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Translocation of Green Fluorescent Protein to Cyanobacterial Periplasm Using Ice Nucleation Protein

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The translocation of proteins to cyanobacterial cell envelope is made complex by the presence of a highly differentiated membrane system. To investigate the protein translocation in cyanobacterium *Synechococcus* PCC 7942 using the truncated ice nucleation protein (InpNC) from *Pseudomonas syringae* KCTC 1832, the green fluorescent protein (GFP) was fused in frame to the carboxyl-terminus of InpNC. The fluorescence of GFP was found almost entirely as a halo in the outer regions of cells which appeared to correspond to the periplasm as demonstrated by confocal laser scanning microscopy, however, GFP was not displayed on the outermost cell surface. Western blotting analysis revealed that InpNC-GFP fusion protein was partially degraded. The N-terminal domain of InpNC may be susceptible to protease attack; the remaining C-terminal domain conjugated with GFP lost the ability to direct translocation across outer membrane and to act as a surface display motif. The fluorescence intensity of cells with periplasmic GFP was approximately 6-fold lower than that of cells with cytoplasmic GFP. The successful translocation of the active GFP to the periplasm may provide a potential means to study the property of cyanobacterial periplasmic substances in response to environmental changes in a non-invasive manner.

***Keywords*:** cyanobacteria, *Synechococcus*, translocation, periplasm, ice nucleation protein, green fluorescent protein (GFP)

Cyanobacteria are oxygenic photosynthetic microorganisms that are ultrastructurally similar to both bacteria and higher plant chloroplasts. Cyanobacterial cell envelope is a combination of Gram-negative and Gram-positive features i.e. outer membrane and plasma membrane separated by a periplasmic space, and the thick peptidoglycan layer, respectively (Hoiczyk and Hansel, 2000). Cyanobacteria have an internal system of thylakoid membranes where the photosynthetic light capture and electron transport take place (Gantt, 1994). The presence of highly differentiated membrane systems lends cyanobacterial cells a unique complexity among eubacteria. The targeting of proteins into and across the correct cyanobacterial membrane systems remains a great challenge; the knowledge on this subject is limited, because (i) no *in vitro* assays are available, the thylakoid and plasma membranes are found to be highly fragmented after isolation (Spence *et al.*, 2003) and (ii) no cyanobacterial protein translocation mutants have been isolated.

Attempts have been made to target the chloramphenical acetyltransferase to periplasmic space and thylakoid lumen of *Synechococcus* PCC 7942 using various signal peptides (Mackle and Zilinskas, 1994), and the green fluorescent protein to periplasmic space of *Synechocystis* PCC 6803 using the Tat pathway (Spence *et al*., 2003). We previously reported that using truncated ice nucleation protein (Inp) containing only the N- and C-terminal portion (InpNC) from *Pseudomonas syringae* KCTC 1832 as an anchoring motif, a minor fraction of organophosphorus hydrolase (OPH) is displayed onto the outermost cell surface of *Synechococcus* PCC 7942, however, a substantial fraction of OPH is buried in the cell wall (Chungjatupornchai and Fa-aroonsawat, 2008). The InpNC has been successfully used for targeting OPH (Shimazu *et al.*, 2001) and green fluorescent protein (GFP) (Li *et al.*, 2004) to cell surface of *E. coli*, and viral antigens to cell surface of *Salmonella* (Lee *et al*., 2000). The N-terminal domain of Inp contains three or four potential transmembrane spans but no signal sequence. The Inp is not linked to an oligosaccharide and therefore not anchored to the outer membrane via phosphatidylinositol as proposed previously (Schmid *et al*., 1997). The secretion mechanism of Inp is still unknown.

GFP is a popular reporter for gene expression and protein localization studies. GFP does not require the addition of any substrate or co-factor, it promises real time visualization of gene expression. The GFPmut2, a GFP variant with a triple amino acid substitution (S65A/V68L/S72A), has been shown to express in *E. coli* as a soluble protein with improved fluorescence (Cormack *et al.*, 1996).

In this study, we have used the GFPmut2 as a reporter to investigate the protein translocation in cyanobacterium *Synechococcus* PCC 7942. The GFP was fused in frame to the carboxyl-terminus of InpNC. We demonstrated that the InpNC was able to direct translocation across the intracellular membranes and that the transported GFP accumulated almost entirely in the periplasm.

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Materials and Methods

Strains, growth conditions, and plasmids

Synechococcus PCC 7942 strain R2-SPc (hereafter, referred to as *Synechococcus*) (Kuhlemeier *et al.*, 1983) was grown on BG-11 medium containing 1.5% agar at 30°C under constant illumination of 3,000 lux (i.e. 38 μE/m^2/sec). *E. coli* strain MC1061 (Casadaban and Cohen, 1980) was grown in LB broth or on agar. Plasmid pGF101, containing the truncated *inaKnc* gene encoding N- and C-terminal domains (InpNC) of ice nucleation protein from *P. syringae* KCTC 1832 (Jung, 1998), was kindly provided by E. J. Kim (Genefocus, Korea). Plasmid pBCgfp harboring *gfp* gene encoding a GFP variant, GFPmut2 (Cormack *et al*., 1996), has been described (Matthysse *et al.*, 1996).

Plasmid construction

The *gfp* gene encoding the GFP was amplified from pBCgfp using primers gfp-F1 and gfp-R2 (Table 1). The *Bam*HI/*Sac*I-digested PCR product was cloned into the corresponding sites in pUC18-GUS (Chungjatupornchai and Fa-aroonsawat, 2008) to obtain pUC-GFP. For translocation expression of GFP, the *inaKnc* gene amplified from pGF101 using primers ina-F1 and ina-R1 was digested with *Sal*I and cloned into the corresponding site in pUC-GFP to obtain pUC-InpNC-GFP. The PCR product containing cassette P_{tRNA}-*inaKnc-gfp* was amplified using primers 24-mer and gfp-R2 with template derived from overlap extension PCR of two PCR products: i) P_{tRNA} promoter amplified using primers 24-mer and E3-R4 with pUC-T1R1 derived from *Bam*HI/*Eco*RI fragment of pKGT-T1R1 (Chungjatupornchai *et al*., 2002) cloned into the corresponding sites in pUC18-GUS as template; ii) *inaKnc-gfp* amplified from pUC-InpNC-GFP using primers Lk-ina-F2 and gfp-R2. For cytoplasmic expression of GFP, the PCR product containing gene cassette P_{tRNA}-*gfp* was amplified using primers T1 and gfp-R2 with template derived from overlap extension PCR of two PCR products: i) P_{tRNA} promoter amplified from pKGT-T1R1 (Chungjatupornchai *et al*., 2002) using primers T1 and E3-R4; ii) *gfp* gene amplified from pBCgfp using primers Lk-gfp-F2 and gfp-R2. The gene cassettes P_{tRNA}-*gfp* digested with *Bam*HI/*Sac*I and P_{tRNA}-*inaKnc-gfp* digested with *Hin*dIII/*Sac*I were cloned into the corresponding sites of pUC18-GUS. The resulting P_{tRNA}-*gfp*-(Nos-ter) digested with *Bam*HI/*Eco*RI and P_{tRNA}-*inaKnc-gfp*-(Nos-ter) digested with *Hin*dIII filled-in/*Eco*RI were cloned into the similar sites of shuttle vector pKGT (Chungjatupornchai *et al*., 2002) resulted in pKT-GFP and pKT-InpNC-GFP, respectively. The resulting plasmids were transformed into *Synechococcus* as described (Kuhlemeier *et al*., 1983).

GFP fluorescence analysis

Synechococcus cells were harvested and resuspended in phosphate-buffered saline (PBS). The intensity of fluorescence was measured using spectrofluorometer (Model FP-6300, Jasco, Japan) with excitation at 488 nm and emission measured at 510 nm. Specific fluorescence intensity was defined as whole cell fluorescence intensity divided by the optical density measured at 730 nm. For pronase accessibility assays, cells were suspended in 1 ml (OD_{730}=1) of PBS containing 16.24 units of pronase (Sigma, USA), and then incubated at 37°C for 2 h. The treated cells were washed with PBS and the fluorescence intensities were measured as described above. For measurement of cell fractions, *Synechococcus* cells were suspended in 50 mM Tris-HCl, 50 mM NaCl, 5% glycerol, pH 8.0, and subjected to three rounds of French press treatment. The whole cell lysate was centrifuged to obtain supernatant (soluble fraction) and pellet (total membrane fraction). The fluorescence intensity of cell fractions was measured as described above. The images of GFP fluorescence in *Synechococcus* cells were obtained using a confocal laser scanning microscope (CLSM) (Fluoview FV1000, Olympus) with excitation at 488 nm and emission measured at 520 nm.

Western blot analysis

Proteins from whole cell extracts were separated by 15% SDS-PAGE and immobilized onto a polyvinylidene-difluoride membrane. For immunodetection, primary GFP monoclonal antibody (Clonetech, USA) was used at 1:2,000 dilution. The secondary antibody, goat anti-mouse IgG conjugated with horseradish peroxidase (Sigma), was used at 1:5,000 dilution. Reaction of horseradish peroxidase was detected using ECL plus Western Blotting Detection System (GE Healthcare, UK) as described by manufacturer.

Results and Discussion

Construction of plasmids harboring the *gfp* gene

We reported previously that using the InpNC as an anchoring motif, a minor fraction of OPH is displayed onto the outermost surface of cyanobacterial cells, but a substantial frac-

Table 1. Primers used in this study. Locations of the primers are indicated in Fig. 1. The restriction sites *Bam*HI, *Sac*I, *Sal*I, and *Sma*I are underlined

Primer	Sequence (5'-3')	Target sequence
T1	CGGGATCCTTGCCCTCGCCTCCTAGTCC	P_{tRNA}
E3-R4	CATAAGGGACTGACCACCCGGGGATCGTGACAAGTTACCAATGTAGC	P_{tRNA}
Lk-gfp-F2	GATCCCCGGGTGGTCAGTCCCTT/ATGAGTAAAGGAGAAGAAC	P_{tRNA} / *gfp*
gfp-F1	CGGGATCCATGAGTAAAGGAGAAGAAC	*gfp*
gfp-R2	CGCGAGCTCTCATTTGTATAGTTCATCCATGC	*gfp*
Lk-ina-F2	GATCCCCGGGTGGTCAGTCCCTT/ATGACTCTCGACAAGGCG	P_{tRNA} / *inaK*
ina-F1	ACGCGTCGACATGACTCTCGACAAGGCG	*inaK*
ina-R1	ACGCGTCGAC CCCGGGCTTTACCTCTATC	*inaK*
24-mer	CGCCAGGGTTTTCCCAGTCACGAC	pUC18

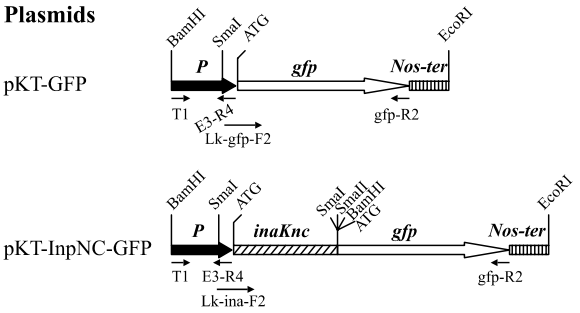

Fig. 1. Constructs encoding GFP. Plasmid pKT-GFP and pKT-InpNC-GFP were used for cytoplasmic and translocation expression of GFP in *Synechococcus*, respectively. P, tRNA promoter; *inaKnc,* truncated ice nucleation protein gene; *gfp*, green fluorescent protein gene; Nos-ter, nopaline synthase terminator. Locations of primers used in this study are indicated. The figure is not drawn to scale.

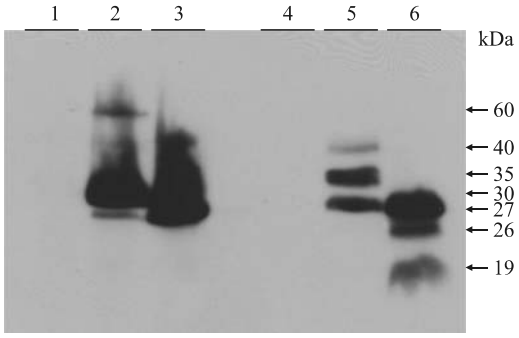

Fig. 3. Western blot analysis of GFP expressed in *Synechococcus*. Proteins from whole cell extracts of *E. coli* at OD_{600}=1.2 (lanes 1, 2, and 3) and *Synechococcus* at OD_{730}=2.0 (lanes 4, 5, and 6) were analyzed by immunoblotting using GFP monoclonal antibody. Lanes 1 and 4 are from cells harboring pKGT; lanes 2 and 5 are pKT-InpNC-GFP; lanes 3 and 6 are pKT-GFP.

tion of OPH is buried in the cell wall (Chungjatupornchai and Fa-aroonsawat, 2008). In this study, in order to investigate the protein translocation in *Synechococcus* PCC 7942, the same construct of InpNC (Chungjatupornchai and Fa-aroonsawat, 2008) was fused in frame with the coding region of a GFP variant, GFPmut2. The GFPmut2 was chosen because it is more fluorescent than wild-type GFP and has been shown to express in *E. coli* as a soluble protein (Cormack *et al*., 1996). Plasmids pKT-InpNC-GFP harboring gene cassette P_{tRNA}-*inaKnc-gfp* for translocation of GFP was constructed (Fig. 1) and transformed into *Synechococcus*. Plasmid pKT-GFP harboring gene cassette P_{tRNA}-*gfp* for cytoplasmic expression of GFP was used as control. Both plasmids contained cyanobacterial $tRNA^{pro}$ promoter (P_{tRNA}) (Chungjatupornchai *et al.*, 1999; Chungjatupornchai *et al*., 2002) enabling constitutive expression of GFP.

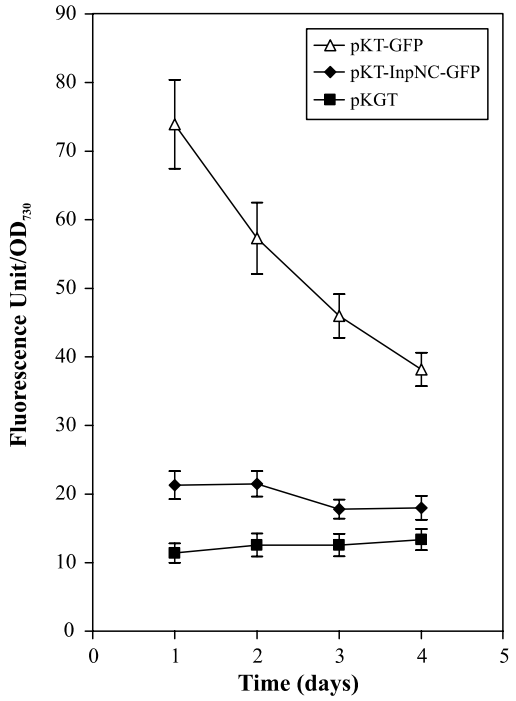

Fig. 2. Fluorescence intensity of GFP in *Synechococcus* cultures at various time courses. Cell cultures of *Synechococcus* harboring pKGT, pKT-GFP, and pKT-InpNC-GFP were harvested each day. The fluorescence intensity of whole cells was measured using spectrofluorometer. Each value and error bar represents the means of three independent experiments and its standard deviation.

Expression of the InpNC-GFP fusion protein in *Synechococcus*

To determine the optimal expression of GFP in *Synechococcus*, the fluorescence intensities of cell cultures harvested at various time courses were measured. The results in Fig. 2 showed that for cells harboring pKT-GFP, the levels of fluorescence intensity reached a maximum in 1-day culture and decreased thereafter in 2-, 3-, and 4-day cultures. For cells harboring pKT-InpNC-GFP, the levels of fluorescence intensity were not significantly different in 1- and 2-day cultures, and then slightly decreased in 3- and 4-day cultures. Therefore, all subsequent GFP fluorescence determination was performed using 1-day culture. Very little background fluorescence was detected in control cells harboring pKGT vector. After background fluorescence was subtracted, the levels of fluorescence intensity of cells harboring pKT-GFP are approximately 6-fold (for 1-day culture) and 5-fold (for 4-day culture) higher than that of corresponding cells harboring pKT-InpNC-GFP, indicating that the GFP of cells harboring pKT-GFP degraded faster than that of pKT- InpNC-GFP. The results in Fig. 2 indicated that GFP is functionally expressed in *Synechococcus*. The growth of cells expressing GFP is not significantly different from that of wild type (data not shown).

Western blotting analysis was performed to monitor the expression of GFP. The *E. coli* harboring the same plasmids as *Synechococcus* were used as control. As shown in Fig. 3, no band was detected with the GFP antibody in control extracts derived from cells harboring pKGT vector (lanes 1 and 4). In *E. coli* extracts, a band of expected size of the InpNC-GFP (60 kDa) was detected (lane 2). However, the

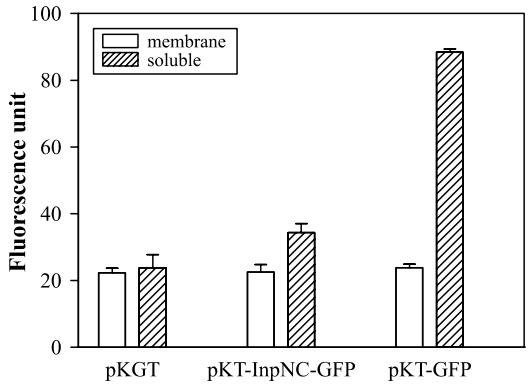

Fig. 4. Fluorescence intensities of *Synechococcus* cell fractions. The fluorescence intensities of soluble and total membrane fractions were measured using spectrofluorometer. The data are means of three experiments with standard deviation. Fluorescence intensities of total membrane fractions of pKT-InpNC-GFP and pKT-GFP were not significantly different from that of pKGT (control). However, fluorescence intensities of soluble fractions of pKT-InpNC-GFP and pKT-GFP were significantly higher than that of pKGT ($P<0.05$, t test).

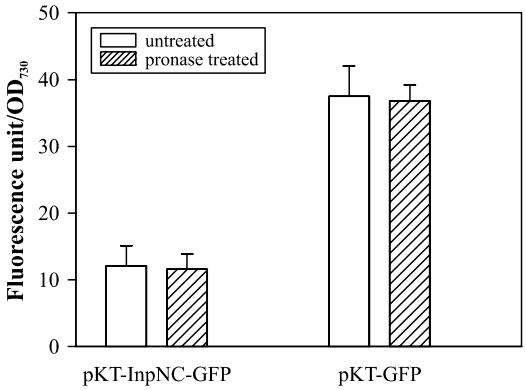

Fig. 5. Pronase accessibility assays of *Synechococcus* expressing GFP. Whole cells harboring pKT-InpNC-GFP and pKT-GFP were treated with pronase prior to determine fluorescence intensities. Values of the control without GFP (pKGT) were subtracted. The data are means of three experiments with standard deviation.

presence of relatively large portion of the degraded InpNC-GFP fusion protein indicated that most of the fusion protein had been subjected to proteolytic degradation. Almost all of the cytoplasmic GFP (with expected size of 27 kDa) had not been subjected to proteolytic degradation (lane 3). In *Synechococcus* extracts, all the bands with molecular weight less than the expected size of the InpNC-GFP fusion protein were detected (lane 5), indicating that almost all of the fusion protein had been subjected to proteolytic degradation. The smallest degradation product was the same size as free GFP (~27 kDa). The degradation products with approximate size of 40 and 35 kDa were also detected. The GFP fluorescence was observed in cells harboring pKT-InpNC-GFP (Fig. 2). Taken together, the results suggest that the degradation products may be GFP conjugated with the C-terminal domain of InpNC. The N-terminal of InpNC-GFP may be susceptible to protease attack and/or structurally unstable. However, most of the cytoplasmic GFP with expected size (~27 kDa) and very small amount of degradation product were detected (lane 6), indicating that most of the cytoplasmic GFP had not been subjected to proteolytic degradation. The immunobloting also showed cytoplasmic GFP (pKT-GFP) bands as thicker than translocated GFP (pKT-InpNC-GFP) bands. The results agreed well with the results in Fig. 2 that total expression of the cytoplasmic GFP is much higher.

In order to determine the fluorescence intensities of cell fractions, *Synechococcus* cells were disrupted by French press treatment and centrifuged to obtain the soluble and total membrane fractions. Figure 4 showed that very little background fluorescence was detected in both fractions of cells harboring pKGT vector without *gfp* gene. For cells harboring translocated GFP (pKT-InpNC-GFP), virtually all of the fluorescence was found in the soluble fraction corresponding to cytoplasmic and/or periplasmic contents. Thus, the translocated GFP is present as a soluble protein. The level of fluorescence detected in total membrane fraction was not significantly different from that of pKGT, indicating that the GFP is not located in cell membrane but in cytoplasm and/or periplasmic space. In cells harboring cytoplasmic GFP (pKT-GFP), high level of fluorescence was found in the soluble fraction. It has been reported that when exported by the Sec pathway, GFP was shown to be completely incapable of folding correctly and no fluorescence was observed (Feilmeier *et al*., 2000), however, the Tat system successfully exported correctly folded GFP to the periplasms of *E. coli* (Santini *et al*., 2001), and cyanobacterium *Synechocystis* PCC 6803 (Spence *et al*., 2003). Our observation of translocated GFP fluorescence in this study strongly suggested that GFP was capable of folding correctly and forming its chromophore in *Synechococcus*.

Probing the translocated GFP

Immunostaining microscopy of whole cells has been used successfully to detect the presence of OPH displayed on the outermost cell surface of *Synechococcus* (Chungjatupornchai and Fa-aroonsawat, 2008). In this study, we used the immunostaining microscopy method described previously (Chungjatupornchai and Fa-aroonsawat, 2008) with GFP monoclonal antibody as first antibody to investigate whether the GFP was present on the outermost cell surface. Under confocal laser scanning microscopy, no immunoreactivity of anti-GFP was detected on the surface of cells harbouring pKT-InpNC-GFP (data not shown). Thus, the GFP was not displayed on the outermost of cell surface.

Pronase accessibility assay of intact cells has been used to provide the evidence for the surface location of GFP protein, since pronase can not readily penetrate through the outer membrane (Shi and Wen Su, 2001; Li *et al.*, 2004). Therefore, *Synechococcus* whole cells were treated with pronase. Figure 5 showed that GFP fluorescence intensities of pronase treated cells harboring pKT-InpNC-GFP and pKT-GFP were not significantly different from those of corresponding untreated cells. The results confirmed that the

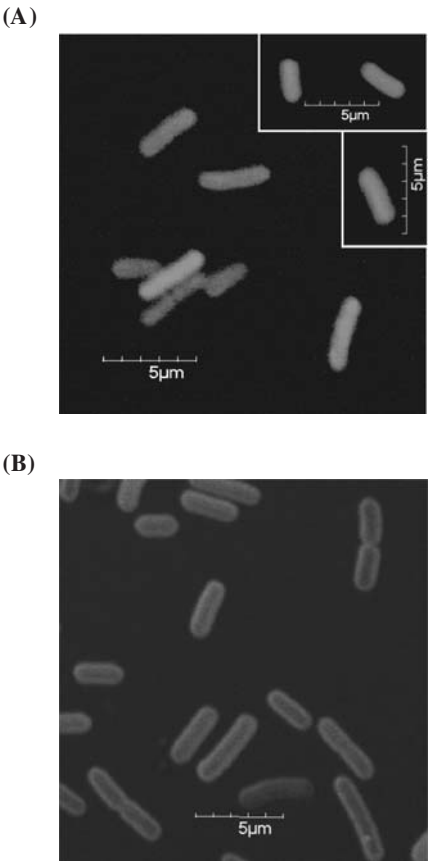

Fig. 6. CLSM images of *Synechococcus* cells expressing GFP. The location of GFP in *Synechococcus* cells harboring pKT-GFP (A) and pKT-InpNC-GFP (B) were visualized under CLSM with excitation at 488 nm and emission measured at 520 nm.

GFP of cells harboring pKT-InpNC-GFP was not located on the outermost cell surface.

The targeting of proteins into and across the correct membrane systems of cyanobacteria is poorly understood because of formidable technical problems that encountered in studies on these organisms. The thylakoid and plasma membranes are highly fragmented after isolation (Spence *et al*., 2003). So far, there are no techniques available to efficiently separate the cytoplasmic and periplasmic content. Therefore, *Synechococcus* harboring pKT-InpNC-GFP was further analyzed to identify the subcellular location of the GFP by CLSM with excitation at 488 nm and emission measured at 520 nm. The autofluorescence of *Synechococcus* photosynthetic pigments in the thylakoid membranes emits primarily in the red region of spectrum, therefore does not interfere with GFP signals. No fluorescence signal was detected in control cells harboring pKGT vector (data not shown). In cells harboring pKT-GFP, very bright fluorescence was distributed uniformly throughout the cytoplasm with no hint of enrichment of the signal at the periphery of the cells (Fig. 6A). In cells harboring pKT-InpNC-GFP, the fluorescence was found almost entirely as a halo in the outer regions of cells which appeared to correspond to the periplasm (Fig. 6B). Very little fluorescence was found in the cell interior, possibly representing untranslocated GFP derived from degradation of InpNC-GFP fusion protein. All of these results collectively suggest that InpNC can direct translocation of GFP across plasma membrane to periplasm but not across outer membrane to display on the outermost cell surface.

Several possibilities might lead to the observed translocation of GFP in cells harbouring pKT-InpNC-GFP. First, the level of periplasmic GFP was much lower than that of cytoplasmic GFP (Fig. 2, 3, 4, 5, and 6), although both plasmids pKT-InpNC-GFP and pKT-GFP contained the *gfp* gene under the control of P_{tRNA} promoter and were derived from pKGT vector with approximately 30 copies in the cells (Monshupanee *et al.*, 2006). Similar results have been observed in the previous study that the surface-expressed InpNC-OPH has a much lower activity than cytoplasmic OPH (Chungjatupornchai and Fa-aroonsawat, 2008). Taken together, these results suggest that some of the instability of InpNC-GFP can be attributed to InpNC moiety. Second, no full-length InpNC-GFP fusion protein was detected (Fig. 3). The N-terminal domain of InpNC may be susceptible to protease attack; the remaining C-terminal domain conjugated with GFP lost the ability to direct translocation across outer membrane and to act as a surface display motif. It has been suggested that the secretion signal may be located in the N-terminal domain of Inp, since truncated Inp containing only the N-terminal domain can be successfully employed as a display motif on *E. coli* cell surface (Li *et al*., 2004). Therefore, better GFP translocation efficiencies might be achieved, if InpNC-GFP fusion protein could be protected from proteolysis. Whether proteolysis of InpNC-GFP fusion protein occurred in cytoplasm and/or periplasm remains to be investigated. Finally, the cell wall of cyanobacteria can be an obstacle; it is a combination of Gram-positive and Gram-negative features. In addition, cyanobacterial outer membranes are normally surrounded by a fibrous sheath and in many cases a surface layer (Hoiczyk and Hansel, 2000).

It has been shown that in *E. coli*, GFP translocated to periplasm can be used as a reporter to study the property of periplasm in response to osmotic up-shock (Santini *et al*., 2001). The successful translocation of the active GFP to the periplasm of *Synechococcus* in this study may provide a potential means to investigate the property of cyanobacterial periplasmic substances in response to environmental changes in a non-invasive manner.

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