# Roles of Signal-input Domain of a Histidine Kinase, Hik33, in *Synechocystis* sp. PCC 6803

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# Abbreviations

**AP: alkaline phosphatase**

**Asp: asparatate**

**ATCC: American Type Culture Collection**

**Chl: chlorophyll**

**DBMIB: 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone**

**DCMU: 3-(3,4-dichlorophenyl)-1,1-dimethylurea**

**EDTA: ethylenediaminetetraacetic acid**

**HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid**

**His: histidine**

**$OD_{730}$: optical density at 730 nm**

**PCC: Pasteur culture collection**

**PCR: polymerase chain reaction**

**TM: transmembrane**

**Tris: tris(hydroxymethyl)aminomethane**

# Abstract

Bacteria, in general, use the two-component system for the acclimation to the environmental change. Two-component system is a signal transduction pathway composed of two proteins, i.e. histidine kinase and response regulator. Histidine kinase phosphorylates a cognate response regulator responding to specific extra- or intracellular conditions, then most of response regulator regulates gene expression depending on its phosphorylation state. The cyanobacterium *Synechocystis* sp. PCC 6803 has a unique histidine kinase, Hik33, which responds to multiple stress conditions such as low temperature, oxidative, high light, high salinity and hyperosmotic stress, but the mechanisms that respond to the stress conditions are not clarified yet. Each histidine kinase possesses divergent N-terminal signal-input domain to perceive specific stimuli and rather conserved histidine kinase domain at the C-terminal region that interacts with the cognate response regulator. Hik33 includes several subdomains at the N-terminal signal-input domain, however function of each subdomain has not been identified. In an attempt to investigate the function of these subdomains I constructed the chimeric histidine kinase between N-terminal signal-input domain of Hik33 and C-terminal histidine kinase domain of SphS, a sensor for phosphate-deficiency in *Synechocystis*, which is named as Hik33n-SphSc. Hik33n-SphSc exhibits expression of the *phoA* gene for alkaline phosphatase under the standard growth conditions and repressed it under the salt and cold stress. To further investigate functions of the subdomains at the signal-input domain of Hik33 a series of subdomain-deletion or -substitution mutations of Hik33n-SphSc was introduced in the cells of *Synechocystis*, then expression of the *phoA* gene was determined under standard growth conditions and under the cold or salt stress. A Hik33n-SphSc that was deleted the membrane-localizing region lost the kinase activity, whereas Hik33n-SphSc of which membrane-localizing region were substituted with membrane-localizing regions from other histidine kinases had functional activities.

These results suggest that localization on the membrane of the kinase, regardless of the sequences of transmembrane helices, might be essential for the function of Hik33. Cells introduced Hik33n-SphScs that were deleted HAMP or PAS domain exhibited approximately 2-fold higher alkaline phosphatase activity than Hik33n-SphSc introduced cells, suggesting that the HAMP and PAS domains might be involved in the regulation of kinase activity of Hik33. Then I attempted to identify the functional residues in the PAS domain that are highly conserved among homologues of Hik33 by substitution in the chimeric constructs, Hik33n-SphScs. Mutations of D300A, W318 and R415E obviously decreased the Hik33n-SphSc activity. These residues might be involved in functions of the PAS domain and might have important role in regulation of Hik33 activity.

# Introduction

Once environmental conditions are changed, living organisms modulate their metabolism to acclimate to the new environment to survive. To modulate the metabolism the organisms have to perceive the environmental changes accurately and the signals are transmitted to downstream protein in the signal transduction pathway such as signal transmitter or transcription factor then sets of stress-inducible genes are expressed. Stress-inducible proteins were then translated to play roles in changing cell metabolism. Hitherto, the proteins that involved in the signal transduction pathway and the set of stress-inducible genes have been well studied and identified [1,2]. However, it is not so clear how the sensor protein regulates the activity of itself depending to the specific stimuli. Regulation of the sensor protein activity are the first step for the cells to acclimate to the fluctuating environment, thus understanding how activity of the sensor protein is regulated in the cells is valuable to understand mechanisms for acclimation to the environment.

**Cyanobacterium *Synechocystis* sp. PCC 6803**

Cyanobacteria are oxygen-evolving photosynthetic prokaryotes. Similar to other photosynthetic eukaryotes, i.e. plants and algae, cyanobacteria have combined photosystems, photosystem I and photosystem II, on the thylakoid membrane. Photosystem I resembles to those of green sulfur bacteria, and photosystem II resembles to those of purple bacteria [3]. Cyanobacteria are very diverse morphologically, biochemically, genomically [4,5]. The taxonomical studies have been curried out by morphological and genomic approaches [4,5]. Rippka *et al.* classified cyanobacteria as five sections according to the form and dividing manor [4]. Genus *Synechocystis* is classified in section II, unicellular, sheath absent and dividing in two or three planes [4]. *Synechocystis* sp. PCC 6803 was originally isolated from fresh water in California, U.S.A., at 1968 by R. Kunisawa as *Aphanocapsa*, and afterward classified as

*Synechocystis* by Rippka *et al.* [4]. This species have been stored in Pasteur Culture Collection of Cyanobacteria, Institute Pasteur, France and also stored as ATCC27184 in American Type Culture Collection, U.S.A. [4]. A glucose-tolerant strain of *Synechocystis* sp. PCC 6803 could be grown photomixotrophically and heterotrophically by addition of glucose in the medium, and thus the photosystem is disrupted by genetic manipulation as described previously [6]. This characteristic is very useful for the research on oxygen-evolving photosynthesis and attracts the researchers. Actually *Synechocystis* sp. PCC 6803 has been subjected to genome sequence project firstly among all known cyanobacteria. In 1996, the chromosome sequence was published [7] and afterward complete-genome sequence including the 7 plasmids was published [8]. *Synechocystis* sp. PCC 6803 is now used as a model organism for the study on photosynthesis, stress response and metabolism of photosynthetic organism. The genome sequence and the study on stress response of *Synechocystis* sp. PCC 6803 using gene knockout line and transcriptomic approach have revealed that the main signal transduction pathway for stress response in *Synechocystis* sp. PCC 6803 is two-component system [2,9]. Hereafter I refer to *Synechocystis* sp. PCC 6803 as *Synechocystis*.

**Two-component system**

Two-component system is a major signal transduction pathway in bacteria to acclimate environment stress [1]. Generally the system is composed of two types of proteins, a histidine kinase and a response regulator [1]. Histidine kinase modulates it autokinase activity according to the intracellular or extracellular conditions, and transfers the phosphate group on the conserved His residue to a conserved Asp residue of a cognate response regulator [1]. Most response regulators act as transcriptional factor and regulate gene expression depending of phosphorylation state of the conserved Asp

residue [1]. The first identified two-component system is EnvZ/OmpR in *Escherichia coli* that regulates expression of the genes for outer membrane porins depending on extracellular osmolarity [10,11]. After the discovery of EnvZ/OmpR, two-component systems have been found in bacteria, archea, fungi, plants, and protists, but not in higher animals [1]. In 1986 Nixon *et al.* have revealed that C-terminal region of NtrB, a nitrogen-responsive histidine kinase, shares homology with C-terminal region of other histidine kinase and N-terminal region of NtrC, a cognate response regulator of NtrB, shares homology with N-terminal region of other response regulators [12]. These findings have contributed to predict the genes for putative histidine kinases and response regulators on the genome sequences [9]. Furthermore the findings have led to the concept that histidine kinases and response regulators are comprised of modules, N-terminal region and C-terminal region. Each histidine kinase has a unique signal-input domain in its N-terminal region and a well-conserved histidine kinase domain in its C-terminal region [1]. It is thought that the unique signal-input domain perceives specific signals and regulates the kinase activity located on the histidine kinase domain [1], and that the histidine kinase domain interacts with the cognate response regulator [13]. Actually the modularity of N-terminal region and C-terminal region of histidine kinase was well studied and characterized previously [14,15]. Some chimeric histidine kinases have been constructed to investigate the functions of signal-input domains. Studies have indicated that chimeric histidine kinases, such as Tar and EnvZ [14], and YtvA and FixL [15] perceive specific stimuli that are originally sensed by the N-terminal region included in the chimeric histidine kinases, and transfer the signals to a response regulator, which is specifically recognized by the C-terminal region of the chimera.

**Cyanobacterial histidine kinase Hik33**

In *Synechocystis* genes for 47 histidine kinases and 43 response regulators were located on the genome sequence. Hik33, a histidine kinase in *Synechocystis*, had been firstly characterized and named as drug sensory protein A (DspA) previously [16]. The mutants that possess the mutations at the *hik33* locus have been more tolerant to drugs than wild-type, however the reason has not been clarified yet. Hitherto each function of histidine kinases in *Synechocystis* has been identified by disrupting the gene for histidine kinase and using transcriptomic approach [2]. Through those studies histidine kinases function as sensors for the stress conditions have been identified. Hik33 is identified as a cold sensor [17,18], then characterized as a sensor for high osmolarity [19,20,21], salt [21,22], high light [23], and oxidative [24] stresses. However, it is not clarified how Hik33 responds to those multiple stress conditions yet. Under these stress conditions Hik33 regulates expression of several genes related to photosystems such as *hliA*, *hliB*, *hliC*, which are encoding high-light inducible proteins which are required for acclimation to high-light stress conditions [25] and *ssr2016* (a homologue of *pgr5* [26] which is required for antimycin A-sensitive cyclic electron flow [27]) [2]. The functions of the Hik33-regulaed genes suggest that Hik33 maintains the activity of photosystems properly to acclimate to the stress conditions. At the present so many genome sequences are available via public databases. Similarity search to deduced protein sequences revealed that orthologues of Hik33 is highly conserved among all known cyanobacteria and some chloroplasts of algae in the red lineage, such as *Porphyra purpurea*, *Porphyra yezoensis*, and *Cyanidium caldarium* [28]. Through the process of intracellular symbiosis most of the plastid genes have been disappeared and some plastid genes have been transferred to the nuclear chromosomes [29]. Algae evolved by secondary symbiosis such Haptophyte *Emiliania huxleyi* and Raphidophyte *Heterosigma akasiwo* also have homologues of the *hik33* gene in the plastids [28,30]. The function and evolutionary conservativeness represent us the importance of Hik33 in those

photosynthetic organisms. Elucidation of the signal perception mechanism of Hik33 contributes to understand how those photosynthetic organisms acclimate to the environmental stresses more deeply. Hence, in this study I aimed to elucidate roles of the signal-input domain of Hik33.

**Subdomains at the signal-input domain of Hik33**

Hik33 possesses several subdomains in the signal-input domain, such as two transmembrane helices, a periplasmic loop region, a HAMP domain and a PAS domain. HAMP domain is amphipathic two alpha helices connected by linker region that is located adjacent to membrane, and this structure is frequently found in histidine kinase, adenylyl cyclase, methyl-accepting chemotaxis protein and phosphatase [31]. HAMP domain of Af1503 in *Archaeoglobus fulgidus* has been observed as a dimer making a bundle form [32]. PAS domain is frequently found in sensor proteins, such as period-circadian protein, aryl hydrocarbon nuclear translocator and single-minded protein [33], included those that perceive light [34], oxygen [35], or redox signals [36]. PAS domains of other histidine kinases also have been observed as a dimer form in the crystals [37,38]. Both the HAMP and PAS domains appear to be involved in self-dimerization of proteins and the regulation of protein activity via conformational changes depending on signal recognition [37,38,39]. However, the roles of each subdomain in signal-input domain of Hik33 remain unclear. Meanwhile, it is thought that the cold signal is perceived by the rigidification of cell membranes, and thus that the sensor for low temperatures is located on the membrane [40]. Martin and colleagues demonstrated that a low-temperature sensory histidine kinase, DesK, in *Bacillus subtilis* might sense decreases in membrane fluidity via its membrane-spanning helix [41]. Hyperosmotic stress is also perceived by changes in turgor, likely via transmembrane helices or the periplasmic loop region of Sln1 in yeast [42]. Conversely, salt stress is

sensed by a change in the concentration of sodium ion in the cytosol via a GAF domain in an adenylyl cyclase from the cyanobacterium *Anabaena* sp. PCC 7120 [43], and other ions, such nickel and manganese, is speculated to be sensed in the periplasmic space via periplasmic region in histidine kinases [44,45], while oxidative stress is sensed by the formation of disulfide bonds in the transcription factor OxyR in *Escherichia coli* [29]. These knowledges, combined with the complexity of the signal-input domain of Hik33, suggest that Hik33 may sense each stress with a different region of its signal-input domain. In an attempt to investigate the role of signal-input domain of Hik33, here I characterized each subdomain of the signal-input domain of Hik33. The results suggest that the transmembrane helices of Hik33 are necessary for kinase activity *in vivo* and that the HAMP and PAS domains are involved in the regulation of kinase activity. These results will contribute to clarification of the signal sensing mechanism of Hik33.

# Materials and Methods

**Strains and culture conditions**

A glucose-tolerant strain of *Synechocystis* sp. PCC 6803 [6] was used as the wild-type. *hik33*-deleted cells and *hik33*-complemented cells were screened and maintained on BG11 media solidified with 1.5% (w/v) agar supplemented with 25 μg/ml kanamycin and 25 μg/ml spectinomycin, respectively. Cells that harbored modified *hik33n-sphSc* genes were screened and maintained on BG11 media solidified with 1.5% (w/v) agar supplemented with 25 μg/ml spectinomycin. The wild-type cells of *Synechocystis* and the genetically modified cell lines were grown photoautotrophically under illumination at 70 μmol photons $m^{-2}$ $s^{-1}$ from incandescent lamps at 34°C in BG-11 medium [5] that was supplemented with 20 mM HEPES-NaOH (pH 7.5) and continuously aerated with 1% (v/v) $CO_2$ in air as under standard growth conditions. Cultures were exposed to 0.5 M NaCl for salt stress, 18°C for cold stress, 500 μmol photons $m^{-2}$ $s^{-1}$ for high light stress, 0.5 M sorbitol for hyperosmotic stress, and 250 μM $H_2O_2$ for oxidative stress.

A strain of *Escherichia coli*, JM109 (TaKaRa Bio, Ohtsu, Japan), was used as a host for construction of the plasmids used in this study. *E. coli* cells harboring engineered plasmids were screened and grown at 37°C in LB medium supplemented with appropriate antibiotics (50 μg/ml ampicillin, 50 μg/ml kanamycin, 50 μg/ml spectinomycin, 25 μg/ml chloramphenicol or 12.5 μg/ml tetracycline).

**Deletion and complementation of the *hik33* gene**

DNA fragments corresponding to the 1-kbp upstream region and the 1-kbp downstream region of the *hik33* gene were amplified by the polymerase chain reaction (PCR) with *Synechocystis* genomic DNA as template and primer pairs Hik33proFHind plus Hik33proRNde and Hik33terFXho plus Hik33terR (Table 1). The resultant fragments were cloned into pT7Blue (Merck Japan, Tokyo) by TA cloning and sequences of the fragments were confirmed. The DNA fragment corresponding to the upstream region of

the *hik33* gene was excised from the plasmid by digestion with *Hind*III and *Nde*I and then inserted into the plasmid that included the downstream region of the *hik33* gene, after cleaved with *Hind*III and *Nde*I, to generate pYS01. I amplified a kanamycin-resistance gene cassette and the *sacB* gene by PCR using a transposon fragment, EZ-Tn5 (Epientre, Madison, WI), and genomic DNA of *Bacillus subtilis* as templates and primer pairs Kan2FNde plus Kan2RNhe and sacBFNhe plus sacBRSal, respectively (Table1). The resultant fragments were cloned into pT7Blue and the functioning of gene products were determined by analyses of kanamycin resistance and sucrose sensitivity of the transformed *E. coli* cells. The kanamycin-resistance gene cassette was excised from the plasmid by digestion with *Nde*I and *Nhe*I and inserted into the plasmid that included the *sacB* gene, after cleavage with *Nde*I and *Nhe*I. The fragment of the kanamycin-resistance gene cassette and the *sacB* gene were excised from the plasmid by digestion with *Nde*I and *Sal*I and inserted into pYS01 that had been cleaved with *Nde*I and *Xho*I to generate pYS02. The sequences of all primers are shown in Table 1, with restriction sites underlined. To obtain the *hik33*-deleted *Synechocystis* cells, I transformed wild-type cells with pYS02 as described by Williams [6]. I screened and segregated the resultant *hik33*-deleted cells using kanamycin.

DNA fragments corresponding to the N-terminal region and the C-terminal region of the coding sequence of *hik33* were amplified by PCR with genomic DNA of *Synechocystis* as template and primer pairs aHik33FNde plus bHik33RNco and HikFNco plus HikRXho (Table 1), respectively. Then they were cloned into pGEM-T Easy (Promega, Tokyo, Japan). The fragment corresponding to the C-terminal region was excised from the plasmid by digestion with *Nco*I and *Xho*I and then inserted into pYS01, after cleavage by *Nco*I and *Xho*I, to generate pYS03. The fragment corresponding to the N-terminal region was excised from the plasmid by digestion with *Nde*I and *Nco*I and inserted into pYS03, after cleavage by *Nde*I and *Nco*I, to generate

pYS04. A spectinomycin-resistance gene cassette was excised by digestion with *Dra*I from pAM1146 [46] and inserted into pYS04 that had been cleaved by *Xho*I and blunted, to generate pYS05. To obtain *hik33*-complemented cells, I transformed *hik33*-deleted cells with pYS05 as described by Williams [6]. I screened and segregated the resultant *hik33*-complemented cells using spectinomycin.

**Purification and resequence of *Synechocystis* genomic DNA**

*Synechocystis* cells were harvested from 500 ml of cultures by centrifugation. The cells were suspended in 2 ml of NaI-saturated water then incubated at 37°C for 30 min. After the incubation the solutions were diluted by addition of 14 ml of $H_2O$ and centrifuged to recover the cells. The cell pellet was suspended with 8 ml of a buffer solution containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5 mM EDTA and added 200 μl of a solution of Lysozyme (10 mg/ml) and 10 μl of RNase (1 mg/ml) then incubated at 37°C for 30 min. After the incubation 800 μl of 10% SDS was added and further incubated at 37°C for 30 min. Then 40 μl of Proteinase K (20 mg/ml) was added and further incubated at 37°C for 30 min. After the incubation 8 ml of Tris-HCl (pH 8.0)-saturated phenol was added and agitated gently by a rotary mixer, RVM-101 (Iwaki Glass, Tokyo, Japan), for 20 min. Then it was centrifuged and the aqueous phase was transferred to a new centrifuge tube. Equal volume of phenol/chloroform/isoamyl alcohol (25:24:1 v/v) was added to the supernatant and agitated for 20 min as described above, then after the agitation the solution was centrifuged and the aqueous phase was transferred to a new tube. This process was repeated twice. Then 13 ml of ethanol and 250 μl of 3 M sodium acetate (pH 5.2) was added and centrifuged to precipitate DNA. The DNA pellet was rinsed with 13 ml of 70% ethanol, and then it was centrifuged and the supernatant was removed. The DNA pellet was resolved in 3 ml of TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) and 3.225 g CsCl was added. Then 100 μl of

ethidium bromide (10 mg/ml) was added to 3 ml of the DNA solution. This DNA solution was centrifuged at $10^6$ x *g* for 20 hours. DNA band was recovered to a new tube, and ehidium bromide was removed by washing with NaCl-saturated propanol. Then the purified DNA was precipitated with ethanol and the resultant DNA pellet was resolved in TE buffer. This DNA sample was used for genome resequence. The genomic DNA was sequenced by Genome Sequencer (illumina, CA, U.S.A.).

**Production of cells containing genes for Hik33n-SphSc or its derivatives**

I produced cells that harbored the gene for a chimeric histidine kinase, Hik33n-SphSc, or its derivatives at the *sphS* locus on their chromosome under control of the native promoter of the *sphS* gene. DNA fragments corresponding to the vector backbone of pSK05 [47], including the approximately 1-kbp upstream and downstream regions and the transmitter domain of the *sphS* gene, and the N-terminal region of the *hik33* gene were amplified by PCR with pSK05 and the genomic DNA of *Synechocystis* as template and the primer pairs SphSHikvecF plus SphSHikvecR and Hik33_F_InF plus Hik33_THP_R_InF (Table 2), respectively. The two resultant fragments were combined using the In-Fusion HD cloning kit (TaKaRa Bio) to yield plasmid pYS06 for introduction of genes for Hik33n-SphSc.

pYS06 was used as the template for PCR to obtain various derivatives of Hik33n-SphSc. To delete the region corresponding to the membrane-localizing region of Hik33n-SphSc (ΔTM), I used the primer pairs Hik33_HAMP_F_InF plus SphSHikvecR, and the resultant fragment was self-cyclized using the In-Fusion kit (TaKaRa Bio) to generate pYS07. I also replaced the membrane-localizing region of Hik33n-SphSc with that from SphS or NrsS ($TM_{SphS}$ or $TM_{NrsS}$). Specifically, an amplified fragment obtained with the primer pair Hik33_TM2_F and SphSHikvecR (Table 2) was conjugated with a DNA fragment that corresponded to the region of SphS

or of NrsS that had been amplified with primer pairs SphS_F_Inf plus SphS_TM_R_Inf2 and NrsS_F_Inf plus NrsS_TM2_R_Inf (see Table 2), respectively, using the In-Fusion kit to generate pYS08 and pYS09. To delete the periplasmic loop region, the HAMP domain, the PAS domain, and both the HAMP and PAS domains together from Hik33n-SphSc, I used primer pairs Hik33_TM1_R plus Hik33_ TM2_F_InF, Hik33_TM2R_InF plus Hik33_HAMP_F, Hik33_TH_R_InF plus SphSHikvecF and Hik33_T_R_InF plus SphSHikvecF (Table 2), respectively. Each fragment was self-cyclized using the In-Fusion kit to generate pYS10, pYS11, pYS12 and pYS13 for production of Δloop, ΔHAMP, ΔPAS, and ΔHAMP/PAS, respectively.

Point-mutated Hik33n-SphSc was obtained by PCR and self-cyclization using the In-Fusion kit with pYS06 as template and the primer pairs listed in Table 3. The resultant plasmids were recovered from *E. coli* cells and the mutated sequences were confirmed.

Cells of the *sphS*-deleted *Synechocystis* (ΔsphS), which was described previously [47], were used as the parental host strain for transformation with plasmids listed in Table 4 that harbored the genes for Hik33n-SphSc and its various derivatives. In ΔsphS cells, the entire coding region of the *sphS* gene was replaced with the kanamycin-resistance gene cassette. Cells that expressed Hik33n-SphSc or its derivatives were screened and segregated on BG11 that contained 25 μg/ml spectinomycin.

**Deletion of the *hik33* or *ssl3451* gene in the cells that have been introduced the gene for Hik33n-SphSc**

The pGEM-T easy plasmid (Promega) that possesses a kanamycin-resistance gene cassette was kindly given from Dr. Satoshi Kimura. The kanamycin-resistance gene cassette had been amplified by PCR using KanF and KanR primers and Ez-Tn5 as

template [47], and then it had been inserted in pGEM-T easy vector by TA-cloning. Thereby the kanamycin-resistance cassette possessed additional *Nde*I and *Sal*I sites at 5' and 3' ends respectively. The kanamycin-resistance gene cassette was excised from the plasmid by digestion with *Nde*I and *Sal*I, and then inserted into the pYS01, after cleaved with *Nde*I and *Xho*I, to generate pYS30. Cells that harbored gene for Hik33n-SphSc were transformed with pYS30 to delete *hik33* gene. The transformants were screened and segregated on BG11 that contained 25 μg/ml kanamycin and 25 μg/ml spectinomycin. In addition, cells that harbored gene for Hik33n-SphSc were transformed with another plasmid for disruption of the *ssl3451* gene [48]. The plasmid that possesses *ssl3451* gene, which had been disrupted by a kanamycin-resistance gene cassette, was kindly given from Mr. Tasuku Sakayori [48]. The transformants were screened and segregated on BG11 that contained 25 μg/ml kanamycin and 25 μg/ml spectinomycin.

**Northern blotting analysis of the *phoA*, *hliB*, and *rnpB* genes**

Levels of expression of the *phoA*, *hliB* and *rnpB* genes were determined by Northern blotting analyses. *Synechocystis* cells were inoculated into fresh BG11 medium at an $OD_{730nm}$ of 0.1 and grown for 16 h under standard growth conditions. Then cells were exposed to each stress or 50 μg/ml rifampicin. *Synechocystis* cells were harvested and total RNA was isolated by the hot phenol method as described previously [49] with slight modifications. Specifically, I changed the duration of centrifugation and the temperature during extraction of RNA to 10 min and 4°C, respectively, and the treatment with Protease K (addition of 50 μl of a mixture that contained 0.5 μg/μl Protease K and 0.5% SDS) to 30 min at 37°C after treatment with DNase I. Aliquots of 10 μg of total RNA were resolved electrophoretically on 1.2% (w/v) agarose gels that contained 2% (w/v) formaldehyde, and RNA was blotted onto Hybond-$N^+$ membranes

(GE Healthcare Japan, Tokyo). DNA fragments for use as *phoA*-, *hliB*- and *rnpB*-specific probes were amplified by PCR with genomic DNA as a template and the primer pairs listed in Table 5. Labeling of probes and hybridization were performed with the AlkPhos Direct kit (GE Healthcare Japan) according to the manufacture's instructions. To detect the hybridization signals, photons transmitted via chemiluminescence due to the reaction catalyzed by alkaline phosphatase (AP) conjugated with the probes and CDP-*Star* as a substrate were measured with the LAS-1000 or LAS-4000 mini system (FujiFilm, Tokyo, Japan).

**Measurement of AP activity**

The AP activities of intact cells were measured in terms of the rate of degradation of *p*-nitrophenyl phosphate, as described previously [50] with slight modifications. *Synechocystis* cells were inoculated into fresh BG11 medium at an $OD_{730nm}$ of 0.2 and grown for 24 h under standard growth conditions. Then 200 µl of the culture were added to 700 µl of 285 mM CAPS buffer (pH 9.5) and 100 µl of 36 mM *p*-nitrophenyl phosphate was added to start the reaction, at 35°C. The reaction was stopped by the addition of 100 µl of 4 M NaOH, and then the samples were centrifuged to remove cells. The absorbance of the supernatant at 410 nm was measured in a UV-1700 spectrophotometer (Shimadzu, Kyoto, Japan) and amount of the product (*p*-nitrophenol) was calculated from a standard curve of *p*-nitrophenol in 0.4 M NaOH solution. The chlorophyll *a* content of cultures was measured as described previously [51]. AP activity was determined in terms of the concentration of *p*-nitrophenol (µmol $ml^{-1}$), the chlorophyll *a* content (µg $ml^{-1}$), and the reaction time (min).

**Quantification of the expression levels of genes for Hik33n-SphSc or its derivatives**

*Synechocystis* cells were inoculated into fresh BG11 medium at $OD_{730nm}$ of 0.1 and the

cells were grown for 16 h under standard growth conditions. *Synechocystis* cells were harvested in the same way for Northern blotting analysis and total RNA was isolated using TRIzol Max Bacterial RNA Isolation Kit (Life Technologies Japan, Tokyo) according to the manufacture's instructions. The isolated total RNA was treated with DNase I as above. To obtain the cDNA, total RNA was reverse transcribed by PrimeScript RT reagent Kit (Perfect Real Time) (Takara Bio). Expression levels of gene for Hik33n-SphSc or its derivatives were quantified by real-time PCR with the cDNAs as templates and primer pair SphSc_RT_F plus SphSc_RT_R (Table 6) using GoTaq qPCR Master Mix (Promega, Tokyo, Japan) and StepOnePlus Real-Time PCR system (Life Technologies Japan). The value was corrected by expression level of the internal standard gene, *rnpB*, using primer pair rnpB_RT_F plus rnpB_RT_R (Table 6).

**Bacterial two-hybrid analysis**

Bacterial two-hybrid analyses were carried out using BacterioMatch II Two-Hybrid System Vector Kit (Agilent Technologies, Santa Clara, CA). DNA fragments corresponding to the PAS domain of the coding sequence of *hik33* were amplified by PCR with pYS6, pYS14 or pYS16 as template and primer pairs Hik33PAS_F_BamHI plus Hik33PAS_R_XhoI (Table 7). The amplified fragments were cloned into pMD19 (Takara Bio). Fragments corresponding to the PAS domain were excised from the plasmids by digestion with *Bam*HI and *Xho*I and then inserted into pBT and pTRG, after cleavage by *Bam*HI and *Xho*I, to generate pBT_hik33_PAS, pBT_hik33_PAS_D300A, pBT_hik33_PAS_W318A, pTRG_hik33_PAS, pTRG_hik33_PAS_D300A, and pTRG_hik33_PAS_W318A (Table 4). To obtain the strains for protein-protein interaction assay, we transformed BacterioMatch II reporter strain with the pBT variants and the pTRG variants. Transformations of the reporter strain and the subsequent protein-protein interaction assays were carried out according

to the instruction provided by the manufacturer.

**Prediction and modeling of tertiary structure of PAS domain**

Amino acids sequence of PAS domain of the Hik33 (A283 to E418) was used for the query sequence. Tertiary structure of the PAS domain was predicted using phyre server (http://www.sbg.bio.ic.ac.uk/~phyre/) [52]. The predicted structure was visualized using open-source software PyMOL (http://www.pymol.org/).

# Results

**Deletion of Hik33 could not be functionally complemented by reintroduction of *hik33* gene**

I first attempted to identify the subdomains of the N-terminal region of Hik33 that are important for the perception of stress and the regulation of kinase activity by replacing the native *hik33* gene with a series of modified *hik33* genes *in vivo*. To express modified forms of Hik33, I deleted *hik33* gene by replacing the entire coding region with a kanamycin-resistance gene cassette and a conditional suicide gene, *sacB*, by homologous recombination via the sequences upstream and downstream of the *hik33* locus. Then, we replaced the kanamycin-resistance gene cassette and the *sacB* gene with the full-length *hik33* gene together with a spectinomycin-resistance gene cassette, for complementation (Figure 1A). *Synechocystis* has multiple chromosomes, and I confirmed segregation of the modified chromosomes from the native chromosomes by PCR and analysis of the tolerance to sucrose due to complete loss of the levansucrase that is encoded by the *sacB* gene (data not shown).

In wild-type cells of *Synechocystis* the expression of *hliB* was induced by salt stress, whereas it was not induced in the *hik33*-deleted cells. These results are in accordance with previously reported phenotypes of cells with mutations in the *hik33* gene (Figure 1B) [22]. However, *hik33*-complemented cells did not respond to salt stress to the same extent as the *hik33*-deleted cells (Figure 1B), even when the genetic structure of the *hik33* gene was equivalent to that of native *hik33* with the exception of the insertion of the spectinomycin-resistance gene cassette downstream of the Hik33-coding sequence (Figure 1A). As reported previously, the Hik33 ortholog in *Synechococcus elongatus* PCC 7942 (hereafter, *Synechococcus*), NblS, cannot be deleted completely without loss of viability [53,54], whereas the *hik33* gene can be deleted (see results of this study and those of [17]). This implies that Hik33 is not essential in *Synechocystis*. In addition, I determined the sequences of the *rre26*

(*slr0947*) and *rre31* (*slr0115*) genes, which encode cognate response regulators of Hik33, in the *hik33*-deleted cells, and found that these sequences did not include any mutations (data not shown). Although Hik33 is a negative regulator that represses the expression of the *hliB* gene under standard growth conditions [55], deletion of the *hik33* gene resulted in total loss of the expression of *hliB*. This result suggests that unexpected mutation(s) that suppress Hik33 activity or the expression of Hik33-regulated genes might occur in *hik33*-deleted cells.

**_hik33_-deleted cells had SNPs**

In an attempt to determine the reason why complementation of the *hik33* gene could not rescue induction of expression of the *hliB* gene under salt stress, I sequenced genomic DNAs of the wild-type strain that have been maintained in my laboratory and the mutants (i.e., two *hik33*-deleted strains, a *hik33*-disrupted strain [17], and a *hik33*-complemented strain), and then compared those. In two *hik33*-deleted strains, the *hik33* genes were deleted by kanamycin-resistance gene cassette and *sacB* gene or by only kanamycin-resistance gene cassette. These results indicated that there was a single point mutation in the *slr1753* gene in the wild-type chromosome comparing the sequence of the gene in *hik33*-deleted strains, which was same as the sequence deposited in the database, Cyanobase [56] (current URL of Cyanobase is http://genome.kazusa.or.jp/cyanobase/). Recently *Synechocystis* genome has been resequenced, and some differences in sequence have been founded [57]. However, any differences in the *slr1753* sequence have not been reported. Slr1753 is annotated as a hypothetical protein, so the function is unknown. Except the *slr1753* there are no difference in open reading frames commonly observed between wild type and *hik33*-deleted strains. In this resequencing experiment it was difficult to determine insertion-deletion mutations, because of each read was so short in this sequencer. It has

been reported that *Synechocystis* cells have several active insertional sequences (IS) in the genome [7,58]. At this moment I can not eliminate the possibility that transposition of these IS might occur in the cells of *hik33*-deleted strains and that might cause suppression of Hik33-regulated genes. Hence I thought the signal-input domain of Hik33 could not be investigated by expression of modified Hik33 *in vivo*. Then, instead of that, I decided to develop a chimeric sensor system to study function of N-terminal subdomains of Hik33 *in vivo*.

**A chimeric construct Hik33n-SphSc regulates expression of the *phoA* gene under stress conditions**

I introduced a chimeric gene for Hik33n-SphSc that encoded the N-terminal region of Hik33 and the C-terminal region of SphS, a phosphate-sensing histidine kinase, by substitution with the coding region of the *sphS* gene within the chromosomes of *Synechocystis* (Figure 2A). SphS regulates the expression of so-called *pho*-regulon, including the genes for acclimation to phosphate-deficient conditions, i.e., periplasmic alkaline phosphatase (AP) and phosphate transporters [59]. SphS is not essential for *Synechocystis* cells, particularly under phosphate-sufficient conditions, such as when growing in standard BG-11 medium [59], and modifying the *sphS* gene is a simple and very useful way to investigate the functions of histidine kinase [47]. The very low level of expression of SphS, which cannot be detected at the protein level [47], is also a benefit of the chimeric sensor system, because the overproduction of the components of signaling pathway, i.e., histidine kinases, perturb the signaling pathways [60,61]. These features of histidine kinase have been useful for screening genes for histidine kinases. Indeed, the *sphS* and *sasA* genes from *Synehococcus* were identified by multicopy suppression of the mutation of certain histidine kinases in *E. coli* cells [50,62]. The gene for Hik33n-SphSc, integrated into the chromosomes, was expressed from the original

promoter of the *sphS* gene and the copy number of each chimeric gene was exactly one per chromosome. I made the assumption that Hik33n-SphSc would be expressed at the same level as the original SphS, and that the chimeric histidine kinase Hik33n-SphSc would respond to stimuli that are perceived by Hik33, given that the N-terminal region was derived from Hik33, and that it would regulate kinase activity, given that its C-terminal region was derived from SphS (Figure 2A). The amount of SphS protein in wild-type cells is quite low, as reported by Kimura *et al.* [47], so it is difficult to determine the levels of SphS with a specific antibody in concentrated cell extracts and, even, in purified thylakoid or plasma membranes. As previously reported [47], I could not detect the SphS and Hik33n-SphSc proteins in both the wild type cells and the cells that harbored the gene for Hik33n-SphSc using the SphS-specific antibody. Therefore, I measured the transcript levels of *sphS* and *hik33n-sphSc* genes by quantitative reverse transcription PCR in these cells of wild type and the cells that harbored the gene for Hik33n-SphSc, respectively. Results revealed a ratio of the transcript of *hik33n-sphSc* to that of *sphS* as 1.1 ± 0.1 (data not shown), indicating that expression levels of these genes might be equal. I, then, assessed the activity of Hik33n-SphSc by measuring levels of expression of the gene, *phoA* for periplasmic alkaline phosphatase, which is normally regulated by the SphS/SphR two-component system in wild-type cells. I measured the transcript levels of the *phoA* gene by Northern blotting analysis under standard growth conditions and salt or cold stress (Figure 2B). Under standard growth conditions, cells possessing the gene for Hik33n-SphSc expressed the *phoA* gene, whereas the expression was repressed under both salt and cold stress (Figure 2B). Neither the wild-type nor the *sphS*-deleted cells expressed the *phoA* gene under any conditions (data not shown). These results suggest that, as predicted, Hik33n-SphSc regulates the expression of the *phoA* gene in accordance with Hik33-response conditions.

I also examined the expression levels of the *hliB* gene, which is regulated by native Hik33, in cells transformed with Hik33n-SphSc. Cells that expressed Hik33n-SphSc did not have any modifications at the *hik33* locus, so the *hliB* gene was regulated by Hik33, as in wild-type cells. The expression of *phoA* and *hliB* gene showed opposite profiles (Figure 2B), likely due to differences in the characteristics of the cognate response regulators of Hik33 and Hik33n-SphSc. RpaB, which is the cognate response regulator of NblS, is phosphorylated under standard growth conditions and represses the expression of stress-inducible genes in *Synechococcus*, whereas RpaB is unphosphorylated by NblS under stress conditions, such that the expressions of the stress-inducible genes are induced [63] (Figure 3). Thus, Rre26 of *Synechocystis* appears to be similar to that of *Synechococcus*. Conversely, SphR, which is the cognate response regulator of SphS, is unphosphorylated under standard growth conditions in wild-type cells, and the unphosphorylated SphR does not induce expression of the *phoA* gene [59]. Once the cells are exposed to phosphate-deficient conditions, SphS phosphorylates SphR, and the phosphorylated SphR induces expression of the *phoA* gene [59]. The native Hik33 and Hik33n-SphSc might phosphorylate Rre26 and SphR, respectively, under standard growth conditions, whereas the proteins may be dephosphorylated under salt or cold stress in cells expressing Hik33n-SphSc (Figure 3). These observations indicate that the regulation of the kinase activities of native Hik33 and Hik33n-SphSc might be similar, and that Hik33n-SphSc could mimic the regulatory mechanism of Hik33. Thus, I concluded that Hik33n-SphSc is a useful tool for studying the N-terminal region of Hik33.

**Activity of Hik33n-SphSc is restored with time under stress**

In order to investigate detailed behavior of Hik33n-SphSc under stress conditions, I measured time course of expression of the *phoA* gene by Northern blotting analysis

under salt stress. Once Hik33n-SphSc cells were exposed to salt stress, expression of the *phoA* gene was decreased, and expression level of *phoA* gene in Hik33n-SphSc cells took minimum level at 30 min after the cells exposed to salt stress (Figure 4). Then, expression levels of the *phoA* gene were restored gradually (Figure 4). Two hours after exposure of the cells to salt stress, expression level of the *phoA* gene was restored to approximately 70% of that under standard growth conditions (Figure 4). Using the same RNA samples, I determined time course of expression of the *hliB* gene under salt stress. The expression profile of the *hliB* gene showed inverse correlation to the expression profile of the *phoA* gene (Figure 4). These results indicate that Hik33n-SphSc is active under the standard growth conditions and is inactivated transiently under the stress conditions and the activity is restored with time similar to Hik33. In addition, I also measured degradation rate of transcripts of the *phoA* after inhibition of the transcription by rifampicin. Transcripts of the *phoA* gene were degraded immediately after inhibition of transcription, and 10 min after the inhibition transcripts of *phoA* gene were disappeared (Figure 5). On the other hand transcripts of the *phoA* gene were not completely disappeared under salt stress even in the minimum level at 30 min (Figure 4). These results indicate that Hik33n-SphSc is not absolutely inactivated under salt stress. Activity of Hik33n-SphSc seems to be fine-tuned under the stress conditions.

**Expression of the *phoA* gene is regulated by Hik33n-SphSc even in *hik33*-deleted cells or *ssl3451*-deleted cells.**

In general, Hik works as a dimer form, so probably Hik33n-SphSc also works as a dimer form in the cells. In my chimeric kinase system both Hik33n-SphSc and the native Hik33 are expressed from different loci of the chromosome, thus, it is thought that both kinases which have same sequences at the N-terminal of them may form heterologous dimer. Thereby I decided to confirm whether Hik33n-SphSc works in

homodimer or forms a heterodimer with native Hik33 in the cells. I deleted the native *hik33* gene in the Hik33n-SphSc introduced cells. The *hik33* disruptant with Hik33n-SphSc expressed the *phoA* gene under the standard growth conditions and the expression level was decreased under salt stress as well as in the cells introduced Hik33n-SphSc (Figure 6). It was concerned that the *hik33* was deleted in the mutant because expression of the *hliB* gene under salt stress was disappeared (Figure 6). These results might indicate that Hik33n-SphSc works as homodimer. In addition, I tested the effect of disruption of the *ssl3451* gene in Hik33n-SphSc cells. Ssl3451 is an accessory protein of Hik33, and it enhances the activity of phosphorylation of Hik33 by interacting with histidine kinase domain of Hik33 [48]. In *ssl3451*-deleted cells Hik33n-SphSc regulated expression of the *phoA* gene responding to salt stress similarly to *ssl3451* non-deleted cells (Figure 7). These results emphasize that Hik33n-SphSc may works independently from Hik33.

**Regulation of expression of the *phoA* gene under high osmolarity, high light and oxidative stress.**

It is known that Hik33 responds to not only salt and cold stress but also high light, high osmolarity and oxidative stress. Therefore I also investigated expression of the *phoA* gene under high light, high osmolarity and oxidative stress in the cells that was introduced the gene for Hik33n-SphSc. Expression level of the *phoA* gene was decreased under high osmolarity stress similarly to under salt and cold stress (Figure 8). However, under high-light stress, expression of the *phoA* gene was increased (Figure8). It have been reported that *phoA* gene is expressed under high-light stress [64] but the expression of the *phoA* gene under the high-light conditions have not been observed by other groups. It is hypothesized that an accessory protein, such as Ssl3451 for Hik33, exists and enhances the kinase activity of SphS under the high-light conditions.

Expression of the *phoA* gene under the high-light conditions might need C-terminal region of SphS. However, in this study, I could not identify any factors that enhance the Hik33n-SphSc activity. Overall, SphS/SphR pathway might be not suitable to study responsibility of the Hik33 to the high-light stress, and further studies using chimeric histidine kinase between N-terminal region of Hik33 and C-terminal region of histidine kinase other than SphS will be necessary to understand the mechanism of Hik33 that respond to the high-light stress. Oxidative stress was given by addition of hydrogen peroxide in the culture. However, in the presence of hydrogen peroxide, cellular RNA was degraded rapidly and I could not detect transcripts of the *phoA* gene 30 min after exposure to oxidative stress by Northern blot (Figure 8). Study regarding experimental condition for oxidative stress is required.

**Location on the membrane is essential for kinase activity**

Then I set out to elucidate which subdomains in the N-terminal region were important for regulating the kinase activity of Hik33 using cells expressing a series of Hik33n-SphScs with deleted or substituted subdomains. To investigate the function of the transmembrane helices of Hik33, I introduced a truncated gene that encoded ΔTM (Figure 9A) that lacked the membrane-localizing region from Hik33n-SphSc at the *sphS* locus and expressed the truncated gene from the *sphS* promoter. ΔTM lacks an N-terminal sequence that extends from the N-terminal Methionine residue to the end of TM2 (amino acid residues 1 through 219; Figure 9A). Cells transformed with the gene for ΔTM did not express the *phoA* gene under all conditions examined (Figure 9C). These results indicated that deletion of the transmembrane helices from Hik33n-SphSc eliminated the kinase activity almost completely. The transcript level of the gene for ΔTM was equivalent to that of the *sphS* gene in the wild-type cells (data not shown), thus, the level of the chimeric protein might be low and similar to that of native SphS.

However, I cannot rule out the possibility that the ΔTM protein was not expressed at a level required for the determination of its activity.

To investigate the roles of transmembrane helices in further detail, I constructed chimeric genes for $TM_{SphS}$ and $TM_{NrsS}$ that substituted the membrane-localizing region of Hik33n-SphSc with those from SphS [47] and NrsS, a $Ni^{2+}$-sensing histidine kinase in *Synchocystis* [45], respectively (Figure 9A). These TM regions localize proteins to the cell membrane (i.e., thet are membrane-localizing regions) [47]. The chimeric proteins $TM_{SphS}$ and $TM_{NrsS}$ contained the N-terminal membrane-localizing regions of SphS (amino acid residues 1 through 27) and of NrsS (amino acid residues 1through 209) fused to ΔTM, respectively. As in the case of the gene for Hik33n-SphSc, we introduced the genes for these variants of Hik33n-SphSc at the *sphS* locus and expressed them from the *sphS* promoter. The cells that expressed $TM_{SphS}$ or $TM_{NrsS}$ induced expression of the *phoA* gene under the standard growth conditions but repressed it under salt or cold stress, similar to the cells that expressed intact Hik33n-SphSc (Figure 9C). These results suggest that localization of Hik33n-SphSc on the membrane might be necessary for kinase activity *in vivo*, regardless of the actual sequence and number of transmembrane helices. According to previous observations, cold stress is perceived as a decrease in membrane fluidity (i.e. rigidification) by DesK and perception is dependent on the amino acid residues in the transmembrane helices [65]. The present my results suggest that the mechanism for sensing cold stress in Hik33 is different from that in DesK. However, the cells that harbored the gene for $TM_{SphS}$ or $TM_{NrsS}$ demonstrated approximately two-fold AP activity compared to cells that harbored the gene for Hik33n-SphSc (Figure 9B), suggesting that the transmembrane helices or the periplasmic loop region of Hik33 might play a role in control of the extent of kinase activity under standard growth conditions. However, truncated Hik33 that lacks a membrane-localizing region does

have kinase activity *in vitro* [48]. Thus, my observations provide insight into the *in vivo* function of the membrane-localizing region of Hik33.

The product of the *phoA* gene, AP, is localized in the periplasmic space in *Synechocystis* cells. It is likely that the AP in the periplasmic space is not degraded by proteases in the cytoplasm and that proteolytic activity might be absent from the periplasmic space. In fact, AP activity that was expressed is not immediately decreased when the transcription of the *phoA* gene is stopped. The half-life of the AP expressed in *Synechocystis* was approximately 24 h (data not shown). Therefore, AP activity was not suitable for assessment of immediate or short-term changes in the expression of *phoA* that was regulated by variants of Hik33n-SphSc. I measured AP activity only as an indicator of the activity of the variants of Hik33n-SphSc under standard growth conditions.

**Removal of the HAMP or PAS domains from Hik33n-SphSc influences its kinase activity**

I investigated, next, the functions of the periplasmic loop region and the cytosolic portions of the N-terminal region of Hik33. Specifically, I produced cells that harbored genes for a series of subdomain-deleted variants of Hik33n-SphSc as follows: Δloop, in which the periplasmic loop region was deleted; ΔHAMP, in which the HAMP domain was deleted; ΔPAS in which the PAS domain was deleted; and ΔHAMP/PAS, in which both the HAMP and the PAS domain were deleted (Figure 10A). These genes were introduced at the *sphS* locus and their expression were driven by the *sphS* promoter. The transcript levels of the modified chimeric genes were equivalent to that of the *sphS* gene in the wild-type cells (data not shown), thus, the level of the modified chimeric protein might be low and similar to that of the native SphS.

The Δloop, ΔHAMP, and ΔPAS-expressing cells expressed *phoA* under

standard growth conditions, and the level of expression of *phoA* fell under salt and cold stress, as also occurred in Hik33n-SphSc-expressing cells. However, the ΔHAMP/PAS cells did not express *phoA* under any condition examined (Figure 10C). Removal of both the HAMP and the PAS domain might drastically change the structural conformation of Hik33n-SphSc. Thus, ΔHAMP/PAS might not form dimers or might be unstable inside cells, and these defects might explain the loss of the kinase activity. Δloop-expressing cells had similar AP activity to that of cells that expressed Hik33n-SphSc. This observation suggests that the periplasmic loop region might not be required for the regulation of kinase activity under standard growth conditions and, therefore, that the aforementioned elevated kinase activities of $TM_{SphS}$ and $TM_{NrsS}$ might be due to substitutions of the transmembrane helices. Meanwhile, both the ΔHAMP and ΔPAS cells had significantly elevated in AP activity as compared to cells that expressed intact Hik33n-SphSc (Figure 10C). These results indicate that the HAMP and PAS domains are involved in the elevated kinase activity of Hik33 or the depressed phosphatase activity of Hik33.

The ΔHAMP construct had characteristics similar to those of Hik33n-SphSc when the latter's membrane-localizing region was that of SphS or of NrsS (Figures 9B, 9C, 10B, and 10C). In general, the HAMP domain consists of two amphipathic α-helices that form a bundle [32], and most of the HAMP domain is located adjacent to the transmembrane helix [31]. The structure and location of the HAMP domain indicate that this domain transmits a signal from outside the cell or at the membrane via transmembrane helices. The transmembrane helices and HAMP domain in Hik33 might work cooperatively.

**Substitutions of amino acid residues in the PAS domain influence kinase activity**

Even though the HAMP domain and the transmembrane helices appeared to play roles

in the kinase activity of Hik33, I focused on the PAS domain in this study because NblS, an ortholog of Hik33 in *Synechococcus*, was originally identified from a mutation in the PAS domain-coding sequence of the gene. The mutation results in an amino acid substitution in the PAS domain (G379D), and *Synechococcus* with mutated NblS does not undergo bleaching under nutritional starvation conditions [53]. Therefore, we attempted to identify other mutations in the PAS domain that would affect the function of Hik33 and, thus, to identify the residues in the PAS domain that are important for the regulation of its kinase activity. I introduced genes that encoded substitutions of amino acid residues in the PAS domain of Hik33n-SphSc, focusing on the residues that are well conserved among the orthologs of Hik33 (Figure 11). I introduced these genes at the *sphS* locus on the chromosome, under control of the *sphS* promoter, in the same way as we had introduced the gene for Hik33n-SphSc.

Most of the cell lines that harbored genes for point-mutated Hik33n-SphScs expressed *phoA* under standard growth conditions but expression of *phoA* was repressed under salt stress. This response was similar in all cell lines that harbored a gene for Hik33n-SphSc, with the exception of cells that harbored genes for D300A-, W318A- or R415E-mutated Hik33n-SphS (Figure 12 and 13). In the case of these three mutated forms of Hik33n-SphSs, AP activity was depressed (Figure 12 and 13), suggesting that these residues are important for full Hik33 kinase activity. In particular, among these three mutant lines, cells expressing R415E-mutated Hik33n-SphSc did not exhibit any AP activity. Thus the R residue might be the most important residue for the appropriate function of the PAS domain. We further analyzed expression levels of the genes for D300A-, W318A- or R415E-mutated Hik33n-SphS variants by quantitative reverse transcription PCR. The ratios of relative expression levels of these genes in the cells transformed with the mutated chimeric genes to expression level of *sphS* in the wild-type cells were approximately 1.0, 1.3, and 1.3 respectively (data not shown).

Removal of the PAS domain, including the residues that we mutated, did not decrease kinase activity (Figures 10B and 10C). Thus, the effects of deletion of the PAS domain and substitution of the three above-mentioned amino acid residues on the kinase activity of Hik33n-SphSc were different. Histidine kinase functions as a dimer and both the HAMP and PAS domains contribute to the formation of homodimers of the proteins containing each of the domains, i.e., these domains have been observed as dimers [32,37,38]. In addition, both the HAMP and the PAS domain are assumed to be involved in the regulation of activity of their parent protein via conformational changes under specific circumstances [37,38,39]. Given that the HAMP or PAS domain-deleted Hik33n-SphScs exhibited higher kinase activities than the intact form of Hik33n-SphSc, the HAMP and PAS domains might associate with themselves, and thereby could also affect the self association or conformation of the counterpart, thus exerting a negative effect on the kinase activity of the dimer form (Figure 14). I also confirmed dimerization of PAS domain of Hik33 *in vivo* by bacterial two-hybrid assay. D300A- or W318A-mutated PAS domain of Hik33 also makes dimer in the assay (data not shown). However the intensity of the dimerization could not be clearly determined by the assay. The D300A, W318A, and R415E mutations might interfere with dimerization of PAS domain but might not influence the inhibitory effect to the HAMP domain. Alternatively, since the PAS domain might accommodate two types of dimer [37,38], D300A, W318A, and R415E might change the dimeric configuration of the PAS domain, thereby enhancing the negative effect on kinase activity (Figure 14).

# Discussions

**Essentiality of Hik33 under photoautotrophic conditions**

Function of Hik33 homologues has been mainly investigated in two cyanobacteria species, *Synechocystis* and *Synechococcus* [2,16,23,53,54,55,63]. In *Synechococcus* NblS is thought to be a negative regulator of stress-inducible genes, however in *Synechocystis* it has been not clear how Hik33 regulate stress-inducible genes. In the present study, I attempted to investigate functions of the subdomains at signal-input domain of Hik33 by modifying the *hik33* gene *in vivo*, however I could not express the mutated forms of Hik33 because of that the *hik33*-deleted mutant could not be complemented (Figure 1). Although I could delete the *hik33* gene photoautotrophic condition in the present study, another group has reported that the *hik33* gene could not be deleted completely under photoautotrophic conditions, but it could be deleted photo-heterotrophic condition [23]. In addition, there have been some discrepancies in expression pattern of the Hik33 regulated genes between *hik33* mutants independently produced by different research groups [2,23]. One group has reported that a *hik33*-disrupted mutant, which had been transformed photoautotrophic conditions, did not express the *hli* genes under any conditions [2]. Consistent with those results, the *hik33*-deleted mutant, which was produced under photoautotrophic conditions in this study, did not express the *hliB* gene under any conditions I examined. However, Hsiao *et al.* have reported that the *hik33*-disrupted mutant, which had been transformed under photo-heterotrophic conditions, expressed all four *hli* genes under any light conditions [23]. Although I could not rescue phenotype of the *hik33*-deleted mutant by reintroduction of the native *hik33* gene, Hsiao *et al.* have reported that phenotype of the *hik33*-disrupted mutant in *Synechocystis* could be rescued by introduction of the native *nblS* gene of *Synechococcus* [23]. It has the possibility that the *hik33* gene is necessary under photoautotrophic conditions, and if the *hik33* genes in the chromosomes are almost disrupted under photoautotrophic conditions some unexpected mutation(s) that

compensate *hik33*-mutation might occur. Different from *Synechocystis*, the *nblS* gene in *Synechococcus* could not be disrupted [54,66]. *Synechocystis* has 127 genes for putative transposase [7,8] but *Synechococcus* has only 1 transposase and the number is exceptionally few among fresh water cyanobacteria [67]. Some putative transposases in *Synechocystis* actively transpose [58] and the uncharacterized mutations in the *hik33* mutant might be caused by the transpositions. Transposase number might be the reason of differences between the essentiality of Hik33 and NblS in the two cyanobacteria, *Synechocystis* and *Synechococcus*.

**Hik33 seems to be active under standard growth conditions and inactive under stress conditions**

Hik33 is thought to transfer the phosphate group to the cognate response regulators Rre26 (Slr0947) and Rre31 (Slr0115) [48]. The homologue of Rre26 is known to be RpaB (regulator of phycobilisome association) in *Synechococcus*, and Rre26 is also evolutionally conserved among cyanobacteria and some chloroplast of algae in red lineage as well as Hik33. Rre31 mutant could have been segregated, however, Rre26 mutant could not have been segregated [68]. Rre26 and RpaB have been reported to bind a motif, HLR1 [69]. The HLR1 sequence, TTACAA-$N_4$-TTACAA, is originally found at upstream of the *psbA2* gene in *Synechocystis* [69], then afterward redefined as a pair of imperfect direct repeat of (G/T)TTACA(T/A)(T/A) separated by two nucleotides [54]. HLR1 motif is a negative element required for repression of *psbA2* gene and *hliB* gene in *Synechocystis* [55,69] and *hliA* gene and *rpoD3* gene in *Synechococcus* [54,63,70] under the low-light conditions. Conversely HLR1 motif functions as positive element on photosystem I genes [71]. It is proposed that role of the HLR1 motif depends the location in the promoter sequence. Binding of Rre26/RpaB to HLR1 motif in core promoter region or 5'-untranslated region probably prevents

interaction between RNA polymerase and core promoter. On the other hand, binding of Rre26/RpaB to HLR1 motif in the upstream of the core promoter probably recruits RNA polymerase on the core promoter. Rre26 binds to the HLR1 motif which locates on the core promoter region of the *hliB* gene and repress the expression under the low-light conditions [55]. In addition, it have been indicated that the phosphorylated-RpaB binds to HLR1 in *Synechococcus* [63]. If the role of Rre26 might be the same as RpaB, *hik33* mutant should express *hliB* gene even under the low-light conditions. The results by Hsiao *et al.* [23] consist with this hypothesis, however the *hik33* mutant that I produced did not express *hliB* gene under any conditions (Figure 1). The *hik33* mutant which was produced under photoautotrophic conditions might contain extra suppressor mutation as I aforementioned. In addition, Hik33n-SphSc exhibits kinase activity under the standard growth conditions (Figure 2) supporting that Hik33 is active under the standard conditions as well as NblS. Regulatory role of Hik33 (NblS) and Rre26 (RpaB) seems to be conserved between *Synechocystis* and *Synechococcus*.

**Characteristics of the signal-input domain and the subdomains included in signal input domain of Hik33**

When SipA, an orthologue of Ssl3451, was discovered, a hypothesis had been proposed that internal signal, probably related with photosynthetic activities, modulate the interaction between SipA and NblS to acclimate stress conditions [72]. Meanwhile, a result that Hik33 could have responds to stress conditions in *ssl3451*-deleted mutant have indicated responsibility of Hik33 to the stress do not depend on interaction with Ssl3451 [48]. In the present study Hik33n-SphSc could respond to the salt, cold and hyperosmotic stress conditions as well as Hik33. These results suggest that Hik33 respond to these stress conditions via the function of the N-terminal signal-input domain, but not via the kinase domain, which associates with Ssl3451 (Figure 2 and 8).

Additionally I could characterize subdomains at signal-input domain of Hik33 by using Hik33n-SphSc. Although the membrane-binding region-deleted Hik33 and NblS have exhibited autokinase activity *in vitro* [48,66], the membrane-binding region-deleted Hik33nSphSc has no autokinase activity *in vivo* (Figure 9). Thus, the membrane-binding region seems to be necessary for autokinase activity *in vivo*. It has been observed that the membrane-binding region-truncated SphS do not exhibit any autokinase activity *in vivo* [47]. Because the amount of histidine kinases in a cell seems to be very low [47], the association with certain membrane may help to align the orientation of histidine kinase and to form dimer with suitable partner even in the very low concentration of the proteins.

Although periplasmic loop regions of Hik33 orthologues showed high conservativeness as well as other subdomains, the periplasmic loop region-deleted Hik33n-SphSc revealed no significant difference to intact Hik33n-SphSc (Figure 10). Periplasmic loop region of Hik33 seems to be redundant to sense at least cold and high salt conditions.

It has been reported that NblS, which did not contain both HAMP and PAS domain, have exhibited autokinase activity *in vitro* [66], whereas in accordance with my present results Hik33n-SphSc that was deleted both HAMP and PAS domain did not exhibit autokinase activity *in vivo* (Figure 10). HAMP and PAS domains are involved in dimerization of proteins [32,37,38], so it may be that both HAMP and PAS domain deleted-Hik33n-SphSc might not form dimer. Otherwise Hik33 might require either HAMP or PAS to take proper structure *in vivo*.

HAMP and PAS domains of Hik33 seem to be involved in the regulation of autokinase activity, because Hik33n-SphSc that was deleted HAMP domain or PAS domain revealed significantly higher autokinase activities than Hik33n-SphSc (Figure 10). It has been suggested that the HAMP and PAS domains might be involved in

phosphatase activity of NblS by *in vitro* experiments [66]. High autokinase activities of HAMP or PAS domain-deleted Hik33n-SphSc could also be explained less phosphatase activity. Distinction between the mutational effects on the kinase activity and the phosphatase activity necessitates further investigations. Hik33 might perceive some unidentified signal with either HAMP or PAS domain. HAMP domain had been thought as a domain that transmits signal from periplasmic region or membrane to cytosolic region [32], whereas recently it has been shown that HAMP domains of a fungal histidine kinase perceive hyperosmotic stress [73]. HAMP domain can perceive signals in some cases. Besides, PAS domains are known to perceive light [34], oxygen [35] and redox [36] signal. How HAMP and PAS domain of Hik33 perceive the signals is remained to be clarified, either HAMP or PAS domains seems to perceive the signals for Hik33.

**PAS domain might be involved in dimerization of Hik33**

In this study I especially focused on PAS domain of the Hik33. PAS domains frequently bind cofactors such as a flavin mononucleotide (FMN) [34], a flavin adenine dinucleotide (FAD) [36,74] or a heme [35], and cofactors may be required for the function of these PAS domains. However, there are no reports that the PAS domain of Hik33 binds any cofactors. Roles of the PAS domain of Hik33 might be different to those cofactor-binding type of PAS domains. Through the point mutation analysis D300, W318 and R415 were identified as important residue for the autokinase activity of Hik33 (Figure 12 and 13). In a predicted structure of the PAS domain of Hik33, D300 seems to locate dimerization surface of the PAS domain (Figure 15). In addition, G405, which is corresponding to G395 of NblS, located closely to D300 in this model (Figure 15). In the present my study, substitutions of G405 did not affect the autokinase activity of Hik33n-SphSc. Nevertheless, G395 might be involved in the dimerization of PAS

domain of NblS. I also revealed that the PAS domain of Hik33, even in D300A or W318A-mutated forms, formed dimers in the bacterial two-hybrid assay, but the intensity of the dimerization could not be clearly determined by the assay. Further studies are required to confirm that D300 has important roles in the dimerization.

**What is the signal for Hik33?**

It is known that expression of *hliA* and *hliB* gene expression is highly induced by addition of 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) than 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) [75]. Both DBMIB and DCMU are inhibitors of photosynthetic electron transport but their inhibitory mechanisms are different. DBMIB inhibits the electron flow from plastquinol to cytochrome $b_6/f$ complex then tends to reduce plastquinone pool. Whereas DCMU inhibits the electron flow from photosystem II to plastquinone at $Q_B$ site then tends to oxidize plastquinone pool. Hik33 responds to multiple stress conditions and several Hik33-regulating genes are induced by reduction of plastquinone pool than oxidation of plastquinone pool. These facts suggest that Hik33 might respond to reduction of plastquinone pool, which is caused by those multiple stress conditions. In the present study I found that the responsiveness of all variants of Hik33n-SphSc to salt and cold stress was quite similar. Therefore, I am not yet able to identify the effects of these stresses on Hik33n-SphS. It is possible that these stresses are integrated by a common stimulus, such over-reduction of plastquinone pool, in the cell, or that Hik33 perceives the stresses via the inhibition of self-assembly or conformational changes of the HAMP and the PAS domains, which enhances the inhibitory effects on kinase activity. Further studies are necessary to verify this hypothesis.

**Potency of the chimeric histidine kinase on studies of sensor proteins**

In this study I could reveal the usefulness of expression of chimera histidine kinase for studying Hik33, of which native gene could not be modified *in vivo*. Hik33n-SphSc has functional N-terminal region derived from Hik33 and functional C-terminal region derived from SphS. The modularity between the N-terminal region and the C-terminal region of a histidine kinase allows this technical strategy. Most histidine kinases have transmembrane domain(s) and, therefore, are located on the cytoplasmic membrane. *In vitro* analyses of membrane binding proteins are still limited by the difficulty to solubilize those proteins with its functions. Hik33 has a membrane-localizing region including two putative transmembrane helices, and its function had not been investigated even in the homologue, NblS. In this study I could investigate the function of transmembrane domain of Hik33 *in vivo* using chimeric histidine kinase. Producing chimeric protein is a powerful tool to investigate membrane-localizing regions of the sensor proteins.

I am now confident of that chimeric histidine kinase between two histidine kinases is also useful for study on the other histidine kinases, which could not be modified *in vivo*. It is known that there are some essential histidine kinases in bacteria. In *Synechocystis* 3 genes for histidine kinases (*hik2*, *hik11*, and *hik26*) could not be deleted [2], and it has been reported that in a gram-positive bacterium *Bacillus subtilis* a histidine kinase WalK (YycG) is essential for the cells [76]. These histidine kinases seem to have important roles in the organisms and involved in viability. Although biologists are highly interested in the function of these proteins, the studies on these essential histidine kinases have not been progressed because of the difficulty to modifying these proteins *in vivo*. I suppose that my chimeric kinase system can solve this difficulty.

# References

1. Stock AM, Robinson VL, Goudreau PN (2000) TWO-COMPONENT SIGNAL TRANSDUCTION. Annual Review of Biochemistry 69: 183-215.
2. Murata N, Suzuki I (2006) Exploitation of genomic sequences in a systematic analysis to access how cyanobacteria sense environmental stress. J Exp Bot 57: 235-247.
3. Blankenship RE (1992) Origin and early evolution of photosynthesis. Photosynth Res 33: 91-111.
4. Rippka R, Deruelles J, Waterbury JB, Herdman M, Stanier RY (1979) Generic Assignments, Strain Histories and Properties of Pure Cultures of Cyanobacteria. Journal of General Microbiology 111: 1-61.
5. Stanier RY, Kunisawa R, Mandel M, Cohen-Bazire G (1971) Purification and properties of unicellular blue-green algae (order Chroococcales). Bacteriol Rev 35: 171-205.
6. Williams JGK (1988) Construction of Specific Mutations in Photosystem-II Photosynthetic Reaction Center by Genetic-Engineering Methods in Synechocystis-6803. Methods in Enzymology 167: 766-778.
7. Kaneko T, Sato S, Kotani H, Tanaka A, Asamizu E, et al. (1996) Sequence analysis of the genome of the unicellular cyanobacterium Synechocystis sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. DNA Res 3: 109-136.
8. Kaneko T, Nakamura Y, Sasamoto S, Watanabe A, Kohara M, et al. (2003) Structural analysis of four large plasmids harboring in a unicellular cyanobacterium, Synechocystis sp. PCC 6803. DNA Res 10: 221-228.
9. Mizuno T, Kaneko T, Tabata S (1996) Compilation of all genes encoding bacterial two-component signal transducers in the genome of the cyanobacterium, Synechocystis sp. strain PCC 6803. DNA Res 3: 407-414.
10. Mizuno T, Wurtzel ET, Inouye M (1982) Cloning of the regulatory genes (ompR and envZ) for the matrix proteins of the Escherichia coli outer membrane. J Bacteriol 150: 1462-1466.
11. Hall MN, Silhavy TJ (1981) The ompB locus and the regulation of the major outer membrane porin proteins of Escherichia coli K12. J Mol Biol 146: 23-43.
12. Nixon BT, Ronson CW, Ausubel FM (1986) 2-Component Regulatory Systems Responsive to Environmental Stimuli Share Strongly Conserved Domains with the Nitrogen Assimilation Regulatory Genes Ntrb and Ntrc. Proceedings of the National Academy of Sciences of the United States of America 83: 7850-7854.
13. Skerker JM, Perchuk BS, Siryaporn A, Lubin EA, Ashenberg O, et al. (2008) Rewiring the specificity of two-component signal transduction systems. Cell 133: 1043-1054.
14. Yoshida T, Phadtare S, Inouye M (2007) The design and development of Tar-EnvZ chimeric receptors. Methods Enzymol 423: 166-183.

15. Moglich A, Ayers RA, Moffat K (2009) Design and signaling mechanism of light-regulated histidine kinases. J Mol Biol 385: 1433-1444.
16. Bartsevich VV, Shestakov SV (1995) The dspA gene product of the cyanobacterium Synechocystis sp. strain PCC 6803 influences sensitivity to chemically different growth inhibitors and has amino acid similarity to histidine protein kinases. Microbiology 141 ( Pt 11): 2915-2920.
17. Suzuki I, Los DA, Kanesaki Y, Mikami K, Murata N (2000) The pathway for perception and transduction of low-temperature signals in Synechocystis. EMBO J 19: 1327-1334.
18. Suzuki I, Kanesaki Y, Mikami K, Kanehisa M, Murata N (2001) Cold-regulated genes under control of the cold sensor Hik33 in Synechocystis. Mol Microbiol 40: 235-244.
19. Mikami K, Kanesaki Y, Suzuki I, Murata N (2002) The histidine kinase Hik33 perceives osmotic stress and cold stress in Synechocystis sp PCC 6803. Mol Microbiol 46: 905-915.
20. Paithoonrangsarid K, Shoumskaya MA, Kanesaki Y, Satoh S, Tabata S, et al. (2004) Five histidine kinases perceive osmotic stress and regulate distinct sets of genes in Synechocystis. J Biol Chem 279: 53078-53086.
21. Shoumskaya MA, Paithoonrangsarid K, Kanesaki Y, Los DA, Zinchenko VV, et al. (2005) Identical Hik-Rre systems are involved in perception and transduction of salt signals and hyperosmotic signals but regulate the expression of individual genes to different extents in synechocystis. J Biol Chem 280: 21531-21538.
22. Marin K, Suzuki I, Yamaguchi K, Ribbeck K, Yamamoto H, et al. (2003) Identification of histidine kinases that act as sensors in the perception of salt stress in Synechocystis sp. PCC 6803. Proc Natl Acad Sci U S A 100: 9061-9066.
23. Hsiao HY, He Q, Van Waasbergen LG, Grossman AR (2004) Control of photosynthetic and high-light-responsive genes by the histidine kinase DspA: negative and positive regulation and interactions between signal transduction pathways. J Bacteriol 186: 3882-3888.
24. Kanesaki Y, Yamamoto H, Paithoonrangsarid K, Shoumskaya M, Suzuki I, et al. (2007) Histidine kinases play important roles in the perception and signal transduction of hydrogen peroxide in the cyanobacterium, Synechocystis sp. PCC 6803. Plant J 49: 313-324.
25. Wang Q, Jantaro S, Lu B, Majeed W, Bailey M, et al. (2008) The high light-inducible polypeptides stabilize trimeric photosystem I complex under high light conditions in Synechocystis PCC 6803. Plant Physiol 147: 1239-1250.
26. Munekage Y, Hojo M, Meurer J, Endo T, Tasaka M, et al. (2002) PGR5 is involved in cyclic electron flow around photosystem I and is essential for photoprotection in Arabidopsis. Cell 110: 361-371.

27. Yeremenko N, Jeanjean R, Prommeenate P, Krasikov V, Nixon PJ, et al. (2005) Open reading frame ssr2016 is required for antimycin A-sensitive photosystem I-driven cyclic electron flow in the cyanobacterium Synechocystis sp. PCC 6803. Plant Cell Physiol 46: 1433-1436.
28. Puthiyaveetil S, Allen JF (2009) Chloroplast two-component systems: evolution of the link between photosynthesis and gene expression. Proc Biol Sci 276: 2133-2145.
29. Martin W, Stoebe B, Goremykin V, Hapsmann S, Hasegawa M, et al. (1998) Gene transfer to the nucleus and the evolution of chloroplasts. Nature 393: 162-165.
30. Duplessis MR, Karol KG, Adman ET, Choi LY, Jacobs MA, et al. (2007) Chloroplast His-to-Asp signal transduction: a potential mechanism for plastid gene regulation in Heterosigma akashiwo (Raphidophyceae). BMC Evol Biol 7: 70.
31. Aravind L, Ponting CP (1999) The cytoplasmic helical linker domain of receptor histidine kinase and methyl-accepting proteins is common to many prokaryotic signalling proteins. FEMS Microbiol Lett 176: 111-116.
32. Hulko M, Berndt F, Gruber M, Linder JU, Truffault V, et al. (2006) The HAMP domain structure implies helix rotation in transmembrane signaling. Cell 126: 929-940.
33. Ponting CP, Aravind L (1997) PAS: a multifunctional domain family comes to light. Curr Biol 7: R674-677.
34. Salomon M, Christie JM, Knieb E, Lempert U, Briggs WR (2000) Photochemical and mutational analysis of the FMN-binding domains of the plant blue light receptor, phototropin. Biochemistry 39: 9401-9410.
35. Monson EK, Weinstein M, Ditta GS, Helinski DR (1992) The FixL protein of Rhizobium meliloti can be separated into a heme-binding oxygen-sensing domain and a functional C-terminal kinase domain. Proc Natl Acad Sci U S A 89: 4280-4284.
36. Repik A, Rebbapragada A, Johnson MS, Haznedar JO, Zhulin IB, et al. (2000) PAS domain residues involved in signal transduction by the Aer redox sensor of Escherichia coli. Mol Microbiol 36: 806-816.
37. Lee J, Tomchick DR, Brautigam CA, Machius M, Kort R, et al. (2008) Changes at the KinA PAS-A dimerization interface influence histidine kinase function. Biochemistry 47: 4051-4064.
38. Ayers RA, Moffat K (2008) Changes in Quaternary Structure in the Signaling Mechanisms of PAS Domains. Biochemistry 47: 12078-12086.
39. Unnerstale S, Maler L, Draheim RR (2011) Structural characterization of AS1-membrane interactions from a subset of HAMP domains. Biochim Biophys Acta 1808: 2403-2412.

40. Los DA, Murata N (1999) Responses to cold shock in cyanobacteria. J Mol Microbiol Biotechnol 1: 221-230.
41. Martin M, Albanesi D, Alzari PM, de Mendoza D (2009) Functional in vitro assembly of the integral membrane bacterial thermosensor DesK. Protein Expr Purif 66: 39-45.
42. Reiser V, Raitt DC, Saito H (2003) Yeast osmosensor Sln1 and plant cytokinin receptor Cre1 respond to changes in turgor pressure. J Cell Biol 161: 1035-1040.
43. Cann M (2007) A subset of GAF domains are evolutionarily conserved sodium sensors. Mol Microbiol 64: 461-472.
44. Yamaguchi K, Suzuki I, Yamamoto H, Lyukevich A, Bodrova I, et al. (2002) A two-component Mn2+-sensing system negatively regulates expression of the mntCAB operon in Synechocystis. Plant Cell 14: 2901-2913.
45. Lopez-Maury L, Garcia-Dominguez M, Florencio FJ, Reyes JC (2002) A two-component signal transduction system involved in nickel sensing in the cyanobacterium Synechocystis sp. PCC 6803. Mol Microbiol 43: 247-256.
46. Tsinoremas NF, Kutach AK, Strayer CA, Golden SS (1994) Efficient gene transfer in Synechococcus sp. strains PCC 7942 and PCC 6301 by interspecies conjugation and chromosomal recombination. J Bacteriol 176: 6764-6768.
47. Kimura S, Shiraiwa Y, Suzuki I (2009) Function of the N-terminal region of the phosphate-sensing histidine kinase, SphS, in Synechocystis sp. PCC 6803. Microbiology 155: 2256-2264.
48. Sakayori T, Shiraiwa Y, Suzuki I (2009) A Synechocystis homolog of SipA protein, Ssl3451, enhances the activity of the histidine kinase Hik33. Plant Cell Physiol 50: 1439-1448.
49. Kiran MD, Annapoorni S, Suzuki I, Murata N, Shivaji S (2005) Cis-trans isomerase gene in psychrophilic Pseudomonas syringae is constitutively expressed during growth and under conditions of temperature and solvent stress. Extremophiles 9: 117-125.
50. Aiba H, Nagaya M, Mizuno T (1993) Sensor and regulator proteins from the cyanobacterium Synechococcus species PCC7942 that belong to the bacterial signal-transduction protein families: implication in the adaptive response to phosphate limitation. Mol Microbiol 8: 81-91.
51. Demarsac NT, Houmard J (1988) Complementary Chromatic Adaptation - Physiological Conditions and Action Spectra. Methods in Enzymology 167: 318-328.
52. Kelley LA, Sternberg MJ (2009) Protein structure prediction on the Web: a case study using the Phyre server. Nat Protoc 4: 363-371.
53. van Waasbergen LG, Dolganov N, Grossman AR (2002) nblS, a gene involved in controlling photosynthesis-related gene expression during high light and nutrient stress in Synechococcus elongatus PCC 7942. J Bacteriol 184: 2481-2490.

54. Kappell AD, Bhaya D, van Waasbergen LG (2006) Negative control of the high light-inducible hliA gene and implications for the activities of the NblS sensor kinase in the cyanobacterium Synechococcus elongatus strain PCC 7942. Arch Microbiol 186: 403-413.
55. Kappell AD, van Waasbergen LG (2007) The response regulator RpaB binds the high light regulatory 1 sequence upstream of the high-light-inducible hliB gene from the cyanobacterium Synechocystis PCC 6803. Arch Microbiol 187: 337-342.
56. Nakamura Y, Kaneko T, Hirosawa M, Miyajima N, Tabata S (1998) CyanoBase, a www database containing the complete nucleotide sequence of the genome of Synechocystis sp. strain PCC6803. Nucleic Acids Res 26: 63-67.
57. Tajima N, Sato S, Maruyama F, Kaneko T, Sasaki NV, et al. (2011) Genomic structure of the cyanobacterium Synechocystis sp. PCC 6803 strain GT-S. DNA Res 18: 393-399.
58. Okamoto S, Ikeuchi M, Ohmori M (1999) Experimental analysis of recently transposed insertion sequences in the cyanobacterium Synechocystis sp. PCC 6803. DNA Res 6: 265-273.
59. Suzuki S, Ferjani A, Suzuki I, Murata N (2004) The SphS-SphR two component system is the exclusive sensor for the induction of gene expression in response to phosphate limitation in synechocystis. J Biol Chem 279: 13234-13240.
60. Krall L, Reed JW (2000) The histidine kinase-related domain participates in phytochrome B function but is dispensable. Proc Natl Acad Sci U S A 97: 8169-8174.
61. Ehira S, Ohmori M (2006) NrrA directly regulates expression of hetR during heterocyst differentiation in the cyanobacterium Anabaena sp. strain PCC 7120. J Bacteriol 188: 8520-8525.
62. Nagaya M, Aiba H, Mizuno T (1993) Cloning of a sensory-kinase-encoding gene that belongs to the two-component regulatory family from the cyanobacterium Synechococcus sp. PCC7942. Gene 131: 119-124.
63. Seki A, Hanaoka M, Akimoto Y, Masuda S, Iwasaki H, et al. (2007) Induction of a group 2 sigma factor, RPOD3, by high light and the underlying mechanism in Synechococcus elongatus PCC 7942. J Biol Chem 282: 36887-36894.
64. Bhaya D, Vaulot D, Amin P, Takahashi AW, Grossman AR (2000) Isolation of regulated genes of the cyanobacterium Synechocystis sp. strain PCC 6803 by differential display. J Bacteriol 182: 5692-5699.
65. Cybulski LE, Martin M, Mansilla MC, Fernandez A, de Mendoza D (2010) Membrane thickness cue for cold sensing in a bacterium. Curr Biol 20: 1539-1544.

66. Lopez-Redondo ML, Moronta F, Salinas P, Espinosa J, Cantos R, et al. (2010) Environmental control of phosphorylation pathways in a branched two-component system. Mol Microbiol 78: 475-489.
67. Sugita C, Ogata K, Shikata M, Jikuya H, Takano J, et al. (2007) Complete nucleotide sequence of the freshwater unicellular cyanobacterium Synechococcus elongatus PCC 6301 chromosome: gene content and organization. Photosynth Res 93: 55-67.
68. Ashby MK, Mullineaux CW (1999) Cyanobacterial ycf27 gene products regulate energy transfer from phycobilisomes to photosystems I and II. FEMS Microbiol Lett 181: 253-260.
69. Eriksson J, Salih GF, Ghebramedhin H, Jansson C (2000) Deletion mutagenesis of the 5' psbA2 region in Synechocystis 6803: identification of a putative cis element involved in photoregulation. Mol Cell Biol Res Commun 3: 292-298.
70. Hanaoka M, Tanaka K (2008) Dynamics of RpaB-promoter interaction during high light stress, revealed by chromatin immunoprecipitation (ChIP) analysis in Synechococcus elongatus PCC 7942. Plant J 56: 327-335.
71. Seino Y, Takahashi T, Hihara Y (2009) The response regulator RpaB binds to the upstream element of photosystem I genes to work for positive regulation under low-light conditions in Synechocystis sp. Strain PCC 6803. J Bacteriol 191: 1581-1586.
72. Espinosa J, Fuentes I, Burillo S, Rodriguez-Mateos F, Contreras A (2006) SipA, a novel type of protein from Synechococcus sp. PCC 7942, binds to the kinase domain of NblS. FEMS Microbiol Lett 254: 41-47.
73. Meena N, Kaur H, Mondal AK (2010) Interactions among HAMP domain repeats act as an osmosensing molecular switch in group III hybrid histidine kinases from fungi. J Biol Chem 285: 12121-12132.
74. Bibikov SI, Biran R, Rudd KE, Parkinson JS (1997) A signal transducer for aerotaxis in Escherichia coli. J Bacteriol 179: 4075-4079.
75. Hihara Y, Sonoike K, Kanehisa M, Ikeuchi M (2003) DNA microarray analysis of redox-responsive genes in the genome of the cyanobacterium Synechocystis sp. strain PCC 6803. J Bacteriol 185: 1719-1725.
76. Fukuchi K, Kasahara Y, Asai K, Kobayashi K, Moriya S, et al. (2000) The essential two-component regulatory system encoded by yycF and yycG modulates expression of the ftsAZ operon in Bacillus subtilis. Microbiology 146 ( Pt 7): 1573-1583.

# Tables and Figures

Table 1. Primers used for the deletion or complementation of *hik33* during plasmid construction.

| Primer | Sequence (5'-3') |
|---|---|
| Hik33proFHind | *AAGCTT*CTAGCACCCACATGGG |
| Hik33proRNde | *CATATG*CTCCTTGCCAAAACTCCTAT |
| Hik33terFXho | *CTCGAG*TTTCGCTTTGACAACCCTGA |
| Hik33terR | *GAATAG*CGTAAATTTTCCAC |
| Kan2FNde | *CATATG*TCTCTTATACACATCTCA |
| Kan2RNhe | *GCTAGC*CACGGTTGATGAGAGCTTTG |
| sacBFNhe | *GCTAGC*AACCCATCACATATACCTGC |
| sacBRSal | *GTCGAC*ACCTTTATGTTGATAAGAAA |
| aHik33FNde | *CATATG*GGGACTTCTGTGTCCAA |
| bHik33RNco | *CCATGG*CCGGGGGAAAACAGCAA |
| HikFNco | *CCATGG*GCCTCAGGAAGAGGAGCAGG |
| HikRXho | *CTCGAG*CTAGCCCACCACCATCAACA |

Table 2. Primers used for producing cells expressing Hik33n-SphSc or its domain-deleted or domain-substituted variants during plasmid construction.

| Primer | Sequence (5'-3') |
|---|---|
| SphSHikvecF | GGTAGGGATCAAGCTTTTTC |
| SphSHikvecR | ATGCTAGCAGTTGCCTAGTC |
| Hik33_F_InF | GGCAACTGCTAGCATATGGGGACTTCTGTGTCCAA |
| Hik33_THP_R_InF | AGCTTGATCCCTACCGGCTTCATTTAATTCCACTT |
| Hik33_HAMP_F_InF | GACTAGGCAACTGCTAGCATATGCTAACTATTACCCA GCCCAT |
| Hik33_TM2_F | CTAACTATTACCCAGCCCAT |
| SphS_F_Inf | GGCAACTGCTAGCATATGGAAATAATTACATTGGC |
| SphS_TM_R_Inf2 | CTGGGTAATAGTTAGTTTATTATTGAGCCGAAACC |
| NrsS_F_Inf | GGCAACTGCTAGCATATGAATACCCGTCGCCTCTT |
| NrsS_TM2_R_Inf | CTGGGTAATAGTTAGTAAGCCCCAACTGGAGAAGG |
| Hik33_TH_R_InF | AGCTTGATCCCTACCCGCCTTCTCCGCAGTCAACT |
| Hik33_T_R_InF | AGCTTGATCCCTACCTAGGGCATTGAATACAGCTC |
| Hik33_TM1_R | ACGGGTATCCACCAATTGGG |
| Hik33_TM2_F_InF | TTGGTGGATACCCGTGATGTGACCATTGCAGTTTT |
| Hik33_TM2_R_InF | CTCTTCAATATTTTGTAGGGCATTGAATACAGCTC |
| Hik33_HAMP_F | CAAAATATTGAAGAGTTGAC |

Table 3. Primers used for producing cells expressing point-mutated Hik33n-SphScs during plasmid construction.

| Primer set | Primer | Sequence (5'-3') |
|---|---|---|
| 1 | 33PAS_D300A_F | TTAGTG*GCA*ACCAATTTGCAACTTTTG |
| | 33PAS_D300A_R | ATTGGT*TGC*CACTAACATGGCCCCATC |
| 2 | 33PAS_N309A_F | TTGGTC*GCA*CCCACTGCCCGTCGCCTA |
| | 33PAS_N309A_R | AGTGGG*TGC*GACCAACAAAAGTTGCAA |
| 3 | 33PAS_W318A_F | TTCGCC*GCA*GAAAATAAGCCAATTATT |
| | 33PAS_W318A_R | ATTTTC*TGC*GGCGAATAGGCGACGGGC |
| 4 | 33PAS_R377A_F | GAATTT*GCA*ATTAGCCTGACCCAACCG |
| | 33PAS_R377A_R | GCTAAT*TGC*AAATTCTTCCGGGGCGTA |
| 5 | 33PAS_R389A_F | ACCATT*GCA*CTGATGTTGACCCAGGTG |
| | 33PAS_R389A_R | CATCAG*TGC*AATGGTGCGGGGAAACGG |
| 6 | 33PAS_R404A_F | AATTTA*GCA*GGCATTGTCATGACGGTG |
| | 33PAS_R404A_R | AATGCC*TGC*TAAATTTTCCCTGTTCTG |
| 7 | 33PAS_G405S_F | TTACGG*TCC*ATTGTCATGACGGTGCAG |
| | 33PAS_G405S_R | GACAAT*GGA*CCGTAAATTTTCCCTGTT |
| 8 | 33PAS_G405A_F | TTACGG*GCA*ATTGTCATGACGGTGCAG |
| | 33PAS_G405A_R | GACAAT*TGC*CCGTAAATTTTCCCTGTT |
| 9 | 33PAS_V395A_F | ACCCAG*GCA*TTGGATCAGAACAGGGAA |
| | 33PAS_V395A_R | ATCCAA*TGC*CTGGGTCAACATCAGACG |
| 10 | 33PAS_T409V_F | GTCATG*GTG*GTGCAGGATATTACTAGG |
| | 33PAS_T409V_R | CTGCAC*CAC*CATGACAATGCCCCGTAA |
| 11 | 33PAS_Q411E_F | ACGGTG*GAA*GATATTACTAGGGAAGTG |
| | 33PAS_Q411E_R | AATATC*TTC*CACCGTCATGACAATGCC |
| 12 | 33PAS_D412N_F | GTGCAG*AAT*ATTACTAGGGAAGTGGAA |
| | 33PAS_D412N_R | AGTAAT*ATT*CTGCACCGTCATGACAAT |
| 13 | 33PAS_T414V_F | GATATT*GTG*AGGGAAGTGGAATTAAAT |
| | 33PAS_T414V_R | TTCCCT*CAC*AATATCCTGCACCGTCAT |
| 14 | 33PAS_R415E_F | ATTACT*GAA*GAAGTGGAATTAAATGAA |
| | 33PAS_R415E_R | CACTTC*TTC*AGTAATATCCTGCACCGT |
| 15 | 33PAS_E416Q_F | ACTAGG*CAA*GTGGAATTAAATGAAGGT |
| | 33PAS_E416Q_R | TTCCAC*TTG*CCTAGTAATATCCTGCAC |

Table 4. Plasmids used.

| Plasmid | Description | Reference or source |
|---|---|---|
| pT7Blue | Cloning vector, $Ap^r$ | Merck KGaA |
| pGEM-T easy | Cloning vector, $Ap^r$ | Promega |
| pAM1146 | Source of $Sp^r$ cassette, $Sp^r$ | Tsinoremas et al. (1994) |
| pSK5 | $P_{sphS}$-*SphS*$^+$, $Ap^r$, $Sp^r$ | Kimura et al. (2009) |
| pYS01 | $P_{hik33}$, $Ap^r$ | This study |
| pYS02 | $P_{hik33}$, *hik33*$^-$, *sacB*, $Ap^r$, $Km^r$ | This study |
| pYS03 | $P_{hik33}$, $Ap^r$, | This study |
| pYS04 | $P_{hik33}$-*hik33*$^+$, $Ap^r$ | This study |
| pYS05 | $P_{hik33}$-*hik33*$^+$, $Ap^r$, $Sp^r$ | This study |
| pYS06 | $P_{sphS}$-[*Hik33n-SphSc*]$^+$, $Ap^r$, $Sp^r$ | This study |
| pYS07 | $P_{sphS}$-[*Hik33n-SphScΔTM*]$^+$ , $Ap^r$, $Sp^r$ | This study |
| pYS08 | $P_{sphS}$-[*Hik33n-SphScTM*$_{sphs}$]$^+$ , $Ap^r$, $Sp^r$ | This study |
| pYS09 | $P_{sphS}$-[*Hik33n-SphScTM*$_{NrsS}$]$^+$, $Ap^r$, $Sp^r$ | This study |
| pYS10 | $P_{sphS}$-[*Hik33n-SphScΔloop*]$^+$, $Ap^r$, $Sp^r$ | This study |
| pYS11 | $P_{sphS}$-[*Hik33n-SphScΔHAMP*]$^+$ , $Ap^r$, $Sp^r$ | This study |
| pYS12 | $P_{sphS}$-[*Hik33n-SphScΔPAS*]$^+$, $Ap^r$, $Sp^r$ | This study |
| pYS13 | $P_{sphS}$-[*Hik33n-SphScΔHAMP/PAS*]$^+$, $Ap^r$, $Sp^r$ | This study |
| pYS14 | $P_{sphS}$-[*Hik33n-SphSc(D300A)*]$^+$, $Ap^r$, $Sp^r$ | This study |
| pYS15 | $P_{sphS}$-[*Hik33n-SphSc(N309A)*]$^+$, $Ap^r$, $Sp^r$ | This study |
| pYS16 | $P_{sphS}$-[*Hik33n-SphSc(W318A)*]$^+$, $Ap^r$, $Sp^r$ | This study |
| pYS17 | $P_{sphS}$-[*Hik33n-SphSc(R377A)*]$^+$, $Ap^r$, $Sp^r$ | This study |
| pYS18 | $P_{sphS}$-[*Hik33n-SphSc(R389A)*]$^+$, $Ap^r$, $Sp^r$ | This study |
| pYS19 | $P_{sphS}$-[*Hik33n-SphSc(R404A)*]$^+$, $Ap^r$, $Sp^r$ | This study |
| pYS20 | $P_{sphS}$-[*Hik33n-SphSc(R400S/R404A)*]$^+$, $Ap^r$, $Sp^r$ | This study |
| pYS21 | $P_{sphS}$-[*Hik33n-SphSc(V395A)*]$^+$, $Ap^r$, $Sp^r$ | This study |
| pYS22 | $P_{sphS}$-[*Hik33n-SphSc(G405S)*]$^+$, $Ap^r$, $Sp^r$ | This study |
| pYS23 | $P_{sphS}$-[*Hik33n-SphSc(G405A)*]$^+$, $Ap^r$, $Sp^r$ | This study |
| pYS24 | $P_{sphS}$-[*Hik33n-SphSc(T409V)*]$^+$, $Ap^r$, $Sp^r$ | This study |
| pYS25 | $P_{sphS}$-[*Hik33n-SphSc(Q411E)*]$^+$, $Ap^r$, $Sp^r$ | This study |
| pYS26 | $P_{sphS}$-[*Hik33n-SphSc(D412N)*]$^+$, $Ap^r$, $Sp^r$ | This study |
| pYS27 | $P_{sphS}$-[*Hik33n-SphSc(T414V)*]$^+$, $Ap^r$, $Sp^r$ | This study |
| pYS28 | $P_{sphS}$-[*Hik33n-SphSc(R415E)*]$^+$, $Ap^r$, $Sp^r$ | This study |
| pYS29 | $P_{sphS}$-[*Hik33n-SphSc(E416Q)*]$^+$, $Ap^r$, $Sp^r$ | This study |
| pYS30 | $P_{hik33}$, *hik33*$^-$, $Ap^r$, $Km^r$ | This study |

| | | |
|---|---|---|
| pBT_hik33_PAS | [Bacteriophage λ repressor-PAS], Cmr | This study |
| pBT_hik33_PAS_D300A | [Bacteriophage λ repressor-PAS(D300A)], Cmr | This study |
| pBT_hik33_PAS_W318A | [Bacteriophage λ repressor-PAS(W318A)], Cmr | This study |
| pTRG_hik33_PAS | [N-terminal domain of the α-subunit of RNA polymerase-PAS], Tcr | This study |
| pTRG_hik33_PAS_D300A | [N-terminal domain of the α-subunit of RNA polymerase-PAS(D300A)], Tcr | This study |
| pTRG_hik33_PAS_W318A | [N-terminal domain of the α-subunit of RNA polymerase-PAS(W318A)], Tcr | This study |

Table 5. Primers used for producing probes for Northern blotting.

| Gene | Primer | Sequence (5'-3') |
|---|---|---|
| *phoA* | phoA_int1_F | CGGGAATTGTGGCTGTATCT |
| | phoA_int1_R | TGCTGGACAAGAGCATTGAG |
| *hliB* | hlib-1544F | ATTGTGGGTTGGTTGCTCTC |
| | hlib-1544R | TAACCCCAGATGAAAGTGGC |
| *rnpB* | rnpBF | GAGAGTTAGGGAGGGAGTTG |
| | rnpBR | AGAGTTAGTCGTAAGCCGGG |

Table 6. Primers used for quantitative reverse transcription PCR

| Primers | Sequences (5'-3') |
|---|---|
| SphSc_RT_F | GGCCAACCAAGTTGAACCTA |
| SphSc_RT_R | ATCCCTTCTGTGCCTTGATG |
| rnpB_RT_F | GTAAGAGCGCACCAGCAGTATC |
| rnpB_RT_R | TCAAGCGGTTCCACCAATC |

Table 7. Primers used for generation of cells for the bacterial two-hybrid assay.

| Primer | Sequence (5'-3') |
|---|---|
| Hik33PAS_F_BamHI | GGATCCACGATCGCCGATGGGGCCAT |
| Hik33PAS_R_XhoI | CTCGAGAGTAATATCCTGCACCGTCA |

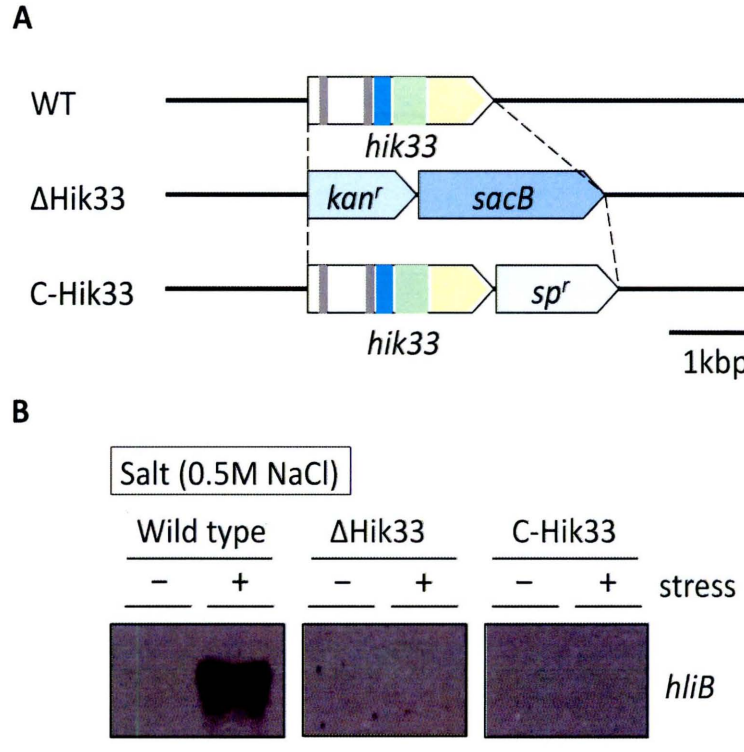


**Figure 1. Complementation of the *hik33* gene mutation by the native *hik33* gene could not rescue expression of the *hliB* gene.**

A) Schematic view of the *hik33* loci on the chromosomes of wild type, ΔHik33 and C-Hik33 cells. Segments and pentagons indicate chromosomes and genes respectively. B) Expression of the *hliB* gene expressions in wild type, ΔHik33 and C-Hik33 cells under the standard growth conditions (N) or the salt stress conditions (S) were analyzed by Northern blot.

ΔHik33: *hik33*-deleted cells, C-Hik33: *hik33*-complemented cells, *kan*$^r$: kanamycin-resistant gene cassette, *sp*$^r$: spectinomycin-resistant gene cassette, -: under non-stressed conditions, +: 30 minutes after exposure to 0.5 M NaCl for salt stress.

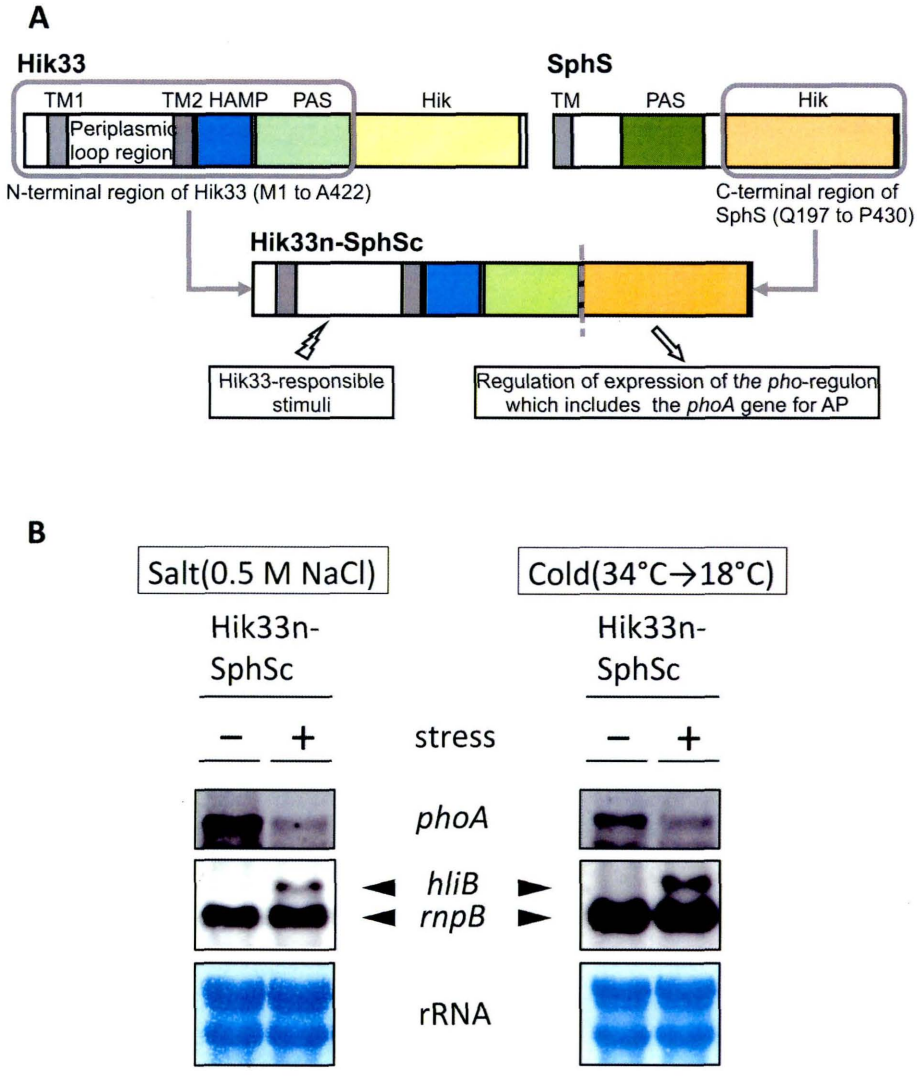


**Figure 2. A chimeric histidine kinase system.**

A) Schematic view of a chimeric histidine kinase, Hik33n-SphSc. Hik33n-SphSc is a chimeric protein between N-terminal region of Hik33 (M1 to A422) and C-terminal region of SphS (Q197 to P430). Each rectangle indicates length and domain organizations of the histidine kinases. Gray rectangles shows putative transmembrane domains (TM); rectangles between two putative transmembrane domains are periplasmic regions; blue rectangles are HAMP domains (HAMP); green rectangles are PAS domains (PAS); yellow or orange rectangles are histidine kinase domains (Hik).

B) Northern blotting analysis of the *phoA, hliB and rnpB* gene in the cells transformed a gene for Hik33n-SphSc. -: standard growth conditions, +: 30 minutes after exposure to 0.5 M NaCl for salt stress or 18°C for cold stress. rRNA: ribosomal RNAs stained by methylene blue.

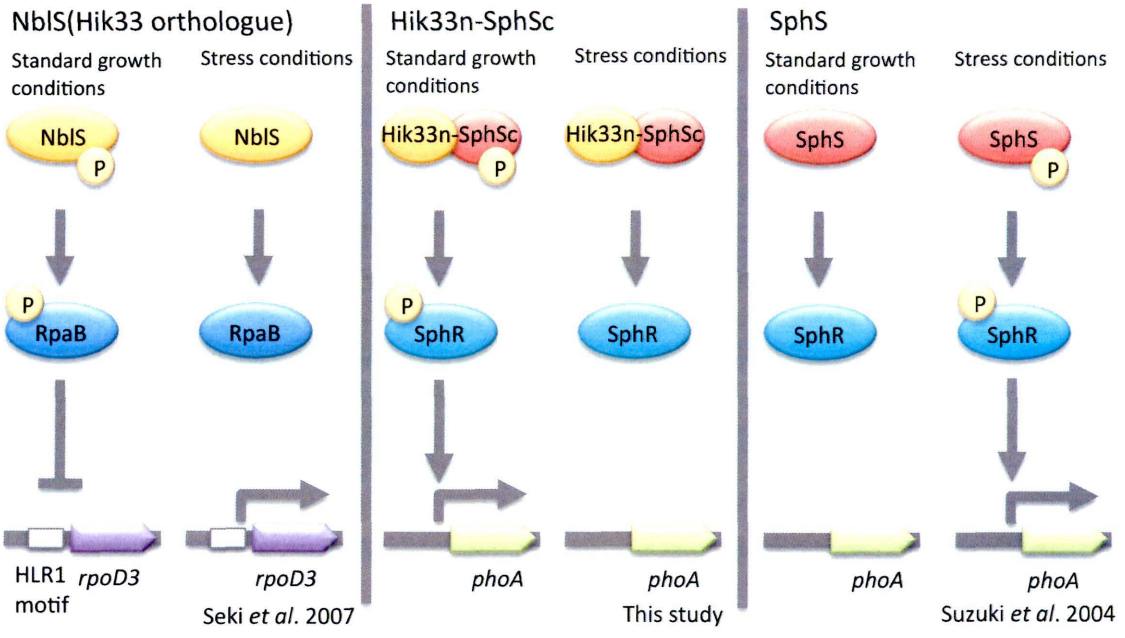


**Figure 3. Regulation of expression of the *phoA* gene by Hik33n-SphSc.**
NblS exhibit autokinase activity under standard growth conditions and phosphorylated RpaB repress expression of the *hliB* gene in *Synechococcus*. Contrariwise NblS is unphosphorylated under stress conditions, and then expression of the *hliB* gene is derepressed. SphS does not phosphorylate SphR under standard growth conditions and phosphorylates SphR under phosphate deficient stress. Then phosphorylated SphR induce expression of the *phoA* gene. Hik33n-SphSc exhibit autokinase activity under standard growth conditions, and the autokinase activity is decreased under Hik33-responsible stress conditions such salt or cold. Hik33n-SphSc phosphorylate SphR and thereby regulate expression of the *phoA* gene.

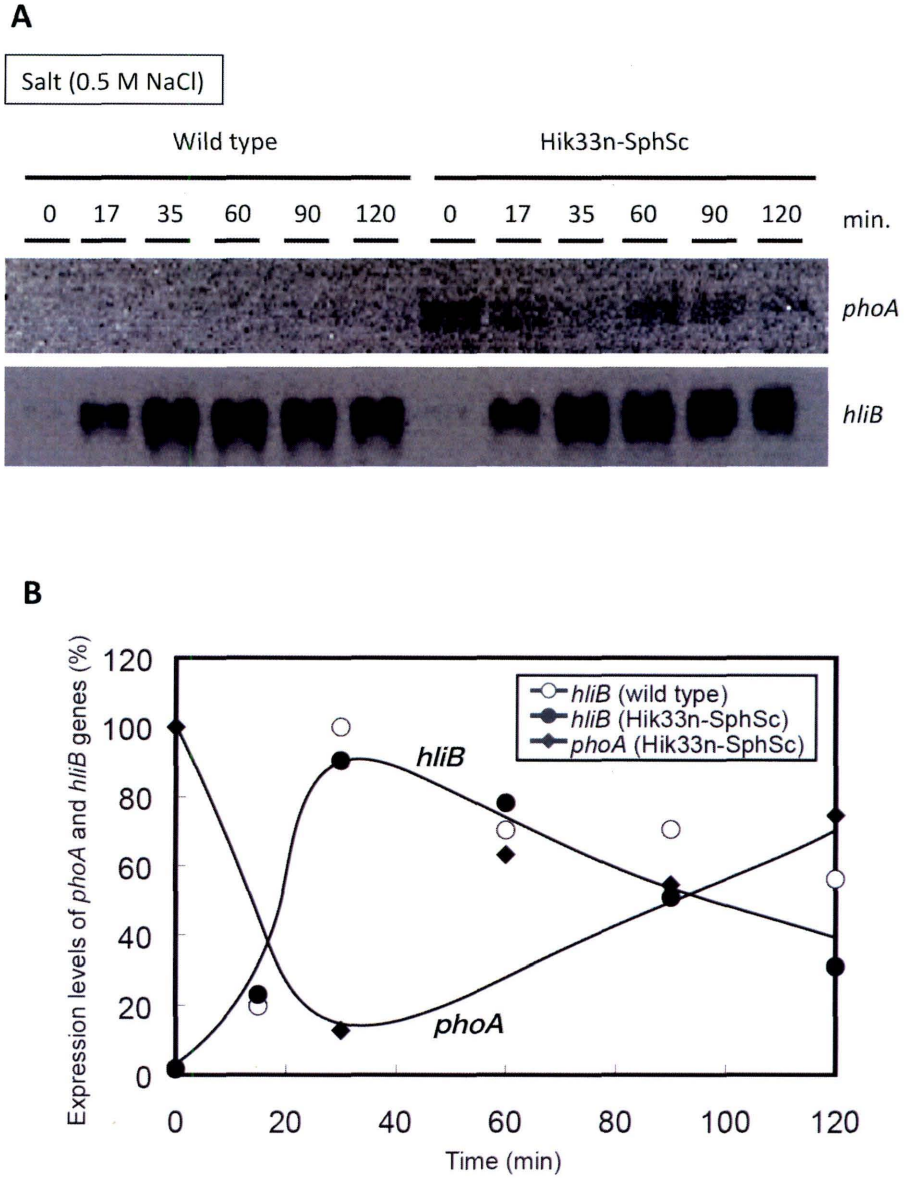


**Figure 4. Time course of expression of the *phoA* gene under salt stress.**

A) Northern blotting analysis of the *phoA* and *hliB* genes after exposure to 0.5 M NaCl for salt stress. Hik33n-SphSc: cells transformed a gene for Hik33n-SphSc.

B) Graph of relative expression levels of the *phoA* and *hliB* genes in wild type cells and the cells transformed a gene for Hik33n-SphSc after exposure to 0.5 M NaCl for salt stress.

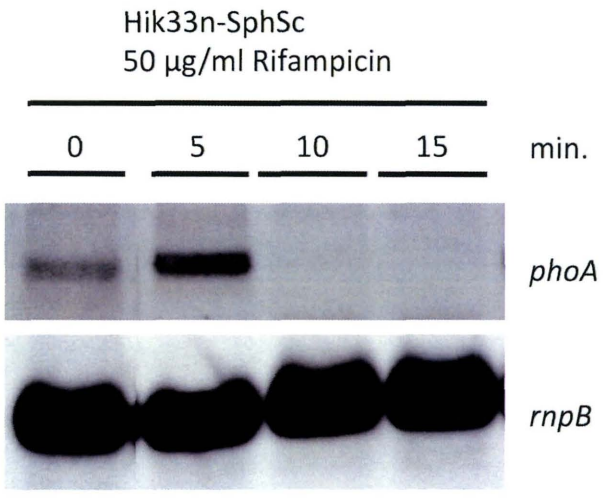


**Figure 5. Degradation rate of the *phoA* gene transcripts.**
Degradation of the *phoA* gene transcripts in the cells transformed a gene for Hik33n-SphSc after inhibition of transcription was analyzed by Northern blot. Transcription was inhibited by addition of rifampicin to the culture (50μg/ml).

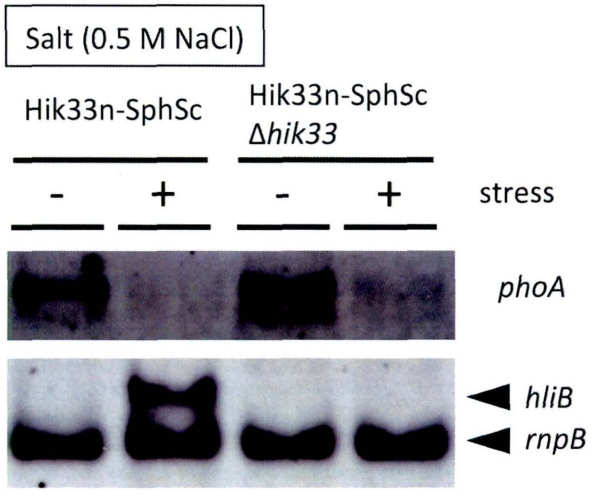


**Figure 6. Regulation of expression of the *phoA* gene by Hik33n-SphSc in *hik33*-deleted cells.**

Expression levels of the *phoA*, *hliB* and *rnpB* geness in the cells were analyzed by Northern blot. -: under non-stressed conditions, +: 30 minutes after exposure to 0.5 M NaCl for salt stress.

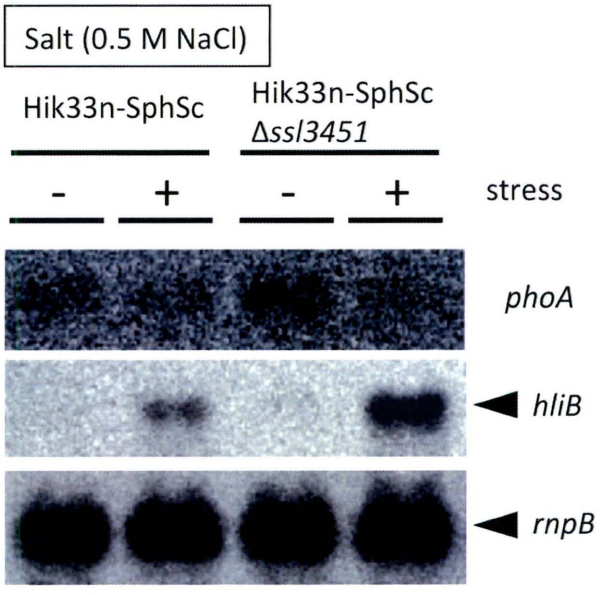


**Figure 7. Regulation of expression of the *phoA* gene by Hik33n-SphSc in *ssl3451*-deleted cells.**

Expression levels of the *phoA*, *hliB* and *rnpB* genes in the cells were analyzed by Northern blot. -: under non-stressed conditions, +: 30 minutes after exposure to 0.5 M NaCl for salt stress.

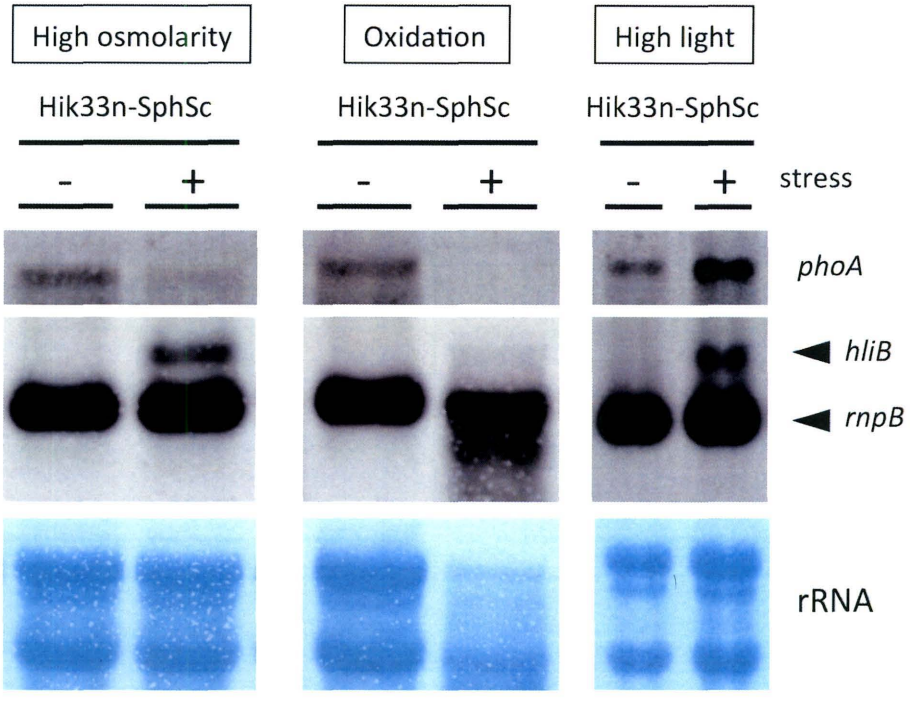


**Figure 8. Response of Hik33n-SphSc to hyperosmotic, oxidative and high light stresses.**

Northern blotting analysis of the *phoA, hliB and rnpB* gene in the cells transformed a gene for Hik33n-SphSc. -: standard growth conditions, +: 30 minutes after exposure to 0.5 M sorbitol for hyperosmotic stress, 250 μM $H_2O_2$ for oxidative stress or 500 μmol photons $m^{-2}$ $s^{-1}$ for high light stress. rRNA: ribosomal RNAs stained by methylene blue.

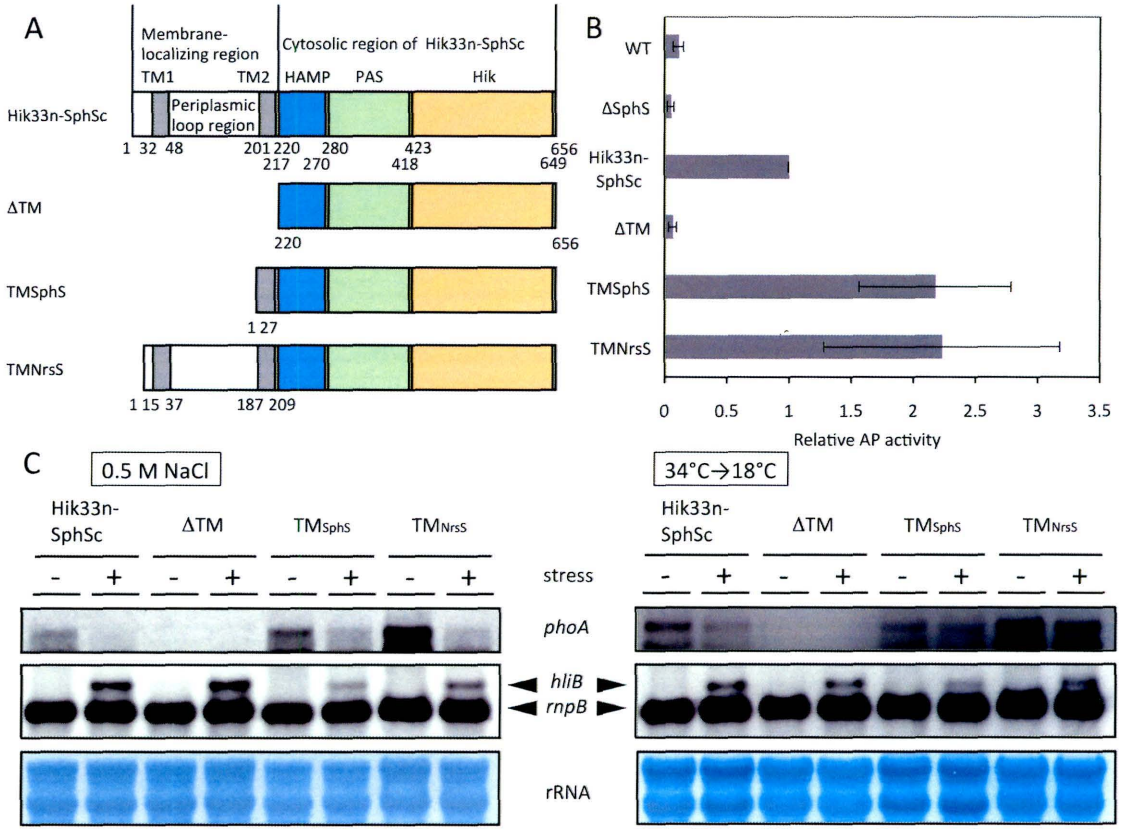


**Figure 9. Properties of membrane-localizing region-modified Hik33n-SphScs.**

A) Schematic view of membrane-localizing region-deleted or -substituted Hik33n-SphSc variants. ΔTM lacks M1 to A219 of Hik33n-SphSc. $TM_{SphS}$ and $TM_{NrsS}$ were fused the membrane-localizing regions from SphS (1 – 27) and NrsS (1- 209) with ΔTM.

B) AP activities of the cells of each variant (n=3). AP activities of each cells under standard growth conditions were measured three times. Average AP activity of Hik33n-SphSc (0.86 mmol/mg chlorophyll *a*/min) was defined as 1.0.

C) Expressions of the *phoA, hliB and rnpB* genes. The expression levels of the genes in the cells were analyzed by Northern blot. N: under non-stressed conditions, S: 30 minutes after exposure to 0.5 M NaCl for salt stress or 18°C for cold stress. rRNA: ribosomal RNAs stained by methylene blue.

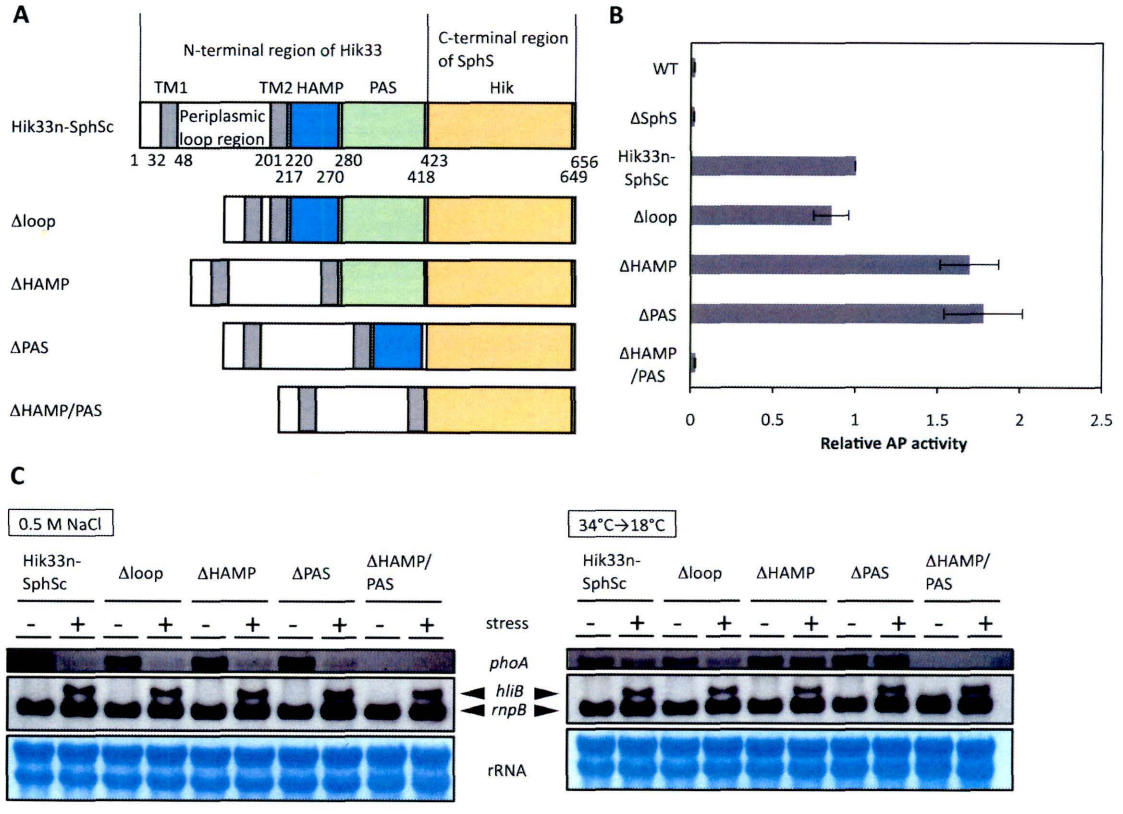


**Figure 10. Properties of the subdomain-deleted Hik33n-SphSc variants.**

A) Schematic view of subdomain-deleted Hik33n-SphSc variants. Δloop is periplasmic loop-deleted Hik33n-SphS which lacks F66 to R197. ΔHAMP is HAMP domain-deleted Hik33n-SphS which lacks T221 to A272. ΔPAS is PAS domain-deleted Hik33n-SphSc which lacks K284 to A422. ΔHAMP/PAS is both HAMP and PAS domain-deleted Hik33n-SphSc which lacks T221 to A422.

B) AP activities of the cells of each variant (n=3). AP activities of each cells under standard growth conditions were measured three times. Average AP activity of Hik33n-SphSc (2.02 mmol/mg chlorophyll *a*/min) was defined as 1.0.

C) Expressions of the *phoA, hliB and rnpB* genes. The expression levels of the genes in the cells were analyzed by Northern blot. N: under non-stressed conditions, S: 30 minutes after exposure to 0.5 M NaCl for salt stress or 18°C for cold stress. rRNA: ribosomal RNAs stained by methylene blue.

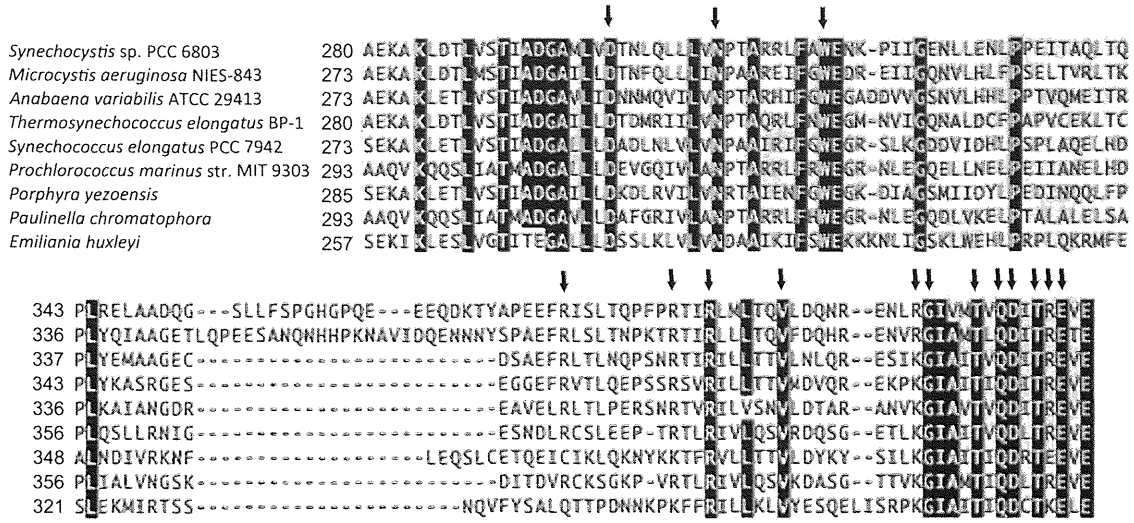


**Figure 11. Alignment of the PAS domains of Hik33 homologues from cyanobacteria and plastid genomes.**

Alignment was generated by ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and visualized by the BoxShade program version 3.21 (http://www.ch.embnet.org/software/BOX_form.html). Highly conserved residues were shaded. Arrows indicate amino acid residues which were substituted in this study, and bold arrows indicate amino acid residues significantly affected to activity of Hik33n-SphSc when substituted (D300, W318, R415). *Synechocystis* sp. PCC 6803 (Genbank accession: BAA16687), *Mycrocystis aeruginosa* NIES-843 (BAG03430), *Anabaena variabilis* ATCC 29413 (ABA22834), *Thermosynechococcus elongatus* BP-1 (BAC07989), *Synechococcus elongatus* PCC 7942 (ABB56954), *Prochlorococcus marinus* str. MIT 9303 (ABM77297), a red alga *Porphyra yezoensis* (BAE92516.1), a filose testate amoeba *Paulinella chromatophora* (ACB43125), a haptophyta *Emiliania huxleyi* (AAX13891).

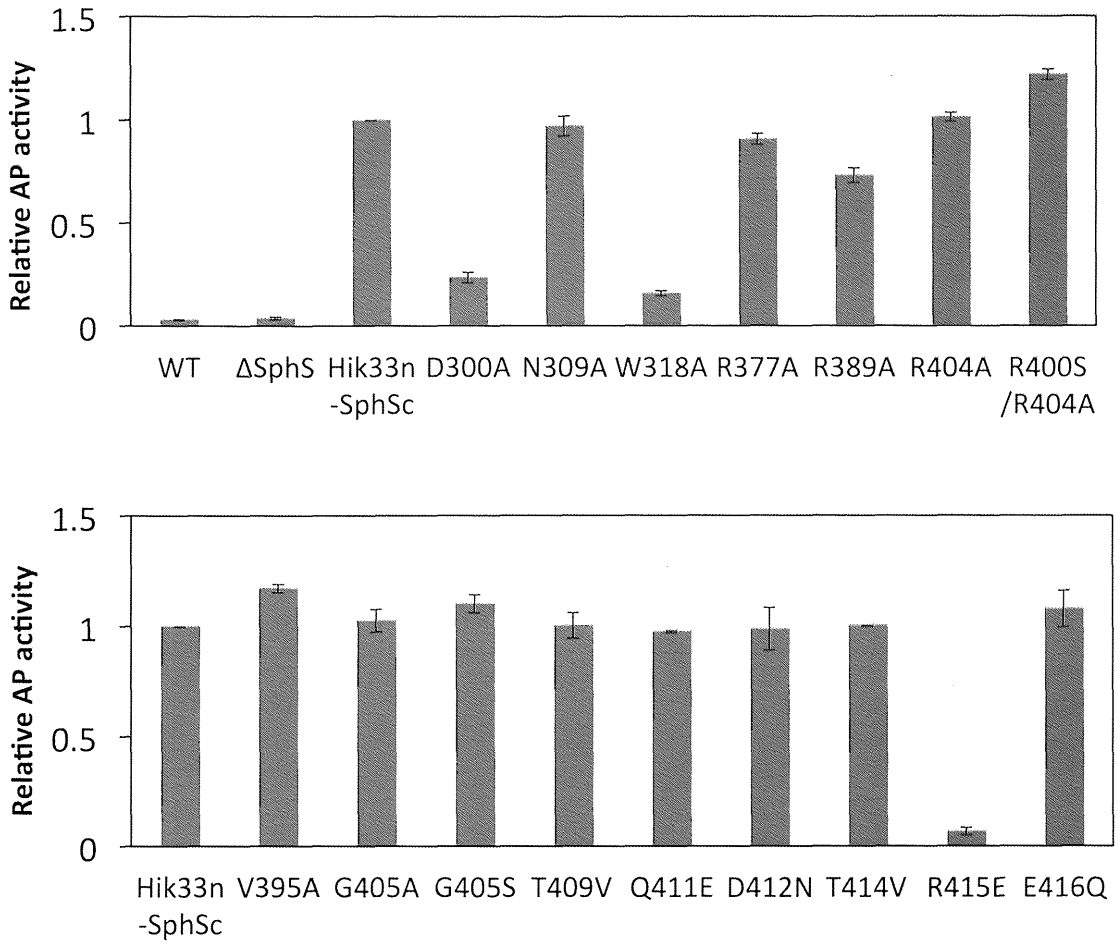


**Figure 12. Effect of the amino acid substitutions at PAS domain of Hik33n-SphSc on the kinase activity.**

AP activities of the cells expressing full Hik33n-SphSc or point-mutated Hik33n-SphSc variants. AP activities of each cells under standard growth conditions were measured three times. Average AP activity of Hik33n-SphSc was defined as 1.0, and average AP activities of each cells which is relative to Hik33n-SphSc and standard deviations were represented. Average AP activities of Hik33n-SphSc were 2.06 and 1.20 (mmol / mg chlorophyll *a* / minute) in upper and in lower graph respectively. Hik33n-SphSc: Hik33n-SphSc expressing cells, D300A, N309A, W318A, R377A, D389A, R404A, V395A, G405A, G405S, T409V, Q411E, D412N, T414V, R415E, E416Q : point-mutated Hik33n-SphSc exprssing cells. R400S/R404A: R400S and R404A double mutated Hik33n-SphSc expressing cells. n=3.

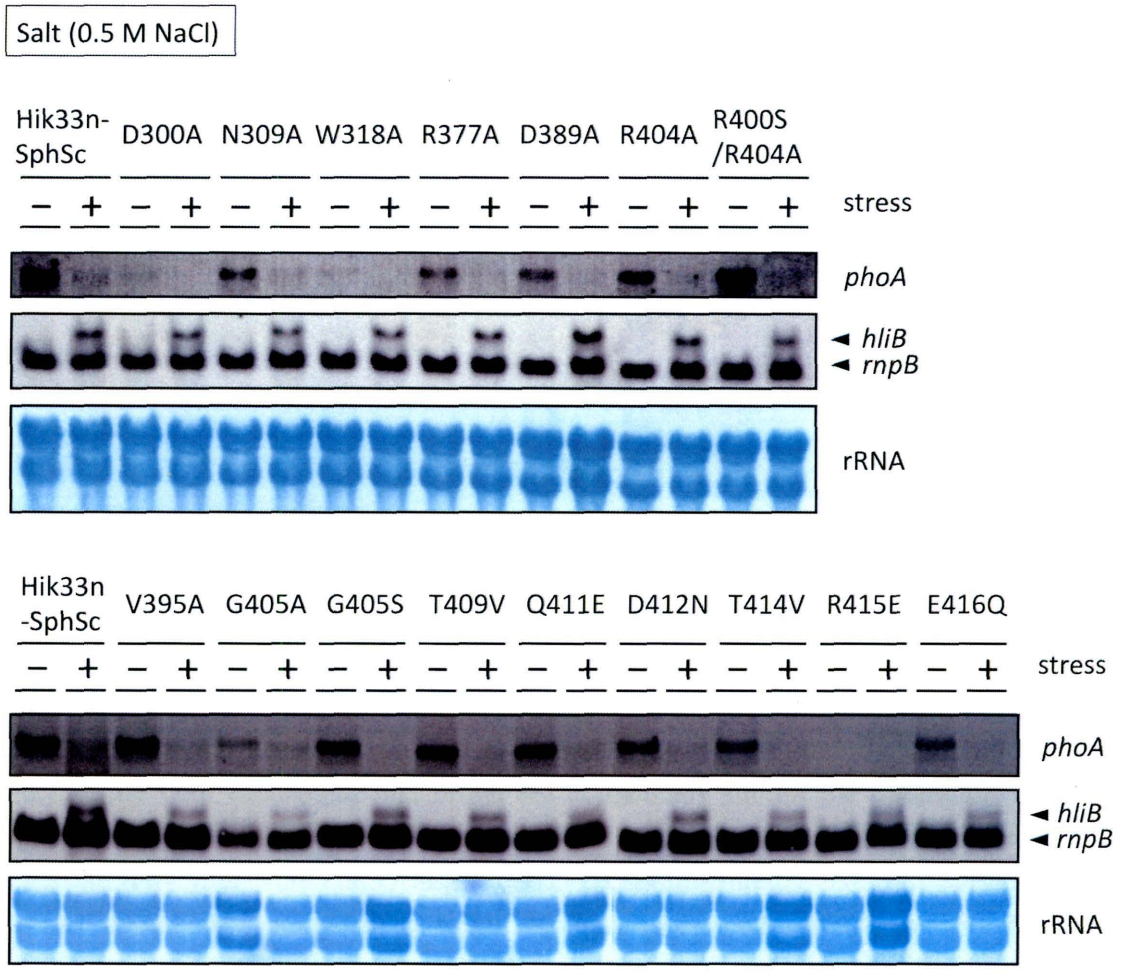


**Figure 13. Effects of the amino acid substitutions at PAS domain of Hik33n-SphSc**
The expression levels of the *phoA* gene in the cells expressing full Hik33n-SphSc or point-mutated Hik33n-SphSc variants were analyzed by Northern blot. The expression levels of the *hliB* and *rnpB* genes ware also analyzed as Hik33-regulating stress-inducible gene marker and as endogenous control respectively. Hik33n-SphSc: Hik33n-SphSc expressing cells, D300A, N309A, W318A, R377A, D389A, R404A, V395A, G405A, G405S, T409V, Q411E, D412N, T414V, R415E, E416Q : point-mutated Hik33n-SphSc exprssing cells. R400S/R404A: R400S and R404A double mutated Hik33n-SphSc expressing cells. N: under non-stressed conditions, S: 30 minutes after exposure to 0.5 M NaCl for salt stress. rRNA: ribosomal RNAs stained by methylene blue

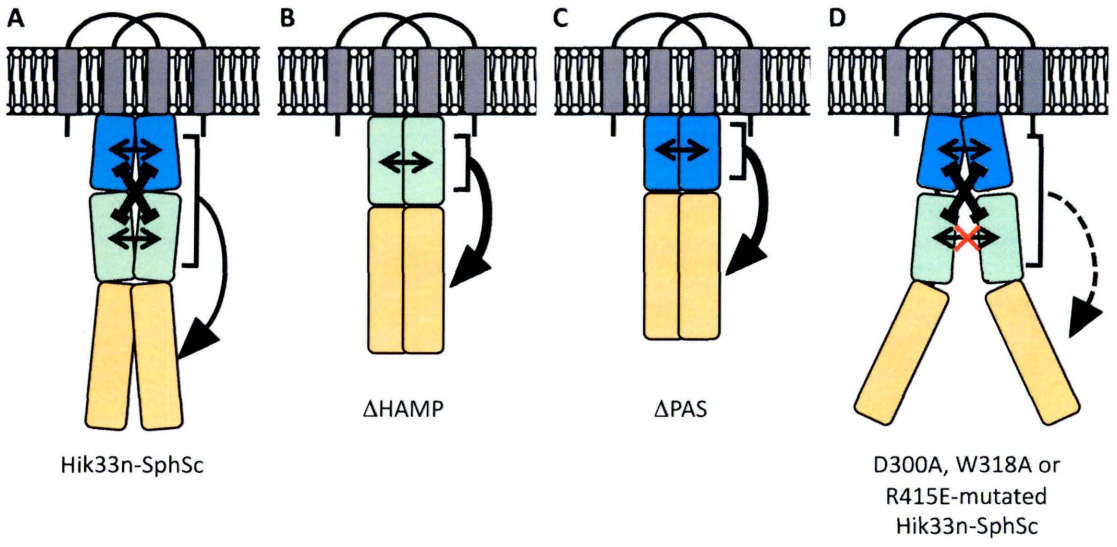


**Figure 14. Model of the interaction between HAMP and PAS domains in the dimer form Hik33-SphS variants.**

Hik33 probably functions as a dimer form, and the HAMP and PAS domains might contribute configurations of the dimer. Namely, the HAMP and PAS domains might also interact with each other and might simultaneously inhibit the dimerization of the counterpart (A). ΔHAMP and ΔPAS might associate more tightly and exhibit higher kinase activity than full Hik33n-SphSc (B, C). Point-mutated Hik33n-SphScs which revealed decreased kinase activity might decrease in the interaction between two PASs, but it might still have inhibitory effect on dimerization of the HAMP domain (D). Dimerization and inhibitory effect on the dimerization are indicated by arrow and bar respectively.

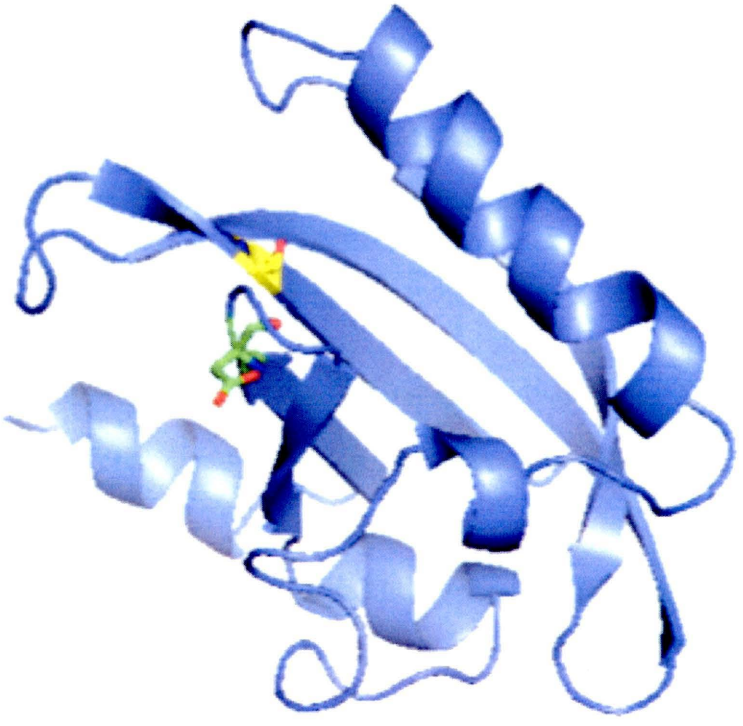

**Figure 15. predicted tertiary structure of the PAS domain of Hik33**
Amino acids sequence of PAS domain of the Hik33 (A283 to E418) was used for query sequence. Tertiary structure of the PAS domain was predicted using phyre server (current URL is http://www.sbg.bio.ic.ac.uk/~phyre/). Reference structure was a chain B of a crystal structure of the heme PAS sensor domain of *Escherichia coli* Dos (oxygen-bound form) (PDB code: 1VB6). The predicted structure was visualized using open-source software PyMOL (current URL is http://www.pymol.org/). D300 and G405 are represented in green and yellow sticks respectively.

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