-2} s^{-1}) every 2 min to cyanobacterial cells for reduction of all PSII centres (Fig. 31). The measurement of the major fluorescence parameter $F_V/F_M = (F_M – F_0)/F_M$, which correlates well with changes in functional activity of PSII and is used as an index of the maximal photochemical efficiency of PSII, showed that the efficiency of photochemical processes in mutant cells was 1.25 time lower than in wild-type (Table 6). The determination of another parameter, photochemical quenching (q_P) coefficient, showed that it was also lower in *Δctlp40* mutant cells (Table 6). This parameter reflects ratio between open and closed reaction centres and shows the balance between excitation and reoxidation of PSII. Furthermore, as shown on Fig. 31 the mutant strain could not fully oxidise the PQ pool after repeated pulses of saturating light. The determination of plastoquinone pool size, PQ ($PQ = (F_S – F_O)/(F_M – F_O)$) showed that it was twice higher in the mutant strain (Table 6). PQ coefficient reflects the rate of reduction and reoxidation of plastoquinone pool.

Table 6. Analysis of the major photosynthetic parameters by room temperature fluorescence.

	$F_V/F_M = F_M – F_O/F_M$	$q_p = (F_M – F_S)/(F_M – F_O)$	$PQ = (F_S – F_O)/(F_M – F_O)$
wt	0.437	0.859	0.141
Δctlp40	0.348	0.687	0.313

F_V/F_M – an index of the maximal photochemical efficiency of PSII; $F_V/F_M = (F_M – F_O)/F_M$. q_p – photochemical quenching coefficient; $q_p = (F_M – F_S)/(F_M – F_O)$. PQ – the size of the plastoquinone pool; $PQ = (F_S – F_O)/(F_M – F_O)$.

3.2.5 Light-dependent photoinhibition in wild-type and *Δctlp40* strain

As shown above (Section 3.2.4) the *Δctlp40* mutant had a slightly lower F_V/F_M ratio than the wild-type. For wild-type grown under LL a value of $F_V/F_M = 0.37 – 0.42$ was typical, the *Δctlp40* mutant showed a slightly lower $F_V/F_M = 0.3 – 0.33$. Since the fluorescence parameter F_V/F_M reflects the maximum efficiency of PSII photosynthetic activity, the experiment for the

analysis of PSII photoinhibition was performed. Cyanobacterial cells of wild-type and *Δctlp40* were normalised by chlorophyll *a* and preadapted to dim light (DL; ~ 10 µE m^{-2} s^{-1}) for 3 h.

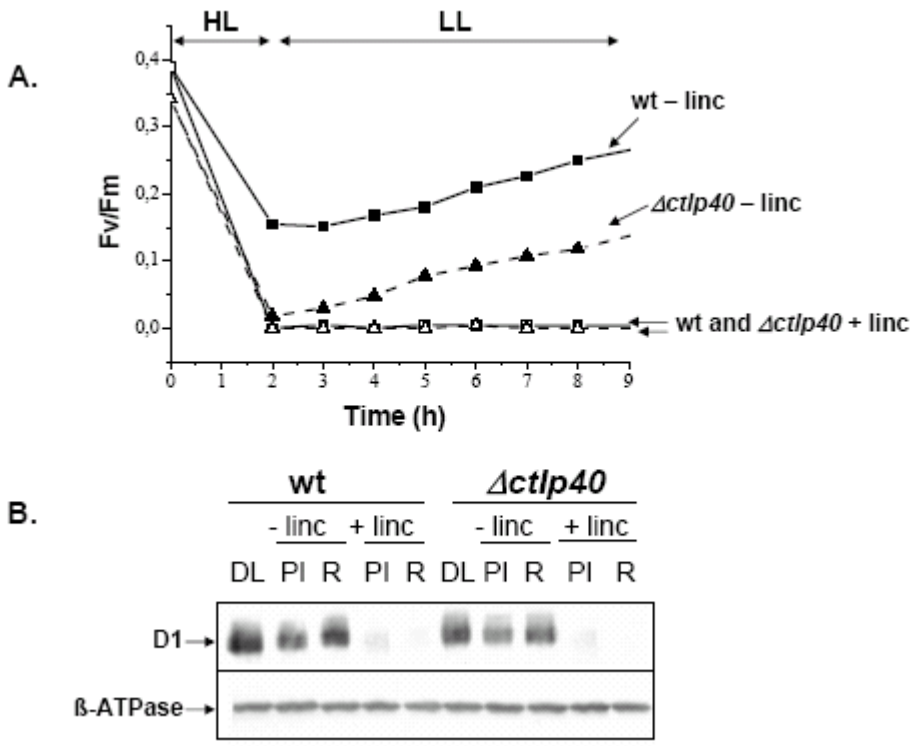


Figure 32. Analysis of PSII photoinhibition. (**A**) Photoinhibition assay of wild-type (solid line) and mutant cells (dash line) in the absence (closed quadrates and triangles) and in the presence of lincomycin (linc.; opened quadrates and triangles). Cells were normalised by chlorophyll *a*, preadapted to dim light and then exposed to HL for 2 h. The recovery of both strains was performed by cell transfer to LL for 6 h. The F_V/F_M ratio reflects PSII activity. (**B**) Immunological analysis of the D1 degradation rate during the photoinhibition experiment. Thylakoid membrane proteins from wild-type and *Δctlp40* mutant were isolated, separated on 4 M urea containing 10% SDS-PAG and transferred onto PVDF membranes. The turnover rate of D1 was analysed immunologically. The β subunit of ATP synthase was used as a control for equal loading.

Then cultures were incubated for 2 h under strong photoinhibitory light (600 µE m^{-2} s^{-1}) in the absence and in the presence of the inhibitor of protein synthesis, lincomycin (end concentration 200 µg/ml). Afterwards, the wild-type and mutant cells were transferred for recovery of PSII activity to low light for 6 h and F_V/F_M was measured every hour in the presence and in the absence of lincomycin (Fig. 32, panel A). After HL treatment mutant *Δctlp40* lost most of its PSII activity although under the same conditions wild-type cells lost only about 60%. This demonstrated that PSII in the *Δctlp40* strain was very sensitive to HL and highly

photodamaged. The recovery phase of both strains from HL to LL was quite similar (Fig. 32, panel A). The recovery of PSII after photoinhibition includes usually two phases: recovery by replacement of damaged D1 protein molecules and by functional reactivation of PSII centres. The mutant could normally recover in the absence of lincomycin proving that the reaction centres that were reversibly damaged could be recovered in the mutant strain as well (Ohad *et al*., 1990). To evaluate another reason for the strong photoinhibition of PSII, an immunological analysis of D1 protein turnover was performed. Since degradation and lacking replacement of damaged D1 protein could cause a high rate of PSII photoinhibition, the turnover rate of D1 protein was detected immunologically before (DL; dim light), after photoinhibition (PI), and after 3 h of recovery to LL (R) in the absence and in the presence of lincomycin (Fig. 32, panel B). In the presence of lincomycin D1 was fully lost in both strains. In the absence of lincomycin, only 50 – 60% of the wild-type D1 protein, which corresponded to 50 – 60% loss of PSII photosynthetic activity, was degraded. The *Δctlp40* mutant strain revealed a similar rate of D1 degradation (Fig. 32, panel B).

3.2.6 Fluorescence analysis of the *Δctlp40* mutant strain under LL and HL conditions by 77K fluorescence spectra

Since PSII fluorescence detected by room temperature fluorescence analysis can be masked by the high fluorescence of phycobiliproteins, we measured additionally the 77K fluorescence in order to compare the PSII and PSI fluorescence and to check the contribution of PC/APC fluorescence as well. Cyanobacterial cells were grown under LL and acclimated afterwards to HL for 24 h. The excitation of chlorophyll *a* at 435 nm induced the emission of PSII at 695 nm and PSI at 725 nm. No changes in PSI fluorescence emission were observed in wild-type and mutant *Δctlp40* under both, LL and HL conditions (Fig. 33, panel A). However, the fluorescence of PSII was higher in *Δctlp40* cells under both regimes (Fig. 33, panel A).

The emission of phycobiliproteins and PSII was preferentially measured by excitation of phycobiliproteins at 570 nm (Fig. 33, panel B). The excitation of phycobiliproteins at 570 nm produced emission peaks of PBSs not attached to photosynthetic complexes (650 - 665 nm), PBSs associated with PSII (695 nm) and with PSI (730 nm). No significant difference in the emission of PSI in the mutant and wild-type normalised to chlorophyll *a* (2 μg/ml) could be noted under both conditions (Fig. 33, panel B). The major differences were found in the emission of „free" versus PSII-associated PBSs. The fluorescence of PBSs associated and not

associated with PSI and PSII was much higher in the mutant strain than in wild-type (Fig. 33, panel B).

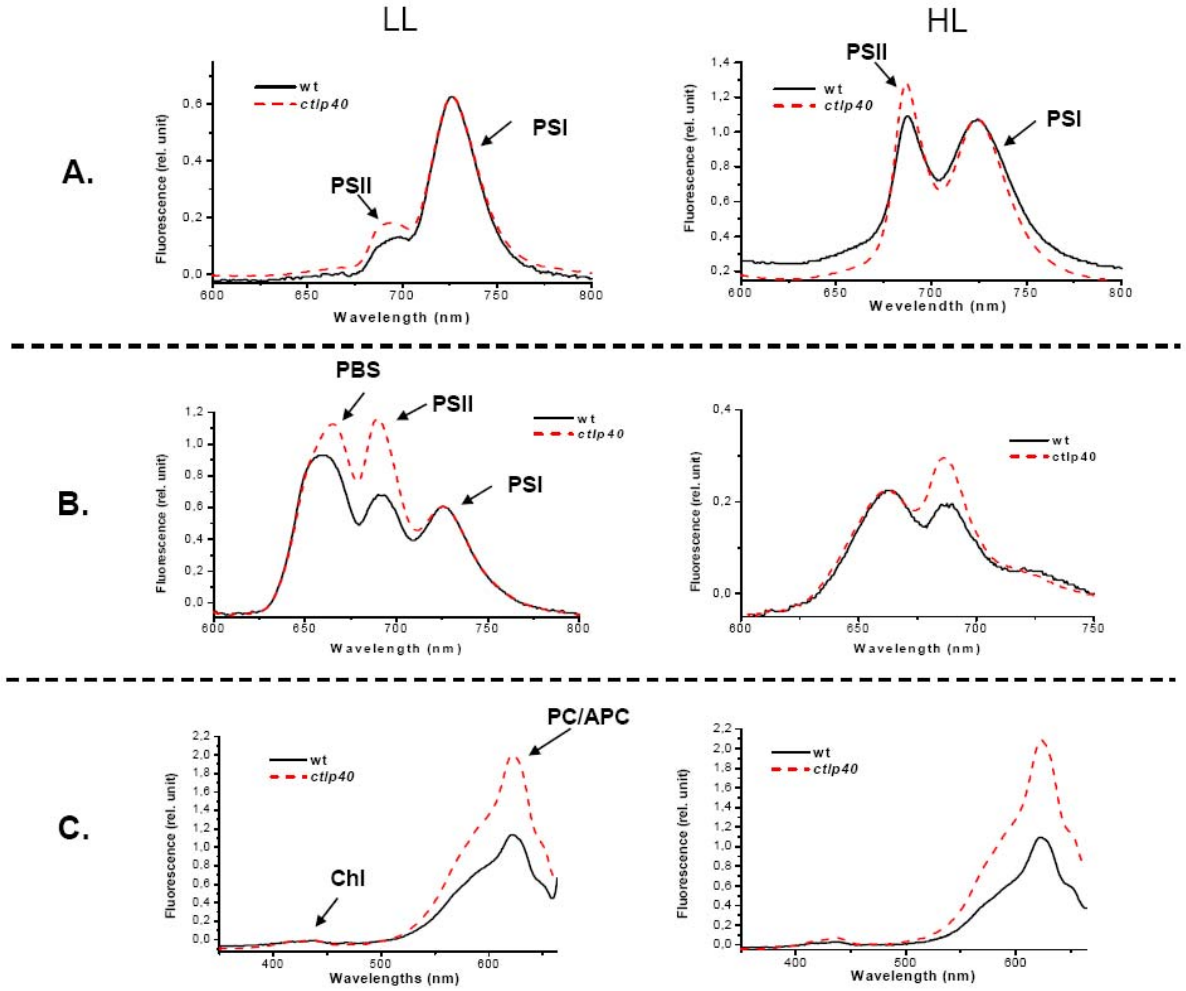


Figure 33. Low-temperature (77K) fluorescence analysis of the whole wild-type and *Δctlp40* mutant cells. The spectra of the wild-type (solid line) and *Δctlp40* mutant (dashed line) cells were recorded for the LL and HL acclimated (24 h) cyanobacterial cells. (**A**) 77K fluorescence emission spectra by excitation of chlorophyll *a* at 435 nm. (**B**) 77K fluorescence emission spectra by excitation at APC/PC absorbtion wavelength (570 nm). (**C**) 77K fluorescence excitation spectra by emission of PSII (695 nm).

The pigment composition of PSII was analysed by recording the excitation spectra by emission of PSII fluorescence at wavelength 695 nm (Fig. 33, panel C). Cyanobacterial cell cultures of wild-type and *Δctlp40* were normalised by chlorophyll *a* (2 µg/ml). The excitation spectra resulted in two peaks, a minor 440 nm peak of chlorophyll *a* and a major peak of 625 nm of PC/APC fluorescence. In the *Δctlp40* mutant strain the fluorescence of PC/APC was

substantially higher than in the wild-type under both light conditions (LL and HL; Fig. 33, panel C).

Thus, the excitation spectra also demonstrated that the level of PBSs attached to PSII in the mutant strain was higher than in wild-type cells. These data confirmed the measurements of pigment concentrations (Sections 3.2.3.2.1 and 3.2.3.2.2) showing a higher amount of PBSs in *Δctlp40* strain, especially of the PSII associated PBSs pool.

3.2.7 Analysis of photosynthetic proteins in the *Δctlp40* mutant under LL and HL regimes

In order to examine a possible role of cTLP40 protein in the biogenesis of photosynthetic complexes, protein contents were analysed immunologically using antisera raised against different photosynthetic components. Since a lower reduction of chlorophyll *a* under HL in the *Δsll0408* mutant strain may reflect a difference in the amount of chlorophyll *a*-binding proteins of PSI or/and PSII, the content of the major photosynthetic proteins was analysed in cells of both strains acclimated to LL or HL. The cell cultures grown under LL until an A_{750} = 0.6 – 0.8 were transferred for acclimation to HL for 24 h. Thylakoid membrane proteins from wild-type and *Δsll0408* cells were isolated as described in Section 2.2.10.1, separated by denaturing 12.5% SDS-PAGE and transferred onto nitrocellulose membranes. The thylakoid membrane proteins were immunodetected with antisera raised against various photosynthetic proteins. No significant differences in the content of proteins of PSI, cytochrome b_6f complex and ATP synthase between the two strains could be noted under the LL and HL (Fig. 34). However, the core subunits of PSII (CP43, CP47, D1 and D2) and the 33 kDa subunit of water-evolving complex of PSII were reduced approximately to 20 – 25% under LL (Fig. 34). After acclimation to HL the amount of PSII proteins in the wild-type was reduced, but this reduction was less pronounced in the mutant strain. The amounts of reaction centre proteins of PSII, D1 and D2, under HL was comparable with those under LL in the mutant. Thus, in *Δctlp40* mutant cells these polypeptides remained more or less stable under HL. These data showed that the higher content of chlorophyll *a* in *Δctlp40* cells after 48 h of acclimation to HL was mainly associated with differences in PSII protein amount.

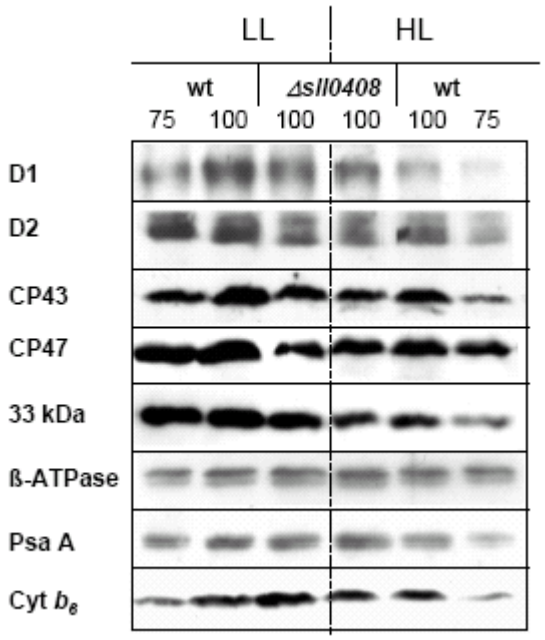


Figure 34. Immunological analysis of the major photosynthetic complexes in wild-type and *Δctlp40* mutant at LL and HL. Thylakoid membrane proteins were isolated from cells grown at LL or acclimated to HL for 24 h. Proteins were separated on a 12.5% SDS-PAG and transferred onto nitrocellulose membranes. Proteins were detected with antisera raised against the β subunit of the ATP synthase, the PsaA reaction centre protein of PSI, the PSII proteins (D1, D2, CP43 and CP47), the 33 kDa protein of the water-evolving complex and cytochrome b_6 of the cytochrome b_6f complex. 100% was equal to 5 µg of loaded chlorophyll amount.

3.2.8 Localisation and contents of the cTLP40 under different stress conditions

3.2.8.1 Analysis of the steady state levels of the cTLP40 under various growth conditions

It has previously been shown that the *Δctlp40* mutant exhibits strong phenotypical modifications under various growth conditions (Section 3.2.3.2). Consequently, protein expression of cTLP40 protein could be regulated by environmental changes. In order to test a possible correlation of cTLP40 quantity with modifications in growth conditions, its protein amount was monitored under different light and temperature stress treatments. Thylakoid membrane proteins were isolated from wild-type cells adapted to LL, HL, cold or double stresses and afterwards immunologically tested with antisera against cTLP40 protein. Western analysis showed that the amount of cTLP40 was independent from these stress conditions. The quantity of the protein was almost constant and comparable to LL, with the exception of double stress treatment when the amount of cTLP40 was slightly higher (Fig. 35, panel A). Immunodetection of the β subunit of the ATP synthase complex, which was presented in the same quantity under various stress conditions (Pojidaeva *et al*., 2004), was used as a loading control.

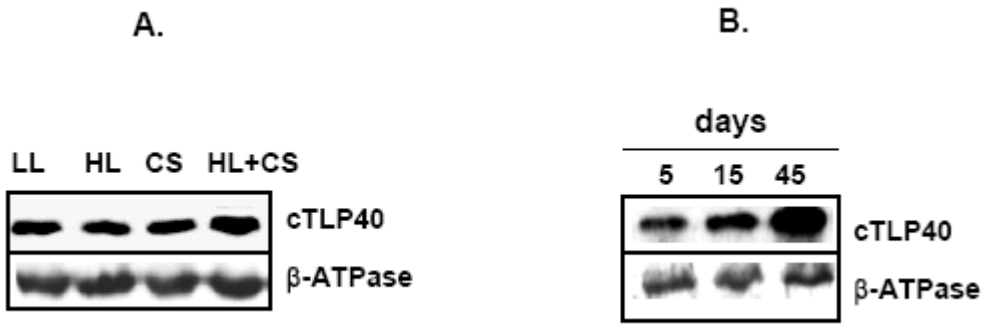


Figure 35. Analysis of cTLP40 protein under various stress conditions (A) and during long-term growth (B). (**A**) Wild-type cells were adapted to standard growth conditions (LL) and transferred to high light (HL), cold stress (CS) and double-stress (HL + CS). Thylakoid membrane proteins were separated by 12.5% SDS-PAGE. The cTLP40 protein was immunologically detected with corresponding antisera. (**B**) Thylakoid membrane proteins were isolated from *Synechocystis* wild-type grown for 5, 15 and 45 days and probed with cTLP40 antisera. The β subunit of ATP synthase was used as a control for equal loading.

To define any relationship between age of culture and quantity of cTLP40 protein, thylakoid membrane proteins were isolated from wild-type cells at 5, 14, and 45 days of cell growth and the amounts of cTLP40 were determined during cell „aging". Immunological analysis with antisera against cTLP40 protein demonstrated that its amount increased upon longer cell growth. The cells of old cultures contained substantially more cTLP40 protein than those of younger cell cultures (Fig. 35, panel B).

3.2.8.2 Localisation of the cTLP40 in thylakoid membrane under various light and temperature stress conditions

The presence of cTLP40 protein in two forms, membrane-associated and free in lumen, raised the question, whether association with the membrane could be modified depending on conditions. To check this hypothesis wild-type and mutant cells were grown under LL and acclimated for 24 h to HL, cold and double stress. Thylakoids were isolated from cyanobacterial cells and separated into soluble lumenal and thylakoid membrane proteins after the sonication procedure. Proteins were immunologically detected with antisera raised against cTLP40 and 33 kDa proteins. The 33 kDa subunit of water-evolving complex of PSII was used as a control for extraction of lumenal proteins. The cTLP40 protein was found in both, membrane-associated and soluble lumenal fractions, although about 50 – 60% of the protein was found associated with thylakoid membranes, when cyanobacterial cells were grown under

standard conditions. Under HL and cold stress the major part of cTLP40 protein was bound to thylakoid membranes, while under double stress it was completely associated with thylakoid membrane (Fig. 36). As it was expected, the lumenal 33 kDa protein was exclusively found in thylakoid lumen under all tested regimes. One can conclude that association/disassociation of cTLP40 protein to thylakoid membranes was strongly correlated with changes in growth conditions.

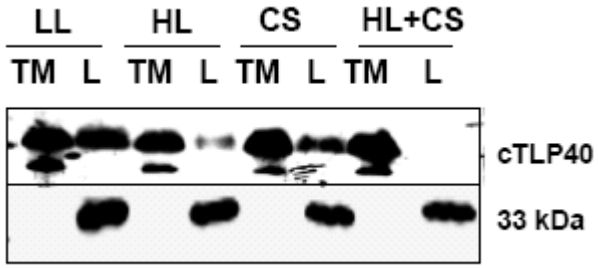


Figure 36. Localisation of cTLP40 protein in thylakoids during acclimation of cyanobacterial cells to different stress regimes. Wild-type *Synechocystis* cells were grown at LL to an A_{750} of 0.6 and then transferred to HL, CS or double stress (HL + CS) for 24 h. Cells were fractionated into thylakoid membrane (TM) and lumenal (L) proteins. After separation of proteins by 12.5% SDS-PAGE and their transfer onto nitrocellulose membranes, proteins were immunodetected with antisera raised against cTLP40 protein. The 33 kDa protein of the oxygen-evolving complex was used as a control for lumenal proteins.

3.2.9 Protein domain analysis of the cTLP40 *in vivo*

3.2.9.1 Construction of various mutant strains of the *sll0408* gene

3.2.9.1.1 Construction of mutant strain lacking the putative phosphatase-binding site of the cTLP40 protein

Previous studies of spinach TLP40 showed that its protein sequence possesses two probable phosphatase-binding domains, which could be involved in the binding of protein phosphatase that dephosphorylates the major PSII core proteins. Two potential phosphatase binding sites that flank two leucine-zipper consensus sequences share high degrees of sequence homology with proteins which bind the catalytic subunits of PP2A and PP2B phosphatases (Vener *et al*., 1999). Sequence analysis of the cTLP40 protein revealed homology only to one of the two phosphatase-binding domains of spinach TLP40 (Fig. 37).

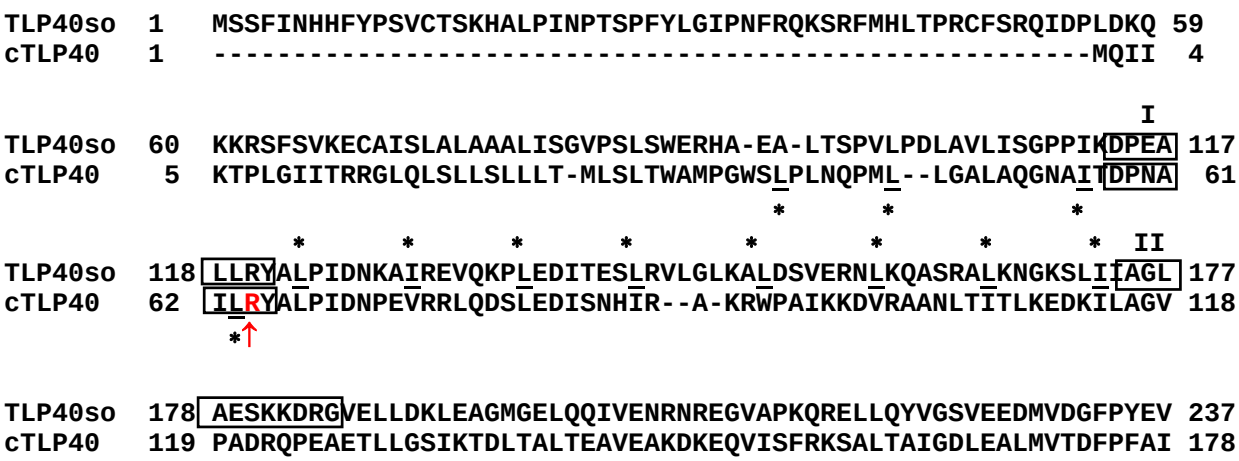


Figure 37. Comparison of the N-terminal protein sequence of TLP40 homologues. TLP40so – spinach thylakoid lumen PPIase, cTLP40 – homologous PPIase from *Synechocystis* sp. PCC 6803. The proposed phosphatase-binding domains are framed and mutated arginine (R) of the phosphatase-binding site in cTLP40 is indicated by an arrow. Leu/Ile amino acid residues of the predicted leucine zipper motifs are underlined and marked by asterisks.

The highly conservative amino acid residue Arg64 (R64) was suggested to be responsible for phosphatase-binding in the *Synechocystis* protein sequence. Since cyanobacteria lack the phosphorylation of PSII proteins, we tried to analyse, whether this residue could be involved in the regulation of some other phosphatases. The Arg64 has been mutated into Ala64 by site-directed mutagenesis *in vivo*. The control strain, $\Delta sll0408^{K}$ that contained the *Km* resistance cassette (1.2 kBp) from pUC4K, was constructed by insertion of the resistance cassette in the neutral site of the non-coding gene region directly behind the *sll0408* gene (postions 2548127 on *Synechocystis* genome; Fig. 38, panel A).

The open reading frames of *slr0434, sll0408* and *sll0409* genes were amplified from wild-type genomic DNA with primers sll0408N, and sll0408C containing *Eco*RI and *Xba*I restriction sites and specific to the 5' and 3' ends of *slr0434* and *sll0409* genes, respectively (Section 2.1.5). The amplified fragment (2.049 kBp) contained 447 bp of 5' and 459 bp of 3' regions located upstream and downstream from *sll0408*, respectively, which are required for homologous recombination. The fragment was cloned into the *Eco*RI and *Xba*I sites of pBluesckript SKII (pBSC SKII) vector that lacked the *Sal*I site. The resulting plasmid (pBSC SKIIΔ :: sll0408) was used as a vector for the construction of the control strain $\Delta sll0408^{K}$. The QuikChange mutagenesis kit (Stratagene, USA) and primer pair sll0408Mfor/sll0408Mrev (Section 2.1.5) were used for introducing the *Sal*I-site into the non-coding region between *sll0408* and *sll0409* of the plasmid pBSC SKIIΔ :: sll0408. The *Km* resistance gene from

plasmid pUC4K was digested with *Sal*I and cloned into the unique *Sal*I-site of previously obtained plasmid (Fig. 38, panel A).

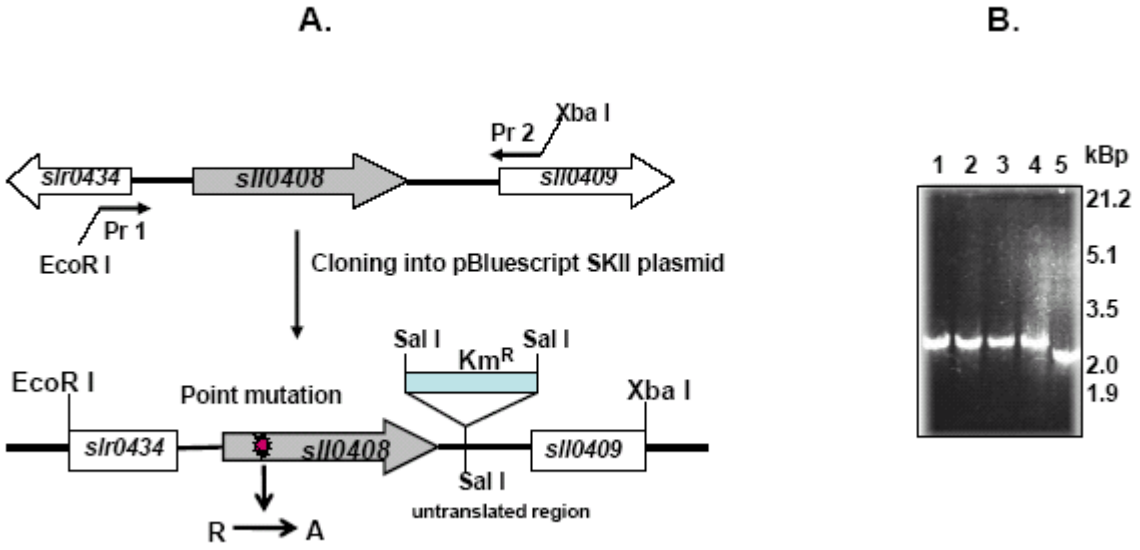


Figure 38. Scheme of point mutagenesis of the potential phosphatase-binding domain in the *sll0408* gene. (**A**) The full-length *sll0408* gene (1.143 kBp) and upstream/downstream regions (447 bp and 459 bp, respectively) were amplified on wild-type genomic DNA with primers sll0408N and sll0408C (indicated as Pr 1 and Pr 2, respectively) which contained *Eco*RI and *Xba*I restriction sites. The amplified fragment (2.049 kBp) was cloned into pBSC SKII vector and point mutations were performed with QuikChange mutagenesis kit. The 1.2 kBp *Km* resistance gene was inserted into the *Sal*I site of the region between *sll0408* and *sll0409*. (**B**) PCR analysis of *Δsll0408Ala* and *Δsll0408*^{K} mutant strains for mutant homozygosity. DNA from mutant *Δctlp40* was used as a control (lane 5). The gene regions containing point mutations were amplified with primers specific to 5' end of *sll0408* (sll0408p1) and to *sll0409* (sll0408C) (Section 2.1.5). The amplified fragment of the *Δctlp40* mutant (lane 5) was 0.35 kBp smaller than the size of the PCR fragment obtained from point mutation strains (lanes 1 – 4; lanes 1 – 3 shows independent transformant lines) due to differences in *Km* (1.2 kBp) and *Gm* (0.85 kBp) cassettes. The PCR fragment from the control DNA with *Km* resistance gene is presented in the lane 4. Molecular weight marker (λ digested with *Eco*RI/*Hind* III) is shown at the right.

As a result, the DNA of the control plasmid pBSC SKIIΔ :: sll0408_Km^{R} was used for transformation of *Δsll0408* mutant cells and control strain *Δsll0408*^{K} carrying the *sll0408* gene of wild-type and the *Km* resistance gene. The mutant strain *Δsll0408Ala* containing the point-mutation (Arg64 → Ala64) was constructed by point mutagenesis of pBSC SKIIΔ :: sll0408_Km^{R} plasmid and QuikChange mutagenesis kit with primers (sll0408Fmut/ sll0408Rmut, Section 2.1.5) containing the desirable point mutation. DNA of this plasmid was

also transformed into *Δsll0408* cells. The mutagenised DNA fragment was recombined with chromosomal DNA and restored the *sll0408* gene interrupted by the *Gm* resistance cassette. Transformants were selected on BG11 medium containing 100 µg/ml of kanamycin and 3 µg/ml of gentamycin. Sequence analysis of the mutated gene region with primers located 90 bp upstream and downstream from the point mutation (sll0408p1/sll0408p2f; Section 2.1.5) confirmed the correctness of the mutation (Arg → Ala) in the constructed strain. PCR analysis with the specific primer pair sll0408p1/sll0408C, specific to the 5' end of *sll0408* and the 3' end of *sll0409*, respectively, showed differences in the amplification size from the *Gm*-containing *Δsll0408* mutant strain (2.35 kBp) and the *Km*-containing mutagenised strains *Δsll0408Ala* and *Δsll0408*^{K} (2.7 kBp) due to differences in *Km* (1.2 kBp) and *Gm* (0.85 kBp) resistance cassettes (Fig. 38, panel B).

3.2.9.1.2 Construction of N- and C-terminal deletion mutants

Further analysis was performed to determine the functional significance of the N- and C-terminally located domains of *sll0408* that include leucine-zipper/phosphatase-binding and immunophilin motifs, respectively.

The C-terminal part of cTLP40 protein shows a high homology to immunophilins which possess PPIase activity and may catalyse the folding of proline-containing proteins. To understand the role of this domain in cTLP40, the N-terminal domain was deleted in order to keep the C-terminal part only (Fig. 39, panel A). The nucleotide sequence containing the transit peptide sequence of the *sll0408* gene (1 – 75 bp from ATG codon) was amplified on genomic DNA of the wild-type using sll0408Ntermfor and sll0408NCfusionrev primers. The sll0408Ntermfor primer was raised to the 5' untranslated region of *sll0408* at the position of – 174 bp (from the ATG codon) and contained a *Sma*I restriction site. The sll0408NCfusionrev primer contained a 24 bp stretch that overlapped to the 5' chain of the C-terminal domain. The entire C-terminal domain (0.682 kBp) was amplified by PCR with sll0408Ctermfor and sll0408Ctermrev primers. The latter one carried a *Hind*III restriction site and a TGA stop-codon. The third PCR was performed on two previously described amplification products with the primers sll0408Ntermfor and sll0408Ctermrev that contained restriction sites *Sma*I and *Hind*III, respectively. The amplified fragment (0.931 kBp) carried the transit peptide fused with the C-terminus of cTLP40. It was cloned into the unique *Sma*I and *Hind*III sites in the kanamycin gene of the vector pVZ323, which resulted in the abortion of the kanamycin

resistance. The correctness of the plasmid obtained was tested by PCR and sequence analysis. The plasmid was transferred into the *Δsll0408* mutant strain by triparental conjugation. Transconjugants (*ΔN-termsll0408*) were selected on BG11 plates containing Gm (3 μg/ml) and Cm (15 μg/ml).

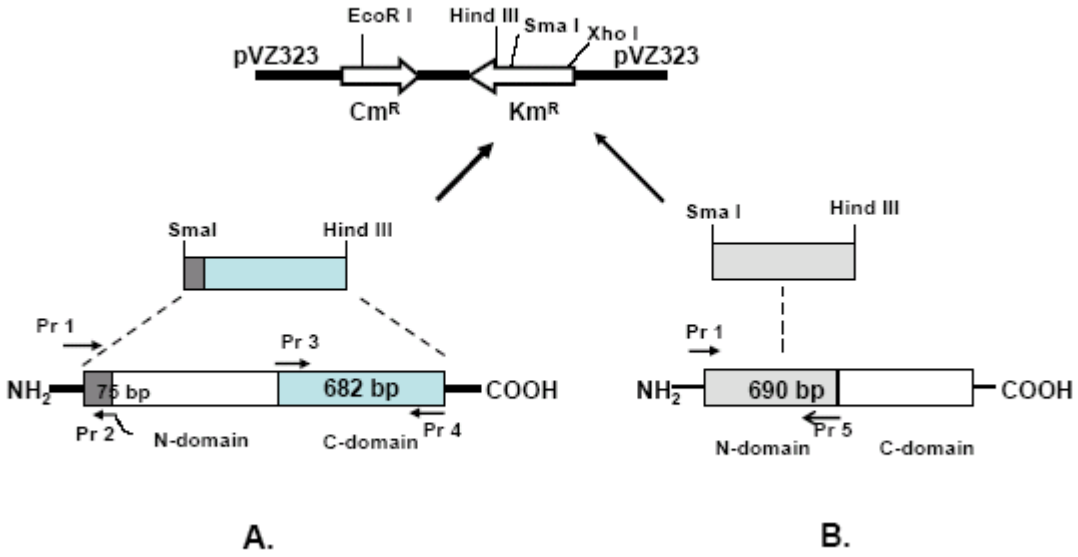


Figure 39. Schemes of the C-terminal (A) and N-terminal (B) deletion constructs of the *sll0408* gene. (**A**) The complementation strain carrying the wild-type copy of the C-terminal domain and the transit peptide of the N-terminal part of *sll0408* gene on the self-replicating plasmid pVZ323 was constructed using primers sll0408Ntermfor, sll0408NCfusionrev, sll0408Ctermfor and sll0408Ctermrev, which are indicated as Pr 1, Pr 2, Pr 3 and Pr 4, respectively. The C-domain was inserted into *Sma*I and *Hind*III sites at the *Km* gene of plasmid pVZ323. (**B**) The primers sll0408Ntermfor and sll0408Ntermrev (indicated Pr 1 and Pr 5, respectively) were used to amplify the N-terminal domain of *sll0408* gene. The fragment was cloned into *Sma*I/*Hind*III sites of the *Km* gene of plasmid pVZ323.

The N-terminal domain of plant TLP40 has been suggested to be responsible for protein binding to the inner surface of thylakoid membranes and regulation of a membrane protein phosphatase (Vener *et al*., 1999). In order to examine the role of the N-terminal domain in the cyanobacterial TLP40 homologue the mutant strain lacking the C-terminal part has been constructed. To obtain such a construct the fragment corresponding to the N-terminal domain (0.690 kBp) was amplified on wild-type genomic DNA with primers sll0408Ntermfor and sll0408Ntermrev carrying *Sma*I and *Hind*III sites, respectively, and TGA stop-codon in sll0408Ntermrev primer (Fig. 39, panel B). The sll0408Ntermfor primer was located 174 bp downstream from the ATG codon. The amplified fragment (0.864 kBp) was cloned into the

*Sma*I and *Hind*III sites of the kanamycin resistance gene of pVZ323 plasmid leading to its inactivation. Sequence analysis and PCR were used to confirm the correctness of the construct. The plasmid was mobilized into the *Δsll0408* mutant strain *via* triparental crossing. Transconjugants (*ΔC-termsll0408*) were selected on BG11 medium; Gm and Cm were used as selective markers under photoautotrophic growth conditions.

3.2.9.1.3 Point mutagenesis of functional domains of the cTLP40

The plasmid pBSC SKIIΔ :: sll0408_Km^R was used as a basis to construct vectors pVZ323 carrying the point mutation in the N- and C-terminal parts of the *sll0408* gene. Desirable point mutations were made by amplification of the gene with primers carrying point mutations and the resulting products were cloned with the QuikChange® mutagenesis kit. The plasmids made carried the point mutations in the second leucine residue of the leucine-zipper domain. The second leucine residue of leucine-zipper domain was modified to the neutral amino acid alanine (CTG → GCG) with primers sll0408L6for and sll0408L6rev.

The C-terminus of cTLP40 carries the immunophilin domain that shows highest homology to immunophilins of the cyclophilin class. This class usually possesses typical amino acid residues, arginine (Arg), asparagine (Asn) and glutamine (Glu) forming the catalytically active pocket. The transition from the *cis*- to the *trans*-form of the proline residue changes the peptide conformation which may get a more extended structure (Hur and Bruice, 2002). The cyanobacterial TLP40 contains two of these conserved amino acid residues (Arg202 and Glu210). To check, whether cTLP40 belongs to a cyclophilin PPIases, the conservative amino acid residues Arg and Glu were mutated *in vivo*. In the first construct, the arginine was replaced by leucine (CGT → CTT), and in the second one, glutamine was mutated to leucine (CAA → CTA). These two constructs were obtained in a similar way as described for the mutants of the leucine-zipper domain. The amplification primer pairs, CtermRpmF/CtermRpmR and CtermQpmF/CtermQpmR carried mutations Arg (CGT) → Leu (CTT) and Glu (CAA) → Leu (CTA), respectively. Afterwards, the double mutant strain with mutations of both, Arg and Glu amino acids residues was obtained. The vector containing the double mutations of both conservative amino acid residues in the C-terminal part was constructed using the QuikChange® mutagenesis kit. The second point mutation of residue Glu (CAA) → Leu (CTA) was performed on vector pBSC SKIIΔ :: sll0408_Km^R carrying the single amino acid substitution Arg (CGT) → Leu (CTT). The point mutation was introduced with the primers

CtermQpmF/CtermQpmR. The mutagenised plasmid pBSC SKIIΔ :: sll0408_Km^R was digested with *Hind*III and the resulting fragment of 1.8 kBp was cloned into the unique *Hind*III site of pVZ323. The resulting chimeric DNA was transferred into *Δsll0408* mutant cells *via* triparental crossing. Transconjugants were selected on BG11 medium containing Cm, Gm and Km antibiotics.

3.2.9.2 The molecular characterisation of the mutants. Analysis of complemented strains for the presence of the cTLP40 protein

The presence of cTLP40 protein in wild-type, *Δctlp40*, *Δsll0408pVZ323*, *ΔC-termsll0408*, *Δsll0408pmL2*, *ΔN-termsll0408*, *Δsll0408pmR*, *Δsll0408pmQ*, *Δsll0408pmR/Q*, *Δsll0408Ala* and *Δsll0408^K* strains was analysed immunologically with anti-cTLP40 serum.

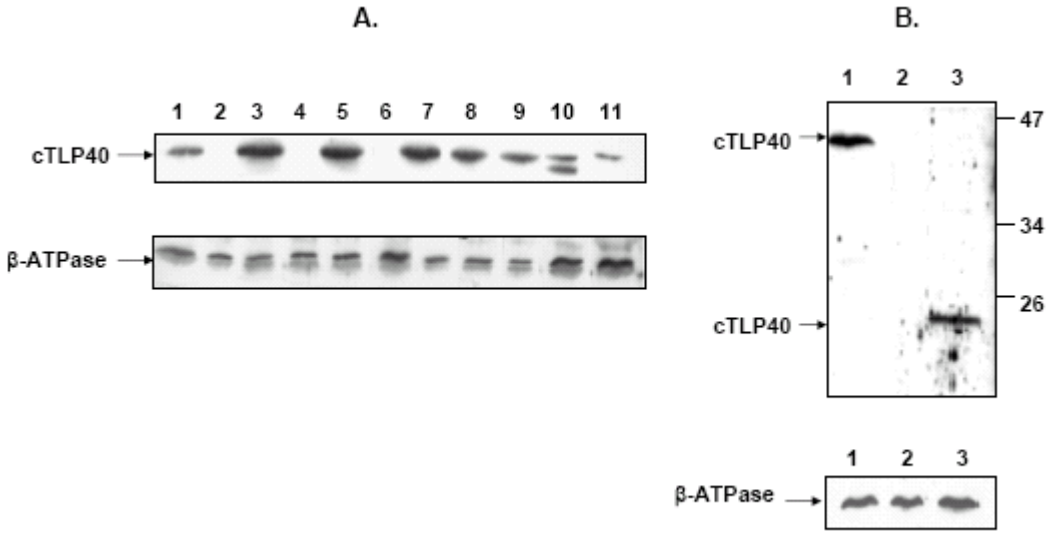


Figure 40. Analysis of complemented strains for the presence of cTLP40 protein. (**A**) Thylakoid-enriched proteins were isolated from wild-type (lane 1), *Δctlp40* (lane 2) and complementation strains (*Δsll0408pVZ323*; lane 3), *ΔC-termsll0408* (lane 4), *Δsll0408pmL2* (lane 5), *ΔN-termsll0408* (lane 6), *Δsll0408pmR* (lane 7), *Δsll0408pmQ* (lane 8), *Δsll0408pmR/Q* (lane 9), *Δsll0408Ala* (lane 10) and *Δsll0408^K* (lane 11). After 12.5 % SDS-PAGE the proteins were transferred onto nitrocellulose membrane and immunologically detected with antisera against cTLP40 and β subunit of ATP synthase as a control. (**B**) The thylakoid proteins from wild-type (lane 1), *Δctlp40* (lane 2) and *ΔN-termsll0408* (lane 3) were separated by 12.5% SDS-PAGE and immunodecorated with antisera against cTLP40 and β subunit of ATP synthase.

The full-length cTLP40 protein was found in the wild-type, complementation strain *Δsll0408pVZ323*, *Δsll0408pmL2*, *Δsll0408pmR*, *Δsll0408pmQ*, *Δsll0408pmR/Q*, and *Δsll0408^K* but not in the *ctlp40* depleted strain (Fig. 40, panel A, lane 2) and in C-terminally deficient strain *ΔC-termsll0408* (Fig 40, panel A, lane 4). Expectedly, the full-length *sll0408* product was not found also in the strain lacking N-terminal part (*ΔN-termsll0408*, Fig. 40, panel A, lane 6), but showed a crossreaction with the antiserum in a 25 kDa protein band. This molecular weight corresponded to the theoretically calculated length of the C-terminal part of cTLP40 protein (Fig. 40, panel B). Lack of crossrecativity of N-terminal domain with antisera suggested that the antigenic epitopes were located at the C-terminal part of the protein. The inactivation of the phosphatase-binding site (Arg64 → Ala64) in *Δsll0408Ala* strain resulted in the appearance of two protein bands crossreacting with cTLP40 and molecular weights of 37 (38) and 40 kDa (Fig. 40, panel A, lane 10). It is likely that the exchange of the amino acid residue Arg64 to Ala64 generated an additional protease cleavage site (L**R**YALP → L**AYA**LP; von Heijne, 1983, 1985).

3.2.9.3 Phenotypical characterisation of the *Δsll0408Ala* mutant strain

The growth of the control and mutant strains *Δsll0408Ala* (point mutation of Arg64 to Ala64) did not show any significant phenotypical differences compared to wild-type. Under double

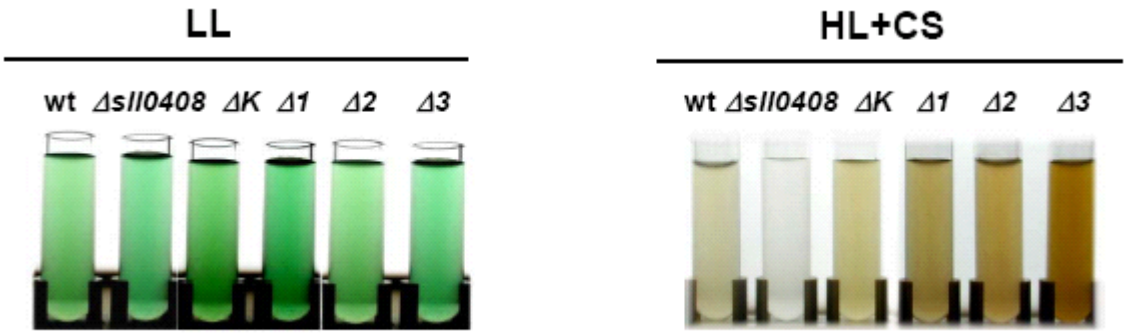


Figure 41. Phenotypical modification of *Δsll0408*, *Δsll0408Ala* and *Δsll0408^K* strains under standard (LL) and double-stress (HL + CS) regimes. Wild-type and mutant strains were grown under LL until an A_{750} of 0.6 and were acclimated to double-stress (400 μE m^{-2} s^{-1}; 17°C) for 24 h. Phenotypical changes were monitored after 24 h for control strain *Δsll0408^K* (*ΔK*) and for three independent transformants of *Δsll0408Ala* (point mutation of Arg64 → Ala64) mutant strain *(Δ1*, *Δ2* and *Δ3*).

stress treatment (400 µE m^{-2} s^{-1}; 17°C) the *Δsll0408Ala* strain showed some phenotypical modifications, but different from those of *Δsll0408*-deficient strain (Fig. 41). The cell culture of the strain with the point mutation displayed a yellow-brown colour under double stress, whereas those of the wild-type and *Δsll0408*^{K} were almost colourless. Thus, the replacement of the conservative amino acid arginine to neutral amino acid residue alanine mostly complemented the phenotype of *Δsll0408* mutant.

3.2.9.4 Photoinhibition analysis of strains containing different mutations in the *sll0408* gene

As demonstrated above (Section 3.2.5), PSII was highly sensitive to HL in the *Δctlp40* strain and HL-induced photodamage. The possibility of mutant strains (*Δsll0408pVZ323*, *ΔC-termsll0408*, *Δsll0408pmL2*, *ΔN-termsll0408*, *Δsll0408pmR*, *Δsll0408pmQ*, *Δsll0408pmR/Q*, *Δsll0408Ala* and *Δsll0408*^{K}*)* to complement the *ctlp40*-deficient phenotype was monitored through analysis of PSII photoinhibition. During photoinhibiting HL (600 µE m^{-2} s^{-1}) the *Δctlp40* mutant lost most of its PSII activity (a value of fluorescence parameter F_V/F_M ~ 0.01), while the wild-type lost only 60% of its PSII activity (F_V/F_M ~ 0.12) (Sections 3.2.4 and 3.2.5). To check which of the point mutation or deletion mutant shows a phenotype similar to *Δctlp40* strain, cyanobacterial cells of wild-type, *Δctlp40* and other mutants were normalised by chlorophyll *a* and preadapted to dim light (DL; ~ 10 µE m^{-2} s^{-1}) for 3 h. Afterwards, cells were exposed to strong photoinhibitory light (600 µE m^{-2} s^{-1}) for 2 h. PSII photosynthetic activity was monitored by measurement of the F_V/F_M ratio before and after photoinhibition (Table 7). It was observed that the mutants with N- or C-terminal domain deletions (*ΔN-termsll0408* and *ΔC-termsll0408* mutants) were sensitive to HL-induced photodamage and their PSII activity was also almost lost as in the *ctlp40*-deficient strain. Other mutants exhibited a F_V/F_M ratio similar to that of the wild-type, although it was slightly lower. The complementation strain *Δsll0408pVZ323* showed exactly the same Fv/Fm ratio as the wild-type (Table 7). These data suggested that both, N- and C-termini, of the *ctlp40* gene are important for PSII activity under strong photoinhibory light, while the point mutations did not influence strongly PSII activity under HL.

Table 7. Photoinhibition of photosynthetic membranes of wild-type, *Δctlp40* and all deletion and point mutants (*Δsll0408pVZ323*, *ΔC-termsll0408*, *Δsll0408pmL2*, *ΔN-termsll0408*, *Δsll0408pmR*, *Δsll0408pmQ*, *Δsll0408pmR/Q*, *Δsll0408Ala*, *Δsll0408^{K}*) under HL.

Strains	**F_V/F_M before photoinhibition**	**F_V/F_M after photoinhibition**
wt	0.426	0.109
Δsll0408	0.34	**0.02**
*Δsll0408*pVZ323	0.451	0.114
*Δ*C-term*sll0408*	0.42	**0.021**
*Δsll0408*pmL2	0.38	0.07
*Δ*N-term*sll0408*	0.417	**0.01**
*Δsll0408*pmR	0.44	0.092
*Δsll0408*pmQ	0.434	0.06
*Δsll0408*pmR/Q	0.39	0.075
*Δsll0408*Ala	0.39	0.076
Δsll0408^{K}	0.37	0.08

Cell cultures were grown under standard light regime (LL ~ 40 μE m^{-2} s^{-1}) and afterwards preadapted to dim light (DL ~ 10 μE m^{-2} s^{-1}). Cells were normalised by chlorophyll *a*. Photosynthetic activity of the strains was measured by F_V/F_M ratio after preadaptation to DL and after exposure of cells for 2 h to photoinhibiting light (HL ~ 600 μE m^{-2} s^{-1}).

4. DISCUSSION

4.1 Phosphorylation of photosynthetic proteins in wild-type *Synechocystis* sp. PCC 6803

4.1.1 Phosphorylation of phycobilisome linker proteins in *Synechocystis* sp. PCC 6803

The reversible phosphorylation of serine, threonine, histidine and tyrosine amino acid residues of proteins belongs to universal molecular regulatory mechanisms that can modulate structural and functional protein features. In higher plants these phosphoproteins belong mainly to the PSII complex as well as the two major polypeptides of the LHCII complex. The reversible phosphorylation of major antenna protein complex LHCPII has been recognized as the molecular basis for state transitions. The dephosphorylation of LHCII subunits is also critical in their recognition for protein degradation.

Various *in vivo* and *in vitro* labelling studies have indicated phosphorylation of thylakoid membrane proteins in photosynthetic prokaryotic organisms (summarized in Mann, 1994), like *Calothrix* sp. PCC 7601 (Schuster *et al*., 1984), *Synechococcus* sp. PCC 6301 (Allen *et al*., 1985; Harrison *et al*., 1991), *Synechococcus* sp. PCC 7942 (James *et al*., 1993) and *Synechocystis* sp. PCC 6803 (Bloye *et al*., 1992). The labelling of proteins in *Calothrix* sp. PCC 7601 revealed three proteins in thylakoid membranes phosphorylated at serine and threonine residues (Schuster *et al*., 1984). In *Synechococcus* sp. PCC 6301 labelling experiments *in vivo* with [^{32}Pi] orthophosphate demonstrated phosphorylation of an 18.5 kDa protein which was also found in a purified PBS fraction and has been proposed to represent the ß subunit of phycocyanin (Sanders and Allen, 1987; Sanders *et al*., 1989). Since these early observations of protein phosphorylation in thylakoid membranes only one additional protein, the PsbH component of photosystem II, was claimed to be phosphorylated in cyanobacteria (Race and Gounaris, 1993) although conflicting observations were reported ten years later (Komenda *et al*., 2002).

The phosphoproteins of *Synechocystis* thylakoid membranes were analysed by three strategies. The first ones were based on an immunological detection with antisera raised against phosphorylated amino acid residues and on the application of a fluorescent dye recognising phosphate groups attached to tyrosine, serine and threonine residues. The third one consists in an *in vivo* incubation of *Synechocystis* cells with radiolabelled orthophosphate. Using these

approaches, approximately 15 phosphoproteins could be detected in the thylakoid membranes of *Synechocystis*. The profiles of phosphoproteins detected by these procedures were qualitatively and quantitatively different. Phospho-specific antisera and the fluorescent dye detect proteins, which carry phosphorylated amino acid groups that may be quite long-lived, while radiolabelling with [^{32}Pi] identifies those proteins which are transitorily post-translationally modified, i.e. during the incubation time, in particular those residues that are reversibly phosphorylated.

Using the serological approach it was not possible to determine which exactly, threonine or serine amino acid residues, are phosphorylated in thylakoid proteins. The serological analysis with inhibitors blocking phospho-threonine or -serine antisera revealed that the antisera crossreact with each other. Immunologically, it has been shown that inhibitor treatments did not change significantly the profile of phosphorylated proteins, and thus, both serine and/or threonine amino acid residues could be phosphorylated.

The most easily detectable and highly phosphorylated proteins detected by the immunological approach turned out to be FNR and linker proteins (L_{CM}^{95}, L_R^{35}, L_R^{33} and L_{RC}). The phosphoprotein with a molecular weight of about 30 kDa, previously detected with a phospho-threonine antisera (Pursiheimo *et al*., 1998), could correspond to one of the rod linker proteins. We did not observe any phosphorylation of the major bilin-containing antenna proteins, APC and PC, which contrasts with a previously published report (Harrison, 1990). FNR, which was shown to be associated with PBSs (Schluchter and Bryant, 1992), was substantially phosphorylated in our antenna preparations. The phosphorylation of FNR from higher plant chloroplasts at serine and threonine residues has been observed, but the function of this process remains poorly understood (Hodges *et al*., 1990). The profile of phosphorylated proteins in wild-type and cyanobacterial mutant strains deficient in PBSs (PAL strain) or only rod linker proteins (CK strain) differed in these bands corresponding to the missing linkers in the mutants. This proved that the signals with phospho-threonine antisera corresponded to linker proteins.

The detection of substantial signals of linker proteins with anti-phospho-threonine sera contrasted with their poor phosphorylation by orthophosphate [^{32}Pi]. This indicated that linker phosphorylation at serine or/and threonine residues is quite a stable posttranslational modification. Surprisingly, the rod linkers were poorly sensitive to dephosphorylation treatment and their dephosphorylation in PBS preparations by alkaline phosphatase did not

exceed 50%. This could be explained by the localisation of linker proteins, connecting phycocyanin segments in the internal cavities of the disks (Reuter *et al*., 1999; Stec *et al*., 1999). Therefore, externally added enzymes have little access to the linkers within fully assembled PBS structures. The separation of partially disassembled PBSs that were pre-treated with alkaline phosphatase demonstrated that indeed only partially or completely dissociated PBSs can be fully dephosphorylated while linkers in fully assembled PBSs are not accessible. Moreover, it was observed that dephosphorylation of linkers in partially assembled PBSs further disassembled their constitutive subunits. This experiment suggested that dephosphorylation of linkers could participate in the mechanism governing the remodelling and turnover of the PBSs in *Synechocystis*. In addition, the dephosphorylation of linker proteins led to higher rates of their degradation at 37°C. Non-dephosphorylated linker proteins were more stable, although it seems that PBS preparation contained cyanobacterial phosphatase(-s) as well.

In higher plant chloroplasts phosphorylation/dephosphorylation of light harvesting antenna is involved in state transitions and in antenna degradation, two phenomena that are controlled by changes in light intensity and light quality. By contrast, no changes in the rate of the phosphorylation/dephosphorylation of linker peptides during state transitions could be observed in *Synechocystis* cells.

It is known that high light, as well as nitrogen deprivation, induces a down-size of the PBS antenna that in part results from changes in expression of phycobiliprotein genes (Belknap and Haselkorn, 1987; de Lorimier *et al*., 1991) and in part from degradation of PBS subunits (Grossman *et al*., 1993a; Pojidaeva *et al*., 2004). In particular, protease SppA1 causes the cleavage of linker proteins under acclimation to higher light regimes with subsequent release of distal PC rod segments (Pojidaeva *et al*., 2004). Upon acclimation to nitrogen deprivation, it was observed that membrane associated PBSs lost completely L_R^{33} and L_R^{35}, which results in shortening of the PC rods. The preservation of the L_{RC} linkers and of a significant fraction of the L_{CM}^{99} linker in the membrane fraction argues for the association of smaller PBSs with the thylakoid membrane under these conditions. Indeed, it was observed that the rate of linker protein phosphorylation decreased with environmental cues that cause restructuring, disassembly and degradation of PBSs. The acclimation of cells for at least 24 h to high light or to nitrogen starvation induced a marked decrease in the amount of phosphorylation of the L_{RC} and L_{CM}^{99} linkers. The net dephosphorylation of these two linkers, as compared to that of L_R^{33}

and L_R^{35} could be due to their slower degradation rate after full dephosphorylation. This observation argues for a role of linker dephosphorylation in PBS remodelling *in vivo*.

Linker dephosphorylation may act as a signal for protein degradation once PBS disassembly has started, while protein phosphorylation could occur before or during assembly of PBS hexamers (Fig. 42).

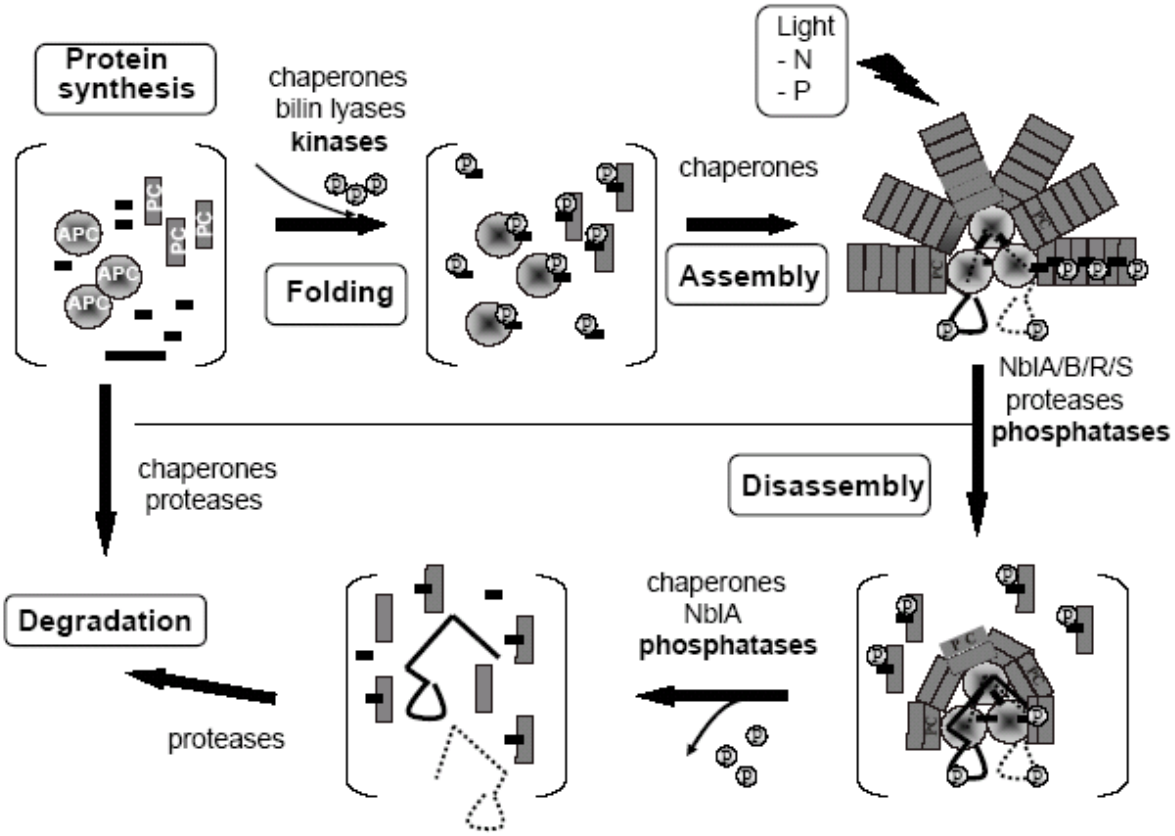


Figure 42. Model for the putative role of protein linker phosphorylation in the biogenesis of PBS structures. Synthesized linker proteins are phosphorylated by some intracellular kinase(-s) either when partially assembled with PC and APC or directly after synthesis. The whole PBS structures are fully assembled afterwards in a step-by-step process (monomers, trimers, hexamers and whole PBSs; not shown). Various stress conditions like high light and nutrient deprivation initiate PBS degradation. Linker dephosphorylation may occur either in partially disassembled PBS or within truncated forms of PC-L_R. Later on the dephosphorylation of linkers can serve as a signal for protein degradation.

The initial steps of biliprotein biosynthesis include competition between protein biosynthesis and degradation. The biliprotein subunits with no attached bilins or those lacking partners can be rapidly subjected to degradation (summarised in Anderson and Toole, 1998). The mechanisms controlling biliprotein degradation are unknown although one of the suggested

models is the binding of chaperone proteins that would activate a degradation pathway (Anderson and Toole, 1998). In that case phosphorylation may play an important role in the stabilisation of linker polypeptides in the PBS assembly pathway, while their dephosphorylated state could be a signal for their degradation. Changes in protein phosphorylation modify folding that in turn changes affinity of these proteins for proteolytic enzymes and assembly partners. The role of linker (de)phosphorylation in cyanobacteria would then be similar to that of the phosphorylation of LHCII and D1 protein in higher plant chloroplasts that are considered to be targeted to degradation upon dephosphorylation (Koivuniemi *et al*., 1995; Yang *et al*., 1998). A long phase of acclimation to higher light intensities of at least 24 h is required for the detection of PBS protein dephosphorylation. Such a lag period in the acclimation was also observed for the light harvesting complex of higher plants (Yang *et al*., 1998). This argues for an activation of some substrates or/and enzymes involved in the protein dephosphorylation and/or cleavage.

Up to now, it is not clear how PBS structures are reduced during nutrient deprivation or light stress, conditions which cause a massive degradation of cyanobacterial antenna, leading to a shortening of PBS rods and degradation of PBS structures (Yamanaka and Glazer, 1980; Duke *et al*., 1989; Collier and Grossman, 1992; Grossman *et al*., 1993a, 2001). The *nbl* gene family encodes the major protein family that is known to be involved in the degradation of PBSs. One of the components, NblA, is directly involved in PBS degradation under nutrient deprivation (Collier and Grossman, 1994; Baier *et al*., 2001, 2004). It has been suggested that binding of NblA to PC can mark proteins for their further recognition by proteases or can soften the PBS structure by increasing the distances between single PC segments. It is then expected that proteases and phosphatases will get better access to their substrates. These and other questions could be approached by studies on NblA and cyanobacterial phosphatases.

4.1.2 Enzymes involved in phosphorylation/dephosphorylation of *Synechocystis* sp. PCC 6803 phycobilisomes

Serine/threonine protein phosphatases and kinases are required for reversible protein phosphorylation and have been shown to play an important role in metabolic regulation and signal transduction in eukaryotes (Cohen, 1992; Hunter, 1995). The importance of serine/threonine protein kinases and phosphatases in bacterial signal transduction has only recently been appreciated, as they are not involved in regulating signal transduction in *E. coli*

(Bork *et al*., 1996; Zhang *et al*., 1996; Adler *et al*., 1997; Leonard *et al*., 1998; Shi *et al*., 1998). However, many bacteria that exhibit complex interactions with their environment or alternative morphological forms do utilize these enzymes (Munoz-Dorado *et al*., 1991; Adler *et al*., 1997; Leonard *et al*., 1998; Kamei *et al*., 2001; Treuner-Lange *et al*., 2001). It has been shown by phosphorylation of PII protein in cyanobacteria (Forchhammer and Tandeau de Marsac, 1994; Irmler and Forchhammer, 2001) that these enzymes are also involved in the regulation of central metabolic processes. Nevertheless, our knowledge of the molecular networks of bacterial signal transduction systems involving protein O-phosphorylation and their function in the cell regulation is still preliminary (Bakal and Davies, 2000). The observations demonstrated that cyanobacteria contain much less of reversibly phosphorylated proteins than higher plants in their thylakoid membranes (Harrison *et al*., 1991) and that the degree of phosphorylation is not very high.

Previous inspections of the *Synechocystis* sp. PCC 6803 genome revealed eight genes for potential members of the PP2C phosphatase family and one for PPP-type phosphatase (Shi *et al*., 1998; Zhang *et al*., 1998b). PP2C-like protein phosphatase dephosphorylates Ser and Thr residues and is known to be important for signal transduction in eukaryotes, but have also been identified in several bacteria including *Pseudomonas aeruginosa*, *Bacillus subtilis* and cyanobacteria (Cohen *et al*., 1990; Barford, 1995; Bork *et al*., 1996; Adler *et al*., 1997). The signal transduction protein PII is dephosphorylated in *Synechocystis* 6803 by protein phosphatase PphA, a member of the 2C family (PP2C) (Irmler and Forchhammer, 2001; Ruppert *et al*., 2002). To find enzymes (kinases and phosphatases), which can be involved in the phosphorylation/dephosphorylation of linker proteins of PBSs, several mutant strains deficient in serine/threonine kinases and phosphatases were analysed. Since the rate of linker protein phosphorylation/dephosphorylation strongly depends on environmental conditions, phosphorylation/dephosphorylation of PBSs were analysed under low light and under nitrogen deprivation. Both, *Δsll1771* and *Δsll1033* phosphatase-deficient mutants, were analysed for their ability to dephosphorylate phospho-linker proteins. The analysis of these mutants revealed a reduced rate of linker protein dephosphorylation under nitrogen starvation and a higher stability of PBS proteins in the mutant deleted in the *sll1033* gene encoding a protein phosphatase. Thus, the phosphatase Sll1033 is probably involved (directly or indirectly) in the dephosphorylation of PBS linker proteins. It was also observed that another phosphatase (PphA; Sll1771) displays a higher level of phosphorylation of L_{CM}^{99} linker under both LL and nitrogen deprivation conditions when compared to wild-type and *Δsll1033* mutant. At the same

time the rate of degradation of all linker proteins including L_{CM}^{99} was unchanged in comparison to wild-type. Previously it was demonstrated that the Sll1771 gene product is responsible for dephosphorylation of the PII signalling protein in the cyanobacterium *Synechocystis* sp. PCC 6803 (Irmler and Forchhammer, 2001). The PII homologues in different organisms are involved in various aspects of nitrogen control (Ninfa and Atkinson, 2000). PphA was the first member of the PP2C phosphatases for which the physiological substrate has been identified. It has been shown that the PII homologue from the cyanobacteria was modified in response to the nitrogen and carbon status of the cells through phosphorylation of a serine residue (Forchhammer and Tandeau de Marsac, 1994; Forchhammer and Tandeau de Marsac, 1995b; Ruppert *et al*., 2002). For example, the PII homologue from the *Synechococcus* 7942 was modified by phosphorylation of Ser49 (Forchhammer and Tandeau de Marsac, 1995b) in the vicinity of the conserved Tyr51. It was shown that the phosphorylation state of PII protein is maximal under conditions of nitrogen deprivation (Forchhammer and Tandeau de Marsac, 1995a). It was shown that the mutant lacking PphA was impaired in an efficient utilization of nitrate as nitrogen source and that non-phosphorylated PII controls the utilization of nitrate in response to low light (Kloft and Forchhammer, 2005). To test, whether Sll1771 (PphA) phosphatase can use other substrates than PII, the level of dephosphorylation of linker proteins was analysed in the *Δsll1771* mutant. No differences in the rate of dephosphorylation and degradation of linker proteins could be observed under condition of nitrogen deprivation, with the exception of L_{CM}^{99} that showed an increased level of phosphorylation.

The analysis of three serine/threonine kinase mutants did not show any decrease of the phosphorylation rate of linker proteins. However, the mutant deleted in *slr0559* gene demonstrated a higher level of phosphorylation of L_{CM}, L_R^{35}, and L_{RC} linker proteins under nitrogen starvation. This resulted in a higher stability of linker proteins. Thus, one can conclude that Slr0559 kinase cannot be directly involved in the phosphorylation of linker proteins, and (or) may be required in signal transduction pathways for regulation of protein phosphatase or other components participating in linker dephosphorylation.

4.2 Characterisation of the cTLP40

4.2.1 Characterisation of the cTLP40 in *Synechocystis* wild-type cells

Peptidyl-proline-isomerases (PPIases) or rotamases is a widely distributed group of proteins that are involved in the isomerisation of proline imidyl bonds and thus participate in the protein folding. A unique rotamase-like protein was found in the chloroplast lumen and it was suggested to be essential for regulation of protein phosphatase which dephosphorylates the reaction centre subunits of PSII (Fulgosi *et al*., 1998; Vener *et al*., 1999). The chloroplast TLP40 homologue has been found in cyanobacteria and its physiological role was not studied up to now. Although the structure of cTLP40, a product of gene *sll0408*, is similar to higher plant TLP40 and includes the N-terminal domain with one leucine-zipper motif, one possible phosphatase-binding site and the C-terminal rotamase domain (Fulgosi *et al*., 2002), a comparable role of cTLP40 in the regulation of a phosphatase was excluded due to lack of phosphorylation of PSII proteins in cyanobacteria (Pursiheimo *et al*., 1998). Similarly to higher plant chloroplasts the product of the *sll0408* gene is targeted to the thylakoid lumen compartment in cyanobacterial cells and found in two forms, free and membrane associated. The cTLP40 protein is only peripherally attached to thylakoid membranes and can be washed out by treatment with NaSCN and alkaline solution. Proteom analysis of soluble periplasma and membrane proteins from *Synechocystis* revealed this component in the thylakoid fraction with a predicted N-terminal *Tat* signal peptide (Srivastava *et al*., 2005). This indicated that the cTLP40 protein is located in the thylakoid lumen. Presence of highly homologous rotamase-like components in plant chloroplasts and cyanobacterial thylakoids raised the question on the functional properties of the corresponding component and its possible evolutionary differences in photosynthetic prokaryotic and eukaryotic organisms.

4.2.2 Physiological role of the cTLP40

Cyclophilins have been showed to act as stress response proteins (Sykes *et al*., 1993; Luan *et al*., 1994b; Marivet *et al*., 1994). Localisation of immunophilins in chloroplasts suggested their possible role for these proteins in the assembly of the photosynthetic apparatus (Luan *et al*., 1994a). PPIases can act as chaperones or cooperate with other chaperone proteins (Pratt *et al*., 2001). In plants, chloroplast-localized molecular chaperones have been characterized and shown to be regulated by light (Gatenby and Ellis, 1990; Chou and Gasser, 1997).

The interposon mutagenesis of *sll0408* in the *Synechocystis* sp. PCC 6803 genome produced a viable photoautotrophic mutant strain with similar phenotypical features of the wild-type under low light (LL) regime. Electron microscopic observations of *Δctlp40* cells revealed that their thylakoid membranes had an irregular morphology and were more frequently segmented compared to wild-type under LL conditions (Fulgosi *et al*., 2002). Previously it has also been reported that *ctlp40*-deficient cyanobacteria cells were impaired in photosynthesis and its photosynthetic activity was light dependent. The photosynthetic rate of *Δctlp40* cells nearly equals the rate of their respiration under HL conditions (Fulgosi *et al*., 2002). The measurements of photosynthetic activities by a Clark-type oxygen electrode revealed that the oxygen production rate in *Δctlp40* mutant cells was nearly zero after 40 min of HL treatment, while in the wild-type strain the decline of oxygen production started slowly after 70 min of HL treatment (Canino, 2006). Such a drop in photosynthetic activity could indicate the defects in PSII activity.

Different environmental parameters can modify the activities of photosynthetic complexes and the levels of pigments and proteins associated with these complexes (Grossman *et al*., 1995). The photosynthetic apparatus is highly dynamic and alteration of light harvesting complex synthesis and degradation occurs in response to environmental stimuli including changes in light quality (Tandeau de Marsac, 1977; Bryant and Cohen-Bazire, 1981), light intensity (Horton *et al*., 1996), and nutrient availability (Allen and Smith, 1969; Yamanaka and Glazer, 1980; Collier and Grossman, 1992). Such an alteration helps efficiently to balance the absorption of excitation energy. An excess of absorbed energy by photosynthetic reaction centres can result in the production of toxic oxygen species, which can induce photooxidative damage within the thylakoid membrane and of other cellular processes (Asada, 1994a, 1994b). The biochemical analysis of major photosynthetic proteins from wild-type and *Δctlp40* mutant cells which were acclimated to LL (low light) and HL (high light) demonstrated a slightly lower content of PSII subunits in the mutant when the cells were grown under LL. After acclimation to HL the amount of PSII proteins of wild-type markedly declined, while PSII subunits of the *Δctlp40* strain were less reduced when compared to LL. The amounts of PSI and cytochrome b_6f complex subunits were comparable in wild-type and mutant strain under the conditions tested.

Measurement of the major PSII parameters of photosynthetic complexes by chlorophyll fluorescence at room temperature revealed changes in the redox state of PSII. Analysis of

cyanobacterial cells grown under standard low light conditions showed a lower F_V/F_M ratio in the *Δctlp40* mutant. This parameter, which reflects the functional activity of PSII, could result from defects in PSII or from changes in electron flow from PSII to PSI. Furthermore, another index of photosynthetic capacity is the photochemical quenching coefficient (q_P), which was also lower in *Δctlp40* mutant cells (Table 6). This parameter reflects the ratio of open and closed reaction centres and shows the balance between excitation and reoxidation of PSII. When photochemical quenching of fluorescence is maximal ($q_p = 1$), the fluorescence yield of PSII is low (all PSII centres are opened) and photochemical quenching is minimal ($q_p = 0$) when all PSII centres are closed. Another possibility could be a slower rate of reoxidation of the PQ pool in the mutant strain, because the size of the PQ pool was larger in the mutant strain (Fig. 31 and Table 6). This suggested that electrons were not efficiently transferred from the PQ pool to the cytochrome b_6f complex, and as a consequence, the rate of PQ pool reoxidation was slowed down. Thus, the transfer of electrons from the PQ pool to PSI through the cytochrome b_6f complex might also be defected in the mutant strain.

Additionally, photoinhibition of wild-type and mutant cells which was monitored by room temperature fluorescence revealed that the *ctlp40*-depleted strain was highly sensitive to photoinhibition. The loss of PSII activity in *Δctlp40* cyanobacterial cells was much faster than in wild-type under high light, a process which leads to the damage of the D1 protein, its degradation and resynthesis of new D1 molecules (Ohad *et al*., 1984, 1990; Greenberg *et al*., 1987; Adir *et al*., 1990). In most cases the high photoinhibition rate is caused by retention of damaged D1 protein in the PSII core that prevents an exchange of the damaged molecules to newly synthesized ones (Ohad *et al*., 1990; Aro *et al*., 1993; Tyystjärvi *et al*., 1996; Adir *et al*., 2003). In the *Δctlp40* strain, D1 was degraded with a similar rate as in wild-type and PSII could recover under low light with a similar rate as in the wild-type. An addition of lincomycin, an inhibitor of the protein synthesis, caused a strong photoinhibition in both, wild-type and *Δctlp40*. The photoinhibition rate in the mutant strain in the presence and in the absence of lincomycin was quite similar. Despite a strong photoinhibition without lincomycin, the D1 protein was degraded with a similar rate as in the wild-type. Strangely, the inhibition of PSII activity up to 95% led only to degradation of about 50% of D1 protein. The restoration of PSII photosynthetic activity under low light was going on with similar rate in wild-type and mutant. These observations confirm findings of G. Canino (2006) that cTLP40 is not involved in the degradation of D1, but is involved in other steps of PSII biogenesis. The assembly studies of

PSII demonstrated that *Δctlp40* is affected in the dimerisation or monomerisation of PSII (Canino, 2006).

High light causes an increased turnover of D1 protein of the PSII reaction centre and LHCII proteins in higher plants (Prasil *et al*., 1992; Aro *et al*., 1993; Lindahl *et al*., 1995). This degradation event is connected with a release of free chlorophyll molecules. Binding of free chlorophyll molecules and preventing the formation of toxic oxygen species through triplet quenching *via* carotenoids would consequently be very significant for protection against oxidative damage in chloroplasts under light stress (Adamska *et al*., 1999). In cyanobacteria similarly to higher plants the acclimation to HL leads to a decrease in the content of photosystems (Murakami and Fujita, 1991; Sonoike *et al*., 2001; Muramatsu and Hihara, 2003). The shift of cyanobacterial cells to HL leads to a decrease of chlorophyll *a* and phycocyanin contents per cell, which reflects the decrease in PBS and chlorophyll *a* binding proteins (Hihara, 1998). Such reduction of the antenna size and amounts of photosystem content is necessary for protection against absorption of excess of light energy.

The pigment analysis revealed that under low light the *ctlp40* deletion has essentially no effect on the pigment composition of the cells, although the amount of PBSs was slightly increased and the chlorophyll concentration was slightly lower. PBSs in cyanobacterial cells can be found in a membrane-associated and free forms. Previously, it has been shown that PBSs diffuse quite rapidly on the surface of the thylakoid membrane. Interactions of PBSs with reaction centres are transient and unstable. PBSs can be assembled and membrane-associated even in the absence of PSII (Bittersmann and Vermaas, 1991), PSI (Shen *et al*., 1993), or both reaction centres (Yu *et al*., 1999). Although most PBSs are attached to reaction centres at any given moment, PBSs frequently decouple, diffuse, and bind to another reaction centre (Mullineaux *et al*., 1997; Sarcina *et al*., 2001). The nature of the association of PBS with membranes and functional role of PBSs mobility is not clear.

The analysis of PBS association to thylakoid membrane showed that the level of phycobiliproteins bound to the membrane under LL was higher in the mutant cells than in wild-type. The same analysis also demonstrated that under stress conditions (HL, CS and double stress), the ratio of PBSs associated to the membrane and free in wild-type cells decreased, while at the same time the more harsh double stress lead to a decrease in the ratio of the membrane-bound versus free PBSs in mutant cells, but to a much lesser extent (for example in

the wild-type this ratio was 2.5 times and in mutant cells 1.5 time less under double stress than under LL). However, other stresses such as a HL and CS did not lead to a decrease of this ratio in *Δctlp40* mutant cells. Moreover, association of PBSs with thylakoid membranes under these conditions became even stronger in the mutant strain. Such a strong connection of PBSs with the membrane could mean that disassociation of PBSs from thylakoid membranes in *Δctlp40* cells was impaired under stress regimes as well as under LL. These data were confirmed by low temperature fluorescence measurements under LL and HL. Fluorescence emission spectra at 77K with excitation of phycobiliproteins at 570 nm showed the ratio of PBSs bound to reaction centres of PSII and PSI versus free PBSs. No significant changes in the emission of PSI under both conditions (LL and HL) could be observed, while the ratio of PBPs associated with the reaction centre of PSII to free phycobiliproteins was increased in *Δctlp40* cells comparably to wild-type under HL as well as under LL regimes. This indicated that the connection between PBSs and PSII in the cTLP40 mutant was significantly stronger than in wild-type.

The *Δctlp40* mutant strain that phenotypically did not differ significantly from the wild-type under standard growth conditions grew with a twofold enhanced rate when kept under a HL regime. Moreover, the microscopic observations revealed that after 24 h of acclimation to HL the size of *Δctlp40* mutant cells was smaller than that of the wild-type and under the same growth conditions the amount of mutant cells per ml of the cell culture also increased. Acclimation of cyanobacterial cells to HL led to degradation of PSII proteins and thus to a decrease of the pigment content. After HL treatment the wild-type cells were bleached, while *Δctlp40* mutant cells remained dark green. The chlorophyll *a* and phycocyanin content in the wild-type decreased up to 35 and 30%, respectively, when cells were transferred for 48 h to HL. Under the same conditions the mutant strain reduced only 15 and 21% of chlorophyll *a* and phycocyanin. The drop of pigment content upon transfer from LL to HL reflects the activation of the photoprotection mechanisms in thylakoid membranes, e. g. the decrease of the PSII/PSI reaction centres and PBS antenna (Grossman *et al*., 1993a, b; Rakhimberdieva *et al*., 2001; Joshua and Mullineaux, 2004). The higher rate of chlorophyll *a* reduction upon transfer from LL to HL in the mutant strain was accompanied by a lower reduction of the steady state levels of PSII reaction centre proteins. Interestingly, is that mutant cells showed the same quantity of chlorophyll *a* during the first 24 h of degradation as the wild-type. However, after 24 h the degradation of major pigments was arrested in mutant cells. As a consequence, during next 24 h the amount of these pigments remained constant in the mutant. Degradation of the

PBSs is an ordered process, by which the terminal hexamers of the PBS rods and their associated linker polypeptides are degraded first. This degradation is followed by degradation of the next phycocyanin hexamers and their associated linker polypeptides. These proteolytic events result in a decrease of the number of rods associated with the PBS core. After the loss of rods, the entire complex is destroyed (Yamanaka and Glazer, 1980; Collier and Grossman, 1992; Grossman *et al*., 1993a). This finding suggested that the inactivation of *sll0408* leads to an impairment of PBS degradation during the second phase of HL acclimation. The fact that the process of degradation was not completely blocked in *Δctlp40* cells but was slowed down suggested that the product of the *ctlp40* gene is involved directly or indirectly in PBS degradation.

The most significant differences in pigment analysis were found in the amounts of carotenoids upon HL acclimation. The carotenoids increased up to 58% upon 48 h HL acclimation in the wild-type. In the *Δctlp40* mutant it even slightly decreased to about 4% after 48 h of HL acclimation. The reduction of the carotenoid pool in mutant cells under HL indicates a defect in the photoprotective mechanism of photosynthetic membranes.

In photosynthetic organisms carotenoids play a protective role against photooxidative damage upon HL illumination (Frank and Cogdell, 1996; Ort, 2001). Wild-type *Synechocystis* is highly protected against light-mediated photooxidation (Schafer *et al*., 2005). Changes in carotenoid content and composition, which occur in response to light intensity and quality, is one of the major adaptation mechanisms to HL that was evolved in cyanobacteria (Kellar and Paerl, 1980; Codd, 1981). It was demonstrated that mutant cells of *Synechococcus* sp. PCC 7942, which contained less carotenoids, were more sensitive to photoinhibition (Sandmann *et al*., 1993). It has also been shown that acclimation to HL was associated with a decrease in the chlorophyll to carotenoid ratio, in addition to a decline in both, chlorophyll and phycobiliprotein content (Raps *et al*., 1983). Analyses of carotenoids in PSII core complexes from wild-type *Synechocystis* 6803 identified *β*–carotene as the major carotenoid and minor components such as a *β*–cryptoxanthin, zeaxanthin, myxoxynthophyll and (or) echinenone (Tracewell *et al*., 2001; Bautista *et al*., 2005). The PSII core complex of the cyanobacterium contains ~13 carotenoid molecules dominated by *β*–carotene. Pigment analyses have identified two *β*-carotene molecules bound to the D1 and D2 polypeptides (Kobayashi *et al*., 1990; Eijckelhoff and Dekker, 1995; Tomo *et al*., 1997). Both molecules have been reported to be redox-active (Telfer *et al*., 2003; Tracewell and Brudvig, 2003). It was also suggested that *β*–carotene is

required for assembly of an active PSII but not for PSI (Masamoto *et al*., 2004). Thus, the destruction of PSII structure could influence the binding of the carotenoids to the PSII reaction centre.

Besides, the cyanobacteria contain specific carotenoid-binding proteins (CPs). These proteins, which function in photoprotection, are water-soluble and their abundance increases under HL treatment (Wu and Krogmann, 1997; Kerfeld, 2004). The fact that *Δctlp40* apparently cannot regulate carotenoid levels under HL illumination implies that the photoprotection mechanisms were strongly affected in the mutant.

Furthermore, it was observed that under any stress conditions (heat stress, high light and cold stresses) for 24 h and nitrogen starvation for at least 8 days the *Δctlp40* cells remained more green than that of the wild-type. Acclimation responses that occur during nitrogen deprivation involve degradation of the photosynthetic pigments. The first step of an acclimation process is the rapid and almost complete degradation of the light-harvesting pigment-protein complexes, PBSs. The loss of PBSs and reduction of chlorophyll levels per cell during nutrient-limited growth helps growing cells to differentiate into non-pigmented cells which are able to endure long periods of starvation (Allen and Smith, 1969; Collier and Grossman, 1992, 1994; Grossman *et al*., 1993b; Gorl *et al*., 1998). Degradation of PBSs may provide limited nitrogen and serve for reduction of the light absorbed by the photosynthetic apparatus during starvation conditions (van Waasbergen *et al*., 2002). It has been shown that starvation of wild-type cells for nitrogen triggers the disconnection of the PBSs from PSII reaction centres, the degradation of the light-harvesting complex, and the down-regulation of PSII activity (Collier and Grossman, 1992, 1994; Collier *et al*., 1994). It has been suggested that chaperones may be essential in the first step of PBS assembly when the degradation of biliproteins competes with protein biosynthesis (Anderson and Toole, 1998). It was suggested that cTLP40 might be a one candidate for this role.

In addition, it was observed that long incubation of wild-type and *Δctlp40* cells under LL (about two months) also leads to stronger pigmentation of the mutant cells, while the wild-type cells were completely bleached. Such phenotypical modification of the „aged" cells of *Δctlp40* was observed also by Fulgosi *et al*. (2002). Electron microscopic studies revealed that after such a long period of incubation, the wild-type ultrastructure remained almost unchanged, with the exception of thylakoids that became slightly disintegrated (Fulgosi *et al*., 2002).

Interestingly, despite the fact that *Δctlp40* cultures retained dark green coloration, the thylakoid system of aged *Δctlp40* cells was almost completely degraded, except for a few PBSs still visible in the cytoplasm. The explanation of this phenotypical modification was that *Δctlp40* cells are impaired in the degradation of chlorophyll molecules (Fulgosi *et al*., 2002). On the other hand, at the same time cyclophilins have been proposed to act as an apoptotic nuclease (Montague *et al*., 1997) and can be involved in the degradation of the genomic DNA during apoptosis. Apoptosis, or programmed cell death, is a type of necrosis that is highly ordered and follows a series of distinct steps that eventually lead to the non-immune-responsive death of the cell. One characteristic of apoptosis is the specific degradation of the cellular nucleic acids. It was observed that cyclophilins are activated to degrade chromatin during apoptosis and has been shown that they can degrade both single-stranded and double-stranded DNA and also display endonucleolytic activity, demonstrated by their ability to degrade supercoiled DNA (Montague *et al*., 1997). In this case the absence of the cTLP40 protein in the mutant could disturb the chlorosis process in the *Δctlp40* cells.

Another question that can be followed from cTLP40 studies is the mechanism of its regulation under HL. Since it has been observed previously that the cTLP40 spinach homologue can differently bind to the membrane depending on modification of temperature conditions (Rokka *et al*., 2000), it was checked whether light could also affect cTLP40 binding to thylakoid membranes. Although cTLP40 can be found under normal growth light in two forms (membrane-associated and free in lumen) with large fraction of free protein, under light stress most of cTLP40 binds to the membrane. Another stress that also leads to the same result was cold stress, and finally cTLP40 was fully bound to the thylakoid membrane under double stress (ligh and cold stresses together).

The steady-state levels of cTLP40 under different stresses were not changed comparably to standard growth conditions. The level of cTLP40 protein was slightly increased when cells were exposed to double stress. However, the amount of cTLP40 was significantly increased in thylakoid membranes of „aged" *Δctlp40* cells.

4.2.3 Structural analysis of the cTLP40 *in vivo*

Despite the fact that TLP40 of higher plants belongs to the class of PPIases, localized in the chloroplast thylakoid lumen, the major prolyl isomerase activity in the thylakoid lumen is not

connected with the TLP40 protein (Edvardsson *et al*., 2003). Most likely TLP40 has a specialized regulatory function. It has been shown to interact with the thylakoid membrane and regulate the activity of PSII-specific protein phosphatase (Vener *et al*., 1999; Rokka *et al*., 2000). TLP40 is a complex PPIase with a C-terminal cyclophilin-like domain that possesses the PPIase catalytic function and an N-terminal domain with a number of protein-binding modules. The N-terminal binding modules of TLP40 are likely to be responsible for the reversible binding of the protein to the inner surface of the thylakoid membranes and regulation of a membrane protein phosphatase (Fulgosi *et al*., 1998; Vener *et al*., 1999, 2001).

The gene *sll0408* of *Synechocystis* sp. PCC 6803 shares a relatively high sequence similarity with the corresponding gene encoding TLP40 isomerase of higher plants. However, the cyanobacteria lack the protein phosphorylation of PSII subunits. This would mean that cTLP40 of *Synechocystis* should have some other functions or another mechanisms of regulation than the homologous component from higher plants. In order to gain knowledge about the role of the C- and N-terminal domains and some conservative amino acid residues in these domains that were proposed to have a functional significance in TLP40 and/or other PPIases, the genetic approach was used to construct different mutant strains. The cyanobacterial cTLP40 possesses the structural elements of the N-terminal part (phosphatase-binding and leucine zipper motifs) similar to plant TLP40. Unlikely to the plant TLP40, the N-terminus of cTLP40 contains one of two potential phosphatase-binding domains and just one leucine-zipper motif. The C-terminal part of the cTLP40 protein determines its proline isomerase activity. The transfer of proline peptide bonds from *cis* to *trans* positions was shown to influence the secondary structure of a protein and to make it more prominent when the proline residue was isomerised to the *trans* position (Hur and Bruice, 2002). These modifications in protein structure may influence protein-protein interactions, protein localisation, and access to substrates. It is not quite clear to which class of PPIases the cTLP40 protein belongs. However, its C-terminus contains two of three conservative amino acids Arg202 and Glu210, which take part in forming the catalytically active pocket in immunophilins of the cyclophilin class. The substitution of these amino acid residues can help to determine the family of cTLP40 component.

The mutant strains generated carried deletions of N- and C-termini of cTLP40 and substitutions in the leucine-zipper and phosphatase binding domains located at the N- and two amino acid residues at the C-termini. Preliminary analysis by PSII photoinhibition assays showed that deletions of the C- and N-termini lead to strong photodamage and loss of PSII activity under

HL similar to the *ctlp40*-deficient strain (Table 7). This means that both N-terminal and C-terminal parts, exert functionally important roles. Single amino acid substitutions in the N- and C-terminal parts did not exhibit significant differences compared to the wild-type under photoinhibitory light.

Immunological analysis showed that the cTLP40 protein was transported into thylakoids in all newly constructed strains. The only strain where the exact target could not be detected was the mutant with the deleted C-terminus (*ΔC-termsll0408).* This was caused by a specific reaction of cTLP40 antisera only with the N-terminal part of the protein. In the N-terminal deficient strain (*ΔN-termsll0408*) cTLP40 was immunologically detected as a protein with a molecular weight of 25 kDa that corresponded to the theoretically calculated length of the C-terminal part. Recent proteomic studies of the thylakoid membrane of *Synechocystis* sp. PCC 6803 (Srivastava *et al*., 2005) revealed that cTLP40 has an N-terminal *Tat* signal peptide with the recognition cleavage site for the leader processing peptidase. The predicted signal peptide contains 33 amino acid residues and was different from the signal peptide containing 12 amino acid residues predicted in the course of our work with a possible AVA recognition cleavage site (von Heijne, 1983, 1985). Despite of this controversial prediction the cTLP40 C-terminal part could be translocated into thylakoid membranes of *ΔN-termsll0408* cells. One explanation could be that the AVA motif was also recognized by protease(-s) as a cleavage site.

Since phosphatase-binding modules in TLP40 of higher plants are involved in signal transduction cascades and have important functional roles (Vener *et al*., 1999; Rokka *et al*., 2000), it has been proposed that the phosphatase-binding domain of cTLP40 is required for interaction with some structural elements. It has been shown previously that some classes of immunophilins possess conservative amino acid residues of Arg that can be responsible for phosphatase binding (Aldape *et al*., 1992). The strain *Δsll0408Ala* carrying a mutation of conserved Arg residues in the phosphatase binding sites was constructed and analysed phenotypically under strong double stress (DS; 400 μE m^{-2} s^{-1}, 17°C). Under DS treatment the *Δsll0408Ala* strain did not recover the phenotype of the *Δsll0408* mutant, however, it demonstrated some other phenotypical modification, that were different from wild-type. It was concluded that the Arg residue is not involved and might be not critical for the functions of the cTLP40 protein. However, the biochemical analysis of the strains with this substitution showed the appearance of two protein bands of 37 and 40 kDa (Fig. 40, panel A, lane 10). The appearance of this doublet could result from the generation of an additional protease cleavage

site AVA when Arg was mutated to Ala (von Heijne, 1983, 1985). Thus, the *Δsll0408Ala* mutant cells appear to possess two forms of the cTLP40 protein.

5. SUMMARY

1. The analysis of protein phosphorylation from the photosynthetic apparatus of *Synechocystis* sp. PCC 6803 has been performed using few detection tools that included various immunological and *in vivo* labelling approaches. The set of phosphoproteins detected with these methods include ferredoxin-NADPH reductase (FNR) and the linker proteins of the phycobilisome antenna.

1.1 Using mutants that lack specific set of linker proteins and were affected in the PBS assembly, it was demonstrated that the phosphoproteins correspond to the core-membrane (L_{CM}^{99}), rod ($L_R^{35, 33}$) and rod-core (L_{RC}) linkers. These proteins were in a phosphorylated state within the assembled PBSs and their phosphorylation rate was stable. Their dephosphorylation required partial disassembly of the PBSs and further contributed to their complete disassembly *in vitro*.

1.2 The linker dephosphorylation occurs *in vivo* upon *long-term* exposure to HL and under nitrogen limitation, two conditions that lead to remodelling and turnover of PBS antenna. It was concluded that phosphorylation process is instrumental in the regulation of assembly/disassembly of PBSs and could be involved in signalling for their proteolytic cleavage and degradation.

1.3 The analysis of the enzymes involved in phosphorylation/dephosphorylation of *Synechocystis* PBSs showed that phosphatase Sll1033 is probably involved (directly or indirectly) in the dephosphorylation of PBS linker proteins. It was also observed that another phosphatase Sll1771 could be involved in the dephosphorylation of L_{CM}^{99} linker that showed a higher level of phosphorylation under standard growth conditions and nitrogen deprivation. The analysis of three serine/threonine kinase mutants demonstrated that kinase Slr0559 may be involved in the regulation of protein phosphatase or other components participating in the linker dephosphorylation.

2. The homologous component of chloroplast lumenal complex immunophilin (PPIase or rotamase), TLP40 (thylakoid lumenal protein) has been found in *Synechocystis* sp. PCC 6803 thylakoids. In higher plants TLP40 was suggested to regulate the protein

phosphatase that dephosphorylats the core proteins of PSII. Since in cyanobacteria the major PSII core proteins are not phsophorylaed the role of cTLP40 (cyanobacterial TLP40) was unclear. The properties and functions of cTLP40 was analysed biochemically *in vitro* and by insertion mutagenesis of corresponding gene *in vivo.*

2.1 The product of *sll0408* gene was found in cyanobacterial thylakoids in two forms, free in lumen and peripherally associated with thylakoid membranes. The association of cTLP40 protein with thylakoid membranes was stronger when cells were exposed to various stress conditions. The analysis of the intramembrane localisation of cTLP40 showed that the protein could be partially associated or comigrated with cytochrome b_6f complex.

2.2 The expression of *sll0408* gene and corresponding product is not regulated by stress conditions. The protein cellular amount was increased only when cells were grown for long time (about 40 days) under standard growth conditions.

2.3 Inactivation of the *sll0408* gene on *Synechocystis* genome resulted in the viable photoautotrophic mutant strain *Δsll0408* (or *Δctlp40*) with similar phenotypical features of the wild-type when grown under LL. The analysis of pigment content of cells grown under LL showed that the mutant contained higher amount of phycocyanin, lower rate of chlorophyll *a* but similar amounts of carotenoids. Biochemical analysis of photosynthetic proteins confirmed slightly lower content of PSII subunits in the mutant under LL.

2.4 Acclimation of cyanobacterial cells to different stress conditions (high light, cold and heat stresses, nitrogen deprivation and long-term growth) led to phenotypical modifications of mutant cells. These modifications were accompanied by differences in the cell absorption spectra which reflect the pigment content of cyanobacterial cells.

2.5 Detailed functional analysis of mutant cells was performed during acclimation to HL. Comparative analysis of pigment concentrations showed that pigment degradation occurred more slowly in the mutant strain than in the wild-type. Immunological analysis of the major photosynthetic proteins showed that PSII proteins of *Δctlp40* strain were less reduced after acclimation to HL when compared to LL. These data showed that the higher content of chlorophyll *a* in *Δctlp40* cells after acclimation to HL was mainly associated with differences in the PSII protein amount.

2.6 The *Δctlp40* strain was highly sensitive to photoinhibition and was able to loose the PSII activity under photoinhibitory conditions much faster than the wild-type. The restoration of PSII photosynthetic activity under LL was similar to the wild-type. The *Δctlp40* mutant strain revealed similar rate of D1 degradation that suggested that cTLP40 is not involved in the degradation of D1 and rather influences other steps of PSII biogenesis.

2.7 In order to examine the role of N-terminal and C-terminal domains in cTLP40 several mutants lacking N- and C-terminal parts and containing point mutations in leucine zipper, phosphatase-binding and immunophilin domains were constructed *in vivo*. It was demonstrated that both, N- and C-terminal parts of cTLP40, are essential for its function. The modification of leucine zipper motif did not lead to any significant changes in cTLP40 protein functions. Mutation of arginine and glutamine residues in immunophilic domain showed that cTLP40 probably does not belong to typical cyclophylin type and contained other (different from Arg and Glu) amino acid residues involved in the substrate recognition. The modification of arginine residue that hypothetically could be involved in the phosphatase binding could restore the wild-type phenotype.

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Zinchenko V, **Piven I**, and Melnik V, Shestakov SV (1999) Vectors for the complementation analysis of cyanobacterial mutants. *Mol Genetics* **35:** 228-232

Yamaguchi K, Suzuki I, Yamamoto H, Lyukevich A, Bodrova I, Los D, **Piven I**, Zinchenko V, Kanehisa M, Murata N (2002) A two-component Mn^{2+}-sensing system negatively regulates expression of the mntCAB operon in *Synechocystis*. *Plant Cell* **14:** 2901-2913

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Ehrenwörtliche Versicherung

Hiermit erkläre ich, daß ich die vorliegende Dissertation selbständig und ohne unerlaubte Hilfe angefertigt habe. Sämtliche Experimente wurden von mir selbst durchgeführt, außer wenn explizit auf dritte verwiesen wird. Ich habe weder anderweitig versucht, eine Dissertation oder Teile einer Dissertation einzureichen bzw. einer Prüfungskommission vorzulegen, noch eine Doktorprüfung durchzuführen.

Irina Piven