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Overexpression of the *groESL* Operon Enhances the Heat and Salinity Stress Tolerance of the Nitrogen-Fixing Cyanobacterium *Anabaena* sp. Strain PCC7120[▽]

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The bicistronic *groESL* operon, encoding the Hsp60 and Hsp10 chaperonins, was cloned into an integrative expression vector, pFPN, and incorporated at an innocuous site in the *Anabaena* sp. strain PCC7120 genome. In the recombinant *Anabaena* strain, the additional *groESL* operon was expressed from a strong cyanobacterial P_{psbA1} promoter without hampering the stress-responsive expression of the native *groESL* operon. The net expression of the two *groESL* operons promoted better growth, supported the vital activities of nitrogen fixation and photosynthesis at ambient conditions, and enhanced the tolerance of the recombinant *Anabaena* strain to heat and salinity stresses.

Nitrogen-fixing cyanobacteria, especially strains of *Nostoc* and *Anabaena*, are native to tropical agroclimatic conditions, such as those of Indian paddy fields, and contribute to the carbon (C) and nitrogen (N) economy of these soils (22, 30). However, their biofertilizer potential decreases during exposure to high temperature, salinity, and other such stressful environments (1). A common target for these stresses is cellular proteins, which are denatured and inactivated during stress, resulting in metabolic arrest, cessation of growth, and eventually loss of viability. Molecular chaperones play a major role in the conformational homeostasis of cellular proteins (13, 16, 24, 26) by (i) proper folding of nascent polypeptide chains; (ii) facilitating protein translocation and maturation to functional conformation, including multiprotein complex assembly; (iii) refolding of misfolded proteins; (iv) sequestering damaged proteins to aggregates; and (v) solubilizing protein aggregates for refolding or degradation. Present at basal levels under optimum growth conditions in bacteria, the expression of chaperonins is significantly enhanced during heat shock and other stresses (2, 25, 32).

The most common and abundant cyanobacterial chaperones are Hsp60 proteins, and nitrogen-fixing cyanobacteria possess two or more copies of the *hsp60* or *groEL* gene (http://genome.kazusa.or.jp/cyanobase). One occurs as a solitary gene, *cpn60* (17, 21), while the other is juxtaposed to its cochaperonin encoding genes *groES* and constitutes a bicistronic operon *groESL* (7, 19, 31). The two *hsp60* genes encode a 59-kDa GroEL and a 61-kDa Cpn60 protein in *Anabaena* (2, 20). Both the Hsp60 chaperonins are strongly expressed during heat stress, resulting in the superior thermotolerance of *Anabaena*, compared to the transient expression of the Hsp60 chaperonins in *Escherichia coli* (20). GroEL and Cpn60 stably associate with thylakoid membranes in *Anabaena* strain PCC7120 (14) and in *Synechocystis* sp. strain PCC6803 (15). In *Synechocystis* sp. strain PCC6803, photosynthetic inhibitors downregulate, while light and redox perturbation induce *cpn60* expression (10, 25, 31), and a *cpn60* mutant exhibits a light-sensitive phenotype (http://genome.kazusa.or.jp/cyanobase), indicating a possible role for Cpn60 in photosynthesis. GroEL, a lipochaperonin (12, 28), requires a cochaperonin, GroES, for its folding activity and has wider substrate selectivity. In heterotrophic nitrogen-fixing bacteria, such as *Klebsiella pneumoniae* and *Bradyrhizobium japonicum*, the GroEL protein has been implicated in *nif* gene expression and the assembly, stability, and activity of the nitrogenase proteins (8, 9, 11).

Earlier work from our laboratory demonstrated that the Hsp60 family chaperonins are commonly induced general-stress proteins in response to heat, salinity, and osmotic stresses in *Anabaena* strains (2, 4). Our recent work elucidated a major role of the *cpn60* gene in the protection from photosynthesis and the nitrate reductase activity of N-supplemented *Anabaena* cultures (21). In this study, we integrated and constitutively overexpressed an extra copy of the *groESL* operon in *Anabaena* to evaluate the importance and contribution of GroEL chaperonin to the physiology of *Anabaena* during optimal and stressful conditions.

Anabaena sp. strain PCC7120 was photoautotrophically grown in combined nitrogen-free ($BG11^-$) or 17 mM $NaNO_3$-supplemented ($BG11^+$) BG11 medium (5) at pH 7.2 under continuous illumination (30 $\mu E\ m^{-2}\ s^{-1}$) and aeration (2 liters min^{-1}) at 25°C ± 2°C. *Escherichia coli* DH5α cultures were grown in Luria-Bertani medium at 37°C at 150 rpm. For *E. coli* DH5α, kanamycin and carbenicillin were used at final concentrations of 50 $\mu g\ ml^{-1}$ and 100 $\mu g\ ml^{-1}$, respectively. Recombinant *Anabaena* clones were selected on $BG11^+$ agar plates supplemented with 25 $\mu g\ ml^{-1}$ neomycin or in $BG11^-$ liquid medium containing 12.5 $\mu g\ ml^{-1}$ neomycin. The growth of cyanobacterial cultures was estimated either by measuring the chlorophyll *a* content as described previously (18) or the turbidity (optical density at 750 nm). Photosynthesis was measured as light-dependent oxygen evolution at 25 ± 2°C by a Clark electrode (Oxy-lab 2/2; Hansatech Instruments, England) as described previously (21). Nitrogenase activity was

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TABLE 1. Plasmids, strains, and primers used in this study

Plasmid, strain, or primer	Feature or sequence[a]	Source or reference
Plasmids		
pFPN	Integrative expression vector	6
pFPNgro	pFPN with *groESL* operon	This study
Strains		
An7120	Wild-type *Anabaena* sp. strain PCC7120	R. Haselkorn
AnFPNgro	GroESL-overexpressing *Anabaena*	This study
Primers		
*groESL*fwd	5′-GGA ATT CCA TAT GGC AGC AGT ATC TCT AAG-3′	This study
*groESL*rev	5′-CGC GGA TCC TTA GTA ATC GAA GTC ACC GCC-3′	This study
P_{psbA1}fwd	5′-GAG CTG CAG GGA TTC CCA AAG ATA GGG-3′	6
P_{psbA1}rev	5′-CTC GGA TCC CCA TAT GTT TTT ATG ATT GCT TTG-3′	6

[a] The underlined nucleotides in the primer sequences represent the incorporated restriction endonuclease sites.

estimated by acetylene reduction assays, as described previously (3). Protein denaturation and aggregation were measured in clarified cell extracts containing ~500 μg cytosolic proteins treated with 100 μM 8-anilino-1-naphthalene sulfonate (ANS). The pellet (protein aggregate) was solubilized in 20 mM Tris–6 M urea–2% sodium dodecyl sulfate (SDS)–40 mM dithiothreitol for 10 min at 50°C. The noncovalently trapped ANS was estimated using a fluorescence spectrometer (model FP-6500; Jasco, Japan) at a $\lambda_{excitation}$ of 380 nm and a $\lambda_{emission}$ of 485 nm, as described previously (29).

The complete bicistronic *groESL* operon (2.040 kb) (GenBank accession no. FJ608815) was PCR amplified from PCC7120 genomic DNA using specific primers (Table 1) and the amplicon cloned into the NdeI-BamHI restriction sites of plasmid vector pFPN, which allows integration at a defined innocuous site in the PCC7120 genome and expression from a strong cyanobacterial P_{psbA1} promoter (6). The resulting construct, designated pFPNgro (Table 1), was electroporated into PCC7120 using an exponential-decay wave form electroporator (200 J capacitive energy at a full charging voltage of 2 kV; Pune Polytronics, Pune, India), as described previously (6). The electroporation was carried out at 6 kV cm^{-1} for 5 ms, employing an external autoclavable electrode with a 2-mm gap. The electroporation buffer contained high concentrations of salt (10 mM HEPES, 100 mM LiCl, 50 mM $CaCl_2$), as have been recommended for plant cells (23) and other cell types (27). The electrotransformants, selected on $BG11^+$ agar plates supplemented with 25 μg ml^{-1} neomycin by repeated subculturing for at least 25 weeks to achieve complete segregation, were designated AnFPNgro.

The transfer of pFPNgro to PCC7120 resulted in the integration of an extra copy of *groESL* (P_{psbA1}-*groESL*) into the PCC7120 genome. PCR amplification (Fig. 1I) with the P_{psbA1} forward and *groESL* reverse primer pairs showed the additional copy of *groEL* juxtaposed downstream to the P_{psbA1} promoter (lane 6) in the recombinant *Anabaena* strain, while the native *groESL* operon found in the wild-type strain (lane 3) remained intact in the AnFPNgro strain (lane 5).

Under normal growth conditions, the recombinant AnFPNgro cells expressed about 8.7- to 9.9-fold higher levels of GroEL protein than that detected in the PCC7120 cells (Fig. 1II), indicating a strong constitutive expression of the GroEL protein from the P_{psbA1} promoter. In PCC7120, the wild-type copy of the GroEL protein was induced by both heat shock (Fig. 1IIA, lane 2) and salt stress (Fig. 1IIB, lane 2). GroEL levels in the recombinant strain were found to be about 2.5-fold higher under heat stress (Fig. 1IIA, lane 4) and approximately 1.7-fold higher under salinity stress (Fig. 1IIB, lane 4) than that expressed by PCC7120 under these stresses (Fig. 1IIA and IIB, lanes 2). The exposure of AnFPNgro cells to heat stress resulted in a further increase of approximately sixfold in GroEL levels (Fig. 1IIA, lane 4), while salt stress enhanced GroEL levels by approximately threefold (Fig. 1IIB, lane 4), compared to the constitutively expressed GroEL level in this strain (Fig. 1IIA and IIB, lanes 3). The constitutive expression of GroEL protein in AnFPNgro under ambient conditions (Fig. 1IIA and IIB, lanes 3) was from the P_{psbA1} promoter (Fig. 1I, lane 6). We assume that the additional increase in GroEL levels observed under heat and salt stress (Fig. 1IIA and IIB, lanes 4) was due to the native stress-induced *groESL* operon, functional from its own promoter.

The diazotrophically grown PCC7120 did not grow during prolonged exposure to heat stress (42°C) (Fig. 2A) and showed poor growth during salinity stress (150 mM) (Fig. 2B). Salinity stress was particularly severe for photosynthetic pigments in PCC7120 and bleached the cells (data not shown). In contrast, the recombinant strain AnFPNgro showed a higher content of major photosynthetic pigments (Fig. 2C) and presented a healthier blue-green phenotype (data not included). Strain AnFPNgro also showed better growth than wild-type PCC7120, both under unstressed and stressed conditions (Fig. 2A and B).

The photosynthetic activity decreased with time during heat stress in PCC7120 but was maintained at comparatively higher levels in AnFPNgro cells (Fig. 3A) than in PCC7120. The dinitrogenase activity in PCC7120 was severely inhibited after 4 h of heat stress (Fig. 3B). In contrast, the dinitrogenase activity of the recombinant strain (AnFPNgro) was about 1.5-fold higher than PCC7120 under ambient conditions (25°C ± 2°C, no NaCl) and more than 3-fold higher than that of PCC7120 after 4 h of heat stress (Fig. 3B). Prolonged exposure to salinity stress inhibited photosynthesis and nitrogen fixation in PCC7120 (Fig. 3C and D). However, strain AnFPNgro displayed significant protection of these activities, possibly due to overexpressed GroES/GroEL proteins. The recombinant

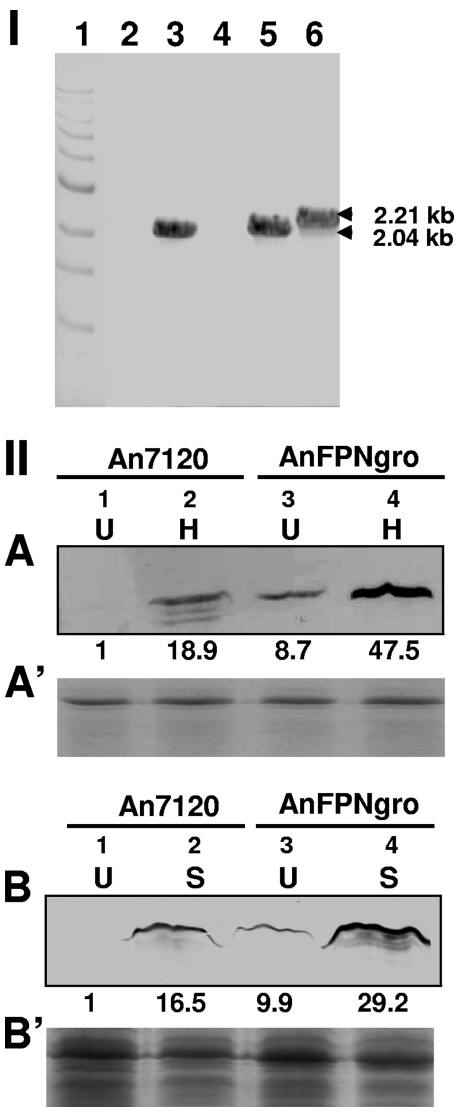

FIG. 1. Integration and constitutive expression of an additional *groESL* operon in *Anabaena* strain PCC7120. (I) Integration of an additional *groESL* operon in the PCC7120 genome. The electrophoretogram shows the transfer and integration of P_{psbA1}-*groESL* in strain AnFPNgro. Lane 1, 1-kb DNA marker; lane 2, PCR control template without primer; lane 3, PCR product from wild-type *Anabaena* using the *groESL*fwd and *groESL*rev primers; lane 4, PCR product from PCC7120 using the P_{psbA1}fwd and *groESL*rev primers; lane 5, PCR product from AnFPNgro using the *groESL*fwd and *groESL*rev primers; lane 6, PCR product from AnFPNgro using the P_{psbA1}fwd and *groESL*rev primers. (II) Expression of the *groESL* operon in the wild-type and recombinant *Anabaena* strains during stress. PCC7120 (An7120) and AnFPNgro were grown for 3 days and then subjected to either heat stress (42°C) for 4 h (A and A′) or salinity stress (150 mM NaCl) for 3 days (B and B′). GroEL levels were estimated by Western blotting of 10% SDS-polyacrylamide gel electrophoresis-resolved whole-cell proteins, followed by immunodetection using anti-AnGroEL antiserum and densitometry (A and B). Panels A′ and B′ depict SDS-polyacrylamide gel electrophoresis-resolved and Coomassie blue-stained proteins to show equal sample loading. Various lanes contained protein samples under unstressed-control (U), heat (H), or salt (S) stress conditions. Numbers below panels A and B show GroEL quantitation by densitometry.

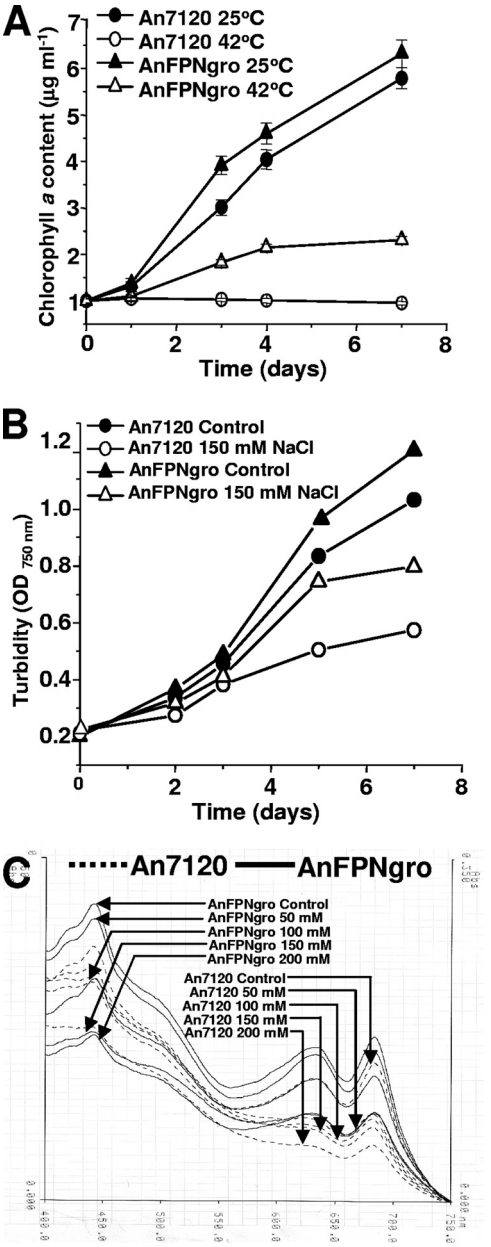

FIG. 2. Effect of *groESL* overexpression on thermotolerance and salinity tolerance of diazotrophically grown *Anabaena* strains. (A) Growth (measured as chlorophyll *a* content) of strains during prolonged exposure to 42°C. (B) Growth (turbidity measured at an optical density at 750 nm) during prolonged exposure to 150 mM NaCl. (C) Absorption spectra of a dilute suspension of whole filaments after 7 days of exposure to various NaCl concentrations.

strain (AnFPNgro) exhibited much-reduced protein aggregation after 4 h of heat stress or after prolonged exposure (10 days) to salinity stress than PCC7120 (Fig. 4).

This study evaluated the possible benefits of *groESL* overexpression for the general stress tolerance of PCC7120. The recombinant AnFPNgro strain harbored two *groESL* operons, one native stress-inducible *groESL* and a second *groESL* operon integrated at a defined innocuous site and placed downstream of a constitutive P_{psbA1} promoter (Fig. 1). The

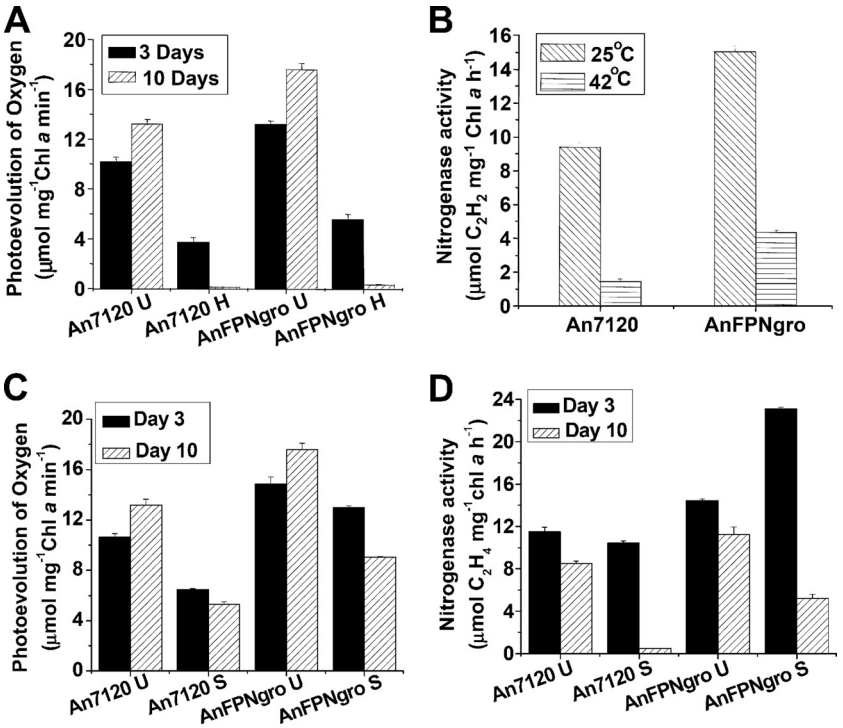

FIG. 3. Effect of *groESL* overexpression on photosynthesis and nitrogen fixation in *Anabaena*. Photosynthesis (A and C) and nitrogenase activity (B and D) in wild-type *Anabaena* strain PCC7120 (An7120) and recombinant AnFPNgro strains exposed to heat stress for 10 days (A) or 4 h (B) or to salinity stress (150 mM) for 10 days (C and D). Letters U, H, and S denote unstressed-control, heat stress, and salt stress conditions, respectively.

recombinant AnFPNgro strain showed an 8- to 10-fold higher constitutive expression of GroEL under ambient conditions than PCC7120, while its inherent stress-induced GroEL expression was not impaired and resulted in 30- and 48-fold more GroEL under salt and heat stress, respectively (Fig. 1).

The AnFPNgro cells exhibited better growth (Fig. 2), photosynthesis, and nitrogen fixation (Fig. 3) than PCC7120, suggesting a possible limitation on the availability of GroEL under ambient conditions. The protection of photosynthetic pigments and oxygen photoevolution during salinity stress were particularly impressive. Nearly 2- to 2.5-fold higher GroEL levels in AnFPNgro under heat or salt stress, compared to those of PCC7120 (Fig. 1), lowered the stress-triggered protein aggregation (Fig. 4) and had beneficial consequences for photosynthesis and nitrogen fixation in the recombinant strain (Fig. 3). An overall improvement in the aforesaid vital metabolic activities eventually resulted in the superior tolerance of recombinant AnFPNgro to heat and salt stresses.

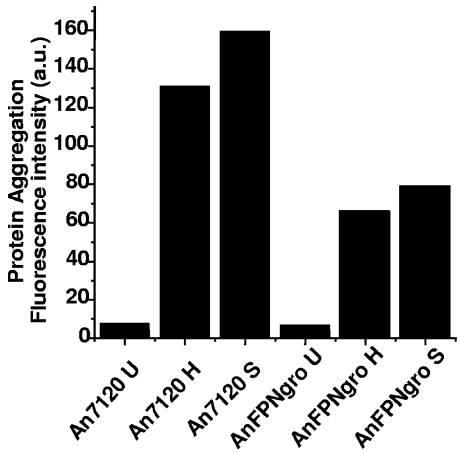

FIG. 4. Protein aggregation in *Anabaena* strains during exposure to heat and salinity stress. The protein aggregation was monitored by ANS fluorescence after 4 h of exposure to 42°C (H) or 10 days of exposure to 150 mM NaCl (S) and compared with the unstressed controls (U) of recombinant strain AnFPNgro and the wild-type *Anabaena* strain PCC7120 (An7120). The fluorescence intensity output from the spectrofluorimeter is expressed as arbitrary units (a.u.).

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