In the cyanobacterium *Synechocystis* sp. PCC 6803, the histidine kinase Hik33 regulates the expression of several stress-inducible genes. Recently, a yeast two-hybrid screen revealed a specific interaction between Hik33 and a small protein, Ssl3451. To investigate the function of Ssl3451, we developed an assay to monitor the autophosphorylation of Hik33 in vitro. Addition of Ssl3451 to the reaction mixture dramatically enhanced the autophosphorylation activity of Hik33. Pulse–chase experiments revealed that Ssl3451 stimulated the autophosphorylation of Hik33 but did not affect its dephosphorylation. These findings indicated that Ssl3451 might be an activator of Hik33. When the amount of Hik33 was kept constant and the amount of Ssl3451 was increased in the reaction mixture, the extent of autophosphorylation of Hik33 reached a plateau when equimolar concentrations were present, suggesting that Ssl3451 enhances the activity of Hik33 by associating with it with a 1:1 stoichiometry. Disruption of the gene for Ssl3451 resulted in increased expression of the *hliB* gene, which is induced by Hik33 under standard growth conditions, but it did not affect the levels of the *hliB* mRNA at low temperature. Together, these results suggest that Ssl3451 might enhance the activity of Hik33 both in vitro and in vivo.

**Keywords:** Autophosphorylation • Cyanobacterium • Gene expression • Response regulator • Signal transduction • Two-component system.

**Abbreviations:** EST, expressed sequence tag; Hik, histidine kinase; Rre, response regulator; TCS, two-component signal transduction system.

**Introduction**

In order to acclimatize to fluctuations in environmental conditions, all living organisms need to be able to perceive changes in their physical and/or chemical environment and to regulate gene expression and protein synthesis appropriately. Two-component systems (TCSs) are involved in many signal transduction pathways in various organisms, including prokaryotes, fungi, slime molds and plants (Stock et al. 2000). In bacteria, TCSs are major signaling systems that respond to numerous environmental stimuli. In general, a TCS consists of a histidine kinase (Hik) that senses a certain stimulus, and a cognate response regulator (Rre) which in most cases is a transcription factor. Signaling is initiated when a specific stimulus triggers the autophosphorylation of a conserved histidine residue in the transmitter domain of Hik. The phosphoryl group is then transferred to a conserved aspartate residue on the receiver domain of the cognate Rre. This modulates the activity of the Rre and thereby alters gene expression (Stock et al. 2000).

Comparative genomic analysis has revealed that cyanobacteria have a larger number of TCSs per unit genome size than other bacteria (Minezaki et al. 2005). This suggests that TCSs might play important roles in the acclimation of cyanobacteria to environmental conditions. The cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) contains a total of 47 genes for Hiks and 45 genes for Rres on its chromosome and plasmids (Kaneko et al. 1996, Mizuno et al. 1996, Kaneko et al. 2003). These Hiks and Rres together form TCSs and are probably involved in the acclimation to various environmental conditions via the regulation of gene expression. Indeed, it has already been demonstrated that some Hiks and Rres in *Synechocystis* are involved in the regulation of gene expression in response to a number of stress conditions, including low temperature (Suzuki et al. 2000, Suzuki et al. 2001), hyperosmotic stress (Paithoonrangsarid et al. 2004), high concentrations of NaCl (Marin et al. 2003, Shoumskaya et al. 2005), depletion of nutritional elements such as phosphate (Hirani et al. 2001, Suzuki et al. 2004) or manganese (Ogawa et al. 2002, Yamaguchi et al. 2002), the presence of glucose (Kahlon et al. 2006), and heavy metal stress (Lopez-Maury et al. 2002). Among the Hiks in...
Suzuki et al. identified Hik33 in Synechocystis, a cyanobacterium, as a low-temperature sensor. Since then, Hik33 has been shown to regulate the expression of stress-inducible genes under high-salt stress, hyperosmotic stress, and oxidative stress. However, the mechanisms of perception of these various signals are not yet fully understood.

In cyanobacteria, like Synechococcus elongatus PCC7942, Hik33 has been identified as a regulator of the nblA gene. NblA is involved in the degradation of phycobilisome proteins under nitrogen- or sulfur-depleted conditions and in strong light. Recently, it was speculated that under standard growth conditions, NblS is active and facilitates binding of an Rre, RpaB, to the HLR-1 regulatory sequence in the upstream regions of stress-inducible genes, thereby repressing their expression. When cells are exposed to stress, NblS is immediately inactivated, thereby repressing their expression.

Yeast-two hybrid screening revealed that a novel small protein, SipA, of approximately 8 kDa, interacts specifically with NblS, which leads to the refolding of the recombinant protein by gradual removal of urea by sequential dialysis.

We report here that Ssl3451, a homolog of SipA in Synechocystis, drastically enhances the autophosphorylation of Hik33 in vitro, via association with Hik33 with a 1:1 stoichiometry. Our finding indicates that Ssl3451 acts directly to enhance the autophosphorylation of Hik33. We also expressed the cytosolic portion of Hik2, which is also strongly conserved among known cyanobacteria, and the entire Ssl3451 protein in E. coli. These proteins did not have any negative effects on the growth of host cells and accumulated in the cells as soluble proteins.

**Ssl3451 stimulated the autophosphorylation of truncated Hik33**

When the purified truncated form of Hik33 was incubated with [γ-32P]ATP in vitro, radioactivity due to 32P was detected in association with Hik33 after SDS–PAGE. This suggests that the truncated form of Hik33 was capable of autophosphorylation in vitro. The incorporation of radioactivity into Hik33 proceeded in a time-dependent manner.

**Expression of recombinant Hik33, Hik2 and Ssl3451 in E. coli**

When the entire Hik33 protein, which contains two membrane-spanning domains, a HAMP domain (Aravind and Ponting 1999), a PAS domain (Hefti et al. 2004) and a Hik domain (Perego and Hoch 1996) (Fig. 1; Suzuki et al. 2000), was expressed in *Escherichia coli*, the growth of host cells was severely retarded. Moreover, we could obtain only very small amounts of recombinant Hik33 protein from extracts of these cells. These observations suggest that the expression of intact Hik33 is detrimental to *E. coli* and inhibits protein synthesis in *E. coli*. Therefore, we decided to express a truncated form of Hik33, namely the cytoplasmic portion of the protein that includes the HAMP, PAS and Hik domains (Fig. 1). Although this recombinant protein accumulated in *E. coli* cells, most of the protein was incorporated into aggregates, as so-called inclusion bodies. We purified the recombinant protein on Ni-NTA columns under denaturing conditions with 6M urea and obtained the refolded recombinant protein by gradual removal of urea by sequential dialysis.

**Results**

**Fig. 1 Schematic representation of the structures of Hik33, Hik2 and Ssl3451. Rectangles indicate lengths of proteins or indicated domains. Black rectangles indicate putative transmembrane (TM) regions; the horizontal striped box indicates the HAMP domain; other domains are identified within rectangles. Numbers below the rectangles indicate the positions of amino acid residues at the beginning and end of each domain.**
for at least 40 s, after which levels remained constant during incubation for up to 5 min (Fig. 2A, B). When Ssl3451 was added to the reaction mixture, the incorporation of $^{32}$P into Hik33 not only increased dramatically but also continued for a longer period, for example 5 min. As a result, the extent of phosphorylation of Hik33 after a 5 min incubation was >10-fold higher with Ssl3451 than without (Fig. 2A, B). These results indicate that Ssl3451 enhances the phosphorylation of Hik33 in vitro.

In order to investigate the effect of Ssl3451 on the autophosphorylation activity of another Hik, we also analyzed the autophosphorylation in vitro of a truncated form of Hik2, with or without Ssl3451. During incubation with [$\gamma$-$^{32}$P]ATP, purified recombinant Hik2 incorporated $^{32}$P in a time-dependent manner, revealing that it also has autophosphorylation activity (Fig. 2C, D). However, unlike truncated Hik33, the rate and extent of incorporation of $^{32}$P into truncated Hik2 were not affected by the addition of Ssl3451 to the reaction mixture (Fig. 2C, D). These results together indicate that Ssl3451 might affect the activity of Hik33, but not Hik2.

**Ssl3451 did not affect the dephosphorylation of Hik33**

The enhancement of Hik33 phosphorylation in the presence of Ssl3451 in vitro could be due to activation of Hik33 autophosphorylation, or the retardation of dephosphorylation. To distinguish between these alternatives, we chased the radioactivity of $^{32}$P that had been incorporated in the presence or absence of Ssl3451 by 700-fold dilution of the [$\gamma$-$^{32}$P]ATP with non-radiolabeled ATP. Although the incorporation of $^{32}$P into Hik33 was clearly enhanced by Ssl3451 prior to addition of non-labeled ATP (Fig. 2), the rates of dephosphorylation of $^{32}$P-labeled Hik33 were similar in the reaction mixtures with and without Ssl3451 (Fig. 3A, B). These results indicated that the ability of Ssl3451 to enhance the phosphorylation of Hik33 might be due to enhancement of the autophosphorylation and hence the kinase activity of Hik33, and not due to an inhibition of Hik33 dephosphorylation.

**Activation of Hik33 by Ssl3451 was dependent on stoichiometry**

To investigate the mechanism of activation of Hik33 by Ssl3451, we varied the relative levels of Ssl3451 and Hik33 in the reaction mixture. If Ssl3451 acts as a specific chaperone for Hik33, then it might be expected to enhance Hik33 activity when added even in small amounts, with larger amounts enhancing the activity more rapidly. When Ssl3451 and Hik33 were incubated at molar ratios of 0.1:1, 0.2:1, 0.5:1 and 1:1, the extent of autophosphorylation of Hik33 increased linearly and depended directly on the amount of Ssl3451 (Fig. 4). However, when Ssl3451 and Hik33 were incubated at molar ratios of 2:1 or 5:1, the extent of autophosphorylation of Hik33 did not exceed that at a molar ratio of 1:1 (Fig. 4). This plateau of activation at a molar ratio of 1:1 suggests that Ssl3451 might associate with Hik33.

*Fig. 2* Autophosphorylation of Hik33 and Hik2 in vitro. The truncated form (1 µM) of Hik33 (A and B) and of Hik2 (C and D), with and without an equimolar amount of Ssl3451, were incubated at 30°C for the indicated times (0–5 min) in kinase buffer that contained 37 kBq of [$\gamma$-$^{32}$P]ATP at a final concentration of 2.5 µM. Radioactivity incorporated into Hik33 (A) and Hik2 (C) on the gels was quantified with a BAS analyzer (Fuji Photo Film). The arrows indicate the apparent molecular masses of the truncated Hik33 and Hik2. (B) and (D) show the time-courses of accumulation of phosphorylated Hik33 (B) and Hik2 (D) in the presence (filled symbols) or absence (open symbols) of recombinant Ssl3451.
with a 1:1 stoichiometry, and that a stable complex of the two proteins is important for enhancement of Hik33 activity. Ssl3451 might not function as a specific chaperone for Hik33.

Conserved residues of Ssl3451 are essential for the activation of Hik33

We identified homologs of Ssl3451 in 26 strains of cyanobacteria for which genomic sequences are available in public DNA databases. We also found genes for homologs in the genomes of higher plants, such as *Arabidopsis thaliana* and *Oryza sativa*, and also in expressed sequence tag (EST) libraries of several green plants (Viridiplantae) and the yellow fever mosquito *Aedes aegypti* (Fig. 5 and Supplementary Fig. S1). The eukaryotic homologs have longer extensions at their N-termini (Fig. 5), which suggests that these homologs might be localized in organelles such as chloroplasts or mitochondria. An unrooted phylogenetic tree indicated that there are two groups of Ssl3451 homologs in cyanobacteria; one group consists mainly of proteins from freshwater and terrestrial cyanobacteria, such as *Synechocystis* and *Anabaena*, while the other group consists mainly of proteins from marine cyanobacteria, such as species of *Prochlorococcus* and *Synechococcus*. A single exception is *Trichodesmium erythraeum*, whose protein homolog of Ssl3451 is located at the base of a branch that includes all homologs from green plants (Supplementary Fig. S1). Since Hik33 is also conserved in all strains of cyanobacteria (Ashby et al. 2002), it is reasonable to predict that the targets of these Ssl3451 homologs might be Hik33 homologs. The homologous proteins from green plants and the mosquito formed a third clade (Supplementary Fig. S1).

To clarify the mechanism by which Ssl3451 activates Hik33, we investigated the contribution of amino acid residues that are most strongly conserved in the various Ssl3451 homologs. In our search for conserved amino acid residues, we identified three hydrophilic amino acid residues in Ssl3451, namely Lys20 which is conserved in all the homologs of Ssl3451, Thr21 which is replaced by serine in the homologs from a few species of cyanobacteria, and Arg29 which is conserved in all the homologs except in *Amborella trichopoda* (Fig. 5). We substituted the individual conserved residues of Ssl3451 with alanine to produce K20A, T21A and R29A mutants, respectively, and examined the autophosphorylation of Hik33 in the presence of native and mutant Ssl3451 proteins. We found that relative to native Ssl3451, the K20A, T21A and R29A substitutions decreased the ability of Ssl3451
Fig. 5 Alignment of the deduced amino acid sequences of homologs of Ssl3451, generated by ClustalW version 1.8 (Higgins et al. 1996) and visualized by the BoxShade program version 3.21 (http://www.ch.embnet.org/software/BOX_form.html). Arrows indicate strongly conserved amino acid residues in the homologous proteins. These residues were replaced by alanine in Ssl3451 in this study (see Fig. 6). Asterisks indicate amino acid residues that are conserved in all the homologous sequences. Underlined sequences are deduced from the sequenced genomes of cyanobacteria and plants; proteins from genomes of cyanobacteria that are currently being sequenced are indicated in plain characters; proteins from EST libraries of various organisms are indicated by dots after the name of the respective organism. Nucleotide sequences of the EST clones were translated to yield the longest putative protein sequence for clustering analysis. Some EST clones have suitable initiation codons, others do not.

Abbreviations: ssl3451, *Synechocystis* sp. PCC 6803 (GenBank accession No. BAA17314); Crocosphaera, *Crocosphaera watsonii* WH 8501 (ZP_00518394); CYB_2902, *Synechococcus* sp. JA-2-3B’a (2–13) (ABD03821); CYA_1355, *Synechococcus* sp. JA-3-3Ab (ABC99527); Ava_0486, *Anabaena variabilis* ATCC 29413 (ABA20110); asl2557, *Anabaena* sp. PCC 7120 (BAB74256); Npun02001810, *Nostoc punctiforme* PCC 73102 (ZP_00110733); Tery_0416, *Trichodesmium erythraeum* IMS101 (ZP_00675440); tsl2428, *Thermosynechococcus elongatus* BP-1 (BAC09980); gsr1914, *Gloeobacter violaceus* PCC 7421 (BAC89855); RS9917_11840, *Synechococcus* sp. RS9917 (ZP_01080149); RS9916_29119, *Synechococcus* sp. RS9916 (EAU73651); PMT0593, *Prochlorococcus marinus* str. MIT 9313 (CAE20768); PMT9312_0752, *Prochlorococcus marinus* str. MIT 9312

Continued
to activate Hik33 by 90, 70 and >95%, respectively (Fig. 6). These results indicate that these conserved amino acid residues in Ssl3451, in particular Lys20 and Arg29, might play important roles in activating the autophosphorylation of Hik33 or in stabilizing the conformation of Ssl3451 itself and/or the conformation of an Ssl3451–Hik33 complex.

Inactivation of Ssl3451 enhanced the level of the transcript of the hliB gene

In order to evaluate the function of Ssl3451 in vivo, we disrupted the ssl3451 gene by insertion of a kanamycin resistance cassette. Using Northern blotting, we analyzed the level of the hliB transcript, whose expression is regulated by Hik33 at low temperature (Murata and Suzuki 2006) (Fig. 7). Levels of the hliB transcript in the disrupted were approximately twice as high as in wild-type cells under standard growth conditions. However, levels of the transcript were similar in both types of cell after exposure to low-temperature conditions. Similar results were also observed through transcriptomic studies using a DNA microarray (data not shown).

Discussion

It has been reported that Ssl3451 interacts with Hik33 in Synechocystis, and that the respective Synechococcus homologs SipA and NblS also interact with one another (Espinosa et al. 2006, Sato et al. 2007). In this study, we found evidence that Ssl3451 might interact with the cytoplasmic portion of Hik33 with a 1:1 stoichiometry and enhances the autophosphorylation of Hiks both in vitro and in vivo (Figs. 2, 3, 4, 7). Our results indicate that Ssl3451 is a novel regulator of Hik33. The autophosphorylation reactions of Hiks might depend on the formation of homodimers of Hiks. Moreover, the phospho-transfer reactions from phosphorylated Hiks to their cognate Rres must depend on protein–protein interactions between the Hiks and Rres. However, the activities of some Hiks also require association with other factors. For example, the PhoR Hik in E. coli, which is a phosphatase sensor, appears to associate with a regulatory protein, PhoU (Wanner 1993). PhoU is believed to be an inhibitory factor of PhoR, since, in PhoU mutant cells, PhoR is constitutively active despite the availability of phosphate. In Bacillus subtilis, two membrane-bound Hiks, KinB and YycG, are regulated in vivo by KapB and YycH, respectively (Dartois et al. 1997, Szurmant et al. 2005). KapB is a lipoprotein while YycH is a periplasmic excreted protein, and thus it seems plausible that they might be involved in the perception of environmental signals. Yeast two-hybrid screening has also confirmed that some Hiks interact with other proteins. For example, in Synechococcus cells, the Hik SasA interacts with a component of the central oscillator of circadian rhythm, KaiC (Iwasaki et al. 2000). A sasA deletion mutation abrogates the response of several promoter activities to circadian rhythm, suggesting that SasA might be a regulatory component of the central oscillator (Iwasaki et al. 2000). Espinosa et al. (2006) reported that SipA associated with NblS by yeast two-hybrid screening. Ssl3451, an ortholog of SipA, might also interact with the ATP-binding motif of the transmitter domain of Hik33 (Sato et al. 2007). These earlier results suggested that Ssl3451 might modulate the autophosphorylation activity of Hiks via direct binding, but might not regulate...
A regulator of histidine kinase in Synechocystis

**Materials and Methods**

**Overexpression and purification of truncated forms of Hik33 and Hik2 and intact Ssl3451**

To construct plasmids for the overproduction of a truncated form of Hik33 (containing the HAMP, PAS and catalytic Hik domains), two-hybrid screening indicated that the interaction between Hik33 and Ssl3451 is specific (Sato et al. 2007).

We found genes for homologs of Ssl3451 for the most part in the genomes and EST libraries of photosynthetic organisms (Fig. 5 and Supplementary Fig. S1). In cyanobacteria, the respective homologs of Ssl3451 might regulate the activities of homologs of Hik33. However, green plants do not contain homologs of Hik33. In green plants, homologs of Ssl3451 might play other roles, perhaps via association with other kinases in organelles. On an unrooted phylogenetic tree, the homologs of Ssl3451 from the scaly green flagellate Mesostigma viride, the moss Physcomytrella patens and the early angiosperm Amborella trichopoda were located at the base of a clade that included homologous proteins from green plants (Supplementary Fig. S1). It has been reported that *M. viride* might be a candidate for the ancestor of the green lineage (McCourt et al. 2004), that the moss *P. patens* might be a primitive land plant and that *A. trichopoda* might be the basal angiosperm (Baroux et al. 2002). Thus, the homologs of Ssl3451 in green plants appear to have been conserved during evolution from their ancestors, rather than having been introduced recently by some form of horizontal gene transfer. It seems likely, moreover, that the conserved homologs of Ssl3451 might play an essential role in the lives of green plants.

Curiously, the EST library of the yellow fever mosquito *A. aegypti* (GenBank accession No. EB095336) also contains a homolog of Ssl3451. This homolog was located in a subclade that includes monocotyledonous plants, such as *Oryza sativa* (rice), *Hordeum vulgare* (barley) and *Triticum aestivum* (wheat) (Supplementary Fig. S1). We cannot explain why an insect protein is grouped together with proteins from these monocotyledonous plants, but the insect protein is the only example found to date of an Ssl3451 homolog in a non-photosynthetic organism.

The substitution of conserved residues in Ssl3451 clearly demonstrated that residues Lys20 and Arg29 play important roles in the activation of Hik33 (Fig. 6), but we do not know how these residues are involved in the function of Ssl3451. To elucidate the mechanism of activation of Hik33 by Ssl3451, we need to determine the structure of Ssl3451. We hope to elucidate the three-dimensional structure of a homolog of Ssl3451 from the thermophilic cyanobacterium *Thermosynechococcus elongatus* BP-1, Tsl2428 (Nakamura et al. 2002), and our findings might help us to understand the mechanism of activation of Hik33.

**Supplementary Fig. S1**

Expression of the *hliB* genes in wild-type and ΔSsl3451 cells, with or without exposure to low temperature conditions. Both types of cells grown at 34°C were transferred to 22°C and incubated for a further 20 min. Total RNA was extracted, and 20 μg of total RNA was loaded onto the gel in each case. Values (mean ± SD of triplicate measurements) indicate ratios in the extent of *hliB* gene expression after normalization of signal intensity using the *mpB* gene as a standard.

![Fig. 7 Expression of the *hliB* genes in wild-type and ΔSsl3451 cells, with or without exposure to low temperature conditions. Both types of cells grown at 34°C were transferred to 22°C and incubated for a further 20 min. Total RNA was extracted, and 20 μg of total RNA was loaded onto the gel in each case. Values (mean ± SD of triplicate measurements) indicate ratios in the extent of *hliB* gene expression after normalization of signal intensity using the *mpB* gene as a standard.](image-url)
domains), a truncated form of Hik2 (containing the catalytic Hik domain) and full-length Ssl3451, we amplified DNA fragments that contained the coding regions of the respective proteins using LA Taq polymerase (TAKARA BIO INC., Ohtsu, Japan). We extracted chromosomal DNA from Synechocystis and used it as a template with the following pairs of primers: hik33nde 5′-CATATGGATAACTTACCAGCCCCAT and hik33xho 5′-CTTCCGAGCTGACCCACACACTAA; hik2nde 5′-ACCATATGGACCAACATTGGGCTGA and hik2xho 5′-CTCTCAGGTTAACATTGTCTCCAGAGC; and 3451nde 5′-ATCATATGGCTGTATTGTTGTCGG and 3451xho 5′-CTTCCAGTAAATCGGAGACTTTCCGT. Forward and reverse primers contained a synthetic NdeI site and an XhoI site, as indicated by underlining. We cloned the amplified fragments separately into the pT7Blue vector (Novagen, Madison, WI, USA) and determined the nucleotide sequences of the inserts. The inserted fragments of the plasmids, corresponding to the coding regions of the respective proteins, were excised with Ndel and XhoI, and the resultant DNA fragments were inserted into the expression vector pColdII (TAKARA BIO INC.), which had been digested with Ndel and XhoI. The resultobal recombinant proteins included an extra 11 amino acid residues (MNHKVHHHHHHH) at their N-termini.

For overexpression of the target proteins, we introduced each plasmid into E. coli strain BL21 (Merck, Tokyo, Japan). Each transformant was grown in 50 ml of Luria–Bertani (LB) broth that contained 50 µg ml⁻¹ ampicillin for 2 h at 37°C until the optical density (OD) at 600 nm reached 0.4–0.5. Cultures were then transferred to 15°C, supplemented with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated at 15°C for a further 24 h to express each recombinant protein. Cells were harvested by centrifugation at 5,000 × g for 10 min at 4°C and stored at −30°C prior to use. Frozen cells were suspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 5 mM EDTA, 5 mM MgCl₂, 100 mM NaCl, 10 mM 2-mercaptoethanol, 6 M urea) and disrupted at 4°C with a sonicator (UD-201, Tomy, Tokyo, Japan). Cell debris was removed by centrifugation at 5,000 × g for 10 min at 4°C.

We examined the solubility of the recombinant proteins by SDS–PAGE, with subsequent staining of protein bands with Coomassie Brilliant Blue R-250 (Nacalai Tesque, Kyoto, Japan). When recombinant proteins were dissolved in cell lysates, each His-tagged protein was purified on Ni-NTA spin columns (QIAGEN, Tokyo, Japan) in accordance with the manufacturer's recommendations. Respective proteins eluted from the columns were pooled and dialyzed against storage buffer [50 mM Tris–HCl, pH 8.0, 200 mM KCl, 10 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol and 50% (v/v) glycerol] to remove imidazole in the eluant, and then stored at −80°C. We determined the concentration of each protein with a Protein Assay kit, using a solution of bovine serum albumin as the standard (Bio-Rad Japan, Tokyo, Japan), and we examined the purity of the proteins by SDS–PAGE. When the recombinant proteins were incorporated into inclusion bodies, the insoluble fraction, including the inclusion bodies, obtained after centrifugation of the lysate of E. coli cells, was washed three times with the lysis buffer and then the inclusion bodies were dissolved in urea-containing lysis buffer (50 mM Tris–HCl, pH 8.0, 5 mM EDTA, 5 mM MgCl₂, 100 mM NaCl, 10 mM 2-mercaptoethanol, 6 M urea) and purified on Ni-NTA spin columns (QIAGEN), as described above. The proteins purified under denaturing conditions were refolded by the gradual removal of urea by dialysis against the storage buffer, in which the concentration of urea was reduced stepwise. During the final dialysis, the purified proteins were dialyzed against lysis buffer without urea for 12 h at 4°C and then used in further experiments as purified proteins.

**Autophosphorylation of truncated Hik33 and Hik2**

To examine the autophosphorylation of purified Hik33 and Hik2 in vitro, we used a previously published method (Yamamoto et al. 2005). In brief, we dissolved each purified protein in kinase buffer (50 mM Tris–HCl, pH 8.0, 50 mM KCl and 10 mM MgCl₂) to a final concentration of 1 µM, and initiated the phosphorylation reaction by the addition of 37 kBq (1 µCi) of [γ-32P] ATP at a final concentration of 2.5 µM, in the presence or absence of 1 µM or the indicated amount of purified Ssl3451 or a point-mutated derivative of Ssl3451. The reaction mixture was incubated at the indicated temperature and aliquots of the reaction mixture were withdrawn at the indicated times and mixed immediately with a 20% (v/v) volume of 6× sample buffer for SDS–PAGE (63 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 75 µM bromophenol blue). The denatured reaction mixtures were fractionated by SDS–PAGE (15% polyacrylamide), and gels were washed three times with a solution of 45% (v/v) methanol and 10% (v/v) acetic acid for 20 min to eliminate the unincorporated [γ-32P] ATP. Finally, gels were dried in a vacuum gel dryer, and the radioactivity incorporated into the proteins on the gels was detected using a BAS 1800 system (Fuji Photo Film, Tokyo, Japan).

**Site-directed mutagenesis of Ssl3451**

We replaced conserved amino acid residues in Ssl3451 with alanine residues using a PCR-based method. First, we separately amplified DNA fragments that corresponded to regions upstream and downstream of sites of substitution in the coding region of the ssl3451 gene, using forward primer 3451nde and the appropriate reverse primers, namely K20Ar (5′-CATGGGATCAGCGGTGCGCA), T21Ar (5′-CATGGGATCACCGGCTTTCA) and R29Ar (5′-TTAAAATCCCGAGAGCCGAC), and 3451xho and forward primers, namely K20Af (5′-CCCCCCCTATTTGGCAACCGC), T21Af (5′-CCCC
CCTATTTGAAAAGCCGC) and R29Af (5'-CCATGCCCCAT GCTCCGTCCT), to produce K20A, T21A and R29A mutant proteins, respectively. The underlined nucleotides show changes from the native gene sequence for substitution of the encoded amino acid residues. The second PCR was performed with a mixture of an equal amount of the corresponding pairs of products of the first PCR as the template and 3451nde and 3451xho as the primers. We cloned the DNA fragments that had been amplified by the second PCR into pT7Blue (Novagen), determined their sequences and then introduced them into the expression vector pColdI (TAKARA BIO INC.), as described above.

Disruption of the ssl3451 gene and expression of the hliB gene
We amplified a DNA fragment that included the ssl3451 gene by PCR, using primers 3451F (CTCGAGGTCCGA CGACGGAGTTA) and 3451R (AAGCTTGGTCATTTACTC GCG) and the chromosomal DNA of Synechocystis as template. We cloned the DNA fragment into pT7Blue (Novagen) and introduced a kanamycin resistance cassette with the EZ::TN <KAN-2> system (Epicentre, Madison, WI, USA). We determined the site of insertion of the cassette by direct sequencing. We used the plasmid to transform Synechocystis by homologous recombination. We selected kanamycin-resistant colonies and confirmed the insertion of the cassette in the coding region of the ssl3451 gene by PCR. Wild-type cells, and cells with a disrupted ssl3451 gene, were grown in BG-11 medium aerated with 1% (v/v) CO2 in air at 34°C under constant illumination by incandescent lamps at 70 µmol photons m-1 s-1. We grew cells for 16 h at 34°C and then transferred some cells to 22°C for a further 20 min. We then extracted total RNA from cells grown at 34°C and from cells exposed to low temperature, as described previously.

Levels of transcripts were determined with an AlkphosDirect kit (GE Healthcare Biosciences, Tokyo, Japan). We amplified a DNA fragment that included the hliB gene Disruption of the ssl3451 gene and expression of the hliB gene

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