An RNA helicase, CrhR, regulates the low-temperature-inducible expression of heat-shock genes *groES*, *groEL1* and *groEL2* in *Synechocystis* sp. PCC 6803

Jogadhenu S. S. Prakash,^{1,2} Pilla Sankara Krishna,¹ Kodru Sirisha,¹ Yu Kanesaki,² Iwane Suzuki,³ Sisinthy Shivaji⁴ and Norio Murata²

Correspondence
Norio Murata
murata@nibb.ac.jp

¹Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad 500 046, India

²National Institute for Basic Biology (NIBB), Okazaki 444-8585, Japan

³Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba 305-8572, Japan

⁴Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India

The *crhR* gene for RNA helicase, CrhR, was one of the most highly induced genes when the cyanobacterium *Synechocystis* sp. PCC 6803 was exposed to a downward shift in ambient temperature. Although CrhR may be involved in the acclimatization of cyanobacterial cells to low-temperature environments, its functional role during the acclimatization is not known. In the present study, we mutated the *crhR* gene by replacement with a spectinomycin-resistance gene cassette. The resultant $\Delta crhR$ mutant exhibited a phenotype of slow growth at low temperatures. DNA microarray analysis of the genome-wide expression of genes, and Northern and Western blotting analyses indicated that mutation of the *crhR* gene repressed the low-temperature-inducible expression of heat-shock genes *groEL1* and *groEL2*, at the transcript and protein levels. The kinetics of the *groESL* co-transcript and the *groEL2* transcript after addition of rifampicin suggested that CrhR stabilized these transcripts at an early phase, namely 5–60 min, during acclimatization to low temperatures, and enhanced the transcription of these genes at a later time, namely 3–5 h. Our results suggest that CrhR regulates the low-temperature-inducible expression of these heat-shock proteins, which, in turn, may be essential for acclimatization of *Synechocystis* cells to low temperatures.

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INTRODUCTION

Low-temperature stress is one of the most important environmental factors that limit various biological activities. Organisms perceive the low-temperature stress and regulate the expression of various genes whose products are assumed to be important for the acclimatization to low-temperature environments (Polissi *et al.*, 2003).

We have been working on the mechanisms of acclimatization to low-temperature stress of the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter, *Synechocystis*). Studies using DNA microarrays demonstrated that low-temperature stress induced the expression of a large number of genes, including genes for ribosomal proteins, RNA-binding proteins, subunits of RNA polymerases, subunits of NADH dehydrogenase, acyl-lipid desaturases,

and proteins of as yet unknown function (Suzuki *et al.*, 2001; Los & Murata, 2002; Murata & Los, 2006). However, the functional role of these proteins during acclimatization to low temperature is not known, except for acyl-lipid desaturases, which are believed to optimize the fluidity in membranes at low temperatures (Los *et al.*, 2008).

The *crhR* gene for RNA helicase is one gene whose expression is highly induced upon low-temperature stress in *Synechocystis* (Suzuki *et al.*, 2001; Los *et al.*, 2008). The database of genome sequences available at <http://genome.kazusa.or.jp/cyanobase/Synechocystis> suggests that *Synechocystis* contains only one gene, *crhR* (ORF *slr0083*), for RNA helicase. Heterologous expression of the *crhR* gene in *Escherichia coli* and subsequent biochemical characterization of the expressed protein demonstrated that this protein catalysed both ATP-dependent unwinding of secondary structures of RNA and annealing of complementary RNA strands (Chamot *et al.*, 2005). Although

Five supplementary figures are available with the online version of this paper.

CrhR *in vitro* is active in unwinding, annealing and exchanging RNA strands, its function during acclimatization of *Synechocystis* cells to low temperature has not been demonstrated.

RNA helicases are ubiquitously distributed in all the biological kingdoms (Rocak & Linder, 2004). Extensive studies have demonstrated that they are active in modulating the secondary structure of RNAs by unwinding RNAs in an ATP-dependent manner (Tanner & Linder, 2001). They participate in various cellular processes in which RNAs are involved. In model experimental organisms, such as *E. coli*, a cold-inducible RNA helicase, CsdA, has been suggested to participate in the assembly of ribosomes (Peil *et al.*, 2008). Another RNA helicase is a component of the 'cold-shock degradosome complex' and enhances the degradation of mRNAs by unwinding the secondary structures so as to facilitate cleavage of mRNA by RNase E (Carpousis *et al.*, 1999; Prud'homme-Genereux *et al.*, 2004).

The cyanobacterium *Anabaena* sp. PCC 7120 contains two genes for RNA helicases; the *crhC* gene is induced only under low-temperature stress whereas the *crhB* gene is induced by salt, low-temperature and light stress, as well as by nitrogen limitation (Chamot *et al.*, 1999). Biochemical characterization and cellular localization of CrhC suggested that it is a membrane-bound protein and may be involved in translocation of proteins across the plasma membrane under low-temperature conditions (El-Fahmawi & Owttrim, 2003).

In this work, we aimed to elucidate the functional role of RNA helicase, CrhR, during acclimatization of *Synechocystis* cells to low temperatures by investigating changes in the genome-wide expression of genes. We demonstrated that the most important change in the gene expression by mutation of the *crhR* gene appeared in low-temperature inducibility of heat-shock genes *groESL* and *groEL2*, at both transcript and protein levels; namely, the *crhR* mutation converted these genes from low-temperature-inducible to low-temperature-non-inducible.

METHODS

Cells and culture conditions. A strain of *Synechocystis*, which is tolerant to glucose (Williams, 1988), was originally obtained from Dr J. G. K. Williams (Dupont de Nemours). Wild-type cells were grown at 34 °C in BG-11 medium (Stanier *et al.*, 1971), buffered with 20 mM HEPES/NaOH (pH 7.5), under continuous illumination from incandescent lamps, as described previously (Wada & Murata, 1989). Δ *crhR* mutant cells (see below), in which the *crhR* gene had been replaced by the spectinomycin resistance gene (*Sp^r*) cassette in the genome, were grown under the same conditions as described above with the exception that the culture medium contained spectinomycin at 25 µg ml⁻¹ during the pre-cultures. Δ *crhR* mutant cells were then transferred to the above-mentioned BG-11 medium, which did not contain spectinomycin, for the final cell culture for the experiments.

Deletional mutagenesis of the *crhR* gene for RNA helicase to generate Δ *crhR* mutant cells. We generated a Δ *crhR* mutant by

replacing the *crhR* gene (*slr0083*) by the *Sp^r* cassette (see Supplementary Fig. S1, available with the online version of this paper): 767 bp upstream and 960 bp downstream flanking regions of ORF *slr0083* from the genomic DNA were amplified using primer sets UF (5'-AAT CTA GAG TCG ATA TTC CTT GGA TTC GTA TT-3')/UR (5'-AAA GGC CTG ACG GTT TAG TGG GCA AAT AAT T-3') and DF (5'-AAA GGC CT AAC TCC TCC AGA ACT AAG ACC-3')/DR (5'-AAG AGC TCC ATC GAA CCC ATT GAC CTA GAG-3'), respectively. An *Xba*I site and a *Sac*I site (underlined) were created in primers UF and DR, respectively, during primer synthesis. A *Stu*I site (underlined) was created in the primers UR and DF. The PCR fragments generated using UF-UR and DF-DR primer sets were cloned separately into pT7Blue T-A cloning vector and named pTcrh767 and pTcrh960, respectively. The DF-DR fragment released from pTcrh960 after digestion with *Stu*I and *Sac*I restriction enzymes was ligated to the same sites on pTcrh767. The resultant construct was named pTcrh⁻. The *Dra*I-digested Omega *Sp^r* cassette was cloned onto the *Stu*I site of the pTcrh⁻ construct by blunt end ligation. The final construct, in which the *slr0083* ORF had been replaced by the Omega *Sp^r* cassette, was used to transform wild-type cells of *Synechocystis*. Genomic DNA extracted from mutant cells was used as the template and UF and DR were used as primers to examine, by PCR, the extent of replacement of the wild-type copy of the chromosome by the mutated copy of the chromosome (Supplementary Fig. S1b). This analysis indicated that the wild-type copy of the *crhR* gene had been completely replaced by the mutated copy in Δ *crhR* cells. The resultant mutant was designated Δ *crhR*.

Complementation of Δ *crhR* mutation. We complemented Δ *crhR* cells with a derivative of the cyanobacterial autonomously replicating plasmid pVZ321 (Zinchenko *et al.*, 1999) harbouring the *crhR* gene. A DNA fragment that included the *crhR* gene and its upstream 400 bp was amplified, by PCR, with the genomic DNA from wild-type *Synechocystis* cells, with the forward primer 5'-GCC CAA AGC TTT GCC CGA AGA AGT AAT G-3' and the reverse primer 5'-CCG TTC TCG AGG AGT TAT TTT TTT CCG AGT C-3'. A *Hind*III and an *Xho*I site (underlined) were created in the forward and reverse primers, respectively. The product amplified by PCR was digested with *Hind*III and *Xho*I and the resulting fragment of 2007 bp was inserted into pVZ321 which had been digested with the same enzymes. The resultant plasmid was introduced into Δ *crhR* cells by triparental gene transfer (Zinchenko *et al.*, 1999). The complemented strain was designated *crhR*⁺.

Preparation of cDNAs for DNA microarray analysis. *Synechocystis* cells that had been exposed to low-temperature stress were killed instantaneously by the addition of 50 ml ice-cold phenol/ethanol (1:20, w/v) to 50 ml of the cell suspension and then total RNA was extracted as described previously (Los *et al.*, 1997). The RNA was treated with DNase I (Nippon Gene) to remove contaminating DNA. cDNAs, labelled with fluorescent dyes (Cy3 and Cy5; Amersham Pharmacia Biotech), were prepared from 10 µg total RNA with an RNA Fluorescence Labelling Core kit (M-MLV, version 2.0; Takara) according to the manufacturer's instructions.

DNA microarray analysis. Genome-wide analysis of transcript levels was performed with DNA microarrays, as described previously (Kanesaki *et al.*, 2002). In brief, we used the *Synechocystis* DNA microarray (CyanoCHIP, Takara), which covered 3079 of the 3168 ORFs (97% of total genes except transposon-related genes) of the *Synechocystis* genome. Hybridization of the labelled cDNA to the DNA microarray was carried out at 65 °C for 16 h. After hybridization, the microarrays were rinsed with 2 × SSC (1 × SSC is 150 mM NaCl and 15 mM sodium citrate) at room temperature. They were washed with 2 × SSC at 60 °C for 10 min and 0.2 × SSC, 0.1% SDS at 60 °C for 10 min and then rinsed with distilled water at room temperature for 2 min. Moisture was removed with the air

spray prior to analysis with the array scanner (GMS418; Affimetrix). Each signal was quantified with the ImaGene version 4.0 program (BioDiscovery). The signal from each gene on the microarray was normalized with reference to the total intensity of signals from all genes with the exception of genes for rRNAs. Then we calculated changes in the level of the mRNA of each gene relative to the total level of mRNA.

Northern blotting analysis. Total RNA was extracted from cells, and Northern blotting analysis was performed as described by Los *et al.* (1997). In the case of mRNA stability experiments, wild-type and $\Delta crhR$ cells were incubated in the presence of a transcriptional inhibitor, rifampicin, at a final concentration of 100 $\mu\text{g ml}^{-1}$ to determine the stability of the *groESL* co-transcript and *groEL2* transcript. At various time points after addition of rifampicin, cells were harvested for RNA extraction and subsequent Northern blotting analysis. DNA fragments corresponding to *groESL*, *groEL2* and *rnpB* mRNAs were conjugated with alkaline phosphatase (Alkphos Direct kit; Amersham Pharmacia Biotech) and the resultant conjugates were used as probes. After hybridization, the blots were soaked with the CDP-star solution (Amersham Pharmacia Biotech) and signals from hybridized mRNAs were detected with a luminescence image analyser (LAS-1000; Fuji-Photo Film).

Preparation of antibodies against CrhR. Antibodies against CrhR were raised in rats with His-tagged CrhR of *Synechocystis*, which had been overexpressed in *E. coli*. First, the *crhR* ORF was amplified, by PCR, with the forward primer 5'-GCCATATGACTAATACT-TTGACTAGTAC-3' and the reverse primer 5'-GCGTCGAC-TTACTGTTGGCGATCACTATAG-3' and purified by agarose gel electrophoresis. The amplified ORF of *crhR* was eluted from the gel and was inserted into pET-28a(+) at the *NdeI* and *SalI* sites to generate pET-CrhR. The N-terminally His-tagged CrhR protein was expressed in BL21(DE3)pLysS, which had been transformed with pET-CrhR, and was purified using HIS-Select Nickel Affinity gel (Sigma – P6611) according to the supplier's instruction. The expression of CrhR protein was induced by addition of 400 μM (final concentration) IPTG. Bacterial cells were collected by centrifugation at 10 000 *g* for 10 min and pelleted cells were disrupted with a sonic oscillator (model, UV2070; probe, MS-72; Bandelin Electronic) operated for 10 min at 50 % power, with 1 min pulse interval, in 100 mM Tris/HCl (pH 8.0) and 200 mM NaCl. Insoluble materials (precipitates) were removed by centrifugation at 20 000 *g* for 20 min at 4 °C. The supernatant was loaded onto a HIS-Select Nickel Affinity column. The column was washed with 100 mM Tris/HCl (pH 8.0), 200 mM NaCl and 10 mM imidazole and sequentially with the same buffer containing 40 mM imidazole. Then His-tagged CrhR was eluted with 100 mM Tris/HCl (pH 8.0), 200 mM NaCl and 200 mM imidazole. The purity of each fraction was examined by SDS-gel electrophoresis. The fractions which gave a single band at the expected region on the gel were combined and dialysed against 5 mM Tris/HCl (pH 8.0). The resultant protein was used to generate anti-CrhR antibodies in rats (by TakaraBio).

Western blotting analysis. Soluble proteins were extracted from *Synechocystis* cells by mechanical disruption of cells using glass beads (106 μm diameter; Sigma). *Synechocystis* cells in 200 μl 50 mM Tris/HCl (pH 8.0) buffer were mixed with 330 mg glass beads in a thick-walled glass tube and disrupted by vigorous vortexing at maximum speed on a vortex mixer for 1 min followed by 2 min cooling on ice. Vortex mixing and cooling on ice was repeated 10 times to ensure maximum disruption of cells. Then, mechanically disrupted cells were centrifuged at 25 000 *g* for 20 min at 4 °C to separate soluble proteins from insoluble materials. The resultant supernatants, which contained soluble proteins from control and treated cells, were loaded onto SDS-PAGE gels on an equal protein basis (25 μg soluble protein in each well). After electrophoresis, the separated proteins were blotted

onto a PVDF membrane (Millipore) in a semidry transfer apparatus (Atto). Levels of CrhR and GroEL were determined immunologically with an enhanced chemiluminescence Western-blotting kit with specific antibodies against CrhR and GroEL according to the protocol supplied with the kit (Amersham International). To investigate the level of CrhR, we used horseradish peroxidase-linked antibody raised in goat against rat immunoglobulin G as the secondary antibody. To investigate the level of GroEL1 and GroEL2, we used antibodies raised in rabbits against *E. coli* GroEL and purchased from Sigma (catalogue no. G-6532) as the primary antibody and horseradish peroxidase antibody raised in donkey against rabbit immunoglobulin G as the secondary antibody. A luminescence image analyser (LAS-1000; Fuji-Photo Film) was used to monitor signals from blotted membranes.

RESULTS AND DISCUSSION

Expression of the *crhR* gene is induced upon a downward shift in temperature

The expression of the *crhR* gene is induced under low-temperature, salt and hyperosmotic stress in *Synechocystis* (Vinnemeier & Hagemann, 1999; Suzuki *et al.*, 2001; Kanesaki *et al.*, 2002). Fig. 1(a) shows changes in the level of *crhR* mRNA during exposure of *Synechocystis* cells, which had been grown at 34 °C, to 24 °C for designated periods of time. A low level of *crhR* mRNA was detected before the exposure, indicating that this gene was constitutively expressed in *Synechocystis*. The downward shift in temperature transiently increased the level of *crhR* transcript, with the maximum attained at 30 min during the incubation at 24 °C (Fig. 1a, c).

Induction of CrhR after a downward shift in temperature

To examine how the level of CrhR protein reflects the transient increase in the level of *crhR* mRNA during incubation at low temperature, we performed Western blotting analysis of changes in the CrhR level during exposure to 24 °C of *Synechocystis* cells which had been grown at 34 °C for 16 h (Fig. 1b, c). The anti-CrhR antibody raised in rats detected CrhR at a molecular mass of approximately 52 kDa (Fig. 1b). Fig. 1(c) shows that CrhR was present at a certain level before the downward shift in temperature. Then the level of CrhR gradually increased during incubation at 24 °C and reached a maximum level that was 3.5 times higher than the original after incubation at 24 °C for 180 min (Fig. 1c). These observations indicated that *Synechocystis* cells induced the expression of the *crhR* gene for RNA helicase and accumulated CrhR after the temperature was shifted downward by 10 °C from growth conditions. Our previous DNA microarray study demonstrated that almost all the low-temperature-inducible genes in *Synechocystis* are under the control of the sensory kinase Hik33, except the *crhR* gene for RNA helicase (Suzuki *et al.*, 2001). This indicates that the low-temperature-inducible expression of the *crhR* gene might be regulated by a mechanism other than two-component signal transduction.

Fig. 1. Northern and Western blotting analyses of changes in the expression of the *crhR* gene upon downward shift in temperature in wild-type cells of *Synechocystis*. (a) Northern blotting of *crhR* mRNA. Total RNA was extracted from wild-type cells that had been grown at 34 °C for 16 h or had been grown at 34 °C for 16 h and then incubated at 24 °C for 10, 20, 30, 40, 50, 60 and 120 min. Aliquots (20 µg) of the extracted RNA were electrophoresed on 1.2% agarose gels that contained 1.4 M formaldehyde. (b) Western blotting of CrhR. Soluble proteins were extracted from wild-type cells that had been grown at 34 °C for 16 h or had been grown at 34 °C for 16 h and then incubated at 24 °C for 30, 60, 180 and 300 min. Samples equivalent to 25 µg proteins were loaded in each well of a polyacrylamide gel (12%) that contained 0.1% SDS. CrhR antibody produced in rat was used to detect CrhR on the blot. (c) Quantitative expression of *crhR* mRNA and CrhR upon the downward shift in temperature (means $\pm$ SD of three independent experiments).

Mutation of the *crhR* gene and complementation of the Δ *crhR* mutant

In order to confirm that the changes in phenotype seen in the Δ *crhR* mutant, as will be discussed in the next sections, were caused by mutation of the *crhR* gene, we compared

Fig. 2. Mutation and complementation of the *crhR* gene affected growth profiles at low temperature. The wild-type (●), the Δ *crhR* mutant cells (○) and the complemented *crhR*⁺ strain (△) were grown photoautotrophically (70 µmol photons m⁻² s⁻¹) at 34 °C (a) or 24 °C (b). Growth was monitored by measuring the OD₇₃₀. Similar results were obtained in three independent experiments, and the data are represented as means $\pm$ SD.

the growth profile of wild-type, Δ *crhR* mutant and *crhR*-complemented *crhR*⁺ cells at 24 °C and 34 °C. At 34 °C these three types of cell revealed similar profiles of growth (Fig. 2a). At 24 °C, however, growth of Δ *crhR* cells was much slower than that of wild-type cells (Fig. 2b), indicating that the low-temperature-induced expression of the *crhR* gene and synthesis of CrhR were important for *Synechocystis* cells to grow at low temperature. Complementation of Δ *crhR* cells *in trans* with a functional CrhR expressed from pVZ-*pcrhR* (*crhR*⁺ strain) restored growth at the low temperature, which was similar to that of wild-type cells (Fig. 2b). The functional complementation of Δ *crhR* cells by the *crhR* gene clearly demonstrated that CrhR plays an important role in the physiology of *Synechocystis* cells at low temperatures.

Genes whose expression at low temperature was affected by mutation of the *crhR* gene

E. coli has two genes for RNA helicases. CsdA is a low-temperature-inducible RNA helicase that resumes translation by unwinding the base-paired regions of mRNA that are formed at low temperatures (Jones *et al.*, 1996). Another RNA helicase, RhlB, is a component of the degradosome complex and is involved in controlling the level of mRNAs (Carpousis *et al.*, 1999). Therefore, it seems likely that the mutation of the *crhR* gene in *Synechocystis* would result in changes in gene expression at the transcript level. To examine genes whose expression by low temperature is regulated by CrhR, we analysed the genome-wide expression of genes in the $\Delta crhR$ mutant cells by a DNA microarray method.

First, we investigated the effect of *crhR* mutation on the genome-wide expression of genes under normal growth conditions. Our previous study with the DNA microarray, using RNA extracted from wild-type cells of *Synechocystis* that had been grown under optimal growth conditions (growth temperature, 34 °C; light intensity, 70 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$; CO₂ concentration, 1%) indicated that most of the genes appeared at induction factors between 2.0 and 0.5. These genes are assigned as those whose expression was not significantly affected by low temperature or mutation. The data points that appeared above induction factor 2.0 or below induction factor 0.5 represent those genes whose expression was induced or repressed, respectively, due to the mutation or by stress conditions (Suzuki *et al.*, 2001; Kanesaki *et al.*, 2002). Microarray analysis indicated that the mutation in $\Delta crhR$ cells had no

significant effect on the genome-wide expression of genes under isothermal conditions at 34 °C (Supplementary Fig. S2).

Next, we performed DNA microarray analysis to investigate whether the mutation in $\Delta crhR$ cells would have any effect on the low-temperature-induced expression of genes. Supplementary Fig. S3(a), (b) and (c) represent the gene expression profiles in wild-type cells after exposure of cells to low temperature for 20, 60 and 180 min, respectively. Supplementary Fig. S3(d), (e) and (f) represent the gene expression profiles in $\Delta crhR$ cells under similar conditions. The inducibility by low temperature of the majority of genes was not much affected by the mutation of *crhR*. Only a very small number of genes were significantly affected by the mutation in $\Delta crhR$. The genes for molecular chaperonins, *groES*, *groEL* and *groEL2*, are indicated by black data points in the scatter plots in Supplementary Fig. S3.

Table 1 lists genes whose low-temperature inducibility was significantly affected by mutation of the *crhR* gene. The mutation significantly enhanced the low-temperature-inducible expression of genes *pyrB*, *gifA*, *gifB* and *slr0082*, whereas it decreased that of *groES*, *groEL1*, *groEL2* and *slr1611*. Although the *slr0082* gene for a hypothetical protein, which is located upstream of the *crhR* (*slr0083*) gene in the *Synechocystis* genome, and the *crhR* gene are transcribed from their own promoters (Vinnemeier & Hagemann, 1999), the mutation in the *crhR* gene enhanced the expression of *slr0082* (Table 1). These observations might suggest that there is a regulatory mechanism that interacts with the expression of these two genes. The *pyrB*

Table 1. Genes whose inducibility by low temperature was affected by the mutation in $\Delta crhR$

Wild-type and $\Delta crhR$ cells were grown at 34 °C for 16 h and then incubated at 24 °C for 20, 60 and 180 min. Each value indicates the ratio of levels of the mRNA from cold-stressed cells to those from control cells. The values shown are the means $\pm$ range of two independent experiments. The numbering of ORFs corresponds to that of Kaneko *et al.* (1996). The complete list of gene expression in wild-type and $\Delta crhR$ mutant can be accessed at http://www.genome.jp/kegg-bin/get_htext?htext=Exp_DB&hier=1

ORF no.	Gene	Product	24 °C/34 °C							
			Wild-type cells			$\Delta crhR$ cells		$crhR^+$ cells		
			20 min	60 min	180 min	20 min	60 min	180min	180 min	
Genes whose inducibility by low temperature was depressed by mutation of <i>crhR</i>										
<i>sll1611</i>		Hypothetical protein	4.0 ± 0.4	8.4 ± 1.5	6.7 ± 1.3	2.1 ± 0.1	2.3 ± 0.1	1.9 ± 0.1	3.7 ± 0.02	
<i>slr2076</i>	<i>groEL1</i>	60 kDa chaperonin 1	0.6 ± 0.0	0.7 ± 0.0	4.4 ± 1.2	0.2 ± 0.0	0.2 ± 0.0	1.8 ± 0.2	3.5 ± 0.12	
<i>sll0416</i>	<i>groEL2</i>	60 kDa chaperonin 2	0.6 ± 0.1	0.7 ± 0.1	3.1 ± 0.4	0.2 ± 0.0	0.2 ± 0.0	1.1 ± 0.1	2.7 ± 0.07	
<i>slr2075</i>	<i>groES</i>	10 kDa chaperonin	0.5 ± 0.1	0.5 ± 0.0	3.9 ± 1.0	0.2 ± 0.0	0.2 ± 0.0	1.6 ± 0.5	2.9 ± 0.12	
Genes whose inducibility by low temperature was enhanced by mutation of <i>crhR</i>										
<i>slr0082</i>		Hypothetical protein	9.3 ± 0.8	2.3 ± 0.1	1.0 ± 0.1	11.6 ± 0.8	8.7 ± 1.1	8.8 ± 0.2	1.3 ± 0.04	
<i>slr1476</i>	<i>pyrB</i>	Aspartate carbamoyltransferase	1.9 ± 0.2	1.5 ± 0.1	1.4 ± 0.4	4.1 ± 0.9	3.8 ± 0.9	3.7 ± 1.1	1.2 ± 0.16	
<i>ssl1911</i>	<i>gifA</i>	Glutamine synthetase	1.3 ± 0.7	0.9 ± 0.3	0.4 ± 0.2	2.4 ± 0.3	3.1 ± 0.6	2.3 ± 0.7	1.5 ± 0.01	
		inactivating factor								
<i>sll1515</i>	<i>gifB</i>	Glutamine synthetase	1.5 ± 0.5	1.5 ± 0.1	0.9 ± 0.4	3.4 ± 0.9	2.7 ± 0.4	1.8 ± 0.5	2.2 ± 0.08	
		inactivating factor

gene encodes aspartate carbamoyltransferase, an enzyme that catalyses the regulatory step of pyrimidine biosynthesis (Kafer & Thornburg, 1999). This gene was not induced by low temperature in wild-type cells, but was induced by low temperature in $\Delta crhR$ cells (Table 1). The expression of *gifA* and *gifB* genes for glutamine synthetase-inactivating

Fig. 3. Northern blotting analysis of the low-temperature-induced expression of *groESL* and *groEL2* genes in wild-type and $\Delta crhR$ cells. RNAs were extracted from wild-type and $\Delta crhR$ cells that had been grown at 34 °C for 16 h and then incubated at 24 °C for designated periods of time. Aliquots (15 µg) of the extracted RNA were fractionated on 1.2% agarose gels that contained 1.4 M formaldehyde. (a) The *groEL2* gene. A 1646 bp DNA fragment that included the *groEL2* gene was amplified by PCR using primer set 5'-TTT CCT TTA AGG ATG AAT CCA G-3'/5'-TTA CAT CAT GCC CAT GCC-3' and used as a probe. (b) The *groESL* operon. A 954 bp DNA fragment that included both *groES* and *groEL* open reading frames was amplified by PCR and used as a probe. ○, Wild-type cells; ●, $\Delta crhR$ cells. For normalization, signals obtained with *groEL2* or *groESL* were compared with signals of the *rnpB* mRNA. Similar results were obtained in two independent experiments, and the figure shows the results of one of these experiments (means ± range).

factors 1 and 2, respectively, was slightly enhanced by low temperature in $\Delta crhR$, whereas the same genes did not respond to low temperature in wild-type cells (Table 1). The *sl1611* gene for a hypothetical protein was induced in wild-type cells by low temperature, whereas the low-temperature-induced expression of this gene was insignificant in $\Delta crhR$ cells (Table 1).

The expression of the *groES*, *groEL1* and *groEL2* genes in wild-type cells was unaffected during incubation at 24 °C for the first 60 min. However, the expression of these genes was enhanced three- to fourfold during incubation at 24 °C from 60 min to 180 min (Table 1). In contrast, the low-temperature-inducible expression of the same genes was significantly repressed in $\Delta crhR$ cells during incubation at 24 °C for 60 min and it recovered to almost the original level at 180 min (Table 1). Functional complementation of CrhR *in trans* reversed the effect of $\Delta crhR$ mutation, although the recovery was incomplete (Table 1, last column). To examine whether the incomplete recovery of low-temperature-induced expression of genes was due to a low level of CrhR, we determined the level of CrhR by Western blotting (Supplementary Fig. S4). The result revealed that the level of CrhR was approximately the same for wild-type and *crhR*⁺ cells.

Northern blotting analysis of the low-temperature-inducible expression of the *groESL* and *groEL2* genes

Changes in the low-temperature-induced expression of *groESL* and *groEL2* genes due to the $\Delta crhR$ mutation were further examined by Northern blotting analysis (Fig. 3). We used primers for specific amplification of a DNA fragment corresponding to the *groEL2* gene for detection of *groEL2* mRNA (Fig. 3a). As observed by the microarray analysis (Table 1), the expression of the *groEL2* gene in wild-type cells was unaffected during incubation at 24 °C for 30 min. However, the expression of this gene was

gradually enhanced during further incubation of wild-type cells at 24 °C, and an approximately fivefold increase in the levels of *groEL2* mRNA was observed during further incubation of wild-type cells at 24 °C for 180 min and this high level was maintained during incubation at 24 °C for 360 min in wild-type cells (Fig. 3a). In contrast, the low-temperature-inducible expression of the *groEL2* gene was significantly repressed in $\Delta crhR$ cells during incubation of cells at 24 °C for 30 min and 60 min, and had recovered to the original level at 180 min (Fig. 3a).

The DNA probe that covered genes *groES* and *groEL1* of a dicistronic operon detected a single band on Northern blotting (Fig. 3b). We observed a slight decrease in the mRNA level of *groESL* during incubation at 24 °C for 30 min in wild-type cells. However, the transcript level gradually increased during further incubation of wild-type cells at 24 °C. We observed that the mRNA level increased approximately fourfold during incubation of wild-type cells at 24 °C for 180 min and maintained the high level at 360 min (Fig. 3b). In contrast, the low-temperature-inducible expression of the *groESL* monocistronic operon was significantly repressed in $\Delta crhR$ cells during incubation at 24 °C for 30 min and 60 min and then recovered to the original and slightly higher than the original level in the $\Delta crhR$ cells (Fig. 3b).

CrhR is not involved in the heat-induced expression of the *groEL2* gene

To examine whether CrhR is involved in the heat-induced expression of *groEL* genes, we compared the heat-induced expression of the *groEL2* gene in wild-type and $\Delta crhR$ mutant cells by Northern blotting analysis (Supplementary Fig. S5). The level of *groEL2* mRNA in wild-type and $\Delta crhR$ cells increased eightfold during incubation at 42 °C for 30 min and then, after a slight decrease, maintained a high level during further incubation at 42 °C for 240 min (Supplementary Fig. S5). There was no significant difference in the time-course of the mRNA levels between wild-type and $\Delta crhR$ cells. These observations suggested that CrhR was not involved in the regulation of heat-inducible expression of the *groEL2* gene in *Synechocystis*. Thus, it is very likely that CrhR is not a regulator of the expression of the *groEL2* gene at high temperatures.

Transcripts of *groESL* and *groEL2* are stabilized under low-temperature conditions

The level of mRNA is a result of balance between the rate of transcription and the rate of degradation. Therefore, the low-temperature-induced changes in levels of *groESL* and *groEL2* mRNA due to the mutation of the *crhR* gene could be related to changes in the rate of transcription and/or changes in the stability of mRNAs. To elucidate whether CrhR regulates the *groESL* transcripts post-transcriptionally at the level of mRNA stability, we analysed the decay kinetics of the *groEL2* transcript and *groESL* co-transcripts

in the presence of rifampicin, an inhibitor of transcription. In this experiment, wild-type and $\Delta crhR$ mutant cells, which had been grown at 34 °C for 16 h (Fig. 4a, b), were incubated at 24 °C for 5 min (Fig. 4c, d) or for 180 min (Fig. 4e, f) before the assay of decay kinetics. In cells grown at 34 °C, mRNA of both *groESL* and *groEL2* degraded to zero at 30 min after the addition of rifampicin; there was no difference between the rate of degradation of *groEL2* and *groESL* mRNAs between wild-type and $\Delta crhR$ cells (Fig. 4a, b). By contrast, the decay of *groEL2* and *groESL* transcripts was much slower in cells that had been incubated at 24 °C than those grown at 34 °C; moreover, the degradation was faster in $\Delta crhR$ cells than in wild-type cells (Fig. 4c, d). The half-decay times for *groEL2* and *groESL* transcripts were 8 min and 12 min, respectively, in wild-type cells, whereas they were 3.6 and 5 min, respectively, in $\Delta crhR$ cells. These results indicated that the stability of *groEL2* and *groESL* transcripts decreased to less than half of the original just after the shift in temperature from 34 °C to 24 °C (Fig. 4c, d). Taken together, the analysis of *groESL* expression by Northern blotting and mRNA stability assays suggested that the marked decrease in levels of mRNA during the initial phase of incubation at low temperature in the $\Delta crhR$ mutant cells (Fig. 3a, b) could be attributed to the decrease in stability of these transcripts (Fig. 4c, d). These observations suggest that CrhR regulates the stability of *groEL2* and *groESL* mRNAs at the early phase of incubation at low temperature.

Fig. 4(e) and (f) depict the stability of *groEL2* transcripts and *groESL* co-transcripts in wild-type and $\Delta crhR$ cells after incubation of these cells at 24 °C for 180 min. The *groEL2* and *groESL* transcripts were equally stable (Fig. 4e, f). But, at this time point, we observed a significant increase in levels of *groEL2* and *groESL* transcripts in wild-type cells (Fig. 3a, b). In contrast, in $\Delta crhR$ mutant cells, there was no increase in levels of *groEL2* transcript and a slightly higher than original level of *groESL* transcript was observed (Fig. 3a, b). These findings suggested that the differential expression of *groEL2* and *groESL* genes during the late phase of incubation at low temperature (Fig. 3a, b) was not due to the differential stability of transcripts of these genes (Fig. 4e, f). It seems likely that CrhR might also be involved in the regulation of *groEL2* and *groESL* expression at the level of transcription during the late phase.

Low-temperature-inducible synthesis of GroEL depends on the presence of CrhR

To examine whether the level of GroEL proteins reflects the alteration in the expression of *groESL* genes at the transcript level, we followed, by Western blotting analysis, changes in the level of GroEL1 and GroEL2 in wild-type and $\Delta crhR$ cells during incubation at low temperature. Since antibodies that had been raised against GroEL1 of *E. coli* immunologically reacted with both GroEL1 and GroEL2 of *Synechocystis*, our results demonstrated changes

Fig. 4. Effects of *crhR* mutation on the stability of *groEL2* transcript and *groESL* co-transcript in wild-type (○) and $\Delta crhR$ (●) cells. (a, b) Levels of *groEL2* and *groESL* transcripts after growth at 34 °C for 16 h. (c, d) Levels of *groEL2* and *groESL* transcripts immediately after the shift in temperature from 34 °C to 24 °C. (e, f) Levels of *groEL2* and *groESL* transcripts after growth at 34 °C for 16 h followed by incubation at 24 °C for 180 min. Rifampicin (100 $\mu\text{g ml}^{-1}$) was added to the control or low-temperature-treated cells and the cells were harvested at various periods of time for extraction of RNAs. Signals detected with a luminescence image analyser (LAS-1000; Fuji-Photo Film) were used for calculating the relative abundance of *groEL2* and *groESL* transcripts and were plotted on a logarithmic scale against the time after addition of rifampicin (means $\pm$ SD of three independent experiments).

in the level of GroEL1 plus GroEL2 (hereafter, GroEL). As shown in Fig. 5, the level of GroEL in wild-type cells increased 1.5-fold during incubation at 24 °C for the first 60 min and about twofold at 180 min. In contrast, the level of GroEL reduced to 50 % in $\Delta crhR$ mutant cells during incubation at 24 °C for 60 min and then recovered to approximately the original level during further incubation at 24 °C for 300 min. As a result, the mutation of *crhR* abolished the low-temperature-inducible accumulation of GroEL (Fig. 5). The phenotype of slow growth at low temperature of $\Delta crhR$ cells could be due to the lowered level of GroEL proteins at low temperature.

Concluding remarks

Our results suggest that, in *Synechocystis* cells, RNA helicase CrhR regulates the low-temperature inducibility of molecular chaperonins GroES, GroEL1 and GroEL2, which are, in turn, essential for growth at low temperatures. Low-temperature inducibility of genes for the heat-shock proteins has been reported in other organisms. In the

Antarctic bacterium *Oleispira antarctica*, the cold-inducible expression of GroES and GroEL is important in acclimatization to low temperatures (Ferrer *et al.*, 2003). In the thermophilic cyanobacterium *Thermosynechococcus elongatus*, GroEL2 is essential for acclimatization to low temperatures, as well as to high temperatures (Sato *et al.*, 2008).

There are two possible mechanisms for the functional role of chaperonins in acclimatization to low temperature. First, low temperature may cause denaturation and/or aggregation of some specific proteins in the cell (Ray, 2006). Proper folding and maintenance of structure are essential for the enzymic activity of proteins at low temperatures (Strocchi *et al.*, 2006). Since chaperonins assist the folding of proteins, their expression at low temperatures might protect the proteins against denaturation and aggregation.

The second possible mechanism at low temperatures involving GroES and GroEL is the quality control of proteins. GroESLs enhance degradation of truncated proteins that are produced due to inhibition of translation

Fig. 5. Changes in the level of GroEL in wild-type and $\Delta crhR$ cells after a downward shift in temperature. Soluble proteins were extracted from wild-type and $\Delta crhR$ cells that had been grown at 34 °C and then incubated at 24 °C for 30, 60, 180 and 300 min. Samples equivalent to 25 µg proteins were loaded in each well. The experiment was performed twice and the results are presented as the means $\pm$ range of the two independent experiments.

at low temperatures (Kandror *et al.*, 1994). The low-temperature-inducible trigger factor (TF) binds to GroEL and enhances GroEL's affinity to unfolded proteins, and hence promotes degradation of certain polypeptides (Kandror & Goldberg, 1997). Thus, the folding and degradation of proteins seem to play a major role in the process of acclimatization to low temperature. Chaperonins, in association with other proteins, contribute to such quality control of proteins. Evidence supports the possibility of chaperonin involvement in both stimulation of protein refolding and enhancement of protein degradation at low temperatures.

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