RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2022.079

ScienceAsia 48 (2022): **496–505**

Chlamydomonas plastid chaperonin subunits expressed in *E. coli* can interact with one another inside the bacterial cell and putatively confer enhanced tolerance toward singlet oxygen

Rungdawan Wongsamart[a], **Chotika Yokthongwattana**[b], **Kittisak Yokthongwattana**[a,*]

[a] Department of Biochemistry and Center for Excellence in Protein and Enzyme Technology, Faculty of Science, Mahidol University, Bangkok 10400 Thailand

[b] Department of Biochemistry, Faculty of Science, Kasetsart University, Bangkok 10900 Thailand

[*]Corresponding author, e-mail: kittisak.yok@mahidol.ac.th

Received 5 Sep 2021, Accepted 28 Jan 2022
Available online 5 May 2022

ABSTRACT: Chaperonins are a group of molecular chaperones with a primary role in assisting folding of other proteins. The most recognized member of the chaperonins is the GroEL/GroES complex. While most eubacteria as well as mitochondria of eukaryotes have a single copy of the *groEL* gene, plants and algae contain multiple versions that have been shown to assemble into a hetero-oligomeric complex. We report here evidence suggesting that *Chlamydomonas* plastid chaperonin 60 subunits, when expressed in *E. coli* cells, can interact with each other and assemble into a high-molecular-weight protein complex. Stress challenge assays also revealed that exogenous expression of all alpha, beta1, and beta2 subunits of *Chlamydomonas* chaperonin 60 in *E. coli* also confer additional tolerance and recovery of cells from singlet oxygen stress.

KEYWORDS: chaperonin, *Chlamydomonas*, Cpn60, *E. coli*, environmental stresses

INTRODUCTION

Chaperonins, a group among many types of molecular chaperones, are ubiquitous and have a primary role in assisting folding of other proteins [1]. Group 1 chaperonins are found in prokaryotes (GroEL), mitochondria (Hsp60 or mtCpn6), and plastids such as chloroplast (Cpn60 or chCpn60), while group 2 chaperonins are found in Achaea (thermosome) and the cytosolic compartment of eukaryotes (CCT/TriC) [2, 3]. Group 1 chaperonins with GroEL being a notable example consist structurally of a large double-ring or barrel-like protein complex requiring ATP hydrolysis for operation [4]. Each of the rings is composed of 7 identical subunits of about 60 kDa in size. It has a co-chaperone called GroES also consisting of 7 homo-subunits, which functions like a lid for the core barrel complex. In contrast to group 1, group 2 chaperonin does not require any co-chaperone for operation.

While most eubacteria as well as mitochondria of eukaryotes have a single copy of the *groEL* chaperonin gene, cyanobacteria usually have 2 or 3 *groEL* genes in their genome [5]. Chloroplasts of algae and plants also possess multiple or more diversified versions of the chaperonin 60 subunits. For example, the green alga *Chlamydomonas reinhardtii* contains one gene coding for an α subunit and 2 genes for β subunits of the plastid chaperonin 60 in its genome [3]. *Arabidopsis thaliana* has 2 genes encoding α subunits along with 4 β genes [6]. Bacterial and cyanobacterial GroELs or plant Cpn60 subunits not only share high sequence similarity [7], but their structures are also similar [8]. With such similarity, it is not beyond expectation that Cpn60 subunits could compensate for the loss of GroEL function in bacteria. Indeed, complementation of GroEL mutants of *E. coli* with cyanobacterial GroEL or *Chlamydomonas* Cpn60s has been demonstrated [9–12].

It has been proposed that evolution brings about novel functions for the additional copies of the chaperonins. While most bacteria have a single copy of the *groEL* chaperonin gene, cyanobacteria usually have 2 genes (*groEL1* and *groEL2*). The *groEL1* gene, which is in operonic arrangement with its co-chaperonin *groES*, is essential for cell survival. On the other hand, the non-essential *groEL2*, which is located outside the operon, is believed to be non-essential under physiological conditions but can provide protection from stresses [13–16]. Chloroplast Cpn60 has also been shown to confer divergent functions other than refolding its natural substrate, the RuBisCo enzyme [2, 17, 18]. Genetic studies revealed that *Arabidopsis* mutants defective in the *cpn60A* or *cpn60B* genes manifested defective phenotypes such as aberrant growth [19] and abnormal development of chloroplasts [20] or the embryo [21].

From evidence in the literature, it can be speculated that the diversified versions of the chaperonin 60 proteins may provide additional fitness for photosynthetic organisms to proliferate and to cope with additional oxidative and environmental stresses. We hypothesized that the plastid version of chaperonin 60 could also provide extra benefit when expressed in bac-

teria. In this study, *Chlamydomonas cpn60* genes were exogenously expressed in *E. coli*. Interactions among subunits as well as their abilities to confer enhanced tolerance against imposed environmental stresses were investigated.

MATERIALS AND METHODS

Vector cloning and protein preparation

Total RNA was extracted from the *Chlamydomonas reinhardtii* strain CC503 cw92 mt+, using TRIzol reagent (Thermo Fisher Scientific, USA). ProtoScript® II reverse transcriptase (New England Biolabs, USA) and Oligo-dT primers were used to reverse transcribe the mRNA to cDNA. The *CrCpn60α*, *CrCpn60β1*, and *CrCpn60β2* genes were amplified using PCR from the cDNA (with primers shown in Fig. 1) and cloned into pETduet-1 vectors (Merck Millipore, USA) using the restriction sites presented in Fig. 1 under standard ligation conditions (4 °C overnight). The constructed vectors were subjected to DNA sequencing verification before being transformed into BL21(DE3) *E. coli*. The transformed cells were cultured at 37 °C until reaching an OD_{600} value of about 0.6 before adding IPTG to the final concentration of 1 mM. The cultures were further incubated at 25 °C for another 3 h before harvesting using centrifugation at 5000 × *g* for 5 min. The cell pellets were stored at −80 °C until use. To obtain crude proteins, cell pellets were thawed at room temperature, followed by the addition of lysis buffer (30 mM Tris-HCl pH 7.5, 30 mM NaCl, and 1 mM EDTA) and 1 mM PMSF. The mixture was suspended and sonicated for 30 s (5 s on and 9 s off for 6 repeats). Centrifugation at 12 000 × *g* and 4 °C for 15 min yielded the supernatant containing crude protein, which was collected and kept at −80 °C.

Co-immunoprecipitation

Total "crude" proteins of about 500 μg were mixed with pull-down antibody at 50:1 v/v ratio and incubated for 16 h. Protein A agarose bead (40 μl) was added to the mixtures and incubated for a further 3 h. After that, the mixtures were centrifuged at 12 000 × *g* for 5 min. The supernatants were collected as "unbound" fractions. The bead-containing precipitates were washed 5 times with 500 μl wash buffer (30 mM Tris-HCl pH 7.5 and 50 mM NaCl), followed by centrifugation at 12 000 × *g* for 1 min, the supernatants of which were discarded. One additional washing step was carried out, and the supernatants were collected as the "last wash" fraction. The washed bead pellets were added with 40 μl 5 × SDS loading dye, followed by vigorous vortexing and incubation in a boiling water bath for 10 min. The "eluted" fractions were collected from supernatants after centrifugation at 12 000 × *g* for 5 min. All steps were carried out at 4 °C.

Gel filtration

Superdex-200 beads, ∼30 ml, were soaked with 20 mM MOPS-NaOH pH 7.2 and 100 mM NaCl and packed into a 1.0 cm diameter column. The column flow rate was set at 0.5 ml/min. A standard curve was created using thyroglobulin 669 kDa, catalase 232 kDa, aldolase 158 kDa, and albumin 66 kDa. To separate the proteins, about 22.5 mg protein (approximately 100 μl of the standards and 500 μl of the crude isolated proteins) was loaded onto the column. Then, the fractions were collected every 1 min and detected by measuring absorbance at 280 nm. The collected fractions were subjected to further analyses.

Native PAGE, SDS-PAGE, and Western blot

Native gels (6% acrylamide separating gel and 4% acrylamide stacking gel) were prepared from a typical 29:1 acrylamide:crosslinker stock solution and run in buffer containing 25 mM Tris-HCl pH 7.6 and 192 mM glycine at 120 V for 90 min at 4 °C. SDS gels (7.5% acrylamide separating gel and 4% acrylamide stacking gel) were run in buffer containing 25 mM Tris-HCl pH 7.6, 192 mM glycine, and 0.1% SDS at 120 V for 90 min at room temperature. The proteins in the gel were transferred to a PVDF membrane using Trans-Blot® Turbo™ (Bio-rad, USA) at 25 V and 1.0 A for 20 min. Next, the membrane was incubated in a blocking buffer (5% skimmed milk in TBST) at room temperature for 1 h. After that, the membrane was incubated with primary antibody dissolved in a new blocking buffer at 1:3000 fold. Then, the membranes were washed twice with TBST (20 mM Tris-HCl pH 7.6, 0.8% NaCl, and 1% Tween) for 10 min and then for 10 min with TBS (20 mM Tris-HCl pH 7.6 and 0.8% NaCl) before and after adding a secondary antibody. The primary antibodies used in this study were HA tag rabbit mAb (C29F4, Cell Signaling, USA), His tag mouse mAb (ab1818, Abcam, UK), S tag rabbit mAb (D2K2V, Cell Signaling, USA), and GroEL mouse mAb (ab82592, Abcam, UK). Goat anti-rabbit conjugated to HRP (AP132P, Merck Millipore, USA) and goat anti-mouse conjugated to HRP (62-6520, Thermo Fisher Scientific, USA) were used as secondary antibodies. Detection substrates, enhanced chemiluminescent (ECL) HRP substrate, were purchased from Thermo Fisher Scientific, USA.

Stress response: time-course assays

The transformants were inoculated in 5 ml of LB containing 100 μg/ml ampicillin and incubated with shaking at 37 °C overnight. The overnight cultures were pipetted into 10 ml of new LB medium (ratio 1:70) and allowed to grow at 37 °C in a shaking incubator for 3.5 h. After that, the cultures were added with IPTG at a final concentration of 1 mM before incubation at 25 °C until reaching an OD_{600} value of approximately 1.25 (after about 1 h). Then, the transformant cells

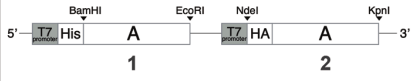
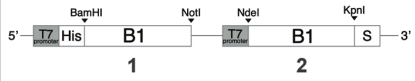
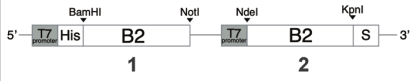
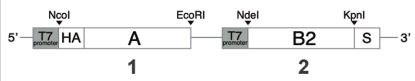
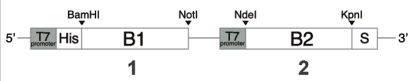
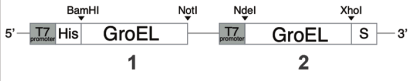
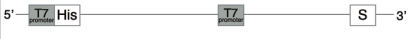
Vector		Primer
2A		**Forward1:** TTGGATCCTGCTGCTGACGCTAAGG **Reverse1:** TTGAATTCTTAGATGGTCATGCCGGAGGG **Forward2:** TTCATATGTACCCATACGATGTTCCAGATTACGCTGCTGCTGACGCTAAG **Reverse2:** TTGGTACCTTAGATGGTCATGCCGGAGGGC
2B1		**Forward1:** TTGGATCCAGCTGCCAAGGGCGGCGTGAG **Reverse1:** TTGCGGCCGCTTACAGGCCGCCGCCGAAGC **Forward2:** TACATATGGCTGCCAAGGGCGGCGTGAGCCG **Reverse2:** AAGGTACCCAGGCCGCCGCCGAAGC
2B2		**Forward1:** TAGGATCCAGTTCAGGCCGCCAAGG **Reverse1:** TTGCGGCCGCTTAGTAGTCGTAGTCACCGC **Forward2:** TTCATATGGTTCAGGCCGCCAAGG **Reverse2:** TTGGTACCGTAGTCGTAGTCACCGCC
AB1		**Forward1:** TTGGATCCAGCTGCCAAGGGCGGCGTGAG **Reverse1:** TTGCGGCCGCTTACAGGCCGCCGCCGAAGC **Forward2:** TTCATATGTACCCATACGATGTTCCAGATTACGCTGCTGCTGACGCTAAG **Reverse2:** TTGGTACCTTAGATGGTCATGCCGGAGGGC
AB2		**Forward1:** TTGGATCCTGCTGCTGACGCTAAGG **Reverse1:** TTGAATTCTTAGATGGTCATGCCGGAGGG **Forward2:** TTCATATGGTTCAGGCCGCCAAGG **Reverse2:** TTGGTACCGTAGTCGTAGTCACCGCC
B1B2		**Forward1:** TTGGATCCAGCTGCCAAGGGCGGCGTGAG **Reverse1:** TTGCGGCCGCTTACAGGCCGCCGCCGAAGC **Forward2:** TTCATATGGTTCAGGCCGCCAAGG **Reverse2:** TTGGTACCGTAGTCGTAGTCACCGCC
AB1B2		**Forward1:** TTGGATCCAGCTGCCAAGGGCGGCGTGAG **Reverse1:** TTGCGGCCGCTTACAGGCCGCCGCCGAAGC **Forward2:** TTCATATGGTTCAGGCCGCCAAGG **Reverse2:** TTGGTACCGTAGTCGTAGTCACCGCC **Forward3:** TTAATTAACCCTCTAGAAATAATTTTGTT **Reverse3:** CCTAGGTTAGATGGTCATGCC
GroEL		**Forward1:** GAATTCGGCAGCTAAAGACGTAAAA **Reverse1:** GCGGCCGCTTACATCATGCCGCCCAT **Forward2:** TTCATATGGCAGCTAAAGACGT **Reverse2:** TTCTCGAGCATCATGCCGCCCA
Empty		

Fig. 1 Diagram showing vector map of CrCpn60 subunit expression vectors and primer sequences used for construction. Genes encoding for the CrCpn60 proteins are illustrated as A for α, B1 for β1, and B2 for β2 subunits, respectively. Each construct contains the ampicillin-resistant gene as a selectable marker, and the transgene is individually driven by T7 promoter except for AB1B2 where Cpn60α and β1 genes share the same promoter. GroEL serves as a control where 2 additional copies of the *groEL* genes are expressed. Empty represents negative control. Positions of T7 promoters, epitope tags, and restriction sites used for cloning are also illustrated.

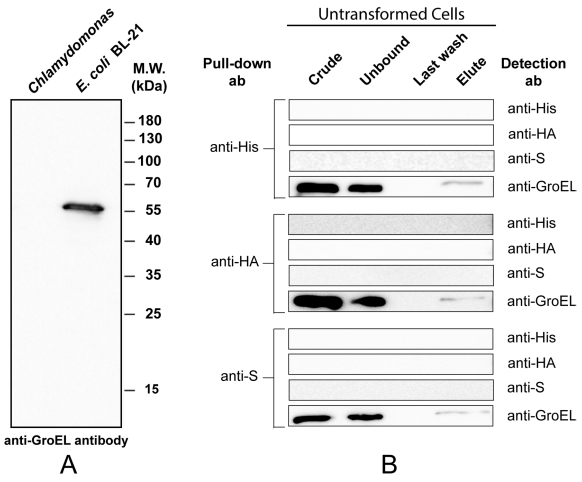


Fig. 2 Western blot analyses showing specificity of antibodies used in this study. (A) Anti-GroEL antibodies were tested against proteins from *C. reinhardtii* and untransformed *E. coli* cells. (B) Each epitope tag-specific antibody as well as GroEL antibodies were used to probe co-IP experimentation of untransformed *E. coli* cells.

were challenged with specified stresses, namely, exposure to heat at 50 °C, 0.5 μM methyl viologen (MV), 1.5 mM hydrogen peroxide (H_2O_2), 5 mM *tert*-butyl hydroperoxide (TBHP), and 0.5 mM Rose Bengal (RB). Cells were collected after 1, 2, and 3 h of treatment for cell density analysis at OD_{600}.

Stress response: spot-test assays

For the spot-test assays, the transformant cultures were serially diluted (10 × dilution for each step) to reach final concentrations of 10^9, 10^8, 10^7, and 10^6 cells/ml according to the Agilent *E. coli* cell culture Biocalculator (OD_{600} of 1.0 is 8×10^8 cells/ml). About 3 μl of the serially diluted cultures above were spotted on LB plates containing 100 μg/ml ampicillin plus one of the following: 150 mM NaCl, 300 mM sorbitol, 0.05 mM Cd^{2+} (cadmium), 0.05 mM Pb^{2+} (lead), 5 μM Hg^{2+} (mercury), 1 mM Cu^{2+} (copper), or 5 mM Rose Bengal. The spotted plates were incubated at 37 °C for 16 h.

RESULTS

CrCpn60 subunits expressed in *E. coli* could interact with one another

To verify that the *Chlamydomonas* CrCpn60 subunits could interact with one another inside the *E. coli* cells, we tagged each of the proteins with different epitopes: histidine tag (His), HA tag (HA), or S tag (S) as shown in Fig. 1. The first 2 copies of the same CrCpn60 subunits, each tagged with different epitopes, were generated to test the possibility of self-interaction and named 2A, 2B1, and 2B2, respectively, as shown in Fig. 1. Alternatively, we also created constructs with combinations of the 3 subunits as: AB1, AB2, B1B2, and AB1B2. In the combination constructs, the α subunit was tagged with the HA tag, while β1 and β2 were tagged with the His and S tags, respectively. Notably, the N and C termini of GroEL and the chaperonins are located in the same area in the 3-dimensional structure, and their tagging has previously been reported not to affect the overall structure of the protein [10, 22, 23].

Interaction between each of the subunits was assessed using co-immunoprecipitaion. *E. coli* cells expressing each of the vector constructs were harvested and subjected to protein isolation. Antibodies against one of the tags were used to pull-down the tagged proteins before separation using SDS-PAGE and detection using Western blot analysis. We also performed the reverse pull-down step using antibodies against another tag present in each construct. To ensure that the Western blot signals observed in this study had not originated from a nonspecific cross-reaction, all antibodies were subjected to verification. As it could be argued that antibodies specific to GroEL could also detect chaperonin proteins due to their relatively high sequence similarity, we tested the antibodies with total proteins isolated from *Chlamydomonas* as well as *E. coli*. The result in Fig. 2A clearly showed that the GroEL antibodies could only detect a protein band of about 60 kDa from *E. coli* with no discernible cross-reaction being identified with proteins from *Chlamydomonas*. In addition, antibodies against the tag epitopes were tested with proteins isolated from untransformed *E. coli* cells (Fig. 2B). Total proteins from the untransformed cells were subjected to the immuno pull-down assay as described in the methods section above with anti-His, anti-HA, and anti-S antibodies. Collected individual fractions were subjected to SDS-PAGE, followed by Western blots using the tag-antibodies. It was clear (Fig. 2B) that none of the tag antibodies could recognize any protein around 60 kDa from untransformed *E. coli* cells in any of the collected fractions (Crude, Unbound, Last Wash, and Elute). Notably, when anti-GroEL was used to probe the same immuno pull-down blots, a faint protein band of about 60 kDa could be observed in the Elute fraction, suggesting that the GroEL protein could bind nonspecifically with either the agarose beads or with other proteins bound to the beads (Fig. 2B).

For the *E. coli* transformant 2A, we first pulled down the CrCpn60 α subunit with anti-His antibodies. The pulled-down proteins were separated and detected by Western blot analyses using anti-His, anti-HA, and anti-GroEL (Fig. 3A). In the crude total protein and unbound fractions, the presence of both copies of the CrCpn60 α subunit and GroEL could be expected, while no protein was observable in the Last Wash fraction. After elution, both copies of the α subunits

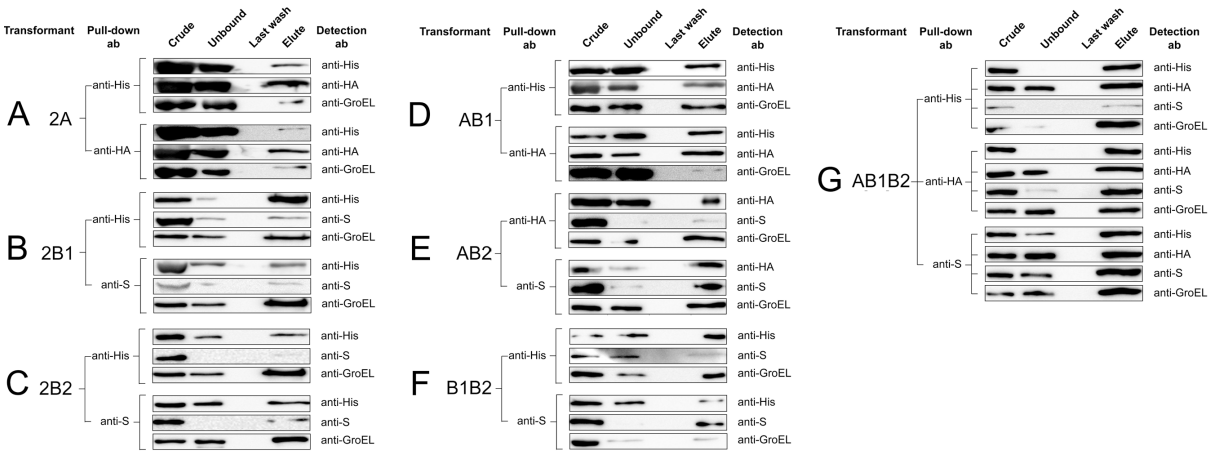


Fig. 3 Co-immunoprecipitation assays of *E. coli* transformants expressing CrCpn60 subunits. Total crude proteins "crude" from each transformant were isolated and incubated with pulled-down antibodies specific to the tag epitope attached to CrCpn60 subunits (Pull-down ab). Unbound fraction "unbound" was obtained after adding Protein A agarose beads to the mixture followed by centrifugation. Agarose beads containing bound proteins were washed 5 times before collecting the "last wash" fraction. Bound proteins were "eluted" from the beads by incubating with 5-SDS loading dye. All collected samples were subjected to SDS-PAGE followed by Western blot analyses using specified antibodies. Individual panels represent proteins isolated from transformant 2A (A), 2B1 (B), 2B2 (C), AB1 (D), AB2 (E), B1B2 (F), and AB1B2 (G).

were clearly discernible by the cross-reactions of the antibodies (Fig. 3A), suggesting that the 2 copies could interact with one another. To verify this, we performed the reverse pull-down using anti-HA antibodies, and the same result was observed. As GroEL could also be detected in the negative control experiments in Fig. 3B, we have refrained from further discussion on its detection in Fig. 3. The same experiments were repeated for the *E. coli* transformants 2B1 (Fig. 3B), 2B2 (Fig. 3C), AB1 (Fig. 3D), AB2 (Fig. 3E), B1B2 (Fig. 3F), and AB1B2 (Fig. 3G). In all tested combinations, the CrCpn60 subunits were able to co-precipitate among themselves (α-α, β1-β1, and β2-β2) or between subunit combinations (αβ1, αβ2, β1β2, and αβ1β2). However, notably, this immunoprecipitation experiment could not distinguish whether such observable interactions originated from protein dimerization, trimerization, oligomerization, or from the full-functional complex. To address this, further analyses were performed showing the high-molecular-weight complex assembly.

CrCpn60 subunits are assembled into a large protein complex in *E. coli*

The native complex of GroEL is a tetradecameric structure consisting of 14 homo-subunits, and that of the CrCpn60 is very similar. Gel filtration is one of the classical methods to determine the size and to purify a native complex of proteins. Therefore, this technique was applied to assess whether CrCpn60 subunits could assemble into a large protein complex inside the *E. coli* cell. As the calculated tetradecameric structure of the CrCpn60 is about 840 kDa, it was expected that its native complex would be eluted at around fraction #12 compared to the elution standard curve (Fig. 4A). From the elution chromatogram (Fig. 4B), high MW proteins could be detected by measuring absorbance at 280 nm from fraction #10 onward. Thus, we collected eluted proteins from fractions #12–16 for further investigation. The native PAGE profile (Fig. 4C) showed a distinct high-molecular-weight band above the 669 kDa native marker (noted as * in Fig. 4C).

To ensure that such a high-molecular-weight band really contained the CrCpn60 subunits, immunoblotting was carried out using antiserum against the epitope tag present in each of the proteins. From the immunoblots of the native PAGE, we could not detect any cross-reaction with any of the antibodies. However, it has been shown that immunoblots of proteins resolved by native PAGE could be complicated due to hindrance of the epitopes by protein conformation [24]. In this case, both N and C terminal tags are located inside the tetradecameric barrel structure and thus could not be readily accessed by the antibodies on the native immunoblot. To verify this, we excised the high-molecular-weight band(s) from the native PAGE of representative transformants (2α, 2β1, and 2β2) and placed them on top of a denaturing SDS-PAGE followed by Western blot analysis. This time, we could detect the presence of the CrCpn60 α subunit (Fig. 4D), β1 subunit (Fig. 4E), and β2 subunit (Fig. 4F). Notably, such cross-reaction could not be detected in the lane where the high-MW native band was excised from the transformants carrying the empty vector (empty) that served as the negative control. The total cell lysates from the transformants were also used as the positive

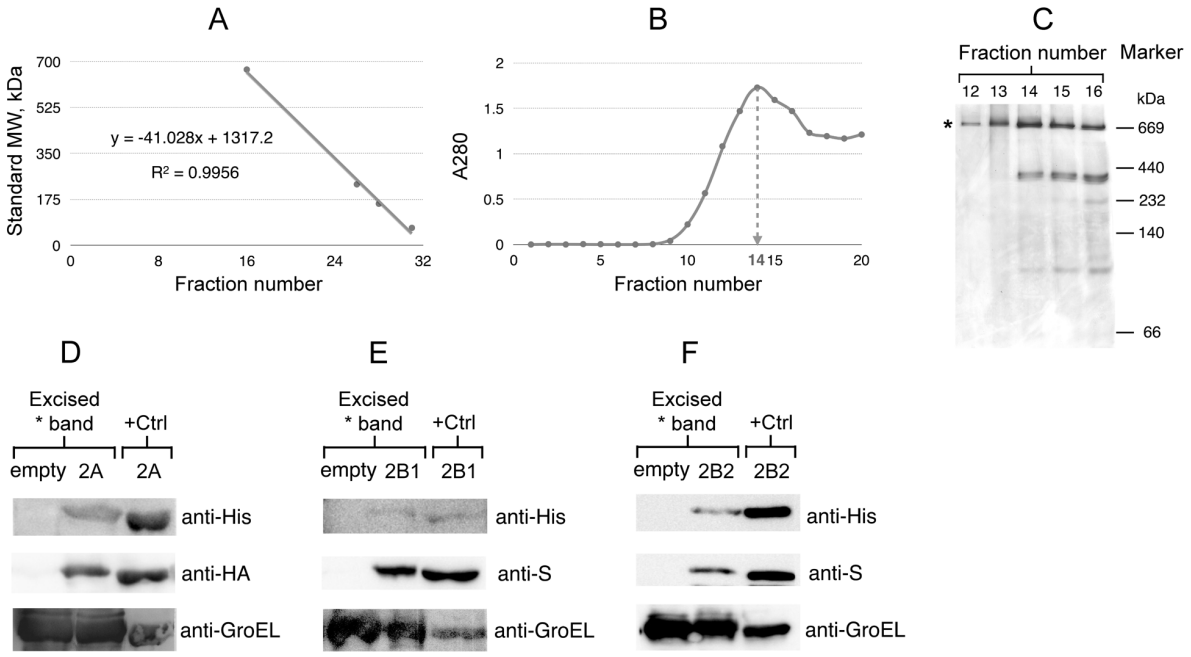


Fig. 4 Investigation of high-molecular-weight native complex in *E. coli* transformants expressing CrCpn60 subunits. (A) Gel filtration standard curve. (B) Gel filtration chromatogram of total proteins isolated from 2B2 transformant. (C) Native PAGE profile of fraction numbers 12–16. * denotes the presence of a high-molecular-weight protein complex (D-F) 2nd-dimension SDS-PAGE of excised high-molecular-weight band marked as * from native PAGE followed by Western blot analyses using specific antibodies against corresponding tag epitopes of 2A transformant (D), 2B1 transformant (E), and 2B2 transformant (F).

control (+Ctrl in Fig. 4D-F). Detection of GroEL in this experiment was not surprising as the native structures of GroEL and CrCpn60s are similar in size. Such a high MW protein band appearing in native PAGE could contain both the native GroEL as well as the protein complex containing the CrCpn60 subunits. These results strengthened our hypothesis that CrCpn60 subunits could putatively cross-assemble into a high-molecular-weight protein complex in *E. coli*.

E. coli expressing CrCpn60 subunits can tolerate environmental stresses

We further investigated our hypothesis regarding whether the exogenous expression of the CrCpn60 subunits could provide any additional tolerance to the *E. coli* cells in response to environmental stresses. We subjected the *E. coli* transformants carrying combinations of the CrCpn60 subunits as well as the empty vector to common stresses that photosynthetic organisms often encounter such as heat and oxidative stresses from H_2O_2, superoxide, hydroxyl radicals, and singlet oxygen. To rule out the beneficial effects of having extra copies of the molecular chaperone proteins, we also created a transformant containing 2 extra copies of the *groEL* gene (GroEL) for comparison. For this experiment, we challenged each of the transformants with stress inducers at specified concentrations. Cells were collected at time 0 and after 1, 2, and 3 h of treatment, and their relative cell densities were assessed via OD_{600}. It was expected that the cell densities of all cultures would decrease as the treatment times progressed. First, cells were challenged with a high temperature of 50 °C. We could clearly see that the cell density of *E. coli* containing only the empty vector (no exogenous expression of any molecular chaperone) dropped significantly faster than the others (Fig. 5A). In this experiment, no significant differences in terms of cell density kinetics were observed among the transformants exogenously expressing the chaperonin proteins. This finding suggested that extra copies of the heat shock proteins, regardless of GroEL or CrCpn60s, could help protect cells from heat stress. Treating the transformants with H_2O_2 (Fig. 5B), TBHP (a hydroxy radical generator, Fig. 5C), and MV (or paraquat, a superoxide generator, Fig. 5D) resulted in similar observations to those from the heat stress. Although not statistically significant, we noticed that transformants harboring all 3 types of the CrCpn60 subunits (AB1B2) performed best in terms of H_2O_2 and superoxide tolerance.

Next, we attempted to challenge the transformants with a unique ROS often faced by photosynthetic organisms, namely, singlet oxygen. Treating the cells with Rose Bengal (RB) under low light intensity could generate such ROS, leading to protein and cell damage. However, the experiment was not successful when per-

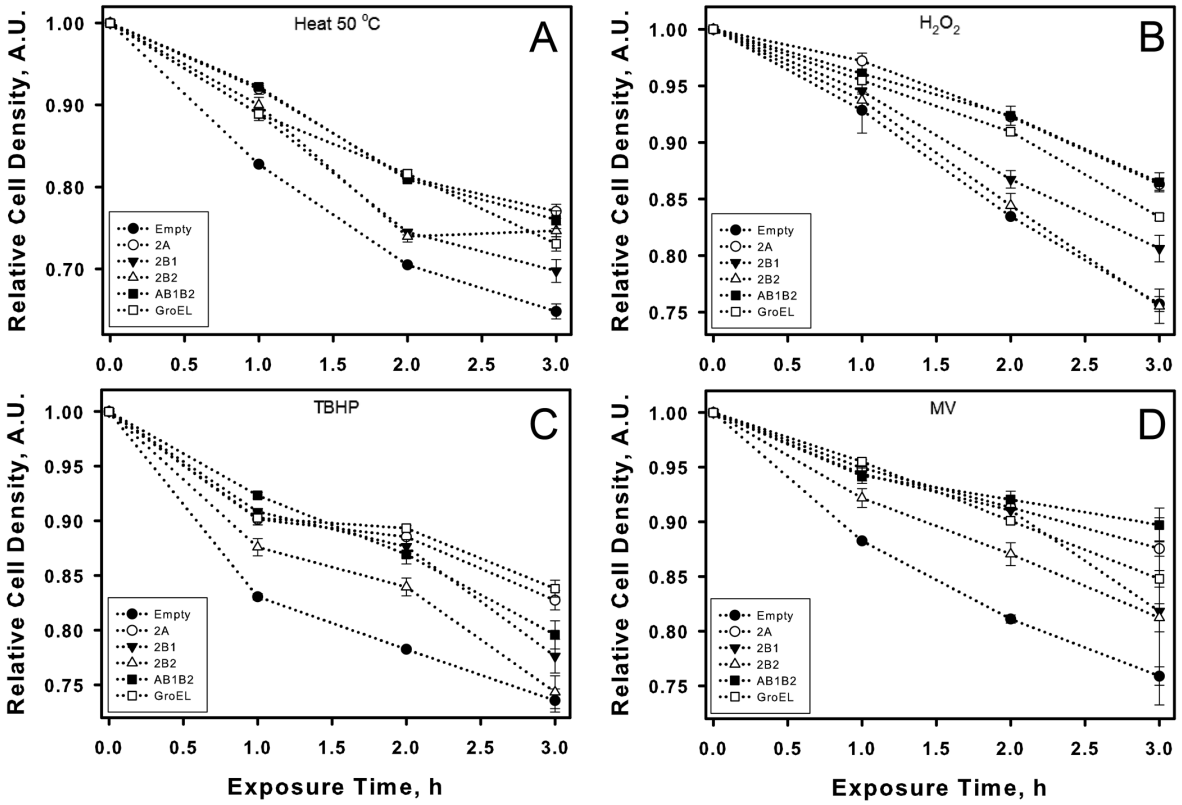


Fig. 5 Short-term stress treatment of *E. coli* transformant expressing combinations of CrCpn60 subunits as well as extra copies of the *groEL* genes. Cells were challenged with stress conditions for 0, 1, 2, and 3 h. (A) Heat treatment at 50 °C, (B) 1.5 mM H_2O_2, (C) 5 mM TBHP, and (D) 0.5 μM MV. Cell densities at each time point were assayed at OD_{600} and normalized to initial cell densities. Data are averages of 3 independent experiments ± SD.

formed in solution as in the preceding studies probably due to the interference of the RB spectral absorption shift in the membrane environment [25]. Then, we used an alternative approach by challenging the cells in solution before spotting them onto LB plates and allowing them to grow for 16 h. As RB is very toxic and could quickly kill the cells, we only challenged them for up to 90 min. Notably, the results in Fig. 6A showed that most transformants were effectively killed after 30 min of treatment as no growth could be observed, except for the AB1B2 *E. coli* transformant for which several colonies of bacteria were discernible even after 60 min. Extending the treatment time to 90 min surprisingly allowed additional CrCpn60 transformants such as AB1, AB2, and B1B2 to recover and grow as well. We further verified this study by spotting serial dilution of the transformant cells (10^8, 10^7, and 10^6 cells/ml) onto LB plates containing 5 mM of RB. Cells were allowed to grow at 37 °C for 16 h under dim light. At 10^8 cells/ml density, there was growth observed in most transformants except 2A with the AB1B2 transformants being the best performer (Fig. 6B, 10^8 lane). Only growth of AB1 and AB1B2 could be detected when the cell density was diluted to lower than 10^7 and 10^6 cells/ml (Fig. 6B). These results strongly suggested that exogenous expression of CrCpn60 subunits could confer enhanced tolerance of *E. coli* cells to singlet oxygen.

We also performed assays for other common environmental stresses by spotting the transformant cells onto LB plates containing stress inducers such as 290 mM NaCl (salt stress), 300 mM sorbitol (osmotic stress), 0.05 mM cadmium, 0.05 mM lead, 5 μM mercury, and 0.1 mM copper (heavy metal stress). At 10^8 cells/ml density, 190 mM NaCl did not affect the viability of any transformants, including the empty and +GroEL controls (Fig. 6C, 10^8 lane). Nevertheless, at 10^7 cells/ml, deteriorated growth was observed for all transformants, albeit AB1B2 performed slightly better than the others (Fig. 6C, 10^7 lane). At 10^6 cells/ml, none of the transformants could grow on LB plates containing 190 mM NaCl (Fig. 6C, 10^6 lane). For sorbitol (Fig. 6D), a clear effect was observed when cells were diluted to 10^6 cells/ml with the AB1B2 and GroEL transformants having higher growth than the others. Treating the cells with cadmium, lead, or

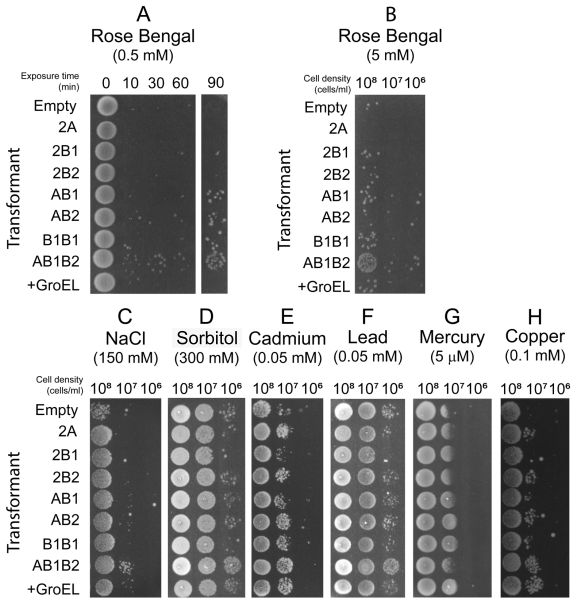


Fig. 6 Spot test assay after challenging *E. coli* transformants expressing combinations of CrCpn60 subunits. Cells were grown and challenged with stress conditions as described in the material and methods. (A) In-solution time-course of RB challenge before being spotted on regular LB-ampicillin plates, (B) transformants serially diluted before being spotted onto LB-ampicillin + 5 mM RB, (C) + 0.05 mM cadmium, (D) + 300 mM sorbitol, (E) + 0.05 mM lead, (F) + 5 μM mercury, and (G) + 0.1 mM copper. Images are representative of 3 independent replicates.

mercury did not result in any significant difference in terms of growth for all transformants. Copper challenge showed that transformants containing additional copies of the chaperonin protein could tolerate the metal better than the empty vector control with the GroEL transformant being the best.

DISCUSSION

The evidence provided here showed that chloroplast chaperonin 60 subunits from the model green alga *Chlamydomonas reinhardtii* could interact with each other and assemble into a high-molecular-weight complex when expressed in *E. coli*. In the co-IP experiment (Fig. 3), regardless of the antibodies used for the pull-down step, one or more other subunits were always co-precipitated, supporting the hypothesis that they interact with each other. As the immuno pull-down assay requires that the epitope for the antibodies needs to be accessible, one could assume that the interactions among CrCpn60 subunits observed in this experiment may not be the fully assembled complex, of which the N- and C-termini are hidden inside the barrel structure. As the IPTG-induced expression of the CrCpn60 proteins in our transformants yielded a vast abundance of the transgene products (results not shown), it is possible that the observed interaction from the co-IP could be the unassembled or partially assembled subunits. However, one could argue that the chaperonin subunits could unspecifically bind to the agarose beads like that of the GroEL shown in Fig. 2. Yet, in most experiments, relative band intensity between the eluted and the crude/unbound fractions of the co-IP CrCpn60 proteins were significantly higher than that of the unspecific GroEL binding to the beads shown in Fig. 2. Furthermore, the results from the native PAGE band excision followed by SDS-PAGE and Western blot also supported the hypothesis that the exogenously expressed proteins could assemble into a high-molecular-weight protein complex of the same electrophoretic mobility as that of GroEL (Fig. 4). Assembly of the chloroplast Cpn60 in *E. coli* into a functional tetradecameric native complex is not without precedence. Cloney et al [26] demonstrated that Cpn60 α and β subunits from *Brassica napus* could also assemble in *E. coli* and function to refold RuBisCO enzyme.

Notably in our study, we observed the high-molecular-weight native complex in the 2A transformant expressing 2 copies of the CrCpn60 α subunit. As it has been shown in the literature that the Cpn60 α subunit cannot assemble into a stable homo-tetradecameric structure without the β subunit [18, 26], there are 2 possible explanations for this observation. First, it is possible that the observed high-molecular-weight protein complex in the 2A transformant may not be stable enough to function. Indeed, the stress-test experiments in Fig. 6 also revealed that on many occasions, the 2A transformants exhibited similar response to that of the transformant carrying the empty vector. The second plausible explanation is that the Cpn60 α subunit in the 2A transformant could cross-assemble with GroEL. Hybrid assembly between the GroEL and Cpn60 subunits has previously been demonstrated [27]. However, whether the GroEL and the Cpn60 α subunit could actually assemble into the functional tetradecameric structure still needs further investigation.

Functionally, the primary role of the GroEL/GroES complex in bacteria is to assist in folding and refolding other proteins. In plants and algae, diversification of the chloroplast Cpn60 subunits could be crucial for evolution. Recent reports have shown the involvement of the chloroplast chaperonin 60 in various activities beyond protein folding and refolding. Examples include RNA maturation [28] and protein targeting into the thylakoid membrane [29]. Our results from in the current study strongly suggest that *E. coli* expression of all types of CrCpn60 subunits (AB1B2 transformant) could tolerate and recover from singlet oxygen stress imposed by RB better than the others (Fig. 6A and 6B).

This could suggest another aspect of the Cpn60 function in plant chloroplasts. As a site of photosynthesis, the chloroplast is frequently exposed to singlet oxygen generated in photosystem II, which is an unnatural situation for *E. coli*. Glatz et al [30] reported that expression of *groEL* in cyanobacteria was modulated by darkness-to-light transition. Such a condition also promotes singlet oxygen formation, especially immediately after the transition from darkness to light. It could be possible that the protein damage inflicted by the singlet oxygen attack might have unique properties recognizable by the Cpn60s. Thus, *E. coli* expressing the CrCpn60 subunits could readily deal with such a condition and help refold the protein faster, leading to better tolerance and recovery from singlet oxygen stress.

In conclusion, this paper has provided additional evidence for the interaction between chaperonin 60 subunits from *Chlamydomonas* chloroplasts when expressed in *E. coli* cells. In addition, we also showed that *E. coli* expressing combinations of CrCpn60 subunits could tolerate and recover from singlet oxygen better than the control cells.

Acknowledgements: This work was financially supported in part by Mahidol University and the Thailand Research Fund [grant #RSA5680032] to KY. CY thanks Kasetsart University Research and Development Institute for financial support [grant #v-t(d)49.59]. RW received scholarship support from the Thailand Research Fund through the Royal Golden Jubilee PhD program [grant #PHD/0074/2556].

REFERENCES

1. Gething M-J, Sambrook J (1992) Protein folding in the cell. *Nature* **355**, 33–45.
2. Boston RS, Viitanen PV, Vierling E (1996) Molecular chaperones and protein folding in plants. *Plant Mol Biol* **32**, 191–222.
3. Schroda M (2004) The *Chlamydomonas* genome reveals its secrets: chaperone genes and the potential roles of their gene products in the chloroplast. *Photosynth Res* **82**, 221–240.
4. Horwich AL, Fenton WA, Chapman E, Farr GW (2007) Two families of chaperonin: physiology and mechanism. *Annu Rev Cell Dev Biol* **23**, 15–145.
5. Lund PA (2009) Multiple chaperonins in bacteria – why so many? *FEMS Microbiol Rev* **33**, 785–800.
6. Hill JE, Hemmingsen SM (2001) *Arabidopsis thaliana* type I and II chaperonins. *Cell Stress Chaperones* **6**, 190–200.
7. Hemmingsen S, Woolford C, van der Vies S, Tilly K, Dennis DT, Georgopoulos CP, Hendrix RW, Ellis RJ (1988) Homologous plant and bacterial proteins chaperone oligomeric protein assembly. *Nature* **333**, 330–334.
8. Zhao Q, Zhang X, Sommer F, Ta N, Wang N, Schroda M, Cong Y, Liu C (2019) Hetero-oligomeric CPN60 resembles highly symmetric group-I chaperonin structure revealed by Cryo-EM. *Plant J* **98**, 798–812.
9. Kovács E, van der Vies SM, Glatz A, Török Z, Varvasovszki V, Horváth I, Vígh L (2001) The chaperonins of Synechocystis PCC 6803 differ in heat inducibility and chaperone activity. *Biochem Biophys Res Commun* **289**, 908–915.
10. Zhang S, Zhou H, Yu F, Bai C, Zhao Q, He J, Liu C (2016) Structural insight into the cooperation of chloroplast chaperonin subunits. *BMC Biol* **14**, ID 29.
11. Furuki M, Tanaka N, Hiyama T, Nakamoto H (1996) Cloning, characterization and functional analysis of *groEL*-like gene from thermophilic cyanobacterium *Synechococcus vulcanus*, which does not form an operon with *groES*. *Biochim Biophys Acta* **1294**, 106–110.
12. Tanaka N, Hiyama T, Nakamoto H (1997) Cloning, characterization and functional analysis of *groESL* operon from thermophilic cyanobacterium *Synechococcus vulcanus*. *Biochim Biophys Acta* **1343**, 335–348.
13. Nakamoto H, Kojima K (2017) Non-housekeeping, non-essential GroEL (chaperonin) has acquired novel structure and function beneficial under stress in cyanobacteria. *Physiol Plant* **161**, 296–310.
14. Rajaram H, Chaurasia AK, Potnis AK (1997) Multiple chaperonins in cyanobacteria: why one is not enough! In: Kumar CMS, Mande SC (eds) *Prokaryotic Chaperonins. Heat Shock Proteins 11*, Springer Nature Singapore, pp 93–109.
15. Sato M, Nimura-Matsune K, Watanabe S, Chibazakura T, Yoshikawa H (2007) Expression analysis of multiple *dnaK* genes in the cyanobacterium *Synechococcus elongatus* PCC 7942. *J Bacteriol* **189**, 3751–3758.
16. Sato S, Ikeuchi M, Nakamoto H (2008) Expression and function of a *groEL* paralog in the thermophilic cyanobacterium *Thermosynechococcus elongatus* under heat and cold stress. *FEBS Lett* **582**, 3389–3395.
17. Feiz L, Williams-Carrier R, Wostrikoff K, Belcher S, Barkan A, Stern DB (2012) Ribulose-1,5-bis-phosphate carboxylase/oxygenase accumulation factor1 is required for holoenzyme assembly in maize. *Plant Cell* **24**, 3435–3446.
18. Zhao Q, Liu C (2018) Chloroplast chaperonin: an intricate protein folding machine for photosynthesis, *Front Mol Biosci* **4**, ID 98.
19. Tiwari LD, Grover A (2019) Cpn60β4 protein regulates growth and developmental cycling and has bearing on flowering time in *Arabidopsis thaliana* plants. *Plant Sci* **286**, 78–88.
20. Suzuki K, Nakanishi H, Bower J, Yoder DW, Osteryoung KW, Miyagishima SY (2009) Plastid chaperonin proteins Cpn60 alpha and Cpn60 beta are required for plastid division in *Arabidopsis thaliana*. *BMC Plant Biol* **9**, ID 38.
21. Ke X, Zou W, Ren Y, Wang Z, Li J, Wu X, Zhao J (2017) Functional divergence of chloroplast Cpn60α subunits during *Arabidopsis* embryo development, *PLoS Genet* **13**, e1007036.
22. Teshima T, Mashimo S, Kondo A, Fukuda H (1998) Affinity purification and immobilization of fusion chaperonin GroEL-(His)6 and its utilization to mediate protein refolding. *J Ferment Bioeng* **86**, 357–362.
23. Roh SH, Hryc CF, Jeong HH, Fei X, Jakana J, Lorimer GH, Chiu W (2017) Subunit conformational variation within individual GroEL oligomers resolved by Cryo-EM. *Proc Natl Acad Sci USA* **114**, 8259–8264.
24. Yokthongwattana K, Chrost B, Behrman S, Casper-Lindley C, Melis A (2001) Photosystem II damage and repair cycle in the green alga *Dunaliella salina*: involve-

ment of a chloroplast-localized HSP70. *Plant Cell Physiol* **42**, 1389–1397.

25. Chang C-C, Yang Y-T, Yang J-T, Wu H-D, Tsai T (2008) Absorption and emission spectral shifts of rose bengal associated with DMPC liposomes. *Dyes Pigm* **79**, 170–179.
26. Cloney LP, Bekkaoui DR, Wood MG, Hemmingsen SM (1992) Assessment of plant chaperonin-60 gene function in *Escherichia coli*. *J Biol Chem* **267**, 23333–23336.
27. Cloney LP, Wu HB, Hemmingsen SM (1992) Expression of plant chaperonin-60 genes in *Escherichia coli*. *J Biol Chem* **267**, 23327–23332.
28. Balczun C, Bunse A, Schwarz C, Piotrowski M, Kück U (2006) Chloroplast heat shock protein Cpn60 from *Chlamydomonas reinhardtii* exhibits a novel function as a group II intron-specific RNA-binding protein. *FEBS Lett* **580**, 4527–4532.
29. Klasek L, Inoue K, Theg SM (2020) Chloroplast chaperonin-mediated targeting of a thylakoid membrane protein. *Plant Cell* **32**, 3884–3901.
30. Glatz A, Hor'ath I, Varvasovszki V, Kovács E, Török Z, Vigh L (1997) Chaperonin genes of the *Synechocystis* PCC 6803 are differentially regulated under light-dark transition during heat stress. *Biochem Biophys Res Commun* **239**, 291–297.