Ferrodoxin: NADP+ reductase and photosynthetic energy transduction in Cyanobacteria
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The N-terminal CpcD homologous domain of Ferredoxin:NADP⁺ reductase from *Synechocystis* PCC 6803 is required for salt-shock induced increase in Photosystem I dependent cyclic electron transport

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Abstract

The influence of the genetic removal of the N-terminal, CpcD homologous, domain of ferredoxin:NADP⁺ reductase (FNR) on the electron transport characteristics of the oxygenic photoheterotrophic cyanobacterium *Synechocystis* PCC 6803 was studied. It was found that this domain directs association of the enzyme with the thylakoids as well as with the phycobilisome complexes. The main effect of the mutation on photosynthetic electron transport was observed in assays of PSI-dependent cyclic electron transport: Both photoacoustic determination of the storage of Farred (PSI specific) light energy, and measurement of the re-reduction kinetics of P700⁺, indicate that induction of cyclic electron transport around PSI as a result of salt shock is absent in this mutant. This induction was correlated in the wildtype with the increased abundance of (wildtype) petH mRNA. The maximal rate of oxygen evolution was found to be significantly higher in the mutant, as compared to the wildtype, indicating an effect of cyclic electron transport, operating under white actinic light of moderate intensity, on the redox poise of the plastoquinone pool. *In vitro* NADPH-cytochrome c reduction assays, using thylakoid membranes, reveal the contribution of FNR, in plastoquinone reductase activity, that is dependent on the presence of its N-terminal domain. It was established that the truncated ΔpetH mRNA in the mutant accumulates, as the wildtype transcript does, as a result of salt shock. In addition, NADP⁺ photoreduction measurements performed with either ferredoxin or flavodoxin, indicate that truncated recombinant FNR has an activity comparable to that of the wildtype recombinant protein, in linear electron transport. From measurements with the ΔpetH truncation mutant and the NAD(P)H dehydrogenase deficient mutant M55 (Ogawa, 1991. *PNAS* 88: 4275-4279), we conclude that both FNR and the NAD(P)H dehydrogenase complex contribute to PSI-dependent cyclic electron transport. Since a salt shock leads to overexpression of both FNR and flavodoxin (Fulda and Hageman, 1995. *J. Plant. Physiol.* 146: 520-526), we propose that, *in vivo*, FNR catalyses flavodoxin-mediated plastoquinone reduction. During photoautotrophic growth the majority of FNR is present in the phycobilisomes, whereas the accumulation of FNR as a result of salt shock results in association of these additional copies with the thylakoid membranes. It is therefore proposed that a physiological function of the localisation of FNR in the phycobilisome is to maintain a fixed population of the enzyme that is competent in linear electron transport from reduced ferredoxin to NADP⁺.
**Introduction**

Cyanobacteria are prokaryotic organisms that perform oxygenic photosynthesis, primarily by transferring electrons from water, via photosystem II (PSII) and photosystem I (PSI), and a connecting electron transfer chain, to NADP⁺. Like green plants and eukaryotic algae, they also perform a second type of photosynthetic electron transfer, that is (light-) driven by photosystem I only, and is cyclic in nature, meaning that electrons produced by PSI photochemistry return from the stromal side to the luminal side of the PSI reaction center via a chain of electron transport carriers (for a recent review see Bendall and Manasse, 1995). It has been demonstrated that ferredoxin, the cytochrome b₆f complex, the plastoquinone pool, and luminal electron carriers (plastocyanin and/or cytochrome c) are part of this chain of electron transport carriers, and are therefore involved in both linear and cyclic electron transport (Bendall and Manasse, 1995). In addition, these components (with the exception of plastocyanin) are also part of the respiratory electron transport chain, that is present in the thylakoids of cyanobacteria as well (for a review see Schmetterer, 1994).

During linear electron transport, requiring both photosystems, the turnover of photosystem I is about 35 - 140 s⁻¹ for cyanobacteria (Maxwell and Biggins, 1976). This can be determined by measuring the rate of re-reduction of P700⁺ in the dark, after actinic illumination with white light. It was noted that the P700⁺ re-reduction rates, after selective illumination of photosystem I with farred light (>710 nm), were much slower in the case of cyanobacteria (+/- 4 s⁻¹) and green algae (Maxwell and Biggins, 1976), as compared to the observed kinetics with white-light excitation. With farred-light excitation the kinetics reflect the re-reduction of the plastoquinone-pool and cytochrome b₆f complex, since these have the highest control on the flux of cyclic electron transport in vivo. The kinetics of reduction of P700⁺ by plastoquinone were determined directly in vivo for a thermophilic Synechococcus species (Nanba and Satoh, 1983). The slowest rate associated with this reaction path was the reduction of the Rieske protein by plastoquinone (~500 s⁻¹). All subsequent reactions were observed to proceed at faster rates (Nanba and Satoh, 1983).

The ‘efficiency’ (a relative measure) of PSI-dependent cyclic electron transport in oxygen-evolving photosynthetic organisms has also been determined by photoacoustic spectroscopy (Herbert et al., 1990; Ravenel et al., 1994). The ‘energy storage’ of farred (PSI-specific) light that is measured with this technique is presumably due to the production of long-lived photochemical products, such as ATP, that is synthesized by means of PSI-dependent cyclic electron transport.

The largest physiological difference between cells assayed by the photoacoustic method and the P700⁺ re-reduction assay, is the redox poise of the components involved in cyclic electron transport. The photoacoustic method determines a quantum efficiency during steady state turnover of electrons in the cyclic route, whereas the other method relies on pre-oxidation of the plastoquinone-pool and cytochrome b₆f complex in order to measure the subsequent reduction of P700⁺ in the dark. Nevertheless, both techniques have been used to record the ‘efficiency’ of PSI-dependent cyclic electron transport in a compatible fashion (Ravenel et al., 1994).
In *Synechocystis* PCC 6803 reduction of the plastoquinone-pool and the cytochrome *b*f complex in the dark is predominantly taking place via respiratory oxidation of NADPH, mediated by a bacterial NAD(P)H dehydrogenase complex, that is present in the thylakoid membrane (Ogawa, 1991). A mutant lacking NdhB (designated M55), and therefore lacking functional NAD(P)H dehydrogenase, was found to show a dramatically decreased rate of P700* re-reduction in the dark. It was therefore concluded that the stromal oxidation of NADPH constitutes the largest contribution to PSI-dependent cyclic electron transport and respiratory activity (Ogawa, 1991; Mi et al., 1992a; Mi et al., 1992b). This mutant was also defective in uptake of inorganic carbon, when present at low concentrations (Ogawa, 1991). It was recently shown that salt-induced photosystem I-dependent cyclic electron transport restores the growth of this mutant in media containing low concentrations of carbonate (0.03% (v/v) CO₂ in air; Jeanjean et al., 1998). Therefore, P700* reduction, that does not require the presence of the NAD(P)H dehydrogenase complex, is responsible for the salt-stress induced cyclic electron transport.

Recently, also the NAD(P)H dehydrogenase complex in the thylakoids of green plants has been implicated in the reduction of plastoquinone (Endo et al., 1997; Teicher and Scheller, 1998). The rates of NAD(P)H oxidation that are observed in these plant cells, however, are not as high as the, earlier documented, ferredoxin-dependent route of cyclic electron transport (Bendall and Manasse, 1995). Both the NAD(P)H dependent- and the ferredoxin-dependent reactions can be part of the cyclic routes of electron transport. In the case of the ferredoxin dependent reaction, oxidation and reduction of ferredoxin by other processes than photoreduction by PSI and oxidation during cyclic electron transport and NADP⁺ reduction, have to be considered. Oxidation of carbohydrates can also produce NADPH. In addition, FNR is able to catalyse NADPH-dependent ferredoxin reduction (given high NADPH:NADP⁺ and Fd(ox):Fd(red) ratios). It is therefore not possible to separate the two contributions to cyclic electron transport operating under physiological conditions.

In chloroplasts, participation of ferredoxin:NADP⁺ reductase in PSI-dependent cyclic electron transport has been suggested by the inhibitory effects of sulphydryl-modifying agents (that are known to inhibit the interaction between ferredoxin and FNR), and heparin (Mills et al., 1979; Shahakef et al., 1981; Hosler and Yocum, 1985; Ravenel et al., 1994). In theory, FNR could contribute to ferredoxin:quinone-oxidoreductase activity (FQR), or plastoquinone-reductase activity, by catalysing 'reverse' electron transport from either reduced ferredoxin, or flavodoxin, or NADPH as electron donor. An NADPH-plastoquinone reductase function, essentially equivalent to the reaction catalysed by the NAD(P)H dehydrogenase complex, has also been proposed for FNR in vegetative- and heterocyst cells of the cyanobacterium *Anabaena* sp. as well (Gonzalez de la Vara and Gomez-Lorejo, 1986; Scherer et al., 1988).

Here we provide evidence, based on molecular-, genetic- and physiological analysis, that FNR is indeed involved in reduction of the plastoquinone pool. It was observed that this 'reverse' reaction requires the N-terminal domain of FNR, whereas the function of FNR in linear electron transport is unaffected by this domain.
Materials and Methods

**Media and Culture Conditions**

Wildtype *Synechocystis* PCC 6803 and mutants strains derived thereof were cultured photoautotrophically in BG-11 medium at 34°C, under continuous illumination using two TL tubes, which provided an average light intensity of 70 μE.m⁻².s⁻¹ (Rippka *et al.*, 1979). Transformations were performed as described previously (Pakrasi *et al.*, 1988). Mutant M55 (Ogawa, 1991) was grown in medium supplemented with 50 μg/ml kanamycin and 10 mM NaHCO₃. Transformants were selected on BG-11 medium containing 1.5% (w/v) agar (Difco) plates, supplemented with 10 mM NaHCO₃, 10 mM N-Tris[hydroxymethyl]-2-aminoethanesulfonic acid (TES)/KOH (pH 8.2), 0.3% Sodium thiosulphate and either 10 μg/ml streptomycin or 50 μg/ml kanamycin, depending on the antibiotic resistance cassette that was used. A salt-stressed culture was obtained by the addition of NaCl to 0.55 M, to a photoautotrophically grown culture (van Thor *et al.*, 1998c). For the analysis of salt-adapted cultures, cells were harvested 12 hours after the addition of NaCl.

**RNA and DNA Analysis**

For the isolation of total RNA from *Synechocystis* 6803 suitable for Northern analysis the RNeasy spin column protocol (Qiagen) was adapted: Cells (10-20 ml of OD₇₅₀ = 1) were collected by centrifugation and resuspended in 600 μl lysis-buffer containing 1% β-mercaptoethanol (Qiagen). Alternatively, this buffer could be substituted by 4.5 M Guanidinium Isothiocyanate (Fluka), 2% (w/v) Sodium-N-Laurylsarcosine (Sigma), 1% (v/v) β-mercaptoethanol and 50 mM Tris-Cl pH 7.5. There was no need for additional homogenization when this protocol was used. 600 μl of phenol/chloroform (1:1) (equilibrated with Tris-Cl pH 7.5) was added, mixed and incubated at 65°C for 5 minutes. The sample was centrifuged, and the aqueous phase was extracted with chloroform. Next, 600 μl of 70% ethanol was added to the sample, and the RNA was isolated according to the specified protocol (Qiagen). Typical yields were 50 - 200 μg RNA. Northern hybridisation with a *Synechocystis petH* probe was performed as described previously (van Thor *et al.*, 1998c).

DNA was isolated by adapting a glass-milk kit (Pharmacia). Utilising the specific DNA binding properties of silica-gel particles, DNA was purified from peptidoglycan contaminations. A cell suspension was lysed with lysozyme and digested with RNAse and Proteinase K, and was extracted with phenol twice. Subsequently, 2 volumes of a buffer containing 6 M NaI and 50 mM Tris-Cl pH 8.0 was added (gel-solubilisation buffer; Pharmacia), and DNA was purified by gentle handling of the glass-milk particles, with elution of the DNA at 50°C.

**Construction of the 5'-Truncated petH Gene**

The *Synechocystis* PCC 6803 petH gene was cloned on a 2.9 kbp *BamHI/HindIII* fragment, resulting in pSP35-9 (van Thor *et al.*, 1998c). The truncation of 75 amino acid residues of the N-terminus of PetH required the in-frame deletion of 225 bp. This was achieved with a loop-out mutagenesis strategy, combined with negative selection against the non-mutagenised, uracil containing, complemen-
tary strand produced by the dut ung E. coli strain CJ236 (Kunkel et al., 1987). To this end, a 830 bp EcoRI/Smal fragment from pSP35-9 was cloned in M13mp19. U-DNA from this construct was isolated from E. coli CJ236. The loop-out oligonucleotide SPETTRUNC (5'-GGAGCAATTAACCCATGGAGGGAGATTCGCCTT-3’) was phosphorylated and annealed to the template. Subsequent second-strand synthesis and transformation yielded clones with the correctly reduced size of the EcoRI/Smal insert. The 605 bp insert was sequenced and cloned into the 5.5 kbp EcoRI/Smal fragment from pSP35-9, resulting in construct pSP35-9A. The Ω cassette, encoding streptomycin and spectinomycin resistance was cloned as a 2.0 kbp BamHI fragment from pHPr5Ω (Prentki and Kirsch, 1984) in the BamHI site of pSP35-9A, producing pSP35-9ΔΩ. A fragment encoding the CpcD-homologous N-terminal domain of PetH and 213 bp untranslated region of pSP35-9 was cloned as a 437 bp XbaI/SpeI fragment from pSP35-9 into the unique XbaI site of pSP35-9ΔΩ. An orientation of this cloned fragment, equal to the truncated petH gene on the construct was selected. The resulting construct (see Figure 2) was linearised with NciI before subsequent transformation.

**GENETIC CONSTRUCTION OF PETH-GFP TRANSCRIPTIONAL AND TRANSLATIONAL FUSIONS**

A fusion was made between the coding region of the N-terminal domain of petH with the *Aequorea victoria* gfp-gene, encoding the Green Fluorescent Protein (GFP), resulting in a translational fusion construct. Likewise, a fusion was made of the petH promoter/operator (P/O) region to the gfp gene, resulting in a transcriptional reporter construct.

A 1.6 kbp EcoRI/Sall fragment from pKOK6 (Kokotek and Lotz, 1989), containing the nptII gene, encoding kanamycin resistance, and the phage fd bidirectional transcription terminator, was cloned into pBluescript SK+, and digested with EcoRI and Sall. The resulting vector was digested with EcoRI and BamHI, and ligated with the 0.8 kbp EcoRI/XbaI fragment from pGFPuv (Clontech) containing the gfp-uv gene, together with the 0.85 kbp XbaI/BamHI fragment from pSP35-9 (van Thor et al., 1998c), encoding the petH P/O region and the N-terminal region of the petH gene. This allowed the in-frame fusion between the PetH N-terminal 75 residues, comprising the CpcD homologous domain (van Thor et al., 1998c), in frame with GFP, by ligation of the XbaI site present at the desired position in the petH gene, with the XbaI site present at the 5’ end of the gfp-uv gene. For the construction of the transcriptional fusion construct, the same fragments were ligated except for the 0.85 kbp XbaI/BamHI fragment from pSP35-9, which was replaced by a 0.6 kbp XbaI/BamHI digested PCR product generated with the M13REVERSE (5’-CAGGAAACAACAGTATGAC-3’) and XBAPETPR (5’-GCTTATAGATCCGGTTAATGGCTCCTACTC-3’) oligonucleotides, with pSP35-9 as a template. This ligation allowed a fusion, directly downstream of the petH initiation codon with gfp, by use of the engineered XbaI site, resulting in a transcriptional fusion of gfp with the petH P/O region and Shine-Dalgarno sequence. Both constructs were transformed as circular DNA. Transformants were selected as kanamycin resistant colonies.
It was noted that transformation of similar DNA constructs, based on the wildtype *Aequorea victoria gfp* gene, did repeatedly result in kanamycine resistant transformants with normal frequencies. However, those transformants could not be grown further than colonies or small-scale liquid cultures. Presumably, cytoplasmic wildtype GFP is toxic for *Synechocystis* cells, and segregation of the engineered genotype caused the arrest of growth.

Western blot analysis was performed with a mouse-α-GFP monoclonal antibody (Clontech) at 1:10,000 dilution. Detection was with chemiluminescent reagent SuperSignal (Pierce).

**In vivo assays of PSI-dependent cyclic electron transport: P700⁺ re-reduction kinetics and photoacoustic energy storage measurements**

*Synechocystis* cells (about 10 µg chlorophyll a) were deposited on a nitrocellulose membrane and illuminated with light specific for PSI (e.g. λ > 715 nm; farred light). The formation of P700⁺ was detected by the pulse-modulated measurement of the increase in absorbance at 820 nm (Ravenel *et al.*, 1994). The kinetics of the subsequent P700⁺ re-reduction in the dark was taken as a measure for the capacity of PSI-dependent cyclic electron transport. Under these conditions electron donation by photosystem II did not significantly contribute to the rates obtained, since the addition of 5 µM of DCMU did not influence the measurements.

The photoacoustic measurement of PSI-dependent cyclic electron transport capacity also depends on the use of farred light (Herbert *et al.*, 1990; Ravenel *et al.*, 1994). Cells (about 50 µg chlorophyll a) were packed on a Millipore nitrocellulose membrane with 3 µm pore size and a diameter of 12 mm by filtration and placed in the photoacoustic chamber. The sample was illuminated with 8 W.m⁻² farred light, modulated at a frequency of 20 Hz. The photoacoustic signal was measured with a lock-in amplifier. PSI reaction centers were closed by illumination with 3000 µmol photons s⁻¹ m⁻² white light. Under these conditions, the pulse-modulated measurement of heat release, produced by absorption of farred light, was taken as 100% (i.e. the Energy Storage was 0%). Including DCMU at a concentration of 5 µM did not influence the measurements, demonstrating the specific excitation of photosystem I by farred light. The photoacoustic chamber was flushed with nitrogen to ensure that the terminal oxidase activity and photochemical side-reactions, such as reduction of oxygen, did not influence the level of the determination.

**In vitro NADP⁺ photoreduction assays**

Recombinant *Synechocystis* plastocyanin, flavodoxin and ferredoxin-NADP⁺ reductase were heterologously expressed and purified as described elsewhere (van Thor *et al.*, 1998b). Recombinant 47 kDa wildtype FNR and a truncated protein with a sequence identical to the truncated ΔpetH gene on pSP35-9A were produced and purified.

NADP⁺ photoreduction assays were performed according to (van Thor *et al.*, 1998b). Photosynthetic membranes (5 µg/ml Chla) were dissolved in 50 mM Tricine pH 8.0, 10 mM MgCl₂, 0.05% (w/v) n-dodecyl β-maltoside, 6 mM ascorbic acid. Recombinant plastocyanin (15 µM) was used at near saturating concentra-
tion. Recombinant FNR was used at 1 µM concentration. Recombinant flavodoxin, spinach ferredoxin (Sigma) and NADP+ were included at a concentration of 15, 5 and 500 µM respectively. Light from a 150 W Xenon lamp was passed through a 650 nm high-pass filter, and used at saturating intensity. The photomultiplier was protected with a 340 nm bandpass filter (Oriel). NADPH formation was assayed from the increase in absorbance at 340 nm during illumination, using an Aminco DW2A spectrophotometer (Silver spring, MA, USA).

Under these conditions the rate of NADP+ formation is considered to reflect the acceptor side-limited rate of electron transport, since reduction of P700+ by reduced plastocyanin is sufficiently rapid (van Thor et al., 1998b).

**Biological preparations, native PAGE and assays**

Cell-free extracts were prepared with a French Press (Aminco Corp, USA), operated at 20,000 psi (°C) in a buffer containing 50 mM Tricine pH 7.9, 10 mM NaCl, 5 mM MgCl2 and 0.5 mM PMSF. Whole cells and cell debris were subsequently removed by centrifugation. Membranes were prepared by precipitation and collection by centrifugation after the addition of 1% (w/v) streptomycin sulphate. After two subsequent precipitations, membranes no longer contained detectable amount of phycobilisomes. Cell-free extracts, thylakoid membranes and cytoplasmic fractions were loaded on native 12.5% acrylamide gels, containing 500 mM Tris/Glycine pH 8.8 and 0.05% Triton X-100. Phycobilisomes were prepared according to the method of Glazer (1988). NADPH dependent diaphorase activity was detected after electrophoresis by incubating the gel in a buffer containing 0.2 µg/ml nitrotetrazolium blue (NBT), 0.5 mM NADPH, 25 µg.ml-1 glucose-6-P dehydrogenase, 5 mM glucose-6-P and 50 mM Tris pH 8.0.

Diaphorase assays for the quantification of FNR in subcellular fractions were performed essentially as in (van Thor et al., 1998c), with the exception of the pH of the TAPS (N-tris(hydroxymethyl)methyl-3-amino-propanesulfonic acid) buffer, which was 9.5, and the addition of 0.025% (v/v) Triton-X100 (Sigma), which destroys the diaphorase activity of the NAD(P)H dehydrogenase complex as it does in native gels. At pH 9.0 no influence on the diaphorase activity due to thylakoid association was observed, in accordance with previous observations with the chloroplast FNR (Davies and San Pietro, 1977).

Cell-free extracts and thylakoid membranes for Western analysis after SDS-PAGE were prepared using the same procedures. GFPuv and GFPuv fusion proteins were detected with a mouse-α-GFP monoclonal antibody (Clontech) at 1:10,000 dilution.

Cytochrome c reductase assays were performed according to (Kraushaar et al., 1990), using a buffer containing 50 mM Tricine, 10 mM NaCl, 10 µM DCMU, 50 µM KCN, and 50 µM horse-heart cytochrome c (Boehringer, Mannheim). Thylakoid membranes were prepared by sucrose density centrifugation in 10 mM TES-NaOH buffer, containing 10 mM NaCl, at pH 7.0 according to (Omata and Murata, 1983). Photosynthetic membranes were added at a concentration of 1 µg Chl a.ml-1, and NADPH (Boehringer, Mannheim) was added at 200 µM concentration. Reduction of cytochrome c was recorded spectrophotometrically. An extinction coefficient of E550-540 = 19.5 mM-1 cm-1 was used (Yonetani, 1965). Oxidation of
NADPH was recorded simultaneously at 340 nm, using an extinction coefficient of $E_{340} = 6.2 \text{ mM}^{-1}\text{cm}^{-1}$. Anaerobicity was achieved by bubbling the reaction mixture with $N_2$ gas, and including 6 mM glucose, 4 U.mL$^{-1}$ glucose oxidase, and 400 U.mL$^{-1}$ catalase, according to (Cleland and Bendall, 1992). KCN was omitted in anaerobic measurements.

Chlorophyll $a$ concentration was determined after extraction with methanol at room temperature of either a cell pellet, or of a concentrated thylakoid membrane preparation, according to (Marker, 1972).

**Electron Microscopy**

Cells of *Synechocystis* PCC 6803 were fixed with 3% (w/v) gluteraldehyde in phosphate buffered saline, pH 7.5 (PBS), dehydrated in a graded ethanol series and embedded in Unicryl (BioCell). Ultra-thin cryo sections were labelled with a 1:1,000 dilution of a polyclonal rabbit-α–GFP antibody (Molecular Probes). The secondary antibody was a goat-α–rabbit 15 nm gold-labelled antibody (Amersham). The thin sections were negative stained with 1% (w/v) uranyl acetate and examined with a Philips CM10 electron microscope. The primary antibody was incubated with a preparation of thylakoid membranes from wildtype *Synechocystis* PCC 6803 in order to reduce background labelling of the thylakoids.

**Results and Discussion**

**Salt Stress Increases the Flux of PSI-Driven Cyclic Electron Transport, Independently of the NAD(P)H Dehydrogenase Complex**

The capacity of PSI-dependent cyclic electron transport can be assayed *in vivo* by a photoacoustic determination of the fraction of absorbed PSI specific, farred light, that is not released immediately as heat, but is converted into photochemical products (Ravenel *et al.*, 1994). Since this determination is performed at the second- to minute-timescale, it reflects the rate of electron transport in steady state. Also, the rate of P700$^+$ re-reduction in the dark after continuous illumination with farred light, is a measure for the capacity of PSI-dependent cyclic electron transport. This rate is usually given as the reaction half-time. However, we observed that this half-time is largely independent of the concentration of P700$^+$ that is accumulated during actinic illumination. This reaction can therefore mathematically be described as a (pseudo-) first order reaction. Its rate constant $k$ ($s^{-1}$) equals $\ln 2/t_{1/2}$.

Wildtype *Synechocystis* PCC 6803 can grow in media supplemented with high concentrations of NaCl (Jeanjean *et al.*, 1993). An increase of the capacity for cyclic electron transport in cells grown in high concentrations of NaCl is evident from the increase in energy storage of farred light (as already shown for the wildtype; Jeanjean *et al.*, 1993), and a corresponding decrease in the half-time of reduction of P700$^+$ (Table 1). In addition, the growth rate is decreased in media containing 0.55 M NaCl (Table 1).

The *Synechocystis* PCC 6803 mutant 'M55' (Ogawa, 1991), lacking functional NAD(P)H dehydrogenase activity, is able to grow in high-salt media as well...
Table 1. Growth characteristics and PSI-dependent cyclic electron transport measurements in wildtype and NdHB deficient (mutant M55) Synechocystis PCC 6803 cultures in (N)ormal and (S)alty (0.55 M NaCl) media.

Growth is given as the time required for cell division under photoautotrophic growth conditions at 34°C, with continuously bubbling of 3% CO₂ in air. The capacity of PSI-dependent Energy Storage is the percentage of absorbed farred light that is converted into photochemical products at 8 W.m⁻². Rates of dark re-reduction of P₇₀₀⁺, accumulated in 8 W.m⁻² farred light, are given as pseudo first-order reaction constants.

<table>
<thead>
<tr>
<th>(N)ORMAL/(S)ALT</th>
<th>WILDTYPE (N)</th>
<th>WILDTYPE (S)</th>
<th>M55 (N)</th>
<th>M55 (S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth (h)</td>
<td>5.5 +/- 0.5</td>
<td>8.5 +/- 0.5</td>
<td>6.5 +/- 0.5</td>
<td>10.5 +/- 0.5</td>
</tr>
<tr>
<td>Energy Storage (%)</td>
<td>18 +/- 1.2</td>
<td>24 +/- 1.7</td>
<td>7 +/- 0.6</td>
<td>11 +/- 1</td>
</tr>
<tr>
<td>P₇₀₀⁺ re-reduction (s⁻¹)</td>
<td>2.31 +/- 0.3</td>
<td>3.85 +/- 0.3</td>
<td>0.71 +/- 0.04</td>
<td>1.20 +/- 0.06</td>
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</table>

(Jeanjean et al., 1998, Table 1). Its doubling time increases from 6.5 h to 10.5 h in medium with added NaCl. This is remarkable in the light of its well-documented phenotype in PSI-dependent cyclic electron transfer (Ogawa, 1991; Mi et al., 1992a; Mi et al., 1992b; Mi et al., 1994). Measurements of energy storage and P₇₀₀⁺ re-reduction kinetics indicate that in this mutant the increase of cyclic electron transport is also induced in high salt conditions (Table 1). Thus, the induction of cyclic electron transport observed during salt stress conditions, is not mediated by the NADPH dehydrogenase complex.

Induction of PSI-dependent cyclic electron transport after a salt shock is preceded by overexpression of petH mRNA

Wildtype Synechocystis PCC 6803 adapts to media containing high salt by overexpressing a number of proteins. Among these are flavodoxin and proteins involved in osmoregulation (Hageman et al., 1990). It was shown previously that also the petH transcript, encoding ferredoxin:NADP⁺ reductase, is overproduced during salt stress (van Thor et al., 1998c).

The time-course of these adaptations of Synechocystis PCC 6803 following transfer to high salt medium was studied by measuring the changes in cyclic electron transport activity and relative expression levels of petH. The increase in the energy storage capacity and the decrease in P₇₀₀⁺ re-reduction rate show identical kinetics. This confirms that both techniques reflect the same physiological event. The adaptation is completed in almost 8 hours after the addition of 0.55 M NaCl (Figure 1). This timescale most probably reflects the time required for transcription and translation of a component(s) responsible for the increase in activity. Quantitative Northern blot analysis of the petH transcript shows that the overexpression precedes the increase in activity of cyclic electron transport by approximately 2 hours (Figure 1). This might indicate a causal relation between the petH overexpression and the increase in activity of the cyclic route.

Mutagenesis of petH: Truncation of the N-terminal CpcD homologous domain

Since the induction of PSI-dependent cyclic electron transport in high-salt media is accompanied by the overexpression of petH, FNR may be involved in the route of electron transport. A deletion of petH proved not to segregate (van Thor
At time 0 h, NaCl was added at a concentration of 0.55 M. The capacity of cyclic electron transport was assayed for 25 h both by photoacoustic farred light energy storage determinations and P700* re-reduction measurements. The accumulation of petH mRNA, encoding ferredoxin:NADP+ reductase, was assayed by Northern hybridisation. The relative abundance was determined from the relative density of the hybridising bands, corrected for the total amount of RNA from ethidium-bromide stained gels.
Modification of the enzyme via deletion of the non-catalytic part of the protein was achieved through truncation of the N-terminal domain that is homologous with the phycobilisome linker polypeptide CpcD. Initially, a strategy was chosen that relied on the integration, by single recombination, of a construct harbouring the in-frame N-terminal deletion together with a C-terminal truncation. However, the resulting genotype proved not to be stable, since recovery of one or more copies of the wildtype genotype by recombination proved possible even after apparent complete segregation. This resulted in an unstable and incomplete phenotype (data not shown). Integration by double homologous recombination of the construct shown in Figure 2 was predicted to be more stable. The prk-petH intergenic region is duplicated, but recombination at this site would lead to the loss of the only copy of the petH N-terminal

**Figure 2. Strategy and Southern analysis of replacement mutagenesis resulting in the 5' in-frame deletion of 225 nucleotides from the open reading frame of petH.**

The gene organisation around petH is shown for the wildtype. Restriction sites are indicated (B=BanHI; El=EcoRI; Sp=SpeI; X=XbaI; Sm=SamI; EV=EcoRV; H=HindIII). The construct shown was linearised before transformation. Southern hybridisation analysis with the entire petH gene as a probe, is shown for EcoRI/EcoRV double digestions of genomic DNA and the controls, plasmids pSP35-9 and pSP35-9 Δ trunc.

**Wildtype genome**

```
prk                   petH
```

**Transformed construct (linear)**

```
prk                               petH
```

**Type B mutant genome**

```
prk                       petH - trunc
```

**EcoRI / EcoRV digestions**

<table>
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<th>Digestion</th>
<th>Type A (partial)</th>
<th>Type B (complete)</th>
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<tr>
<td>2.1 kbp</td>
<td>1.9 kbp</td>
<td>2.1 kbp</td>
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coding sequence that is left in the mutant genome (Figure 2).

Integrations can result in insertion of the marker, leaving the wildtype petH gene intact. This can happen if the first recombination event occurs upstream of the N-terminal coding region, and the second recombination takes place upstream of the in-frame deletion. The resulting genotype is essentially wildtype (Figure 2; type A). Only if the second recombination event is downstream of the deletion, the truncated gene replaces the wildtype (Figure 2; type B). Southern analysis showed that both genotypes resulted from transformation of the linearised construct. Complete segregation of the mutant genotype did not require selective growth conditions.

**The truncated petH transcript is overexpressed under salt stress conditions**

The petH transcription start site was mapped 523 bp upstream of the initiation codon. The resulting 5' untranslated region proved to overlap 223 bp with the divergently transcribed prk mRNA. Both the expression of petH and prk showed light regulation, whereas only the petH transcript was overexpressed when cells were challenged with high salt (van Thor et al., 1998c). The hypothesis to explain these observations includes the presence of regulatory elements that control petH transcription in the sequence upstream of the wildtype gene. The upstream sequence was therefore included in the mutant genotype (see also Figure 2). In particular, the response to salt shock should be conserved, if the effect of the truncation on cyclic electron transport is to be studied. To test this, 0.55 M NaCl was added to a growing culture of the mutant, and the expression level of the truncated petH transcript was followed, during the subsequent hours, by Northern hybridisation. Overexpression was observed already after 1 hour in the mutant. The increase in mobility of the 225 nucleotides truncated transcript is apparent (Figure 3). Thus, salt stress mediated transcriptional regulation of the truncated gene is conserved in the mutant genotype. In addition, diaphorase activity determined in cell-free extracts of the wildtype and the mutant, grown under standard conditions, were comparable (see also Figure 4).

**Figure 3. Northern analysis of wildtype petH and accumulation after salt shock of ΔpetH mRNA.**

Northern analysis of RNA from wildtype and SM8 'ΔpetH' cells was performed with a petH DNA probe identical to the one used for Southern analysis (Figure 2).
As shown above, the wildtype petH gene can be replaced with a truncated form, using a directed mutagenesis strategy. Since segregation of this genotype can be achieved under photoautotrophic growth conditions, this already indicates that the truncated enzyme is active in photosynthetic linear electron transport. To test this further, recombinant forms of the wildtype and the truncated enzyme were assayed in vitro for NADP⁺ photoreduction activity. For this purpose also recombinant Synechocystis plastocyanin and flavodoxin were produced heterologously and purified. The concentration of plastocyanin was chosen such that the rate-limiting reactions in the photoreduction assays were on the acceptor side of PS1. Under these conditions, an apparent $K_m$ of 0.7 μM was determined for the wildtype FNR (van Thor et al., 1998b). The activities of the wildtype- and the truncated enzyme were compared at a 1 μM concentration, using either spinach ferredoxin or recombinant Synechocystis flavodoxin (Table 2). No obvious difference in the activity between the two forms of FNR could be demonstrated with this photoreduction assay. This is in apparent agreement with the observation that recombinant forms of wildtype (49 kDa) and truncated (36 kDa) FNR from Anabaena sp. PCC 7119 show comparable turnover numbers in ferredoxin-NADP⁺ reductase assays at low ionic strength (Martínez-Júnuez et al., 1996).

**Truncated FNR does not bind to thylakoid membranes nor to phycobilisomes**

Native PAGE at pH 8.8 was used to separate the wildtype and truncated proteins and to allow detection of catalytic activity after electrophoresis. When cleared cell lysates of the wildtype- and the SM8 strains are separated on native PAGE, the resulting patterns show distinct differences in the mobility of FNR (Figure 4). In the lysate of the wildtype, FNR hardly migrates into the gel, whereas the truncated FNR, present in the lysate of SM8, shows a high mobility. For comparison, the mobilities of the purified wildtype and the truncated recombinant proteins are shown. Thus, wildtype FNR is retained in this gel-system whereas truncated FNR is not. The mobilities of the recombinant proteins are in agreement with
their predicted pI and molecular masses. The calculated pI of the 46 kDa wildtype protein, based on the amino acid sequence, is 5.62, whereas the lowering to 4.99 for the 38 kDa truncated protein, is the result of the loss of the positively charged N-terminal domain. Their charges at pH 8.8 are calculated to be -12.3 and -18.1 respectively. Fig. 4 shows that the truncated protein has a mobility that is 1.7 times that of the wildtype protein, that is the effect of both an increased charge and a decreased molecular mass of truncated FNR.

An additional band is observed in lysates of both the wildtype and the mutant. This band is not related to FNR, since its mobility is not affected by the truncation. In addition, it is no longer observed after one freeze-thaw cycle, which FNR is known to withstand (not shown). Also, the \textit{drgA} gene-product was recently identified in cell-free extracts of \textit{Synechocystis} PCC 6803 to possess NADPH-dependent diaphorase activity, which can also be demonstrated in native gels (Matsuo \textit{et al.}, 1998). The \textit{drgA} gene (slr1719 Cyanobase identifier) shows sequence homology to a

\begin{center}
\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure4.png}
\caption{Native gel-electrophoresis and NADPH-NBT reductase activity staining}
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\end{center}

Native gel-electrophoresis at pH 8.8 of cell fractionation preparations (see M&M) of wildtype and \textit{ΔpetH} truncation mutant SM8, and recombinant 47 kDa wildtype and 38 kDa truncated FNR. 3 μg Chl \textit{a} was loaded in the case of the cell-free extract and thylakoid preparations. The cytoplasmic fractions were the equivalent of cell-free extracts (3 μg Chl \textit{a}), after removal of the thylakoids by streptomycin sulfate precipitation. 5 μg protein was loaded in the case of phycobilisome preparations, and approximately 100 ng protein was loaded in the case of the recombinant proteins.
nitroreductase, a NADH dehydrogenase, and a NAD(P)H-flavin oxidoreductase.

Activity of the NAD(P)H dehydrogenase complex is not expected to produce signals in our system, since Triton X-100 is included in the gels, which is known to disrupt a multi-subunit complex such as the NAD(P)H dehydrogenase complex. Solubilisation of active complexes is only possible with mild detergents such as 3-[(3-chloramidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS; Matsuo et al., 1998). Accordingly, no signals other than FNR were detected in thylakoid preparations (Figure 4). In addition, cell-free extracts and thylakoid membranes from the NAD(P)H dehydrogenase deficient mutant M55 produced the same bands as the wildtype in our native gel system (not shown).

The FNR concentration was determined in these cell-free extracts by measuring the diaphorase activity at pH 9.5, in an assay with Triton X-100 included, and comparison with the activity of the purified recombinant 47 kDa wildtype protein. The concentration was 6.9 pmol FNR \( \mu \text{g} \) Chl \( a^{-1} \), which corresponds to approximately \( 0.9 \text{ FNR} : \text{PSI} \).

Purification of intact phycobilisomes by sucrose-density gradient centrifugation has been applied to many cyanobacteria and red algae. Preparations of a number of cyanobacteria have shown to contain FNR, although in some cases no, or only low amounts are present (Schluchter and Bryant, 1992; Sidler, 1994). Preparations of phycobilisomes of wildtype Synechocystis PCC 6803 invariably contain a detectable amount of FNR. It is calculated from the enzymatic activity present in such preparations that each phycobilisome complex contains approximately 2.4 copies of FNR. As expected FNR is no longer present in the phycobilisomes of the SM8 mutant, since the phycobilisome-binding domain is removed from this protein (Figure 4).

When the amount of FNR present in isolated phycobilisome complexes is compared to the concentration in cell-free extracts, it is concluded that 74% of the FNR molecules is bound to phycobilisomes. The extinction coefficient at 625 nm for wildtype Synechocystis PCC 6803 phycobilisome complexes was determined to be approximately 29,500 mM\(^{-1}\) cm\(^{-1}\), based on deconvoluted absorption spectra for phycocyanin chromophores (Demidov and Mimuro, 1995) and the structural characterisation of the wildtype complexes (Elmorjani et al., 1986). Using this molar extinction coefficient, and assuming a Chl \( a^{-1} \):F700 ratio of 150:1, a phycobilisome:PSI ratio of 0.3:1 is determined, using absorption spectra of whole cells. This is in reasonable agreement of the measured value of Ashby and Mullineaux (1998). The 2.4 copies of FNR per phycobilisome complex then correspond to 0.7 FNR:PSI. Thus, two independent types of calculation arrive at the conclusion that most of the FNR copies are bound to the phycobilisome.

When the thylakoids are removed by streptomycin precipitation, the cytoplasmic fraction of both the wildtype and the mutant contain most of the FNR. As a result of the removal of the thylakoids the wildtype protein is no longer retarded in the gel. This may suggest that the majority of the FNR molecules are associated with the membranes in suspension, even when Triton X-100 is present.

Both recombinant proteins show a small fraction of a highly mobile species, that is most probably the result of proteolytic degradation. This band is present in both preparations of recombinant proteins, as well as in the cytoplasmic frac-
tion of the wildtype and mutant *Synechocystis* strains (Figure 4; bands with highest mobility). Therefore, it is probably the result of an N-terminal cleavage, yielding the same product in the case of both the wildtype and the truncated proteins. A potential cleavage site was found upon examination of the deduced amino acid sequence of PetH: a ‘PEST-site’ was detected at position 91-110 in the wildtype protein. This sequence (PSQSEGSGSEAVANPAPESN) is rich in proline, glutamic acid, serine and threonine residues. In addition, it is not too hydrophobic, and is flanked by (positively charged) lysine residues at both sides. These features are diagnostic for a PEST site. This particular site scored 7.33 with the algorithm of Rogers *et al.* (1986), which is considered to be significant. This site is also present in the truncated protein, which lacks 75 N-terminal residues, at position 16-35. It can not be predicted what the site of cleavage is, based on the sequence only. However, the wildtype would probably gain most mobility after cleavage, since it would lose the positively charged N-terminal region. Another argument is that in a cleared lysate of wildtype *Synechocystis*, this cleaved species is not retarded, and therefore probably does not contain the N-terminal domain. It is noted that in the mutant SM8 most FNR is present as the ‘PEST-processed’ species, whereas in the wildtype this represents only a fraction. However, prolonged incubation at 0°C of the extract did convert a significant fraction of the wildtype protein into the ‘PEST-processed’ species (not shown). Others have detected exclusively proteolytically degraded FNR in extracts of *Synechocystis* PCC 6803 (Matsuo *et al.*, 1998). These authors determined the N-terminal sequence ‘MTTTPKEK’ (position 114-121 in the Open Reading Frame), for this degraded form of FNR. This N-terminus would result from cleavage at one residue on the C-terminal side of the positively charged lysine residue, that is part of the ‘PEST’ signature. We therefore suggest that the protein that was characterized in their study is identical to the ‘PEST-processed’ species observed in our native gels. A molecular weight of 34 kDa, a pI of 5.22, and a charge of -14 at pH 8.8 was calculated for this polypeptide.

Both the recombinant truncated protein and the protein present in the *Synechocystis* mutant cell extracts are detected as a triplet. The band with the highest mobility has been assigned as a ‘PEST-processed’ species. The remaining doublet is assigned to truncated FNR. The nature of the difference in mobility between the two bands is not known.

When thylakoids were precipitated with 1% (w/v) streptomycin sulfate twice, phycobilisome free membrane preparations were obtained. Only in wildtype membranes some FNR was retained after this treatment (Figure 4). Quantification of the thylakoid bound FNR was 0.9 pmol FNR μg Chl a⁻¹, which corresponds to about 0.12 FNR:PSI.

In conclusion, we found that the N-terminal CpcD homologous domain is a thylakoid binding domain, as well as a phycobilisome binding domain. When cell-free extracts of the wildtype were prepared in low ionic strength buffers, phycobilisome-associated FNR is released and shows affinity for the thylakoid, in contrast to the truncated protein. Copies of FNR that are not associated to the phycobilisome complexes are firmly bound to the thylakoid membranes via the N-terminal CpcD homologous domain, and are recovered in the membrane frac-
tion, whereas the bulk of the proteins is not. Membranes isolated from a sucrose step-gradient after ultracentrifugation (Omata and Murata, 1983) contain about 70% of the enzymatic activity that is present in a cell-free extract, in contrast to thylakoids from mutant SM8, demonstrating the affinity of the wildtype FNR for thylakoid membranes specifically, independent of the native gel technique.

Two modes of interaction, that are only detected for the 47 kDa wildtype protein with the thylakoid membrane, seem apparent from these experiments: One mode of tight interaction that allows copurification of FNR with the membrane fraction, when using a precipitation protocol. These copies represent a small fraction of the FNR pool, and are presumably not bound to the thylakoid in the intact cell. The other mode of interaction is a complexation with unknown thylakoid components that can exist in the presence of Triton X-100, but is broken during precipitation of the membranes, and presumably relies on a modification of the N-terminal domain. Mutants M55 (lacking the NAD(P)H dehydrogenase complex) and a PsaE deficient mutant were tested for this interaction, and were found to show wildtype FNR-thylakoid interactions (not shown).

**Green Fluorescent Protein binds thylakoids and phycobilisomes only when fused to the N-terminal domain of FNR**

Fusions were made between the *Aequorea victoria* gfp-uv gene and both the promoter region of petH and the N-terminal 75 codons of petH, resulting in a transcriptional and a translational fusion construct, respectively. Both constructs were transformed to the wildtype *Synechocystis* PCC 6803 and the *ApetH* truncation mutant ‘SM8’. Transformants were selected for their kanamycin resistance.

Western blot analysis showed that both the 38 kDa PetH'-GFP fusion and GFP itself were expressed in the kanamycin resistant transformants (Figure 5A). Purified recombinant GFP was used as a marker for quantification (Figure 5A, lane 1). Both in the wildtype and in the SM8 mutant background it was found that the PetH'-GFP fusion was more abundant than the corresponding, transcriptionally fused, GFP expressed in transformants. Since the only difference between both constructs is the 75 N-terminal PetH' codons (see: Materials and Methods), this suggests that this region has an effect on the stability of either the mRNA or the protein. Additionally, it is observed that the abundance of both GFP and the fusion protein in the SM8 background, is lower than in the wildtype background. Since the genotype of SM8 includes a duplication of the petH P/O region (Figure 2), this suggests that this is a transcriptional effect of the ‘dilution’ of P/O regions by the introduction of additional copies.

Fluorescence of neither GFP nor the GFP fusion protein was observed by fluorescence spectroscopy of either cell suspensions or cell-free extracts. The concentration of GFP in these samples, as quantified by western detection, is well within the detectable range for purified recombinant GFP for which a high fluorescence quantum yield was determined: 200-500 ng.ml⁻¹. Since Western blot analysis of non-denatured samples showed that most GFP was present as the auto-oxidised species (not shown), it was concluded that fluorescence was quenched in the cells and cell-free lysates.

When (phycobilisome-free) thylakoid membranes were prepared by two sub-
(A) Cell-free extracts (1.5 µg Chl a)  

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<td>WT</td>
<td>WT</td>
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<td>SM8</td>
<td>SM8 (Background)</td>
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(B) Thylakoid membranes (3 µg Chl a) 

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<tr>
<td>WT</td>
<td>WT</td>
<td>WT</td>
<td>SM8</td>
<td>SM8 (Background)</td>
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**Figure 5**

Western detection of GFP and PetH'-GFP fusion proteins in recombinant Synechocystis strains

(A) GFP and GFP fusion proteins in cell-free extracts

Cell-free extracts (1.5 µg Chl a) were loaded per lane. 120 ng (3.75 pmole) recombinant purified GFPuv was loaded as a reference. Lane 2: wildtype. Lane 3: WT-GFPuv. Lane 4: WT-GFPcrip. Lane 5: SM8-GFPuv. Lane 6: SM8-GFPcrip

(B) GFP and GFP fusion proteins in thylakoids

Phycobilisome-free thylakoid preparations (3 µg Chl a) were loaded per lane. 120 ng (3.75 pmole) recombinant purified GFPuv was loaded as a reference. Lane 2: wildtype. Lane 3: WT-GFPuv. Lane 4: WT-GFPcrip. Lane 5: SM8-GFPuv. Lane 6: SM8-GFPcrip

Sequent streptomycin sulfate precipitations, only the PetH'-GFP fusion proteins were detected with the monoclonal antibody, both in the case of the wildtype and in the SM8 background (Figure 5B). GFP from the WT-GFPcrip and SM8-GFPcrip strains do not copurify with the thylakoids (Figure 5B). This demonstrated independently from the behaviour of the wildtype and the truncated FNR proteins, that the N-terminal domain of PetH has specific affinity for the thylakoid membranes. Most PetH'-GFP fusion protein is present as the species that binds thylakoid membranes: about 5.3 pmol/µg Chl a⁻¹ (equivalent to approximately 0.7 GFP:PSI) in the case of the wildtype background, which is comparable to the amount of wildtype FNR present in thylakoid membranes. It is noted that most, or all, fusion protein is recovered in the thylakoid fraction, both in the case of the wildtype and the mutant background. This suggests that there is no detectable competition for binding sites between FNR and the PetH'-GFP fusion protein. As predicted, some fusion protein is also copurified with the phycobilisome complexes, demonstrating the specific phycobilisome-binding properties of the N-terminal domain as well.

Furthermore, cells from the WT-GFPuv strain were analysed for the sub-celular localisation of the GFP fusion protein by immuno-gold labelling and subsequent analysis by electron microscopy. Using a polyclonal α-GFP antibody specific labelling of the thylakoid membranes was observed (Figure 6). However, some labelling of the thylakoid membranes of cells of wildtype *Synechocystis*...
PCC 6803 was also observed (not shown). Western blot analysis identified an unknown component in the thylakoid membranes with a molecular mass of approximately 90 kDa (not shown). Pre-incubation of the primary antibody with isolated thylakoid membranes from the wildtype strain increased the labelling specificity (the average number of gold particles per cell of the wildtype decreased to approximately 20-30% relative to the number of gold particles per cell of the WT-GFPat strain). Hardly any labelling of the cytoplasm was observed, confirming the quantitative analysis of the Western blots performed with cell fractions of the recombinant strains expressing GFP fusion proteins. From both these in situ labelling- and from the cell-fractionation experiments we conclude that most of the fusion protein is localised at the thylakoid membranes, regardless of the presence of wildtype FNR. Whether some molecules of the GFP fusion protein were localised in the cytoplasm could not be established with these techniques. It was calculated that approximately 10% of the GFP fusion protein is bound to the phycobilisome complexes in the WT-GFPat strain. Therefore, most of the molecules are associated with the thylakoids via binding sites other than the phycobilisome complexes.

**Figure 6. In situ localisation of the GFP-fusion protein**

Immuno-gold (15 nm) labelling of a thin section of gluteraldehyde fixed cells of the WT-GFPat strain. An α-GFP polyclonal antibody was used with a 1:1,000 dilution. The thin section was negative stained with uranyl acetate. The bar represents 0.5 μm.
The NAD(P)H dehydrogenase complex catalyses both respiratory activity and an NADPH-dependent form of cyclic electron transport (Mi et al., 1992b; Mi et al., 1994). In the Synechocystis PCC 6803 mutant ‘M55’, lacking the NAD(P)H dehydrogenase complex, the plastoquinone pool is less reduced than in the wildtype, under conditions other than prolonged dark starvation. As a result, photochemical quenching (q_p) of PSII fluorescence measured in the dark is significantly higher in the mutant (Mi et al., 1994). For the wildtype Synechocystis PCC 6803 it was shown that respiratory electron donation to the plastoquinone pool is significant when photoautotrophically grown cells are directly analysed in the dark, whereas starvation induces a sharp decline of respiratory oxygen uptake (Mi et al., 1994). Thus, under conditions of photoautotrophic growth, oxidation of NADPH by the NAD(P)H dehydrogenase complex is expected to contribute to the total flux of electrons through the plastoquinone pool. In particular, the NADPH dependent route of cyclic electron transport around PSI is expected to be part of photosynthetic electron transport under conditions of phototrophic growth in white light.

The dependency of the rate of oxygen evolution on the (white) light-intensity was measured for the wildtype and mutants M55 and SM8, in order to determine the effects of the redox poise of plastoquinone on Q_A, via the Q_B site(Figure 7). In the dark, mutant M55 shows a decreased rate of respiratory oxygen uptake, relative to the wildtype. The ‘remaining’ 20-30% of the activity reflects electron donation catalysed by mechanisms other than via the NAD(P)H dehydrogenase complex. Interestingly, only the M55 mutant shows a sigmoidal dependence of the oxygen evolution rate on the light intensity. It was shown previously for this mutant that, contrary to expectations, at low (blue) light intensities, the stationary PSII fluorescence yield increased to relatively high levels, reflecting the accumulation of electrons in the intersystem chain (Schreiber et al., 1995). Using the same technique this was also observed with low intensities of phycobilisome absorbed light (not shown). This may reflect the imbalance in excitation of both photosystems that is the result of a ’lock’ in light-state 1, due to the over-oxidation of the plastoquinone pool of this mutant (Schreiber et al., 1995; van Thor et al., 1998a). This apparent overreduction, surprisingly, does not lead to the formation of a light-state 2 on the minutes timescale (Schreiber et al., 1995; van Thor et al., 1998a). Thus, at low light intensities, when the rate of light absorption is limiting for photosynthetic electron transport, the plastoquinone pool becomes over-reduced due to the increased (chlorophyll- and phycobilisome associated) absorption cross-section of PSII relative to PSI. (According to the ‘mobile-antenna model’ a state 2 - state 1 transition is the consequence of an increased chlorophyll- and phycobilisome associated absorption cross-section of PSII. However, in the ‘spill-over model’ it is assumed that a state 2 - state 1 transition involves a decrease in the spill-over (excitonic coupling by weak interactions) from PSII to PSI. In this case PSII does not acquire additional absorption cross-section, but increases its quantum efficiency. In either case the above mentioned arguments
Photosynthetic (P) electron transport rates were determined as the light intensity (I) dependent rate of oxygen evolution. At low light intensities net oxygen uptake is due to respiratory terminal oxidase activity. The activities are expressed on the basis of chlorophyll a. Wildtype (filled circles) and mutant strains M55 (NdhB-; open squares) and SM8 (ApetH truncation mutant; open circles) were grown photoautotrophically to mid-logarithmic phase.

Mutant M55 clearly is able to reach significantly higher rates of oxygen evolution, at saturating light intensity, than the wildtype can under comparable growth conditions. This effect is observed at high light intensities, when electron transport has become rate-limiting. Specifically, differences in the redox poise of the plastoquinone pool that are developed under light-saturated conditions can be demonstrated, since a higher rate of oxygen evolution is the result of increased photochemical quenching. This argument holds when (i) the PSII:PSI ratio is not changed drastically, and (ii) ‘pseudocyclic’ electron transport does not occur by the photochemical reduction of oxygen at the high-light induced over-reduced acceptor-side of the PSI reaction centers. The PSII:PSI ratio’s were determined by comparing the fluorescence emission spectra taken at 77K with 440 nm excitation of the wildtype in state 1 and the M55 mutant grown in standard conditions. It was found that in M55 the PSII:PSI ratio is increased by approximately
However, this cannot explain the large increase in the maximal rate of oxygen evolution. The 'pseudocyclic' reaction involving oxygen reduction is known to be relatively unimportant in cyanobacteria at the light intensities that were used (Campbell et al., 1998). With *Synechocystis* wildtype and mutant strains a decline of the oxygen evolution rate was observed at light intensities of 500 μmoles photons m⁻²s⁻¹ and higher (Figure 7). A three-fold increase of the maximal rate of oxygen evolution of the mutant M55, as compared to the wildtype, would suggest a significant contribution (when compared to PSII dependent rate of plastoquinone reduction) of the NAD(P)H dehydrogenase complex to reduction of the intersystem chain. Perhaps cells of mutant M55 are 'more' in state 1 than the wildtype at high light intensities, and the higher rate of oxygen evolution can partly be explained by an increased quantum efficiency of PSII.

Mutant SM8 shows a significant increase in the maximal rate of oxygen evolution as well (Figure 7). Similarly, this is interpreted as a decrease in the rate of plastoquinone reduction that is a result of the truncation of PetH. Since the rate of dark respiration of SM8 is intermediate between the wildtype and M55, as is the maximal rate of oxygen evolution (Figure 7), the truncation of PetH may affect respiratory as well as PSI-dependent cyclic electron transport. In mutant SM8 the PSII:PSI ratio was found to be increased by approximately 10%, as compared to the wildtype. The double mutant DM4, lacking both the NAD(P)H dehydrogenase complex and the PetH N-terminal domain, showed characteristics similar to M55 in these tests.

The maximal rate of oxygen evolution that is determined experimentally is considered to be related to the maximal growth rate of oxygenic photosynthetic algae (Geider and Maclntyre, 1996). The characteristics of mutants deficient in plastoquinone reduction pathways shows that this statement must be refined. Presumably, fluxes of electrons through PSI and the terminal oxidase are not significantly altered under conditions of reduced rates of plastoquinone reduction via respiratory electron transport or PSI-dependent cyclic electron transport activity. Photosystem II activity is proposed to become limited at high light intensities by the redox poise of the plastoquinone pool. The effect of respiratory electron donation to the plastoquinone pool on the quantum efficiency of PSI has been investigated extensively in both chloroplasts and blue-green algae (Weis and Berry, 1987; Dominy and Williams, 1987). We propose that the maximal flux of (linear and cyclic) photosynthetic electron transport through the cytochrome $b_{6}f$ complex may be a more appropriate measure for the maximal sustainable growth rate of oxygenic phototrophic organisms.
adapted to salt stress did not show induction of cyclic electron transport as is observed in the wildtype and mutant M55 (Figure 8). Thus, even in the presence of functional NAD(P)H dehydrogenase complex, the adaptation to 0.55 M NaCl requires a function of ferredoxin:NADP\(^+\) reductase that is dependent on the presence of the N-terminal domain.

A phycocyanin deficient mutant '4R' (Plank et al., 1995a) was also tested for its capacity of cyclic electron transport, and was found to closely resemble the wildtype both with and without salt stress. Since preparations of the allophycocyanin core from this mutant do not contain substantial amounts of FNR, most probably no complex between FNR and the phycobilisome exists in vivo in this mutant, just as in mutant SM8. Since induction of cyclic electron transport due to salt stress is observed in mutant 4R, the fraction of the FNR molecules that is bound to the phycobilisomes is not responsible for the function in cyclic electron transport.

The double mutant DM4 (carrying the M55 as well as SM8 mutation) did not grow under salt stress conditions, indicating that both NAD(P)H dehydrogenase complex activity and wildtype PetH activity contribute to cyclic electron transport under salt stress conditions. After prolonged growth of the double mutant DM4 under standard photoautotrophic growth conditions it was found that suppressors arose that gained the ability to grow under salt stress conditions. A revertant of DM4 was tested for energy storage and P\(700^+\) re-reduction kinetics. It was found that the energy storage in salt stressed cultures was also not increased above the level that was determined under standard growth conditions. This level, however, was significantly lower than the level determined for the M55 single mutant (Figure 8 A). The P\(700^+\) re-reduction kinetics, however, were comparable under standard conditions for M55 and the revertant of double mutant DM4, whereas the double mutant in high salt cultures showed significantly decreased rates of electron transport (Figure 8 B). Presumably an additional route of plastoquinone reduction...
was induced in the revertants of DM4, that allowed the strains to grow under salt stress conditions. However, since this route was not induced under salt stress conditions, it did not involve the function of wildtype FNR.

**DEMONSTRATION OF FNR:PLASTOQUINONE OXIDOREDUCTASE ACTIVITY IN VITRO WITH ASSAYS OF ELECTRON TRANSFER FROM NADPH TO CYTOCHROME C**

Cyanobacterial thylakoids oxidise NADH as well as NADPH. It has been established that these reactions are part of both the respiratory electron transport pathway and PSI-dependent cyclic electron transport, catalysed mainly via the NAD(P)H dehydrogenase complex. Moreover, the *Synechocystis* PCC 6803 NAD(P)H dehydrogenase complex was shown to oxidise NADPH preferentially over NADH (Mi et al., 1992b). In order to separate the contributions to plastoquinone reductase activity made by, respectively the NAD(P)H dehydrogenase complex and FNR, *in vitro* NADPH:cytochrome c oxidoreductase assays were performed according to (Kraushaar et al., 1990).

When thylakoids are prepared by sucrose density flotation centrifugation, according to Omata and Murata (1983), these membranes are isolated separately from the cytoplasmic membranes. The latter are known to support respiratory oxidation of NADH and NADPH as well. When oxidised horse-heart cytochrome c was added to a reaction mixture containing isolated thylakoid membranes, no reduction of cytochrome c was observed. When NADPH was added at a concentration of 200 µM, however, rapid reduction of cytochrome c was observed (Figure 9). Simultaneously, oxidation of NADPH, monitored at 340 nm, proceeded with a stoichiometry of 1 NADPH : 2 cytochrome c (not shown). When cytochrome c was added to thylakoid membranes of mutant M55, oxidation of the small fraction of reduced cytochrome c proceeded until no reduced cytochrome c could be detected spectrophotometrically anymore (Figure 9). This indicated that (i) membranes from mutant M55 were more oxidised than membranes from the wildtype when they were isolated from cell extracts, and (ii) oxidation of cyt c proceeded via direct interaction with the thylakoid membrane, most probably with the cytochrome b,f complex. Addition of NADPH to membranes from M55 resulted in reduction of cyt c with significantly lower rates than observed with membranes from the wildtype (Figure 9), demonstrating the contribution of the NAD(P)H dehydrogenase complex to the activity. Membranes from mutant SM8 do not support oxidation of cyt c as in the case of mutant M55, but showed significantly reduced cyt c reductase activity, with NADPH present, as well (Figure 9). Membranes isolated from the double mutant DM4 essentially show the same activity as mutant M55.

A ‘direct’ cyt c reductase activity of FNR is well-known (Knaff and Hirasawa, 1991). Cyt c can be reduced by FNR efficiently if ferredoxin is present, or by a reduced oxygen intermediate. Isolated thylakoid membranes are essentially ferredoxin-free. The cellular localisation of ferredoxin was found to be cytoplasmic in the case of the cyanobacterium *Synechococcus* sp. PCC 7942 (van der Plas et al., 1988).

Direct reduction of cyt c by FNR under aerobic conditions was investigated for the recombinant wildtype 47 kDa protein. About 0.32 mol cyt c . mol FNR⁻¹
NADPH:CYTOCHROME C OXIDOREDUCTASE ASSAYS OF WILDTYPE, SM8, M55 AND DM4 THYLAKOID MEMBRANES

Thylakoid mediated NADPH-cytochrome c reduction was monitored spectroscopically, by following the oxidation of NADPH at 340 nm as well as the reduction of cytochrome c (A550-A540). Cytochrome c reduction kinetics are shown for the measurements with thylakoid membranes, isolated by sucrose density flotation centrifugation, from wildtype, mutants SM8, M55 and a revertant of DM4. At t=0 cytochrome c is added in mostly oxidised form. NADPH is added at 200 μM concentration at t=180 s. Rates are given in nmoles cytochrome c reduced.mg Chl a^−1.min^−1.

was reduced at 50 μM cyt c, with NADPH present. The K_m for cyt c was approximately 15 μM in these assays. When the reactions were performed anaerobically, the activity decreased to about 0.06 mol cyt c.mol FNR^−1.sec^−1, indicating that this reaction proceeded mainly via oxygen radicals. When NADPH:cyt c oxidoreductase assays of thylakoid membranes were performed anaerobically, no decrease in activity was observed for any type of membrane preparation, indicating that the assays are not dominated by side-reactions involving oxygen reduction. This is further supported by the observation that 80 pmol FNR.μg Chl a^−1 (3.8 μg FNR.μg Chl a^−1, corresponding to about 12 times the amount present in cell-free extracts) has to be present in these thylakoid preparations in order to be able to catalyse these rates of cyt c reduction via oxygen. Clearly, much less is present in the thylakoid membranes, or in the cell-free extracts. Since a significant decrease in the cyt c reductase activity was assayed in the case of mutant SM8 membranes, it is concluded that in the wildtype membranes FNR catalyses oxidation of NADPH with a relatively high turnover number via reduction of the plastoquinone pool and the cytochrome b_{6f} complex. If the activity of thylakoids of SM8 is subtracted from the activity determined in the wildtype membranes (Figure 9), a turnover number of 0.63 e^−.s^−1 is calculated, assuming that all FNR present in the membrane fraction participates in this reaction. The turnover number becomes 3.7 e^−.s^−1 if only the "tightly bound" FNR participates in cytochrome c reduction.
Isolation of thylakoid membranes from salt-stressed cultures was problematic due to a low yield of recovery. In addition, electron transport in such membranes could not be demonstrated, not even with material isolated from the wildtype. After salt shock, FNR accumulates to higher levels than under standard conditions (van Thor et al., 1998c). Phycobilisome complexes isolated from such cultures do not contain more FNR than corresponding cultures grown under standard conditions. Therefore, this additional protein will be free to interact with the thylakoid membrane and contribute to plastoquinone reductase activity.

General discussion

The FNR:P700 ratio that was determined for Synechocystis PCC 6803 is comparable to previously reported ratio's in algae and chloroplasts. The highest ratio that was reported for chloroplasts was 3:1 for spinach (Böhme, 1978). The PSI:PSII ratio in Synechocystis is higher in chloroplasts, suggesting that under saturating light conditions the turnover numbers of chloroplast PSI reaction centers are at least as high as PSI centers of cyanobacteria. The turnover number determined for cells of green algae and blue-green algae with white actinic illumination were shown to be in the same order of magnitude ($k = 70 - 115 \text{ s}^{-1}$) (Maxwell and Biggins, 1976). The copies of FNR present under standard photoautotrophic growth conditions probably mainly support linear electron transport from reduced ferredoxin to NADP$^+$. Furthermore, three quarters of the total pool is present in the phycobilisome. Under conditions of salt stress the levels increase, and these additional molecules are associated with ‘reverse’ electron transport to plastoquinone.

The N-terminal domain of FNR, as predicted, binds the protein to the phycobilisome complexes. It was found that a specific interaction of the N-terminal domain with the thylakoid membrane directs the copies that are not bound to the phycobilisome to the thylakoid membrane. In agreement with these results it was shown by analysis of cell fractions, and in situ localisation experiments, that a fusion protein of GFP with the N-terminal domain of FNR, also binds to the thylakoid membrane.

In the wildtype, most of the FNR is present as the 47 kDa full-length species, although cleavage at a ‘PEST’ site yields small amounts of a processed species that lacks the N-terminal CpcD homologous domain. Since the N-terminal domain specifically directs the protein to both the thylakoid and the phycobilisome, it is speculated that processing of FNR may constitute a regulatory mechanism. However, in other systems, cleavage of proteins at ‘PEST’ sites leads to an increased rate of proteolytic turnover of the protein (Rogers et al., 1986). In wildtype Synechocystis cells, only small amounts of the cleaved species are observed, whereas most of the truncated form is ‘PEST’ processed. This indicates that the processed species is not highly unstable, since it can accumulate at levels comparable to the full-length wildtype protein. Therefore, ‘PEST’ processing may have another function in the case of Synechocystis FNR.
The \textit{ApetH} mutant SM8 shows a clear phenotype in assays, designed for assaying the contributions to plastoquinone reductase activity \textit{in vivo} as well as \textit{in vitro}. Measurements of farred light energy storage capacity and assays of $\text{P700}^+$ re-reduction indicate that in the mutant induction of cyclic electron transport is absent. Moreover, this inducible flux of cyclic electron transfer correlated with overexpression of flavodoxin (Fulda and Hageman, 1995), and FNR (van Thor \textit{et al.}, 1998c) in the wildtype strain.

\textit{In vitro} assays show that thylakoid membranes, containing wildtype FNR, catalyse higher rates of NADPH dependent cytochrome $c$ reduction than the mutants lacking the NAD(P)H dehydrogenase complex (M55) or FNR (SM8). Since the wildtype thylakoid membrane preparations contain (additional) full-length FNR, that was released from the phycobilisomes during the isolation procedure, this assay may in fact have been the result of a reconstitution of FNR. Therefore, the assay may have mimicked salt stress conditions, when additional copies of FNR accumulate and are directed to the thylakoid.

As a result of salt shock the FNR concentration increases above the level that is normally present in the phycobilisomes, providing a possible mechanism for control of the pool size that can engage either in ‘forward’ or in ‘reverse’ electron flow. For the purpose of this discussion ferredoxin: NAD$^+$ oxidoreductase activity is considered ‘forward’ electron transport, and FNR: plastoquinone oxidoreductase activity is considered ‘reverse’ electron transport. The binding of FNR to the phycobilisome might simply be a mechanism that is present in order to prevent the binding of FNR to the thylakoids and therefore the participation in plastoquinone reductase activity. Since the FNR:phycobilisome and the phycobilisome:PSI ratios are relatively constant, this could ensure that enough copies are present to catalyse linear electron transport. Accumulation of FNR, above the level present in the phycobilisomes, is presumably the result of a specific transcriptional induction of the \textit{petH} promoter (van Thor \textit{et al.}, 1998c). This could regulate the number of molecules that are available for reverse electron transport.

\textit{In vivo} FNR most probably catalyses, during reverse electron transport, ferredoxin-dependent (and/or flavodoxin-dependent) plastoquinone reduction, converting a one-electron redox reaction into a two-electron reaction. NADPH:plastoquinone oxidoreductase activity of FNR can conceivably contribute to the flux, since this reaction can be demonstrated \textit{in vitro}. The free energy difference between the ferredoxin (flavodoxin) / plastoquinone couple (-500 and -450 mV, respectively) is higher than between the NADPH / plastoquinone couple (-420 mV).

The rate of $\text{P700}^+$ re-reduction is experimentally determined from the halftime of re-reduction, which is largely independent of the concentration of accumulated $\text{P700}^+$. This implies that the reaction can be described in terms of a (pseudo) first-order reaction rate constant. If the kinetics of the individual partial reactions are considered, the reduction of components present in the thylakoid membrane presumably has the highest control on the rate of $\text{P700}^+$ reduction (Nanba and Katoh, 1983; Maxwell and Biggins, 1976). This situation could in principle develop if the second-order rate constant for a plastoquinone...
reductase and the electron donor was high, and the first order rate-constant for electron transport was slow. It is interesting to note that in the case of ferredoxin and flavodoxin, the second order rate constant is indeed very high ($>10^8 \text{ M}^{-1}\text{s}^{-1}$; Batie and Kamin, 1984; Hurley et al., 1996), as well as for NADPH ($>10^8 \text{ M}^{-1}\text{s}^{-1}$; Batie and Kamin, 1984), while the turnover number that was determined for FNR with the NADPH-cytochrome c reductase assay was relatively low, indicating that the first order-rate constant for electron transport was indeed small. Variations in the plastoquinone reductase concentration would then consequently lead to changes in the first order rate constant for $P_{700}^+$ reduction. Thus, the forward rate constant for electron transport from FNR to an acceptor in the thylakoid membrane is small. Consequently, the reaction constant for the corresponding back reaction must very small, considering the free energy difference.

It was concluded from the behaviour of mutants M55 and SM8 in the oxygen evolution experiments, that fluxes of plastoquinone reduction (cyclic electron transport and/or ‘pseudo-cyclic’ route involving NADPH generated via the pentose phosphate pathway) are considerable during white-light saturated assay conditions, even with cells grown under standard photoautotrophy. It was indeed observed that the maximal oxygen evolution rates decreased in salt stressed cultures, demonstrating the effect of increased rates of plastoquinone reduction on the activity of PSII via the reduction state of the plastoquinone pool. It must be concluded that the assays of $P_{700}^+$ re-reduction and farred energy storage measurements do not necessarily reflect the total rate of cyclic electron transport that operates under conditions of white-light driven photosynthetic electron transport, since exclusive excitation of PSI results in a different redox poise of the components involved. It would be difficult to predict what the differences in concentration of reduced and oxidised components will become upon excitation of either PSII and PSI, or exclusively PSI. However, complete oxidation of the plastoquinone pool and the cytochrome $b_{f}$ complex seems likely under conditions of PSI excitation only. We propose that the ratio of the dark re-reduction rates of $P_{700}^+$ after excitation with either white or farred light, do not directly reflect the rates of linear and cyclic electron transport, as was proposed by Maxwell and Biggins (1976). On the other hand it can not directly be calculated what the fluxes of electron transport actually are, since the relation between oxygen evolution by PSII and the redox state of the plastoquinone pool, in addition to the plastoquinone pool size, is not known.

It should be mentioned that we could not detect any ferredoxin:plastoquinone oxidoreductase activity (‘FQR’; Cleland and Bendall, 1992) with isolated thylakoid membranes. Even under anaerobic conditions the rate of oxidation of ferredoxin was faster than the rate of its photochemical reduction by PSI. As a result no reduced ferredoxin accumulated. However, we do not rule out that in vivo FNR could be part of a ‘FQR’ pathway, catalysing reverse electron flow in a manner that is dependent on the presence of the N-terminal thylakoid binding domain.

The oxygen consumption rate of mutant M55 in the dark was significantly lower than that of the wildtype. However, some electron transport remains, indicating the presence of mechanisms of plastoquinone reduction other than the NAD(P)H dehydrogenase complex activity, in intact Synechocystis cells. The dou-
ble mutant DM4, lacking both the NAD(P)H dehydrogenase complex and the N-terminal domain of FNR, showed both the decreased capacity for cyclic electron transport (which is also observed for mutant M55) and the absence of induction after salt shock, that is the result of truncation of FNR. Initially the strain did not grow in high salt media. However, revertants were selected under normal photoautotrophic conditions. We observed a relatively high rate of cytochrome c reduction with thylakoid membranes isolated from such revertants. Probably an additional route of plastoquinone reduction is induced in this mutant. A possible candidate is NDH2, for which three putative genes have been identified in the genome sequence of *Synechocystis* PCC 6803: slr1743, slr0851 and sll1484 (Kaneko et al., 1996).

This is the first report of the involvement of FNR in photosystem I-dependent cyclic electron transport, via its plastoquinone reductase activity, that is not based on the specific inhibitory effects of modifying agents (Mills et al., 1979; Shahak et al., 1981; Hostler and Yocum, 1985), but is based on the characterization of the phenotype of a mutant that was genetically engineered. In addition, *in vitro* assays confirmed the observed decrease in plastoquinone reductase activity that is the result of the removal of the N-terminal domain that is required for thylakoid association as well as for phycobilisome binding.

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