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FtsH protease is required for induction of inorganic carbon acquisition complexes in *Synechocystis* sp. PCC 6803

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Summary

Cyanobacteria possess a complex CO_2-concentrating mechanism (CCM), which is induced by low inorganic carbon conditions. To investigate the involvement of proteases in the processes of induction and degradation of the CCM complexes, we studied the FtsH2 (ΔSlr0228) and Deg-G (ΔSlr1204/ΔSll1679/ΔSll1427) protease mutants of *Synechocystis* sp. PCC 6803. WT and protease mutant cells were grown under high CO_2 and then shifted to low CO_2, followed by a proteome analysis of the membrane protein complexes. Interestingly, in the FtsH2 protease mutant, inducible CCM complexes were not detected upon shift to low CO_2, whereas the Deg-G mutant behaved like WT. Also the transcripts of the inducible CCM genes and their regulator *ndhR* failed to accumulate upon shift of FtsH2 mutant cells from high to low CO_2, indicating that the regulation by the FtsH2 protease is upstream of NdhR. Moreover, functional photosynthesis was shown a prerequisite for induction of CCM in WT at low CO_2, possibly via generation of oxidative stress, which was shown here to enhance the expression of inducible CCM genes even at high CO_2 conditions. Once synthesized, the CCM complexes were not subject to proteolytic degradation, even when dispensable upon a shift of cells to high CO_2.

Introduction

For aquatic photosynthetic organisms like cyanobacteria, inorganic carbon (Ci) is an essential but often limited substrate. In order to overcome the low affinity of Rubisco for CO_2, cyanobacteria have developed an elaborate CO_2-concentrating mechanism (CCM), including an uptake of both CO_2 and HCO_3^-, which elevates the CO_2 concentration in carboxysomes (Price *et al*., 1998; Kaplan and Reinhold, 1999; Badger and Price, 2003; Badger *et al*., 2006).

The CCM involves a set of protein complexes, which facilitate cells to acquire Ci from environment. At least five modes of Ci uptake have been identified in model laboratory strains of cyanobacteria (Price *et al*., 2002; Shibata *et al*., 2002b; Ogawa and Kaplan, 2003; Badger *et al*., 2006). Two of them function in CO_2 uptake via specialized type I NAD(P)H dehydrogenase (NDH-1) complexes located in the thylakoid membrane. Inactivation of some specific *ndh* genes leads to abolishing not only the CO_2 uptake but the cells are also to a large extent deficient in the cyclic electron flow around PSI (Ogawa, 1991a,b; 1992; Mi *et al*., 1992; Marco *et al*., 1993; Yeremenko *et al*., 2005), suggesting that the NDH-1-dependent cyclic electron transport might be essential to energize CO_2 uptake. Both genetic and proteomic analysis have demonstrated that diverse forms of NDH-1 complexes, besides canonical respiratory NDH-1, exist in cyanobacteria, and function in CO_2 uptake (Klughammer *et al*., 1999; Ohkawa *et al*., 2000; Herranen *et al*., 2004; Zhang *et al*., 2004; 2005). The diversity of NDH-1 complexes results from modification of the subunit composition during evolution by harbouring different products of the *ndhD* and *ndhF* genes, which constitute multigene families in cyanobacteria (Cyanobase). These alternative NDH-1 complexes include a constitutively expressed low-affinity CO_2 uptake system containing the NdhD4, NdhF4 and CupB proteins, and a low Ci-induced CO_2 uptake system containing the NdhD3, NdhF3, CupA and sll1735 proteins (Shibata *et al*., 2001; Maeda *et al*., 2002; Zhang *et al*., 2004). The other three Ci acquisition systems are involved in bicarbonate transport and reside on the plasma membrane. An ATP-binding cassette (ABC)-type HCO_3^- transporter encoded by *cmpABCD* operon (Omata and Ogawa, 1986; Omata *et al*., 1999) as well as a Na^+/HCO_3^- symporter encoded by the *sbtA* gene (Shibata *et al*., 2002a) and Na^+-dependent HCO_3^- transporter BicA (Price *et al*., 2004)

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have been identified. They are driven by ATP and Na^+ gradient across the cytoplasmic membrane respectively. In *Synechocystis* 6803, SbtA is the major HCO_3^- transporter (Shibata *et al*., 2002a).

The CCM is regulated by environmental Ci supply. The CCM is constitutively expressed at relatively low level under Ci rich conditions, whereas the maximum CCM activity is induced under Ci limitation. Among five identified Ci uptake systems in *Synechocystis* 6803, three are induced by low Ci conditions, and two of them (NDH-1S and SbtA complexes) were recently identified in *Synechocystis* membrane proteome (Herranen *et al*., 2004; Zhang *et al*., 2004). Induction of the inducible CCM is a fast response upon transfer of cells from high CO_2 to low CO_2. In general, it takes 1–4 h for cells to adapt to the new growth condition and to synthesize proteins *de novo* (Omata and Ogawa, 1985; Kaplan *et al*., 1994). LysR family proteins CmpR and NdhR regulate the *cmpABCD* operon and the *ndhF3/ndhD3/cupA* operon, respectively (Figge *et al*., 2001; Omata *et al*., 2001). A recent study showed that NdhR is a universal CCM regulator, which not only controls the transcription of *ndhF3/ndhD3/cupA*, but also regulates *slr2006-slr2013* and *sbtA/sbtB*, therefore suggested a name 'CcmR' (Wang *et al*., 2004). Transcript accumulation from the *ndhF3*, *sbtA* and *cmpA* genes, representatives of the three inducible Ci uptake systems, showed a fast response under Ci limitation whereas the regulators *cmpR* and *ndhR* are more moderately expressed (McGinn *et al*., 2003).

Although considerable progress has been made during the past few years in the analysis of the molecular components, evolution and induction of CCM in cyanobacteria, little is known about the life cycle of the CCM protein complexes once synthesized. In order to investigate the induction and degradation of the CCM complexes induced in low Ci environment, we have studied *Synechocystis* FtsH2 (Δ*slr0228*) and Deg-G protease mutants (Δ*slr1204*/Δ*sll1679*/Δ*sll1427*). These proteases have previously been reported to be involved in degradation of membrane proteins and protein complexes (Silva *et al*., 2003; Barker *et al*., 2006; Komenda *et al*., 2006).

Membrane proteomics, however, revealed that the CCM complexes are not subject to degradation, even though they are not required after shifting of cells to high CO_2. On the contrary, our results show that the FtsH2 protease is crucial for full induction of CCM and that the FtsH2 protease may target the regulator protein NdhR, which negatively regulates the transcription of inducible CCM genes as well as *ndhR*. Consequently, the FtsH2 protease is likely to be needed for degradation of the repressor protein NdhR, thus allowing the activation of transcription of these inducible CCM genes upon shift to low Ci condition.

Results

Induction of the CCM complexes in WT and protease mutants upon shift from high to low CO_2 environment

In order to address the involvement of proteases in the induction and degradation of the CCM complexes, *Synechocystis* WT and protease deficiency mutant strains FtsH2 (Δ*slr0228*) and Deg-G (Δ*slr1204*/Δ*sll1679*/Δ*sll1427*) were investigated by growing of cells at high CO_2 and then shifted to low CO_2 for 48 h. Membrane protein complexes were separated by 2-D BN/SDS-PAGE (Fig. 1). In all three strains grown under high CO_2 condition, the CCM complexes NDH-1S and SbtA as well as CmpA protein were not detectable, and the proteome pattern was very similar. The only clear difference observed was a strong accumulation of the CP43-less PSII monomer in the FtsH2 mutant. Upon shift to low CO_2 growth condition, the NDH-1S and NDH-1M as well as SbtA and CmpA were strongly induced in WT to enhance the CO_2 and HCO_3^- uptake respectively. Similar phenomenon was observed in the Deg-G mutant. However, the deletion of *ftsH2* (*slr0228*) abolished the induction of these CCM complexes upon a shift of cells from high to low CO_2 condition, because no apparent changes in the membrane proteome were detected as compared with the cells grown at high CO_2 condition.

Turnover of the CCM complexes

The induction of the CCM complexes upon shift to low Ci environment is a well known phenomenon, whereas very little is known about the fate of these complexes once synthesized. To get insights into the lifetime of the CCM complexes, following experiments were performed with WT *Synechocystis* cells. The high CO_2 grown cells were shifted to low CO_2, and a radioactive pulse was given by supplementing the culture medium with $^{[35]}$S-Met for 1 h. Then the pulse was terminated, and the cells were chased both at low and high CO_2 condition for 3 h. Tracking of radioactive label in the membrane proteins is shown in Fig. 2.

Upon 1 h pulse, most radioactive label was incorporated into PSII complexes, and in lesser amounts also in PSI, ATP synthase, cytochrome b_6f (Cyt b_6f), NDH-1 and SbtA complexes. This indicated that the CCM complexes (NDH-1S and SbtA) were induced and actively synthesized after 2 h shifting from high to low CO_2. Due to a difference in the turnover rates of proteins, the relative amount of radioactive label in various membrane proteins had changed after a 3 h chase (Table 1). The D1 protein is known to be rapidly degraded and synthesized during the PSII-damage repair cycle, and clearly the ratio of label in D1 to that in D2 decreased during the chase. Using the label accumulated in the D2 protein of PSII as a reference

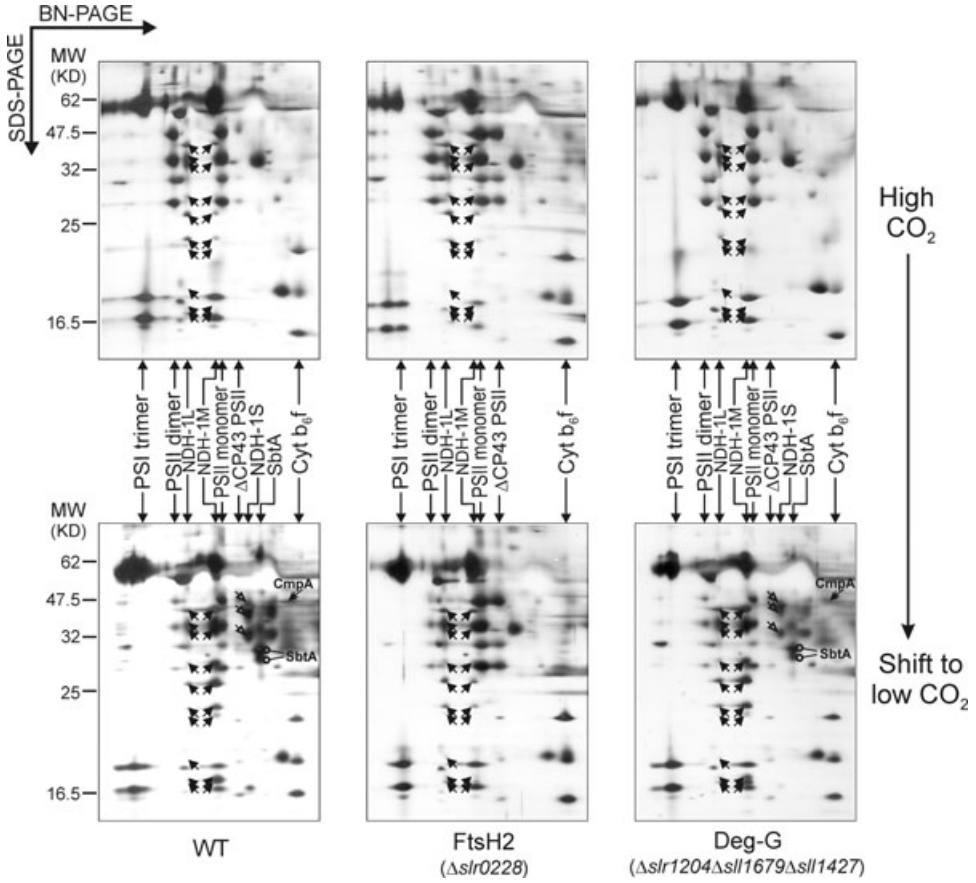

Fig. 1. Proteome of the membrane protein complexes of the FtsH2 and Deg-G mutants as compared with the WT strain grown under similar conditions. The *Synechocystis* cells were grown under high CO_2 and shifted to low CO_2 for 48 h. Total membranes were isolated and subjected to 2-D BN/SDS-PAGE. The major protein complexes are indicated on the silver-stained gels. The spots of the subunits belonging to NDH-1L and NDH-1M complexes are marked with black-head arrows, the spots of the subunits belonging to NDH-1S complex (CupA, NdhF3 and NdhD3 proteins) are marked with white-head arrows, the spots corresponding to SbtA protein are indicated by white nods, and the spot corresponding to CmpA protein is marked by double-arrow. Identification of the protein spots by mass spectrometry, please see Herranen *et al.* (2004) and Battchikova *et al.* (2005).

also for the $Cytb_6f$ complex (subunit b_6), the NDH-1S complex and the SbtA complex, we could observe that the label in the CCM complexes did not decrease even when cells were shifted to high CO_2 for 3 h. Thus, the CCM complexes, once synthesized, are not subjected to rapid degradation by membrane proteases when not needed for carbon acquisition.

To examine the time-courses for the turnover of the CCM complexes, the low CO_2 grown WT *Synechocystis* cells were shifted to high CO_2 and concomitantly supplemented with lincomycin to interrupt protein synthesis. Degradation of PSII, SbtA and NDH-1S complexes was investigated at time points 0, 6 h, 1 day, 3 days and 8 days using antibodies against membrane proteins D1, SbtA and NdhD3 respectively.

By supplementing lincomycin in the culture medium, new protein synthesis and cell growth were interrupted. Due to damage and lacking of new synthesis of D1, extensive degradation of PSII was observed. As demonstrated in Fig. 3A, the D1 protein was degraded quickly and only about 15% D1 was left after 1 day. After 3 days, there was no intact D1 left in the cells and a small amount of D1 fragments appeared. In dying cells (8 day sample when the bleaching of cells was obvious), fragments of D1 accumulated. On the contrary, the amount of the NdhD3 and SbtA protein was constant until the cells started dying. In the control culture without lincomycin, the amount of the D1 protein remained stable by compensation with *de novo* synthesized D1, whereas the amount of the NdhD3 and SbtA decreased due to dilution by newly growing cells (Fig. 3A). The proteome patterns of the membrane protein complexes isolated from the cells before and after 3 days lincomycin treatment are shown in Fig. 3B. The PSII complex was totally degraded after 3 days, and only the free CP47 protein was present, whereas all NDH-1 complexes, SbtA and CmpA remained stable as well as PSI, $Cytb_6f$ and ATP synthase.

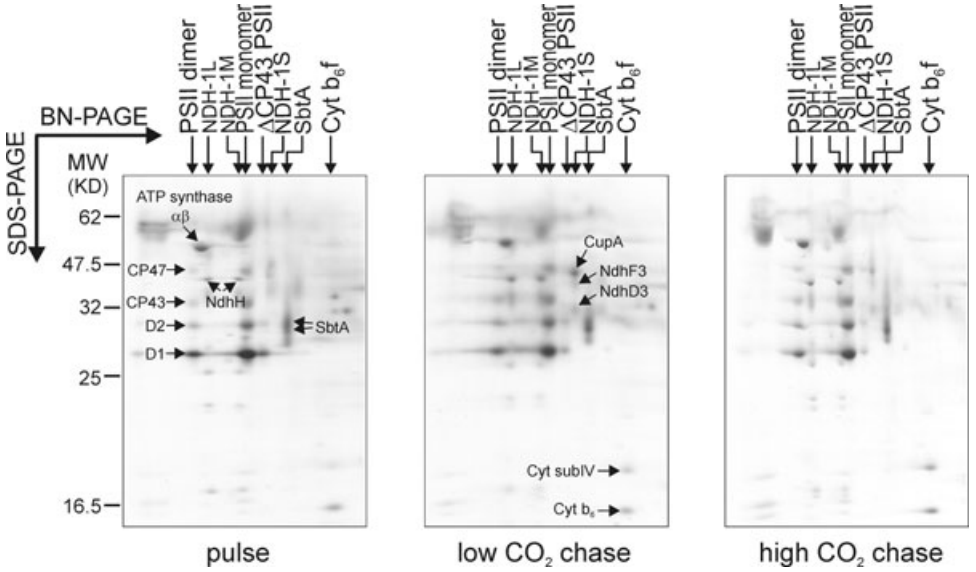

Fig. 2. Autoradiograph of membrane proteins separated by 2-D BN/SDS-PAGE. *Synechocystis* WT cells were grown at high CO_2. After shift to low CO_2 for 2 h, a radioactive pulse was given by $^{[35]}$S-Met for 1 h. The cells were chased in low and high CO_2 conditions for 3 h. The proteins with incorporated radioactive label (major PSII proteins CP47, CP43, D2 and D1, the NdhH protein of the NDH-1L and NDH-1M complexes, the CupA, NdhF3 and NdhD3 proteines of the NDH-1S complex, $Cytb_6$ and subIV proteins of the $Cytb_6f$ complex, the α and β subunits of the ATP synthase as well as the SbtA protein) are indicated by arrows.

Effect of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) on the induction of the CCM complexes

WT *Synechocystis* cells were grown at high CO_2 condition and then shifted to low CO_2 and concomitantly supplemented with various concentrations (0–15 μM) of DCMU. After DCMU treatment for 24 h, the expression of the NDH-1S and SbtA complexes was semi-quantified by immunoblotting with specific antibodies against NdhD3 and SbtA, respectively (Fig. 4A). The PSII activity of the cells was monitored by measuring steady state rate of oxygen evolution (Fig. 4B).

DCMU inhibited PSII activity and prevented a proper induction of NDH-1S and SbtA upon shifting cells from high to low CO_2. Small amount of DCMU (0.1 μM) allowed considerable PSII activity (60%) and the NdhD3 and SbtA proteins were induced about 85% compared with control cells. Supplementing 0.3 μM DCMU remained 40% of PSII activity and allowed only very little of induction of NDH-1S and SbtA. Further increasing the DCMU concentration allowing less than a quarter of maximal PSII activity, completely inhibited the induction of both NDH-1S and SbtA.

Table 1. Relative amount of radioactive label accumulated in the membrane protein complexes (a representation protein of each complex was used as indicated).

	PSII (D1/D2)*	Cytb_6f b_6/D2	SbtA/D2	NDH-1S/D2
Pulse	2.87 ± 0.29	0.237	0.795	0.425
LC chase	1.47 ± 0.09	0.141	0.802	0.481
HC chase	1.49 ± 0.26	0.237	0.775	0.461

The D2 protein was used as a reference. LC and HC, low and high CO_2 respectively.
*Label accumulated in the D1 and D2 proteins of the PSII dimer, PSII monomer and CP43 minus monomer is calculated, the results are shown as mean ± SD.

Transcript accumulation of the CCM complexes upon environmental Ci changes

To get further insights into the regulation of the expression of CCM complexes, we studied transcripts of *ndhF3*, *ndhJ*, *sbtA*, *cmpA*, *ftsH2* (*slr0228*) and *ftsH3* (*slr1604*) of both the WT and FtsH2 strains upon changes in environmental CO_2 condition. Because it has been suggested that the expression of some inducible CCM genes is negatively regulated by the regulatory protein NdhR (Figge *et al*., 2001; Wang *et al*., 2004), the transcripts of regulator *ndhR* were also monitored. Cells were grown at high CO_2 condition, and then shifted to low CO_2 for 2 h. Real-time polymerase chain reaction (PCR) analyses of *ndhF3*, *ndhJ*, *sbtA*, *ndhR*, *cmpA*, *ftsH2* (*slr0228*) and *ftsH3*(*slr1604*) are shown in Fig. 5A, *rnpB* is a housekeeping gene included here as a reference gene.

In WT cells, the transcripts of all genes investigated were expressed at a relatively low level at high CO_2 (data not shown). However, the expression of *ndhF3*, *ndhJ*, *sbtA* and *cmpA* as well as the regulator gene *ndhR* was dramatically increased upon shifting the cells from high to low CO_2 (Fig. 5A). Compared with high CO_2 condition, *cmpA* gene was induced several thousand folds, *sbtA* and

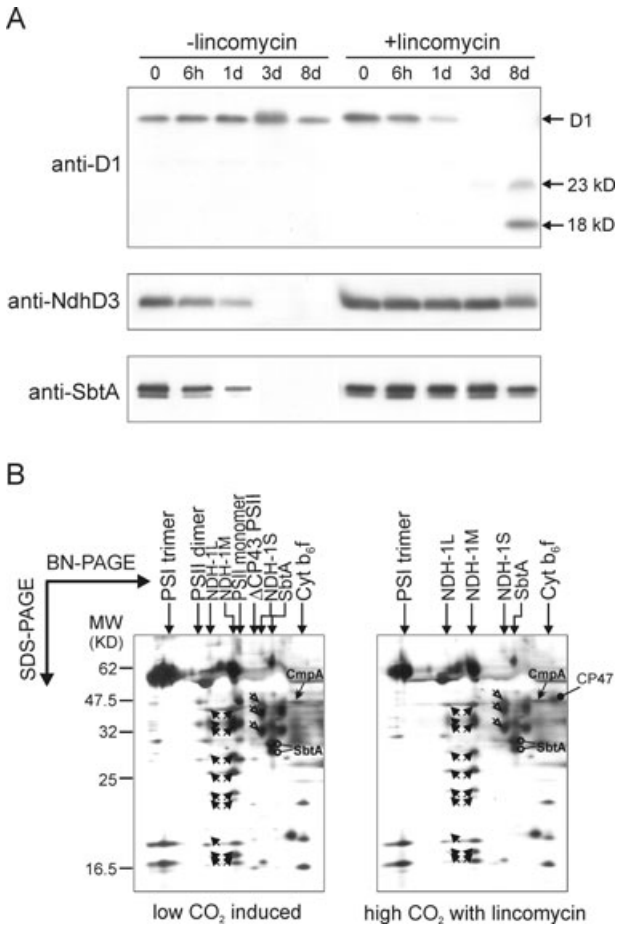

Fig. 3. Degradation of the CCM complexes upon shift of cells from low to high CO_2. Low CO_2 grown WT *Synechocystis* cells were transferred to high CO_2, simultaneously supplementing the growth medium with/without lincomycin. The membranes were isolated from the cells collected at time points 0, 6 h, 1 day, 3 days and 8 days.
A. Immunoblots of anti-D1, anti-NdhD3 and anti-SbtA. The membrane samples (15 μg protein) were separated by SDS-PAGE, transferred to a PVDF membrane, and then probed with the N-terminal D1 antibody to trace the PSII complex, with the NdhD3 antibody to trace the NDH-1S complex, and SbtA antibody to trace the SbtA complex.
B. Silver-stained gels. The membranes isolated at time points 0 and 3 days with lincomycin were separated in 2-D BN/SDS-PAGE. The spots of the subunits belonging to NDH-1L and NDH-1M complexes are marked with black-head arrows, the spots of the subunits belonging to NDH-1S complex (CupA, NdhF3 and NdhD3 proteins) are marked with white-head arrows, the spots corresponding to SbtA protein are indicated by white nods, and the spot corresponding to CmpA is marked by double-arrow. Free CP47 indicating degradation of the PSII complex is shown by a black nod.

ndhF3 were induced more than hundred folds, *ndhR* and *ndhJ* were more moderately induced about fivefold, and no apparent induction of *ftsH2* and *ftsH3* genes was observed (Fig. 5A). Expression of *ndhJ* was also induced upon high to low CO_2 shift, because NdhJ is one of the subunits of the NDH-1M complex, which together with NDH-1S form a large CCM complex (Zhang *et al*., 2005). In sharp contrast to WT cells, the expression of none of the investigated genes showed conspicuous changes upon shift of cells to different CO_2 conditions in the FtsH2 mutant (Fig. 5A).

Expression of FtsH2 and Ci acquisition proteins upon shift to low CO_2

Expression of FtsH, NDH-1S and SbtA at protein level upon shift of high CO_2 grown WT cells to low CO_2 was monitored by specific antibodies. Two clear bands were recognized by the FtsH antibody (Fig. 5B), and the lower one (missing from the FtsH2 mutant) seemed to respond to CO_2 condition. There are four *ftsH* genes in *Synechocystis*, of which only *slr0228* (*ftsH2)* and *slr1604* (*ftsH3)* respond to a shift to low CO_2 (Wang *et al*., 2004). Indeed, the expression of Slr0228 and Slr1604 is co-ordinated and the FtsH2 and FtsH3 proteins comigrated in the BN gel, which suggested that they possibly form a high molecular mass supercomplex, an FtsH2-FtsH3 heterocomplex together with the PSII complex in *Synechocystis* (Fig. 5C). In *Arabidopsis*, it has been shown that FtsH2 and FtsH8 (homo-

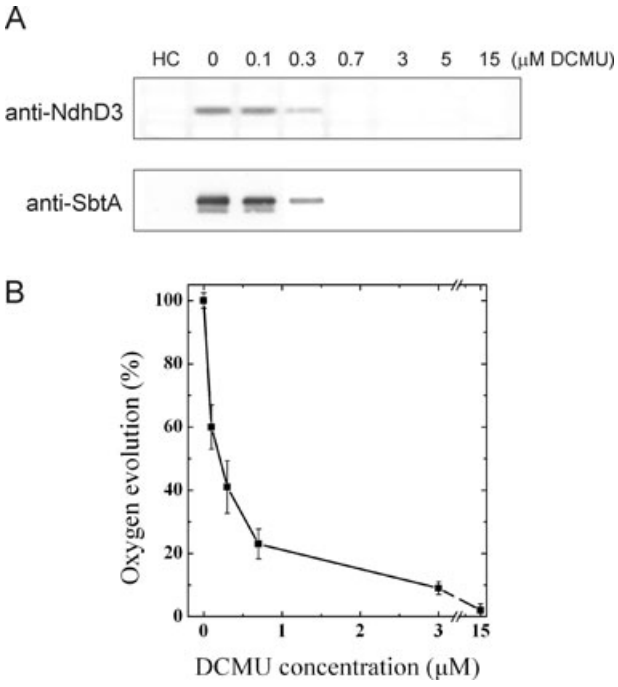

Fig. 4. Induction of the CCM proteins in the presence of DCMU. The high CO_2 grown WT *Synechocystis* cells were transferred to low CO_2. At the same time, DCMU was added to the culture medium in various concentrations (0–15 μM), and the cells were incubated under low CO_2 growth condition for 24 h.
A. Immunoblots with anti-NdhD3 and anti-SbtA.
B. The relative PSII activity measured as steady state oxygen evolution. The result is shown as a percentage of the oxygen evolution of the cells without DCMU (492 μmol O_2 per mg of Chl per hour set at 100%) ± SD for three independent experiments.

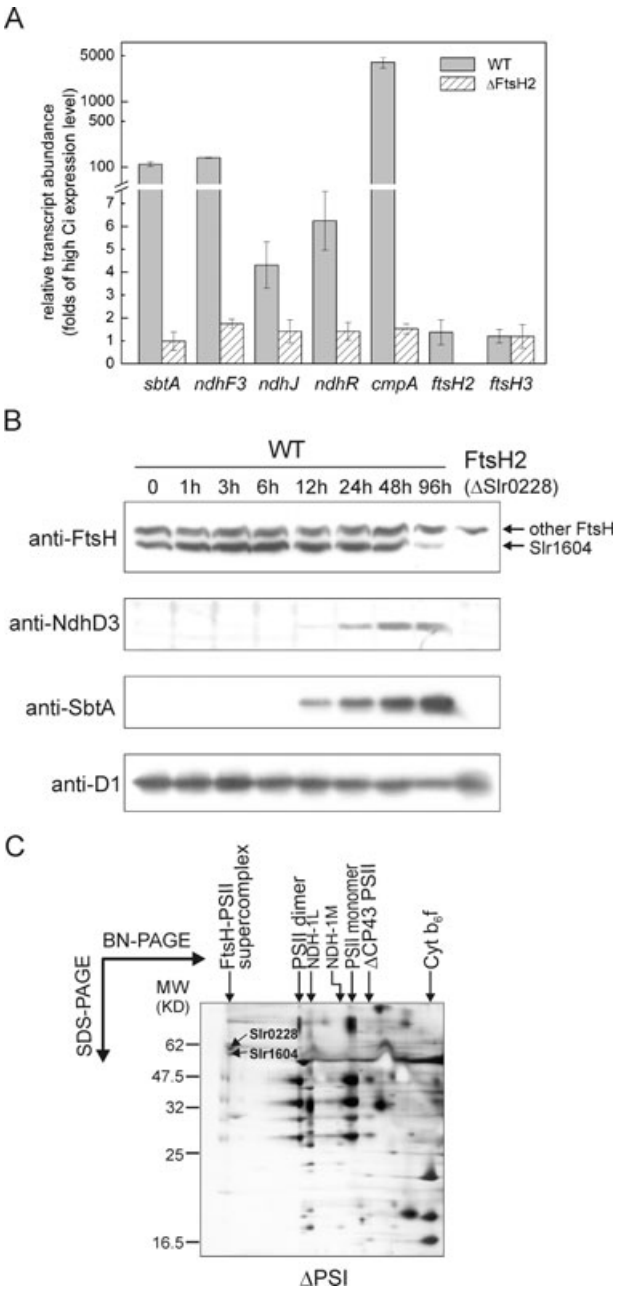

Fig. 5. Transcription and translation of the CCM and *ftsH* genes upon changes in environmental CO_2.
A. *Synechocystis* WT and FtsH2 mutant were grown at high CO_2, then transferred to low CO_2 for 2 h. Relative transcription of *sbtA*, *ndhF3*, *ndhJ*, *ndhR*, *cmpA*, *ftsH2* (*slr0228*), and *ftsH3* (*slr1604*) were analysed by RT-Q-RT-PCR. The transcript level of *rnpB* is used as a reference. Bars represent the relative expression after the shift to low Ci as a fold of high Ci amount ± SE for three independent experiments. Note that no *ftsH2* (*slr0228*) transcripts were detected in the FtsH2 mutant.
B. *Synechocystis* WT cells were grown at high CO_2 and then shifted to low CO_2. The cells were collected after the shift at points 0, 1 h, 3 h, 6 h, 12 h, 24 h, 48 h and 96 h. The membranes were isolated and probed with specific antibodies against the FtsH, NdhD3, SbtA and D1 proteins respectively. The membranes isolated from the FtsH2 mutant (Δ*slr0228*) cells grown at high CO_2, and shifted to low CO_2 for 48 h served as a reference.
C. Membrane proteome of PSI mutant. FtsH2 (Slr0228) and FtsH3 (Slr1604) were identified in a complex by mass spectrometry.

logues of *Synechocystis* FtsH2) interact with FtsH5 and FtsH1 (homologues of *Synechocystis* FtsH3) and constitute hetero-oligomer FtsH complexes (Yu *et al*., 2004; Adam *et al*., 2006). It is conceivable that closely related FtsH proteins form hetero-complexes also in cyanobacteria, like the FtsH2/FtsH3 in Fig. 5C. In this complex, two proteins were found at approximately 70 kDa region, and the bigger one was identified by mass spectrometry as Slr0228 and smaller one as Slr1604 (Fig. 5C). Because Slr1604 and Slr0228 seem to form a complex *in vivo*, it is logical that the lower FtsH band in Fig. 5B, corresponding to Slr1604 protein, was also missing in the FtsH2 (Δ*slr0228*) mutant, whereas the upper band contains Slr0228 and most probably also the other two FtsH proteins (Fig. 5B). The FtsH proteases were expressed in considerable amount in high CO_2 grown cells, and their amount slightly increased upon shift to low CO_2. The maximum expression was achieved by 3–6 h of low CO_2 incubation and thereafter decreased gradually. In steady state low CO_2 grown cells (96 h), the expression of FtsHs was at a relatively low level. On the other hand, the NdhD3 and SbtA proteins were observed only 12 h after shift of cells to low CO_2, and they accumulated further in the course of low CO_2 incubation. The expression of both the NdhD3 and SbtA proteins was completely repressed in the FtsH2 mutant (Fig. 5B).

Effect of methyl viologen (MV) on the expression of inducible CCM genes

Synechocystis cultures of WT and FtsH2 mutant grown in low CO_2 concentration were supplemented with 2 μM MV to enhance oxidative stress. After 4 days, the expression of the CCM complex proteins was detected by NdhD3 and SbtA antibodies. The amounts of both the NdhD3 and SbtA proteins were slightly higher in the WT of MV treated cells than control cells, whereas no expression of these proteins was observed in the FtsH2 mutant (Fig. 6A). These results suggested that additional oxidative stress might be involved in CCM regulation.

In the next set of experiments, the WT *Synechocystis* cells were grown at high CO_2 condition. The cells were then treated with 50 μM MV for 30, 60 and 120 min. The treatment condition was according to Havaux *et al*. (2005) with slight modification. The transcription of the two *ftsH* (*slr0228* and *slr1604*) genes, *sbtA*, *ndhF3*, *ndhR*, and *ndhD1* was quantified by RT-Q-RT-PCR. As shown in Fig. 6B, the two *ftsH* genes were the most responsive ones. Their expression was coupregulated 17 times after 1 h MV addition, and slightly slowed down after that. Tran-

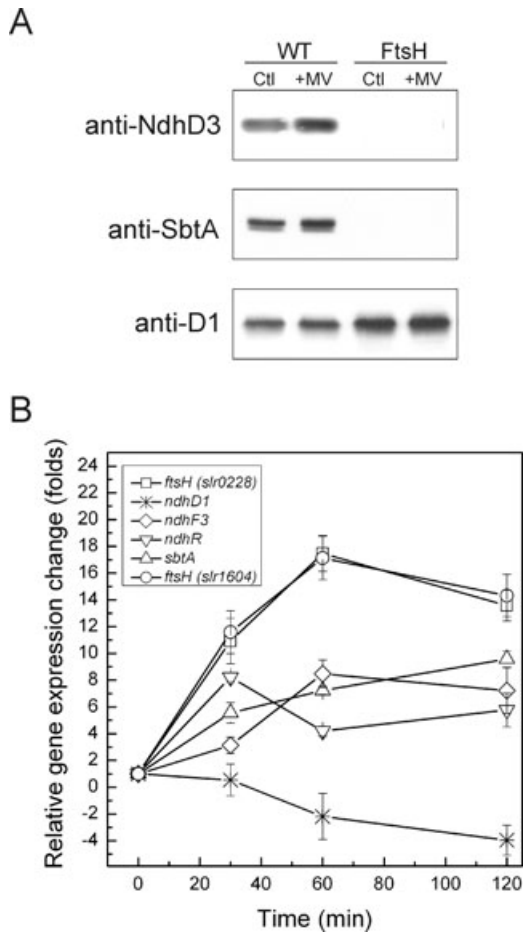

Fig. 6. Expression of the CCM and *ftsH* genes in the presence of MV.
A. The cells of *Synechocystis* WT and FtsH2 mutant were grown at low CO_2 with or without 2 μM MV, and the expression of the CCM complexes was monitored by specific antibodies against NdhD3 and SbtA respectively. Immunoblot of D1 protein was used as a reference.
B. *Synechocystis* WT cells were grown at high CO_2 and treated with 50 μM MV for 0, 30, 60 and 120 min. Relative transcription of *sbtA*, *ndhF3*, *ndhR* and two *ftsH* genes (*slr0228* and *slr1604*) as well as *ndhD1* were analysed by RT-Q-RT-PCR. The transcript level of *rnpB* is used as a reference. Each point represents the relative expression as a fold of MV treated cells to untreated cells ± SE for three independent experiments.

scription of *sbtA*, *ndhF3* and *ndhR* moderately responded to MV. They were upregulated four to eight times during the MV treatment. Notably, the expression of *ndhD1* was not induced by MV (Fig. 6B).

Discussion

Dispensable Ci acquisition complexes are not degraded

It has long been known that cyanobacteria can induce an efficient CCM under low Ci conditions. The experimental data presented here for the first time clearly shows from a proteomic perspective that the CCM protein complexes of *Synechocystis*, actively synthesized after short adaptation at low CO_2 (Fig. 2), remain stable in the membrane after shift of cells from low to high CO_2 (Fig. 3B). These protein data strongly corroborate previous functional studies, which demonstrated that the induction of CCM takes a few hours at low Ci (Omata and Ogawa, 1985; Kaplan *et al*., 1994), whereas Ci uptake capacity remains high for days when cells are shifted from low to high Ci (Benschop *et al*., 2003). Because newly growing cells do not express inducible CCM protein complexes NDH-1S, SbtA and CmpA in high CO_2, the Ci acquisition activity then goes down gradually by dilution of the enzymes upon progress of cell divisions.

FtsH2 protease is specifically required for induction of CCM

Contrary to WT, the FtsH2 mutant could not induce the NDH-1S and SbtA protein complexes or the CmpA protein, upon shift of cells from high to low CO_2. The CCM induction, on the other hand, was normal in the Deg-G mutant (Fig. 1), indicating that the FtsH2 protease has a specific function in the expression of the CCM complexes, which includes a series of events from sensing mechanisms to transcriptional activation, translation of the CCM proteins, and the assembly of the Ci acquisition complexes. In the FtsH2 mutant, there was no induction of the transcripts of the inducible CCM genes upon shift from high to low CO_2 (Fig. 5A), indicating that the FtsH2 protease is a prerequisite for transcriptional activation of the CCM complexes. Thus, the FtsH2 protease is involved in the regulation of CCM induction before the CCM genes are transcribed. The target might be found at the level of transcription regulation or at the level of the low Ci signal sensing.

Small amounts of transcripts of *ndhF3*, *sbtA* and *cmpA* were present in the FtsH2 mutant cells, at levels similar to those in WT cells grown at high CO_2 (data not shown). However, such low transcript levels did not result in detectable accumulation of the NDH-1S, SbtA and CmpA proteins in the FtsH2 mutant (Figs 1, 5B and 6A).

Signalling for CCM

CO_2-concentrating mechanism is a strategy for cyanobacteria to assimilate Ci and achieve optimal acclimation under a variety of CO_2 environments. The Ci uptake is directly linked to photosynthetic light reactions and carbon fixation. Indeed, both darkness and supplementation of the culture medium with glucose or DCMU restrain Ci uptake (Ogawa *et al*., 1985; Miller *et al*., 1990; Kaplan *et al*., 1991; McGinn *et al*., 2003) and the expression of the inducible CCM complexes (Herranen *et al*., 2004) in

cyanobacteria. Thus, an efficient CCM can be induced only in autotrophically grown cells with functional photosynthesis.

Specific requirement of the FtsH2 protease gave insights into the mechanisms involved in the induction of Ci acquisition. The FtsH proteases are involved in PSII damage-repair cycle in both plants and cyanobacteria (Bailey *et al*., 2002; Silva *et al*., 2003; Nixon *et al*., 2005), and therefore it is conceivable to presume that the failure in the induction of the CCM complexes in the FtsH2 mutant would result from the decrease of PSII activity. However, the PSII activity of the FtsH2 mutant is still at a significant level, which allows the mutant cells autotrophic growth and division with active photosynthetic machinery (Mann *et al*., 2000). The active photosynthesis creates the need of an active repair cycle that in turn requires the presence of FtsH proteases. Further, the Calvin cycle was not blocked in the FtsH2 mutant (data not shown), and thus the FtsH2 mutant has a functional photosynthetic machinery normally required for induction of CCM.

We have shown here that the amount of the FtsH proteases (Slr0228 and Slr1604) slightly increases in WT upon a shift of cells from high to low CO_2 (Fig. 5B), but there is no significant change in the transcriptional level of the *ftsH2* and *ftsH3* genes (Fig. 5A). It seems that increase of the FtsH protease amount is not a prerequisite for the expression of inducible CCM proteins upon shift of cells to low CO_2. However, the presence of FtsH2 is crucial at least for initial CCM induction (Fig. 5A). Taken together with the fact that the NdhR repressor must become inactive by either degradation or conformational change, or both, before the upregulation of the CCM complexes or NdhR itself, it is conceivable that the FtsH2 protease has a specific role in modulation of NdhR function. Indeed, the RT-Q-RT-PCR showed that the transcripts of neither the inducible CCM genes nor the *ndhR* gene accumulated in the FtsH2 mutant upon shift to low CO_2 (Fig. 5A). Therefore, it is likely that regulation by the FtsH2 protease is located upstream of NdhR in signalling of CCM. Because FtsH2 (Slr0228) is a protease, and does not have any DNA binding site, it is unlikely to directly function in gene transcription. Further, the FtsH2 protease belongs to the AAA-protein family, and is integrated into *Synechocystis* thylakoid membrane (Komenda *et al*., 2006).

Considering the fact that NdhF3 and NdhD3 are very hydrophobic proteins with many transmembrane helices, the transcription, translation and assembly of the NDH-1S complex might be temporally linked and spatially close to the thylakoid membrane. It is conceivable that a thylakoid located protease FtsH2 would degrade the DNA-bound repressor NdhR and thereby activates the transcription of the operon. Also free NdhR, not bound to specific CO_2 responsive promoters, can be a target of FtsH2, thus changing the functional equilibrium between bound and unbound repressor. Besides the direct regulatory role of FtsH2 in induction of CCM, the possibility that FtsH2 indirectly regulates the induction of CCM cannot be completely excluded.

Whether the FtsH2 protease works on NdhR directly or indirectly has not yet been solved, as no direct interaction between these proteins has so far been reported (Stirnberg *et al*., 2007). It should be noted, however, that the *in vitro* assay used by Stirnberg *et al*. (2007) also failed to detect the well-known FtsH2 targets like the PSII core subunit D1, thus leaving open the possibility that NdhR is a direct proteolytic target of FtsH2. Expression of the *cmpA* gene, regulated by CmpR activator protein, behaved similar to the other inducible CCM genes at both the protein and the transcriptional level (Figs 1 and 5A) in WT and the FtsH2 mutant, suggesting that both NdhR and CmpR are regulated under the control of the FtsH2 protease. This indicated that the FtsH2 protease is required for global low Ci response and operates upstream of the regulators NdhR and CmpR.

Regulation of CCM in cyanobacteria, at both the transcriptional (Woodger *et al*., 2005a,b) and translational (Zhang *et al*., 2004) level, is determined to a large extend by the internal Ci concentration. Here we show for the first time that the FtsH2 protease modulates the expression of inducible CCM. Given the nature of the NdhR repressor (member of the LysR family), the binding of small molecules can cause a conformational change that makes them to attach or release from promoters (Schell, 1993). We therefore propose that internal HCO_3^- level is detected directly by the repressors and affects their function. High internal Ci level is postulated to cause the bicarbonate signal molecule to bind to the repressor, which in turn will bind to its specific promoter and block transcription. When the internal Ci level is decreased, the availability of the bicarbonate molecules decreases and inducible CCM operons become active due to a release of the repressor by a conformational change. The free repressor is likely to become a target for the FtsH2 protease and is degraded. If the FtsH2 protease is missing (in FtsH2 mutant), the released repressor is available to bind new signal molecules, change to the active conformation and repress the CCM operon. It is also important to point out that with functional FtsH2 protease, the CCM can be induced only under low Ci condition (Figs 1 and 5B). However, the induction of CCM is further enhanced by additional oxidative stress. We observed that the expression of inducible CCM was increased at protein level when the WT cells were treated with MV at low Ci condition (Fig. 6A). Even under high Ci condition, addition of MV induced a transient increase of the transcripts of the CCM genes (Fig. 6B). Also the expression of FtsH2 and FtsH3 are enhanced by MV treatment (Fig. 6B) and under high light

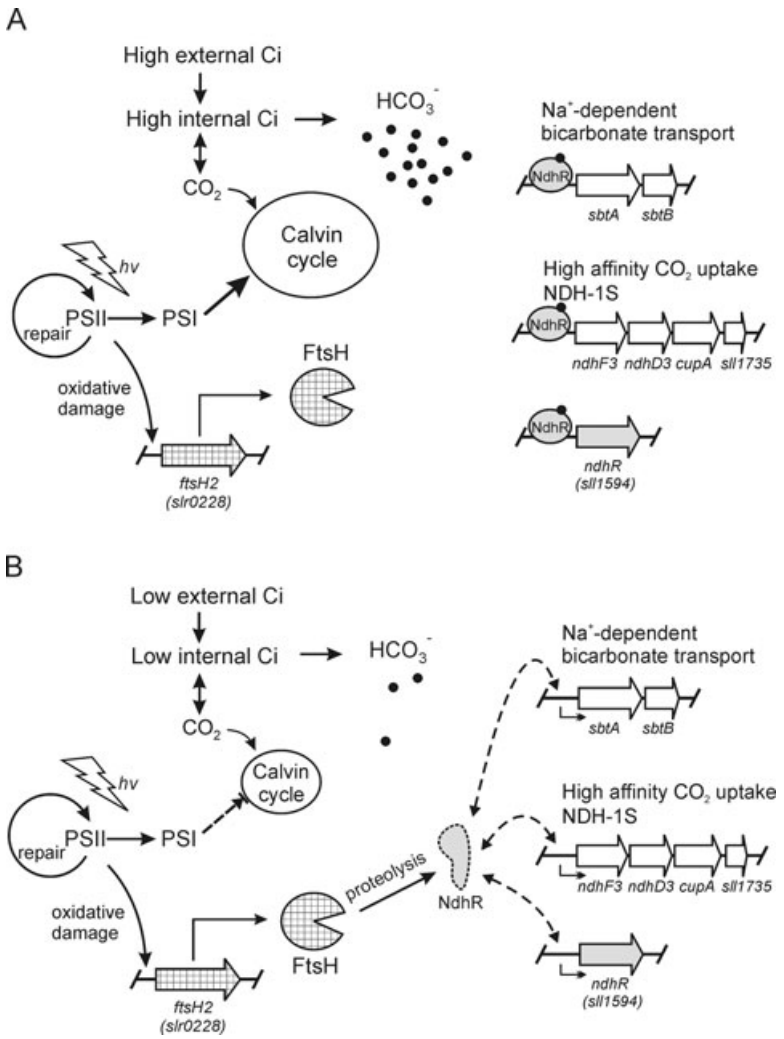

Fig. 7. A hypothetical signalling pathway for the induction of CCM. This model describes the involvement of the FtsH2 protease in low Ci signalling and expression of inducible CCM genes.
A. NdhR represses the transcription of inducible CCM genes under high Ci conditions via conformational modulation by HCO_3^- and binding to the promoter region.
B. Proteolysis of NdhR activates the transcription of the inducible CCM genes upon shift from high to low Ci. Under Ci limitation, a deficiency of internal HCO_3^- molecules causes a conformational change in the repressor protein NdhR and release from the promoters making them become as targets of the FtsH2 protease. Proteolysis of the repressor protein enhances the transcription of the inducible CCM operons.

(Hihara *et al*., 2001), suggesting that the expression of the FtsH protease is mediated by the redox state of the cell, which thereby plays a role also in CCM signalling.

An intriguing question is the nature of the signal(s) that primarily induces the CCM. It has been reported that the full transcriptional induction of CCM is regulated by the internal Ci level but occurs only in the light (Woodger *et al*., 2003; 2005a,b). How to link light and internal Ci level together as CCM signalling? We assume that light represents the prerequisite for photosynthetic activity and HCO_3^- is the signalling molecule. When photosynthesis is blocked by anaerobiosis or the presence of DCMU under light or by darkness, no oxidative pressure from electron transfer is present, and no induction of CCM at low Ci takes place. These conditions are also characterized by no damage and turnover of the D1 protein, the primary target of the FtsH protease. On the other hand, enhanced D1 protein turnover and FtsH protease expression as well as enhanced CCM are induced at low Ci by the MV treatment, suggesting a yet unidentified functional connection between these phenomena. Also the presence of glucose prevents the induction of CCM proteins at low CO_2 condition (Herranen *et al*., 2004), but this is likely to result from a high internal Ci level induced by external glucose, thus keeping the repressors bound to the promoter. In conclusion, we suggest that the induction of the CCM is dynamically regulated by a balance between two components: the active photosynthetic machinery that ensures the presence/activity of the FtsH protease also involved in light-dependent D1 protein degradation and the internal Ci level of the cell. A hypothetical scheme for the regulation of CCM is summarized in Fig. 7.

Experimental procedures

Cell growth and experimental treatment conditions

Synechocystis 6803 glucose-tolerant strain (WT) and the protease mutants FtsH2 (Δ*slr0228*) (Silva *et al*., 2003) and ΔDeg-G (D*slr1204*/D*sll1679*/Δ*sll1427*) (Barker *et al*., 2006) as well as PSI mutant (Δ*psaA/psaB*) (Shen *et al*., 1993) were

grown in BG-11 medium at 30°C under gentle agitation (Williams, 1988). The cells were grown at high CO_2 (3%) or low CO_2 (air level) condition. Appropriate antibiotics were supplemented in the culture of the mutant strains. PSI mutant was grown in the medium supplemented with 15 μM glucose under 5 μmol photons m^{-2} s^{-1} light condition. WT cells were routinely cultivated at illumination of 50 μmol photons m^{-2} s^{-1}. However, when comparisons were made between the WT and protease mutant strains, the cells were grown at 30 μmol photons m^{-2} s^{-1}.

Lincomycin treatment. To study the degradation of membrane proteins and protein complexes, WT cells were grown at high CO_2 until logarithmic phase, and shifted to low CO_2 to induce CCM complexes for up to 48 h. The cell suspension was adjusted to chlorophyll a concentration of 10 μg ml^{-1}, and transferred back to high CO_2. New protein synthesis was inhibited by supplementing the culture medium with lincomycin (150 μg ml^{-1}).

DCMU treatment. High CO_2 grown WT cells were resuspended in BG-11 at 10 μg ml^{-1} chlorophyll a, and transferred to low CO_2 environment with the presence of 0–15 μM DCMU in the culture for 24 h.

Methyl viologen treatment. WT and FtsH2 mutant cells were grown at low CO_2 with or without 2 μM MV for 4 days. WT cells were also grown at high CO_2 until logarithmic phase (6 μg ml^{-1} chlorophyll) and directly treated with 50 μM MV for 30, 60 and 120 min in high CO_2 environment.

Membrane isolation, electrophoresis and immunodection

Total membrane fraction from *Synechocystis* cells was isolated as described before (Zhang *et al*., 2004). The cells were harvested and suspended in 50 mM Hepes-NaOH, pH 7.5, 30 mM $CaCl_2$, 800 mM sorbitol, 1 mM ε-amino-n-caproic acid. The cells were broken by vortexing 8 × 1 min at 4°C in the presence of glass beads. The glass beads and unbroken cells were removed by centrifugation at 3000 *g* for 5 min. The membrane fraction was pelletted at 18 000 *g* for 20 min, and resuspended in 50 mM Hepes-NaOH, pH 7.5, 600 mM sucrose, 30 mM $CaCl_2$, 1 M glycinbetaine.

Membrane protein complexes were separated by 2-D blue-native (BN)/SDS-PAGE (Zhang *et al*., 2004). The membrane was solubilized by 1.5% n-dodecyl-β-D-maltoside in 25 mM BisTris pH 7.0, 20% (w/v) glycerol, 1 mM pefabloc, 10 mM $MgCl_2$, 0.01 U μl^{-1} Dnase RQ1 (Promega, USA) for 10 min on ice and 10 min at room temperature. Insoluble material was removed by centrifugation at 18 000 *g* for 15 min. The supernatant was mixed with 1/10 volume of 5% Serva blue G, 200 mM BisTris pH 7.0, 75% sucrose, 1 M ε-amino-n-caproic acid, and loaded on 0.75 mm-thick 5–12.5% acrylamide gradient gel (150 μg protein per well). Electrophoresis was performed at 4° for 5.5 h by gradually increasing voltage from 50 V to 200 V. The BN-gel lane was cut out and solubilized in SDS sample buffer (125 mM TrisCl pH 6.8, 4% SDS, 25% glycerol, 6 M urea, 5% β-mercaptoethanol) at room temperature for 1 h. After brief wash, the lane was laid on the top of 1 mm-thick 14% SDS-PAGE (Laemmli, 1970) with 6 M urea and subjected to electrophoresis. Separated proteins were visualized by silver staining (Blum *et al*., 1987) or immunodetection with protein-specific antibodies. For that, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Immolilon P, Millipore, USA) using semidry apparatus (Pharmacia), and detected by protein specific antibodies using the CDP-Star® Chemiluminescent Detection Kit (New England Biolabs, USA). The D1 antibody was prepared against amino acids 58–84 of spinach D1 protein. The NdhD3 antibody was raised against amino acids 185–196 and 346–359 of *Synechocystis* NdhD3 (Eurogentec, Belgium). The SbtA antibody was raised against amino acids 184–203 of *Synechocystis* SbtA, and was a kind gift from T. Ogawa. The FtsH antibody was raised against FtsH protein of *Escherichia coli* (a kind gift from T. Ogura, Kumamoto University, Japan).

Pulse-labelling of membrane proteins in vivo

Synechocystis WT cells were grown at high CO_2 to logarithmic phase and the chlorophyll a concentration was adjusted to 10 μg ml^{-1}. Cell suspension was transferred to low CO_2 condition for 2 h, and then pulse labelled with 6 μCi ml^{-1} $^{[35]}$S-L-Met (Amersham Biosciences UK) for 1 h. Additional 1 mM non-radioactive Met was added to terminate labelling. The cells were pelletted down, resuspended in BG-11 medium and chased at low or high CO_2 conditions for 3 h. The samples were cooled down rapidly on ice and harvested by centrifugation at 4°C. Membranes were isolated and applied to BN/SDS-PAGE as described. The proteins were visualized by autoradiography from dried gels.

Oxygen evolution measurements

Steady-state rates of oxygen evolution were measured with Hansatech DW1 O_2 electrode at saturating light intensity in the presence of 1 mM 2,6-dimethyl-*p*-benzoquinone (DMBQ) as an artificial electron acceptor. In each measurement, 1 ml cell suspension at 10 μg ml^{-1} chlorophyll a was used. For each condition, three biological replicates were measured. For each sample, three replicates were measured.

RNA isolation and RT-Q-RT-PCR assay

Total RNA of *Synechocystis* cells was isolated from 10 ml of culture by Trizol (Invitrogen) method (McGinn *et al*., 2003), and treated with 1 units DNase (Ambion Turbo DNase kit, USA) to remove genomic DNA.

First strand cDNA synthesis from 1 μg of purified RNA was performed using the Bio-Rad iScript cDNA Synthesis kit (Bio-Rad Laboratories). The primer pairs were designed to generate amplicons of similar length (400–500 bases) from each transcript pool. House-keeping gene *rnpB* encoding RNase P subunit B was used as a reference. The expression level of *rnpB* is within the range of the genes of interest. The primer pairs were tested *in silico* using the Amplify software (B. Engels, 2004, University of Wisconsin) and were compared using BLAST against the *Synechocystis* genome (http://www.kazusa.or.jp/cyano) in order to ensure their gene specificity. The annealing temperature for the primer pairs was optimized by RT-Q-RT-PCR on WT cDNA as template, using a 10

Table 2. Oligonucleotide sequences used to perform RT-Q-RT-PCR.

Gene name	Forward primer (5′→3′)	Reverse primer (5′→3′)
sbtA (slr1512)	TTGACGGAAATGCTCCTACC	GCCAGATTTTGACCCGATTA
ndhF3 (sll1732)	ATCTTTCCTTCCCCTGGCTA	TCCAGCAATTAAGGCCAAAC
ndhJ (slr1281)	CTCCCCCAATGAAGCTGTTA	ACAATGCCGAACATGTCGTA
ndhD1 (slr0331)	GCTGACCACGATCATCCTTT	ACTGGCACCAACTCCAATTC
ndhR (sll1594)	CGCTAGACATGGCAGTTTCA	ACGGGCAATGACCACTAAAG
cmpA (slr0040)	CGAGCGGATTGGGTAGATAA	GCTTCTCGCCACAAATCTTC
ftsH2 (slr0228)	CTTTGGTCCCTACCCCTGTT	GCCACATCATCGAACATGAC
ftsH3 (slr1604)	GTTCCCAAGCCATGAACTTT	ACTGGTTGAGGGTCTGTTCC
rnpB	CCAATTTCCCAAGACTACGG	GGCAGGAAAAAGACCAACCT

degree temperature gradient. The primer pairs used in this study are summarized in Table 2.

The RT-Q-RT-PCR was performed on a Bio-Rad IQ5 system using iQ SYBR Green Supermix. The efficiencies of each individual reactions and the relative change in gene expression relative to control was calculated as described earlier (Sicora *et al*., 2006). Melting curve analysis was performed for each run to ensure the specificity of the amplification product (data not shown).

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