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Nitrogen status and heat-stress-dependent differential expression of the *cpn60* chaperonin gene influences thermotolerance in the cyanobacterium *Anabaena*

Hema Rajaram and Shree Kumar Apte

Molecular Biology Division, Bhabha Atomic Research Centre, Trombay, Mumbai 400 085, India

Correspondence
Shree Kumar Apte
aptesk@barc.gov.in

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Heat stress caused rapid and severe inhibition of photosynthesis and nitrate reduction in nitrate-supplemented cultures of the cyanobacterium *Anabaena* sp. strain L-31, compared to nitrogen-fixing cultures. *Anabaena* strains harbour two *hsp60* family genes, *groEL* and *cpn60*, respectively encoding the 59 kDa GroEL and 61 kDa Cpn60 chaperonin proteins. Of these two Hsp60 chaperonins, GroEL was strongly induced during heat stress, irrespective of the nitrogen status of the cultures, but Cpn60 was rapidly repressed and degraded in heat-stressed nitrate or ammonium-supplemented cultures. The recovery of photosynthesis, nitrate assimilation and growth in heat-stressed, nitrate-supplemented cultures were preceded by resynthesis and restoration of cellular Cpn60 levels. Glutamine synthetase activity, although adversely affected by prolonged heat stress, was not dependent on either the nitrogen status or Cpn60 levels during heat stress. Overexpression of the Cpn60 protein in the closely related *Anabaena* sp. strain PCC7120 conferred significant protection from heat stress to growth, photosynthesis and nitrate reduction in the recombinant strain. The data favour a role for Cpn60 in carbon and nitrogen assimilation in *Anabaena*.

INTRODUCTION

The heat-shock response (HSR) involves *de novo* synthesis of heat-shock proteins (Hsps) comprising several chaperones (Hsp70, Hsp60) and proteases (Lon, Clp). HSR is a universal stress response that living organisms use to restore homeostasis during short-term temperature up-shifts and other environmental stresses (Morimoto *et al.*, 1994; Yura *et al.*, 2000). Although studied in great detail in several bacteria and higher organisms, the HSR is not so well understood in nitrogen-fixing cyanobacteria. Most bacteria possess a single bicistronic *groESL* operon harbouring the *groES* and *groEL* genes encoding a 10 and a 60 kDa chaperonin, respectively (Yura *et al.*, 2000). Occurrence of additional *hsp60* genes in *Streptomyces* species (Guglielmi *et al.*, 1991), *Mycobacterium tuberculosis* and *M. leprae* (Rinke de Wit *et al.*, 1992), and of multiple *groEL* genes in rhizobia (Rusanganwa & Gupta, 1993) and *Bradyrhizobium japonicum* (Fischer *et al.*, 1993) has also been reported. All cyanobacteria whose genomes have been completely sequenced exhibit two distinct *hsp60* genes, a *groEL* gene as part of the *groESL* operon and a solitary *cpn60* gene (Chitnis & Nelson, 1991; Lehel *et al.*, 1993; www.kazusa.or.jp/cyano).

Earlier work from our laboratory showed that (a) the two Hsp60 protein-encoding genes are expressed abundantly in the nitrogen-fixing cyanobacterium, *Anabaena* sp. strain L-31 (hereafter referred to as *Anabaena* L-31) during exposure to heat and other environmental stresses (Apte *et al.*, 1998; Bhagwat & Apte, 1989), and (b) nitrogen-fixing *Anabaena* L-31 cultures recover from prolonged exposure to continuous heat stress as a consequence of continuous synthesis, greater stability and accumulation of the two Hsp60 proteins during heat stress (Rajaram & Apte, 2003). In this study, we have cloned the *cpn60* gene from *Anabaena* L-31 and compared its expression with that of *groEL* (Rajaram *et al.*, 2001; Rajaram & Apte, 2003) in response to nitrogen status and heat stress. Our results demonstrate that loss of Cpn60 during heat stress in nitrate-supplemented cultures of *Anabaena* correlates with the inactivation of the photosynthetic machinery and nitrate reduction, while Cpn60 overexpression enhances the thermal stability of these vital metabolic processes.

Abbreviations: chl, chlorophyll; Hsp, heat-shock proteins; HSR, heat-shock response; MV_r-NR, reduced methyl viologen-dependent nitrate reductase.

The GenBank/EMBL/DDBJ accession number for the sequence of the insert from construct pHR302 is AY328922.

METHODS

Organism and growth conditions. *Anabaena* L-31 (Thomas, 1970) and *Anabaena* sp. strain PCC7120 (hereafter referred to as *Anabaena*

7120) were grown in BG-11 liquid medium, pH 7.0 (Castenholz, 1988) with (BG-11, N^+) or without (BG-11, N^-) combined nitrogen (17 mM $NaNO_3$, or 3 mM NH_4Cl with 5 mM MOPS) under continuous illumination (30 μE m^{-2} s^{-1}) and aeration (3 l min^{-1}) at 27 °C. The transgenic *Anabaena* 7120 strain AnFPNcpn was maintained in BG-11, N^+ supplemented with 25 μg neomycin ml^{-1} (hereafter referred to as BG-11, N^+Neo_{25}). Heat stress involved exposure to 42 °C, other growth conditions being identical. For thermotolerance and recovery experiments, the heat-stressed cultures were inoculated into fresh, BG-11 medium at 1 μg chlorophyll (chl) *a* ml^{-1}. Growth was measured as chl *a* content in methanolic extracts as described by Mackinney (1941).

Measurement of photosynthetic, nitrate reductase and glutamine synthetase activities. Light-dependent photosynthetic oxygen evolution was measured using the Oxygen Monitoring System (OxyLab, Hansatech Instruments). Oxygen evolution measurement involved three alternating cycles of light and dark for 5 min duration each and calculation of the average rate. Cellular nitrate reductase activity was estimated as extracellular nitrite released either in light as natural reductant (L-NR) (Hageman & Hucklesby, 1971) or with reduced methyl viologen (MV_r-NR) (Herrero *et al.*, 1981). The results from both assays were similar. Glutamine synthetase activity was assayed by the transferase assay described by Martin *et al.* (1997).

Two-dimensional IEF/SDS-PAGE. The cell-free protein extract was prepared by repeated freezing–thawing of the cells resuspended in 10 mM Tris buffer, pH 8.0, followed by centrifugation (10 000 ***g***, 5 min) to collect the supernatant. Proteins were subjected to isoelectric focussing (IEF) using ampholines (Amersham Biosciences) of two pH ranges, 3–10 and 3.5–5, mixed in a 2 : 1 ratio in tube gels followed by resolution using 10 % SDS-PAGE (Bhagwat & Apte, 1989).

Genomic DNA isolation, PCR amplification and electrophoresis of DNA fragments. *Anabaena* genomic DNA was isolated as described previously (Apte & Haselkorn, 1990). PCR amplification of genomic DNA (100 ng) was carried out using *Taq* DNA Polymerase (Roche Biochemicals). DNA samples were electrophoretically resolved on 0.7 % agarose gels in TBE (Tris-Borate-EDTA) at 80 V for 2 h.

Cloning of *Anabaena* L-31 *cpn60* gene and *groESL* operon. The 1.7 kb *cpn60* gene was amplified from *Anabaena* L-31 chromosomal DNA using the cpn60F2 and cpn60R1 primers (Table 1), designed on the basis of the genome sequence of *Anabaena* 7120. The PCR product was end-filled with dNTPs and Klenow enzyme and ligated to *Eco*RV-digested plasmid vector, pBluescript SKII. The insert from this construct, designated pHR302, was sequenced and the sequence has been submitted to GenBank (accession no. AY328922). The *groESL* operon from this cyanobacterium was cloned and sequenced previously (Rajaram *et al.*, 2001; accession no. AF324500).

Table 1. List of primers used for PCR amplification

Primer	Sequence (5′–3′)*
cpn60F2	GTATCCTACATGAGCATCAAG
cpn60R1	ACGATCGCTTCGGTGGTG
cpn60OEFwd	GCCATATGGCAAAAATTATTTCA (*Nde*I)
cpn60OERev	GCCTCGAGGAAC ATACCCATACCACC (*Xho*I)
cpn60OERev1	GCGGATCCTTAGAACATACCCATACC (*Bam*HI)
CgroELFwd	GCCATATGGCAAAGCGCATTATTTAC (*Nde*I)
CgroELRev	GCGGATCCTTAGTAATCGAAGTCACCGCC (*Bam*HI)

*Underlined sequences are cut by the restriction enzyme shown in parentheses.

RNA isolation and dot blot hybridization. RNA was isolated from *Anabaena* cultures as described previously (Rajaram *et al.*, 2001) and treated with DNaseI for removing any DNA contamination. Purified RNA (5 μg per spot) was spotted onto nylon membranes (Roche Diagnostics), cross-linked with UV and hybridized with specific DIG-labelled DNA probes as described previously (Rajaram *et al.*, 2001).

Generation of antibodies against purified Cpn60 and GroEL proteins of *Anabaena* L-31. The 1.7 kb *Nde*I/*Xho*I fragment of the *cpn60* gene, amplified from pHR302 using cpn60OEFwd and cpn60OERev primers (Table 1), was cloned at identical sites in the overexpression vector pET29a. The 1.6 kb *Nde*I/*Bam*HI fragment of the *groEL* gene, amplified from *Anabaena* L-31 chromosomal DNA using primers CgroELFwd and CgroELRev (Table 1), was cloned at identical sites in the overexpression vector pET16b. The resulting constructs, pETcpn60 and pETgroEL, were transformed into *Escherichia coli* BL21(pLysS) cells. The Cpn60 and GroEL proteins were overexpressed by induction with 1 mM IPTG at 37 °C for 1 h and purified under denaturing conditions (8 M urea) using NiNTA affinity chromatography (Qiagen). Purified Cpn60 and GroEL proteins of *Anabaena* L-31 were used to generate the corresponding polyclonal antibodies in rabbit.

Western blotting and immunodetection. Proteins were extracted, resolved by 10 % linear SDS-PAGE or by 2-D IEF/SDS-PAGE and electroblotted onto positively charged nylon membranes (Roche Diagnostics), as described previously (Alahari & Apte, 1998). Immunodetection was carried out with anti-GroEL antiserum raised against the purified GroEL protein of *E. coli* (anti-EcGroEL) or with antisera raised against the purified *Anabaena* L-31 Cpn60 (anti-AnCpn60) or GroEL (anti-AnGroEL) proteins, respectively. Cpn60 levels were quantified using a densitometer (Syngene Biotech).

Overexpression of Cpn60 in *Anabaena* 7120. The *cpn60* ORF was PCR-amplified from pHR302 using primers cpn60OEFwd and cpn60OERev1 (Table 1), restriction-digested with *Nde*I/*Bam*HI and ligated to an identically digested *Anabaena* 7120 vector pFPN, developed in our laboratory (GenBank accession no. EF468631), to obtain plasmid pFPNcpn. Plasmid pFPN allows integration of the transgene between positions 4 654 700 and 4 655 900 in the *Anabaena* 7120 genome and its expression from a strong light-inducible promoter, P*psbA*. Plasmid pFPNcpn was electroporated into *Anabaena* 7120 as described by Thiel & Poo (1989) and electrotransformants were selected on BG-11, N^+Neo_{25} plates.

RESULTS

Nitrogen-status-dependent effect of heat stress on major metabolic activities in *Anabaena* L-31

Anabaena L-31 cells were unable to grow upon continuous exposure to heat stress at 42 °C, irrespective of the nitrogen status of the growth medium (data not shown). At 27 °C, photosynthetic activity was comparable in the presence or absence of combined nitrogen, but decreased to undetectable levels within 15 h in nitrogen-supplemented (NO_3^- or NH_4^+) cultures. In comparison, nitrogen-fixing cultures showed very slow inhibition of photosynthesis (Fig. 1a). The nitrate reductase activity, in the absence (L-NR) or

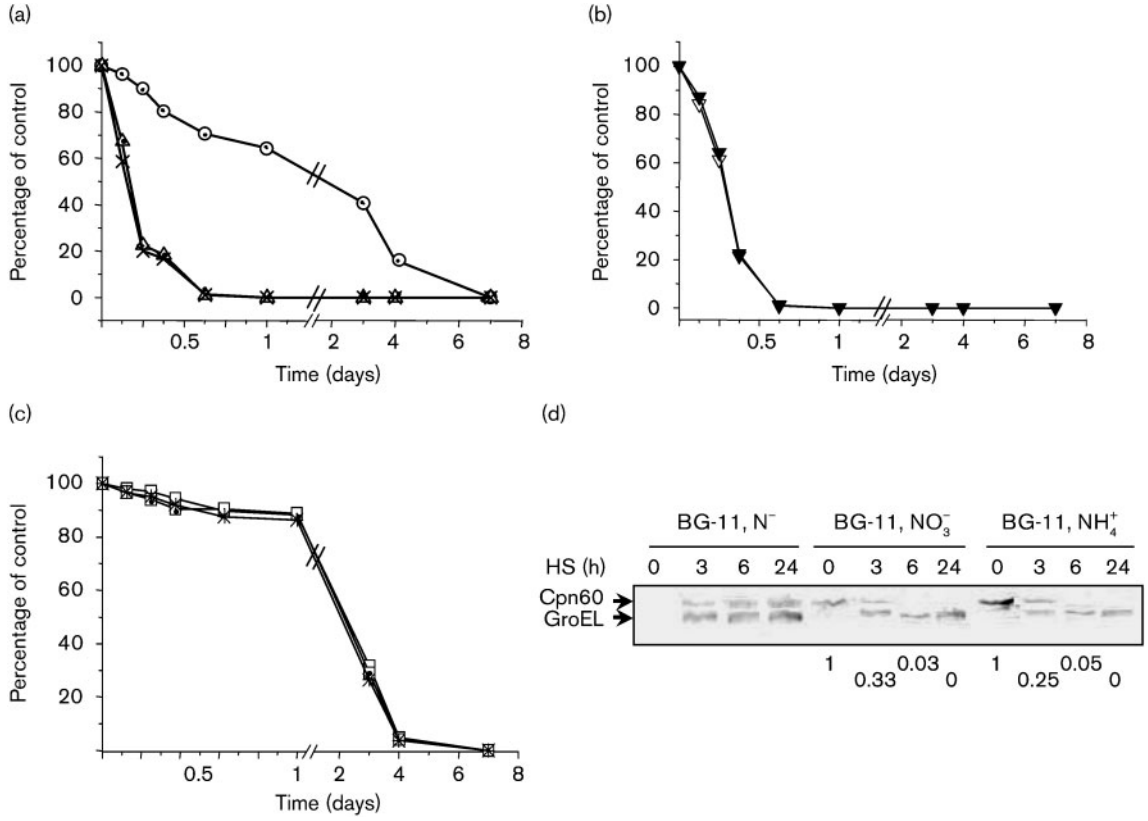

Fig. 1. Effect of heat stress on metabolic activities and Hsp60 synthesis in *Anabaena* L-31 grown with different combined nitrogen sources. Three-day-old *Anabaena* L-31 cultures grown under nitrogen-fixing conditions (BG-11, N^-) or in the presence of nitrate (BG-11, NO_3^-) or ammonium (BG-11, NH_4^+) were inoculated into the respective fresh media, incubated at 27 or 42 °C for 7 days and assessed for different physiological activities. (a) Light-dependent photosynthetic O_2 evolution. The 100 % photosynthetic activity of controls was 7.267 ± 0.153 μmol O_2 (mg chl *a*)$^{-1}$ min^{-1} in nitrogen-deficient medium and 7.417 ± 0.201 μmol O_2 (mg chl *a*)$^{-1}$ min^{-1} and 7.365 ± 0.172 μmol O_2 (mg chl *a*)$^{-1}$ min^{-1} in the presence of nitrate and ammonium, respectively. ⊙, BG-11, N^-; △, BG-11, NO_3^-; ×, BG-11, NH_4^+. (b) Nitrate reductase activity measured in light (L-NR, ▽) or in the presence of MV_r-NR (▼). The 100 % nitrate reductase activity in controls in the absence and presence of MV_r-NR was 2.956 ± 0.58 μmol NO_2^- (mg chl *a*)$^{-1}$ min^{-1} and 3.567 ± 0.039 μmol NO_2^- (mg chl *a*)$^{-1}$ min^{-1} respectively. (c) Glutamine synthetase activity. The 100 % glutamine synthetase activity of controls was 12.193 ± 0.092 μmol γ-glutamyl hydroxamate (γ-GH) (mg chl *a*)$^{-1}$ min^{-1} in nitrogen-deficient medium and 9.645 ± 0.099 μmol γ-GH (mg chl *a*)$^{-1}$ min^{-1} and 10.325 ± 0.088 μmol γ-GH (mg chl *a*)$^{-1}$ min^{-1} in the presence of nitrate and ammonium, respectively. □, BG-11, N^-; ⊡, BG-11, NO_3^-; ✳, BG-11, NH_4^+ (d) Immunodetection of Hsp60 proteins. Proteins were extracted after a specified duration of heat stress, electrophoretically resolved and electroblotted. Immunodetection was carried out with anti-EcGroEL antisera. Values below the BG-11, NO_3^- and BG-11, NH_4^+ lanes depict Cpn60 levels during heat stress relative to the respective unstressed controls at 0 h.

presence of an artificial reductant (MV_r-NR), similarly decreased to very low levels within 15 h of heat stress (Fig. 1b). Inhibition of another assimilatory enzyme, glutamine synthetase, was observed only on prolonged exposure to heat stress, but showed no relationship with the nitrogen source in the growth medium (Fig. 1c). Immunodetection techniques showed that a 59 kDa GroEL protein was undetectable at 27 °C, but was strongly induced during heat stress, irrespective of nitrogen status (Fig. 1d). Cpn60 protein was higher in both nitrate or ammonium-supplemented *Anabaena* cultures compared to nitrogen-fixing cultures, but was undetectable after 6 h of heat stress in nitrogen-supplemented cultures, unlike its accumulation in nitrogen-deficient cultures (Fig. 1d).

Effect of nitrogen status on expression of *hsp60* genes

Detailed analysis of *hsp60* expression in nitrogen-deficient (BG-11, N^-) and nitrate-supplemented (BG-11, NO_3^-) cultures confirmed the results shown in Fig. 1(d). The identities of the 59 kDa (GroEL) and 61 kDa (Cpn60) Hsp60 proteins were confirmed by 2-D electrophoretic resolution (Fig. 2a) and MALDI-TOF-MS-based peptide

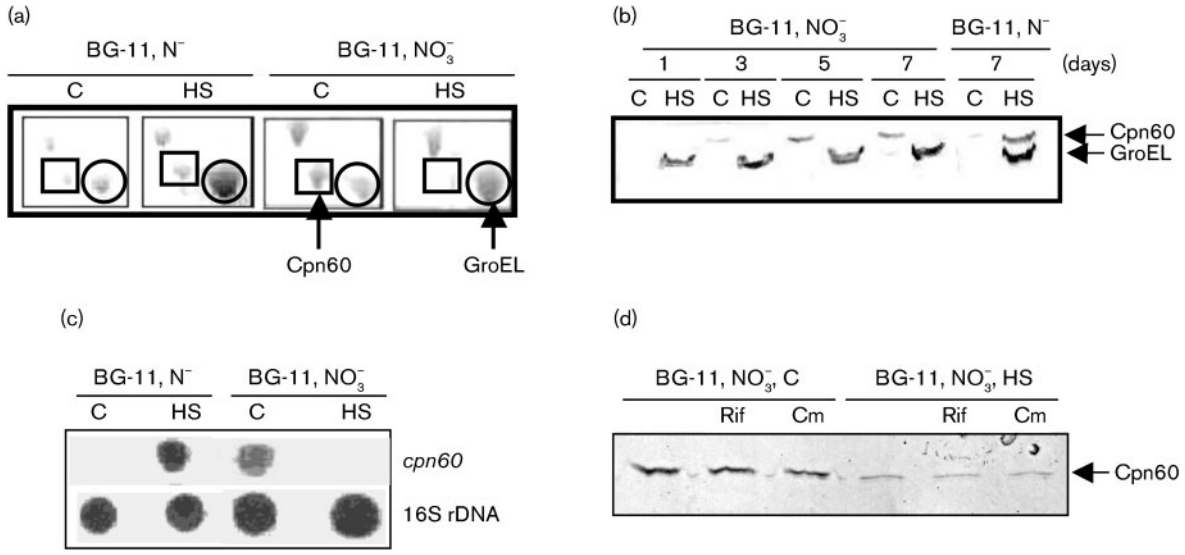

Fig. 2. Expression of *hsp60* genes in heat-stressed *Anabaena* L-31 in the presence or absence of combined nitrogen. (a) Identification of Hsp60 proteins. Proteins were extracted after 24 h incubation at 27 °C (control, C) or 42 °C (heat stress, HS), separated by 2-D IEF/SDS-PAGE and stained with Coomassie brilliant blue. The marked spots were identified by peptide mass fingerprinting. (b) Immunodetection of Hsp60 proteins. Three-day-old *Anabaena* L-31 cultures grown in BG-11, N^+ medium were exposed to heat stress (42 °C) for 7 days. Hsp60 levels detected with anti-EcGroEL antiserum were compared with those in BG-11, N^- cultures heat-stressed for 7 days. Other details were as described in the legend to Fig. 1(d). (c) Detection of *cpn60* transcript. Three-day-old *Anabaena* L-31 cells grown in the presence or absence of nitrate were subjected to heat stress for 1 h. Equal amounts of RNA (5 μg per spot) isolated from control (C) and heat-stressed (HS) cultures were blotted onto nylon membrane and hybridized with a *cpn60* gene probe. A 16S rDNA probe was used as internal control. (d) Effect of transcriptional and translational inhibitors on Cpn60 levels. Rifampicin (Rif, 50 μg ml^{-1}) or chloramphenicol (Cm, 34 μg ml^{-1}) were added to 3-day-old *Anabaena* L-31 cells grown in BG-11, NO_3^- and subsequently cultures were grown either at 27 °C (C) or subjected to heat stress at 42 °C (HS) for 3 h. Cpn60 protein was immunodetected from the specified cell extracts using anti-AnCpn60 antisera.

mass fingerprinting analysis (data not shown). The absence of Cpn60 protein during prolonged heat stress in the presence of combined nitrogen (Fig. 2b) correlated well with the rapid inactivation of photosynthetic machinery and nitrate reduction observed previously (Fig. 1a, b). Heat stress strongly and rapidly repressed the transcription of the *cpn60* gene (Fig. 2c), and also caused degradation of the pre-synthesized 61 kDa Cpn60 protein (Fig. 2a, b) during nitrate-supplemented growth. In comparison, *cpn60* transcription was significantly enhanced by heat stress in nitrogen-fixing cultures (Fig. 2c). Preincubation of nitrate-grown cultures with transcriptional (rifampicin) or translational (chloramphenicol) inhibitors showed that Cpn60 protein was quite stable in controls up to 3 h, but was rapidly degraded during heat stress (Fig. 2d).

Effect of nitrogen status on the recovery of *Anabaena* L-31 cells from heat stress

Previous studies showed that nitrogen-fixing cultures of *Anabaena* L-31 were capable of a remarkable recovery even after 7 days of continuous heat stress at 42 °C (Rajaram & Apte, 2003). When compared to their respective controls, the recovery of nitrogen-fixing cultures was relatively more rapid than that of the nitrate-grown cultures (Fig. 3a), as would be expected if Cpn60 was essential for nitrate-supplemented growth and metabolism. Rapid recovery of photosynthetic activity in the heat-stressed nitrogen-fixing cultures (Fig. 3b) did not transform into equivalent growth recovery (Fig. 3a), since nitrogenase activity takes much longer to recover under such conditions (Rajaram & Apte, 2003). Recovery of nitrate reductase (L-NR) activity followed kinetics very similar to that of recovery of photosynthetic activity in nitrate-grown cultures (Fig. 3b).

The correlation between Cpn60 levels, photosynthesis and nitrate reduction was carefully monitored during recovery of nitrogen-supplemented cultures from a short-term (1 day) heat stress (Fig. 4). About 25 % of the Cpn60 protein was synthesized by 2 h of recovery (Fig. 4a) and measurable recovery of photosynthetic and nitrate reductase (MV_r-NR) activities commenced subsequently (Fig. 4b). Thus the presence of a threshold level of Cpn60 may be a prerequisite for restoration of photosynthesis and nitrate assimilation in nitrate-supplemented cultures following heat stress.

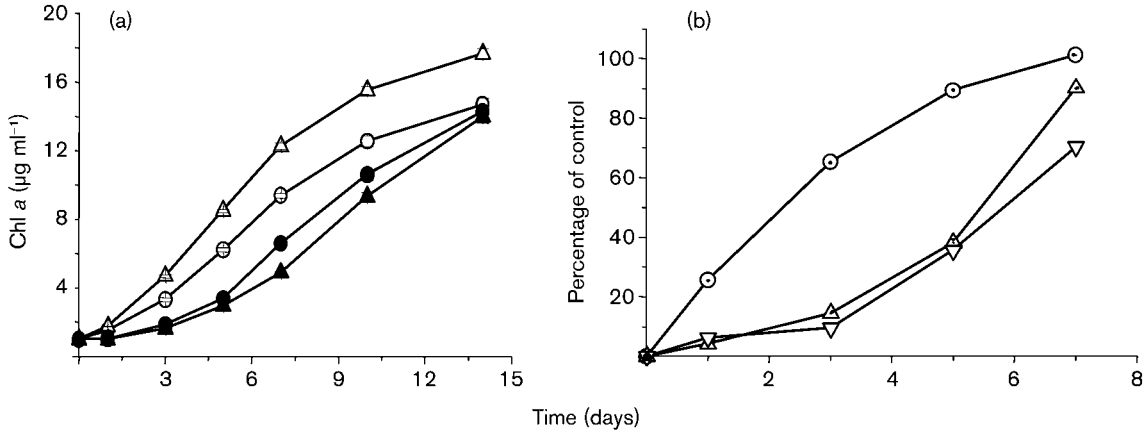

Fig. 3. Effect of nitrogen status on the recovery of 7 day heat-stressed *Anabaena* L-31. Cultures were grown either in the absence (BG-11, N^-) or presence (BG-11, NO_3^-) of nitrate in the medium and incubated either at 27 °C (control, C) or at 42 °C (heat stress, HS) for 7 days. The cells were then washed, inoculated in the respective fresh media and allowed to recover at 27 °C. (a) Growth recovery in terms of chl *a* content of heat-stressed cultures was compared with their respective unstressed control for 14 days. ○, BG-11, N^-, C; ●, BG-11, N^-, HS; △, BG-11, NO_3^-, C; ▲, BG-11, NO_3^-, HS. (b) Recovery of light-dependent photosynthetic O_2 evolution (BG-11, N^-, PS, ⊙) for nitrogen-fixing cultures, and photosynthetic (BG-11, NO_3^-, PS, ◬) and nitrate reductase (BG-11, NO_3^-, L-NR, ▽) activities for nitrate-supplemented cultures. Other details were as given in the legend to Fig. 1.

Overexpression of Cpn60 in *Anabaena* 7120

Attempts were made to overexpress Cpn60 in *Anabaena*. Since *Anabaena* L-31, is not amenable to genetic manipulation, such attempts were made in *Anabaena* 7120, which shows an Hsp60 expression profile similar to that in *Anabaena* L-31 (Figs 1d and 5a). For this, plasmid pFPNcpn was introduced into *Anabaena* 7120 by electrotransformation. Immunodetection with anti-AnCpn60 antiserum confirmed 2.4-fold overexpression of Cpn60 protein in recombinant *Anabaena* 7120 cells (hereafter referred to as AnFPNcpn) (lane C, Fig. 5b) compared to *Anabaena* 7120 (lane C, Fig. 5a). Significantly higher levels of Cpn60 were observed in nitrate-grown AnFPNcpn cultures after 3 days of heat stress (Fig. 5b), unlike in *Anabaena* 7120 (Fig. 5a), and decreased thereafter (Fig. 5b). As expected, the GroEL levels remained similarly enhanced during heat stress in both cultures (Fig. 5a, b).

Effect of overexpression of Cpn60 on metabolic activities in *Anabaena* 7120

The AnFPNcpn cultures grew slowly at 42 °C, unlike *Anabaena* 7120 which failed to grow (Fig. 6a). Correspondingly, the photosynthetic and nitrate reductase activities in AnFPNcpn cultures showed superior thermal stability, while glutamine synthetase activity was not significantly affected by Cpn60 overexpression (Fig. 6b–d). While 1 day of heat stress completely abolished both photosynthetic and nitrate reductase activities in *Anabaena* 7120 (Fig. 6b, c), AnFPNcpn cultures retained over 80–90 % and 30–50 % of their activities on day 1 and day 3, respectively, under the same conditions. The sustenance of photosynthetic and nitrate reductase activities in the transformed cells during heat stress (Fig. 6b, c) closely corresponded with maintenance of higher levels of Cpn60 (Fig. 5b).

The AnFPNcpn cells also showed faster recovery than the wild-type *Anabaena* 7120 cells when exposed to 1 day of heat stress (Fig. 6e). After 4 days of heat stress the difference in recovery was less impressive (Fig. 6f). This correlated well with much higher levels of Cpn60 in AnFPNcpn, compared to *Anabaena* 7120, after 1 day of heat stress than after 4 days (Fig. 5a, b).

DISCUSSION

Information available from genome sequences (www.kazusa.or.jp/cyano) and the limited heat-shock response studies in cyanobacteria have revealed the presence of two *hsp60* genes, the *groESL* operon and the *cpn60* gene, in all of them. Both genes have been cloned and sequenced from the nitrogen-fixing cyanobacterium *Anabaena* sp. strain L-31 by our laboratory (GenBank AF324500 and AY328922). In the unicellular adiazotrophic cyanobacterium *Synechocystis* sp. PCC6803, the two *hsp60* genes show differential expression in response to heat stress and light–dark cycles (Asadulghani *et al.*, 2003; Glatz *et al.*, 1997; Kovacs *et al.*, 2001; Mary *et al.*, 2004) as well as photosynthetic electron transport (Glatz *et al.*, 1997), or during exposure to UV or oxidative stress (Chitnis & Nelson, 1991). Functional differences in the two Hsp60 proteins have been observed in the complementation of the

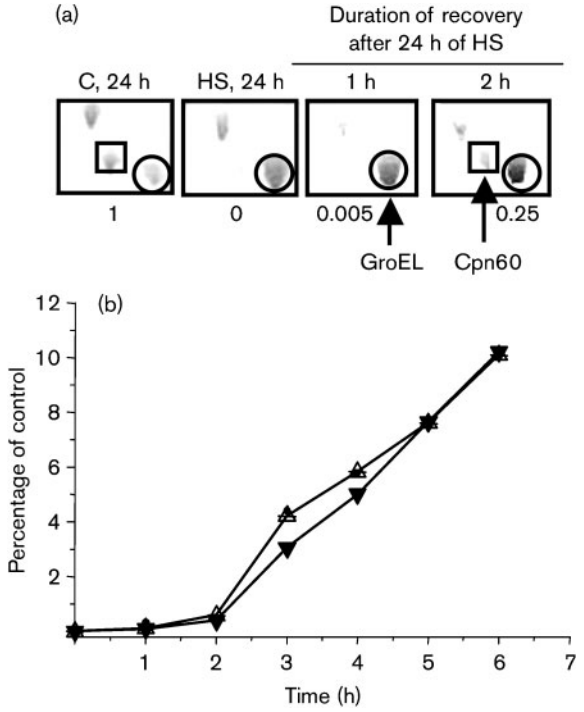

Fig. 4. Recovery of short-term heat-stressed nitrate-supplemented *Anabaena* L-31. Cells heat-stressed (HS) at 42 °C for 24 h were washed and allowed to recover in fresh BG-11, N^+ at 27 °C (C) for 6 h. (a) Hsps resolved by 2-D IEF/SDS-PAGE and immunodetected with anti-EcGroEL antisera. Values below the boxes depict the levels of Cpn60 relative to that in C, 24 h. (b) Photosynthetic (PS, △) and MV_r-NR (▼) activities were measured and expressed as a percentage of the unstressed control. Other details were as described in the legend to Fig. 1.

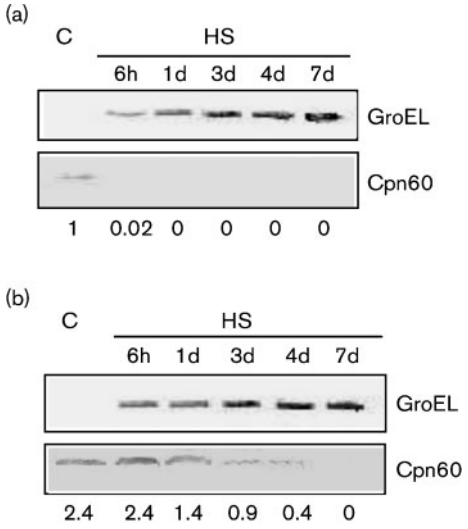

Fig. 5. Levels of Hsp60 proteins in heat-stressed nitrate-supplemented *Anabaena* 7120 and AnFPNcpn cultures. Immunodetection of GroEL and Cpn60 proteins, using specific anti-AnGroEL and anti-AnCpn60 antisera, respectively, in control (C) and heat-stressed (HS) *Anabaena* 7120 (a) and AnFPNcpn (b) cultures. Values below the lanes depict Cpn60 levels relative to the *Anabaena* 7120 unstressed control (a, lane C). Other details were as described in the legend to Fig. 2.

E. coli groEL44 mutant by the *groESL* operon and *cpn60* gene of *Synechocystis* sp. PCC6803 (Kovacs *et al.*, 2001), but their individual roles have remained enigmatic.

Photosynthesis is inactivated during heat stress in cyanobacteria (Eriksson & Clarke, 1996) and the Hsp60 proteins have been found to be associated with carboxysomes (Jager & Bergman, 1990). In *Synechocystis* sp. PCC6803, the *cpn60* gene is not transcribed during heat stress in the dark nor in the presence of DCMU [3-(3,4-dichlorophenyl)-1, 1-dimethylurea] (Glatz *et al.*, 1997), suggesting that it may be required for the assembly of multimeric photosynthetic complexes in light. The non-availability of the Cpn60 protein in nitrogen-supplemented *Anabaena* cultures during heat stress may thus be responsible for the faster inactivation of photosynthesis compared to that observed under nitrogen-fixing conditions (Figs 1, 2, 5 and 6). Overexpression of Cpn60 does confer a higher thermostability of photosynthesis to *Anabaena* (Fig. 6).

This study is the first in which nitrogen-status-dependent regulation of the *hsp60* genes has been studied. Combined nitrogen-supplemented unstressed *Anabaena* cultures exhibit significantly higher levels of Cpn60 compared to nitrogen-fixing cultures. This is perhaps indicative of a higher requirement of Cpn60 for photosynthesis and/or nitrate reductase. Both Hsp60 proteins accumulate during prolonged exposure to heat stress under nitrogen-fixing conditions with GroEL being more strongly induced by heat stress than Cpn60 (Fig. 2; Rajaram & Apte, 2003). Novel features elucidated by the present study are (i) the repression of *cpn60* expression and degradation of Cpn60 by heat stress selectively during nitrogen-supplemented growth of *Anabaena* (Fig. 2) and (ii) apparent correlation between the Cpn60 levels and photosynthetic and nitrate reductase activities (Figs 1, 4, 5 and 6). The data presented clearly show that in nitrogen-supplemented cultures, Cpn60 levels during heat stress are determined by inhibition of transcription (Fig. 2c) and enhanced degradation of the Cpn60 protein (Fig. 2a, b and d). Cpn60 is stable in nitrogen-supplemented cultures grown at 27 °C (Fig. 2d). Using a pulse–chase technique, we have shown previously that the Cpn60 protein is stable up to 24 h of heat stress under nitrogen-fixing conditions (Rajaram & Apte, 2003). The instability during heat stress in nitrogen-supplemented cultures may, therefore, be due to specific proteolytic degradation of the protein.

In cyanobacteria, photosynthesis and nitrate reduction are closely related, since nitrate reductase requires a photo-reduced ferredoxin (Flores *et al.*, 2005). However, nitrate reductase activity was inhibited during heat stress, even when provided with an artificial reductant, MV_r-NR,

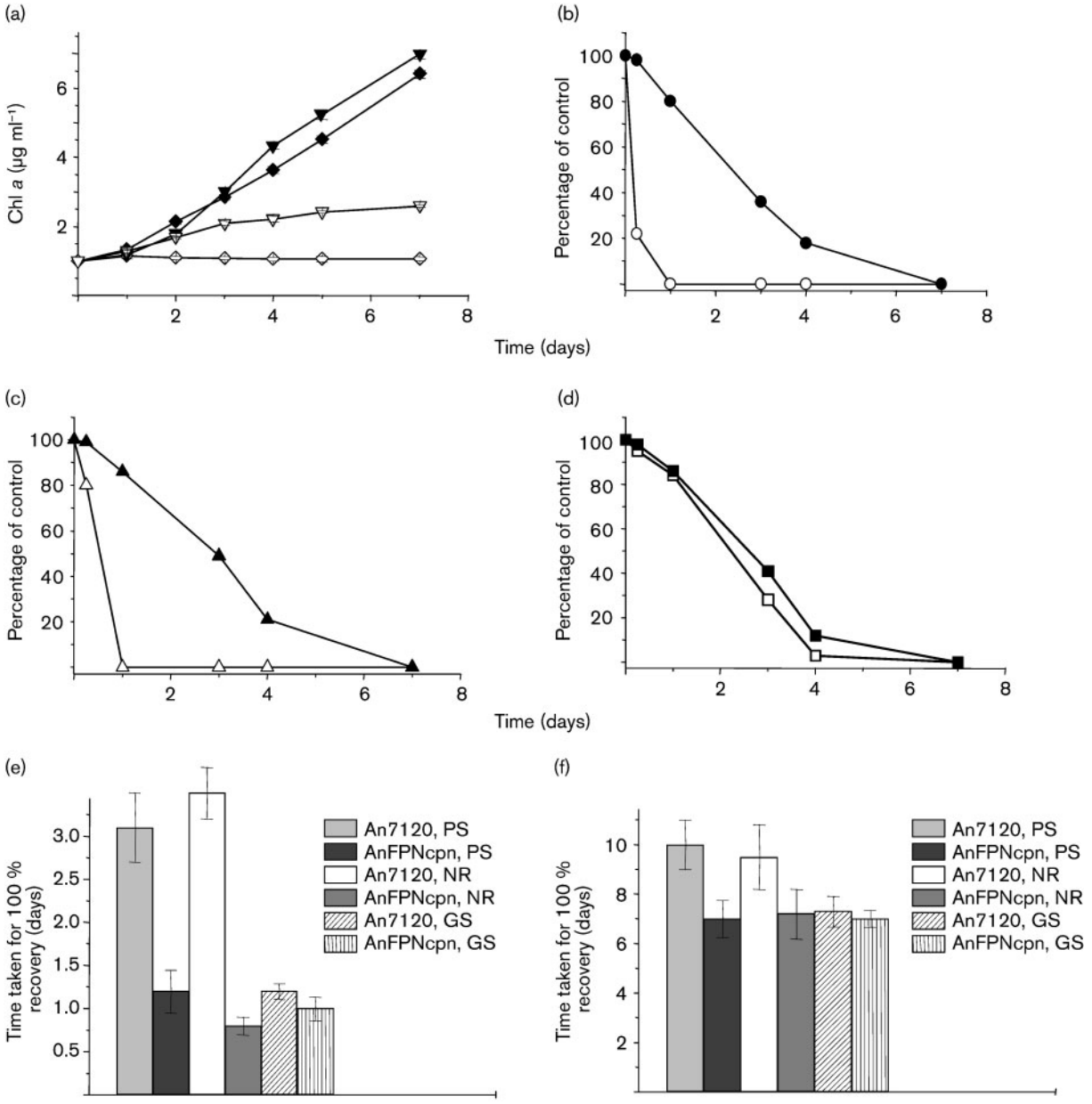

Fig. 6. Comparative analysis of photosynthetic, nitrate reductase and glutamine synthetase activities in *Anabaena* 7120 and AnFPNcpn during heat stress and recovery therefrom. (a–d) Three-day-old nitrate-grown *Anabaena* 7120 and AnFPNcpn cultures were subjected to heat stress at 42 °C and analysed for (a) growth, (b) photosynthetic, (c) nitrate reductase and (d) glutamine synthetase activities. (e, f) Recovery of photosynthetic (PS), nitrate reductase (NR) and glutamine synthetase (GS) activities of nitrate-grown *Anabaena* 7120 and AnFPNcpn cultures, heat-stressed for (e) 1 day and (f) 4 days compared with respective controls. The 100 % control photosynthetic activity was 7.432 ± 0.189 μmol O_2 (mg chl *a*)$^{-1}$ min^{-1} for *Anabaena* 7120 and 7.565 ± 0.215 μmol O_2 (mg chl *a*)$^{-1}$ min^{-1} for AnFPNcpn cultures. The 100 % control nitrate reductase activity (MV_r-NR) was 3.569 ± 0.041 μmol NO_2^- (mg chl *a*)$^{-1}$ min^{-1} for *Anabaena* 7120 and 3.728 ± 0.065 μmol NO_2^- (mg chl *a*)$^{-1}$ min^{-1} for AnFPNcpn cultures. The 100 % control glutamine synthetase activity was 10.471 ± 0.195 μmol γ-GH (mg chl *a*)$^{-1}$ min^{-1} for *Anabaena* 7120 and 13.172 ± 0.136 μmol γ-GH (mg chl *a*)$^{-1}$ min^{-1} for AnFPNcpn cultures. (a) ◆, 7120 C; □, 7120 HS; ▼, AnFPNcpn C; ▽, AnFPNcpn HS. (b) ○, *Anabaena* 7120; ●, AnFPNcpn. (c) △, *Anabaena* 7120; ▲, AnFPNcpn. (d) □, *Anabaena* 7120; ■, AnFPNcpn.

indicating that heat stress influenced nitrate reductase and photosynthesis independently. Thermal inactivation of nitrate reductase has been reported in yeast (Siverio *et al.*, 1993). Interestingly, loss or overexpression of Cpn60 did not directly influence glutamine synthetase activity in *Anabaena* (Figs 1 and 6). In *E. coli*, GroES and GroEL are

reportedly required for the assembly of glutamine synthetase (Fisher, 1994). The decrease in glutamine synthetase activity on prolonged exposure to heat stress in *Anabaena* may be a generic effect of heat stress, which causes denaturation and repression of synthesis of several proteins.

The higher thermosensitivity of nitrate-supplemented *Anabaena* cultures may be due to complete loss of two vital assimilatory processes (Fig. 1) in the absence of Cpn60 (Fig. 2), compared to that of only nitrogen fixation in heat-stressed nitrogen-fixing cultures. The rapid recovery of heat-stressed diazotrophic cultures may be due to the accumulation of both GroEL and Cpn60 chaperonins in them, while the absence of Cpn60 in heat-stressed nitrate-supplemented cultures seems to limit their recovery until adequate Cpn60 is resynthesized. Our attempts to mutagenize *cpn60* did not yield viable mutants (data not shown), but this clearly showed that the *cpn60* gene is essential for normal growth of *Anabaena*. The overexpression of the Cpn60 protein in *Anabaena* 7120 (Fig. 5), on the other hand, resulted in superior thermal stability of both photosynthesis and nitrate reduction compared to the wild-type strain (Fig. 6). The Cpn60 chaperonin thus appears to play a major role in cellular metabolism, especially during nitrogen-supplemented growth of *Anabaena*.

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