# Functional analysis of peptidases from the cyanobacterium *Synechocystis* sp. PCC 6803

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*To my family*

# CONTENT

## ABBREVIATIONS

| | |
|---|---|
| A | absorbance |
| APC | allophycocyanin |
| amp | ampicillin |
| APS | ammonium persulfate |
| ATP | adenosine 5'-triphosphate |
| bp | base pairs |
| BSA | bovine serum albumin |
| cDNA | complementary DNA |
| Cm | chloramphenicol |
| dCTP | desoxy cytosine 5'-triphosphate |
| DMSO | dimethyl sulfoxide |
| DL | dim light |
| DNase | deoxyribonuclease I |
| DNA | deoxyribonucleic acid |
| EtBr | ethidium bromide |
| Gm | gentamycin |
| h | hour |
| HEPES | N-(2-hydroxyethyl)piperazine N'-(2-ethane sulfonic acid) |
| HL | high light |
| HLIP | high light inducible proteins |
| IAA | iodoacetamide |
| IgG | immunoglobulin G |
| IPTG | isopropyl ß-D-thiogalactoside |
| Km | kanamycin |
| kb | kilobases |
| kDa | kilo Dalton |
| LL | low light |
| MES | 2-N-morpholinoethanesulfonacid |
| ML | medium light |
| MOPS | 4-morpholinopropansulfonacid |
| ORF | open reading frame |
| PAA | polyacrylamide |

| | |
|---|---|
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phycobilisome |
| PC | phycocyanin |
| PCR | polymerase chain reaction |
| PSI | photosystem I |
| PSII | photosystem II |
| RCI | reaction centre I |
| rpm | rotations per minute |
| RNase | ribonuclease |
| rRNA | ribosomal RNA |
| RT | room temperature |
| RT-PCR | reverse transcription polymerase chain reaction |
| SDS | sodium dodecylsulfate |
| SSC | standard saline citrate |
| SSC | small single-copy region |
| Synechocystis | *Synechocystis* sp. PCC 6803 |
| TBE | Tris-borate-EDTA |
| TCA | trichloroacetic acid |
| TE | Tris-EDTA |
| TEMED | N, N, N', N'-tetramethylethylene diamine |
| Tris | Tris(hydroxymethyl)aminomethane |
| Tween 20 | polyoxyethylensorbitanmonolaurat |
| WT | wild-type |

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 Proteolytic enzymes and their role in cell homeostasis

In all living organisms protein molecules are continuously synthesized and degraded. The concentration of individual cellular proteins is determined by a balance between rates of synthesis and degradation, which in turn is controlled by a series of regulated biochemical mechanisms. The rates of protein degradation can play an essential role in defining their cellular concentrations and may vary in different compartments depending on environment (Vierstra, 1993; Andersson and Aro, 1997; Adam, 2000).

The key enzymes of the protein degradation machinery are designated as „peptidases“ or “proteases”. Peptidases catalyze the hydrolysis of peptides by digestion of the specific bonds inside target molecule. Peptidases can be divided into two large groups according to their substrate specificities: endopeptidases and exopeptidases. Exopeptidases remove single or several amino acid residues, dipeptides or tripeptides, from N- or C-termini, and accordingly can be classified into mono-, di- and tripeptidases, respectively (Kenny, 1999; Rawling and Barret, 1999). Endopeptidases can also remove single or several amino acid residues, but in contrast to exopeptidases, classification of endopeptidases is based on the active proteolytic residues of the enzymes, not on a substrate. Endopeptidases have been divided into four major groups: serine, cysteine, aspartic and metallo-peptidases (Barret, 1994 and 1995; Callis, 1995; Kenny, 1999).

Investigations of degradation processes in cells have demonstrated that protein turnover occurs in all organelles (mitochondria and chloroplasts) and compartments of a cell (Burgess et al., 1978; Brown et al., 2000; Hicke, 2001). Protein turnover is involved in various cellular key processes, such as in the control of the cell cycle (Pagano, 1997), gene expression (Adam, 2000), cell differentiation (Kinoshita et al., 1995), protein targeting and sorting (Perlman and Halvorson, 1983; Richter et al., 1998), protein quality control (Bradshaw et al., 1998), programmed cell death and senescence (Huffaker, 1990; Matile et al., 1996; Hortensteiner and Fello, 2002).

Eukaryotic cells have evolved different proteolytic systems compared to prokaryotic cells: a membrane-enveloped lysosomal system and selective proteolytic systems consisting of highly

specific proteinases residing in different cellular compartments (Rivett, 1985; Klausner and Sitia, 1990; Heinemeyer et al., 1991). A major selective non-lysosomal proteolytic pathway is mediated by an ubiquitin system (Ciechanover et al., 1980; Ciechanover, 1998; Hershko et al., 1983). In this pathway substrate proteins are proteolyzed by a *proteasome* (Hershko et al., 1983; Hershko, 1988). In various organisms the proteasome represents a highly conserved 2.6 MDa protein complex that degrades protein substrates in an ATP-dependent fashion and yields small peptides and free ubiquitin. Ubiquitin is a highly conserved small polypeptide of 76 amino acids that binds covalently to lysine residues on a target protein. Labelled proteins are recognized by proteases and subjected to proteolysis (Hershko, 1988; Ciechanover and Schwarz, 1989; Vierstra, 1993; Ciechanover, 1998; De Mot et al., 1999; Glickman, 2000). Two known major functions of ubiquitin are the modification of the histones H2A and H2B during interphase cell growth and the modification of cellular proteins for targeting them to proteolytic degradation (Bond et al., 1988). Biochemical and genetic evidence strongly supports the view that the ubiquitin system is primarily responsible for the degradation of heat- or stress-damaged proteins in eukaryotic cells. Previous studies have shown that ubiquitin synthesis is markedly accelerated in a heat-shocked cells. It is possible that ubiquitin acts as a signal target for proteolytic removal of abnormal proteins produced by stresses.

There is no clear evidence for regulation of gene expression by ubiquitin during stress conditions (Parag et al., 1987; Bond et al., 1988). Also, the role of ubiquitin in lower plants and prokaryotes is not well known. Some archea and prokaryotes, for instance the archaebacterium *Thermoplasma acidophilum*, contain simple versions of the proteasome, however, in the absence of ubiquitin and the ubiquitin-conjugating system (Seemüller et al., 1995). In plant tissues ubiquitin appears to be involved in the degradation of phytochrome photoreceptors. Following a brief light pulse phytochrome is converted to another form that is degraded 100-fold faster than the original species. During the phase of rapid degradation, markedly decreased levels of ubiquitin-phytochrome conjugates were observed (Shanklin et al., 1987; Hershko, 1988).

The degradation of proteins takes place in organelles, lysosomes and in higher plants vacuoles. Similar to other proteolytic systems lysosomal protein degradation requires addition of ATP energy (Seglen and Bohley, 1992). Under nutritional or hormonal deprivations the number of lysosomes accelerates rapidly. Lysosomal breakdown appears to be largely non-specific, although it has been suggested that certain proteins are targeted to lysosomes by a

specific amino acid sequence (Chiang and Dice, 1988; Dice, 1992). It has been shown with mammalian cell cultures that activation of a more selective system can take place that transports proteins directly into lysosomes during nutrient deprivation. This pathway is mediated by the heat shock cognate protein of 73 kDa (Hsc73), which binds to a molecular determinant in those cytosolic polypeptides that are destined for lysosomal catabolism (Terlecky and Dice, 1993). The transport processes also involve the recognition of proteins containing the specific sequence motif Lys-Phe-Glu-Arg-Gln (KFERQ) (Dice, 1992). Alteration of the conformation of targeted proteins occurs in an ATP-dependent step that, in turn, facilitates transport of the target into lysosomes. Approximately 25 - 30% of human cytosolic proteins contain domains that react with antibodies produced against the KFERQ sequence, suggesting that many proteins are targeted to lysosomes *via* this mechanism (Dice and Chiang, 1989; Olson et al., 1991; Terlecky and Dice, 1993; Vierstra, 1993). Despite its different localization it is assumed that ubiquitin could be involved in the utilisation of lysosomal proteins (Vierstra, 1993; Hershko and Ciechanover, 1998). In plants precursors of vacuolar proteins are transported from the endoplasmic reticulum into vacuoles where they are proteolytically processed to their mature forms (Hara-Nishimura et al., 1991). Some reports have described vacuolar processing enzymes (VPEs) that are responsible for the maturation of seed proteins (Kinoshita et al., 1995). Recent studies of these proteins showed that seed-type VPEs constitute only one pathway for processing seed storage proteins and that other proteolytic enzymes can also process storage proteins into chains capable of stable accumulation in mature seeds (Gruis et al., 2002).

Chloroplasts, as photosynthetic bacteria, also contain a number of proteolytic enzymes that are directly or indirectly involved in protein utilisation. Among them are peptidases that participate in the turnover of proteins that constitute the photosynthetic machinery in higher plants (Melis, 1991; Murakami and Fujita, 1991). Proteases that have been identified in the organelle include the stroma-located members of Clp family which are well conserved from prokaryotes to eukaryotes (Moore and Keegstra, 1993; Schelin et al., 2002), the stromal and thylakoid-located processing peptidases (VanderVere et al., 1995) that dissect imported or translocated protein precursors, the thylakoid-bound metalloproteases FtsH (Lindahl et al., 1996), and two lumenal components, the heat shock protease HtrA (DegP) (Itzhaki et al., 1998) and CtpA, an enzyme involved in the C-terminal processing of the core protein of the photosystem II reaction centre, the D1 protein (Shestakov et al., 1994; Anbudurai et al., 1994).

At present, little information is available about degradation of proteins in other cellular and organelles compartments. There is some evidence that mitochondria and endoplasmatic reticulum with its connected sectretory system have proteolytic pathways involved in the maturation and function of their proteins.

Mitochondrial proteins as a rule show relatively long half-lives and the proteins of the outer membrane turn over more rapidly than those from inner membrane and matrix (Bohley, 1996). One reason for the long half-lives of mitochondrial proteins could be the relatively low protease concentration in this organelle (Bohley, 1996). Proteolytic processes play an important role in the biogenesis of mitochondria and in quality surveillance of mitochondrial proteins. For instance, Oxa1p is a component of a general mitochondrial export machinery whose role is accompanied by two mitochondrial proteases, the matrix protease Afg3p-Rca1p and the intermembrane space protease Yme1p (Lemaire et al., 2000). The mitochondrial PIM1 protease that cooperates with molecular chaperones appears to be essential in the degradation of misfolded proteins that are generated during heat stress (Wagner et al., 1994). The proteolytic enzymes, caspases (**c**ysteine **as**partyl **p**rote**ases**), are found in mitochondria of animal cells and play a critical role in apoptosis by being involved in a cascade of cleavage events (Zou et al., 1997; Thornberry and Lazebnik, 1998; Kumar and Vaux, 2002). It was proposed that mitochondria, like the Golgi complex, sense and transduce the pro-apoptotic signals through caspases, which regulate different proteins participating in apoptosis (Zou et al., 1987; Mancini et al., 2000).

Secretory proteins are synthesized in the cytosol and usually translocated into endoplasmic reticulum *via* the Sec61 translocon (Matlack et al., 1998). Translocation can proceed either co- or post-translationally and requires the proteins in unfolded state. Protein transport and maturation are inevitably prone to errors, which leads to misfolded and misassembled polypeptides that used to be removed by proteolytic pathways. Selective degradation of proteins at the endoplasmic reticulum (ER-associated degradation) is thought to proceed largely *via* the cytosolic ubiquitin-proteasome pathway (Klausner and Sitia, 1990; Mayer et al., 1998; Kopito and Sitia, 2000; Lee et al., 2004; Kopito, 1997). For instance, in yeast two ubiquitin-conjugating enzymes (UBC6 and UBC7) were found in the endoplasmatic reticulum. It was also shown that they are essential for ER-associated protein degradation (Sommer and Jentsch, 1993; Mayer et al., 1998).

Taken together, peptidases in both eukaryotes and prokaryotes play a crucial role in the following processes:

1. *Protein biogenesis* (keeping in order major cellular functions)

a. Proteases maintain various cell functions by removal of mistranslated or aberrant polypeptides expressed from defective RNAs (Goldberg and Dice, 1974; Keiller et al., 1996) or of post-translationally modified proteins that cannot return to their native, functional form (*e. g.* misfolded or unassembled proteins; Callis, 1995; Grune et al., 1995; Bohley, 1996; Missiakas and Raina, 1997).

Such degradation processes mainly occur in the intracellular space and generally involve unspecific peptidases. The best studied group are proteases that recognize misfolded or mistargeted proteins and subject them to proteolytic degradation (Chuang and Blattner, 1993; Itzhaki et al., 1998; Clausen et al., 2002). Physiological stress, such as increased temperature or light intensity, often results in denaturation of proteins. The expression of heat shock proteins is induced under these conditions in order to diminish damage to organelles or cells. As molecular chaperones, some heat shock proteins can stabilize the folding intermediates prone to aggregation and ensure efficient refolding of native structure under normal conditions (Hartl, 1996; Schwarz et al., 1996). If renaturation can not be achieved by the chaperone function the aberrant proteins will be degraded *via* proteolysis. In some cases, peptidases carry both, molecular chaperone and proteolytic activities. For instance, the chaperone function of the heat shock protein DegP (HtrA) in *E. coli* dominates at low temperatures, while the proteolytic activity is activated at elevated temperatures. These findings illustrate that some cellular components could switch between two key pathways, control of protein stability and turnover (Spiess et al., 1999).

b. *Control of gene expression by direct or indirect interaction with transcription through sigma factors*

Some proteases can be involved in the regulation of sigma factors. For instance, in *E. coli* FtsH protease is responsible for specific degradation of the transcription factor $\sigma^{32}$ (RpoH; Herman et al., 1995; Tomoyasu et al., 1995) and transcriptional activation of the CII protein (Herman et al., 1993). Clp proteases including ClpXP and ClpAP are involved in the degradation of transcription factors such as RpoS and λO (Gottesman et al., 1993; Becker et al., 1999; Hengge-Aronis, 2002). In yeast several other proteases, including homologues of

the Lon protease of *E. coli,* are involved in regulation of transcription or replication of DNA by binding to mitochondrial promoters (Fu et al., 1997; van Dyck et al., 1998).

c. *Control of protein quality*

Some proteases can regulate activation of proteins through processing, for instance, post-translational modification of proteins by processing of apoproteins to form active mediators or holoproteins (Zou et al., 1997; Tang and Guest, 1999). There are two different types of protein processing: carboxyl-terminal processing and amino-terminal processing. Specific degradation of proteins with non-polar C-terminal sequences was first reported for several cytoplasmatic proteins in *E. coli* (Parsell et al., 1990; Keiler and Sauer, 1996). Later, Tsp (**t**ail-**s**pecific **p**eptidase) endopeptidase from *E. coli* was purified (Silber and Sauer, 1994) and shown to be a major periplasmatic peptidase responsible for the specific C-terminal degradation of proteins. Tsp peptidase is involved in the degradation of mistranslated proteins that carry an *ssrA* sequence at their C-termini, which can be specifically recognized by that peptidase (Hara et al., 1991; Silber et al., 1992; Lentsch, 1996; Keiler et al., 1996). The homologues of Tsp peptidase were identified in photosynthetic organisms (Fulda et al., 2000) and were later shown to be ivolved in the C-terminal processing of D1 precursor (Anbudurai et al., 1994). C-terminal processing of D1 is an essential event for the assembly of a manganese cluster, for light-mediated water oxidation and stabilization of a reaction centre of PSII (von Heijne et al., 1989; Anbudurai et al., 1994; Inagaki et al., 2001).

Amino-terminal degradation includes two large groups of peptidases according to different functional properties. Recent investigations deduced a group of aminopeptidases that are involved in N-terminal methionine excision (Bradshaw et al., 1998), a process that often is essential for protein stabilization (Giglione et al., 2003) or translation (Chang et al., 1989; Li and Chiang, 1995). The N-end recognizing proteins that select potential proteolytic substrates by binding to their amino-terminal residues have also been identified as the E3 protein of the ubiquitin-dependent proteolytic system (Callis, 1995; Bohley, 1996; Varshavsky, 1997a,b). The second group includes a number of proteolytic enzymes (processing peptidase family) involved in protein translocation across membranes. A large fraction of proteins is synthesized in a compartment different from the compartment of their destination. Such proteins carry the presequences at their N-termini that are removed by processing enzymes in one or two steps, generally depending on their localization (Hageman et al., 1986; Oblong and Lamppa, 1992; Richter and Lamppa, 1998 and 1999). For example, proteins targeted to

chloroplasts or mitochondria possess a special transit peptides recognized by peptidases associated with the transport machinery (Perlman and Halvorson, 1983; von Heijne et al., 1989; Dalbey, 1991; Houben et al., 2002).

*2. Role in adaptive response to ever changing environment*

Protein degradation play a crucial role in adaptation processes, e.g. during stress conditions such as light stress (Adamska et al., 1996; Porankiewicz et al., 1998; Grossman et al., 2001; Haussuhl et al., 2001; Lensch et al., 2001), heat shock (Lipinska et al., 1990; Spiess et al., 1999; Diamant et al., 2001), salt tolerance (Gerth et al., 1998; Msadek et al., 1998), nutrient starvation (Damerau and St John, 1993; Collier and Grossman, 1994; Katoh et al., 2001) or treatment with chemical reagents (Adam, 1996; Andersson and Aro, 1997). Damage caused by stress can activate specific proteases that are essential for maintenance of physiological processes in living cells. Proteolysis of abnormal proteins leads ultimately to free amino acids that can then be reutilized for protein synthesis or metabolized otherwise.

## 1. 2 Characterization of the protease complement of cyanobacterial cells

Comparison of the number of proteolytic components has shown that cyanobacteria possess a set of proteases such as Clp (Eriksson and Clarke, 1996; Porankiewicz et al., 1998; Panichkin et al., 2001), Deg (Sokolenko et at., 2002), FtsH (Mann et al., 2000; Bailey et al., 2001), Ctp (Shestakov et al., 1994; Ivleva et al., 2002), Gsp (Zuther et al., 1998) and SppA (Lensch et al., 2001) that are also represent in non-photosynthetic prokaryotes. Since cyanobacteria, together with a heterotrophic eukaryotic cell, are ancestors for an endosymbiotic event that led to chloroplasts (Herrmann 1997; Martin and Herrmann, 1998; Martin et al., 1998), this group of organisms can be profitably used for the investigation of proteolytic components in plant cells. Various studies deduced a number of prokaryotic-type peptidases also in higher plants (Adam et al., 2001; Sokolenko et al., 2002), however the function most of them still unknown.

### 1.2.1 The Clp peptidase family

The ATP-dependent Clp peptidases are soluble multisubunit protein complexes present in both, prokaryotes and eukaryotes. A typical Clp complex represents an assembly of two types of subunits that perform distinct functions: a multimeric proteolytically active peptidase moiety, designated ClpP that also exists in catalytically probably inactive versions, ClpR, and

chaperone-type subunits, that regulate the proteolytic activity of the peptidase complex. There are two regulatory ATPase subunits (ClpC and ClpX; Schirmer et al., 1996; Turgay et al., 1998) and a chaperone (ClpB; Squires and Squires, 1992; Weaver et al., 1999). In non-photosynthetic prokaryotes ClpR is encoded by a single gene, whereas in plants multiple ClpR isomers, designated ClpR1, ClpR2, ClpR3 and ClpR4, are present (Nakabayashi et al., 1999; Adam et al., 2001).

Clp subunits are involved either in the regulation of proteolysis or function as molecular chaperones (Squires and Squires, 1992; Schirmer et al., 1996; Gottesman et al., 1997a, b). For instance, in non-photosynthetic bacteria, such as *Escherichia coli* and *Bacillus subtilis*, Clp proteins play a critical role in stress tolerance (Gerth et al., 1998; Msadek et al., 1998) or in responses to nutrient starvation (Damerau and John, 1993). Clp proteins can be directly involved in proteolysis of misfolded or damaged proteins (Frees and Ingmer, 1999; Krüger et al., 2000) and are also required for crucial processes such as cell division, development of sporulation, genetic competence and quality control of protein translation (Jenal and Fuchs, 1998; Msadek et al., 1998; Nanamiya et al., 1998; Turgay et al., 1998; Flynn et al., 2001). While most Clp regulatory subunits are part of high molecular weight Clp complexes, ClpB has not been found in supramolecular assemblies. It acts as a chaperone under conditions of salt and heat stress (Diamant et al., 2001).

In the photoautotrophic organism *Synechoccocus* sp. PCC 7942 ClpP is involved in the steady-state growth and long-term acclimation to high light, cold stress and UV-B (Clarke et al., 1998; Porankiewicz et al., 1998), while ClpB appears to be involved in thermotolerance (Clarke and Eriksson, 2000). In *Chlamydomonas* chloroplasts ClpP peptidase appears to participate in proteolytic disposal of fully or partially assembled cytochrome $b_6f$ and PSII complexes (Majeran et al., 2000; 2001). Some experimental evidence from higher plant chloroplasts suggested that Clp peptidase represents a major housekeeping peptidase in the chloroplast stroma (Shanklin et al., 1995; Halperin et al., 2001). In tobacco chloroplasts, ClpP is involved in chloroplast development and required for cell survival (Shikanai et al., 2001). The expression analysis of *Arabidopsis clp* genes and their products demonstrated the presence of Clp peptidase not only in photosynthetic tissues but also in roots and a constitutive level of the proteins after various short-term stresses and during normal growth (Zheng et al., 2001).

### 1.2.2 The Deg peptidase family

The Deg peptidase family includes a number of ATP-dependent serine peptidases that are widespread among bacteria and eukaryotes. The Deg family also known as the Htr family includes three related members HtrA (DegP), HhoA (DegQ) and HhoB (DegS). In *E. coli* HtrA is a heat stress protease that functions in a homohexameric state in the periplasm (Kim et al., 1999; Sassoon et al., 1999). It is responsible for the degradation of misfolded and abnormal proteins of the cell membrane or periplasmic compartment (Strauch and Beckwith, 1988; Lipinska et al., 1990; Kim et al., 1999) and required for cell viability at elevated temperatures or under oxidative stress (Strauch et al., 1989; Lipinska et al., 1990; Skorko-Glonek et al., 1997; 1999). HtrA also functions as a chaperone at low temperature, while its proteolytic activity develops at high temperatures (Spiess et al., 1999).

HhoA and HhoB were initially identified in *E. coli* as multicopy suppressors of a lethal null mutation in the *prc* gene encoding a C-terminal processing peptidase (Bass et al., 1996). Both, HhoA and HtrA are soluble proteins located in the bacterial periplasmic space (Waller and Sauer, 1996). HhoA can rescue the temperature-sensitive phenotype of an $HtrA^-$ strain, but in contrast to HtrA, it is not required for cell survival at elevated temperatures (Waller and Sauer, 1996). Its homologue, HhoB, is a smaller and a membrane-bound protein, exposing its putative catalytic site to the periplasmic space (Alba et al., 2001). HhoB is involved in the regulation of the $\sigma^E$ -specific anti-sigma factor RseA and extracytoplasmic stress response controlled by the $\sigma^E$ protein (Ades et al., 1999; Alba et al., 2001). This observation could explain why HhoB is important for *E. coli* cells under both normal and extreme growth conditions (Waller and Sauer, 1996).

Fourteen different genes encoding proteins related to DegP were found in the *Arabidopsis* genome (Adam et al., 2001; Sokolenko et al., 2002). Of the nine loci predicted to encode organellar peptidases, four are unambiguously assigned to the chloroplast where they function in distinct sub-locations (Adam et al. 2001). Three DegP peptidases from higher plants have been described recently, DegP1, DegP2 and DegP5. DegP1 and DegP5 are lumenal peptidases where the first one is tightly associated with thylakoid membranes (Itzhaki et al., 1998; Lensch, 2002; Kieselbach and Funk, 2003). This peptidase is expressed constitutively, but its level increases in plants exposed to higher temperatures. The DegP2 peptidase (Adam et al., 2001) is active at the stromal face of the thylakoid membrane and has been suggested to

be involved in the initial step of D1 degradation (Haußühl et al., 2001). Recent proteome analysis of the *Arabidopsis* thylakoid lumen revealed three Deg peptidases (DegP1, DegP5 and DegP8) in that compartment (Schubert et al., 2001).

### 1.2.3 The FtsH peptidase family

Bacterial FtsH is a membrane-integral ATP-dependent peptidase (Tomoyasu et al., 1993). It participates in various intracellular degradation processes ranging from the degradation of the protein translocase subunit SecY to that of the heat stress transcription factor $\sigma^{32}$ and of the transcriptional activator λCII (Herman et al., 1995; Kihara et al., 1995). With one exception, the mitochondrial Yme1p subunit, one of the FtsH components, that spans the membrane only once, all the other FtsH members posses two transmembrane domains in the N-terminal region, which are followed by a large cytoplasmic domain (Tomoyasu et al., 1993; Langer, 2000). Like other ATP-dependent peptidases, FtsH possesses a homodimeric or/and tetrameric structure (Akiyama et al., 1995). The multimeric structure is required for catalytic activity as was shown for AAA-ATPases (Karata et al., 1999). The N-terminal domain is involved in the oligomerization of the peptidase but the transmembrane organization is essential for proteolytic activity against integral membrane proteins (Akiyama and Ito, 2000). The structure and function of AAA peptidases has been well studied from yeast mitochondria. The proteins were found to be a key-player in the destruction of membrane proteins (for review see Langer, 2000).

The peptidase family is encoded by four genes in *Synechocystis* and sixteen genes in *Arabidopsis* (Adam et al., 2001). With few exceptions (FtsH3, FtsH4, FtsH10 and FtsH11) most of the *Arabidopsis* FtsH peptidases and FtsHi subunits are located or predicted to reside in chloroplast membranes. Only two FtsH members were predicted to be targeted to mitochondria (FtsH3 and FtsH10). The chloroplast FtsH peptidase was first identified immunologically in spinach thylakoid membranes and expression of its gene was shown to be light-inducible (Lindahl et al., 1996). It was proposed that this peptidase is involved in the degradation of unassembled thylakoid proteins (Ostersetzer and Adam, 1997). Later, the involvement of FtsH in the second step of degradation of the photosystem II reaction centre core protein D1 was demonstrated by an *in vitro* approach (Lindahl et al., 2000). Inactivation of the *Arabidopsis* FtsH2 (*VAR2*) protein caused a variegated phenotype, suggesting that this protein is required for plastid differentiation to prevent partial photooxidation of developing

chloroplasts (Chen et al., 2000; Takechi et al., 2000). This is consistent with the recent finding that the FtsH2 homologue is required for the cleavage of the D1 polypeptide *in vivo* (Bailey et al., 2002).

All four genes coding for FtsH peptidases were individually inactivated in *Synechocystis* sp. PCC 6803 (Bailey et al., 2001). Two mutations were lethal (FtsH1 and FtsH3), the third one, FtsH4, showed no obvious phenotype while the fourth one caused an altered pigmentation (FtsH2, *slr0228*::Ω). This mutant was impaired predominantly in PSI biogenesis (Mann et al., 2000). It has also been suggested that FtsH2 is essential for controlling the turnover of the D1 protein, similar to the FtsH2 homologue from *Arabidopsis* (Bailey et al., 2001).

### 1.2.4 The Ctp family of carboxypeptidases

The Ctp family of carboxypeptidases belongs to the family of C-terminal processing peptidases. The physiological function of these enzymes is less well understood. The Tsp peptidase from *E. coli* (Hara et al., 1991; Silber et al., 1992) is involved in the degradation of mis-translated proteins that are targeted for degradation by the addition of an *ssrA* sequence at their C-termini which can be specifically recognized by the Tsp peptidase (Jentsch, 1996; Keiler et al., 1996b). This peptidase has a characteristic PDZ domain at its C-terminus that is required for substrate recognition (Ponting, 1997; Beebe et al., 2000).

The family of C-terminal processing peptidases of *Synechocystis* consists of CtpA, CtpB and CtpC that are homologous to the well-studied bacterial Tsp peptidase. Cyanobacterial *ctp* genes share more than 30% identity with those of *E. coli* and encode soluble proteins that reside in the periplasm (Fulda et al., 2000; Zak et al., 2001). The *Arabidopsis* genome carries three *ctp* genes encoding proteins that are predicted to be soluble and to be translocated into the chloroplast. The proteome analysis of *Arabidopsis* lumenal fractions showed that all three Ctp members are translocated into the thylakoid lumen (Schubert et al., 2001), although CtpB and CtpC do not show an Ala-X-Ala motif and lack the highly hydrophobic N-terminal domain typical for lumen-targeted proteins. The CtpA peptidase was isolated from spinach and its chloroplast lumenal localization was verified experimentally (Inagaki et al., 1996; Oelmüller et al., 1996). Evidence for an involvement of this peptidase in biogenesis of the photosynthetic machinery was provided by an analysis of a *Synechocystis ctpA*-deficient mutant that was unable to grow under photoautotrophic conditions (Shestakov et al., 1994) and was impaired in the C-terminal processing of D1. This latter step is essential for correct

integration of D1 into thylakoids (Diner et al., 1988). No complete inactivation has been achieved for the second member of the Ctp-family, *ctpC,* which indicates that its product is required for cell viability (Ivleva et al., 2002). Inactivation of *ctpB* did not lead to any visible phenotypical or physiological changes (Ivleva et al., 2002).

### 1.2.5 The SppA peptidase family

This family includes two members of different molecular mass, with the shorter version representing the C-terminal domain of the longer one. These proteins were given different names depending on the organism. In *E. coli* they correspond to SppA (or peptidase IV) and SohB (Ichihara et al., 1986; Baird et al., 1991), in *B. subtilis* to SppA and TepA (Bolhuis et al., 1999), in *Synechocystis* to SppA1 and SppA2 (Lensch et al., 2001). Peptidase IV in *E. coli* has been characterized as a membrane-associated protein (Regnier, 1981a,b; Pacaud, 1982a,b). In bacteria these two peptidases have been described as signal peptide peptidases that are required for the processing and removal of small peptides (Regnier, 1981a,b; Pacaud, 1982a,b; Ichihara et al., 1984; Novak and Dev, 1988; Bolhuis et al., 1999). The analysis of *E. coli* SppA by gene inactivation and by *in vitro* experiments using the overexpressed protein showed that this enzyme is required for the initial endoproteolytic cut of signal peptides. SohB was discovered in *E. coli* during a search of suppressors of mutants for the HtrA peptidase (Baird et al., 1991). One of the suppressor mutations was capable to complement the temperature-sensitive phenotype of the $HtrA^-$ strain. The corresponding gene was identified as *sohB* that encoded a periplasmic peptidase of 39 kDa (Baird et al., 1991). The temperature-sensitive phenotype of the *htrA* mutant could be caused by accumulation of toxic peptides which could be degraded upon overproduction of SohB. In *B. subtilis* TepA is a cytoplasmic protein (Bolhuis et al., 1999), differently from SohB of *E. co*li which associated with membranes (Baird et al., 1991). Two genes, *sppA1* and *sppA2*, are found in the genome of *Synechocystis* and only one, the homologue to *Synechocystis sppA1*, in *Arabidopsis thaliana*. The latter is light-activated at both, the transcriptional and post-translational levels (Lensch et al., 2001). Experimental data showed that *Arabidopsis* SppA is a membrane-bound, probably monotopic protein with a putative amphipatic helix interacting with the surface of the lipid bilayer. The protein may associates with the thylakoid membrane as a homotetramer (Lensch et al., 2001).

## 1.3 Cyanobacteria as models for the analysis of the photosynthetic machinery

Cyanobacteria are unicellular gram-negative bacteria that, in a row with a higher plants and algae, can perform oxygenic photosynthesis. The presence of an oxygenic photosynthetic thylakoid membrane system similar to that in higher plants, fast growth, known genome sequences and easy transformation present these organisms as appealing models for gene inactivation and modification.

The cyanobacterium *Synechocystis sp*. PCC 6803 that has been used in the present study is a unicellular strain from which the entire genome sequence is known (Kaneko et al., 1996). It has an additional advantage in that it is able to grow under photoautotrophic and photoheterotrophic conditions (Shestakov and Grigorjeva, 1982). This can be profitably used for investigation of photosynthetic genes. The *Synechocystis sp*. PCC 6803 genome is about 3.57 MBp large and encodes 3168 proteins (Kaneko et al., 1996).

### 1.3.1 Structure of cyanobacterial photosynthetic complexes

In cyanobacteria, as in the higher plants and green algae, light energy is converted into the chemical energy by the action of different protein complexes: phycobilisome (PBS), photosystem II, cytochrome $b_6f$, photosystem I and ATP synthase (Bryant, 1994) (Fig. 1).

**PBS** is a large membrane–external and water-soluble complex that is involved in the accumulation of light energy and its transfer to the photosystems of cyanobacteria. The major PBS components are the chromophore-bearing biliproteins: phycocyanin (PC), allophycocyanin (APC) and phycoerythrin (PE). The last one is not present in the *Synechocystis* 6803 strain (Glazer, 1985). Minor components of PBS are linker polypeptides that are involved in the regulation of PBS quaternary structure and optimization of the energy transfer by modifying the light absorption properties of the phycobiliproteins (de Marsac and Cohen-Bazire, 1977; MacColl, 1998). The core-membrane linker $L_{CM}$ with a molecular weight of about 99 kDa participates in the energy transfer from PBS to PSII. The rod-core linkers, $L_{RC}$, attach the peripheral rods to the core of PBS. In addition, rod linkers, $L_R$, and core linkers, $L_C$, are involved in the assembly of rods and core domains of PBS, respectively. Still, the question about the mechanisms of energy absorption and transfer to the photosystems in cyanobacteria has not been clarified. A major step of energy transfer in

cyanobacteria includes transfer from phycocyanin of rods to allophycocyanin in the cores and then from APC to the photosystem *via* the $L_{CM}$ linker protein (Houmard et al., 1990; Macoll, 1998). Also, the mechanism that provides the energy distribution from PBS between PSI and PSII is still not clear. In favor is the model that PBSs are mainly attached to dimer PSII particles and transfer energy directly PSII (Kuhl H. et al., 1999). However, recent studies in *Spirulina* cells showed that 20% of PBS are bound to PSII, while 60% of PBS transfer the energy to the PSI trimer and 20% are associated with PSI monomers (Rakhimberdieva et al., 2001).

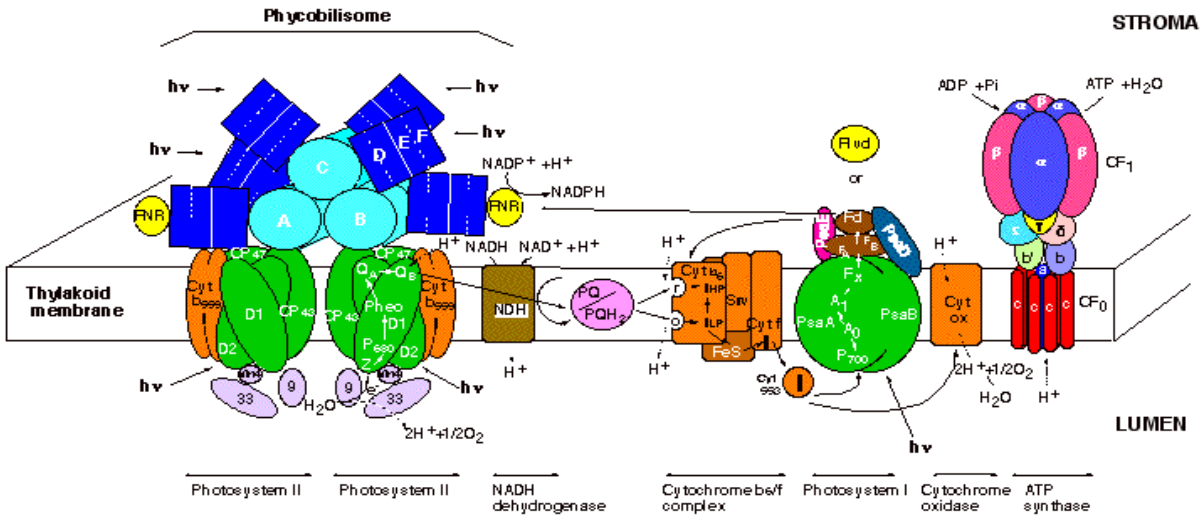


**Figure1. Schematic presentation of the thylakoid membrane of cyanobacteria (adapted from the Bryan, 1994)**

**PSII** is a pigment-protein complex of the thylakoid membrane of oxygenic photosynthetic organisms, including cyanobacteria. It catalyzes the light-induced electron transfer from water to plastoquinone, with associated production of molecular oxygen. PSII is a large complex with polypeptide species, most of which are integral membrane proteins. A number of extrinsic proteins is also associated to PSII. The entire set of electron transfer cofactors, including chlorophyll *a*, pheophytin *a*, plastoquinones, and non-heme iron, is associated with the D1/D2 heterodimer. These two proteins, together with the α- and ß-subunits of cytochrome $b_{559}$ and the PsbI protein, constitute the so-called reaction centre II (RCII), which is the smallest PSII subparticle still able to perform light-induced charge separation. Two large integral membrane proteins, CP43 and CP47, each coordinating a number of chlorophyll *a* molecules, and several low molecular mass (<10 kDa) polypeptides are constituents of the

PSII core complex, which is very similar in higher plants and cyanobacteria (Nanba and Satoh, 1987; Diner et al., 1991; Yu and Vermaas, 1993; Szabo et al., 2001; Kashino et al., 2002).

**The cytochrome $b_6f$ complex** provides the electronic connection between reaction centres of the PSI and of PSII and generally contributes to the transmembrane electrochemical proton gradient for adenosine triphosphate synthesis (Rich and Bendall, 1980; Allen, 2004). In cyanobacteria, the cytochrome $b_6f$ complex is located both in thylakoid and cytoplasmic membranes. It is involved in both photosynthetic and respiratory electron transport, acting as a plastoquinol-cytochrome $c_6$-plastocyanin oxidoreductase and playing a role in electron transfer from PSII or NAD(P)H dehydrogenase to PSI or cytochrome oxidase, respectively. In all organisms capable of oxygenic photosynthesis the cytochrome $b_6f$ complex consists of four major proteins and additional small subunits (Zhang et al., 2003). The 25 kDa cytochrome $b_6$ protein contains two b-type (Widger et al., 1984) and one novel x-type (Kurisu et al., 2003) hemes, and, together with the subunit IV of 17 kDa, is homologous to cytochrome *b* of the cytochrome $bc_1$ complex (Widger et al., 1984). Cytochrome $b_6$ and subunit IV are integral membrane proteins with four and three predicted transmembrane α-helices, respectively. Cytochrome *f* is a 31 kDa c-type cytochrome with a covalently bound heme in the large lumen-exposed domain; it is anchored by a single C-terminal α-helix in the membrane. Similarly, the Rieske iron-sulfur protein has a large hydrophilic lumenal domain attached to a single transmembrane α-helix at the N-terminus (for reference see Kallas, 1994). The genome of *Synechocystis* contains three open reading frames, designated *sll1316* (*petC1*), *slr1185* (*petC2*), and *sll1182* (*petC3*), encoding for three putative Rieske iron-sulfur proteins (Schneider et al., 2002).

**PSI** represents a multimeric polypeptide complex, formed basically around the PsaA/B heterodimer that binds 90 - 120 chlorophyll *a* molecules (for references see Golbeck, 1994; Ben-Shem et al., 2003). The cyclic electron transport around photosystem I plays a role in the generation of ATP required for respiration of cyanobacteria and plants during dark stages (Howitt et al., 2001). Comparison of higher plant PSI with that of cyanobacteria showed that cyanobacteria do not contain the PsaG, PsaH, PsaN and PsaO proteins (Kashino et al., 2002). On the other hand, PsaM protein has been identified only in cyanobacteria, but not in higher plants. PsaM is a low molecular weight protein with a single trans-membrane helix. The function of this protein is still not clear (Scheller et al., 2001; Kashino et al., 2001; Knoetzel et

al., 2002). PSI can exist in two different forms: monomeric and trimeric. The trimeric form is a consequence of the association of monomeric PSI through the PsaL protein (Chitnis and Chitnis, 1993).

**The ATP synthase** is a multisubunit membrane-bound protein complex, which catalyzes the synthesis of ATP from ADP and phosphate (Pedersen and Amzel, 1993; Frasch, 1994; Neisser et al., 1994; Dimroth et al., 2000). The activity of ATP synthase requires a proton gradient ΔpH and depends on the membrane potential $\Delta\psi$ (Kaim and Dimroth, 1999; Wieczorek et al., 1999; Dimroth et al., 2000). It is comprised of two subcomplexes, a membrane-integral part, $F_0$, and an extrinsic appendix, $F_1$.

$F_1F_0$ ATP synthases comprise a huge family of enzymes with members found in the cytoplasmatic membrane of bacteria, inner membrane of mitochondria and thylakoid membranes. The hydrophilic $F_1$ component of the enzymes, which catalyzes the ATP synthesis, is comprised of five subunits (α, β, γ, δ, and ε). The hydrophobic $F_0$ component, made up of four subunits (a, b, b′ and c), forms the proton channel through the membrane (for references see Frasch, 1994; Robertson et al., 1989). The gene coding for ATP synthase subunits are generally encoded by two operons. The *atp1* operon encodes the subunits a, c, b′, b, δ, α, γ. The *atp2* operon contains the genes for subunits β and ε. The *Synechocystis* $F_1F_0$ ATP synthase is also composed of nine subunits (Lill and Nelson, 1991).

The cytoplasmic membrane of cyanobacteria contains some additional type of ATP synthase called P-type ATPase (P-ATPase), present in small amounts. It increases during light-limiting growth when respiration becomes more important for energy generation (Niesser et al., 1994).

### 1.3.2 Adaptation of cyanobacteria to environmental changes

One of the intriguing features of thylakoid membranes and the photosynthetic apparatus in general is the capability of adaptation during or following environmental changes. Peptidases play a crucial role in such processes. Fluctuations of various environmental factors including levels of specific nutrients, the intensity of the incident irradiation and temperature alters growth rates of phototrophic organisms. Dramatic changes in pigment content, activities of various metabolic processes and cell morphology may be observed under extreme conditions (Kehoe and Grossman, 1996; Grossman et al., 2000; He et al., 2001).

#### 1.3.2.1 Light adaptation

Acclimation to light regimes is one of the most essential and complex responses of photosynthetic organisms to varying environmental conditions. Light adaptation is often accompanied by changes in content of pigments and in the composition of thylakoid proteins (Allen, 1995; Anderson et al., 1995) with following modifications in the composition of antenna complexes (Lorimier et al., 1991), redistribution of excitation energy between the photosystems (Murakami and Fujita, 1991), changes in the composition of reaction centres or in $CO_2$ fixation activity (Schmetterer, 1994; Murakami et al., 1997).

Under light-limiting conditions cyanobacteria increase their concentration of phycobiliproteins and chlorophyll *a*. Increasing antenna sizes occurs by elongation of the phycobilisome rods and by an increase in the number of phycobilisomes per unit area of the thylakoid membrane. Under light-saturating conditions cyanobacteria reduce their antenna size and photosystem content which is accompanied with a marked decrease in the chlorophyll *a* and phycobiliprotein levels and a slight decrease in total carotenoid levels (Hihara et al., 1998; He et al., 2001; Havaux et al., 2002). Simultaneously, phycobilisome sizes and photosystem contents are reduced to avoid absorption of excess light energy.

Expression analysis in *Synechocystis* cells showed that chlorophyll *a* and phycocyanin contents decline drastically within 3 h of acclimation to high light. These changes could originate from down-regulation of the genes that encode enzymes for biosynthesis of photosynthetic pigments: *chlorophyll* genes and structural components of the PBS (*aps* and *cpc* genes; Lorimier et al., 1991; Hihara et al., 2001) and from synthesis of proteins that are directly or not directly involved in the degradation of chlorophyll *a* and phycobiliproteins. Expression analysis of genes encoding PBS subunits uncovered that transcription of allophycocyanin genes is down-regulated by light, whereas the mRNA levels of linker proteins are not affected at high light regimes (Lorimier et al., 1991). Simultaneously, the remodeling of the PSII antenna complex is accompanied by a shortening of the PBS rods *via* detachment of external rod segments or whole rods from the PBS core. Various studies indicated that peptidases participate in posttranslational modification of the PBS antenna (Yamanaka et al., 1980).

Later, it was shown that Lhc-like polypeptides (HLIP; high light inducible polypeptides) accumulate during adaptation to light stress in plants and cyanobacteria (Adamska et al., 1999; Funk and Vermaas, 1999; He et al., 2001). Cyanobacterial cells encode five genes (*hliA*, *hliB*, *hliC*, *hliD* and *hemH*) for polypeptides with similarity to Lhc polypeptides of vascular plants. Levels of all Hli polypeptides were elevated in high light and during nitrogen limitation (van Waasbergen et al., 2002). The transcripts of three gene, *hliA*, *hliB*, *hliC*, accumulate to high levels following exposure to sulphur deprivation and low temperature. Therefore, the products of these genes can be involved in the protection against different types of stress, including light stress. The initial accumulation of Hli polypeptides occurs during the phase of acclimation in which cells are unable to divide. The role of HliA protein in photosynthetic electron flow is unclear, although the polypeptides may be involved in the dissipation of excess absorbed light energy or function in a complex that shuttles chlorophylls to sites of degradation and/or pigment protein complex biosynthesis (He et al., 2001; Havaux et al., 2002). Expression studies of *Synechocystis* peptidase genes under acclimation to different light regimes showed that three genes, *clpB2, ftsH1* and *ftsH2*, are induced within 15 min after transfer to high levels of light and become down-regulated within next 15 h (Hihara et al., 2001). As it was noted before, the products of *ftsH1* and *ftsH2* genes were found to be essential for cells since no knock-out mutants for these peptidases could be obtained. FtsH2 protein is essential for controlling the turnover of D1 protein (Bailey et al., 2002; Silva et al., 2003).

In cyanobacterial cells about 110 - 140 molecules of chlorophyll *a* are attached with the reaction centre of PSI, but only 35 - 70 molecules reside in PSII reaction center. As chlorophyll *a* is mainly associated with PSI, the acclimation to high light is accompanied by a decrease in PSI relative to PSII (Fujita et al., 1994, Murakami et al., 1997; McConnell et al., 2002). Such adaptation serves to regulate the distribution of excitation energy between photosystems and to correct any energy imbalances (Hihara et al., 1998). Changes in photosystem contents are regulated by decreasing mRNA levels of genes encoding subunits of photosystems (Hihara et al., 1998; Hihara et al., 2001; Muramatsu and Hihara, 2003) and, simultaneously, by activation of proteolytic components involved in the degradation of photosynthetic proteins during light stress (Lindahl et al., 2000; Bailey et al., 2001; Lensch et al., 2001; Kanervo et al., 2003; Silva et al., 2003). Recently, it has been shown that transcript levels of photosystem I genes rapidly decrease to less than 10% of initial levels within 1 h after a shift to HL, whereas the response of PSII transcript levels was not coordinated (Hihara

et al., 1998; Muramatsu and Hihara, 2003). Fast changes in the transcription of PSI genes, but not PSII genes, seem to be important for *Synechocystis* cells to regulate their photosystem contents in response to high light acclimation. Probably, the content of PSII can be promptly reduced under HL conditions due to an accelerated turnover rate of its reaction centre subunits (Mohamed and Jansson, 1989; Komenda et al., 2000). The turnover process of PSII includes the degradation of damaged D1 polypeptide, *de novo* synthesis of D1 and assembly of the heterodimeric complex with other PSII polypeptides (Melis, 1999; Komenda et al., 2000). There are various proteases and chaperones that are involved in D1 turnover *in vivo*. Two families of proteases are being studied: the FtsH family of $Zn^{2+}$-activated nucleotide-dependent proteases; and the HtrA (or DegP) family of serine-type proteases. Recent evidence showed that the HtrA family of proteases is involved in the resistance of *Synechocystis* to light stress and play a part, either directly or indirectly, in the repair of PSII *in vivo* (Bailey et al., 2001; Silva et al., 2002). Chloroplast FtsH showed light-inducible gene expression (Lindahl et al., 2000). It was proposed that this peptidase is involved in the degradation of unassembled thylakoid proteins (Ostersetzer and Adam, 1997) and, perhaps, in the second step of the degradation of PSII reaction center core protein D1 (Lindahl et al., 2000; Silva et al., 2003).

#### 1.3.2.2 Heat stress

Response to elevated temperature is an environmental factor that is also studied in cyanobacteria. Exposure of cyanobacterial cells to temperatures exceeding 60 - 65°C even for 10 min results in a bleached phenotype. Such alternation is the result of a breakdown of components of PBS (Zhao and Brand, 1989; Nishiyama et al., 1993). Higher temperatures bleach bilin-containing pigments fast, but also resulted in a gradual bleaching of chlorophyll and carotenoids. PSII has been shown to be the most sensitive thylakoid assembly to heat among the photosynthetic activities (Berry and Björkman, 1980). The dissociation of two of the four Mn atoms from the PSII complex by heat results in complete inactivation of oxygen evolution without significant loss of proteins (Nash et al., 1985). Therefore, the mechanism of photosynthetic adaptation to high temperature is related to ability to protect the PSII oxygen-evolving complex against heat-induced inactivation.

Different factors, which are regulated by growth temperature, may contribute to observed adaptation. Early investigations suggested that high temperature increases the level of

saturated fatty acids in membrane lipids and enhances the thermal stability of photosynthesis (Shneyour et al., 1973). Later, it has been shown that thermal stability is not only affected by changes in the saturation level of membrane lipids, but other factors are also responsible for adaptation of photosynthesis to high temperature. There is a number of heat stress proteins, chaperones and peptidases that are involved in the refolding or degradation of polypeptides misfolded by heat. One of the best studied peptidases functioning during heat stress is the bacterial HtrA peptidase (Strauch et al., 1989; Lipinska et al., 1990; Spiess et al., 1999).

#### 1.3.2.3 Nutrient stress

One of the essential requirements to survive in natural environment is the ability to withstand nutrient limitation or even deprivation. Nutrient limitation, like other extreme conditions, is accompanied by expression of special sets of stress proteins (Hagemann et al., 1991; Fulda et al., 1999; Suzuki et al., 2000; Hihara et al., 2001; Görl et al., 1998). These stress proteins can be divided into two groups, special stress proteins that are only induced by defined stresses and general stress proteins, which are induced by several stresses (Hagemann et al., 1999). Such factors can control intercellular protein contents at transcriptional, translational and post-translational levels.

A cyanobacterial cell that undergoes iron, nitrogen or sulfur deprivation contains less than half of the normal complement of thylakoid membranes (Wanner et al., 1986). During sulfur and nitrogen starvation the color of cultures changes from blue-green to yellow, a process known as a chlorosis. It is caused by degradation of phycobiliproteins. Small polypeptides of the Nbl-family were found to trigger proteolytic degradation of phycobiliproteins. Perhaps, the NblA protein can act as an activator of a specific protease that subsequently degrades PBS (Collier and Grossman, 1996; summarized by Grossman et al., 2001). Sulfur deprivation that induces phycobilisome degradation has been reported for *Synechococcus* cells, but not for *Synechocystis* (Richaud et al., 2001). In various studies the time course and extent of PC degradation varied from 50% degradation of PC per volume of culture within 60 h to 90% degradation within 9 h (Lau et al., 1977; Wanner et al., 1986). In contrast, cells deprived of phosphorus show only very limited degradation of PBS, as may be expected since PBS contain no phosphorus. In phosphorus-limited cells of PBS biosynthesis decreased relative to the rate of cell division (Grossman et al., 1993; Sauer et al., 2001). Opinion about degradation of chlorophyll *a* differs. Some studies indicate a dramatic decline of chlorophyll *a*, whereas

other investigations showed only a slightly decreasing or even constant chlorophyll *a* concentration (Allen and Smith, 1969; Wanner et al., 1986; Collier and Grossman, 1992).

Iron deficiency also leads to a faster degradation of PBS and PSI proteins, with accompanying expression of iron-regulated genes (*isiA* and *isiB*) (Pakrasi et al., 1985; Bibby et al., 2001). In cells chlorophyll contents and phycobiliproteins decrease undergoing iron starvation (Trick et al., 1995; Sandström et al., 2002). Intercellular spaces are filled by granules of glycogen and thylakoids are reduced in size. This type of deficiency corresponds predominantly to a decline in chlorophyll content, which can be restored after one or two cell divisions in iron-containing media (Sherman and Sherman, 1983).

In the absence of copper plastocyanin degradation is activated. In the green alga *Chlamydomonas reinhardtii*, the copper-dependent accumulation of plastocyanin is affected *via* an altered stability of the protein in copper-deficient versus copper-sufficient medium. Two mechanisms have been proposed to account for differential degradation of plastocyanin in copper-deficient cells. Copper-deficient cells contain a form of plastocyanin that is more susceptible to proteolysis (*i.e.* apoplastocyanin). Alternatively, copper-deficient cells induce a plastocyanin-specific protease activity in order to ensure that copper might be some available for other, probably indispensable, copper enzymes. Data have shown that apoplastocyanin is preferred substrate for proteolysis, but growth under copper deficiency is additionally required for the degradation of apoplastocyanin (Li and Merchant, 1995).

The main aim of this thesis was to identify proteolytic components in the cyanobacterium *Synechocystis* sp. PCC 6803 which participate in response to environmental changes, such as light stress, heat stress and nutrient deprivation. Initial work was concerned with components of the *Synechocystis* SppA family that contributes to light adaptation.

# 2. MATERIALS AND METHODS

## 2. 1 Materials

### 2.1.1 Chemicals and enzymes

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

Radiochemicals ($\alpha^{32}$P-dCTP, $\alpha^{32}$P-UTP, $^{35}$S-Met, $^{35}$S-Met/Cys cells labeling mix, $^{132}$I-protein A) were from Amersham Pharmacia Biotech (Freiburg i. Br.). Restriction endonucleases, DNA- and RNA-modifying enzymes, DNA-, RNA-polymerases were purchased from Boehringer (Mannheim), MBI Fermentas and New England Biolabs.

### 2.1.2 Molecular weight markers

**RNA- and DNA-length molecular standards**

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

**Protein molecular weight standards**

The apparent molecular weight of proteins in SDS-polyacrylamide gel electrophoresis was determined according to SDS-7 molecular weight marker (14 - 66 kDa) from Sigma Chemical Company (Munich), peqGOLD protein marker (14.4 – 116 kDa) and peqGOLD prestained protein marker (20 - 122 kDa) from PeqLab (Biotechnologie GmbH), Multi-Colored Standard (4 - 250 kDa) from Invitrogen TECH-LINE (USA).

### 2.1.3 Vectors and strains

**Vectors**

pGEM-T (Promega, Madison, USA)

pRSET5a (Rosenberg et al., 1987)

pVZ321, pVZ322, pVZ323, pVZ324 (Zinchenko et al., 1999)

pIVEX-MBP, pIVEX2.4b, pIVEX2.6d (Roche Diagnostics GmbH, Basel, Switzerland)

**Bacterial strains**

*E.coli* R751

*E.coli* DH5a (Hanahan, 1985)

*E.coli* BL21 (DE3) pLysS (Studier and Moffat, 1986)

*E.coli* M15 (Quiagen GmbH, Hilden)

*Synechocystis* sp. PCC 6803 glucose sensitive and non-motile strain (Prof. Shestakov, Genetic department of Moscow State University)

### 2.1.4 Antibodies

Most antisera were used from the collection of Prof. Dr. R.G. Herrmann's laboratory. Polyclonal anti-rabbit IgG peroxidase-conjugate antibodies raised in goat were obtained from Sigma Chemical Company (Munich). Antiserum against SppA2 was produced during this work (dilution 1:1.000). Antisera against phycobiliproteins (phycocyanin, allophycocyanin and linker proteins; 4A - 8A; dilution 1:2.000) were kindly provided by Prof. Dr. A. Grossman (Stanford University, USA). Antisera against subunits of PSI (PsaA, PsaB, PsaF, PsaC; dilution 1:1.000), PSII (D1, D2, PsbJ, CP43, CP47; dilution 1:1.000), Cyt $b_6f$ complex (anti-Rieske Fe/S protein; dilution 1:1.000) and ATP synthase (α- and β-subunit; dilution 1:2.000) were selected for the work presented.

### 2.1.5 Oligonucleotides

All standard primers (T7, T3, M13) and gene-specific oligonucleotides that are listed below were obtained from MWG-BIOTECH (Ebersberg, Germany).

#### 2.1.5.1 Primers for *Escherichia coli* strains

| | |
|---|---|
| pIVEX2.6d_fwd | 5'-GAA GGC CGC TTA ATT AAA-3' |
| PIVEX-MPB_fwd | 5'-ATC GAA GGC AGG CCT GGC CGC-3' |
| T7_fwd | 5'-TAA TAC GAC TCA CTA TAG GG-3' |

#### 2.1.5.2 *Synechocystis* gene-specific primers

SppA1 and SppA2 cloning and sequencing

| | |
|---|---|
| sll1703fwd | 5'-GGT TTC GGC TGA GGC AGA TC-3' |
| sll1703rev | 5'-GCC TTC GAG GTA AAC AAT GGC-3' |
| sll1703complfwd | 5'-GTT TGG GGA TGA TTT TGG GCT GG-3' |
| sll1703complrev | 5'- GAA GGC AGT AGT AAA TCC CGA CCA-3' |
| slr0021_fwd | 5'-TCT ATT GTT GGC GAG GGA AGC AG-3' |
| slr0021_rev | 5'-ATT CTT TGA CTT TTT CCA CTG CCA-3' |

SppA1 and SppA2 overexpression

| | |
|---|---|
| sll1703F_full | 5'-ATT CTC GAG ATG AAG AAC TTT TTC AAC AA-3' |
| sll1703R_full | 5'-TTA CTG CAG TTA AGG ATT AAG AAA TGC CA-3' |
| sll1703F_part | 5'-ATT CTC AGC AAC AAA TGG TGG CTA GTT TC-3' |
| sll1703R_part | 5'-TTA CTG CAG GAT GGT ACT CGC CAA ATT AA-3' |
| slr0021F_full | 5'-ATT CTC GAG ATG ATT TGG CCC CCC TTC AAA-3' |
| slr0021R_full | 5'-TTA CTG CAG TCA TCG ATA AAG CCA GAG-3' |
| slr0021F_part | 5'-ATT CTC GAG TTC AAA ACC AGC ACC CGT-3' |
| slr0021R_part | 5'-TTA CTG CAG ATT GCC TTG CAT CTT CCT-3' |
| slr0021F_comtest | 5'-TAT TGT TGG CGA GGG AAG AAG CAG-3' |
| slr0021R_comtest | 5'-TCT TCA ATG GTA TCC AGT TCT AC-3' |

Overexpression of high-light inducible proteins

| | |
|---|---|
| HliA_fwd | 5'-ATT CTC GAG ATG ACC CGT GGC TCC GC-3' |
| HliA_rev | 5'-TTA CTG CAG ACG ACC ATT CAT TTT TCG GC-3' |
| HliD_fwd | 5'-ATT CTC GAG AAT GAG TGA AGA ACT ACA ACC-3' |
| HliD_fullrev | 5'-TTA CTG CAG CTA GCG CAG TCC CAA CCA GGC-3' |

Primers for analysis of gene expression

| | |
|---|---|
| 16S_RNA fwd | 5’-AAT GGA GAG TTT GAT CCT GGC T-3’ |
| 16S_RNArev | 5’-AGG TGA TCC AGC CAC ACC TTC-3’ |
| apcE_Synforw | 5’-ATG AGT GTT AAG GCA AGT GGT-3’ |
| apcE_Synrev | 5’-CTA ACC GCC CAC TTT TAC TAC T-3’ |
| cpcA_for | 5’-ATG AAA ACC CCT TTA ACT G-3’ |
| cpcA_rev | 5’-CTA GCT CAG AGC ATT GAT GG-3’ |
| apcA_for | 5’-ATG AGT ATC GTC ACG AAA TC-3’ |
| apcA_rev | 5’-CTA GCT CAT TTT TCC GAT AAC-3’ |
| sll1702_for | 5’-ATG GAC GTT ACC ATT TAT CTC-3’ |
| sl1702_rev | 5’-TTT GCG GTC AAG TTT AAT CTC-3’ |
| sll1704_for | 5’-ATG GCA AAT CAG AAT TTT CCA-3’ |
| sll1704_rev | 5’-ACC ACG GCA AAA TTG TGC CAT-3’ |
| sll1704/CMP_revers | 5’-ATG GTC TAA CTC GGC GAT CGC-3’ |

### 2.1.6 General buffers and solutions

| | |
|---|---|
| TE buffer | 10.0 mM Tris-HCl, pH 8.0 |
| | 1.0 mM EDTA |
| STET buffer | 8% sucrose |
| | 5% Triton X-100 |
| | 50 mM EDTA |
| | 50 mM Tris-HCl, pH 8.0 |
| 10 x TBE | 1.0 M Tris |
| | 0.5 M boric acid |
| | 20.0 mM EDTA |
| 20 x SSC | 3.0 M NaCl |
| | 0.3 M $Na_3$-citrate x 2 $H_2O$, pH 7.2 |

| | |
|---|---|
| 20 x SSPE | 0.2 M $NaH_2PO_4$<br>3.0 M NaCl<br>20.0 mM $Na_2EDTA$ x $2H_2O$, pH 7.4 |
| 10 x Transffer buffer | 1.5 M glycin<br>0.2 M Tris-HCl, pH 8.0 |
| 10 x PBS | 750 mM NaCl<br>30.0 mM KCl<br>45.0 mM $Na_2HPO_4$<br>15.0 mM $KH_2PO_4$, pH 7.5 |
| 50 x Denhardt solution | 1% (w/v) BSA<br>1% (w/v) polyvinylpyrrolidon<br>1% (w/v) Ficoll |
| Denaturation solution | 1.5 M NaCl<br>0.5 N NaOH |
| Neutralization solution | 1.5 M NaCl<br>0.5 M Tris-HCl, pH 7.0 |

### 2.1.7 Media for bacterial growth

***E.coli* growth media**

| | |
|---|---|
| LB | 10 g Bacto-Trypton<br>5.0 g Yeast extract<br>10 g NaCl<br>$H_2O$ add till 1 l |
| LBG | 10 g Bacto–Ttrypton<br>5.0 g Yeast extract<br>10 g NaCl |

4.0 g glucose
$H_2O$ add till 1 l

For solid media 15 g/l of agar was added. Antibiotics (100 µg/ml ampicillin, 20 µg/ml chloramphenicol, 40 µg/ml kanamycin or 2 µg/ml gentamycin) were added from stock solution after autoclaving and cooling the media down to 50°C.

Antibiotic stock solutions:

| | |
|---|---|
| ampicillin | 100 mg/ml (in 50% ethanol) |
| kanamycin | 100 mg/ml (in 50% ethanol) |
| gentamycin | 10 mg/ml (in $H_2O$) |
| chloramphenicol | 25 mg/ml (in 50% ethanol) |
| lincomycin | 100 mg/ml (in 50% ethanol) |

**Medium for the *Synechocystis* sp. PCC 6803**

BG-11 medium for cyanobacteria (Rippka et al. 1988).
Macro- and microelements were added in dilution 1 : 1000 and filled with distilled water up to 1 L and autoclaved. When required, sterile glucose solution was added up to 5mM end concentration. For solid medium 1% Agar in BG-11 (Difco, Detrit, USA) was added.

| **Macro-elements (1 x stock)** | **g/l** | **Micro-elements (1000 x stock)** | **g/l** |
|---|---|---|---|
| **$NaNO_3$** | 1.5 | **$H_3BO_3$** | 2.86 |
| **$K_2HPO_4$** | 0.04 | **$MnCl_2 \cdot x\ 4H_2O$** | 1.81 |
| **$MgSO_4$ x $7H_2O$** | 0.075 | **$ZnSO_4 \cdot x\ 7H_2O$** | 0.222 |
| **$CaCl_2 \cdot x\ 2H_2O$** | 0.036 | **$NaMoO_4 \cdot x\ 2H_2O$** | 0.39 |
| **Citric acid** | 0.006 | **$CuSO_4 \cdot x\ 5H_2O$** | 0.079 |
| **Ferric ammonium citrate** | 0.006 | **$Co(NO_3)_2 \cdot x\ 6H_2O$** | 0.049 |
| **EDTA (disodium salt)** | 0.001 | | |
| **$Na_2CO_3$** | 0.02 | | |

### 2.1.8 Transfer membranes

Hybond nitrocellulose membranes for nucleic acid transfers were purchased from Amersham (Braunschweig). PVDF and nitrocellulose PROTRAN membrane for protein transfers were obtained from Schleicher & Schuell (Dasel) or PALL (Portsmouth, England).

### 2.1.9 Plant material

For biochemical analysis samples of chloroplasts, thylakoid membranes and lumen proteins from spinach (*Spinacia oleracea*), *Arabidopsis thaliana* L., ecotype Columbia (Col-0), and *tobacco* were kindly provided by Ms. Gabriele Burkhard.

## 2.2 Methods

Unless otherwise indicated standard molecular biology methods like DNA gel electrophoresis, DNA restriction analysis, DNA phenol/chloroform extraction, DNA ethanol/isopropanol precipitation, DNA dephosphorylation and ligation were performed according to Sambrook *et al.* (1989) or according to protocols provided by enzyme suppliers. DNA fragments were purified from low melting agarose gels using JETSORB Gel Extraction Kit (GENOMED). For sequence analysis DNA fragments were precipitated with 4.5 M of ammonium acetate and 6 volume of 100% ethanol. Approximately 100 ng DNA was used for sequence analysis.

### 2.2.1 Sequence analysis

Genome sequences were obtained from *Synechocystis* (http://www.kazusa.or.jp/cyano/cyano.html) and *Arabidopsis* (http://www.mips.biochem.mpg.de/proj/thal/db/index.html) data banks. Analysis of protein and gene homologies was performed using BLAST search on NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) and ClustalW on EMBL (http://www2.ebi.ac.uk.clustalw/). Protein sequence analysis of peptidases was obtained from MEROPS database (http://merops.sanger.ac.uk/). Hydropathy plots were performed according to Kyte and Doolittle (1982) and the SOSUI program (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html).

### 2.2.2 Strains and growth conditions for *Synechocystis*

A glucose-sensitive wild-type strain of *Synechocystis* was obtained from the strain collection of the Department of Genetics, Moscow State University (Russia). Wild-type and mutant strains were cultivated at 30°C in standard BG-11 medium with 20 mM HEPES-NaOH, pH

7.0 (Rippka, 1988). For dim light conditions (DL; 20 µmol photons $m^{-2}$ $s^{-1}$) cells were grown at 20 µmol photons $m^{-2}$ $s^{-1}$ in the 50 ml flasks covered with aluminium folia. Low light (LL; 50 µmol photons $m^{-2}$ $s^{-1}$) cells were grown at 50 µmol photons $m^{-2}$ $s^{-1}$ for at least 1 day and harvested at a cell density of $A_{750}$ = 0.6 to 1.0. For exposure to middle light (ML; 150 µmol photons $m^{-2}$ $s^{-1}$) and high light (HL; 350 µmol photons $m^{-2}$ $s^{-1}$) cells were grown at 50 µmol photons $m^{-2}$ $s^{-1}$ to a cell density of an $A_{750}$ = 0.6 to 0.8 and then transferred to ML or HL. Mutant strains were grown in medium with kanamycin (50 µg/ml), chloramphenicol (20 µg/ml) or gentamycin (2 µg/ml).

Media lacking either nitrogen (- N), sulphur (- S), phosphorus (- P), iron (- Fe) or copper (- Cu) were prepared by replacing the salts containing these elements with equimolar amounts of equivalent salts lacking the target elements (- N: ferric ammonium citrate, $NaNO_3$ and $Co(NO_3)_2$ were substituted by $FeCl_3$, NaCl and $CoCl_2$; - S: $MgSO_4$, $ZnSO_4$ and $CuSO_4$ by $MgCl_2$, $ZnCl_2$ and $CuCl_2$; - P: $K_2HPO_4$ by KCl; - Fe: depletion of ferric ammonium citrate; - Cu: $CuSO_4$ by $MgSO_4$). For deprivation the cyanobacterial culture was diluted to a cell density of 0.2 at the end of the log-phase and grown until an $A_{750}$ of 0.6. Cells were harvested by centrifugation at 2.500 x g for 15 min and resuspended in 50 ml of medium depleted of nitrogen, sulfur, phosphorus, iron or copper.

Cells of the exponential growth phase were transferred to heat shock conditions at 42°C for 48 h. Cold temperature treatment was performed at 17°C with different periods of cultivation (from 3 up to 12 days).

### 2.2.3 Construction of knock-out mutants in *Synechocystis*

#### 2.2.3.1 Construction of recombinant plasmids

Mutant strains were constructed using the insertion or deletion inactivation approach (Ivleva et al., 2000). To generate the mutants lacking peptidase-encoding genes, a 1.2-kb kanamycin ($Km^R$) resistance gene cassette from the pUC4K plasmid (Amersham Pharmacia Biotech, Piscataway, NJ) and a 0.7-kb gentamycin ($Gm^R$) resistance gene cassette from the pSL762 (Schweizer, 1993) were used. The mutants containing insertions in peptidase-encoded genes (Table 1) and those to complement mutant Δ*sppA1* were constructed in cooperation with Prof. Dr. S.V. Shestakov (Genetic Department of Moscow State University).

*E. coli* strains *DH5α* , *BL21 DE3* (*Lys*) and *M15* were transformed by the $CaCl_2$/heat shock method according to Cohen *et al.* (1972). The strain *JM109* was transformed by electroporation as described by Sambrook *et al.* (1989). After transformation bacteria were plated on LB medium supplemented with the appropriate antibiotica and incubated overnight at 37°C.

The part of the coding region of required gene was amplified from genomic DNA using synthetic primers (Section 2.1.5). PCR products were cloned into the *pGEMT-Easy* vector (Promega). The resulting plasmids were digested and ligated with the kanamycin-resistant cassette. These plasmids were used for transformation of *Synechocystis* (Section 2.2.3.2). For construction of the complementation mutant Δ*sppA1* the conjugal transfer with the corresponding DNA was used (Section 2.2.3.3).

Gene-targeted mutagenesis by inserting the kanamycin or gentamycin resistance gene cassettes into respective region was performed according to Ivleva et al. (2000).

**Table 1. Characteristics of peptidase genes that were inactivated in this work**

| ORF | Gene name | Gene size (bp) | Protein molecular weight (kDa) | Restriction site | Antibiotica resistance |
|---|---|---|---|---|---|
| *slr1641* | *clpB2* | 2619 | 95.92 | *Hind*III | $Gm^R$ |
| *sll0534* | *clpP2* | 681 | 24.86 | *Sma*I | $Km^R$ |
| *sll1703* | *sppA1* | 1833 | 67.1 | *Eco*81I | $Km^R$ |
| *slr0021* | *sppA2* | 833 | 30.47 | *Cla*I | $Km^R$ |
| *sll1679* | *hhoA* | 1185 | 43.34 | *Hind*III | $Gm^R$ |
| *sll1427* | *hhoB* | 1251 | 45.76 | *Sma*I | $Km^R$ |
| *slr0535* | *srp* | 1842 | 67.43 | *EcoR*V | $Km^R$ |
| *sll1343* | *pepN* | 2610 | 98.56 | *Sma*I | $Gm^R$ |
| *sll0136* | *pepP* | 1326 | 48.51 | *BamH*I | $Km^R$ |
| *sll2008* | *prp1* | 1293 | 47.3 | *EcoR*V | $Km^R$ |
| *sll2009* | *prp2* | 1308 | 47.96 | *Stu*I | $Gm^R$ |
| *sll0055* | *prp3* | 1287 | 47.08 | *Kpn*I | $Gm^R$ |
| *slr1331* | *ymxG* | 1542 | 56.43 | *BamH*I | $Km^R$ |
| *sll0915* | *pqqE* | 1575 | 57.64 | *Xba*I | $Km^R$ |

### 2.2.3.2 Transformation of *Synechocystis*

Transformation of *Synechocystis* was carried out as described by Grigorieva and Shestakov (1982). Cells were grown for 3 or 5 days in liquid BG-11 medium supplemented with or without 5 mM glucose. Cells were harvested by centrifugation for 5 min at 5.000 x g at room temperature and resuspended in a small volume of BG-11. Pelleted cells were plated on solid BG-11 with low concentration of antibiotic with or without 5 mM glucose. Plates without

glucose were incubated under LL. Those plates that contained glucose were incubated under DL. Colonies of transformants appeared upon incubation at 30°C under different light conditions within 8 to 10 days. Segregation of mutant cells was carried out with several passages on high concentrations of antibiotica. The transformed *Synechocystis* cells were selected in the presence of increasing amounts of kanamycin (5 – 50 µg/ml), chloramphenicol (0.5 - 20 µg/ml) and gentamycin (0.5 - 3 µg/ml) under LL. PCR analysis of chromosomal DNA isolated from transformed strains was performed to monitor segregation of mutations in the cyanobacterial genome (Section 2.2.4.2).

#### 2.2.3.3 Conjugal transfer of plasmids into cyanobacterial cells

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

Cyanobacterial cells of wild-type or mutant *Synechocystis* strains were grown until late exponential growth phase (to an $A_{750}$ of 1.0 – 1.5). Then the cells were collected by centrifugation and washed with 1 x BG-11. An overnight culture of *E. coli* was washed with 1 x LB to remove antibiotica. Then 1 ml of cyanobacterial cells was mixed with *E. coli* (1 ml), which contained plasmids *pVZ321* and *R751*, centrifuged and resuspended in 100 µl of sterile water. All three parents were spotted onto a nitrocellulose filter resting on a solid cyanobacterial medium containing a mixture of BG-11 and LB agar of 1% and 5%, respectively. After one day on the non-selective medium the filters were transferred to selective medium containing chloramphenicol (0.5 µg/ml) and kanamycin (10 µg/ml).

### 2.2.4 DNA and RNA analysis

#### 2.2.4.1 DNA isolation from *Synechocystis*

For isolation of chromosomal DNA *Synechocystis* cells from 3 – 5 ml of culture at the late exponential phase ($A_{750}$ of 1.0) were collected, washed with 1 ml of TE buffer and resuspended in 270 µl STET buffer (Section 2.1.6). The cell suspensions were mixed with 15

µl of chloroform and the mixture was subjected to vigorous vortexing for 5 min. Then, 30 µl of lysozyme (20 mg/ml) was added and the cell suspension was incubated for 30 min at 37°C. The lysozyme-treated cells were lyzed for 50 min at 65°C after addition of 100 µl of 10% (w/v) SDS and further 10 min after addition of 100 µl 5 M NaCl. The lysate was deproteinized by chloroform extraction. DNA was precipitated with isopropanol, washed with 70% ethanol and dissolved in sterile water.

#### 2.2.4.2 PCR analysis

PCR amplification was performed with 0.1 µg of purified *Synechocystis* DNA according to the following programm: 1 cycle of 94°C denaturation for 5 min; 30 cycles at 94°C (30 s), 54°C (30 s), and 72°C (2 min) and an elongation cycle of 10 min at 72°C using Taq polymerase (Qiagen).

#### 2.2.4.3 Isolation of plasmid DNA from *E. coli*

Mini-preparations of plasmid DNA from *E. coli* were performed using the "boiling lysate" method (Sambrook et al., 1989). Bacteria of 1.5 ml fresh overnight culture were collected by centrifugation (10.000 x g for 5 min at RT). The bacterial pellet was resuspended in 400 µl STET buffer containing 1% (w/v) lysozyme and afterwards incubated for 45 sec at 95°C. The lysed bacteria cells and the denatured genomic DNA were centrifuged (10.000 x g for 10 min at RT) and removed from the supernatant plasmid solution with a sterile toothpick. Plasmid DNA was precipitated with 40 µl of 3 M Na-acetate (pH 5.2) and 420 µl of isopropanol for 5 min at room temperature. After centrifugation (12.000 x g for 5 min at RT) the DNA pellet was washed once with 70% ethanol, centrifuged again and dried. The final pellet was resuspended in 100 µl 1 x TE (pH 8.0) buffer containing 20 µg/ml of RNase A and shaked for 10 min at 50°C. DNA solutions were stored at -20°C.

#### 2.2.4.4 RNA isolation from *Synechocystis*

Total RNA was isolated from mid log-phase liquid culture ($A_{750}$ = 0.5) using TRIZOL reagent according to the manufacturer instructions (GibcoBRL Life technologies). Cyanobacterial cells were harvested by centrifugation at 4°C for 15 min at 12.000 x g. Then, the pellet was quickly frozen in liquid nitrogen and homogenized with 1 ml of Trizol solution. The samples

were incubated for 5 min at room temperature to enhance and insure complete dissociation of nucleoprotein complexes. An equal volume of phenol-chloroform-isoamyl alcohol mixture, pH 6.6 - 7.9 (Ambion) was added to Trizol-containing lysate and kept on ice for 5 min. The suspension was centrifuged at 12.000 x g for 5 min at 4°C and the aqueous phase containing RNA was transferred into the new vessel. The RNA was mixed with 1/10 aqueous phase volume of 3M sodium acetate by shaking for about 10 sec. It was then mixed with acid-phenol-chloroform solution (Ambion) and incubated for 5 min at 4°C, followed by a centrifugation step, as described before. RNA was precipitated from the solution by adding 500 µl isopropanol and following incubation at -20°C for 30 min. It was collected by centrifugation at 12.000 x g for 30 min at 4°C. The RNA pellet was washed once with 75% EtOH in a 1 to 1 ratio with the initial amount of TRIZOL mixed by vortexing and centrifuged at 3.500 x g for 5 min at 4°C. The pellet was dried for 5 - 10 minutes and dissolved in RNAse-free TE-buffer or 5% SDS solution by passing the solution through a pipette tip. RNA was incubated for 10 min at 55 - 60°C to remove secondary structures. RNA concentration was spectrophotometrically determined at 260 nm.

#### 2.2.4.5 Analysis of gene expression

##### 2.2.4.5.1 Northern analysis

Total RNA samples (15 µg of total RNA per lane) were electrophoresed in 1.2% agarose gels in MOPS-buffer with 2.5 M formaldehyde (Sambrook et al., 1989). RNA marker was used for estimation of molecular masses. RNA was transferred to nylone membrane (Hybon-N+, Amersham) with 10 x SSC as previously described (Sambrook et al., 1998).

The hybridization probes were synthesized by a random priming method with α-[$^{32}$P]-dCTP (Amersham) and Klenow fragment of DNA polymerase (Pharmacia). RNA blots were prehybridized for 2 h at 42°C in 5 x Denhardt solution, 5 x SSC, 7% SDS, 50% deionized formamide, 25 mM sodium hydrophosphate buffer, pH 7.2, and 100 µg ml$^{-1}$ denatured salmon sperm DNA (1 x SSC: 150 mM NaCl, 15 mM Na-citrate, pH 7.0). Hybridization was carried out overnight at 42°C. After hybridization membranes were washed twice for 10 min with 2 x SSC, 0.5% SDS at room temperature and once for 10 min with 0.1 x SSC, 0.1% SDS at 65°C.

#### 2.2.4.5.2 RT-PCR

After preparation of RNA the rests of DNA were removed by treatment with DNAse I at 37°C for 30 min. One-single cDNA was synthesized using 1 µg of total RNA with a RT-PCR Kit (Invitrogen) in a final volume of 20 µl, according to the manufacturer's instructions. After denaturation of RNA for 3 min at 70°C (necessary for removing secondary structure of RNA), cDNA synthesis was performed for 30 min at 60°C. As a negative control for DNA presence, 1 µg of RNA without the RT reaction was subjected to PCR amplification of 16S rRNA gene.

### 2.2.5 Overexpression of proteins

#### 2.2.5.1 Overexpression of proteins in *E. coli* cells

For protein overexpression in *E. coli* cells the full-length or partial DNA fragments of genes of interest were cloned into pRSET vectors (Schoepfer, 1993).

| | |
|---|---|
| Buffer A | 20% sucrose |
| | 20.0 mM Tris-HCl, pH 8.0 |
| | 2.0 M EDTA |
| Buffer B | 50.0 mM Tris-HCl, pH 8.0 |
| | 20.0 mM EDTA |

The overexpression vector containing the relevant DNA fragment was transformed into *E. coli* BL21. The transformed cells were selected on LB ampicillin-containing plates. A single colony was inoculated in 3 ml LBG medium with 100 µg/ml of ampicillin and incubated overnight at 37°C. The overnight culture was diluted with fresh medium (1 : 100) and incubated at 37°C until an $A_{600}$ of 0.6. One ml of culture was removed and kept on ice, as a control without expression. The rest of the culture was induced with 1 mM IPTG and incubated for 4 h at 37°C. Cells were pelleted by centrifugation (10.000 x g, for 10 min at 4°C) and resuspended in 1 x Laemmli sample buffer. The samples were denaturated for 5 min at 100°C, briefly centrifuged and analyzed on PAGE.

The non-induced and IPTG-induced *E. coli* cells were fractionated into periplasma (soluble fraction) proteins and inclusion bodies (membrane fraction). For this, cells were sedimented, resuspended in 1 ml of buffer A (for 1 ml initial culture) and incubated for 10 min at 25°C. The cell lysates were centrifugated for 5 min at 10.000 x g. The supernatant (periplasma proteins) was precipitated with 4 – 5 volumes of 100% ice-cold acetone or 1/10 volume of 100% trichloroacetic acid, sedimented for 10 min at 15.000 x g, washed twice with 70% acetone, dried on air and resuspended in 50 µl of 2 x Laemmli sample buffer.

#### 2.2.5.2 Overexpression of proteins in *E. coli* lysates

For overexpression of proteins that could not be overexpressed *in E. coli* cells the Rapid Translation System based on *E. coli* cell lysates (Roche Diagnostics GmbH) was used. The relevant DNA fragments were cloned into pIVEX vectors and the resulting plasmids were used for protein overexpression according to kit instruction.

### 2.2.6 Generation of protein-specific antisera

#### 2.2.6.1 Preparation of probes for rabbit immunization

Overexpressed N-terminal part of SppA2 was prepared as described in Section 2.2.5.1 and loaded onto a 12.5% PAA gel. After separation of proteins the gel was stained with imidazol solution and the band corresponding to overexpressed partial SppA2 protein was excised from the gel. This part of gel was rubbed between two glasses in 300 µl of 1 x PBS solution. The solution was vortexed for 2 min and kept at 4°C, centrifuged for 15.000 x g at 4°C for 15 min and supernatant was used for the injection of rabbits. For the first injection the protein extract was mixed with adjuvant TiterMax™ in a ration of 1 : 1 (the maximal volume was 300 µl). For the second and further injections the Freund's adjuvant was used instead TiterMax™.

#### 2.2.6.2 Injection of rabbits and antibody preparation

The immunization of rabbits and antiserum preparation was performed according to Harlow and Lane (1988). Before antigen injections 5 ml of blood were taken as preimmune serum from each animal, to check unspecific reactions. The antigen-TiterMax™ suspension was then injected, generally at three sites. After two weeks the injection with Frend's adjuvant was

repeated. Serum samples were taken 10 days after the second boost. After collection the blood was allowed to clot for 30 min at 37°C. The clot was kept at 4°C overnight. The serum was separated from the clot by centrifugation at 10.000 x g for 15 min at 4°C. Na azide (0.01%) was added to the supernatant. The antiserum was aliquoted and stored at –20°C.

#### 2.2.6.3 Purification of antibodies

##### 2.2.6.3.1 Purification of antibodies against overexpressed protein on the membrane

Purification of antisera were performed according to Lehto and Virtanen (1983). After electrophoretic transfer of proteins, nitrocellulose membranes were stained by Ponceaus solution in order to visualize the overexpessed protein. The band was excised and incubated in blocking buffer (Section 2.2.9.1) for 1 h at room temperature. The filter was incubated with an antiserum for 5 h at 4°C or 1 h at 37°C. It was then rinsed twice in 1 x PBS solution for 15 min at room temperature. The nitrocellulose band was placed for 20 min on a piece of Parafilm with a small volume of elution buffer (200 - 500 µl of 0.2 M glycine, pH 2.8) in a humidified atmosphere within a Petri dish. The eluted antibody solution was immediately neutralized with 1/10 volume of 1M Tris, pH 8.0, and diluted with 1/10 volume of 10 x PBS and sodium azide (0.02%). The samples were stored at 4°C.

To remove contaminants and suppress unspecific reaction with the nitrocellulose membranes the antiserum was diluted (1:1.000) and the nitrocellulose membrane was incubated with for 1 hour in blocking buffer.

##### 2.2.6.3.2 Purification of antibodies with Protein A-Sepharose

0.5 g of Protein A-Sepharose was resuspended in 5 - 6 ml of distilled water and poured onto a 10 ml column. The column was washed twice with 10 ml of distilled water during 15 min and equilibrated with 10 ml of 1 x PBS buffer. The antiserum solution (2 ml) was mixed with 18 ml of 1 x PBS and centrifuged for 15 min at 10.000 x g. Supernatant was filtrated through 0.2 µm filter and loaded onto the column. The column was washed once with 30 ml of 1 x PBS. Immunoglobilins bound to Protein A-Sepharose were eluted with 10 ml of Na-citrate, pH 3.0. The eluate was immediately neutralized with 200 µl of 3 M Tris-HCl, pH 8.8, and 10 µl of each fraction were used for PAA gel electrophoresis. The fractions containing

immunoglobilines were combined and dialyzed to remove salts. The dialyses containing antibodies were filtered through a 0.2 µm filter, loaded onto a column as described previously and eluted with 2 ml of 0.1 M Na-citrate, pH 3.0. The samples were diluted with water and stored at 4°C.

### 2.2.7 Pigment analysis of *Synechocystis* cells

The growth of cyanobacterial strains was monitored by measuring light scattering at 750 nm in on UV/VIS-2401PC spectrophotometer (SHIMADZU, Japan).

#### 2.2.7.1 Determination of chlorophyll *a* concentrations

The content of chlorophyll *a* was estimated according to Arnon et al. (1974). Cell suspension of 1 ml was centrifuged at 10.000 x g for 10 min at 4°C and the pellet was extracted with 90% (v/v) methanol for 1 h at –20°C, followed by centrifugation at 10.000 x g for 10 min at 4°C. The chlorophyll *a* content was calculated from the absorbance of the methanol extract at 652 and 665 nm using the equation:

$$\text{Chlorophyll (mg/ml)} = 16.82 \times A_{665} - 9.28 \times A_{652}.$$

#### 2.2.7.2 Determination of carotenoid concentrations

Carotenoid concentrations were determined by measuring the absorbance at 470 nm and the concentration of chlorophyll from section 2.2.7.1:

$$\text{Carotenoids (mg/ml)} = (1000 \times A_{470} - 1.91 \times [\text{Chl}])$$

#### 2.2.7.3 Determination of C-phycocyanin concentrations

The content of phycobiliproteins was estimated according to Grossman et al. (1993). The cell samples were heated at 75°C for 10 min and the phycocyanin content was determined according to the following equation:

$$\text{Phycocyanin (mg/ml)} = [A_{620} - A_{750}\ \text{(unheated)}] - [A_{620} - A_{750}\ \text{(heated)}].$$

### 2.2.8 Determination of cell densities

The cell density of *Synechocystis* was estimated by measuring the absorbance of suspensions at 750 nm ($A_{750}$) (spectrophotometer Uvikon, Kontron). The absorption spectra were analyzed using program „Origin“ (version 5.0). Cell densities were also determined by counting *Synechocystis* cells with a Malassez cell chamber. The dependence of cell amounts from the absorbance of wild-type cultures is shown in Fig. 2. Determinations of pigment concentrations per cells were performed with cultures grown at (or diluted to) optical density $OD_{750}$ = 0.3.

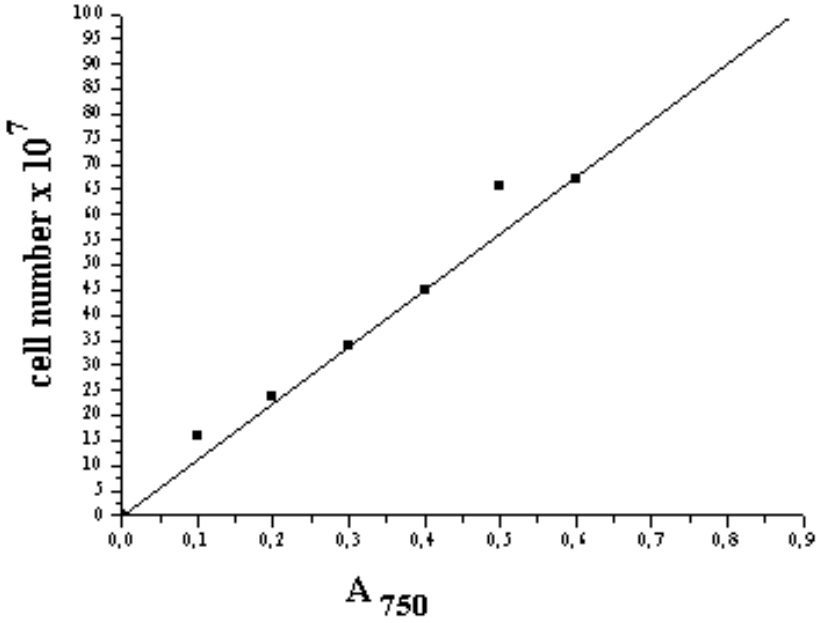


**Figure 2. Graphic illustrating the relation between absorbance ($A_{750}$) and cell density for the wild-type *Synechocystis* strain.** The cell density at different absorbance was determined by counting *Synechocystis* cells with a Malassez cell chamber.

### 2.2.9 Protein analysis

#### 2.2.9.1 Determination of protein concentrations

Protein concentrations were determined according to a modified Lowry procedure (Markwell, 1978). For estimation of cyanobacterial proteins 1.5 ml of cell suspension were precipitated with 10% (w/v) trichloroacetic acid and centrifugated at 10.000 x g for 10 min at 4°C. The pellet was resuspended in 1 N NaOH, boiled for 30 min, cooled down and recentrifugated to

eliminate light-scattering material. For estimation of protein concentrations in membrane fractions 5 µl of membrane were resuspended in 95 µl of water and precipitated with 6 volume of 100% cold acetone. The dry pellet was resuspended in 100 µl of water and used for protein determination as described before.

#### 2.2.9.2 Protein gel electrophoresis

##### 2.2.9.2.1 Gel electrophoresis according to Laemmli et al. (1970)

**Table 2. Pipetting scheme of PAA gels**

| Solution | Separating gel (end concentration) | Stacking gel (end concentration) |
|---|---|---|
| 2 M Tris-HCl, pH 8.8 | 0.375 M | - |
| 1M Tris-HCl, pH 6.8 | - | 0.125 M |
| 40% acrylamide (acrylamide : bisacrylamide – 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 |

<u>10 x Laemmli buffer</u>

0.25 M Tris-HCl, pH 8.5
1.92 M glycine
1% SDS

<u>4 x Laemmli sample buffer</u>

0.25 M Tris-HCl, pH 6.8
8% SDS
40% glycerol
20% ß-mercaptoethanol
0.016% Bromophenol Blue

##### 2.2.9.2.2 Gel electrophoresis according to Kashino et al. (2001)

This gel system was used for separation of polypeptides with molecular masses from below 5 kDa to over 100 kDa on an 18 - 25% urea-containing denaturing gel.

**Table 3. Composition of stock solutions for SDS-PAGE**

| Stock solution | Concentrations of constituents |
|---|---|
| 50% w/v PAA stock mixture for resolving gel | 49.5% acrylamide, 0.5% bisacrylamide |
| 30% w/v PAA stock mixture for resolving gel | 29.2% acrylamide, 0.8% bisacrylamide |
| resolving gel buffer (pH adjusted to 6.8 by HCl) | 3 M Tris, 0.65 M MES, 0.5% SDS |
| reservoir buffer (no adjusted of pH required) | 25 mM Tris, 192 mM glycine, 0.1% SDS |

**Table 4. Gel solution mixtures for of 18 - 24% PAA gradient gels containing 6 M urea**

| Solution or urea | 18% AA mixture | 24% AA mixture | Stacking gel mixture |
|---|---|---|---|
| 50% AA | 3.6 ml | 4.8 ml | - |
| 30% AA | - | - | 1.6 ml |
| resolving gel buffer | 2 ml | 2 ml | - |
| stacking gel buffer | - | - | 1.6 ml |
| urea | 3.6 g | 3.6 g | 2.9 g |
| $H_2O$ | 1.9 ml | 0.7 ml | 2.8 ml |
| 10% APS | 25 - 15 µl | 15 - 8 µl | 65 µl |
| TEMED | 4.5 µl | 4.5 µl | 9 µl |
| end volume | 7.5 ml | 7.5 ml | 7 ml |

The volumes of APS in the resolving gel solutions were changed depending on the surrounding temperature so as to polymerize the acrylamide in about 1 h. In a room of around 10°C 25 µl for 18% AA solution and 15 µl for 24% AA were used. At 30°C 15 and 8 µl for 18 and 24% AA were added, respectively. The running buffer was the same as in the Laemmli system.

#### 2.2.9.3 Staining of PAA gels

##### 2.2.9.3.1 Coomassie Brilliant Blue staining

Staining solution

| | |
|---|---|
| 45% | methanol |
| 9% | acetic acid |
| 0.2% (w/v) | Coomassie Brilliant Blue R 250 (Serva, Heidelberg) |

Destaining solution

| | |
|---|---|
| 25% | methanol |
| 7.5% | acetic acid |

The gel was stained for 30 min in a staining solution which was previously warmed up to 50°C to accelerate the process. It was destained in destaining solution (also previously warmed to about 50°C).

**2.2.9.3.2 Silver staining**

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) | $AgNO_3$ |
| 5% | 37% formaldehyde |

Developing solution

| | |
|---|---|
| 6% (w/v) | $Na_2CO_3$ |
| 0.05% | 37% formaldehyde |
| 4 mg/ml | $Na_2S_2O_3$ |

Stopping solution

| | |
|---|---|
| 50% | methanol |
| 12% | acetic acid |

Silver staining was used for detection of low quantities of proteins in gels. The gel of 1 mm thickness was incubated for 1 h in fixation solution, washed three times for 20 min in 50% ethanol and soaked for 1 min in thiosulfate solution. Afterwards, the gel was washed three

times with water for 30 sec and incubated in silver solution for 20 min in darkness with constant agitation. The gel was then washed with water again and incubated in developing solution until the bands reached the desired intensity. The reaction was stopped by addition of stopping solution.

Destaining solution for silver staining

| | |
|---|---|
| 1% | $K_3Fe(CN)_6$ |
| 1.6% | $Na_2S_2O_3$ |

The gel for destaining was briefly (15 - 30 sec) incubated in destaining solution and washed several times with distilled water.

#### 2.2.9.3.3 Imidazol staining

| | |
|---|---|
| 0.2 M | imidazol |
| 0.3 M | zink sulfate |

This method was used for the quantitative detection of protein bands in a gel before protein transfer. The gel was shaken for 10 min in imidazol-containing buffer (0.2 M imidazol), and then briefly incubated in 0.3 M $ZnSO_4$ before visualization of protein bands. The transparent protein bands were visible in a milk-white background of the gel. Staining was terminated by incubation with water. The gel was stored at 4°C until blotting.

### 2.2.9.4. Isolation and fractionation of thylakoid membranes

#### 2.2.9.4.1 Isolation of total cellular and membrane proteins from *Synechocystis*

Total cell extracts and membrane proteins were isolated as described in Shukla et al. (1992).

Buffer A
- 50 mM HEPES-NaOH, pH 7.0
- 0.5 M sucrose
- 15 mM NaCl
- 5 mM $MgCl_2$
- 0.001 volume of a proteinase inhibitor cocktail (Sigma)

Buffer B
10 mM Tricine, pH 7.5
6% sucrose
30 mM DTT
0.001 volume of a proteinase inhibitor cocktail (Sigma)

Cells were harvested by centrifugation at 4°C, resuspended in 600 µl of buffer A and then broken by vortexing with glass beads (3 times for 2 min each and keeping the samples for 1 min on ice between cycles). Unbroken cells were removed by a brief centrifugation (4.000 x g for 5 min at 4°C). Thylakoids were collected from supernatant by ultracentrifugation at 45.000 x g for 30 min at 4°C. Thylakoids were washed twice in buffer A, finally resuspended in buffer B and immediately frozen at –70°C.

**2.2.9.4.2 Extraction of peripherally associated proteins**

For investigation of protein interactions within the membrane, thylakoid membranes were incubated with chaotropic salts and alkaline solutions. Thylakoids (5 µg/µl chlorophyll) were resuspended in HEPES-NaOH, pH 7.0, 0.5 M sucrose buffer without any additions or containing 2 M NaBr, 2 M NaSCN, 0.1 M $Na_2CO_3$ or 0.1 M NaOH. After incubation on ice for 30 min thylakoids were diluted 1 : 2 with buffer lacking salts and pelleted by centrifugation for 10 min at 30.000 x g. Thylakoid membranes were resuspended in 2 x Laemmli sample buffer and denaturated for 10 min at 80°C. Extracted polypeptides were collected by precipitation with 4 to 5 volumes of 100% ice-cold acetone (2 h at –20°C), dried and resuspended in 2 x LSB.

**2.2.9.4.3 Preparation of phycobilisomes**

Phosphate buffer: 0.75M $K_3PO_4$
(mixture of $K_2HPO_4$ and $KH_2PO_4$ at the molar ratio 3 : 1), pH 7.0

Phycobilisomes were isolated according to Gantt et al. (1976). Cyanobacterial cells were broken by vortexing with glass beads (120 – 210 microns, Sigma) in 0.75 M phosphate buffer, pH 7.0, at room temperature. The cell extract was solubilized with 2% Triton X-100 for 20 min at room temperature and constant rotation. Unbroken cells were removed by centrifugation for 10.000 x g at 30 min at 15°C and the blue-colored supernatant was loaded

onto a sucrose density gradient (0.25 M – 0.79 M sucrose in phosphate buffer). Ultracentrifugation was performed at 130.000 x g for 16 h at 15°C using rotor SW40. The fraction of intact phycobilisomes that form the lower band in the gradient was collected and diluted with 0.75 M phosphate buffer (in 6 - fold) and phycobilisomes were pelleted for 4 h at 80.000 x g at 4°C. The sedimented phycobilisomes were dissolved in $H_2O$ and immediately frozen.

**2.2.9.4.4 Isolation of photosynthetic complexes from *Synechocystis* cells by sucrose gradient centrifugation**

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 growth stage and resuspended in SMN solution containing 0.2 mM PMSF and 5 mM benzamidine. Cells were broken by vortexing with glass beads and thylakoids were isolated by centrifugation at 50.000 x g for 60 min. Thylakoid membranes (200 µg chlorophyll/ml) were incubated for 30 min at room temperature with 1mM $CaCl_2$ in SMN buffer. Thylakoid membranes were solubilized by addition of dodecyl-β-D-maltoside (Sigma) to a final concentration of 1.5% and incubated for 15 min on ice. Insoluble material was pelleted by centrifugation at 20.000 x g for 15 min at 4°C. Solubilized membranes were loaded onto a 12 - 28% sucrose linear gradient in 10 mM MOPS, pH 7.0, 0.05% dodecyl-β-D-maltoside and centrifugated at 160.000 x g for 16 h using 12-ml tubes of a SW40 rotor. Typically, 60% chlorophyll in thylakoids can be recovered as PSI trimers by this procedure.

**2.2.9.4.5 Isolation of photosynthetic complexes from *Synechocystis* cells by non-denaturing gel electrophoresis (blue native PAGE)**

ACA buffer: 750 mM ε-aminocaproic acid
50 mM bis-Tris/HCl, pH 7.0
0.5 mM EDTA-$Na_2$

| | |
|---|---|
| DM in $ddH_2O$: | 10% (w/v) n-dodecyl β-D-maltoside |
| Sample buffer: | 5% (w/v) Coomassie Blue G<br>750 mM ε-aminocapronic acid |
| Cathode buffer 1: | 50 mM Tricine<br>15 mM bis-Tris/HCl, pH 7.0<br>0.02% (w/v) Coomassie G |
| Cathode buffer 2: | 50 mM Tricine<br>15 mM bis-Tris/HCl, pH 7.0 |
| Anode buffer: | 50 mM bis-Tris/HCl, pH 7.0 |
| Denaturation buffer: | 2% (w/v) SDS<br>66 mM DTT<br>66 mM $Na_2CO_3$ |

Supramolecular photosynthetic complexes were isolated according to Schägger et al. (1991). Cells of 100 ml of *Synechocystis* culture were collected by centrifugation at 10.000 x g for 15 min at 4°C and used for extraction of thylakoid membranes (Section 2.2.8.4.1). Isolated thylakoids were resuspended in 60 µl of ACA buffer (end concentration 1 µg/µl chlorophyll) and solubilized by addition of 10 µl of DM [10% (w/v) in $H_2O$] for 15 min at 4°C under constant rotation. Unsolubilized material was collected by ultracentrifugation at 45.000 x g for 15 min at 4°C. Solubilized thylakoids were mixed with 5 µl of sample buffer. The samples were then loaded onto a 4 - 12% gradient gel (Table 5) and electrophoresed at 100 V and 15 mA. After entering the separation gel electrophoresis was continued at 500 - 1000 V. When the front had reached half the gel distance, cathode buffer containing Coomassie Blue G was replaced by the same buffer without staining reagent. All solutions were pre-cooled to 4°C; electrophoresis was performed at 10°C.

For separation of individual proteins from photosynthetic complexes SDS-denaturing gel electrophoresis was performed (Section 2.2.8.2.1). The gel slice was cutted out and incubated at room temperature in denaturation buffer for 30 min. The slice was then horizontally loaded

onto a denaturing 5% stacking gel and fixed with 0.5% agarose (w/v in $H_2O$). SDS-PAGE was performed overnight at room temperature.

**Table 5. Gel solutions for 4 - 12% non-denaturating gradient PAA gels**

| Solution | Resolving gel | | Stacking gel |
|---|---|---|---|
| | 4% | 12% | 4 % |
| 30% acrylamide (acrylamide : bisacrylamide - 30 : 0.8) | 4.1 ml | 9.2 ml | 1.3 ml |
| 1 M ε-Aminocapronic acid | 11.5 ml | 11.5 ml | 5 ml |
| 1 M Bis-Tris/HCl pH 7.0 | 1.2 ml | 1.2 ml | 0.5 ml |
| 100% glycerol | - | 4.6 g | - |
| 10% APS | 44 µl | 44 µl | 190 µl |
| TEMED | 11 µl | 11 µl | 19 µl |
| $H_2O$ | 6.65 ml | 1.1 ml | 2,9 ml |

### 2.2.9.5 Immunological detection of proteins

#### 2.2.9.5.1 Transfer of proteins onto nitrocellulose membranes

**Semi-dry blotting system**

Anode buffer I: 0.025 M Tris (no adjustment of pH required)

Anode buffer II: 0.3 M Tris (no adjustment of pH required)

Cathode buffer: 40 mM ε-aminocaproic acid
0.01% (w/v) SDS

The proteins were transferred onto membranes using a semi-dry blotting system (PHASE GmbH, Lübeck). The transfer membranes and PAA gels were incubated for 10 min in anode buffer II prior to transfer. Three layers of Whatman paper were soaked in cathode buffer and placed onto the bottom (cathode part). Then the gel was placed on paper and covered with the transfer membrane. Gel and transfer membrane assembly was covered by two layers of Whatman paper soaked in anode buffer II and three layers of Whatman paper soaked in anode buffer I. The transfer was performed for 1.5 – 2.5 h at 0.8 mA / $cm^2$.

**Wet-blotting system**

| | |
|---|---|
| Blotting buffer: | 0.02 Tris-HCl, pH 8.0 |
| | 0.15 M glycin |

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

**2.2.9.5.2 Staining blots with Ponceau S**

| | | |
|---|---|---|
| Ponceau S Solution: | 0.2% (w/v) | Ponceau S |
| | 1.0% | acetic acid |

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

**2.2.9.5.3 Western analysis using horseradish peroxidase-conjgated antibodies**

| | | |
|---|---|---|
| Blocking buffer | 1 x | PBS |
| | 5% | dry milk |
| | 1% | Tween 20 |
| Washing buffer | 1 x | PBS |
| | 1% | Tween |

Developing stocks

| | |
|---|---|
| Solution 1 | 2.5 mM luminol (in DSMO) |
| | 0.4 mM p-coumaric acid (in DMSO) |
| | 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, nitrocellulose membranes were incubated in blocking buffer for 1 h at room temperature. The antiserum diluted to the desired concentration in blocking buffer was incubated for 2 h at room temperature or overnight at 4°C. The first antibody was removed by washing the membrane with blocking buffer four times for 10 min. Then, anti-rabbit IgG peroxidase conjugate antibodies (Sigma) were diluted in blocking buffer and incubated with the membrane for 1 h. The membrane was then washed four times in washing buffer. The membrane was developed in a mixture of solutions 1 and 2 (1 : 1) by incubation for 1 min. Then the filters were exposed with X-ray films (Hyperfilm, Amersham Life Science, England).

#### 2.2.9.5.4 Western analysis using $I^{125}$-labeled Protein A antibody

The membrane with transferred proteins was incubated with antiserum diluted to the desired concentration in blocking buffer for 1 h at 37°C. The first antibodies were removed by washing the membrane twice with blocking buffer for 15 min. Then, $I^{125}$-labeled Protein A was diluted (1 : 1000) in blocking buffer and incubated with the membrane for 1 h at 37°C. The membrane was then washed once in blocking buffer and once in washing buffer, each time for 15 min. The membrane was dried and the immunoreaction visualized by exposure of the membrane with X-ray films (Hyperfilm; Amersham Life Science, England) for 3 hours or overnight.

### 2.2.10 Proteolysis activity assay

Overexpressed SppA2 protein was added to one milliliter of sodium phosphate buffer (pH 8.0) containing 5 mg of Azocoll protease unspecific substrate with (or without presence) of

different protease inhibitors (for serine type: 1 mM PMSF and 1 mM DFP; for cysteine type: 1 mM IAA; for metalloproteases: 5 mM EDTA, pH 8.0) and incubated with shaking at 37°C. The absorbance was measured at 520 nm and the rate of proteolysis was expressed as the change in absorbance at 520 nm per milliliter.

### 2.2.11 Protein pulse-labeling with $^{35}$S-methionine

The pulse-chase labeling of the cyanobacterial cells and their thylakoids was performed as described in Komenda et al. (2000) with some modifications. To probe the synthesis of cyanobacterial proteins, pulse-labeling with $^{35}$S-methionine (>1000 Ci/mmol, Amersham, Freiburg) was used. Cells were grown in liquid BG-11 medium till $A_{750}$ of 0.3 to 0.6 and then diluted with BG-11 to a chlorophyll concentration 5 μg/ml ($A_{750}$ of 0.4). $^{35}$S-methionine was added to the medium to a final concentration of 1 μCi/ml. The culture was incubated in a 50 ml flask for 40 min under LL and then the cells were transferred to a ML or HL or kept under LL. The reaction was stopped by addition of chloramphenicol (150 μg/ml) an inhibitor of protein synthesis, and placing samples into ice. For long-time labeling, cells were incubated under different stress conditions, and then an aliquot of 10 ml was diluted till $A_{750}$ of 0.7. $^{35}$S-methionine was added to the medium to a final concentration of 1 μCi/ml and cells were incubated for 1 hour.

### 2.2.12 Measurements of oxygen evolution by the Clark electrode

Oxygen evolution of photosynthetic membranes of *Synechocystis* cells was measured with a Clark-type electrode (Hansatech Instruments Ltd, Reutlingen, Germany). The *Synechocystis* cells (at a chlorophyll *a* concentration of 2.5 μg/ml) were adapted to darkness for 10 min, then continuously stirred at 30°C and illuminated with saturating actinic light (50 μE $m^{-2}$ $s^{-1}$). The zero point for $O_2$ synthesis was determined by calculation of the difference between measurements with oxygen-free water [1 ml $H_2O$ + a few crystals of $Na_2S_2O_4$, and oxygen-saturated water (1ml)]. Using constant values of oxygen content in air-saturated water (Seidlell and Linke, 1965), the amount of released $O_2$ was calculated for 1 ml of solution and 1 cm of recorder print. The whole-cell photosynthetic activity was measured for 30 min.

### 2.2.13 Low temperature (77K) fluorescence analysis

77K fluorescence was recorded using SPEX Fluorolog-2 model F212 spectrofluorometer (Industries, Inc., USA). *Synechocystis* samples grown under different light intensities were used. Cells were equilibrated to 2 μg of chlorophyll *a* (approximately $A_{750}$ of 0.5) and adapted to the dark during 10 min. They were then rapidly frozen in liquid nitrogen. For investigation of energy distribution between phycobiliproteins, PSI and PSII emission spectra at excitations of 435 nm and 570 nm were recorded. To monitor changing quantities of pigments excitation spectra were recorded at an emission of 685 nm for phycobiliproteins and 735 nm for chlorophyll *a*.

# 3. RESULTS

## 3.1 Characterization of peptidases in *Synechocystis*

A systematic gene disruption approach was chosen for functional analysis of peptidases from *Synechocystis* sp. PCC 6803. Twenty peptidase-encoding genes were inactivated by inserting the kanamycin or gentamycin resistance gene cassette into the coding region or by replacing part of the respective coding regions with the antibiotic resistance genes. The mutant strains deficient in protease genes were obtained in collaboration with the laboratory of Prof. S. Shestakov (Department of Genetics, Moscow State University, Russia). The mutagenized genes are presented in the Table 1 of Section 2.2.3. The homozygous mutant strains in which all wild-type gene copies were replaced by the knock-out cassette were achieved after several rounds of selection with antibiotic (approximately 12 rounds of plating). Complete segregation of mutant lines was verified by a PCR approach that allows the detection of even a low number of the wild-type DNA copies. Description of the interrupted genes and their products is presented in the Table 6.

**TABLE 6. Characterization of mutagenized peptidase-encoding genes and their products in *Synechocystis***

| *Gene* | Protein | Protein function | Peptidase type | Predicted localization | Homozygous mutant |
|---|---|---|---|---|---|
| *slr0542* | ClpP1 | Proteolytic Clp subunit | Serine | Cyt | No |
| *sll0534* | ClpP2 | Proteolytic Clp subunit | Serine | Cyt | Yes |
| *slr0165* | ClpP3 | Proteolytic Clp subunit | Serine | Cyt | No |
| *slr0164* | ClpR (ClpP4) | Proteolytic Clp subunit | Serine | Cyt | No |
| *sll0535* | ClpX | Non-peptidase Clp subunit | - | Cyt | No |
| *sll0020* | ClpC | Non-peptidase Clp subunit | - | Cyt | No |
| *slr0156* | ClpB1 | ClpB1 protein | - | Cyt | No |
| *slr1641* | ClpB2 | ClpB2 protein | - | Cyt | Yes |
| *sll1204* | HtrA (DegP) | Heat-shock peptidase | Serine | L | No |
| *sll1679* | HhoA (DegQ) | Heat-shock peptidase | Serine | Per | Yes |
| *sll1427* | HhoB (DegS) | Heat-shock peptidase | Serine | Mem | Yes |
| *sll2008* | Prp1 | Processing peptidase | Metallo- | Cyt | Yes |
| *sll2009* | Prp2 | Processing peptidase | Metallo- | Cyt | Yes |
| *sll0055* | Prp3 | Processing peptidase | Metallo- | Cyt | Yes |
| *slr1331* | YmxG | Processing peptidase | Metallo- | Per | Yes |
| *sll0915* | PqqE | Processing peptidase | Metallo- | Per | Yes |
| *sll1703* | SppA1 | Protease IV | Serine | Mem | Yes |
| *slr0021* | SppA2 | Protease IV | Serine | Cyt | Yes |
| *sll1343* | Ape2 (PepN) | Alanine AP | Metallo- | Cyt | Yes |
| *sll0136* | PepP | Proline AP | Metallo- | Cyt | Yes |

*AP* aminopeptidase, *Cyt* cytoplasm, *Mem* membrane, *Per* periplasm, *L* lumen

Only completely segregated homozygous strains were used for functional analyses. The interposon mutagenesis approach did not recover fully segregated knock-out strains for seven genes including predominantly members of the Clp family (Table 5). Only one mutant strain, Δ*clpP2*, that was not able to grow under photoautotrophic conditions, but could be cultivated in the presence of a carbon source (0.5% glucose), segregated fully.

### 3.1.1 Physiological analysis of peptidase knock-out mutant strains

One of the principal roles of peptidases is the regulation of intracellular processes during or following environmental stress. All peptidase mutants obtained were monitored for their acclimation abilities to various light intensities, temperature regimes and nutrient deprivations. The phenotypical changes were compared with there of wild-type grown under comparable conditions. Peptidase-deficient strains and wild-type were grown under standard conditions till $A_{750}$ = 0.6 and then transferred to different stress regimes (see Section 2.2.2). Results of the phenotypical analysis of mutant strains under different stress conditions are summarized in the Table 7.

**TABLE 7. Characterization of peptidase knock-out mutants**

| Gene | Mutant | Photoautotrophic growth | Visible phenotype | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Growth conditions | | | Nutrient deprivation | | | | |
| | | | LL | HL | HS | -N | -S | -P | -Fe | -Cu |
| Clp family | | | | | | | | | | |
| *sll0534* | Δ*clpP2* | No | B/SS | - | - | -/F | | -/F | -/F | - |
| *slr1641* | Δ*clpB2* | Yes | - | G | - | - | - | - | - | - |
| Deg family | | | | | | | | | | |
| *sll1679* | Δ*hhoA* | Yes | - | - | B | - | - | - | G | - |
| *sll1427* | Δ*hhoB* | Yes | G/F | - | - | - | - | B | - | - |
| Processing peptidases | | | | | | | | | | |
| *sll2008* | Δ*prp1* | Yes | G/F | - | - | - | - | - | - | - |
| *sll2009* | Δ*prp2* | Yes | - | - | B | - | - | - | - | - |
| *sll0055* | Δ*prp3* | Yes | - | - | - | - | - | - | - | - |
| *slr1331* | Δ*ymxG* | Yes | G/F | - | - | - | - | - | - | - |
| *sll0915* | Δ*pqqE* | Yes | - | - | - | - | - | - | - | - |
| *sll1703* | Δ*sppA1* | Yes | -/F | B | - | - | - | - | - | - |
| *slr0021* | Δ*sppA2* | Yes | -/SS | - | - | - | - | - | - | B |
| Aminopeptidases | | | | | | | | | | |
| *sll1343* | Δ*ape2* | Yes | - | - | - | - | - | - | G | B |
| *sll0136* | Δ*pepP* | Yes | B/SS | B | B | - | - | - | - | - |

*B* Bleached phenotype, *Cu* copper, *F* faster growth rate, *Fe* iron, *G* enhanced green pigmentation, *HL* high light *HS* heat stress (42°C), *LL* low light, *N* nitrogen, *P* phosphorus, *S* sulfur, *SS* slower growth rate, - no visible phenotypical modifications

### 3.1.1.1 Acclimation of cyanobacterial cells to different light intensities

To analyse the acclimation of wild-type and mutant strains to different light conditions cyanobacterial cells were grown under LL and then transferred to HL for three days. The response of the wild-type cells to HL was bleaching of the cell culture and increased time of cell duplication. Under standard experimental conditions (LL; 30°C) wild-type strain of *Synechocystis* has a doubling time of about 12 h, while during adaptation to HL the doubling time increased up to 28 - 30 h. The phenotypical changes demonstrated that four peptidases, ClpP2, SppA1, SppA2 and PepP contributed to light acclimation since the corresponding knock-out mutants demonstrated an altered pigmentation phenotype for Δ*sppA1*, Δ*sppA2* and Δ*pepP* or ceased growth for Δ*clpP2* upon transfer to different light intensities (Fig. 3). The Δ*clpP2* mutant was highly sensitive to light and was not able to grow under light intensities higher than 10 μE $m^{-2}$ $s^{-1}$ (Fig. 3A and B). This mutant showed a strong phenotype typical for photosynthetic mutants and it could grow only under heterotrophic conditions (Fig. 3A and B). The Δ*pepP* strain already showed some bleaching phenotype and slower growth rate under LL regime (Fig. 3C). The same sensitive phenotype (slower growth rate and bleaching) under standard light intensity showed Δ*sppA2* mutant (Fig. 3D; see Section 3.3 below). No significant changes were observed phenotypically in the Δ*sppA2* after exposure to HL. The Δ*sppA1* mutant bleached faster than the wild-type after transfer to HL and this phenotype was accompanied by a drastic drop in the growth rate (Fig. 3E).

### 3.1.1.2 Heat stress

To examine the behaviour of wild-type and peptidase knock-out strains under high temperature the cells were acclimated from standard temperature regime (30°C) to heat stress (42°C) for 48 h. Incubation of wild-type cells under 42°C for 6 h led to a bleached phenotype that correspond to in reduced of chlorophyll and phycobiliprotein contents (data not shown). The analysis of phenotypical changes and absorption spectra under heat stress showed that most of the peptidase strains, with the exception of three mutants, Δ*pepP*, Δ*prp2* and Δ*hhoA,* displayed similar responses to heat stress as the wild-type. Three knock-out strains, Δ*pepP*, Δ*prp2* and Δ*hhoA*, were more sensitive to heat stress and bleached rapidly during acclimation to 42°C (Fig. 4).

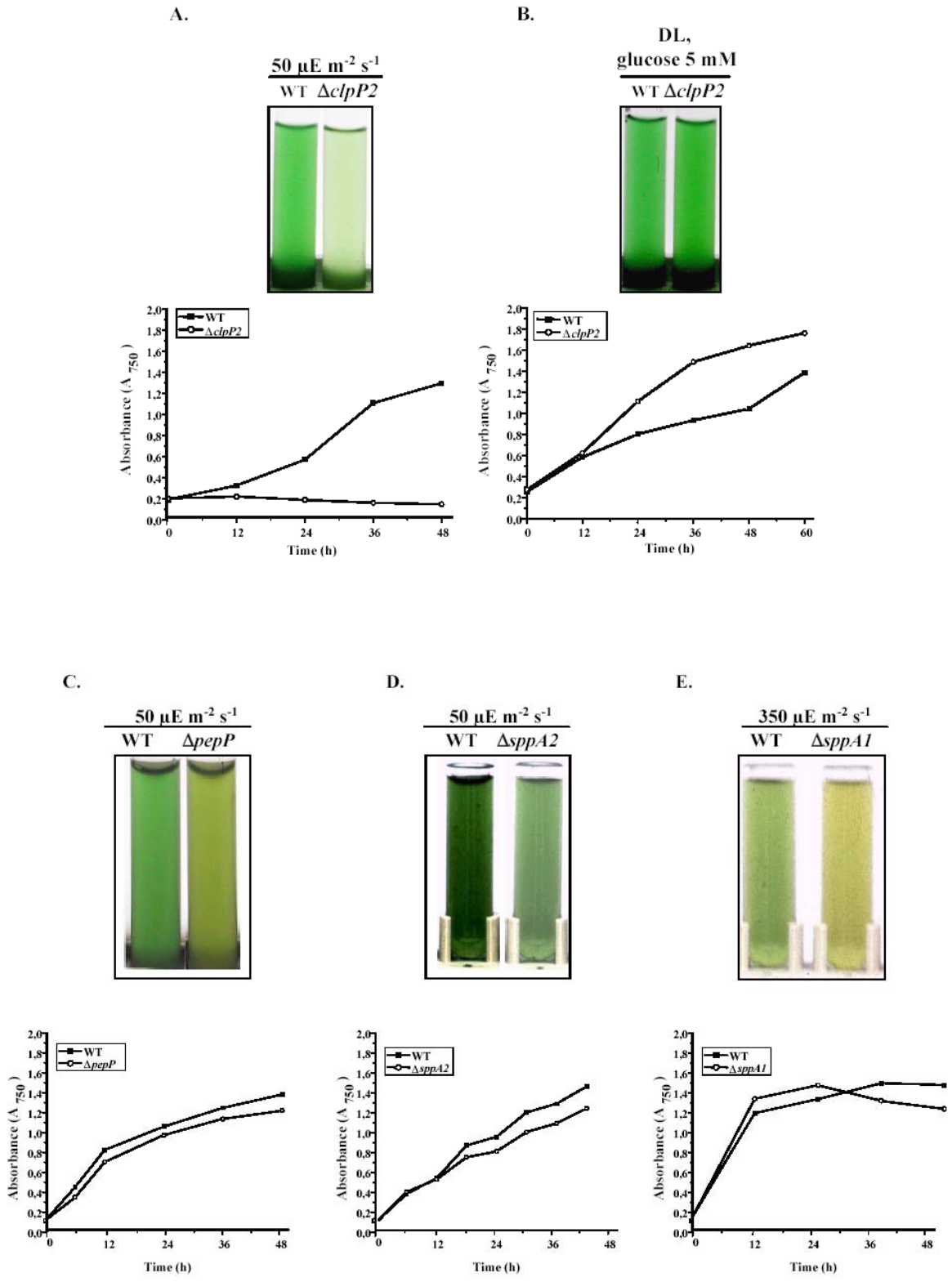


**Figure 3. Analysis of the phenotypical modifications and growth rates of the wild-type and mutant strains under different light regimes.** The wild-type and mutant knock-out strains were grown under LL and then transferred to HL or left at LL. The changes were monitored for 50 h during acclimation to LL for Δ*clpP2* (A and B), Δ*pepP* (C) and Δ*sppA2* (D) and to HL regime for Δ*sppA1* (E).

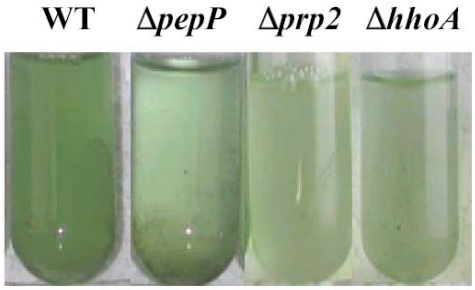


**Figure 4. Phenotypical modification of the wild-type as well as Δ*pepP*, Δ*prp2* and Δ*hhoA* strains under heat stress.** Wild-type and mutant strains were grown under 30°C to an $A_{750}$ of 0.6 and transferred to 42°C. The phenotypical changes were recorded 24 h after heat stress.

### 3.1.1.3 Nutrient deprivation

To test the influence of nutrient deprivations on the behaviour of wild-type and peptidase knock-out mutants the strains were shifted to medium depleted in nitrogen, iron, sulfate, phosphorus or copper. Cells at the exponential growth phase were collected, resuspended in media deficient in different microelements and grown under standard conditions for 3 to 10 days. Wild-type cells grown in the absence of nitrogen, phosphorus or iron showed a bleaching phenotype after 3 days that corresponds to decline in the content of phycobiliproteins. However, the cells grown on sulfur or copper-lacking medium did not exhibit any significant changes in the pigmentation. The Δ*hhoB* mutant bleached more rapidly than the wild-type when grown in a medium depleted in phosphorus, whereas Δ*hhoA* and Δ*ape2* remained more green than the wild-type under iron deprivation. The Δ*sppA2* and Δ*ape2* mutant strains bleached during growth in the absence of copper (Fig. 5).

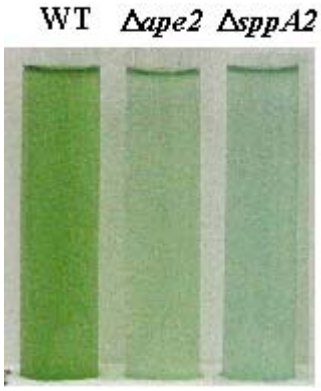


**Figure 5. Response of the wild-type, Δ*ape2* and Δ*sppA2* strains under copper deprivation.** The wild-type and mutant strains were grown under normal conditions in a medium depleted in copper for 10 days.

The analysis of growth of the Δ*clpP2* under nutrient deprivation was difficult to perform since the mutant was only able to grow in the presence of glucose (Fig. 3A and B). It was previously observed that the presence of glucose in the growth medium partially inhibits phycobilisome degradation which is the first response of deprivation of nitrogen or

phosphorus microelements in *Synechocystis*. In that case the growth rate of Δ*clpP2*, in addition to the pigmentation of the cell culture, was monitored (**Fig. 6**). The Δ*clpP2* mutant grew faster than the wild-type during the first two days of nitrogen, phosphorus or iron starvation. However, no significant difference in growth rate to the wild-type could be noted during sulfur starvation (Fig. 7).

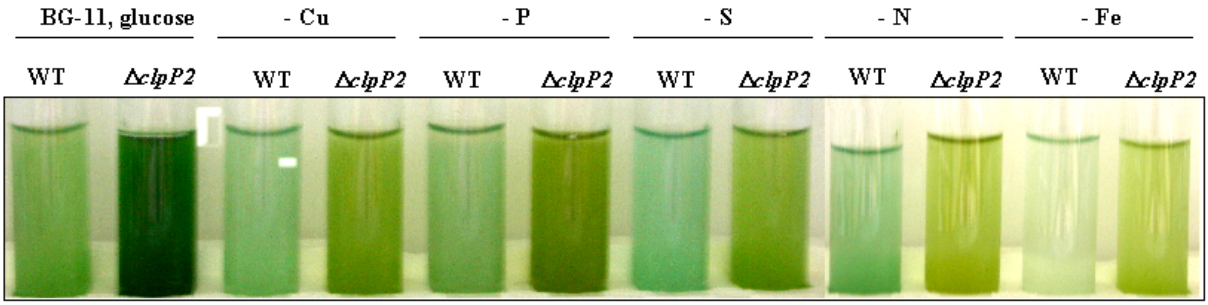


**Figure 6. Phenotypical changes of the wild-type and Δ*clpP2* mutant during nutrient deprivations.** Wild-type and Δ*clpP2* mutant were grown in the glucose-containing BG-11 medium to an $A_{750}$ of 0.6 and transferred for 72 hours to the media depleted in copper (- Cu), iron (- Fe), nitrogen (- N), phosphorus (- P) or sulfur (- S).

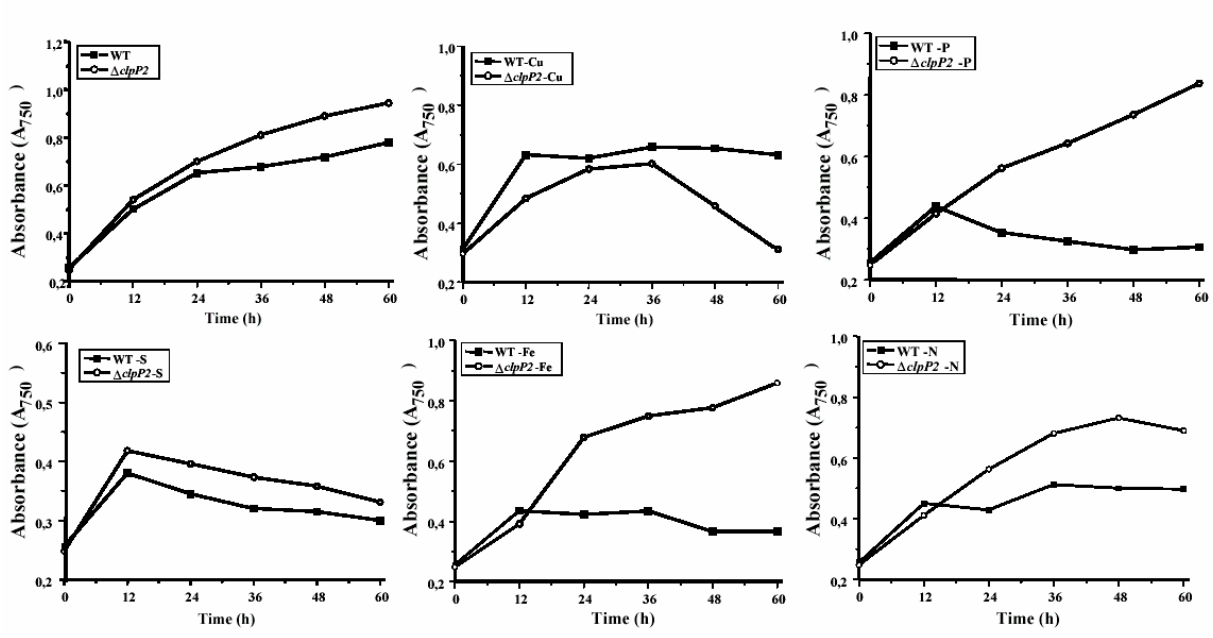


**Figure 7. Growth rates of the wild-type and Δ*clpP2* mutant during nutrient deprivation**. The cultures were normalized to an $A_{750}$ and transferred to the media lacking copper (-Cu), iron (-Fe), nitrogen (-N), phosphorus (-P) or sulfur (-S). Afterwards the strains were incubated for 72 h. The absorbance at $A_{750}$ was measured every 12 h.

### 3.2 Functional analysis of the *SppA* peptidase family in *Synechocystis*

BLAST search analysis revealed two homologous SppA proteins in the genome of *Synechocystis*. The *sll1703* gene encodes an *Arabidopsis* homologue of the 64 kDa thylakoid membrane-associated SppA protease (Lensch et al., 2001). The *slr0021* gene encodes a second SppA-homologue, the SppA2 protein, with a predicted molecular mass of 30 kDa. This gene is present only on prokaryotic genomes. Analysis of the domain structure of SppA proteins based on **p**rotein **fam**ilies database (Pfam) indicated that SppA1 consists of two homologous domains separated by an interdomain region (Fig. 8), whereas SppA2 contains only one domain in its protein structure.

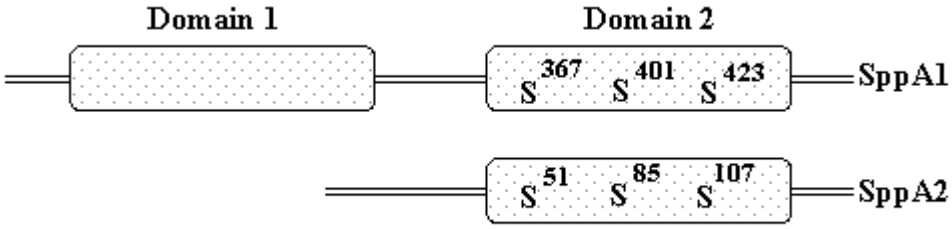


**Figure 8. Domain organization of SppA proteases.** Possible catalytic serine residues in domain 2 are indicated.

SppA proteases belong to the serine type of proteases that possess serines as catalytically active residues. Sequence comparison of SppA proteases from *E. coli*, *Arabidopsis* and *Synechocystis* strongly suggested the localization of catalytic serines within domain 2 (Lensch et al., 2001) at positions $Ser^{367}$, $Ser^{401}$ and $Ser^{423}$ for SppA1 protease and at positions $Ser^{51}$, $Ser^{85}$ and $Ser^{107}$ for the SppA2 protease of *Synechocystis* (Figs. 8 and 9).

#### 3.2.1 Functional analysis of SppA1 protease

##### 3.2.1.1 Analysis of SppA1 protein sequence

Hydrophathy analysis according to Kyte & Doolittle revealed a SppA1 is a hydrophilic protein with a hydrophobic domain of about 50 amino acids at the N-terminus. Inspection revealed a typical ARA sequence for the signal processing peptidase of position of 39 amino acids at the N-terminus. The *Arabidopsis* SppA and the *Synechocystis* SppA1 sequences

contain two possible membrane domains (I and II), predicting association of SppA1 with the thylakoid membrane (Fig. 10).

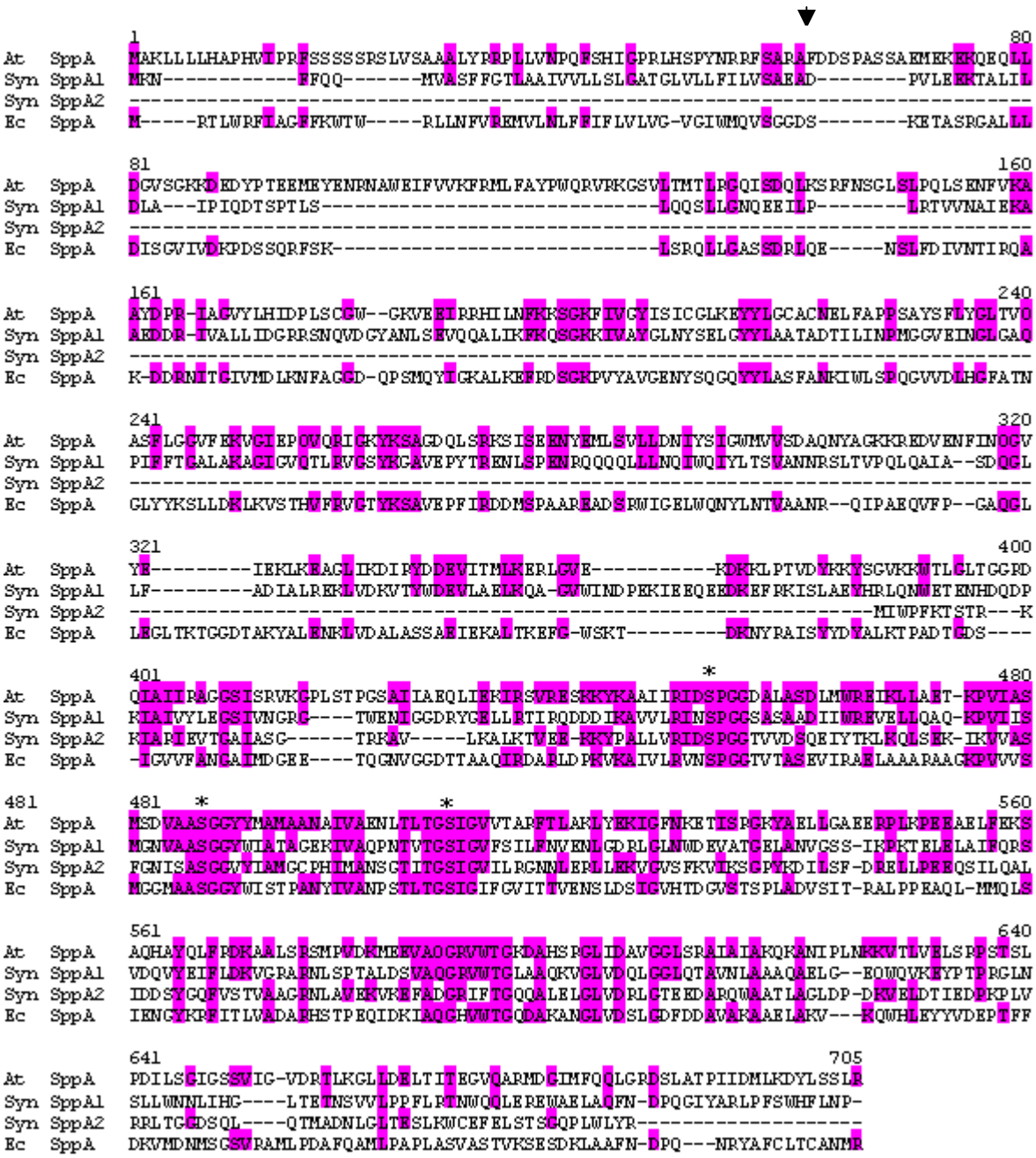

**Figure 9. Amino acid comparison of SppA proteins from different organisms.** Identical amino acid residues are shadowed in red. **At** – *Arabidopsis thaliana*, **Syn** – *Synechocystis*, **Ec** – *Escherichia coli*. Putative catalytic active serine residues are designated by stars. The arrow indicates position of putative processing site.

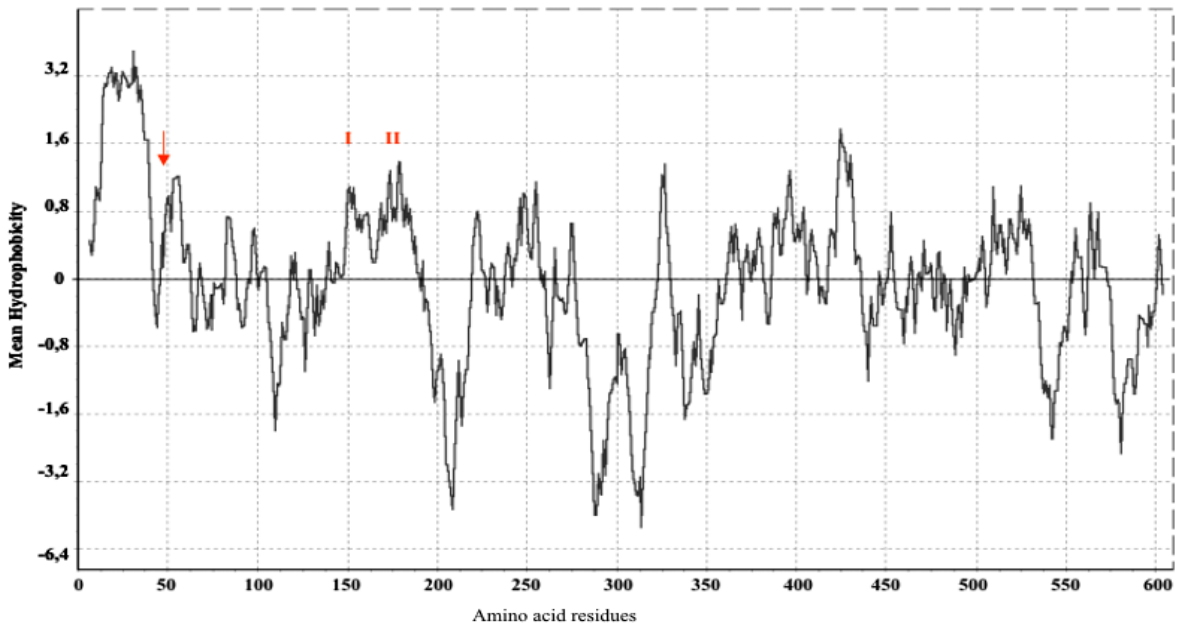


**Figure 10. Hydropathy plot of the *Synechocystis* SppA1 protein according to Kyte and Doolittle (1982).** Amino acid positions are indicated at the bottom of the graph. The scan window size was 13. Hydrophobic amino acid residues are placed above the zero line. The possible cleavage site for transit peptide marked by red arrow. Two hydrophobic domains are designated I and II.

### 3.2.1.2 Construction of Δ*sppA1* and complement *pVZsppA1* mutant strains

The *sll1703* gene encoding the SppA1 peptidase was inactivated by interposon mutagenesis in *Synechocystis* (Fig. 11). Mutagenesis of relevant open reading frame can influence the expression of nearby located genes. It that case the phenotype of a mutant strains can be caused either by depletion of the gene of interest or by site effect on expression of nearby located genes. To exclude the pleiotropic effect of gene disruption and to prove that the phenotype corresponds exclusively to the disrupted gene, a complementation analysis of the Δ*sppA1* mutant strain by an autonomously replicating plasmid pVZ321 carrying the wild-type *sppA1* gene (Fig. 12) was performed. The pVZ321 plasmid based on RSF1010 replicon is able to autonomously replicate in cyanobacterial cells. Conjugal transfer of resultant pVZsppA1 plasmid into the Δ*sppA1* strain was performed *via* triparental mating. Mobilization of plasmid pVZsppA1 into *Synechocystis* was performed with the *E. coli* strain R751. The transconjugants were selected on the BG11-containing plates with 25 μg $ml^{-1}$ chloramphenicol and 100 μg $ml^{-1}$ kanamycin.

During insertion of self replicating resistance cassette the gene of interest can be independently expressed from the vector but can as well be integrated into genomic DNA by reverse recombination. The PCR approach was used to exclude possible reverse recombination between *Synechocystis* chromosomal DNA and the gene fragment cloned in the complementation plasmid. For this analysis, primers corresponding to further sequences upstream and downstream of PrAF and PrAR for amplification of entire coding region *sll1703* were constructed. These primers cannot amplify a fragment on plasmid pVZsppA1, but amplify a fragment with an identical size from the DNA of Δ*sppA1* strain. The DNAs from wild-type, Δ*sppA1* and *pVZsppA1* strains were used for PCR analysis. The size of the amplified fragment differs by 1.2 kb that corresponds to the *Km* resistance gene from the amplification product of the wild-type DNA (Fig. 13). The wild-type fragment appears as a 3.4 kb band, whereas Δ*sppA1* and *pVZsppA1* fragments share the same size of 4.6 kb due to a 1.2 kb *Km* resistance gene.

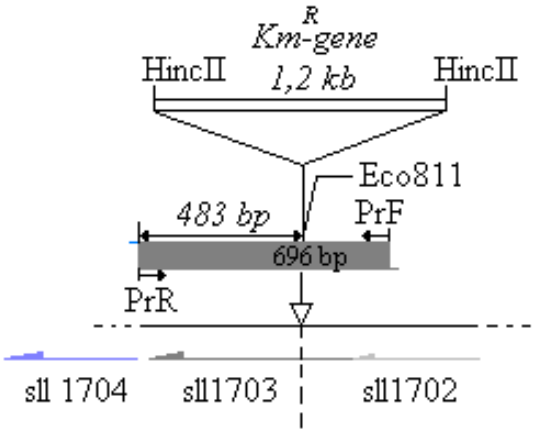


**Figure 11. Construction of the knock out vector for the *sll1703* gene.** The *sll1703* gene encoding SppA1 protease was amplified from genomic DNA of *Synechocystis* with primers PrF and PrR. The resulting PCR product of 696 bp was cloned into the PCR-cloning vector pGEM-T (Promega). The 1.2 kb $Km^R$ cassette excised by HincII from the pUC4K plasmid was inserted into the Eco81I restriction site of *sll1703* gene.

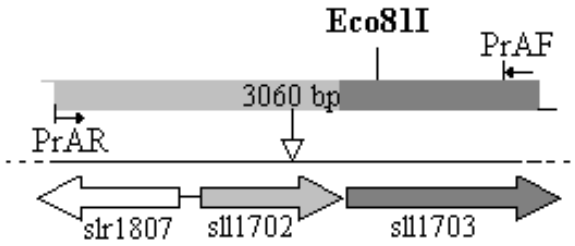


**Figure 12. Construct scheme of the vector for the complementation analysis of the Δ*sppA1* mutant.** For complementation analysis of the *sppA1* gene the 3060 bp fragment, encoding the entire *sll1702/sll1703* region and part of *sll1807* gene was amplified with primers PrAF and PrAR and cloned into the pGEM-T vector. The PCR product was excised from the vector with PvuII and recloned into the unique SmaI site within the $Km^R$ gene of vector pVZ321.

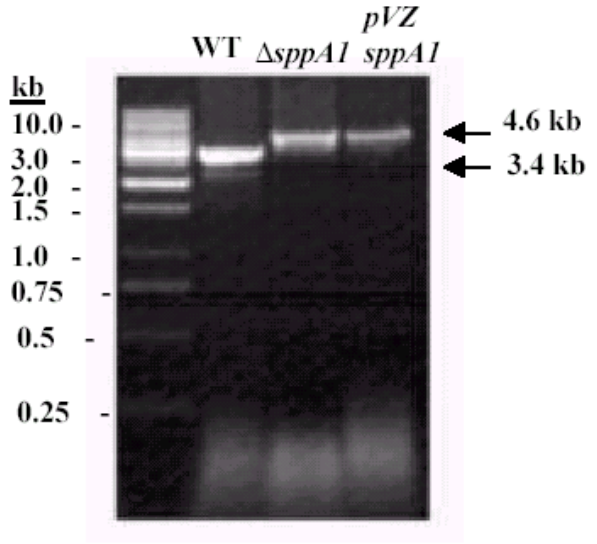


**Figure 13. PCR analysis with DNA from wild-type, Δ*sppA1* and *pVZsppA1* strains to check for possible reverse recombination.** The part of the DNA containing *sll1703/sll1702* and *slr1807* was amplified using sll1704_frw and slr1807_compl_rev primers and the resulting PCR products were separated electrophoretically in a 0.8% agarose gel.

### 3.2.1.3 Analysis of *sppA1* gene

Analysis of the DNA sequence showed that the 1860 bp *sll1703* gene is a member of a gene cluster, encoding *sll1702-sll1703-sll1704*, where the *sll1702* and *sll1703* have four overlapping nucleotides in their termination and starting codons, respectively. Gene s*ll1704* (770 bp) that encodes a dehydrogenase chain, is located downstream of *sll1703* with an intergenic region of 24 bp (Fig. 14).

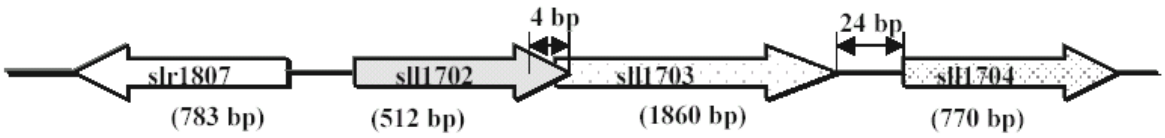


**Figure 14. Physical map of the chromosome region containing the *sppA1* gene.** The *sll1703/sll1702* genes are located in a gene cluster with an overlapping region of 4 bp. The s*ll1704* gene is located down-stream of the *sll1703* gene at a distance 24 bp from the stop codon of *sll1703*.

To check a possible polar effect of disruption of the *sll1703* gene on the expression of the *sll1704* a Northern experiment with RNA from wild-type, Δ*sppA1* and complemented mutant was performed. The RNAs were hybridized with a *sll1704* gene-specific probe (Fig. 15). The *sll1704* transcript of 0.85 kb was found in comparable quantity in all strains.

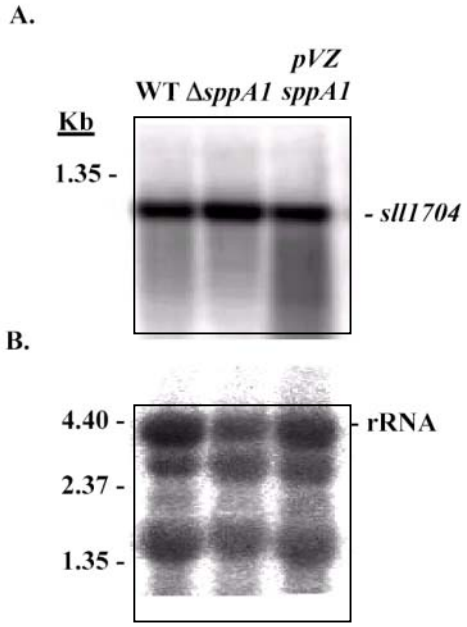


**Figure 15. Analysis of *sll1704* gene expression.** RNA was extracted from cyanobacteria cells of wild-type, Δ*sppA1* and *pVZsppA1* strains grown under LL for 5 days till end of the exponential phase ($A_{750}$ = 1.5), separated in an RNA-denaturing gel and transferred onto Nylon membrane. (**A**) The membrane was hybridized with the radiolabelled *sll1704* gene fragment of 770 bp. (**B**) Hybridization with rRNA was used as a loading control.

Since *sll1702* and *sll1703* are located in one gene cluster two different primer combinations were used to detect the *sll1702* transcript (512 bp) levels in wild-type, Δ*sppA1* and *pVZsppA1*. The sll1702F/sll1703R primer pair was used for the determination of *sll1702/sll1703* transcript of 2.5 kb and *sll1702* gene-specific primers for the detection of only that of *sll1702* gene. RT-PCR did not reveal any transcript of *sll1702/sll1703* that indicated independent transcription of *sll1702* and *sll1703* genes. RT-PCR analysis of expression of *sll1702* demonstrated that its expression in the wild-type and Δ*sppA1* mutant was similar. Transcription of the 16S rRNA was used as a control (Fig. 16).

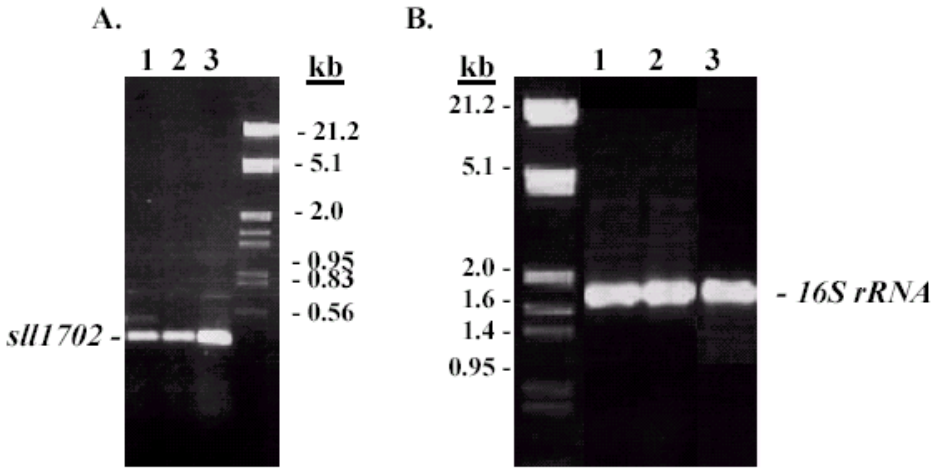


**Figure 16. RT-PCR analysis of the *sll1702* gene.** Total RNA was extracted from wild-type (lane 1), Δ*sppA1* (lane 2) and *pVZsppA1* (lane 3). PCR with *sll1702* - specific primers was performed for amplification of the *sll1702* transcript (**Panel A)** and with primers specific to *16S rRNA* gene for amplification of the 16S rRNA (**Panel B).**

### 3.2.1.4 Expression of the *sppA1* gene under different light intensities

The analysis of the Δ*sppA1* mutant (see Section 3.1.1.1) indicated possible involvement of SppA1 in the acclimation of cyanobacterial cells to higher light intensities. The light-dependent regulation of SppA1 was first analyzed at the gene expression level. For this, RNA was isolated from wild-type cells adapted to different light intensities, such as LL, ML and HL. The *sppA1* transcripts were then detected by Northern analysis. S*ppA1* transcripts were present under all light regimes in almost equal amounts (Fig. 17).

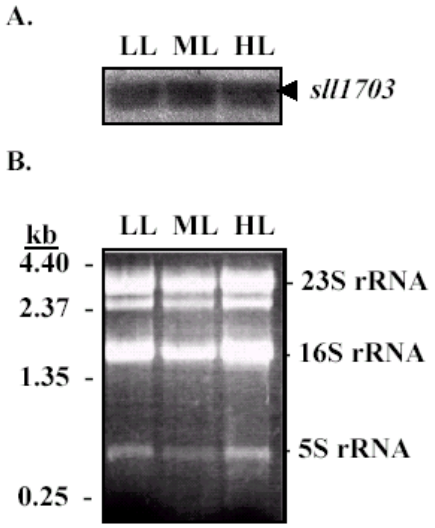


**Figure 17. Transcription levels of the *sll1703* (*sppA1*) gene in the wild-type under different light regimes.** (**A**) RNA was extracted from the cells grown under LL, ML and HL regimes. The *sppA1* transcripts were detected by hybridization with the radiolabelled 1860 bp DNA fragment of the *sll1703* gene. (**B**) The RNA gel was stained with EtBr for rRNA visualization, as a control for equal loading.

### 3.2.1.5 Phenotypical characterization of the Δ*sppA1* mutant

Physiological characterization of the Δ*sppA1* mutant did not show any significant difference in the growth under LL when compare to wild-type (see Section 3.1). To analyze the phenotypical modifications of wild-type, Δ*sppA1* and *pVZsppA1* due to different light intensities the cell batches grown at LL to the end of the exponential phase were diluted to an $A_{750}$ of 0.5 and then transferred to ML and HL or kept at LL for further three days. In contrast to the LL Δ*sppA1* mutant behaved differently from the wild-type when the cells were grown at ML and HL. The Δ*sppA1* mutant exhibited non-bleaching after the shift to ML and bleached faster under HL relative to the wild-type cells (Fig. 18).

Typical absorption spectra of the wild-type cells display a broad absorption peak with several shoulders that correspond to the Soret region of the chlorophyll *a* absorption spectrum (440

nm) and to the absorption of various carotenoid bands (above 450 nm). The red region displays two distinct peaks, one centered around 620 nm due to phycobilin-containing proteins, phycocyanin (PC) and allophycocyanin (APC), the other around 680 nm corresponding to chlorophyll *a*. The absorption spectra of thylakoids extracted from wild-type and mutant strains grown at various light conditions showed that the amounts of phycobilisomes as well as of chlorophyll *a* and carotenoid decreased within 3 – 4 days after transfer of *Synechocystis* cells from LL light to HL (Fig. 19). Under ML and HL the wild-type and *pVZsppA1* strains revealed lower absorbance of the PC peak than at LL. The Δ*sppA1* strain behaved in a similar way at LL and HL. However, although a decrease in chlorophyll *a* absorption relative to that of carotenoids was observed, the PC/APC absorption peak at 620 nm remained higher in Δ*sppA1* under ML. Consequently, the ratio between the chlorophyll *a* and PC/APC absorption peaks in mutant cells grown under ML remained similar to that grown under LL. These observations suggest that the wild-type adapts to ML by losing more of its PC/APC-containing phycobiliproteins than Δ*sppA1*.

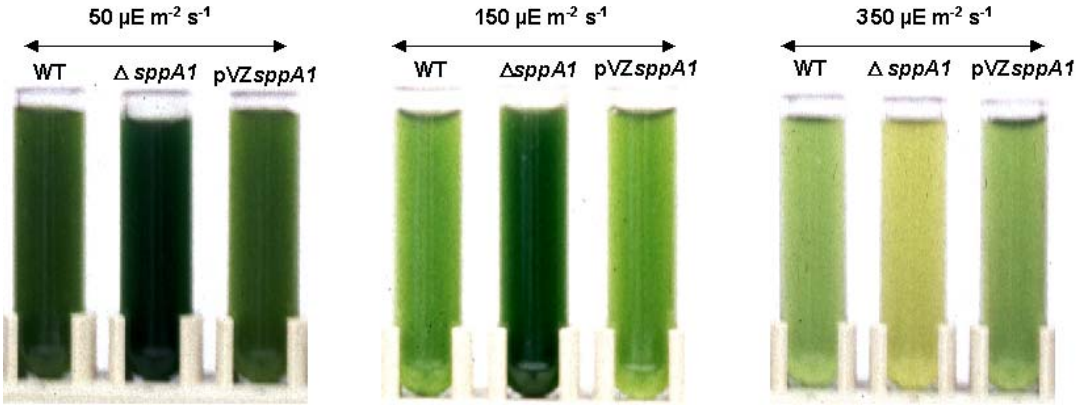


**Figure 18**. **Phenotypical characterization of wild-type, Δ*sppA1* and *pVZsppA1* strains upon acclimation to various light intensities.** Wild-type, Δ*sppA1* and *pVZsppA1* were incubated under LL to an $A_{750}$ of 0.6 and then transferred to LL, ML or HL for 3 days. The phenotypical changes were monitored after 3 days of light acclimation.

Table 8 shows the generation time of wild-type and Δ*sppA1* mutant at three light intensities, DL, LL and ML. It has been observed that cell division was light-limited in the 20 - 50 µE $m^{-2}$ $s^{-1}$ range. The latter intensity was close to saturation since a further increase in light intensity by a factor of three produced only a moderate increase in growth rates for the two strains. That these growth conditions were not limited by $CO_2$ availability is demonstrated by similar generation times observed, independently whether cultures were bubbled with $CO_2$or not.

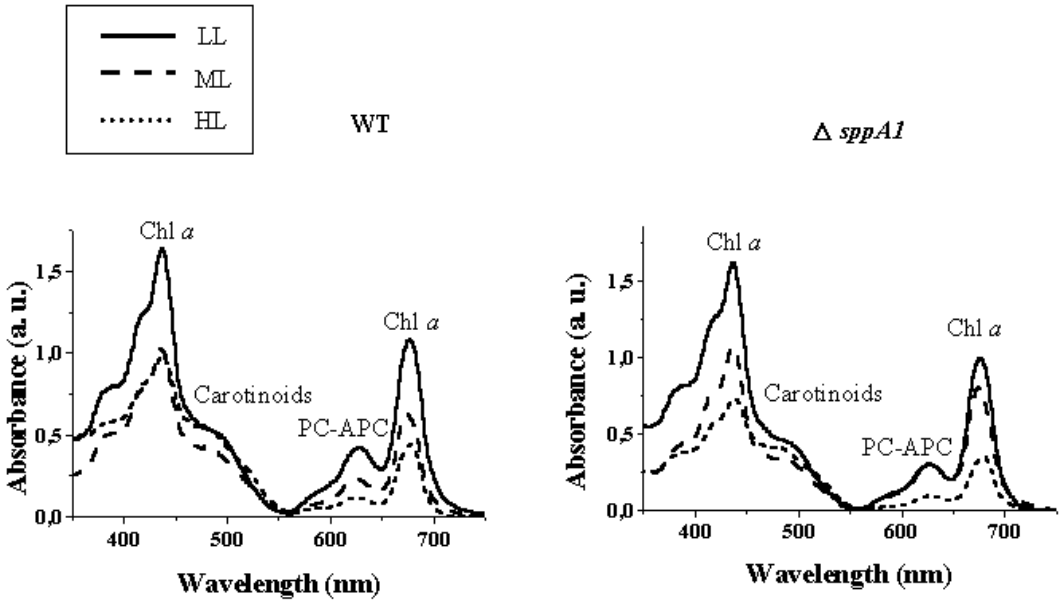


**Figure 19. Absorbance spectra of thylakoids of the wild-type, Δ*sppA1* and *pVZsppA1* during acclimation to different light regimes.** *Synechocystis* cells were grown at LL to an $A_{750}$ of 0.5. Cells were then transferred to ML or HL, or kept at LL for the next three days. Thylakoid membranes were isolated from cultures and adjusted to the same $A_{750}$ before determining the spectra.

**TABLE 8. Doubling time of wild-type and Δ*sppA1* mutant at different light intensities**

| Strain and light intensity | Doubling time (h) | Cell concentration (number/ml) at $T_{72}$[a] |
|---|---|---|
| wild-type (DL; 20 μE $m^{-2}$ $s^{-1}$) | 36.3 ± 3.0 | 8.0 ± 0.3 x $10^8$ |
| Δ*sppA1* (DL; 20 μE $m^{-2}$ $s^{-1}$) | 35.6 ± 1.5 | 8.4 ± 0.4 x $10^8$ |
| wild-type (LL; 50 μE $m^{-2}$ $s^{-1}$) | 11.4 ± 0.9 | 26.0 ± 0.2 x $10^8$ |
| Δ*sppA1* (LL; 50 μE $m^{-2}$ $s^{-1}$) | 10.5 ± 1.8 | 29.5 ± 0.3 x $10^8$ |
| wild-type (ML; 150 μE $m^{-2}$ $s^{-1}$) | 10.0 ± 1.7 | 40.1 ± 0.1 x $10^8$ |
| Δ*sppA1* (ML; 150 μE $m^{-2}$ $s^{-1}$) | 9.8 ± 0.8 | 44.5 ± 0.3 x $10^8$ |
| wild-type (HL; 350 μE $m^{-2}$ $s^{-1}$) | 40.0 ± 0.7 | 17.6 ± 0.8 x $10^8$ |
| Δ*sppA1* (HL; 350 μE $m^{-2}$ $s^{-1}$) | 36.8 ± 0.2 | 24.5 ± 0.2 x $10^8$ |
| wild-type (3% $CO_2$; 50 μE $m^{-2}$ $s^{-1}$) | 12.0 ± 0.3 | 21.7 ± 0.4 x $10^8$ |
| Δ*sppA1* (3% $CO_2$; 50 μE $m^{-2}$ $s^{-1}$) | 11.1 ± 0.8 | 26.1 ± 0.7 x$10^8$ |
| wild-type (3% $CO_2$; 150 μE $m^{-2}$ $s^{-1}$) | 10.3 ± 1.2 | 38.7 ± 0.2 x $10^8$ |
| Δ*sppA1* (3% $CO_2$; 150 μE $m^{-2}$ $s^{-1}$) | 9.1 ± 1.1 | 44.0 ± 0.1 x $10^8$ |

[a] $T_{72}$ - time of cell growth under indicated conditions; values listed are means from at least three experiments

For the analysis of pigment contents in wild-type and Δ*sppA1* strains during adaptation to different light regimes cyanobacterial cells were acclimated to LL, ML and HL for 3 days and the concentration of phycocyanin and chlorophyll *a* was measured for 3 days every 12 h. Measurement of the pigment concentrations per optical density of cells ($A_{750}$ = 0.4) showed that the PC pool in the wild-type decreases when cells are adapted to ML and HL, in the Δ*sppA1* strain its amount remained constant under ML as under LL. Under HL both strains, wild-type and Δ*sppA1*, contained almost equal amount of PC (Fig. 20).

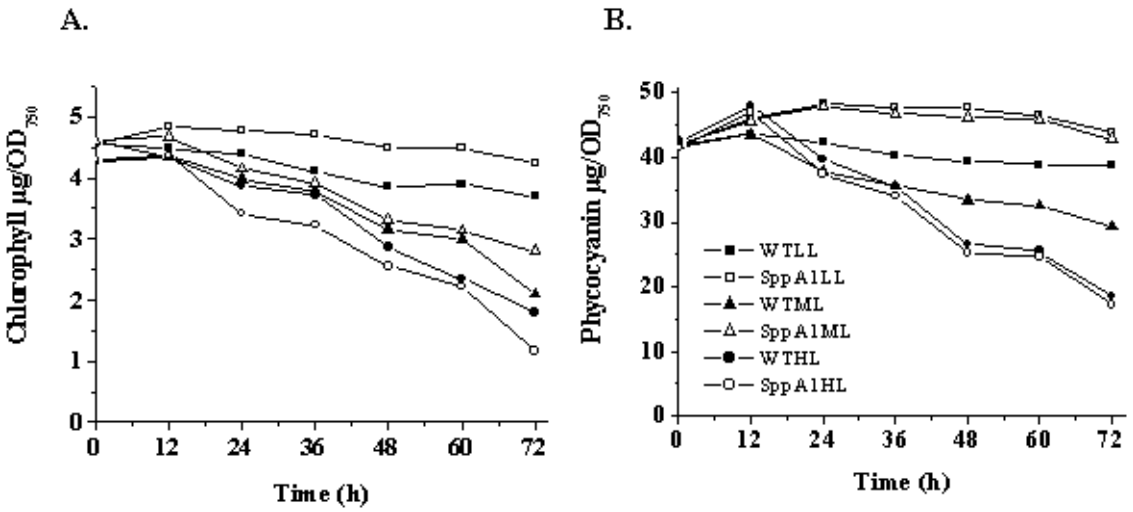


**Figure 20. Contents of chlorophyll and phycobiliproteins in wild-type and Δ*sppA1* cells under different light regimes.** *Synechocystis* cells were grown at LL till log-phase, diluted with BG-11 medium and transferred to LL, ML and HL for 3 days. The measurements were taken from the beginning of acclimation. Chlorophyll (A) and phycocyanin (B) concentrations at $A_{750}$ of 0.4 were measured.

The loss of phycobiliproteins in *Synechocystis* wild-type is well documented under nitrogen deprivation. To check whether SppA1 inactivation also resulted in the preservation of PBS under these conditions, wild-type and Δ*sppA1* were grown in nitrogen-depleted medium for 3 days (Fig. 21). The loss of PBS was visualized by the loss of the PC/APC absorbance peak in the 620 nm region of the spectrum. The same decrease in PC/APC absorbance was observed in both strains showing that SppA1 plays no role in the PBS loss under nitrogen starvation.

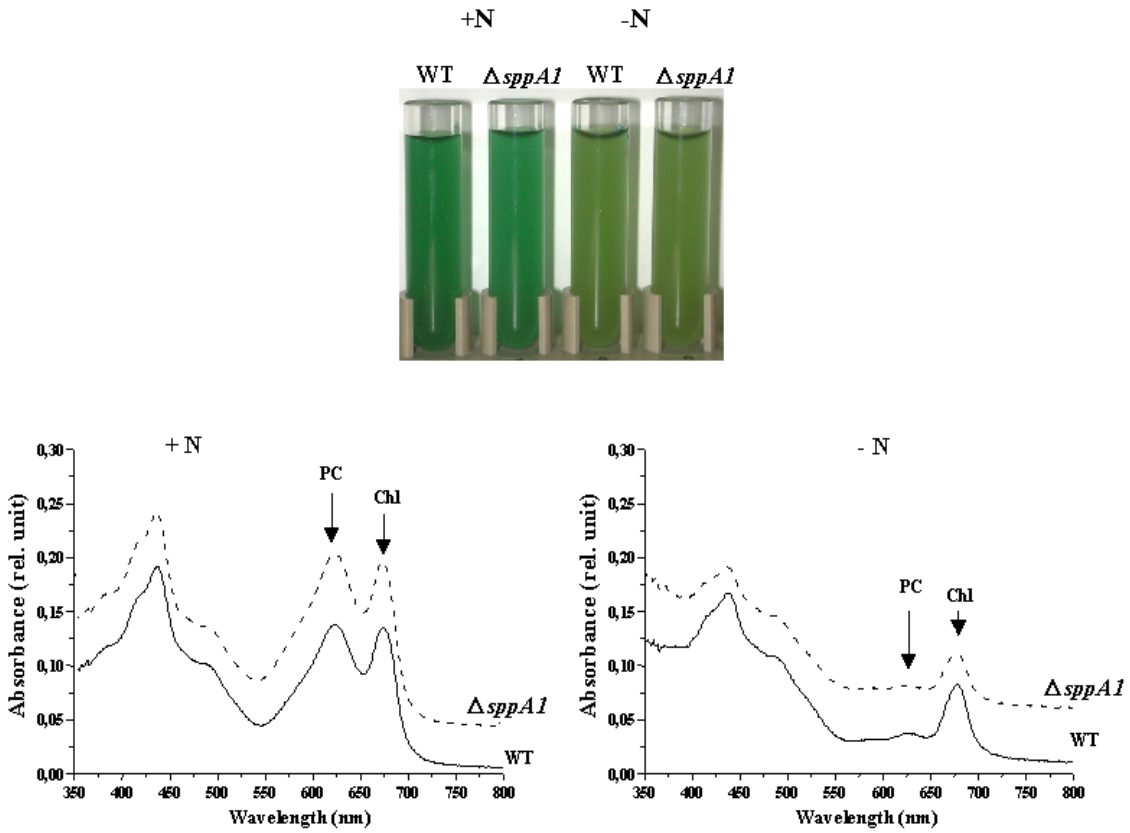


**Figure 21. Phenotypical modifications and absorption spectra of wild-type and Δ*sppA1* mutant during nitrogen starvation.** Wild-type cells and Δ*sppA1* mutant were incubated under LL till $A_{750}$ of 0.6 and then transferred to BG-11 medium depleted in nitrogen. The phenotypical changes and spectra were monitored after 3 days of deprivation.

### 3.2.1.6 Spectroscopic characterization of the Δ*sppA1* mutant

To examine the correlation between changes in pigment content and absorption properties with modifications in the functional organization of antenna in wild-type and Δ*sppA1*, 77K fluorescence analysis was performed on cyanobacterial cells adapted for 36 h to either LL, ML or HL.

The 77 K excitation spectra were recorded by monitoring of the emission of the PSII core at 695 nm. The wild-type spectrum at LL displayed an excitation component in the blue region that corresponds to chlorophyll *a* from the PSII cores and a major 628 nm component that corresponds to those PBS antenna functionally connected to the chlorophyll *a*-containing PSII cores (Fig. 22). When compared to LL conditions, the wild-type grown at ML showed a major decrease in the contribution of PBS sensitization relative to chlorophyll sensitization of the PSII emission. In contrast, the Δ*sppA1* mutant displayed a well preserved contribution of PBS

sensitization to PSII fluorescence at ML. Taken together, absorbance and 77K fluorescence data suggest that light acclimation, based on a decreased PBS content which takes place in the wild-type grown at ML, is prevented in the absence of SppA1. Under HL the contribution of PBS to the fluorescence of PSII was strongly decreased compared to that under ML in the mutant and resembles the low excitation contribution observed in wild-type cells placed under the same experimental conditions (Fig. 22).

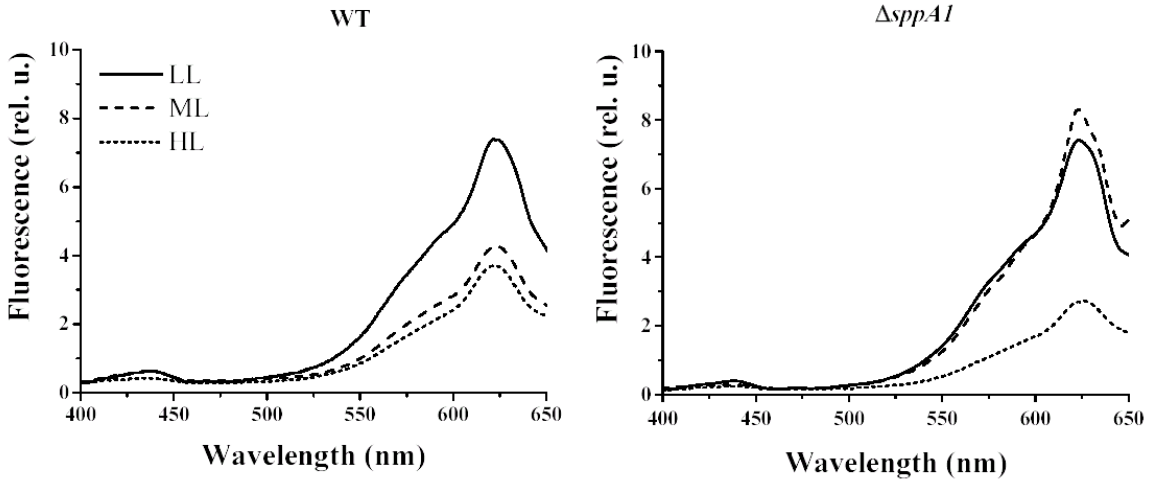


**Figure 22. Low-temperature (77 K) fluorescence excitation spectra of wild-type and Δ*sppA1* strain at LL, ML and HL regimes**. *Synechocystis* cells of wild-type and Δ*sppA1* were grown and adapted to ML and HL for 36 h. The excitation spectra were recorded at emission of 695 nm.

Light intensity and $CO_2$ concentration determine the content of phycobiliproteins in cyanobacterial cells. Increased light intensity, that is similar to decrease of $CO_2$ concentration, leads to a reduction of phycobiliproteins in cells. To check the $CO_2$ effect on changes in PBS content during light acclimation the 77K fluorescence analysis was performed with cells grown under LL and ML with constant $CO_2$ bubbling for 36 h. Cells were diluted to 2 μg of chlorophyll per ml. The excitation of APC at 540 nm resulted in a fluorescence emission of phycocyanin and allophycocyanin of the PBS at 640 - 660 nm, PSII at 695 nm and PSI at 725 nm. No changes in the emission of PBS, PSII and PSI could be observed in wild-type and Δ*sppA1* under LL (Fig. 23). However, the fluorescence of phycobilisomes not associated with PSI and PSII was substantially higher in Δ*sppA1*. The fluorescence from PBS associated with PSII and PSI remained the same. These data showed that SppA1 is not involved in the acclimation to $CO_2$, but in the adaptation to high light intensities.

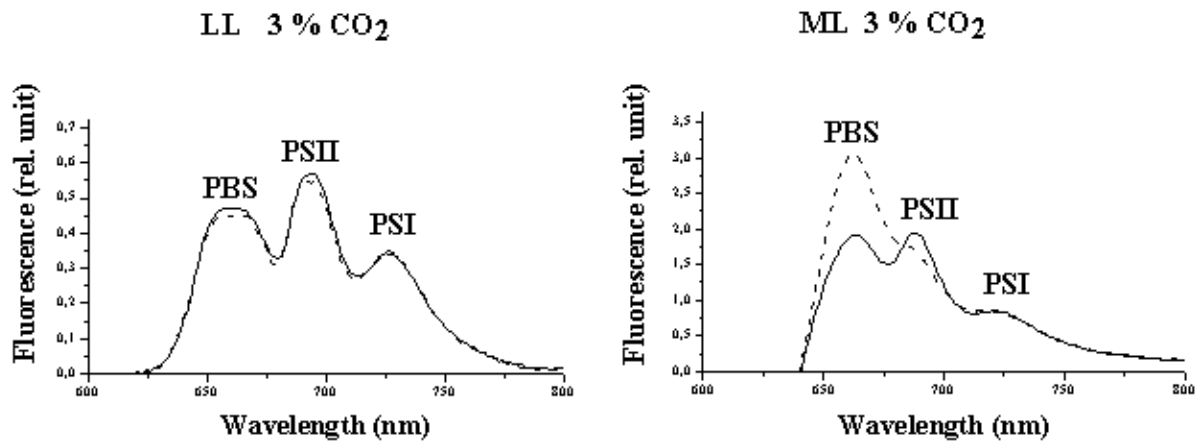


**Figure 23. Low-temperature (77 K) fluorescence emission spectra of cells adapted to different light regimes.** The 77 K fluorescence emission spectra of wild-type (solid line) and Δ*sppA1* mutant (dashed line) are shown after excitation of APC at 540 nm. The spectra were normalized to the emission peak at 725 nm. The cells were incubated under indicated light conditions for 36 hours.

### 3.2.1.7 Analysis of contents of photosynthetic proteins

The changes in intracellular pigment content in the wild-type and Δ*sppA1* under various light regimes could reflect changes in the amount of thylakoid membrane per cell or changes in the relative content of pigment-binding proteins per thylakoid membrane area, or both. Thylakoid membrane proteins isolated from wild-type and Δ*sppA1* grown for three days under LL, ML and HL were compared after SDS-PAGE (Fig. 24). Immunodetection of thylakoid proteins with antisera raised against the ß subunit of the ATP synthase, the PsaA/B proteins of the PSI reaction center, the D1 protein of PSII, the Rieske Fe/S protein of the cytochrome *b/f* complex and major core phycobilisome proteins (PC) is presented in Fig. 25. No significant differences in the content of ATP synthase and the cytochrome *b/f* complex between the two strains were observed at various light regimes. In contrast, the content in chlorophyll *a*-containing reaction centre proteins of PSI and PSII decreased with exposure to higher light intensities, but no differences in the relative contents of these protein complexes were detected between wild-type and mutant strain.

However, the content of phycobiliproteins in thylakoid membranes (Figs. 24 and 25) was markedly different between wild-type and Δ*sppA1* when grown at ML and HL. Amount of PC/APC decreased substantially with increasing light regimes in the wild-type, whereas

Δ*sppA1* retained much of its PC/APC content at ML. Therefore, the Δ*sppA1* is mainly altered in the adaptation of PBS structures to an increase in light intensities.

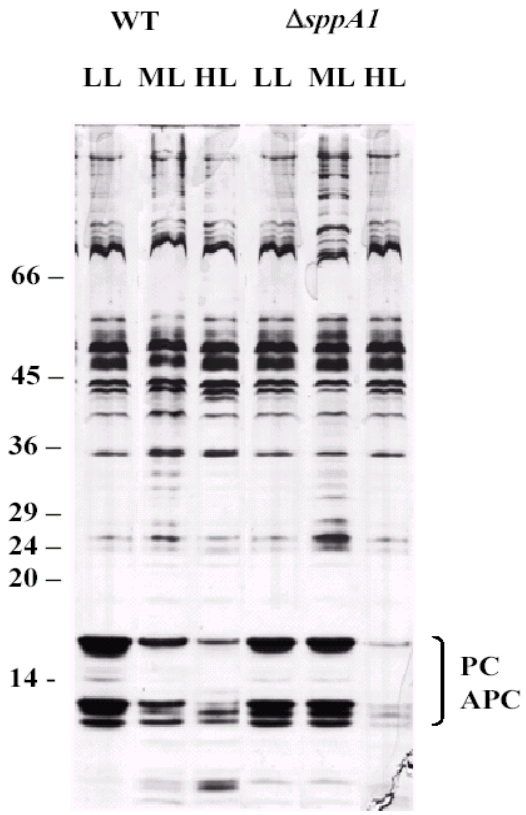


**Figure 24. Separation of thylakoid membrane proteins from *Synechocystis* wild-type and Δ*sppA1* mutant.** Thylakoid proteins were isolated from cyanobacterial cells adapted for 3 days to LL, ML or HL regimes and separated by 12% SDS-PAGE. The proteins were visualized by silver staining. The positions of phycobiliproteins (PC and APC) are indicated by a bracket.

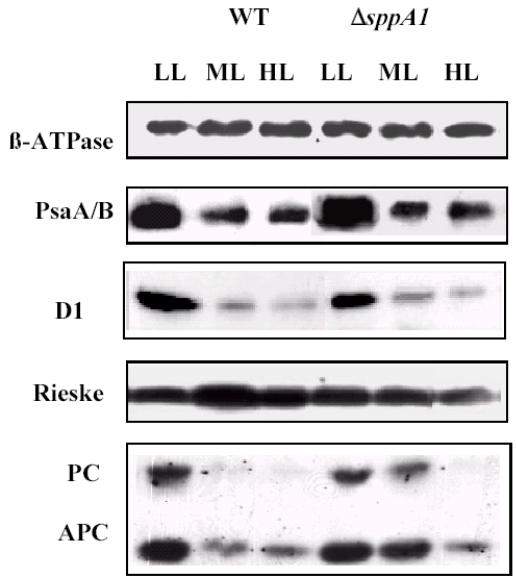


**Figure 25. Biochemical analysis of major photosynthetic complexes.** Thylakoid membrane proteins were isolated from cyanobacterial cells adapted for 3 days to LL, ML or HL and separated by 12% SDS-PAGE. The proteins were visualized by immunodetection with antisera raised against the β subunit of the ATP synthase, PsaA/B reaction centre proteins of PSI, D1 protein of PSII, Rieske FeS protein of the cytochrome *b/f* complex or rods of PC.

### 3.2.1.8 Genes and expression analysis of phycobiliproteins

To check the role of SppA1 in the expression of phycobiliprotein-encoding genes Northern experiments were performed for of *apcABC* and *cpcBAC2C1* transcripts. The transcriptional organization of the allophycocyanin and phycocyanin gene clusters is summarized in Figs. 26

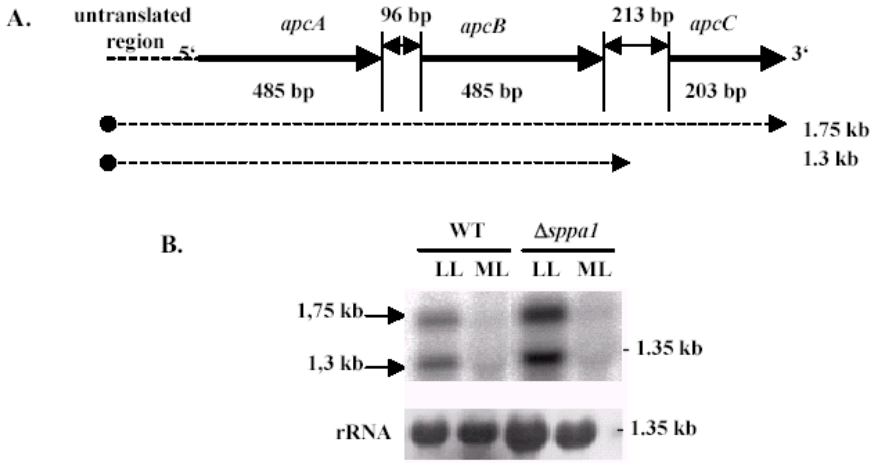


**Figure 26. Northern analysis of the allophycocyanin (*apc*)transcripts in wild-type and Δ*sppA1* mutant under different light regimes.** (**A**) Genetic and transcriptional organization of *apc* operon in *Synechocystis*. The transcript size for the *apcABC* operon is 1.75 kb, for the *apcAB* operon is 1.3 kb. (**B**) Total RNA was prepared from the wild-type cells grown under LL and ML and hybridized with the radiolabelled 0.5 kb DNA fragment of *apcA*. Arrows show the positions of *apcABC* and *apcAB* transcripts. Hybridization with rRNA was used as a quantitative control for equal loading.

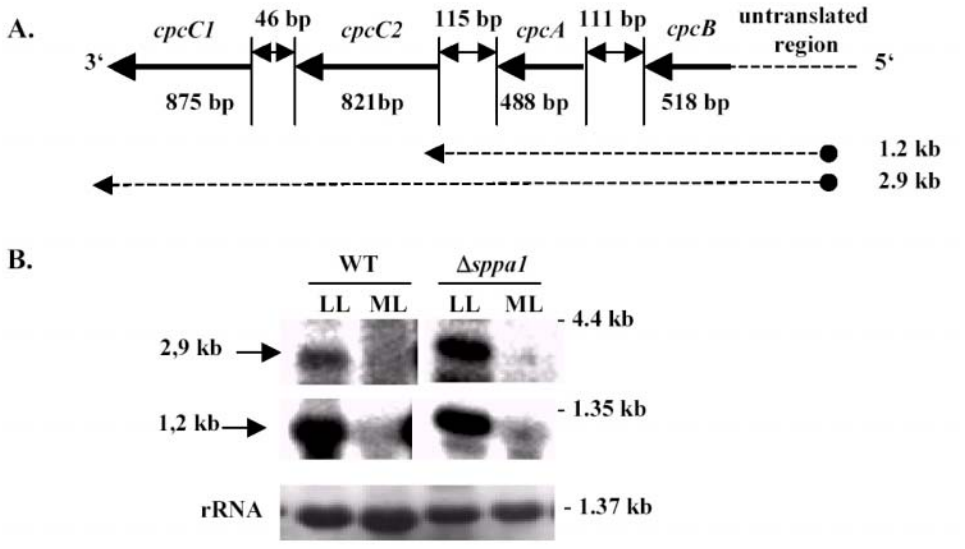


**Figure 27. Northern analysis of the phycocyanin (*cpc*) transcripts in wild-type and Δ*sppA1* mutant under different light regimes.** (A) Genetic and transcriptional organization of *cpc*-operon in *Synechocystis*. Two transcripts with different sizes were observed for *cpcBAC2C1* operon for c*pcBA* (1.2 kb) and for *cpcBAC2C1* (2.9 kb), respectively. (**B**) Total RNA was extracted from wild-type cells grown under LL and ML conditions and subsequently hybridized with *cpcA* (0.5 kb) radioactively labeled probe. Arrows show the positions of the *cpcBA* and *cpcBAC2C1* transcripts. Hybridization with rRNA was used as a control for equal loading.

and 27. The RNAs of the wild-type culture grown under LL and ML conditions were separated electrophoretically, transferred and hybridized with the radiolabelled 0.5 kb DNA fragment of the *apcA* gene (Fig. 26) or the 0.5 kb DNA fragment of *cpcA* gene (Fig. 27). The rates of RNA expression for *apc* and *cpc* decreased in both strains with increasing light intensity. The mRNA levels of biliproteins remained similar in wild-type and mutant under the light conditions tested.

### 3.2.1.9 *In vivo* labeling of wild-type and Δ*sppA1*

Since the Δ*sppA1* mutant showed differences to wild-type in phycobiliprotein contents under ML regimes the rates of synthesis of the major bilin-containing proteins were monitored by pulse-labeling studies using L-[$^{35}$S]-methionine at conditions of LL and ML (Fig. 28). The cells of both strains were grown under LL until the late exponential growth state. They were then diluted to an $A_{750}$ of 0.6 and transferred to LL and ML for 36 h. For labeling 10 ml of cultures from different light conditions were taken every 12 hours and incubated with $^{35}$S-methionine for 40 min. The reaction was stopped by adding cold methionine and the cells were used for isolation of thylakoid membranes. The proteins were separated electrophoretically by 10 - 17.5% gradient SDS-PA gel and Coomassie stained. The gel was dried and exposed with a phosphorimage plate.

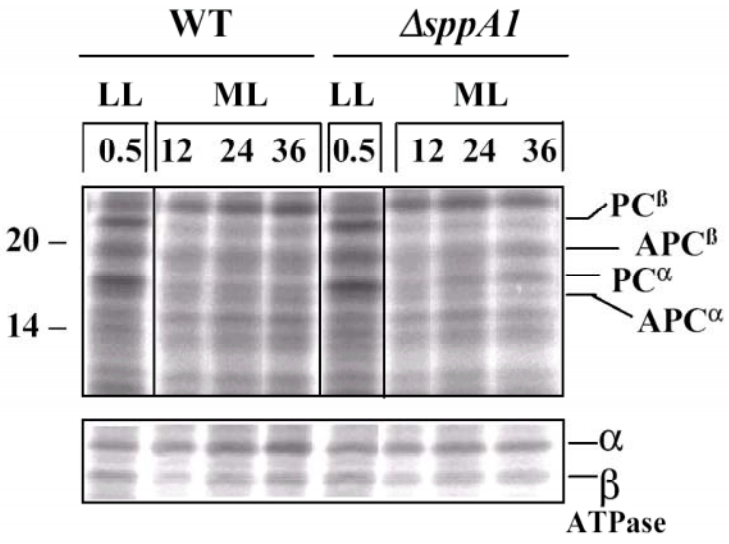


**Figure 28. Analysis of protein translation rate in Δ*sppA1* and wild-type under various light regimes by pulse-labeling with L-[$^{35}$S]-methionine.** Cells were adapted to LL and ML for 12, 24 or 36 h. Cells were labeled with L-[$^{35}$S]-methionine after immediate transfer to various light regimes for 30 min (lane „0.5") and after each time point of incubation at ML. Total cellular proteins were separated by 10 - 17.5% gradient PAGE and the gel was fluorographed in a Fuji phosphorimager. The lower part of the panel with ATP synthase subunits α and β was used as a loading control.

The wild-type and Δ*sppA1* strains displayed the same patterns with respect to changes in light regimes; there was a drop in the rate of synthesis of the major PC and APC polypeptides at ML versus LL in the two strains. This change occurred within the first 12 h of transfer to ML, with no further decrease over the next 36 h of acclimation. These data exclude that the larger decrease in phycobiliproteins in the wild-type when placed in ML is due to a mere translational regulation. The Δ*sppA1* undergoes a similar down regulation of translation although it preserves a higher phycobiliproteins content at ML.

#### 3.2.1.10 Kinetics of phycobiliproteins degradation

Figure 29 demonstrates the kinetics of decrease of the APC/PC proteins for wild-type and Δ*sppA1* under ML and HL over a 36 h period as compared to their content in LL. Thylakoid membranes were extracted from aliquots of cell cultures adapted to ML and HL after 12, 24 and 36 h. Proteins were separated electrophoretically in 12% SDS-PA gels and stained with Coomassie Blue. The α and β subunits of the ATP synthase are presented as a loading control in the bottom panel of the figure. No significant changes were detected in the wild-type during the first 12 h of acclimation to ML. The decrease in $PC^{\alpha}$ and $APC^{\alpha}$ subunits became noticeable only after 24 h and further up to 36 h (see arrows in Fig. 29), a period that corresponds to the bleaching visible in the cultures. In contrast, the Δ*sppA1* displayed only a limited decrease in the upper PC band after 24 h in ML and retained most of the PC/APC subunits even after 36 h exposure to ML. When exposed to HL, both strains displayed an extensive decrease in all PBS subunits after 36 h of acclimation. However, here again the protein loss remained limited during the first 24 h of exposure to HL. These data proved that the loss of PBS under higher light regimes is a delayed process in cyanobacterial cells that requires about 24 h before it can be detected.

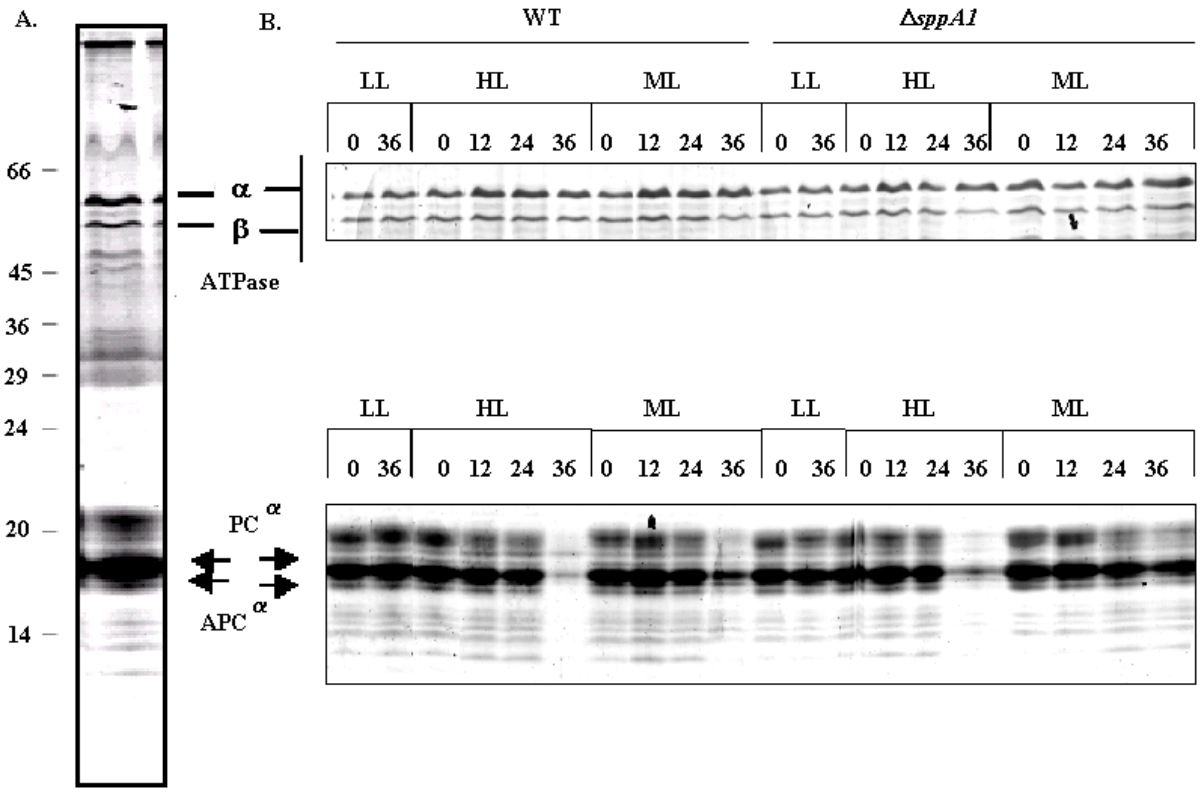


**Figure 29. Kinetics of phycobilisome degradation *in vivo*.** Wild-type and Δ*sppA1* were grown in LL till mid-log phase, then diluted with BG11 to an $A_{750}$ of 0.5 (point 0) and transferred to ML or HL, or kept in LL for 36 h. Cells were taken after 12, 24, 36 h in ML or HL, and after 36 h in LL. Total proteins were extracted from cells grown to the same $A_{750}$ and separated by electrophoresis in 12% SDS-containing gel. Proteins were visualized by Coomassie Blue staining. (**A**) The Coomassie stained cell proteins from the wild-type; (**B**) Visualization of $PC^{\alpha}$ and $APC^{\alpha}$ bands during acclimation to ML and HL. The upper part of the panel with α- and β-ATPase subunits was used as a loading control.

### 3.2.1.11 Analysis of phycobiliproteins

#### 3.2.1.11.1 Characterization and isolation of phycobilisomes

Phycobilisomes were extracted from strains as described in Section 2.2.8.4.3 of Materials and Methods. After centrifugation intact PBS were collected, diluted six times with 0.9 M of potassium phosphate buffer and pelleted by ultracentrifugation at 45.000 x g for 4 h at 4°C. The PBS were diluted in water and used for further spectrophotometrical analysis. Figure 30 demonstrates the model of PBS from *Synechocystis* cells, the profile of phycobiliproteins after separation in a 0.25 – 0.79 M sucrose gradient and the PBS protein patterns after electrophoresis in denaturing SDS-containing gel.

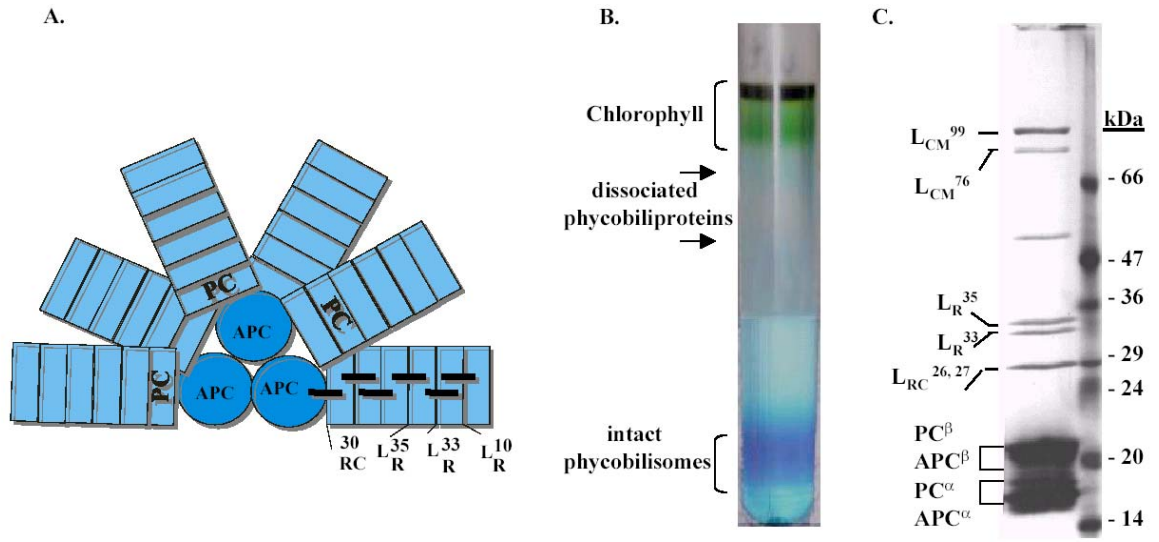


**Figure 30. Characterization of PBS from *Synechocystis*.** (**A**) Schematic presentation of PBS from *Synechocystis*. *APC* – allophycocyanin, *PC* – phycocyanin, $L_R$ – rod linkers 10, 33 and 35 kDa, *RC* – core rod linker of 30 kDa. (**B**) The extraction of PBS by 0.25 - 0.79 M sucrose gradient ultracentrifugation. The lower blue band corresponds to intact PBS that were collected and dissolved in phosphate buffer. (**C**) The proteins of the extracted PBS were separated by 10 - 17.5% gradient SDS-PAGE. The position of linker polypeptides was determined by Western analysis and designated in the picture by arrows at the left side. Molecular weight markers are indicated at the right side.

To measure the ratio of allophycocyanin and phycocyanin polypeptides in phycobilisomes extracted from cells adapted to different light intensities, fluorescence spectra at room temperature (25°C) were taken from isolated PBS. Intact PBS were extracted from wild-type and Δ*sppA1* previously adapted to LL or ML regimes for 48 h and then were diluted with 0.6 M of sodium phosphate buffer to the end concentration of 1 µg/µl. Before measurement the PBSs of both strains were dissociated by incubation at room temperature for 15 min. The excitation spectra (450 – 800 nm) at 25°C were taken and monitoring by the emission at the 695 nm. The fluorescence spectrum revealed two peaks at different wavelengths, a 570 nm peak that corresponds to APC fluorescence, and a 675 nm which corresponds to the fluorescence of PC (Fig. 31). During adaptation to ML, the phycocyanin content decreases relative to the allophycocyanin content. In the wild-type, PBS extracted from cells acclimated to ML showed a decrease in the 675 nm (PC) peak and an increase in APC fluorescence at 570 nm in comparison with the emission spectrum of the wild-type PBS from LL. The Δ*sppA1* mutant did not show any significant difference in the APC/PC ratio when compared with that of the wild-type under LL conditions. The APC/PC fluorescence ratio from PBS extracted from the Δ*sppA1* mutant adapted to ML remained stable (Fig. 31).

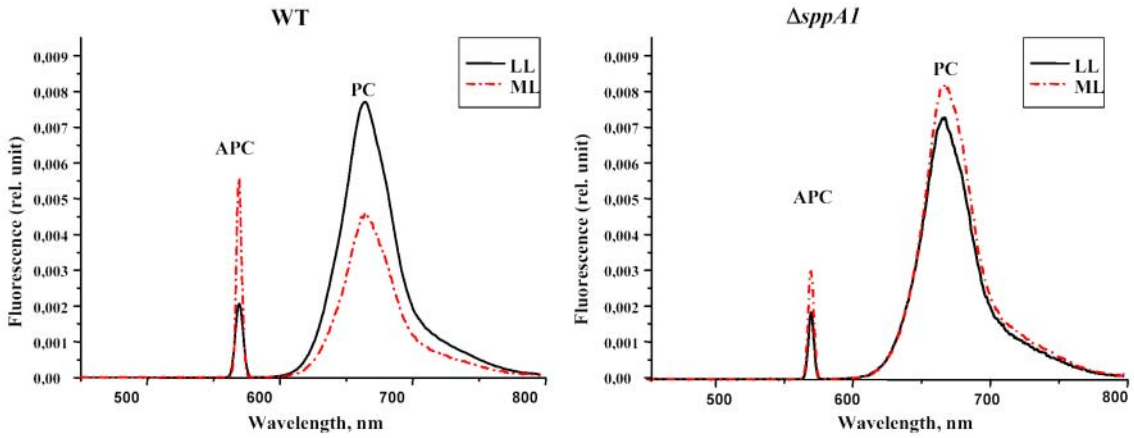


**Figure 31. Fluorescence excitation spectra at room temperature (25°C) of phycobilisomes extracted from wild-type and Δ*sppA1* mutant grown under LL or ML.** Intact PBS of both strains were isolated with sucrose gradients by ultracentrifugation, concentrated and resuspended in 0.6 M phosphate buffer. PBS were incubated for 15 min at room temperature for partial dissociation. The excitation spectra were collected at an emission at 695 nm.

### 3.2.1.11.2 SppA1 is involved in the light-dependent cleavage of rod linkers

A decrease in PBS antenna may occur through a shortening of PBS rods, a change in the number of rods per PBS or in the number of PBS themselves. The amount of linker proteins in PBS bound to the thylakoid membranes in wild-type and mutant adapted to various light conditions for 3 days was therefore analysed immunologically (Fig. 32A). The amount of $L_R^{35}$ was stable in the wild-type under all light conditions, while that of $L_{CM}^{99}$ and $L_R^{33}$ strongly decreased upon acclimation to ML or HL. In marked contrast, these two linker proteins remained stable in the mutant strain at ML, and the content of both linker proteins dropped only at HL. These observations are consistent with the loss of membrane bound APC and PC observed in wild-type but not in the Δ*sppA1* mutant and reflect an SppA1-driven loss in phycobiliproteins in *Synechocystis* during light acclimation to ML.

Earlier studies have stated that isolated PBS are not stable *in vitro* and subject to rapid degradation. In an attempt to link these observations with the present findings, PBS from wild-type, *pVZsppA1* and Δ*sppA1* cells adapted to LL were isolated and subsequently incubated at 4°C in the dark and at 37°C at HL for 3 h (Fig. 32B). Degradation of the membrane ($L_{CM}^{99}$) and rod ($L_R^{33}$) linkers in PBS preparations was observed exclusively in the wild-type and pVZ*sppA1* strains. $L_{CM}^{99}$ and $L_R^{33}$ linker proteins were stable in the Δ*sppA1*

mutant. This demonstrated that at least two groups of linker peptides, $L_{CM}^{99}$ and $L_R^{33}$, are degraded in isolated PBS fractions and that PBS of Δ*sppA1* mutant lacks the peptidase activity(-ies) that controls linker degradation in the wild-type. Taken together, the experimental data from *in vitro* isolated PBS and *in vivo* adapted antenna demonstrate a proteolytic resistance of two major PBS linker polypeptides, $L_{CM}^{99}$ and $L_R^{33}$, in the absence of the SppA1 peptidase.

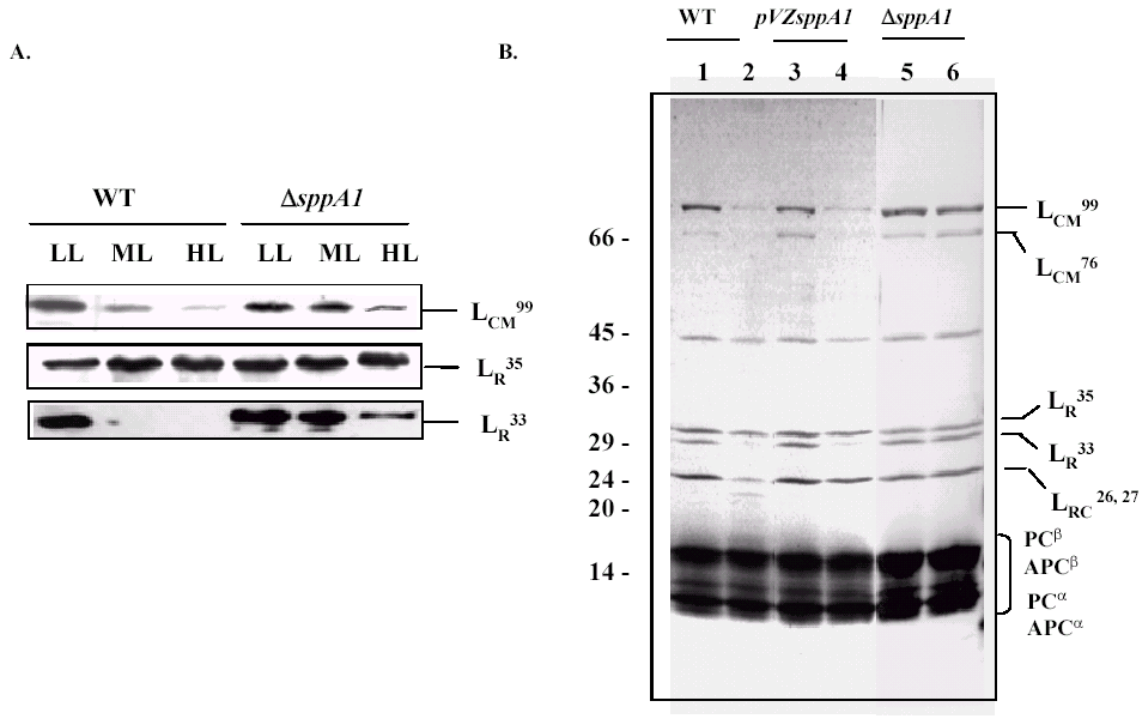


**Figure 32. Degradation of linker polypeptides of PBS antenna.** (**A**) Wild-type and Δ*sppA1* mutant cells were grown at LL and then transferred for the next 72 h to ML or HL. Thylakoid membrane proteins were separated, transferred onto nitrocellulose membranes and immunodetected with antisera against various linker proteins: membrane linker $L_{CM}^{99}$, rod linkers $L_R^{35}$ and $L_R^{33}$. Protein loading was normalized to the ß subunit of ATP synthase. (**B**) PBS were isolated from wild-type, *pVZsppA1* and Δ*sppA1* cells grown under LL. Isolated PBS were incubated at 4°C under dark (lanes 1, 3 and 5) and at 37°C under ML (lanes 2, 4 and 6) for 3h. The reaction was stopped by placing the samples on ice. Proteins were separated by electrophoresis in 12% SDS-PA gels. For protein visualization the gel was silver-stained.

### 3.2.2 Functional analysis of SppA2 protease

#### 3.2.2.1 Analysis of SppA2 protein sequence

SppA2 protein with a predicted molecular mass of 31 kDa is the second component of the *Synechocystis* SppA family. Hydropathy analysis according to Kyte and Doolittle did not deduce any transmembrane domains in the SppA2 sequence and predicted its localization in the cytoplasm (Fig. 33). Sequence analysis of SppA2 showed the absence of an Ala-X-Ala motif typical for recognition of protein precursors by signal processing peptidase, which tends to exclude a possible localization in the thylakoid lumen or periplasmatic space (Fig. 34).

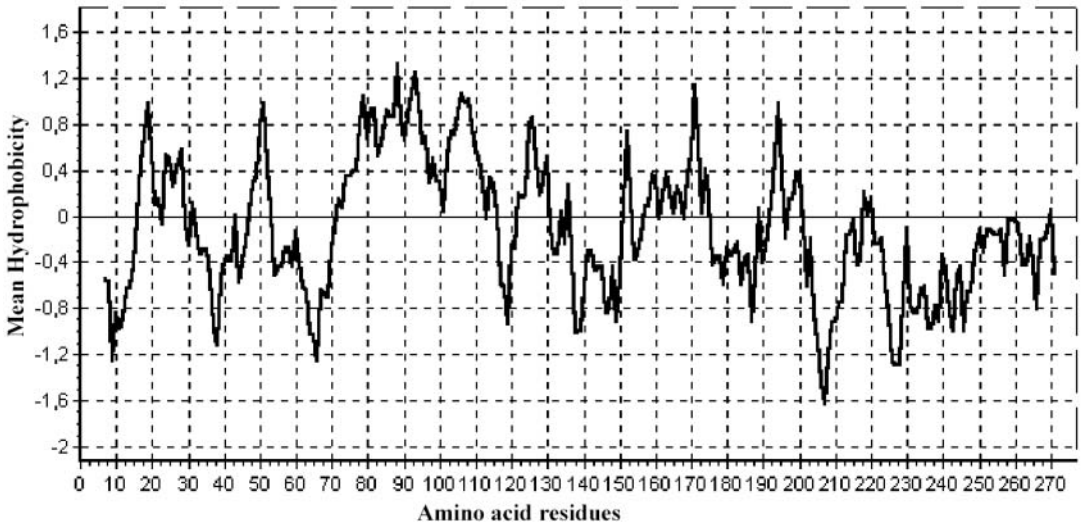


**Figure 33. Hydropathy plot of the *Synechocystis* SppA2 protein according to Kyte and Doolittle (1982).** Amino acid positions are indicated at the bottom of the graph. The scan window size was 13. Hydrophobic amino acid residues reside above the zero line.

The analysis of potential hydrophobic domains for SppA2-1 and SppA2-2 from *Pseudomonas aeruginosa* and SppA2-2 from *Agrobacterium tumefaciens* predicted a possible transmembrane domain close to the N-terminus (Fig. 34). The previously characterized SppA2 proteins from *E. coli* and *B. subtilis* (Baird et al., 1991; Bolhuis et al., 1999) were also targeted to different cell compartments, the outer cell membrane and cytosol, respectively. Sequence analysis of *Synechocystis* SppA2 showed the presence of a hydrophobic part as a possible membrane anchor of 12 amino acid residues (position 110 – 122; Fig. 34). This hydrophobic part includes a sequence that is conservative among SppA proteases from *Arabidopsis* and *E. coli* and this domain has been predicted to be integrated into the membrane (Lensch et al., 2001; Baird et al., 1991).

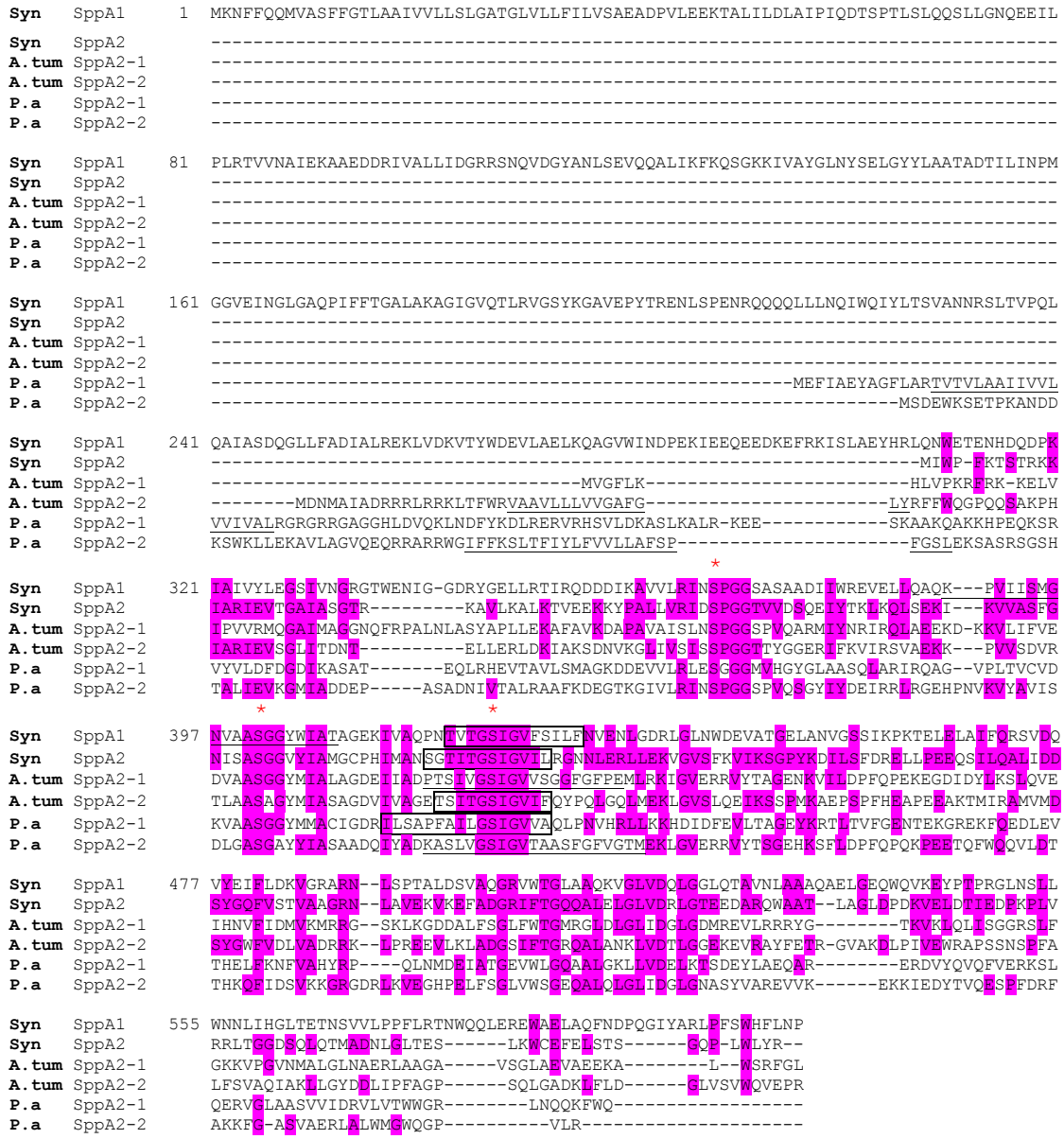


**Figure 34. Amino acid comparison of SppA1 and SppA2 proteases from different organisms.** Identical amino acids residues are shadowed in red. Possible transmembrane domains are underlined, possible membrane anchors indicated by open boxes. Putative catalytic amino acid residues are marked by stars. **Syn** – *Synechocystis*, **A. tum** – *Agrobacterium tumefaciens*, **P. a** – *Pseudomonas aeruginosa*.

### 3.2.2.2 Overexpression of SppA2 protein

To obtain an antiserum against SppA2 protein two different constructions were performed. Full-length (830 bp) and truncate (650 bp) fragment of the *sppA2* gene were amplified by PCR with primer pairs with introduced restriction sites for XhoI and PstI restriction endonucleases (slr0021_full_F, slr0021_full_R, slr0021_part_F and slr0021_part_R, respectively; Fig. 35A). The *sppA2* gene fragments were cloned into the PstI/XhoI sites of pRSTE5α and pIVEX2.4b overexpression vectors. The constructs were analyzed by

restriction (Fig. 35B). The correct plasmids were amplified by T7 forward and reverse primers and the resulting PCR fragments were used for sequence analysis (data not shown).

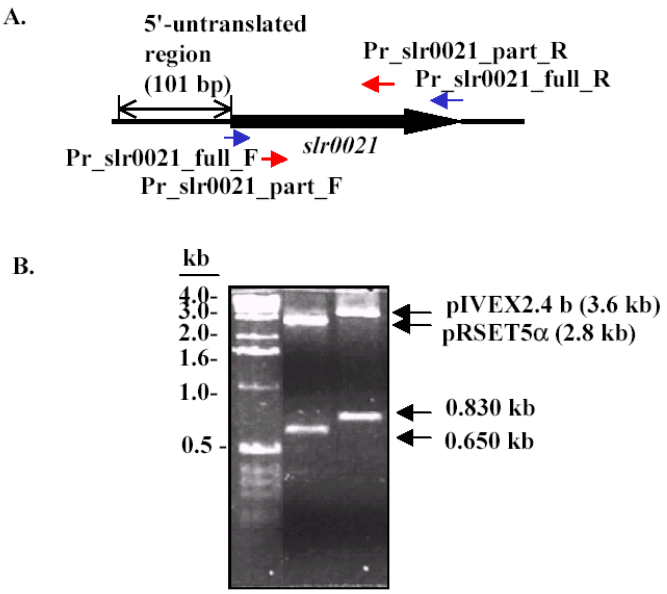


**Figure 35. Scheme of construction of pRSET5α and pIVEX2.4b recombinant plasmids used for SppA2 overexpression. (A)** The truncated form of the *sppA2* gene (650 bp) was amplified by slr0021_part_F and slr0021_part_R primers and cloned into pRSET5α vector. The full-length *sppA2* gene of 830 bp was amplified by slr0021_full_F and slr0021_full_R primers and cloned into pIVEX2.4b vector that contains a His-tag site. **(B)** Results of restriction analysis of recombinant pRSET5α and pIVEX2.4b vectors. The recombinant plasmids were digested by XhoI and PstI and used for sequence analysis. The λ DNA HindIII/EcoRI marker is indicated at the left.

The overexpression procedure for pRSET5α-*sppA2* (truncated) construct was carried out as described in Section 2.2.5.1. After transformation of *E. coli* BL21 (D3Lys) cells with pRSET5α-*sppA2* plasmid and induction with 1 mM IPTG for 3 h the overexpressed polypeptide with its molecular weight of about 23 kDa was detected (Fig. 36A). After separation of the *E. coli* proteins into soluble and insoluble protein fractions the truncated overexpressed SppA2 was found in inclusion bodies (data not shown).

Overexpression of the full-length *sppA2* gene cloned into pIVEX2.4b vector occurred in protein lysates of *E. coli* provided by Rapid Translation Systems from *Roche* (Section 2.2.5.2). The reaction device contains two compartments (reaction and feeding compartments) separated by a semipermeable membrane. The overexpression reaction took place in 1 ml of reaction compartment of the reaction device. At the same time, newly synthesized proteins are constantly diluted *via* diffusion through the semipermeable membrane into 10 ml of the feeding compartment, to prevent a potentially inhibitory reaction by the overexpressed product. The reaction was performed at 30°C for 24 h in the reaction compartment. Aliquots of the overexpressed product from the reaction and feeding compartments were used for Western analysis with anti-His antiserum (Fig. 36B).

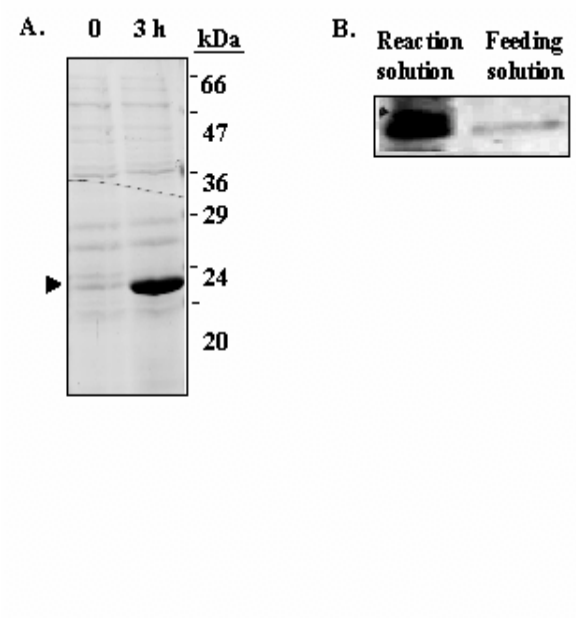


**Figure 36. Overexpression of a full-length and truncated SppA2 protein. (A)** Overexpression was carried out in *E. coli* strain BL21 (DE3Lys) which was transformed with the expression vector pRSET5α containing the truncated *sppA2* gene. Expression was induced by addition of 1mM IPTG for 3 h at 37°C. Accumulation of expressed protein was monitored by SDS-PAGE. Arrow indicates the overexpressed truncated SppA2 protein with a molecular weight of 23 kDa. The molecular weight marker is shown at the right side **(B)** Expression of full-length SppA2 *in vitro.* The protein expression mixture was prepared according to instructions of Roche. Protein synthesis was carried out for 24 h at 30°C. The 29 kDa full-length SppA2 protein from the reaction compartment and feeding compartment were determined by Western analysis with anti-His antibody.

### 3.2.2.3 Analysis of proteolytic activity of SppA2

Proteolytic assays with Azocoll were used for investigation of the proteolytic activity of SppA2 protease. The full-length SppA2 protein was overexpressed in *E. coli* lysates using the Roche overexpression system. The lysate containing the SppA2 protein was added to 1 ml of sodium phosphate buffer (pH 7.5) containing 5 mg of Azocoll, a non-specific protease substrate, and incubated under constant shaking and illumination at 37°C for 24 h.

**Table 9. Colorimetric assay of SppA2 protease in the presence of various inhibitors**

| Addition | Concentration | Relative activity % |
|---|---|---|
| None | - | 100 |
| DFP | 2 mM | 0 |
| PMSF | 2 mM | 0 |
| IAA | 5 mM | 65 ± 8.15 |
| EDTA | 5 mM | 73.6 ± 3.6 |

The data presented are from three determinations and the error bars represent the mean standard error.

Different protease inhibitors, 10 mM DFP, 10 mM PMSF, 1 mM EDTA or 1 mM IAA, were used to determine the nature of SppA2 activity. Azocoll in sodium phosphate buffer was used for background subtraction. The absorption of a supernatant was measured at 520 nm (Table 9). The activity of SppA2 protease (taken as 100%) was partially inhibited by IAA and EDTA but fully inhibited by DFP and PMSF.

### 3.2.2.4 Localization of the SppA2 protein

#### 3.2.2.2.4.1 Intracellular localization of the SppA2 protein

To examine the localization of SppA2 protein in *Synechocystis* cells a polyclonal antiserum raised against the truncated overexpressed SppA2 protein were used. For detection of the *sppA2* gene product proteins from the wild-type were extracted (Section 2.2.9.4.1) and fractionated into membrane and soluble fractions. After transfer of proteins into nitrocellulose membrane SppA2 and D1 proteins were immunologically detected. Immunodetection of D1 protein (a component of PSII reaction center) was used as a control for thylakoid-enriched fraction. Western analysis demonstrated association of the SppA2 protein with the membrane fraction (Fig. 37).

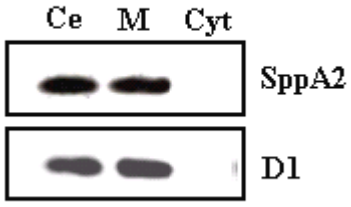


**Figure 37. Localization of SppA2 in *Synechocystis* cells.** Wild-type cells were fractionated into total cellular protein extract (Ce), membrane (M) and cytoplasma (Cyt) proteins. After separation of proteins on SDS-PAGE and transfer onto nitrocellulose membrane they were immunodecorated with antisera raised against SppA2 and D1 proteins. Immunoreaction with antisera against D1 protein was used as a control for thylakoid-enriched fraction.

#### 3.2.2.4.2 Association of SppA2 protein with membrane structures

To determine the association of SppA2 protein with the membranes, isolated thylakoids were concentrated in HM buffer to 200 μg of chlorophyll/ml and treated by chaotropic salts (2 M NaBr, 2 M NaSCN) or alkaline solutions (0.1 M $Na_2CO_3$ and 0.1 N NaOH; Section 2.2.9.4.2).

After incubation on ice for 30 min the thylakoids were recovered by centrifugation and analyzed by SDS-PAGE and Western analysis with antisera against SppA2 and the β subunit of ATP synthase, used as a control for peripheral proteins. Chaotropic salts disturb hydrophobic interactions between proteins and membrane lipids. Alkaline solutions able to destabilize ionic interactions lead to a partial removal of proteins from the membrane. Differently from the predictions SppA2 protein showed a strong stable association with the membrane fraction. SppA2 was partially released from thylakoids only after the treatment with strong reagents such as NaSCN or NaOH, but not with $Na_2CO_3$ and NaBr. The same membrane treatments released the β subunit of ATP synthase that is a part of $CF_1$ and peripherally associated with the membrane-integrate $CF_0$ moiety of the ATP synthase complex (Fig. 38). These data suggest that SppA2 is not peripherally attached but a membrane-associated component.

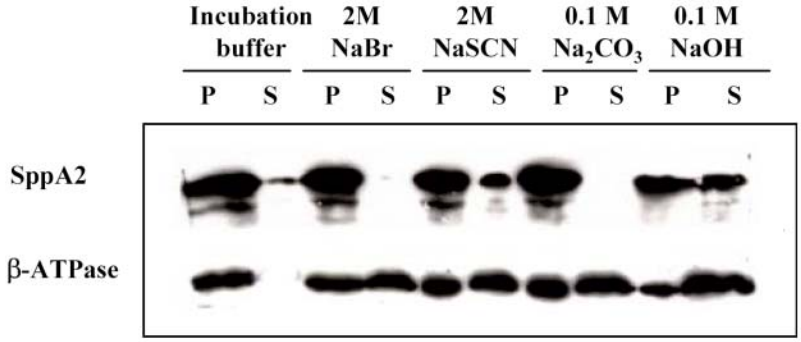


**Figure 38. Nature of the membrane association of SppA2.** Isolated membranes from wild-type cells were incubated on ice for 30 min with chaotropic salts (2 M NaBr and 2 M NaSCN) or alkaline solutions (0.1 N NaOH and 0.1 M $Na_2CO_3$), or with incubation buffer (as a control). After extraction thylakoids were recovered by centrifugation, analyzed by SDS-PAGE and Western analysis with antisera against SppA2 and the ß subunit of ATP synthase (P – pellet, S – soluble fraction).

### 3.2.2.4.3 Localization of SppA2 in a high molecular weight membrane complex

Since SppA2 showed association with membranes, its possible association within or localization in some homo- or heteromeric complex was investigated by isopycnic centrifugation of solubilized membranes on a sucrose gradient. The membranes extracted from the wild-type cells grown at 30°C under LL were separated on the sucrose gradient (Section 2.2.9.4.4). The proteins from the gradient were fractionated and separated by denaturing SDS-PAGE. The position of the major photosynthetic complexes was determined

by immunoreaction with antisera raised against D1 protein (PSII), PsaD (PSI) and phycocyanin, the major rod protein of PBS. Immunodetection showed that SppA2 was a part of a supramolecular complex that did not migrate with one of photosynthetic complexes, but migrated between the monomeric and trimeric forms of PSI (Fig. 39). These data demonstrated that SppA2 protein represents part of a novel high molecular weight complex in the membranes.

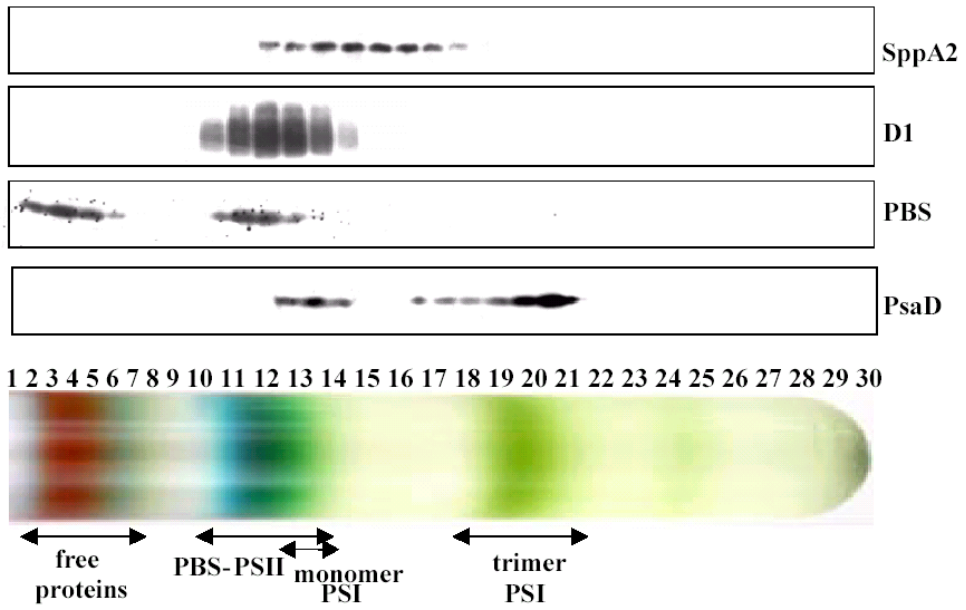


**Figure 39. Isolation of photosynthetic complexes from wild-type *Synechocystis* cells on sucrose gradients.** Thylakoid membranes from wild-type were solubilized with 2% of n-dodecyl-ß-D-maltoside and separated on a linear 12 to 24% MOPS-containing sucrose gradient. Fractions of 0.3-ml were collected for analysis of photosynthetic complexes and SppA2 using antisera directed against SppA2, D1 (PSII), PsaD (PSI) and phycocyanin proteins of phycobilisome rods.

Possible association of SppA2 with SppA1 protein was analyzed in *sppA1*-deficient cells. Photosynthetic complexes were separated from Δ*sppA1* and migration of the SppA2-containing complex was determined immunologically. No migration shift of the complex could be observed in thylakoid membranes extracted from the Δ*sppA1* that argues for no physical association of both proteins at least under solubilization conditions used (data not shown).

### 3.2.2.4.4 Isolation of photosynthetic complexes by non-denaturing „blue-native" PAGE

Possible association of SppA2 with photosynthetic complexes was also analyzed by non-denaturing „blue native" gel electrophoresis (Section 2.2.9.4.5). Thylakoid membranes from

wild-type cells were isolated and resuspended in ACA-buffer (end concentration of chlorophyll 0.336 µg/µl). Thylakoid membrane proteins containing 30 µg of chlorophyll were solubilized with 1, 1.5 and 2% of n-dodecyl-ß-D-maltoside. Solubilized proteins were loaded onto 4 - 12% non-denaturing „blue-native" PAGE (Fig. 40A). Best solubilization was achieved with 2% of n-dodecyl-ß-D-maltoside and, after separation, protein complexes were visualized by Coomassie Blue staining of the gel (Fig. 40B). The position of each complex was determined by immunoreaction with antisera raised against components of PSI (PsaD), PSII (CP47) and ATP synthase (α subunit).

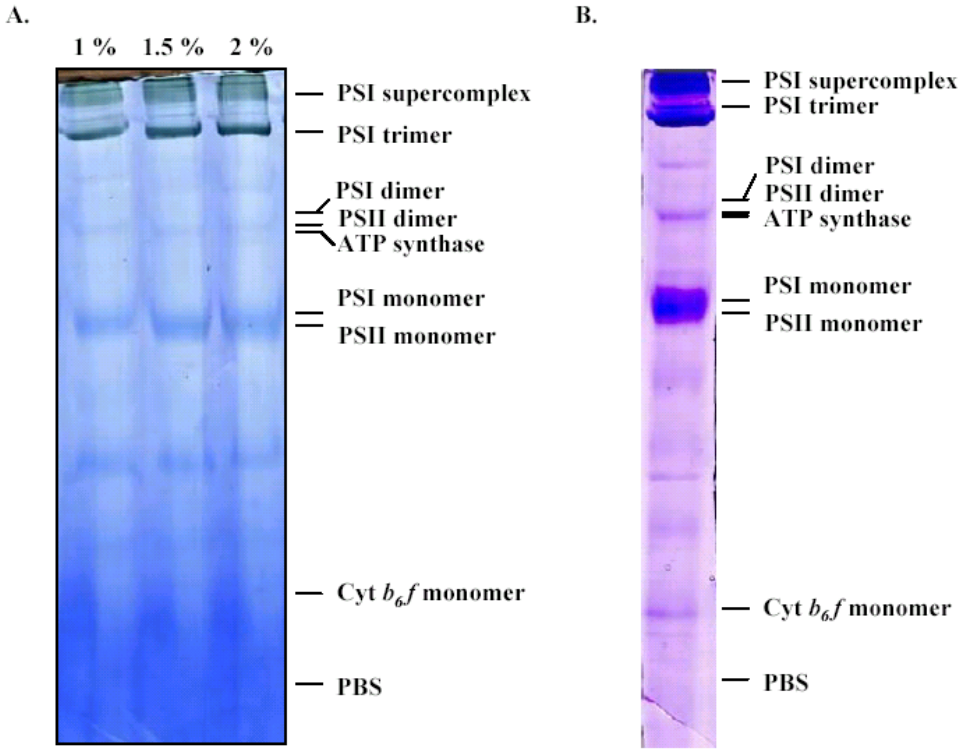


**Figure 40. Isolation of photosynthetic complexes from wild-type *Synechocystis* cells by non-denaturing 4 – 12% „blue native" gel electrophoresis.** (**A**) Thylakoids from the wild-type were solubilized with different concentrations of n-dodecyl-ß-D-maltoside (1, 1.5 and 2%) and used for isolation of major photosynthetic complexes by non-denaturing "blue native" gel electrophoresis. (**B**) Protein complexes were visualized by Coomassie Blue staining.

To identify protein complexes and to resolve their composition, the protein complexes from "blue native" gel lanes were separated in a second dimension by denaturing SDS-PAGE and silver-stained (Fig. 41). Proteins were identified by Western analysis with antisera raised against components of PSI (PsaD and PsaA/B), PSII (CP47) and ATP synthase (α subunit).

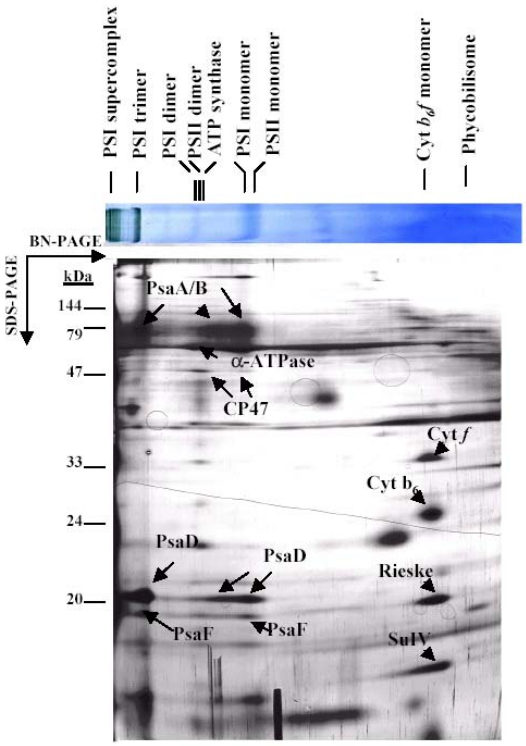


**Figure 41. Separation of membrane complexes from wild-type *Synechocystis* cells by two-dimensional BN/SDS-PAGE.** Thylakoid membranes were isolated from wild-type cells. The major photosynthetic complexes were isolated by non-denaturing blue native gel electrophoresis. The proteins from photosynthetic complexes were separated in the second dimension by denaturing 12% SDS-PAGE. Proteins were visualized by silver staining.

The localization of SppA2 was determined in the second-dimension gel with anti-SppA2 antiserum. The protein of 29 kDa corresponding to SppA2 was identified in the wild-type but not in Δ*sppA2* (data not shown). The SppA2 complex migrated close to the monomeric forms of PSI and PSII. Position of PSI and PSII complexes was detected with antisera raised against PsaD and CP47, respectively. No co-localization of SppA2 complex with cytochrome *b/f* complex and ATP synthase complex could be observed as well (Fig. 42).

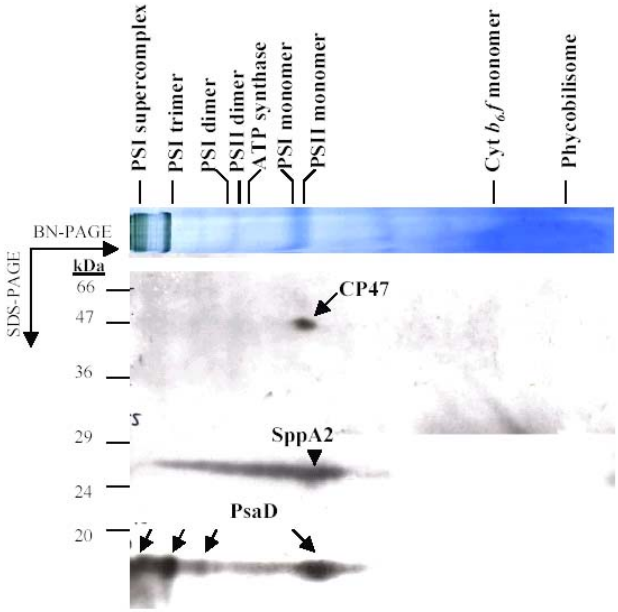


**Figure 42. Localization of SppA2 protein in *Synechocystis* cells by non-denaturing 4 – 12% "blue native" gel electrophoresis.** Thylakoid membranes (0.5 mg/ml chlorophyll) from wild-type were solubilized with 2% n-dodecyl-ß-D-maltoside. Proteins were analyzed by 4 – 12% native gel in the first dimension and by 12.5% SDS-PAGE in the second. For identification of SppA2 and proteins of PSI and PSII complexes the separated proteins were immunoreacted with antibodies against SppA2, CP47 (PSII) and PsaD (PSI).

### 3.2.2.5 Expression analysis of SppA2

#### 3.2.2.5.1 Expression of *sppA2* gene during different light regimes

Previous studies of the SppA family from *Arabidopsis* has shown that the gene expression of SppA protease is light-regulated, however, expression of *Synechocystis sppA1* gene was not induced by light. Since SppA2 shares protein homology to SppA1 and also showed a light-dependent phenotype the expression of the *sppA2* gene was tested under different light regimes. For this, wild-type cells were adapted to DL, LL, ML and HL regimes for 56 h. The isolated RNAs were separated on MOPS-containing agarose gels and quantified by staining with EtBr solution. The filter with the transferred RNAs was used for Northern analysis with *sppA2* gene specific probes labeled with dCTP. Gene expression analysis showed that the amount of *sppA2* transcript of 930 bp accumulated during increased light intensities (Fig. 43).

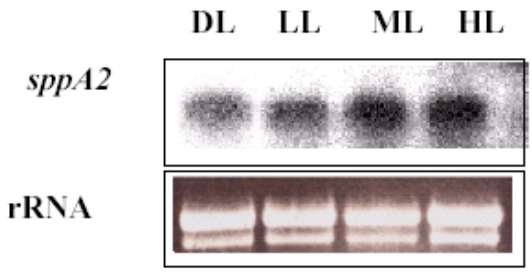


**Figure 43. Expression of *slr0021* (*sppA2)* gene in wild-type under different light regimes.** Wild-type RNA was extracted from cyanobacterial cells grown under different light regimes (DL, LL, ML and HL) and used for Northern analysis with *sppA2* probe labeled with $^{32}$P-dCTP. The RNA gel was stained with EtBr for visualization of rRNA as a control for equal loading.

#### 3.2.2.5.2 SppA2 protein levels under various stress conditions

To analyze the correlation between transcription and translation levels the amount of SppA2 in the wild-type cells under different light conditions was detected by immunoreaction with antibodies raised against SppA2 protein. *Synechocystis* cells were grown under LL and then transferred to ML and HL regimes for 52 h. Thylakoid membranes were separated on denaturing 12% SDS-PAGE and transferred onto nitrocellulose membranes. The immunoreaction was performed with secondary antibodies coupled to $^{125}$I-labeled Protein A (Section 2.2.8.5.4). In contrast with *sppA2* gene expression the amount of SppA2 strongly decreased in cells upon transfer to higher light. During acclimation to ML approximately 40% of the protein was still present, while under HL the protein disappeared completely after 3 days of exposure to HL (Fig. 44A). In order to analyze whether the amount of SppA2 protein was also affected in *sppA1*-deficient cells adapted to high light intensities thylakoid proteins

were extracted from Δ*sppA1* and immunoreacted with antisera against SppA2 protein. In contrast to the wild-type SppA2 protein remained stable in the Δ*sppA1* mutant cells even after acclimation to HL and the accumulation of the protein in Δ*sppA1* thylakoids was proportional to an increase of *sppA2* gene expression under ML and HL (Fig. 44A). To examine whether degradation of SppA2 protein is only specific for HL or also other stress conditions as well cyanobacterial cells were acclimated parallel to LL and HL to nitrogen deprivation, heat and cold stress (Fig. 44B). SppA2 was stable under all tested stress conditions, with exception of HL, proving specific involvement of SppA2 in acclimation upon changes of light intensities.

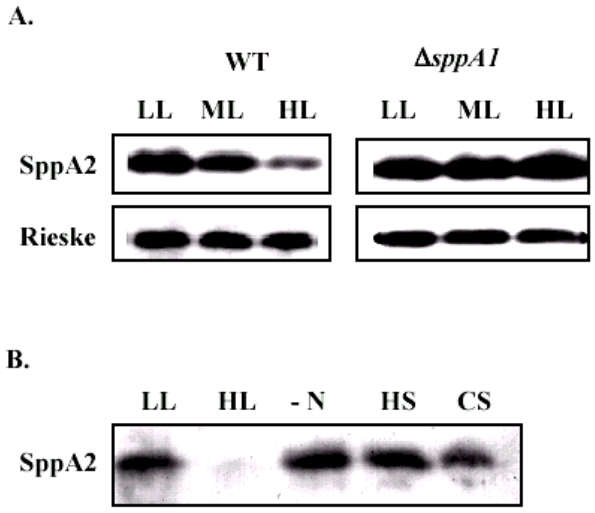


**Figure 44. Analysis of SppA2 in wild-type and Δ*sppA1* cells.** (**A**) Wild-type and Δ*sppA1* cells were adapted to LL, ML and HL for 52 h. Thylakoid membrane proteins were separated by denaturing 12.5% SDS-PAGE. The SppA2 protein was immunodetected with an SppA2 antiserum using $^{125}$I-labeled protein A. Immunoreaction with antisera against Rieske was used as a control for equal loading. (**B**) Accumulation of SppA2 protein under various stress conditions. Wild-type cells were grown under standard regimes to an $A_{750}$ of 0.5 and then transferred for HL, nitrogen starvation (- N), heat stress (HS) and cold stress (CS). SppA2 was immunologically detected in thylakoid membranes extracted from adapted cells.

### 3.2.2.5.3 Kinetics of SppA2 degradation upon HL exposure and its acclimation during recovery from HL to LL

To define the kinetics of SppA2 degradation upon acclimation from LL to HL cells grown under LL were transferred for 1, 2, 3, 12, 24 and 36 h to HL. Thylakoid membrane proteins were then extracted and used for Western analysis with antisera raised against SppA2. The protein was stable within the first 2 h of exposure to HL and reduced up to 60% within the third hour. After 36 h exposure to light stress only about 5 to 10% of protein was left. The behavior of SppA2 protein during the change from HL to LL was tested by reverse adaptation

of HL-acclimated cells to LL for 0.5, 2, 4, 6, 8, 12, 24 and 36 h. Under these conditions, the level of SppA2 protease reached its maximum after 4 h of exposure to LL (Fig. 45A and B). This level was maintained up to 8 h and then it declined to a steady state. The results suggest that SppA2 plays a role during cell recovery from HL to LL and may be involved in the degradation of proteins required for photoprotection under HL which are then rapidly degraded upon adaptation back to LL. In both acclimation experiments from LL to HL and back the β subunit of the ATP synthase complex was used as a control. No changes in the level of this protein were observed under the chosen conditions.

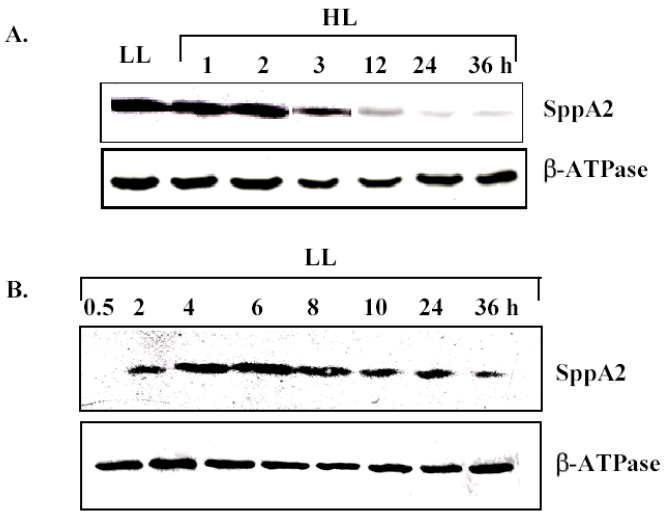


**Figure 45. Kinetics of SppA2 degradation and accumulation from LL to HL and recovery to LL.** (**A**) Wild-type cells were grown under LL and transferred to HL for 1, 2, 3, 12, 24 and 36 h. (**B**) After 36 h of acclimation to HL the adapted cells were transferred back to LL for various periods (0.5, 2, 4, 6, 8, 10, 24 and 36 h). The extracted membrane proteins were immunoreacted with SppA2 antiserum. The β subunit of ATP synthase was used as a control for equal loading.

### 3.2.2.6 Construction of *sppA*2-depleted and complemented mutant strains

To continue the functional analysis of the SppA protease family in *Synechocystis*, a reverse genetic approach has been chosen to understand the function of the *sppA2* gene product. Computer analysis showed that SppA2 is encoded by the *slr0021* gene of 830 bp. *SppA2* is located between two open reading frames *slr0020* and *sll0002* which are most probably not organized in a gene cluster (Fig. 46A).

A fragment of the *Synechocystis slr0021* gene (777 bp) was amplified from genomic DNA with slr0021_F and slr0021_R primers and cloned into pGEM-T vector (Promega, UK). The *Km* resistance gene was excised of the pUC4K plasmid by HincII and then inserted into the ClaI site located in the middle part of *sppA2* (Fig. 46B). The correct plasmid was used for transformation of *Synechocystis* cells. The DNA from wild-type and Δ*sppA2* strains was used for PCR analysis with gene-specific primer pairs. The size of the amplified fragments differs

by 1.2 kb that correspond to the *Km* resistance gene from the amplification product of the wild-type DNA (Fig. 46C). The wild-type fragment appears as a 0.83 kb band, whereas the Δ*sppA2* fragment has a size of 2.1 kb due to 1.2 kb *Km* resistance gene.

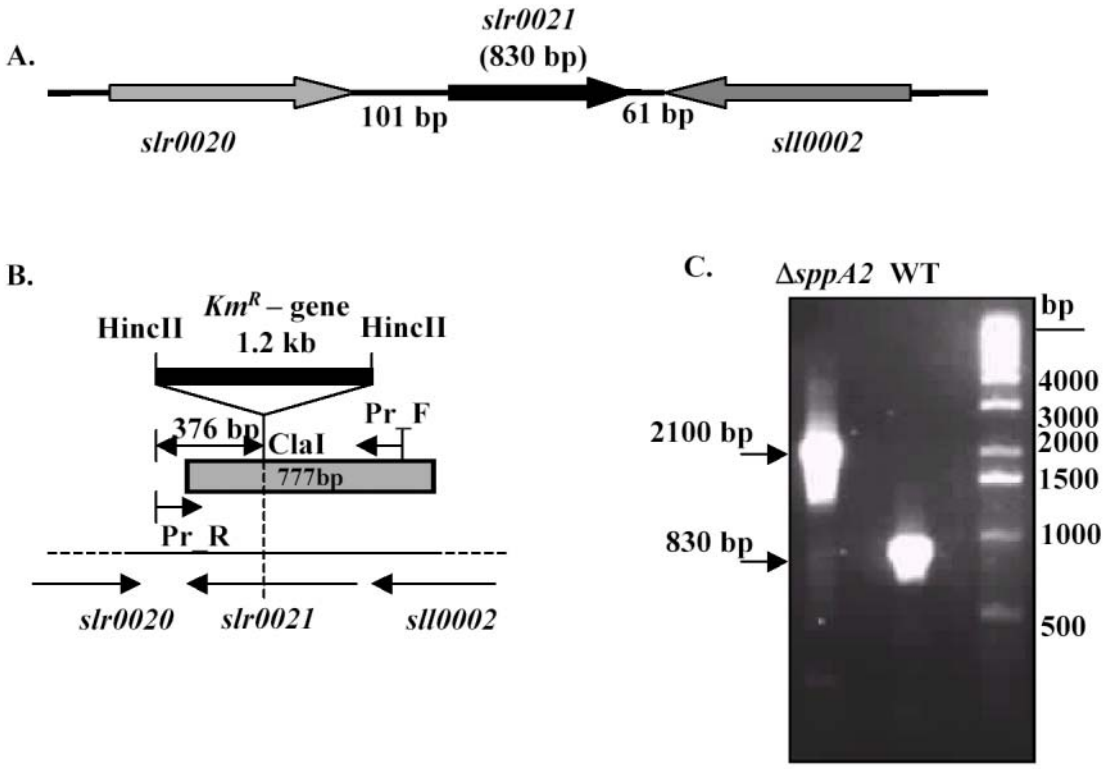


**Figure 46. Construction of the pGEM-T ΔsppA2 plasmid and PCR analysis of the *Synechocystis* Δ*sppA2* strain**. **(A)** Chromosomal localization of the *sppA2* gene. **(B)** Construct of the interrupted *sppA2* gene used for transformation of *Synechocystis*. The 777 bp fragment of gene *slr0021* was amplified by PCR using slr0021_F and slr0021_R primers which are indicated as Pr_F and Pr_R, respectively. Inactivation of *sppA2* was performed by insertion of $Km^R$-gene into internal ClaI site of the fragment. **(C)** PCR analysis of Δ*sppA2* insertion segregated to homozygosity. The wild-type fragment appears as a 0.83 kb band, whereas the Δ*sppA2* fragment has a size of 2.1 kb due to the 1.2 kb *Km* resistance cassette. The last line represents the 1 kb DNA ladder marker.

Mutagenesis of the desired open reading frame can influence the expression of closely located genes. In that case the phenotype of a mutant strains can be caused either by depletion of gene of interest or by site effect on some other genes. To exclude the last possibility and to prove that the phenotype corresponds exclusively to the disrupted gene a complementation analysis of the mutant strain by a self replicating plasmid that carries the wild-type copy of the interrupted gene has been performed. To confirm the correctness of *sppA2* gene targeting the complementation strain *pVZsppA2* of the Δ*sppA2* mutant was generated (see Section 2.2.3). The *slr0021* gene with 5'-untranslated region of 100 bp was amplified using the primers slr0021F_XhoI_com and slr0021R_HindIII_com and cloned into pVZ322 plasmid containing

two resistance genes, *Km* and *Gm*. The fragment was inserted into HindIII/XhoI sites inside of the $Km^R$ –gene that led to inactivation of that, transcription of the $Gm^R$ –gene was not affected. The scheme of the construct and its cloning is shown in Fig. 47A. Clones resistant to gentamycin and sensitive to kanamycin were selected for further analysis.

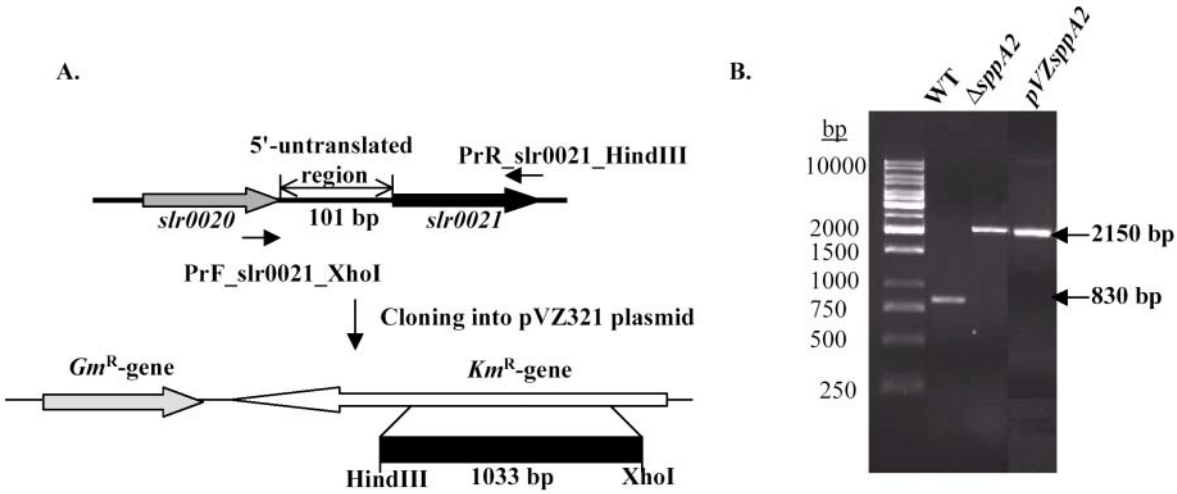


**Figure 47. Construction scheme of the complementation strain for the Δ*sppA2* mutant.** **(A)** Fragment of the *Synechocystis* DNA containing the *sppA2* gene was used for complementation analysis of Δ*sppA2* mutant cells. The DNA fragment containing *slr0021* gene with the 5'-untranslated region was amplified by PCR with primers slr0021_HindIII and slr0021_XhoI. PCR fragment of 1033 bp with introduced restriction sites. The PCR fragment of 1033 bp was digested with HindIII and XhoI and cloned into pVZ321 vector. **(B)** The PCR analysis of wild-type, Δ*sppA2* and *pVZsppA2* strains for possible reverse recombination. The part of DNA containing the *slr0021* gene was amplified using primers slr0021F_comtest and slr0021R_comtest and the resulting PCR products were separated by 0.8% agarose gel electrophoresis. The position of the 1 kb ladder marker is indicated at the left.

During transfer of self replicating plasmid containing the relevant gene the last one can be independently expressed from the vector but can be as well integrate into genomic DNA by reverse recombination. The PCR approach was used to exclude possible reverse recombination between *Synechocystis* chromosomal DNA and the gene fragment cloned into complementation plasmid. For this analysis the primers corresponding to sequences further upstream and downstream of slr0021F_XhoI_com and slr0021R_HindIII_com for amplification of the entire coding region *slr0021* were designed. These primers do not amplify the fragment on the plasmid pVZsppA2 but amplify the fragment with an identical size from the Δ*sppA2* strain. The DNA from wild-type, the Δ*sppA2* and *pVZsppA2* strains

were used for PCR analysis. The size of amplified fragment differs by 1.2 kb that correspond to the *Km*-resistance gene from the amplification product on wild-type DNA (Fig. 47B). The wild-type fragment appears as a 830 bp band, whereas the Δ*sppA2* and *pVZsppA2* fragments possess the same size of 2150 bp.

### 3.2.2.7 Phenotypical characterization of the Δ*sppA2* mutant strain

The analysis of the *sppA2*-deficient strain (Section 3.1.1.1) had demonstrated a slower growth rate and bleaching phenotype under standard growth conditions. However, further adaptation to HL did not show any significant difference in growth rate of Δ*sppA2* in comparison to the wild-type (Fig. 48). To analyze the phenotypical modifications of wild-type and Δ*sppA2* under different light intensities cells grown at LL to the end of the exponential phase were diluted to an $A_{750}$ of 0.4 and then transferred to HL or kept at LL for 46 h. Phenotypical analysis revealed that the Δ*sppA2* exhibited bleaching of culture even when grown under LL but showed less pigment loss in comparison with the wild-type under HL (Fig. 49A). Such changes could be due to differences in pigment content. However, analysis of absorption spectra of wild-type and Δ*sppA2* showed that both strains decreased chlorophyll *a*, phycobiliproteins and carotenoids contents in the same way after transfer to HL (Fig. 46B). Pigment contents were examined in wild-type and Δ*sppA2* strain grown under LL and HL. This demonstrated that Δ*sppA2* had less C-phycocyanin under LL, but more of it under HL. Such differences in ratio between chlorophyll *a* and phycobiliproteins could be responsible for the bleached phenotype of the mutant strain under LL and enhanced pigmentation of mutant cells under HL (Table 10).

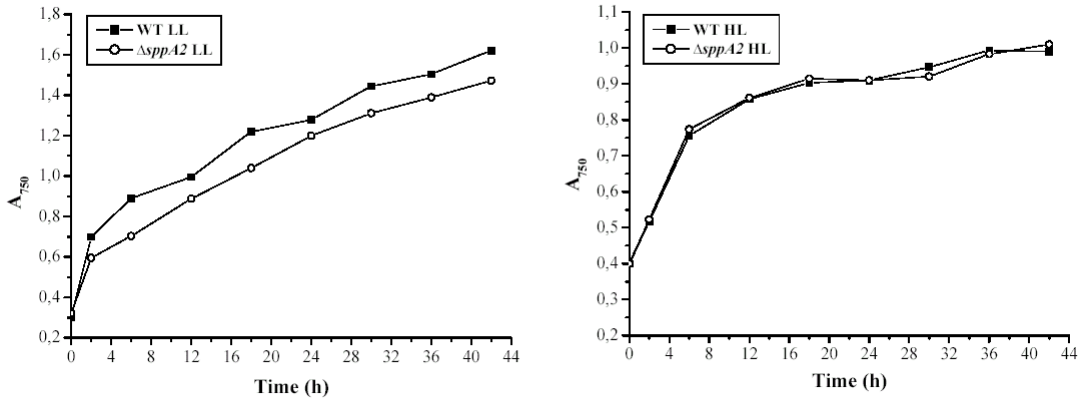


**Figure 48. Growth rate of wild-type and Δ*sppA2* strain under LL and HL.** The cells were grown at LL (right panel) diluted to an $A_{750}$ of 0.4 and transferred to HL (left panel) or kept at LL for 42 h.

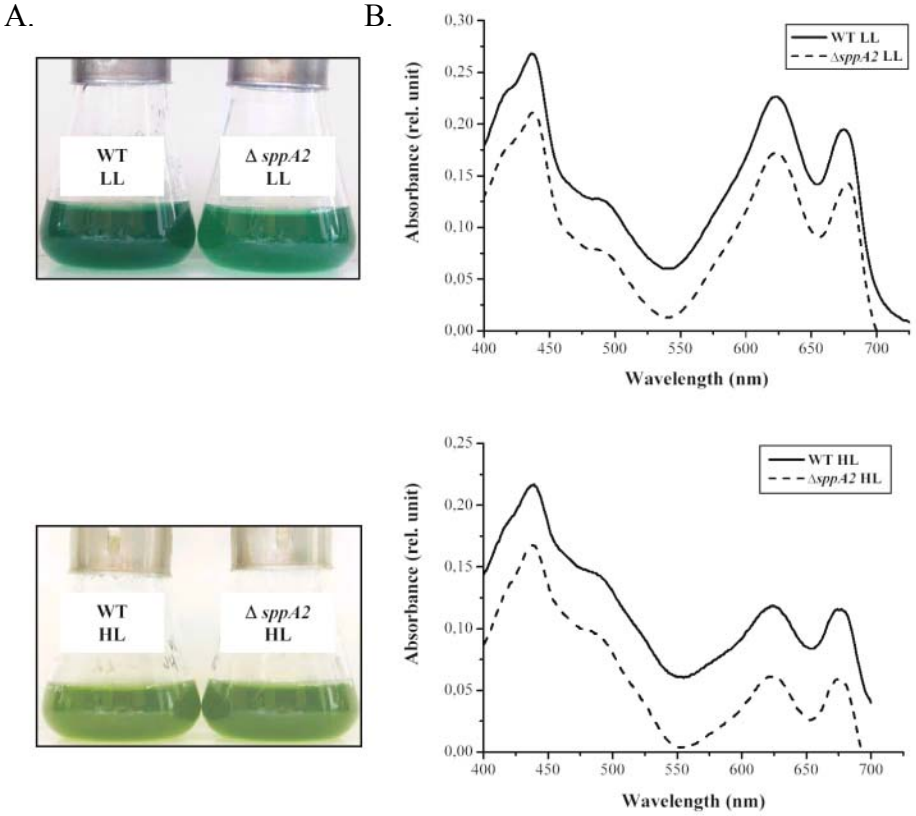


**Figure 49. Characterization of wild-type and Δ*sppA2* under different light regimes.** Cells of both strains were adapted to LL and HL regimes. Phenotypical modification (**A**) and absorption spectra (**B**) were collected after 52 h of exposure to different light intensities.

**Table 10. Pigment contents in wild-type and Δ*sppA2* strains adapted to different light intensities**

| **Pigments** | **WT LL** | **Δ*sppA2* LL** | **WT HL** | **Δ*sppA2* HL** |
|---|---|---|---|---|
| **Chlorophyll *a* (µg/ml)** | 27.5 ± 2.1 | 29.7 ± 2.0 | 24 ±1.5 | 28 ± 2.1 |
| **Phycocyanin (µg/ml)** | 43.9 ± 4.8 | 30.3 ± 1.41 | 20.3 ± 1.88 | 25.2 ± 0.9 |
| **Carotenoid (µg/ml)** | 0.95 ± 0.05 | 0.3 ± 0.09 | 0.57 ± 0.031 | 0.2 ± 0.07 |

Both strains were grown under LL conditions to an $A_{750}$ of 0.5 at LL or HL. The pigment concentrations were measured after 52 h of incubation. The data presented are from four determinations.

### 3.2.2.8 Analysis of protein synthesis in Δ*sppA2* under LL and HL regimes

Since the SppA2-deficient mutant showed a slower growth rate than wild-type under LL and no differences upon adaptation to HL an analysis of protein synthesis rate was performed. For this, the cells of wild-type and Δ*sppA2* cells were labeled with $S^{35}$-methionine for 40 min in DL and then transferred to the LL and HL for 3 h. The thylakoid membranes were extracted from the cells taken after every 1 h of labeling and separated on the SDS-PAGE (Fig. 50). The

analysis did not reveal any significant difference in protein synthesis of Δ*sppA2* strain in comparison with wild-type under both growth conditions.

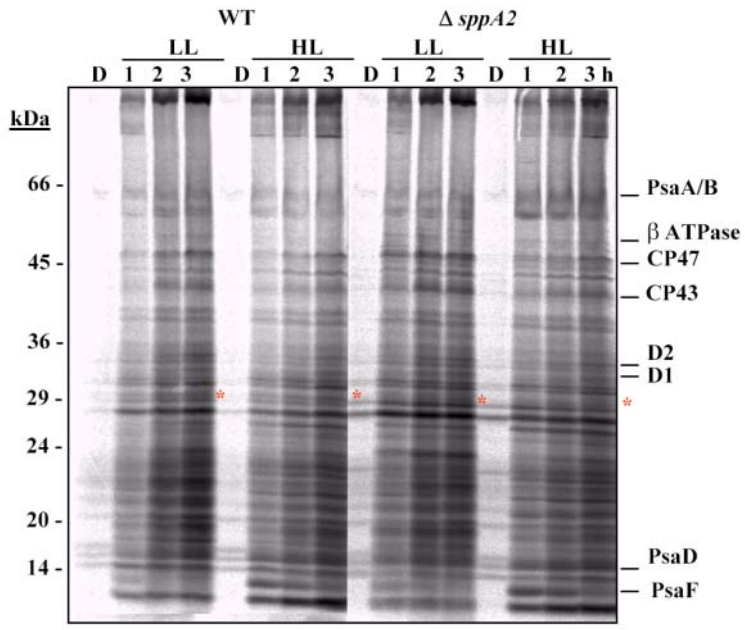


**Figure 50.** Pulse-labeling of proteins with $S^{35}$-methionine under different light regimes. The cells of wild-type and Δ*sppA2* mutant were incubated with $S^{35}$-methionine for 40 min under DL, then the cells were transferred to LL and HL for 1, 2 and 3 h. After labeling the cells were centrifuged, denaturated in sample buffer and the proteins separated by 10 - 17.5% sucrose containing SDS-PAGE. The gel was dried and exposed overnight. The position of molecular weight marker is indicated at the left. The positions of proteins from photosystem I (PsaA/B; PsaF and PsaD) and photosystem II (CP47, CP43, D1 and D2) were detected by Western analysis and shown at the right.

### 3.2.2.9 Biochemical analysis of Δ*sppA2* thylakoid proteins under LL and HL regimes

The steady state level of proteins in wild-type and Δ*sppA2* mutant after adaptation to different light intensities was examined. The cells grown under LL were transferred to HL for 72 h. Thylakoid membrane proteins were separated on denaturing 10 - 17.5 % SDS-PAGE and stained by silver. The protein stained analysis showed higher content of phycobiliproteins and other proteins in molecular weight range of 36 and 45 kDa in the Δ*sppA2* samples in comparison to the wild-type (Fig. 51).

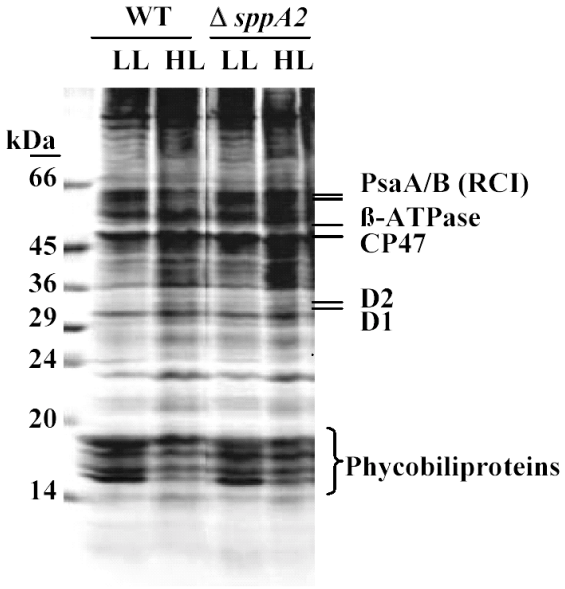


**Figure 51.** Protein profile of wild-type and Δ*sppA2* mutant acclimated to LL and HL. Wild-type and Δ*sppA2* strains were adapted under LL and HL for 36 h. Thylakoid proteins were separated by 10 - 17.5% SDS-PAGE. Proteins were revealed by silver staining. The position of main proteins of ATP synthase (ß subunit), photosystem I (PsaA/B), photosystem II (D1, D2 and CP47) and phycobiliproteins were detected by Western analysis and shown at the right of figure. Molecular weight in kDa is indicated at the left.

To check the content of the major photosynthetic complexes in the Δ*sppA2* mutant in comparison to *Synechocystis* wild-type, thylakoid proteins extracted from cells adapted to LL and HL were tested with antisera against various photosynthetic proteins. In wild-type cyanobacterial cells general response to HL acclimation exhibited the reduction of PBS and degradation of PSII and PSI complexes. No differences in protein amounts from all photosynthetic complexes were observed in the Δ*sppA2* mutant compare to the wild-type (Fig. 52). However, Δ*sppA2* mutant contained slightly more of phycobiliproteins under HL.

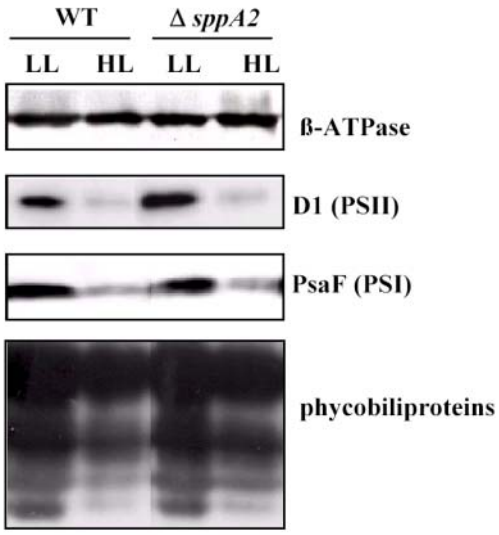


**Figure 52. Western analysis of wild-type and Δ*sppA2* mutant adapted to LL and HL.** Thylakoid proteins from wild-type and Δ*sppA2* mutant were separated on the 12% SDS-PAGE, transferred onto nitrocellulose membrane and incubated with antisera against proteins of ATP synthase (ß subunit), photosystem I (PsaA/B), photosystem II (D1, D2 and CP47) and phycobiliproteins

### 3.2.2.10 Characterization of wild-type and Δ*sppA2* strains after recovery from HL to LL

Physiological analysis revealed that the Δ*sppA2* strain exhibited bleaching of cells and a slower growth rate even under standard light conditions. The detection of SppA2 under LL but not under HL proved that this protease is important for LL and recovery from HL to LL. These data suggest that SppA2 can be involved in turnover of proteins essential under HL but not required under LL conditions.

Photoprotection mechanism of cyanobacteria evolves different ways including the synthesis and recruitment of enzymes essential under stress conditions with followed degradation of that after recovery to the standard conditions. One group of proteins that accumulate upon exposure to HL (as well as under other stress conditions, e.g. cold stress, sulfur and nitrogen deprivation) is the HLIP (**h**igh **l**ight **i**nducible **p**roteins; He et al., 2001). These low molecular weight polypeptides are requires for survival and acclimation of cells to the absorption of excess of light energy, and perhaps could bind and store free chlorophyll specifically when cells are absorbing excess excitation (Havaux et al., 2003). Accumulation of HLIP occurred within 1 h of transfer to HL with maximum peak in abundance over 24 h of light stress and accompanied by decreasing of photosynthetic activities of cells. All HLIP proteins are rapidly degraded during first the hours of recovery of cells from excess excitation (He et al., 2001).

To check whether SppA2 could be involved in the recovery step, wild-type and Δ*sppA2* strains were characterized after exposure to LL and HL, and after recovery of HL treated cells to LL.

#### 3.2.2.10.1 Photosynthetic activity of cells under different light regimes

The photosynthetic activity of the cells was estimated by measuring the $O_2$ evolution under different light regimes with the Clark electrode. Cells of wild-type and Δ*sppA2* strains grown at LL were exposed to HL for 24 h and then after transferred back to LL for further 24 h. The samples for $O_2$ measurements were taken and diluted to a chlorophyll *a* concentration of 2 µg/ml. As shown in Fig. 53, photosynthetic $O_2$ evolution in mutant cells was lower than that measured in the wild-type already under LL. However, the strains adapted to HL showed roughly identical activity of $O_2$ evolution. Measuring the $O_2$ evolution after recovery from HL

to LL showed that both strains could restore photosynthetic activities to LL level, however, in mutant cells this process occurred slower as in wild-type.

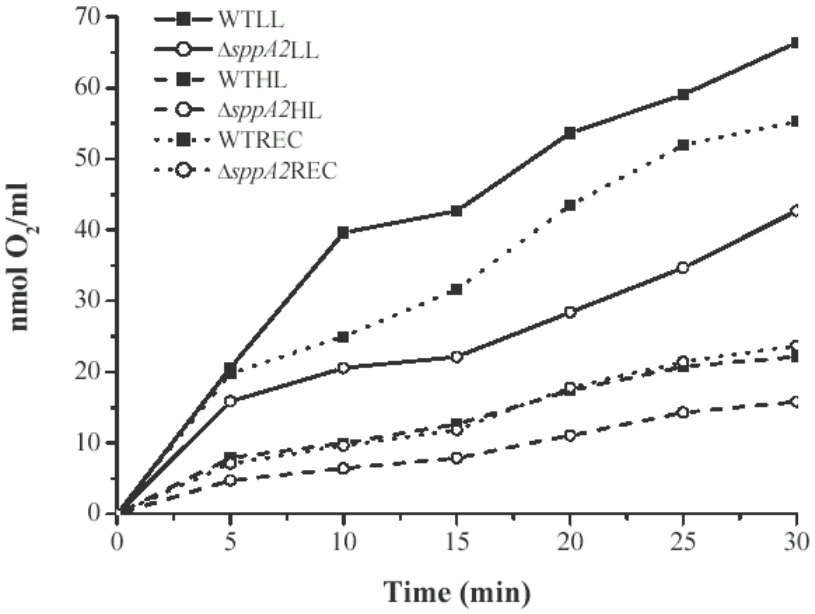


**Figure 53.** Effect of different light intensities on photosynthetic $O_2$ evolution in wild-type and Δ*sppA2* strains. For the measurements cells of both strains from different light regimes were concentrated till chlorophyll concentration of 2 µg/ml. Net $O_2$ evolution of cells was measured with white light of photon flux density 50 µmol $m^{-2}$ $s^{-1}$.

### 3.2.10.2 Biochemical analysis of Δ*sppA2* thylakoid proteins under LL and HL regimes

Figure 54 shows comparison of thylakoid membrane proteins from wild-type and Δ*sppA2* strains grown under LL, HL and recovery from HL to LL regimes. The separation of proteins on denaturing SDS-PAGE and following silver staining revealed accumulation of unknown low molecular weight polypeptides in the Δ*sppA2* as well as in the wild-type under HL. However, in contrast to the wild-type these polypeptides remained stable in the mutant strain after recovery from HL to the LL. The accumulation of polypeptides with molecular masses between 4 and 6 kDa was observed only under HL in the wild-type cells that assumed the light-inducible expression of these proteins.

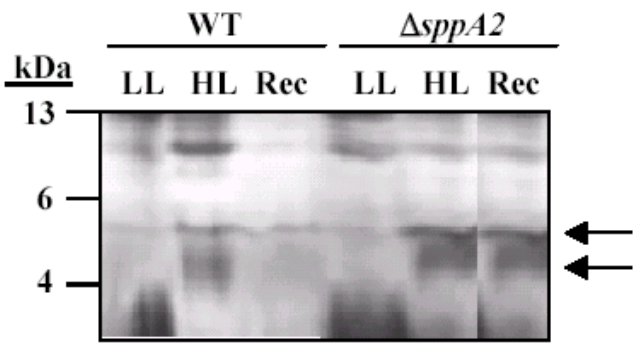


**Figure 54. Separation of thylakoid membrane polypeptides from wild-type and Δ*sppA2* strains grown at different light regimes**. Cells of both strains were grown at LL, transferred to the HL and then cells were recovered back to LL for 24 h (Rec). Proteins were revealed by silver staining. Arrows indicate the positions of two unknown polypeptides, which are absent in wild-type cells after recovery from HL to LL.

# 4. DISCUSSION

## 4.1 Comparison of the protease complement in cyanobacteria and *Arabidopsis* chloroplast

Peptidases, protein kinases, protein phosphatases and chaperones are major regulatory key players in structuring, adapting and maintaining cells under everchanging or even harmful conditions. A set of 62 genes that encodes the entire peptidase complement of *Synechocystis* has been identified in the genome database of that bacterium (Kaneko et al., 1996; Sokolenko et al., 2002). The impact of peptidases for cellular homeostasis becomes apparent especially after impairing proteolytic processes which can lead to drastic alterations in cellular physiology (Kirschner, 1994; Kopito and Sitia, 2000). It is therefore not surprising that work in the genomic era has uncovered a relatively high number of such enzymes and that autotrophic and heterotrophic organisms, despite differences in cell structure and life form, share a basic set of peptidases due to common phylogenetic roots.

Chloroplasts are proposed to derive from an endosymbiotic event between a cyanobacterial and a heterotrophic eukaryotic cell. During the evolution of the resulting plant cell, the genetic potentials of these cell conglomerates were massively rearranged to yield an integrated, compartmentalized genetic system (summarized by Herrmann 1997; Race et al. 1999; Herrmann and Westhoff, 2001). This genomic restructuring included an intracellular translocation of genes, especially from the organelles to the nucleus. It is therefore not too surprising that the proteolytic systems in plastids and mitochondria, although predominantly of nuclear/cytoplasmic origin, relate to those of the respective ancestors, a cyanobacterial-like cell in the former, an α-proteobacterial-like cell in the latter case (Gray et al. 2001), and hence they can at least in part be derived from eubacterial genomic data.

The analysis of the gene complements for peptidases in *Synechocystis* and in chloroplasts revealed a high homology of their proteolytic machinery. The functional and structural analysis of bacterial peptidases showed that some of them represent homo- or heteromeric complexes (Clp, FtsHi and Deg peptidases) of proteolytically, non-proteolytically active and/or regulatory subunits. Although, the exact number of such isoforms in cyanobacteria and *Arabidopsis* is not known, it is possible to calculate the number of peptidase subunits, the homologues that lost their catalytic domains (ClpR and FtsH), and the regulatory subunits that are necessary for activity of a complex. Based on computational and experimental analyses, at

least 49 chloroplast peptidase subunits and their homologous components of bacterial origin were identified in *Arabidopsis thaliana*, used as a model with a fully sequenced nuclear genome. This number is close to that found in the *Synechocystis* genome, which encodes 62 genes for peptidase subunits. This is an intriguing feature of the chloroplast degradation machinery, given the fact that some additional non-cyanobacterial or even non-bacterial peptidases are present in the organelle (e.g. the stromal processing peptidase: VanderVere et al., 1995; Lon: Adam et al., 2001; aspartyl peptidases: A. Sokolenko, unpublished data). Obviously, some of the metabolic functions found in cyanobacteria and controlled by bacterial-type peptidases were either transferred to the nucleo-cytoplasmic compartment in *Arabidopsis* or simply lost, as in the case of the genes controlling the biogenesis of pili, cell walls, or cyanophycin aggregates. The *Arabidopsis* genome lacks some peptidase genes that are found in *Synechocystis*, such as the D-Ala-D-Ala carboxypeptidases. However, the remarkable complexity of the peptidase complement in the chloroplast, which probably exceeds 49 polypeptides subunits, is probably caused by new regulatory functions as a consequence of endosymbiosis. Among these is the need to ensure protein import into the organelle from the nucleo-cytoplasmic compartment. Remarkably enough, the family of peptidases that is expected to serve this function, i.e. processing peptidases, shows a significant reduction in number in *Arabidopsis*. Another chloroplast-based regulation that requires specific proteolytic function is the delivery of nuclear-encoded protein factors, which provide fine-tuning of chloroplast gene expression. This set of proteins has been estimated to comprise approximately several hundred members (Herrmann, 1997; Rochaix, 2001; Herrmann and Westhoff, 2001) that act at the post-transcriptional level on specific chloroplast transcript targets. Because of their regulatory function, they are most likely present in limiting amounts (Green-Willms et al., 2001) and therefore are probably short-lived proteins that mobilize specific peptidases. The peptidases involved in the specific degradation of these proteins are unknown.

The characterization of the *Arabidopsis* peptidase families of cyanobacterial origin showed that, in many cases, these originate in gene duplications that are likely to reflect evolutionary divergence in substrate specificity and final subcellular localization. Three peptidase families show a particularly high number of such genes: (1) the Clp family which has 8 members in *Synechocystis*, but up to 15 chloroplast-located subunits in *Arabidopsis* (Table 11), (2) the Deg family, which possesses only 3 members in *Synechocystis*, but 14 in *Arabidopsis*, with about 6 members for each type of organelle and 2 members in the cytoplasm (Table 12), and

(3) the FtsH family with 4 genes in *Synechocystis* (encoding only FtsH subunits), but at least 12 (FtsH) chloroplast members (Table 13).

**Table 11. Characterization of the Clp protease family in *Synechocystis* and *Arabidopsis***

| Gene | Protein | Protein function | Peptidase type | Predicted localization | Homologous components from *A. thaliana.* | | |
|---|---|---|---|---|---|---|---|
| | | | | | Gene number | Predicted localization | Identity to *Syn*[c] % |
| *slr0542* | ClpP1 | Proteolytic Clp subunit | Serine | Cyt | pClpP (ClpP1)[a] | C, S[c] | 46 |
| | | | | | At5g23140 (ClpP2)[a] | C, M[c] | 52 |
| | | | | | At1g66670 (ClpP3)[a] | C, S[c] | 51 |
| | | | | | At5g45390 (ClpP4)[a] | C, S[c] | 46 |
| | | | | | At1g02560 (ClpP5)[a] | C, S[c] | 62 |
| *sll0534* | ClpP2 | Proteolytic Clp subunit | Serine | Cyt | At1g11750 (ClpP6)[a] | C, S[c] | 39 |
| *slr0165* | ClpP3 | proteolytic Clp subunit | Serine | Cyt | At1g12410 (ClpR2)[a] | C, S[c] | 44 |
| *slr0164* | ClpR (ClpP4) | Clp subunit | Serine | Cyt | At1g49970 (ClpR1)[a] | C, S[c] | 37 |
| | | | | | At1g09130 (ClpR3)[a] | C, S[c] | 41 |
| | | | | | At4g17040 (ClpR4)[a] | C, S | 28 |
| *sll0535* | ClpX | Non-peptidase Clp subunit | - | Cyt | At5g53350 (ClpX1)[a] | M, Mat | 43 |
| | | | | | At5g49840 (ClpX2)[a] | M, Mat | 38 |
| | | | | | At1g33360 (ClpX3) | M, Mat | 42 |
| *sll0020* | ClpC | Non-peptidase Clp subunit | - | Cyt | At5g50920 (ClpC1)[a] | C, S | 77 |
| | | | | | At3g48870 (ClpC2)[a] | C, S | 77 |
| | | | | | At5g51070 (ClpD)[a] | C, S[c] | 44 |
| | | | | | At3g45450 (ClpF)[b] | Cyt | 46 |
| *slr0156* | ClpB1 | ClpB protein | - | Cyt | At2g25030 (ClpZ)[b] | Cyt | 42 |
| *slr1641* | ClpB2 | ClpB protein | - | Cyt | At1g74310 (ClpB1)[b] | Cyt | 51 |
| | | | | | At2g25140 (ClpB2)[b] | Cyt | 61 |
| | | | | | At5g15450 (ClpB3)[b] | C/Cyt | 65 |
| | | | | | At4g14670 (ClpB4)[b] | Cyt | 48 |
| Non-cyanobacterial homologous Clp subunits | | | | | | | |
| - | - | - | - | - | At4g25370 (ClpS1)[b] | C, S[c] | - |
| | | | | | At4g12060 (ClpS2)[b] | C, S | - |
| | | | | | 6728865 (Clp-like 1)[b] | Cyt | - |
| | | | | | At3g53270 (Clp-like 2)[b] | Cyt | - |

[a] proteins annotated in Adam et al. (2001)

[b] proteins annotated in Peltier et al. (2001)

[c] identity score indicates the highest alignment to one of *Synechocystis* homologue

Abbreviations: *C*, chloroplast; *S*, chloroplast stroma; *M*, mitochondria; *Mat*, mitochondrial matrix; *Cyt*, cytoplasm; *A. thaliana*, *Arabidopsis thaliana*; *Syn.*, *Synechocystis*

**Table 12. Characterization of the Deg protease family in *Synechocystis* and *Arabidopsis***

| Gene | Protein | Protein function | Peptidase type | Probable localization | Homologous components from *A. thaliana.* | | |
|---|---|---|---|---|---|---|---|
| | | | | | Gene number | Probable localisation | Identity to *Syn*[b] % |
| *slr1204* | HtrA (DegP) | Heat-shock peptidase | Serine | L | At5g36950 (DegP10)[a] | M, Mat | 17 |
| | | | | | At3g27925 (DegP1)[a] | C, $TM_L$[c] | 30 |
| *sll1679* | HhoA (DegQ) | Heat-shock peptidase | Serine | Periplasm[b] | At2g47940 (DegP2)[a] | C, $TM_S$[c] | 18 |
| | | | | | At1g65630 (DegP3)[a] | M, Mat | 16 |
| | | | | | At1g65640 (DegP4)[a] | M, Mem | 17 |
| | | | | | At4g18370 (DegP5)[a] | C, L[c] | 22 |
| | | | | | At1g51150 (DegP6)[a] | M/C | 13 |
| | | | | | At3g03380 (DegP7)[a] | Cyt | 13 |
| | | | | | At5g39830 (DegP8)[a] | C, L[c] | 24 |
| | | | | | At5g40200 (DegP9)[a] | C/N | 18 |
| | | | | | At5g40560 (DegP13)[a] | ER/P | 13 |
| | | | | | At5g27660(DegP14) | C/M | 15 |
| | | | | | At3g16540 (DegP11)[a] | M, Mem | 15 |
| *sll1427* | HhoB (DegS) | Heat-shock peptidase | Serine | Mem | At3g16550 (DegP12)[a] | M, Mat | 12 |

**Table 13. Characterization of FtsHi family in *Synechocystis* and *Arabidopsis***

| Gene | Protein | Protein function | Peptidase type | Probable localization | Homologous components from *A. thaliana.* | | |
|---|---|---|---|---|---|---|---|
| | | | | | Gene number | Probable localization | Identity to *Syn*[b] % |
| *slr1390* | FtsH1 | Cell division protein FtsH | Metallo- | Membrane | At3g02450 (FtsHi3) | C, Mem | 28 |
| *slr0228* | FtsH2 | Cell division protein FtsH | Metallo- | Membrane | At2g30950 (FtsH2)[a] | C, Mem[c] | 60 |
| | | | | | At5g15250 (FtsH6)[a] | C, Mem | 55 |
| | | | | | At1g06430 (FtsH8)[a] | C, Mem | 58 |
| | | | | | At5g53170 (FtsH11) | C/Others | 55 |
| | | | | | At5g15250 (FtsH12) | C, Mem | 55 |
| *slr1604* | FtsH3 | Cell division protein FtsH | Metallo- | Membrane | At5g64580 (FtsHi4) | C, Mem | 30 |
| | | | | | At1g50250 (FtsH1)[a] | C, Mem[c] | 60 |
| | | | | | At2g29080 (FtsH3)[a] | M, Mem | 36 |
| | | | | | At2g26140 (FtsH4) | Others | 38 |
| | | | | | At5g42270 (FtsH5)[a] | C, Mem | 61 |
| | | | | | At4g23940 (FtsHi1) | C, Mem | 30 |
| | | | | | At3g16290 (FtsHi2) | C, Mem | 30 |
| *sll1463* | FtsH4 | Cell division protein FtsH | Metallo- | Membrane | At3g47060 (FtsH7)[a] | C, Mem | 40 |
| | | | | | At5g58870 (FtsH9) | C, Mem | 39 |
| | | | | | At1g07510 (FtsH10) | M, Mem | 39 |

[a] proteins annotated in Adam et al. (2001)

[b] identity score indicates the highest alignment to one of *Synechocystis* homologue

[c] the localisation of these proteins was proven experimentally (see the text)

Abbreviations: *C*, chloroplast; $TM_S$, thylakoid membrane protein associated from stroma side; $TM_L$, thylakoid membrane protein associated from lumenal side; *S*, chloroplast stroma; L, thylakoid lumen; M, mitochondria; Mat, mitochondrial matrix; Mem, membrane; Cyt, cytoplasm; N, nucleus; PM, plasma membrane; ER, endoplasmic reticulum; *A. thaliana*, *Arabidopsis thaliana; Syn, Synechocystis*

In contrast, other peptidase families have fewer members in the chloroplast than in cyanobacteria, e.g. processing peptidases with 18 cyanobacterial components and only 8 plastid members, or aminopeptidases with 25 in *Synechocystis* and 9 chloroplast components (Tables 14 and 15). It is tempting to relate the opposite variation in the sizes of these peptidase families between chloroplasts and cyanobacteria to physiological changes. The high number of processing peptidases and aminopeptidases in a prokaryote-like *Synechocystis* may reflect its critical dependence on secretory pathways for sensing and adapting to environment. In contrast, the chloroplast appears to have no secretory protein pathway directed to the cytoplasm but has become an intracellular entity mainly devoted to photosynthesis, with a substantial development of its inner, photosynthetic membrane and metabolite exchange systems. The organelle contains approximately ten times more thylakoid membranes per unit volume than a cyanobacterium. This expansion and divaricating of the thylakoid membranes may explain in part why chloroplasts need a larger set of managing peptidases for membrane-associated proteins, as is the case for the members of the FtsH, Deg and Clp families.

An important finding is that the large numbers of paralogues in most peptidase families of bacterial origin are not a matter of functional redundancy (Sokolenko et al., 2002). This finding is at variance with the general view on most *Arabidopsis* multigenic families, whose members are thought to be able to substitute each other. In the case of peptidases, it was noted that several homologous components confined to one compartment - as is the case for various ClpP and FtsH products - cannot functionally replace each other. In other instances, related components are addressed to different subcellular compartments in *Synechocystis*, like lumenal HtrA versus periplasm, i.e. HhoA and membrane-intrinsic HhoB. In *Arabidopsis*, the situation is sometimes even more complicated, as for the Deg family. Its members are located in different organelles and subcompartments. Based on sequence prediction programs, it was found that most peptidases of cyanobacterial origin in plants are confined to the chloroplast, with a few exceptions, like the soluble processing metallo-peptidases, which are excluded from the chloroplast but are present in mitochondria and cytoplasm. At first glance, this latter observation is paradoxical in view of the cyanobacterial origin of the chloroplast. However, the low sequence conservation between the *Arabidopsis* mitochondrial members of the soluble processing metallo-peptidase family and those in *Synechocystis* raises the possibility that plastids have acquired this type of peptidase gene by lateral gene transfer from an α-proteobacterial source that was the ancestor of mitochondria (Gray et al., 2001). One should also wonder about the possible presence of new *Arabidopsis*

**Table 14. Characterization of processing peptidases from *Synechocystis* and *Arabidopsis***

| Gene | Protein | Protein function | Peptidase type | Predicted localization | Homologous components from *A. thaliana* | | |
|---|---|---|---|---|---|---|---|
| | | | | | Gene number | Predicted localization | Identity to *Syn*[b] % |
| Ctp family | | | | | | | |
| *slr0008* | CtpA | Carboxyl-terminal processing peptidase | Serine | Per[a] | At3g57680 | C, L[a] | 30 |
| | | | | | At4g17740 | C, L[a] | 36 |
| *slr0257* | CtpB | Carboxyl-terminal processing peptidase | Serine | Per[a] | - | - | - |
| *slr1751* | CtpC | Carboxyl-terminal processing peptidase | Serine | Per[a] | At5g46390 | C, L[a] | 28 |
| Soluble processing metallo-peptidases | | | | | | | |
| *sll2008* | Prp1 | Processing peptidase | Metallo- | Cyt | At5g56730 | Cyt | 15 |
| | | | | | At3g02090 | M, Mat[a] | 20 |
| *sll2009* | Prp2 | Processing peptidase | Metallo- | Cyt | - | - | - |
| *sll0055* | Prp3 | Processing peptidase | Metallo- | Cyt | At3g16480 | Cyt | 18 |
| | | | | | At1g51980 | M, Mat[a] | 18 |
| *slr1331* | YmxG | Processing peptidase | Mettalo- | Per[a] | - | - | - |
| *sll0915* | PqqE | Processing peptidase | Metallo- | Per[a] | - | - | - |
| Transmembrane processing metallo-proteases | | | | | | | |
| *sll0528* | - | Hypothetical | Metallo- | Mem | - | - | - |
| *slr1821* | - | - | Metallo- | Mem | At1g05140 | C, Mem | 39 |
| | | | | | At2g32480 | C, Mem | 40 |
| Leader peptidases | | | | | | | |
| *sll0716* | LepB1 | Leader peptidase I | Serine | Cyt | At1g06870 | C, Mem | 24 |
| *slr1377* | LepB2 | Leader peptidase I | Serine | Cyt | - | - | - |
| *slr1366* | LspA | Leader peptidase II | Aspartic | Mem | not present in higher plants | | - |
| *slr0807* | Gcp | Putative glycopeptidase | Aetallo- | Cyt | At2g45270 | C/M | 27 |
| | | | | | At4g22720 | Cyt | 28 |
| SppA peptidase family | | | | | | | |
| *sll1703* | SppA1 | Protease IV | Serine | Mem | At1g73990 | C, TM[a] | 29 |
| *slr0021* | SppA2 | Protease IV | Serine | Cyt | not present in higher plants | | - |
| Prepilin peptidase family | | | | | | | |
| *slr1120* | HofD | Prepilin peptidase | Aspartic | Mem | not present in higher plants | | - |
| *sll0146* | - | Non-peptidase | - | Mem | | | |

[a] the localization of these proteins was proven experimentally (see the text)

[b] identity score indicates the highest alignment to one of *Synechocystis* homologue

Abbreviations: *C*, chloroplast; *Mem*, membrane; *Mat*, matrix; *TM*, thylakoid membrane; *Cyt*, cytoplasm; n.d., not done; *A. thaliana*, *Arabidopsis thaliana*; *Syn*, *Synechocystis*

**Table 15. Characterization of aminopeptidases from *Synechocystis* and *Arabidopsis***

| Gene | Protein | Protein function | Peptidase type | Predicted localization | Homologous components from *A. thaliana* | | |
|---|---|---|---|---|---|---|---|
| | | | | | Gene number | Probable localization | Identity to *Syn*[b] % |
| *sll1343* | Ape2 | Alanine AP | Metallo- | Cyt | not present in higher plants | | - |
| *slr1939* | HtpX | AP | Metallo- | Mem | not present in higher plants | | - |
| *sll1280* | - | AP | Metallo- | Me | At3g27110 | C, S | 46 |
| | | | | | At5g51740 | M, Mat | 15 |
| | | | | | At4g01320 | Cyt, Mem | 18 |
| *sll2001* | Lep | Leucine AP | Metallo- | Cyt | At2g24200 | Cyt | 45 |
| | | | | | At4g30920 | Cyt | 45 |
| | | | | | At4g30910 | Cyt | 44 |
| *slr0031* | - | Glycyl-AP | Metallo- | Cyt | not present in higher plants | | - |
| *slr0918* | PepM | Methionyl AP A | Metallo- | Cyt | At2g45240 (MAP1A) | Cyt[a] | 30 |
| | | | | | At1g13270 (MAP1B) | C, S[a] | 43 |
| | | | | | At3g25740 (MAP1C) | C and M[a] | 35 |
| | | | | | At4g37040 (MAP1D) | C and M[a] | 46 |
| | | | | | At2g44180 (MAP2A) | Cyt[a] | 46 |
| | | | | | At3g59990 (MAP2B) | Cyt[a] | 39 |
| *slr0786* | PepM | Methionyl AP B | Metallo- | Cyt | - | - | - |
| *sll0555* | - | Methionyl AP C | Metallo- | Cyt | - | - | - |
| *sll0136* | PepP | Methionyl-AP | Metallo- | Cyt | At1g09300 | Cyt | 29 |
| | | | | | At4g29500 | C, S | 20 |
| *sll0422* | - | Asparaginase | Threonine | Cyt | At3g16150 | Cyt | 29 |
| | | | | | At5g08100 | Cyt | 27 |
| | | | | | At4g00590 | Cyt/M | 18 |
| | | | | | At5g61540 | Secr | 20 |
| *slr1653* | Ama | N-acyl-L-amynoacyl-hydrolase | Metallo- | Cyt | At1g51760 | Secr, Mem | 35 |
| | | | | | At5g56650 | Secr, Mem | 35 |
| | | | | | At1g51780 | ER, Mem | 33 |
| | | | | | At5g56660 | ER | 35 |
| *sll0100* | - | Hydrolase | Metallo- | Cyt | At3g02875 | Secr | 34 |
| *slr1269* | Ggt | Gamma glutamyl-transferase | Threonine | Cyt | At4g39650 | Secr | 26 |
| | | | | | At4g39640 | Secr | 25 |
| | | | | | At4g29210 | Cyt | 23 |
| | | | | | At5g61540 | Secr | 15 |
| *slr0878* | - | Hypothetical | Metallo- | Cyt | not present in higher plants | | - |
| *slr0993* | NlpD | - | Metallo- | Cyt | not present in higher plants | | - |
| *sll0757* | PurF | Glutamine aminotransferase | Cysteine | Cyt | At4g34740 | C, S | 42 |
| | | | | | At2g16570 | C, S | 42 |
| | | | | | At4g38880 | C, S | 41 |
| *sll0236* | - | CP A | Metallo- | Cyt | not present in higher plants | | - |
| *slr1924* | - | D-Ala-D-Ala CP | Serine | Cyt | not present in higher plants | | - |
| *slr0646* | DacB | CP | Serine | Cyt | not present in higher plants | | - |
| *slr0804* | DacB | CP | Serine | Cyt | not present in higher plants | | - |
| | | | | | not present in higher plants | | - |
| *sll1369* | - | CP | Serine | Cyt | not present in higher plants | | - |
| *slr1679* | - | Dipeptidase | Metallo- | Cyt | not present in higher plants | | - |
| *slr0535* | Spr | Tripeptidyl-peptidase | Metallo- | Mem | At3g14067[b] | Secr | 19 |
| | | | | | At5g67360[b] | Secr | 19 |
| *slr0825* | - | OP | Serine | Cyt | At5g36210 | C, S | 44 |

[a] localisation of these proteins was proven experimentally (see the text)

[b] these sequences are given as an example of the cucumusin type peptidases that shows low identity score (up to 19%) to Spr peptidase; the family include about 45 members and all of them are targeted to secretory pathway

Abbreviations: *C*, chloroplast, *S*, chloroplast stroma; *L*, thylakoid lumen; *M*, mitochondria; *Mat*, mitochondrial matrix; *Mem*, membrane; *Cyt*, cytoplasm; *ER*, endoplasmic reticulum; *Secr*, secretory; *A. thaliana*, *Arabidopsis thaliana*; Syn, *Synechocystis; AP*, aminopeptidase; *OP*, oligopeptidase; *CP*, carboxypeptidase

chloroplast-specific peptidases not present in *Synechocystis*. Such candidates could be the thylakoid-processing peptidase known as thylakoid-processing enzyme (Chaal et al., 1998), stromal-processing peptidase (CPE: VanderVere et al., 1995; Koussevitzky et al., 1998) and two bacterial Lon homologues that are predicted to be chloroplast-located components (Adam et al., 2001). Preliminary analysis of the *Arabidopsis* database revealed some examples of aspartate peptidases of eukaryotic origin that are not present in the cyanobacterial genome and might have been targeted to the chloroplast (A. Sokolenko, unpublished data). Further experimental analysis of this peptidase group is required. The continuously appearing data on substrate-specificity and mechanisms of peptidases from different organisms will aid in obtaining a more complete view of the diversity, divergence and interaction of the various peptidases, including their evolution.

A systematic gene disruption approach was chosen to individually inactivate, by customary transformation strategies, the majority of the cyanobacterial genes encoding peptidase subunits that are related to chloroplast enzymes (Shestakov and Reaston, 1987; Thiel, 1994). The set of genes in the *Synechocystis* genome that codes for proteolytic enzymes was determined and searched for their homologues in the *Arabidopsis* genome in order to gain insight into the function, intracellular location and evolution of plastid peptidases. Afterwards, the deduced cyanobacterial peptidase genes were grouped into families of homologous enzymes (Tables 8 - 12) and then various genes were inactivated for this study (Table 5; Section 3.1).

Each cell of *Synechocystis* contains about 12 identical copies of the chromosome and, thus, genes can be randomly inactivated to some extent with (or without) detectable changes in phenotype. The ability of *Synechocystis* cells for the photoheterotrophic growth provided the opportunity for screening of mutants previously not obtained in other organisms. It allowed a distinction to be made between those peptidases that are required for cell viability and those that can be functionally complemented by other peptidases or are involved in stress acclimation processes. Those essential for cell survival in *Synechocystis* are members of the Clp family (ClpP1, ClpP3, ClpR, ClpC, ClpX and ClpB1), several FtsH components (FtsH1 and FtsH3), HtrA, LepB1 and CtpC (Bailey et al., 2001; Ivleva et al., 2002; Sokolenko et al., 2002; also see Tables 8, 9, 10 and 11). Many of the homologous peptidases within each gene family exert a distinct intracellular function. Inactivation of the plastid-encoded ClpP, the homologue of *Arabidopsis* ClpP1, did not lead to a homoplastomic mutant, neither in tobacco

(Shikanai et al., 2001), nor in *Chlamydomonas* (Huang et al., 1994). Two cyanobacterial peptidases, ClpP2 and CtpA, are critical for the function of the photosynthetic machinery since after knock-out of corresponding genes, the cells were not able to grow under phototrophic conditions (Shestakov et al., 1994; Panichkin et al., 2001; Sokolenko et al., 2002). Whereas the essential function of CtpA in the maturation of subunit D1 of the PSII reaction center has long been acknowledged (Shestakov et al., 1994), the function of ClpP2 in photosynthesis remains to be studied. Only two fully homozygous knock-out mutants were obtained after insertional inactivation of *hhoA* and *hhoB* from Deg family in this work. Although fully segregated HtrA mutant could apparent be obtained by another group, which showed the resistance of *Synechocystis* cells to light stress (Silva et al., 2002), no full segregation of the knock-out strain was reached for HtrA under the chosen experimental conditions (Sokolenko et al., 2002). Such distinction might reflect differences of specific strains. Therefore, from this work was observed that *htrA* gene is also essential for cell viability, as are *clpC*, *clpX* and *clpB1*. Two genes encoding metallo-aminopeptidases, the alanine (Ape2) and methionine (PepP) aminopeptidases in *Synechocystis* were successfully inactivated. The presence of four genes encoding methionine aminopeptidases in *Synechocystis* probably explains the non-lethal phenotype of the knock-out strains, which is at variance with the results from gene inactivation of this enzyme in *E. coli* (Chang et al., 1989) and yeast (Li and Chang, 1995).

### 4.2 Role of peptidases in the adaptation of thylakoid membranes to environmental stress

One of the principal roles of peptidases is the regulation of intracellular processes during or following environmental changes. Changes in light quality and intensity, temperature or nutrient supply generally lead to a reorganization of cell morphology, metabolism and even membrane structure. Cyanobacterial cells starved in nutrients or exposed to high levels of light accumulate low levels of thylakoid membranes and phycobilisomes and show a decline in photosystem II activity (Collier and Grossman, 1992; de Marsac and Houmard, 1993; Grossman et al., 2001). These modifications result from intracellular responses that include down-regulation of gene expression, changes in the rate of protein synthesis and proteolytic disposal of altered or temporarily functional proteins. It is well known from bacterial studies that peptidases are involved not only in posttranslational degradation of proteins but also in the control of gene expression through their interaction with transcription factors (for a review see Adam, 2000).

### 4.2.1 Light stress

Cyanobacteria and higher plants have evolved mechanisms that ensure their adaptation to high levels of light (Lorimier et al., 1991; Murakami and Fujita, 1991). Expression studies of *Synechocystis* peptidase genes under acclimation to different light regimes showed that three genes, *clpB2*, *ftsH1* and *ftsH2*, are induced within 15 min after transfer to high levels of light and become down-regulated within the next 15 h (Hihara et al., 2001).

In this study the analysis of knock-out strains of *Synechocystis* revealed that ClpB2 is involved in the acclimation to light stress, since deletion of the corresponding gene led to a non-bleaching phenotype under high light intensities. It was also observed that four peptidases, ClpP2, SppA1, SppA2 and PepP, contribute to light acclimation, because the corresponding knock-out mutants demonstrated an altered pigmentation phenotype for Δ*sppA1*, Δ*sppA2* and Δ*pepP* or ceased growth for Δ*clpP2* upon transfer to higher light intensities (Panichkin et al., 2001; Sokolenko et al., 2002). Further experiments showed that Δ*sppA1* is defective in the cleavage of phycobilisome antenna linker proteins, which is required for reducing the antenna size at high light intensities (Pojidaeva et al., 2004). It was further found that SppA2 protease is involved in the degradation of low molecular weight polypeptides accumulating under HL during recovery of cells from HL to LL (Pojidaeva and Sokolenko, manuscript in preparation). An altered phenotype was found for Δ*pepP* under LL and HL regimes. Major phenotypic changes encompassed slower cell growth and enhanced pigmentation. Since this peptidase might be involved in the N-terminal processing of the initiator methionine, Δ*pepP* should express a set of defective proteins. Experiments with $S^{35}$-methionine revealed a number of proteins whose translation level was decreased in mutant cells (Svetanović, 2003). Sequence analysis should help for identification of possible substrates for PepP protease or for PepP-regulated factors that could influence protein translation.

### 4.2.2 Heat stress

The major response to heat stress is borne out by the induction of heat stress proteins, chaperones and peptidases that are involved in the refolding or degradation of misfolded polypeptides. One of the best studied peptidases functioning during heat stress is the bacterial HtrA peptidase (Strauch et al., 1989; Lipinska et al., 1990; Spiess et al., 1999). The

*Synechocystis htrA* gene was recently inactivated and in contrast to non-photosynthetic bacteria Δ*htrA* showed a strong phenotype of photosynthesis. It was predicted to play a part in the repair of PSII (Silvia et al., 2003). Our studies showed that the periplasmic HhoA peptidase, a HtrA homologue, is probably involved in temperature acclimation of *Synechocystis* cells, since the cyanobacterial *hhoA* mutant bleached rapidly upon transfer to higher temperature. Two other knock-out strains, Δ*pepP* and Δ*prp2*, were sensitive to heat stress and bleached upon transfer to higher temperatures. The role of the PepP aminopeptidase in heat-stress acclimation could be explained by its role in the posttranslational modification of proteins, including those that are highly expressed during heat stress. The role of Prp2 in heat stress remains unclear.

### 4.2.3 Nutrient stress

Nutrient and co-factor deprivation, for instance nitrogen, sulfur, phosphorus, iron, or copper, represents another source of environmental stress. Among the best studied degradation processes in cyanobacteria in response to nutrient deprivation are the degradation of (1) phycobilisomes in the absence of nitrogen or phosphorus (Collier and Grossman, 1992; Sauer et al., 2001) and (2) plastocyanin in the absence of copper (Li and Merchant, 1995). Sulfur deprivation was also reported to induce phycobilisome degradation in *Synechococcus*, but not in *Synechocystis* (Richaud et al., 2001), which suggests some differences in signaling pathways between these two organisms. Iron deficiency also leads to a faster degradation of phycobilisomes and photosystem I proteins (Guikema and Sherman, 1983) and to the expression of iron-regulated genes that help cells to survive (Burnap et al., 1993; Bibby et al., 2001; Boekema et al., 2001). It was previously reported that the family of *nbl* genes controls the adaptation of the cyanobacterial cell to various nutrient deprivations (summarized by Grossman et al., 2001), but little information is yet available about the identification of peptidases involved in stress-induced degradation processes.

The non-photoautotrophic and hyper-light-sensitive Δ*clpP2* strain that depends upon glucose for growth grew faster than the wild-type during the first two days of nitrogen, phosphorus and iron starvation. However, it showed no difference in growth rate to wild-type during sulfur starvation, a nutrient stress that does not cause phycobilisome degradation in *Synechocystis* sp. PCC 6803 (Richaud et al., 2001). These observations suggest that ClpP2 could be involved in phycobilisome degradation during nutrient stress. All other peptidase

knock-out lines which were investigated were photoautotrophic strains and their phenotypes could be studied in the absence of glucose. Mutant Δ*hhoB* bleached more rapidly than the wild-type when grown in a medium depleted in phosphorus, whereas Δ*hhoA* and Δ*ape2* remained greener than the wild-type under iron deprivation. Ape2 is likely to participate also in the response to copper deficiency, together with SppA2, since the corresponding knock-out strains bleached when grown in the absence of copper. The processing Gcp peptidase is involved in salt-stress response and may also participate in cyanophycin degradation in *Synechocystis* cells (Zuther et al., 1998).

## 4.3 The SppA protease family

### 4.3.1 Characterization of SppA1

Generation of a collection of targeted peptidase mutants from *Synechocystis* has revealed four components that are involved in light acclimation of cyanobacterial cells (Sokolenko et al., 2002). One of these components, the SppA1 peptidase, is an integral membrane endopeptidase in bacteria that degrades processed presequences of exported proteins (Novak and Dev, 1988; Bolhuis et al., 1999). It was recently identified as a thylakoid membrane associated protein in *Arabidopsis* that showed light induction both at the transcriptional, translational and possibly post-translational levels (Lensch et al., 2001). Cyanobacteria, as all other bacterial organisms, express two SppA homologues, SppA1 and SppA2 (Sokolenko et al., 2002).

The *sll1703* gene, encoding a homologous thylakoid membrane-associated SppA peptidase from *Arabidopsis*, was inactivated by site-directed mutagenesis in *Synechocystis* sp. PCC 6803 (this work; Sokolenko et al., 2002). Analysis of the DNA sequence showed that the *sll1703* gene with a size of 1860 bp could be a member of the gene cluster *sll1702-sll1703-sll1704*. Since the transcription levels of *sll1702* and *sll1704* genes were not affected after interruption of *sll1703* gene, this suggests that these genes are not organized in one operon. This remains to be verified.

#### 4.3.1.1 Topology of SppA1

Since antisera against SppA1 were not obtained it is possible only to speculate about the topology and localization of this protein in the cyanobacterial cell. From a comparison of the localization for *Arabidopsis* SppA, which is integrated in thylakoid membranes, and *E. coli* SppA, which anchors in the plasma membrane, *Synechocystis* SppA1 can be predicted as well as a membrane-integrated protein (Lensch et al., 2001). Indeed, the amino acid sequence deduced from the *sppA1* gene indicates that SppA1 is a protein of 610 amino acid residues with two hydrophobic stretches at the N-terminal part (domain I is defined by amino acid residues 385 – 405 and domain II – 421 – 434; Fig. 31), where one of them, by analogy with *Arabidopsis*, SppA (Lensch et al., 2001), could interact with the lipid bilayer. Comparison of sequences of SppA1 from various species also revealed a putative cleavage site for a signal peptidase (at position 39 of *Synechocystis* SppA1) that can remove the transit peptide from the protein. The SppA1 protease from *Synechocystis* displays 27% identity to *Arabidopsis* SppA and 34% to *E. coli* SppA. Highest conservation was found in the C-terminal part of protein that also contains the serine residues deduced as possible catalytic amino acid residues (Lensch et al., 2001). According to studies of catalytic domains of serine-type protease (Slilaty and Vu, 1991; Paetzel and Dalbey, 1997) those serine residues involved in catalysis should be found close to histidine or lysine residues to form the so-called catalytic dyad. Based on the results of *Arabidopsis* SppA three possible catalytic serines have been indicated ($S^{367}$, $S^{401}$ and $S^{423}$).

#### 4.3.1.2 The Δ*sppA1* mutant lost part of its light acclimation properties

Under the chosen experimental conditions the wild-type strain of *Synechocystis* has a doubling time of about 11 h when grown at 50 µE $m^{-2}$ $s^{-1}$ under white light. The switch to 150 µE $m^{-2}$ $s^{-1}$ (saturating light) produces only a modest increase in generation time, by about 10%. This generation time is not limited by $CO_2$ availability since bubbling $CO_2$ into the culture does not change the rate of cell division under LL and ML regimes. However, this transition from a close-to-saturating light to saturating light was accompanied by a marked bleaching of the cultures that became visible after 24 h exposure to the new light regime and slowly developed over the next 48 h. This delay in bleaching was observed for initial cell densities of either 0.1 or 0.5 $A_{750}$ as well as in cultures that were either kept at constant cell density by a daily dilution up to an $A_{750}$ of 1. The 40% decrease in phycobilins and

chlorophyll after 3 days under 150 μE $m^{-2}$ $s^{-1}$ was accounted for by the loss of a significant proportion of the PC and APC phycobiliproteins and of the photosystem I and II chlorophyll-binding proteins. The former change reflects an acclimation of *Synechocystis* to increasing light intensities that has been previously attributed to a changes in the expression of genes encoding phycobiliproteins (de Lorimier et al., 1991; Lichtle et al., 1996).

The Δ*sppA1* mutant grows at similar rates as the wild-type - even a little faster under 50 μE $m^{-2}$ $s^{-1}$ and 150 μE $m^{-2}$ $s^{-1}$. In that respect it is similar to other genetically modified cyanobacterial strains that were previously reported to have increased growth rates compared to wild-type (Lomax et al., 1987; Emlyn-Jones et al., 1999). When cultures of the Δ*sppA1* mutant were exposed to 50/150 μE $m^{-2}$ $s^{-1}$ light transition, the same changes were observed in chlorophyll and photosystem contents and the same drop in synthesis of the major phycobiliproteins as in the wild-type. However, the mere comparison of the cultures showed that the pronounced bleaching observed with the wild-type was no longer seen with the mutant. Although a minor part of this difference can be ascribed to the higher pigment/cell content and higher cell density reached by the mutant cultures, the major contribution came from the better preservation of phycobiliproteins in Δ*sppA1* grown at 150 μE $m^{-2}$ $s^{-1}$. The higher content in peripheral antenna in the mutant was documented both at the protein level, relative to other photosynthetic proteins, and by a higher PBS sensitization of PSII fluorescence emission at 77K. Because the two strains showed a similar down regulation of phycobiliprotein synthesis at 150 μE $m^{-2}$ $s^{-1}$, it was concluded that the higher amount of phycobiliproteins in the mutant is due to a hampered degradation process in the Δ*sppA1* strain.

In contrast with light of 150 μE $m^{-2}$ $s^{-1}$ the mutant was more sensitive to light of 350 μE $m^{-2}$ $s^{-1}$ than the wild-type as demonstrated by its stronger bleaching, more pronounced drop in cell growth and larger decrease in chlorophyll content. Such behavior can be explained by an inability of Δ*sppA1* to properly down-regulate photosynthesis through a down-sizing of its phycobilisome antenna: excess light excitation of PSII will then cause strong photodamage leading to protein degradation (Ownby et al., 1979). However, SppA1 could also control the activity of other peptidases or proteins involved in photoprotection of thylakoid membranes (this situation will be discussed later).

#### 4.3.1.3 Δ*sppA1* does not undergo the cleavage of the $L_R^{33}$ and $L_{CM}^{99}$ linker proteins after exposure to increasing light intensities

The participation of SppA1 in the degradation of phycobiliproteins when reaching light intensities immediately above saturation is specific to this acclimation process. The loss in phycobiliproteins and PBS due to other changes in environmental conditions, such as nitrogen deprivation or deprivation in S, P, Fe and Cu (Grossman et al., 1993; Richaud et al., 2001) remained unaltered in Δ*sppA1* (Pojidaeva et al., 2004). PBS degradation responses are thus controlled by different proteases depending on the environmental stimulus. They differ both in amplitude and kinetics, with nitrogen depletion leading to a rather rapid decrease of up to 90% of PBS (Li and Sherman, 2002) while the drop in intracellular phycobiliproteins reported here, upon acclimation to saturating light, is a delayed process and does not exceed 40%. During nitrogen deprivation cell division and synthesis of phycobiliproteins stop, while pre-existing phycobiliproteins are degraded. Therefore, there is a net loss in phycobiliproteins. During light acclimation the synthesis of new phycobiliproteins is decreased and the content in pre-existing phycobiliproteins also declines due to a limited and specific degradation process. However, since the cell cultures continue to grow, there is still a relative increase in phycobiliproteins per unit volume although the content per cell decreases. It has been noted that the degradation of LHCII antenna upon acclimation of the chloroplast to high light conditions is also a slow process, with 30% degradation only, observed after three days of acclimation to high light intensities (Yang et al., 1998).

It was previously demonstrated that linker proteins could be degraded *in vitro* within isolated PBS by some co-isolated proteolytic enzyme(s) (Reuter and Nickel-Reuter, 1993). In this work this observation was confirmed. There was a selective loss of $L_R^{33}$ and $L_{CM}^{99}$ linker proteins in PBS isolated from the wild-type, when incubated at 37°C for three hours. However, this linker degradation was no longer observed when using PBS isolated from Δ*sppA1*. This points to an acclimation process at saturating light regimes that is caused by a linker-targeted and SppA1-mediated degradation process. That linker proteins were degraded *in vitro* under ML, whereas they remained stable in the dark, demonstrated that the protease can be activated by light most likely by some conformational changes. It is relevant to note that an extensive degradation of rod linker $L_R^{33}$, but not of the membrane linker, was also reported during nitrogen starvation (Li and Sherman, 2002), a process that does not require

SppA1. This means that PBS may undergo similar modifications of their structure during degradation through different regulatory mechanisms.

Proteolysis of PBS encompasses a diversity of phenomena from extensive degradation of all PBS subunits, as observed during nitrogen starvation, to some limited modifications in their supramolecular structure due to the selective action of endopeptidases. From this work *in vitro* experiments suggest that such a fine-tuned regulation in antenna organization should start with the cleavage of the distal linker protein $L_R^{33}$ and the membrane linker $L_{CM}^{99}$. Decreased energy transfer from PBS to PSII at saturating light intensities can occur through shortening of the PBS rods via a detachment of the external rod segments, of whole rods from PBS cores, or/and through a decreased ratio of PBS to PSII per photosynthetic membrane due to their detachment from the membrane leading ultimately to their degradation in the cytoplasm. Degradation of $L_R^{33}$ and $L_{CM}^{99}$ can account for both a shortening and a release of PBS from the membranes. The former process should be driven by the loss of $L_R^{33}$-PC that represents the distal chains of the rods. Present work has shown that this loss is controlled by SppA1, an observation which is consistent with previous reports showing that the regulation of $L_R^{33}$ accumulation is not primarily due to transcriptional changes but rather to a control at the translational or post-translational levels (de Lorimier et al., 1991). The release of PBS from the membranes probably involves the other linker, $L_{CM}$, which participates in assembling the PBS structure in an energy transfer-competent position towards photosystem II (Glazer, 1988). $L_{CM}$ linker represents a chimeric protein with a heterogeneous domain structure. The C-terminal part of this protein contains three repeat domains (REP1 – 3) which show high homology to conserved domains of the rod and rod-core linker polypeptides and provides the binding domains that interact with the APC trimer. Sequencing of a 23 kDa peptide that was associated with an APC ($\alpha^{AP}ß^{AP}$) subcomplex showed that it originated from the C-terminal part of this membrane linker (Gottschalk et al., 1994) that carried only the last REP domain. This crucial experiment demonstrates that there is a peptidase able to cleave the C-terminal sequence of the $L_{CM}$ linker that is tightly interacting with APC. Since there are two copies of $L_{CM}$ per PBS structure, each APC trimeric cylinder could potentially be detached, leading to a complete dissociation of PBS from the thylakoid membrane. Thus, one would expect that degradation of $L_{CM}$ should lead to a decrease in the whole cell content of assembled PBS, an observation which was indeed reported for some cyanobacteria during light acclimation (Raps et al., 1985).

PBS linker proteins were protected from degradation in the Δ*sppA1* strain under ML. This is in favour of SppA1 being the peptidase co-isolated with PBS that cleaves the linkers. However, we can not exclude an indirect role of SppA. For instance, it could control the susceptibility of the PBS structure to another peptidase or regulate the expression of this PBS-targeted peptidase. The study of other intracellular targets for SppA1 should provide a better view of the regulatory function of thylakoid bound peptidases in cyanobacteria.

### 4.3.2 Characterization of SppA2 component

#### 4.3.2.1 SppA2 is a serine-type protease

SppA2 protease belongs to the SppA family that includes serine type proteases (Lensch et al., 2001). Although biochemical studies on purified protease IV (SppA1 homologue) from *E. coli* showed their proteolytic activity against substrates as azocoll, synthetic polypeptides and α-casein (Regnier, 1981; Pacaud, 1982a; Palmer and John, 1987) the actual correspondence of these purified enzymes to SppA1 and SppA2 from cyanobacteria or higher plants has never been proven. The SppA1-containing high molecular complex from *Arabidopsis* was proteolytically active against the non-specific substrate gelatin (Lensch et al., 2001). The proteolytic assay with overexpressed *in vitro* SppA2 protein demonstrated that SppA2 is a serine-type protease and can hydrolyze the unspecific substrate azocoll. The proteolytic activity against azocoll was slow and observed only after 12 h of incubation. Typical serine-type inhibitors, DFP and PMSF, inhibited SppA2-mediated activity and these data corresponded to biochemical studies of Pacaud (1982b) for protease V (SppA2 homologue). Analysis of the SppA2 sequence revealed three serines in positions 51, 85 and 107 as possible catalytically active amino acids as it was proposed for SppAs from *E. coli* and *Arabidopsis thaliana* (Lensch et al., 2001).

#### 4.3.2.2 SppA2 is a component of a membrane-associated high molecular weight complex

The second component of the SppA family, SppA2 protease, was found only in the genomes of prokaryotic organisms. In all cases SppA2 has molecular weight of about 30 kDa, that is half of the size of SppA1, and shares 22% identity to the C-terminus of SppA1 (Lensch et al., 2001). Studies of SppA2 in non-photosynthetic organisms showed that this protein is targeted

to the soluble compartment in *B. subtilis* or into the cytoplasmic inner membrane in *E. coli* (Bair et al., 1991; Bolhuis et al., 1999). Further, *Arabidopsis* SppA1 protease showed strong association with thylakoid membranes. Despite of predictions of *Synechocystis* SppA2 protease in the soluble compartment biochemical topological studies proved that it is either a monotopic membrane protein similar to SppA1 *Arabidopsis* or that it is strongly associated with some other membrane component(s). Treatment of the membrane with chaotropic salts and alkaline solutions could only partially (in case of NaOH) release the protein proving its strong interaction with the lipid bilayer. Comparative analysis of *Synechocystis* SppA2 with its homologoues from *Pseudomonas* and *Agrobacterium* and SppA1 proteases from *E. coli* and *Arabidopsis* showed the presence of a hydrophobic region (110 – 122 amino acids residues) which could partisipate in association of SppA2 with membranes or with another membrane-associated protein(s). Association of SppA2 with thylakoid membranes was strengthened by recent proteome analysis of periplasma and outer membrane proteins from *Synechocystis* that did not detect SppA2 protease in the latter subfractions (Huang et al., 2002; Huang et al., 2004). Further analysis of membrane topology showed that SppA2 forms a high molecular weight complex in the membrane which is not associated with the major photosynthetic complexes such as PSI, PSII, cytochrome *b/f* complex and ATP synthase. Two different approaches were chosen for determining the nature of the association of SppA2 with membranes: “blue native” PAGE and sucrose gradient ultracentrifugation of partially solubilized membranes. Isolation of thylakoid protein complexes by “blue native” PAGE excluded a possible co-migration of SppA2 with ATP synthase and cytochrome *b/f* complex and revealed more or less co-migration with the monomer forms of PSI and PSII. Sucrose gradient centrifugation allowed a better resolution of the photosynthetic complexes and showed that, indeed, the SppA2-containing complex is located between the monomer forms of PSI and PSII but is not a constituent part of these complexes. Previous work on protease V from *E. coli* (SppA2 homologue) demonstrated that this protein with a molecular weight of 24 kDa forms a complex of 130 kDa in the cytoplasma membrane (Pacaud, 1982b). The complex organization of SppA1 had also been previously demonstrated in *E. coli* (Pacaud, 1982b; Ichihara et al., 1986) and in *Arabidopsis thaliana* (Lensch et al., 2001). In all cases SppA complexes possess a homotetrameric structure. On „blue native” PAGE the SppA2-containing complex appears as a complex of 260 kDa, that is, twice the size of the homotetramer of SppA2. This suggests the presence of some other protein(s) in the SppA2-containing complex or the complex could represented by duplication of SppA2 tetramer as it was previously suggested for SppA1 from *E. coli* (Pacaud, 1982b; Ichihara et al., 1986).

The homology of SppA1 and SppA2 and their regulation by changes in light intensity proposed also their physical interaction in the cell. Analysis of the SppA2 complex in the mutant strain deficient in the *sppA1* gene did not show any modification in mobility of the SppA2 complex. This suggests that SppA1 and SppA2 are not constituents of the same multimeric protein complex in the membranes or the complex of both components is not stable under the chosen solubilization conditions.

#### 4.3.2.3 SppA2 degrades small polypeptides accumulating under HL after recovery from HL to LL

In this, work it was observed that the Δ*sppA2* mutant showed a slower growth rate, bleached phenotype and lower $O_2$ production in cells grown under LL and after recovery from HL to LL in comparison with the wild-type. However, no differences in behaviour of both strains were detected under HL. Protein analysis revealed accumulation of low molecular weight polypeptides under HL that retained stable in the Δ*sppA2* mutant in contrast to those of the wild-type. The degradation of SppA2 protein during HL and its accumulation during recovery from HL to LL suggested that it could be involved in the specific degradation of low molecular weight polypeptides taking part in adaptive response of cyanobacteria to elevated light intensities. The photoprotection mechanisms of cyanobacteria operates in different ways including the synthesis and recruitment of enzymes essential under stress conditions with their following degradation after recovery to standard conditions (Mohamed and Jansson, 1991; He et al., 2001; Havaux et al., 2003). Previous studies revealed at least two groups of low molecular weight polypeptides that can be involved in cell survival under light stress: Nbl proteins (non-bleached polypeptides; Collier and Grossman, 1994; Schwarz and Grossman, 1998) and HLIP (high light inducible proteins; Montane and Kloppstech, 2000; He et al., 2001; Havaux et al., 2003). Expression of *nbl* genes as well as of *hli* genes depends on light intensity, temperature and nutrient conditions (e.g. sulfur and nitrogen deprivation, cold stress; Collier and Grossman, 1992; Salem and van Waasbergen, 2004; van Waasbergen et al., 2002;). It is interesting that the expression of HLIP is also controlled by Nbl proteins. NblS was found to control the expression of a number of photosynthesis-related genes, including *hliA*, under HL, red-light, blue-light and UV-A light and also to control the expression of the *nblA* gene, the product of which is involved in the degradation of PBS during nitrogen and sulfur starvation (van Waasbergen et al., 2002). A possible effect of *sppA2* interruption on NblR and NblS was excluded since accumulation of NblA1 and NblA2

proteins was not affected in Δ*sppA2* under nitrogen-limiting and light-elevated conditions (Pojidaeva E., unpublished data).

This study proposes that HLIPs could be a substrates for the SppA2 protease. Initial accumulation of HLIP occurs during the phase of acclimation to HL in which wild-type cells are unable to divide (6 – 10 h) and lose approximately 50% of their capacity for PSII activity. The binding of chlorophyll and/or chlorophyll intermediates could protect the cyanobacteria from a potentially phototoxic effect of these free pigments. HLIPs are rapidly degraded during the first hours (1 – 3 h) of recovery of cells from excitation excess (He et al., 2001). However, the disappearance of these proteins proceeds re-greening of the cells and restoration PSII activity (van Waasbergen et al., 2002). In wild-type cells SppA2 protein was detected only within first 12 h of HL. It then completely disappeared within 48 h. The accumulation of newly synthesized protein was observed within 2 h during the recovery phase from HL to LL that proposed the involvement of SppA2 in the expression or degradation of HLIP proteins under LL. High expression of HLIP was detected under different stress conditions (nutrient deprivation, heat and light stresses; He et al., 2001; van Waasbergen et al., 2002; Salem and van Waasbergen, 2004). However, accumulation of SppA2 dropped exclusively under HL and not under other stress conditions. These results suggest that other components and mechanisms could be involved in the degradation of HLIP under other stress regimes as well.

#### 4.3.2.4 Accumulation of SppA2 is regulated by SppA1 protease

A novel and striking finding of this study is the light-activated expression of *sppA2* gene at one side and light-activated turnover of SppA2 protein on the other side. Similar to *sppA1* in *Arabidopsis thaliana* (Lensch et al., 2001), but not to *Synechocystis* (Pojidaeva et al., 2004), *sppA2* transcript was increased when the cells were transferred to higher light regimes. At the same time protein accumulation decreased substantially with a half-life time of 3 h upon HL exposure. Protein recovery from HL to LL was reached within first 2 h with its maximum level between 6 and 8 h after transfer from HL to LL. These data demonstrated that SppA2 is a short-lived protein under HL required probably only under LL or after recovery to LL in cyanobacterial cells. As it was mentioned before, the activity of SppA1 protease correlated with different light intensities and is involved in the acclimation of linker proteins of phycobilisome antenna to acclimation to higher light regimes (Pojidaeva et al., 2004).

Decrease of SppA2 protein during acclimation to HL is correlated with the presence of SppA1 protease. No drop in protein amount upon transfer to HL could be observed in the Δ*sppA1* mutant strain. Therefore, SppA2 protease is another substrate for the SppA1 protease or a SppA1-regulated protease under HL. Although the functions and direct substrates of SppA1 and SppA2 are not yet clarified, this study show an interplay and net regulation between the two members of SppA family.

## 5. SUMMARY

A set of 62 genes that encode the entire peptidase complement of *Synechocystis* sp. PCC 6803 has been identified in the genome database of that cyanobacterium. Sequence comparisons with the *Arabidopsis thaliana* genome uncovered the homologous chloroplast components inherited from their cyanobacterial ancestor. A systematic gene disruption approach was chosen to individually inactivate, by customary transformation strategies, the majority of the *Synechocystis* sp. PCC 6803 genes encoding peptidase subunits that are related to the *Arabidopsis thaliana* chloroplast enzymes. This allowed classification of the peptidases that are required for cell viability or are involved in specific stress responses.

1. *General analysis of various peptidase knock-out mutants*: Using the knock-out strategy it was found that the members of the Clp family (ClpP1, ClpP3, ClpR, ClpC, ClpX and ClpB1) are essential for cell viability since corresponding fully segregated knock-out strains could not be obtained. Only *clpP2* and *clpB2* genes were successfully inactivated in this study. ClpP2 is critical for the function of the photosynthetic machinery since after knock-out of the gene the cells were not able to grow under phototrophic conditions. The work also demonstrated that ClpP2 could be involved in phycobilisome degradation during nutrient deprivation and is required for temperature acclimation.

Only two knock-out strains (Δ*hhoA* and Δ*hhoB*) from the Deg family segregated fully. Physiological analysis revealed that HhoA and HhoB differ in their functions in *Synechocystis*. HhoA is essential under heat stress and iron deprivation, whereas the product of the *hhoB* gene could be important under phosphorus deprivation. The non-successful inactivation of the *htrA* gene suggests that this gene is essential for cell viability. Other peptidases, such as Prp2, SppA1, SppA2, Ape2 and PepP, are also involved in the acclimation to environmental stresses. SppA1, SppA2 and PepP contribute to light acclimation. PepP takes as well part in acclimation to high temperature. Probably, this protease plays a role in the posttranslational modification of proteins, including those that are highly expressed during light and heat stress. Ape2 likely participates in responses to iron and copper deficiency.

2. *Analysis of the SppA family*: Two genes, *sppA1* and *sppA2*, are found in the genome of *Synechocystis* sp. PCC 6803 that encode the serine-type proteases SppA1 and SppA2,

respectively. Inactivation of the corresponding genes showed that both proteins are required for light adaptation of cyanobacterial cells.

Upon acclimation of Δ*sppA1* from 50 to 150 μE $m^{-2}$ $s^{-1}$ light intensities most of the phycobilisome content was preserved, whereas the wild-type developed a bleached phenotype due to the loss of about 40% of its phycobiliproteins. In the Δ*sppA1* strain cleavage of the $L_R^{33}$ and $L_{CM}^{99}$ linker proteins does not occur in medium light. Thus, a major contribution to light acclimation in cyanobacteria rests on a SppA1-mediated cleavage of phycobilisome linker proteins that besides changes in gene expression of the major phycobiliproteins, contributes a mechanism responsible for a reduced content in phycobilisome antenna upon acclimation to higher light intensity.

The second member of SppA protease family, the SppA2 protein, is present in the genome of *Synechocystis* sp. PCC 6803, but not in *Arabidopsis thaliana*. SppA2 represents a protein half of the size of SppA1 and shows domain homology to the C-terminal part of SppA1. The *in vitro* proteolytic assay with the non-specific protease substrate azocoll revealed a serine-type activity of the overexpressed full-length SppA2 protein. SppA2 is a membrane associated protease that forms a high molecular weight complex of 260 kDa in cyanobacterial membranes. Similar to *sppA* from higher plants the expression of *sppA2* (*slr0021*) in *Synechocystis* is enhanced at higher light. By contrast, SppA2 protein showed a strong reduction down to zero levels in cyanobacterial cells that were exposed to high light for two days. The data demonstrated that SppA2 is rapidly degraded with a half-life of 3 h under high light. The degradation of SppA2 is specific to light and not to other stress conditions, like nutrient deprivation, heat or cold stresses. SppA2 protease accumulated under high light in the mutant strain deficient in *sppA1* gene encoding SppA1 protease. This demonstrates that SppA2 protease expression or degradation is regulated by SppA1 protease or SppA1-controlled proteases in a light-dependent manner.

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