Slr0967 and Sll0939 induced by the SphR response regulator in *Synechocystis* sp. PCC 6803 are essential for growth under acid stress conditions ☆

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ABSTRACT

Two-component signal transduction is the primary signaling mechanism for global regulation of the cellular response to environmental changes. We used DNA microarray analysis to identify genes that were upregulated by acid stress in the cyanobacterium *Synechocystis* sp. PCC 6803. Several of these genes may be response regulators that are directly involved in this type of stress response. We constructed deletion mutants for the response regulator genes and compared the growth rates of cells transfected with mutant and wild-type genes in a low pH medium. Of these mutants, deletion of *sphR* affected the growth rate under acid stress (pH 6.0) conditions. We examined genome-wide expression in Δ*sphR* mutant cells using DNA microarray to determine whether SphR was involved in the regulation of other acid stress responsive genes. Microarray and real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analyses of wild-type cells showed that the expression of *phoA*, *pstS1*, and *pstS2*, which are upregulated under phosphate-limiting conditions, increased (2.48-, 1.88-, and 5.07-fold, respectively) after acid stress treatment for 0.5 h. In contrast, *pstS2* expression did not increase in the Δ*sphR* mutant cells after acid stress, whereas the *phoA* and *sphX* mRNA levels increased. Furthermore, qRT-PCR and northern blot analysis indicated that downregulation of the acid-responsive genes *slr0967* and *sll0939* occurred with the deletion of *sphR*. Indeed, mutants of these genes were more sensitive to acid stress than the wild-type cells. Thus, induction of Slr0967 and Sll0939 by SphR may be essential for growth under acid stress conditions. This article is part of a Special Issue entitled: Photosynthesis Research for Sustainability: from Natural to Artificial.

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1. Introduction

Plants exposed to environmental stresses, such as extreme temperatures, light intensity, salt concentrations, and heavy metal densities, often possess superior acclimation abilities to environmental change. Acid rain is currently recognized as a major environmental stress [1–3]; however, extensive research has not been conducted to elucidate the basic set of adaptations required for acid tolerance in plants, algae, or cyanobacteria. *Synechocystis* sp. PCC 6803 (*Synechocystis* 6803) was the first photosynthetic organism to have its complete genome sequenced [4]. DNA microarrays have been used to examine its gene expression in response to different types of stress such as redox, oxidative, osmotic pressure, salinity, and light [5,6].

DNA microarray analysis of *Synechocystis* 6803 cells revealed that acid stress induces the expression of putative stress-related proteins, such as chaperones and regulatory factors, as well as proteins with unknown functions [7]. The expression of two genes, in particular *slr0967* and *sll0939* increased continuously by 7- and 16-fold, respectively, after 4 h of acid stress [7].

Bacteria frequently use two-component signal transduction systems for environmental sensing [8]. These systems consist of a sensor, histidine kinase, and a response regulator [9,10]. When an environmental change is detected, a specific histidine residue in the histidine kinase is autophosphorylated and a phosphoryl group is transferred to an aspartate residue on the cognate response regulator. An activated response regulator can regulate transcription, which alters gene expression in response to the stimulus [11]. A two-component system utilizing PhoP and PhoQ proved to be important for acid-stress tolerance in *Salmonella typhimurium* [12], whereas EvgS/EvgA, RcsCD/RcsB, and RstA/RstB were reported to be involved in the acid-stress tolerance of *Escherichia coli* [13–15]. We have

demonstrated that several members of the two-component systems were significantly upregulated by acid stress [7]. Therefore, we focused on two-component systems to clarify the putative sensing and response mechanisms for acid stress in cyanobacteria.

In general, cyanobacteria perceive and respond to environmental changes through a two-component regulatory system, which is a ubiquitous signal transduction pathway that represents a prevalent signaling mechanism found in all bacteria [16,17]. In *Synechocystis* 6803, different Hik-Rre systems have been identified as regulating the response to different environmental stresses [18]. A previous study reported that two *Synechocystis* 6803 genes, *sll0339* (*sphS*; *hik7*) and *slr0081* (*sphR*; *rre29*), encode proteins with respective homology to PhoR and PhoB in *E. coli*, PhoR and PhoP in *Bacillus subtilis*, and SphS-SphR in *Synechococcus* sp. PCC 7942 [19]. In *Synechococcus* sp. PCC 7942, the SphS-SphR two-component system induces the transcription of *phoA*, which is an alkaline phosphatase, in response to limited phosphate in the medium [20]. Subsequently, an SphS-SphR two-component system was identified as the mode of perception and transduction of phosphate limitation signals in *Synechocystis* 6803 [21,22].

In this study, we constructed deletion mutants of *sphR* for the cyanobacteria *Synechocystis* 6803 and we performed DNA microarray analyses under acid stress conditions. Under acid stress conditions, deletion mutants of *sphR*, which encoded a response regulator involved in phosphate limitation [19,21], were less viable than the wild-type cells. Analyses using qRT-PCR showed that SphR upregulates acid responsive genes (*slr0967* and *sll0939*). This suggests that SphR plays an important role in the growth of *Synechocystis* 6803 under acidic conditions.

2. Materials and methods

2.1. Strain and culture conditions of cyanobacteria

The *Synechocystis* 6803 wild-type strain and *sphR*-disrupted mutants, created by the insertion of a chloramphenicol (Cm)-resistance cassette, were grown at 30 °C in BG-11 medium [23] with 5 mM TES-NaOH (pH 8.0) under continuous illumination using fluorescent lamps. Cells in the exponential growth phase were subjected to acid stress by centrifuging the cell cultures and re-suspending the cell pellets in pH-adjusted BG-11 medium. The BG-11 medium was acidified using MES (pH 6.0) buffer rather than TES (pH 8.0). Cultures were streaked onto pH-adjusted BG-11 plates and cultured for 7 days. Experiments were performed in duplicate at least three times.

2.2. Generation of insertion mutants

The *sphR*, *slr1214*, *slr0967*, and *sll0939* mutants were generated by deletion mutagenesis of *sphR*, *slr1214*, *slr0967*, and *sll0939* using a Cm- or kanamycin (Km)-resistance gene cassette. Specifically, the upstream and downstream regions of *sphR* were amplified by PCR using genomic DNA extracted from the wild-type cells of *Synechocystis* 6803 as the template as well as the primers slr0081-F1, slr0081-R1, slr0081-F2, and slr0081-R2 (Table 1). The upstream and downstream regions of *slr1214* were amplified by PCR using genomic DNA as the template as well as the primers Slr1214-F1, slr1214-R1, slr1214-F2, and slr1214-R2 (Table 1). The upstream and downstream regions of *slr0967* were amplified by PCR using genomic DNA as the template as well as the primers slr0967-F1, slr0967-R1, slr0967-F2, and slr0967-R2 (Table 1). The upstream and downstream of *sll0939* were amplified by PCR using genomic DNA as the template as well as the primers sll0939-F1, sll0939-R1, sll0939-F2, and sll0939-R2 (Table 1). Approximately 400 bp (upstream) and 600 bp (downstream) of the PCR products were cloned into pUC19 (Toyobo, Osaka, Japan) to generate pUC-sphR, pUC-slr1214, pUC-slr0967, and pUC-sll0939. A Cm-resistance gene cassette was obtained from pLysS by PCR amplification using the BamHI site-

Table 1
List of primers.

Name	Sequence (5′→3′)	Source
slr0081-F1	CCGAATTCAATGGAAAGCCAACGGATGG	This work
slr0081-R1	CCGGATCCCAACCAACATATAACACAGA	This work
slr0081-F2	CCGGATCC GCCGGAATATTTAGTCACAG	This work
slr0081-R2	CCAAGCTT ATAACGATTTTCTTCTTTGC	This work
Slr1214-F1	CCGAATTCGCTGCGAATGCCATTGGCTC	This work
slr1214-R1	CCGGATCCGTACTTTGCCACCAGGTATG	This work
slr1214-F2	CCGGATCCTCCCAGGAATTACTGCAAGTC	This work
slr1214-R2	CCAAGCTTTTCATCACGGCGATGAATTGG	This work
slr0967-F1	CCGAATTCGAACTGAAAACGGCCATCAG	This work
slr0967-R1	CCGGATCCTCCAAGTTAGCTTCGTTGAG	This work
slr0967-F2	CCGGATCCTGATTTGACCGGTGCCAATC	This work
slr0967-R2	CCAAGCTTTAGGGCTAATCGCTGTAATC	This work
sll0939-F1	CCGAATTCTGCAAATTTTTGCCCAACGC	This work
sll0939-R1	CCGGATCCGCAAAGTTTTCACTAGCCCG	This work
sll0939-F2	CCGGATCCGGCTAATCGCTGTAATCAGG	This work
sll0939-R2	CCAAGCTTTGCTGGAAACCAATGCCTGC	This work
Cm-F	CCGGATCCCACTGGAGCACCTCAAAAAC	This work
Cm-R	CCGGATCCCTTATTCAGGCGTAGCACCA	This work
Km-F	CCGGATCCGGGAAAGCCACGTTGTGTCT	This work
Km-R	CCGGATCCCCTTCAACTCAGCAAAAGTT	This work
RT-phoAF	GGTAATTACTACGTCCAATCTGGGGATG	This work
RT-phoAR	TTAGACTAAAACGGAGTCAGCCGTGGTT	This work
1247F	AGCGGCAACGGTTAAGCA	[28]
1247R	GTTACGGCGGGCAAAGGT	[28]
0679F	TCGAAGAGCTAAAGCGCATTT	[28]
0679R	TGGTTCCAGCGGGTCAAG	[28]
RT-slr0967F	CTATGGGGAGGAACGGCGAT	This work
RT-slr0967R	CAAATCTGCCCCAATCAG	This work
RT-sll0939F	ATTTAGTCAGCATTACCAGG	This work
RT-sll0939R	GAGCCAACCACAAACCGAAT	This work
RT-RnpBF	GGAGTTGCGGATTCCTGTCA	This work
RT-RnpBR	ACTGTTTACTGGTTGCTGTTTTCTA	This work
slr0967F	GCGAGAGGAGAAAGATGATG	This work
slr0967R	TCCAGTCAAGGCCATCAATC	This work
sll0939F	GCAAAGGAATAAAACTATGG	This work
sll0939R	GCCATGGCTCAATGGTTAAC	This work
RnpBF	TGGGTAACGCCCAGTGCGCG	This work
RnpBR	TTGTTACCAAATTCCTCAAG	This work

containing Cm-F primer and the BamHI site-containing Cm-R primer (Table 1). This product was digested using BamHI and ligated into pUC-SphR and pUC-slr1214. A Km-resistance gene cassette was obtained by PCR amplification from pUC4K using the BamHI site-containing Km-F primer and the BamHI site-containing Km-R primer (Table 1). This product was digested with BamHI and ligated into pUC-slr0967 and pUC-sll0939. The resultant plasmids were used for transformation of *Synechocystis* 6803, as described previously [24]. Transformants were initially selected using a medium containing 10 μg mL^{-1} km and Cm (Wako Pure Chemical, Osaka, Japan), whereas the segregation of clones was performed by re-streaking (at least three transfers) the primary clones onto plates supplemented with 50 μg mL^{-1} km and 30 μg mL^{-1} cm. To examine the extent of the replacement of *sphR*, *slr1214*, *slr0967*, and *sll0939* by the corresponding mutated genes in mutant cells, we amplified the genes using the pairs of synthetic primers and chromosomal DNA from the wild-type *sphR*, *slr1214*, *slr0967*, and *sll0939* cells.

2.3. RNA isolation and real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

RNA was extracted from exponentially growing cultures of wild-type *Synechocystis* 6803 or SphR mutant cells using normal BG-11, acid-treated BG-11, or phosphate-limited BG-11. The cell pellet was collected, frozen in liquid nitrogen, and stored at −80 °C. Total RNA was isolated from *Synechocystis* 6803 cells using an RNeasy Midi Kit (Qiagen), as described previously [6]. The extracted RNA was reverse transcribed using a PrimeScript™ RT Reagent Kit (Takara Bio). *phoA*, *pstS1*, *sphX*, *slr0967*, *sll0939*, and *rnpB* were amplified by PCR using the

primers RT-phoAF, RT-phoAR, 1247F, 1247R, 0679F, 0679R, RT-slr0967F, RT-slr0967R, RT-sll0939F, RT-sll0939R, RT-RnpBF, and RT-RnpBR (Table 1). RT-PCR with SYBR Green I was performed using SYBR Premix Ex Taq (Perfect Real Time) (Takara). Each RT-PCR was performed in triplicate with *rnpB* as the internal standard.

2.4. Northern blot analysis

The extracted RNA was quantified using a NanoDrop ND-1000 spectrophotometer (PeqLab Biotechnology), separated by electrophoresis on 1.5% agarose formaldehyde gels, and blotted onto Hybond-N + (Amersham) membranes [25]. Hybridization and washes were performed as described in the Amersham Instruction Manual. Probes for northern blot hybridization were synthesized by PCR using the oligonucleotide pairs slr0967F–slr0967R, sll0939F–sll0939R, and RnpBF–RnpBR for *slr0967*, *sll0939*, and *rnpB*, respectively (Table 1). As a loading control, a similar probe was prepared that corresponded to the coding sequence of the RNase P RNA gene (*rnpB*). Each corresponding signal was detected using thermostable alkaline phosphatase (AP)-labeled DNA probes. AP was detected using an AlkPhos Direct Labeling and Detection Kit (Amersham) according to the instruction manual.

2.5. DNA microarray analysis

Cells were harvested from 10 mL of culture by centrifugation at $4000 \times g$ for 5 min at 25 °C and broken immediately using a Mini-Bead Beater (Biospec, Bartlesville, OK, USA). Total RNA was isolated using an RNeasy Mini kit (Qiagen) as described by Hihara et al. [6]. A *Synechocystis* 6803 DNA microarray (CyanoCHIP) was obtained from Takara (Kyoto, Japan), which covered 3079 of the 3168 open reading frames (ORFs) in *Synechocystis* 6803, excluding the ORF transposases. The conditions used for the synthesis of Cy3-labeled and Cy5-labeled cDNAs, hybridization, and washing were as described previously [6]. Images were acquired using ScanArray 4000 (GSI Lumonics, Watertown, MA, USA) with the autobalance–autorange feature. This feature allowed the sensitivity of the instrument to be automatically adjusted by changing the laser power and photomultiplier gain settings so the signal remained within 90% of the maximum in order to prevent saturation. The raw data obtained using the ScanArray 4000 was analyzed with the QuantArray version 2.0 program (GSI Lumonics, Tokyo, Japan). The fluorescence intensity of each spot was quantified for Cy3 and Cy5 images before the local background fluorescence levels were subtracted. Cy3 and Cy5 images were normalized by adjusting the total signal intensities of the two images. The results shown are averages of 4–6 biologically independent experiments.

2.6. Inductively coupled plasma-atomic emission spectrometry analysis (ICP-AES)

The samples were prepared by growing *Synechocystis* 6803 cells at 30 °C in BG-11 medium with 10 mM TES-NaOH (pH 8.0) or 10 mM MES-NaOH (pH 6.0) under continuous illumination using fluorescent lamps for 1 week. Approximately 1 g of sub-samples was placed into a test tube and digested with 5 mL of HNO_3 and 2.5 mL of H_2O_2 at 100 °C until dry. Digestion was repeated with an additional 1 mL of HNO_3 to effectively oxidize the protein matrix. Following digestion, the residue was dissolved and diluted to 10 mL with 1 M HCl.

A Perkin Elmer Optima 5300 DV ICP-AES was used for the measurement of elements present in the BG-11 medium. This instrument was used in all the experiments [26] and the emission intensity of each element was measured in the axial view mode at each wavelength. The amount of each element in solution was determined by ICP-AES and a stock sample (Multielement Std. Soln. W-, Wako) was used as the standard element.

3. Results and discussion

3.1. Characterization of the sphR deletion mutant under acidic conditions

We constructed deletion mutant cells based on the results of the DNA microarray analysis under acid stress conditions. Expression of *slr1214* (*patA*; *rre15*), which showed the earliest induction, implying a role in the early response to inorganic carbon limitation [27], was induced under acid stress [7]; however, the *patA* deletion mutants were not less viable than the wild-type cells. This showed that *patA* did not contribute to acid stress tolerance. A plasmid carrying *slr0081* (*sphR*; *rre29*) that had been interrupted with a Cm-resistance gene was used for transformation of *Synechocystis* 6803. PCR analysis of Cm-resistant cells showed that *sphR* was inserted by the Cm-resistance gene because of the segregation of chromosomes (data not shown). SphR is known to be a response regulator in a two-component system that regulates the expression of *phoA* for alkaline phosphatase under phosphate-limiting conditions in *Synechocystis* 6803 [19,21]. In the normal BG-11 medium at pH 8.0, the mutant cells exhibited a similar photoautotrophic doubling time when compared to the wild type (Fig. 1A and C), suggesting that the deletion did not affect growth in normal conditions. However, under acid stress conditions the growth rate of deletion mutants of *sphR* was slower than that of the wild-type cells (Fig. 1B). In addition, the growth of Δ*sphR* mutant cells under acid stress condition (pH 6.0) was significantly inhibited compared with that of the wild-type cells (Fig. 1C). Expression of *sphR* was not induced under acidic conditions [7]; however, *sphR* mutant cells were not grown under acid stress condition. Therefore, SphR may be activated in response to acid stress.

These results suggest that SphR has an important role in the growth of *Synechocystis* 6803 under acid stress conditions. SphR is a response regulator involved in the cellular response to phosphate limitation. Therefore, we suggest that acid stress conditions may induce phosphate limitation in *Synechocystis* 6803 (see discussion in Section 3.3).
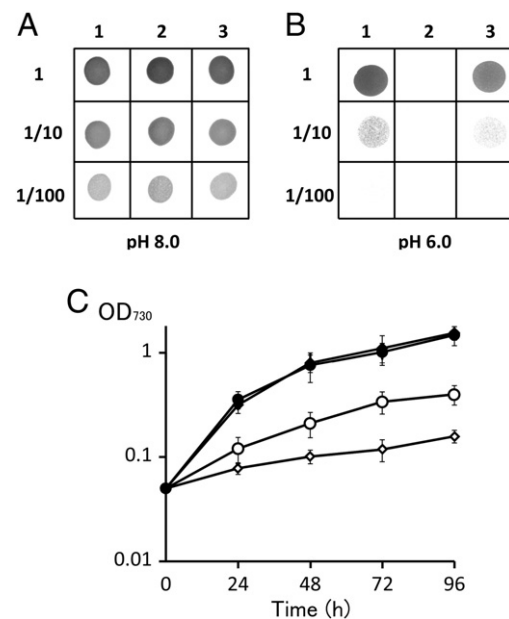

Fig. 1. Growth of wild-type and *sphR* deletion mutant cells at pH 6.0. Typical growth of wild type (1), Δ*sphR* mutant (2), and Δ*patA* mutant (3) cells cultivated on solid BG11 media. Plates were photographed and examined after 7 days of incubation at 30 °C at pH (A) 8.0 and (B) 6.0. (C) Photoautotrophic growth of strains was measured as the optical density at 730 nm in BG-11 (black symbols) or acidic BG-11 (white symbols); wild type (circles) and Δ*sphR* mutant (diamonds).

3.2. Real-time qRT-PCR analysis of pstS2, phoA, and sphX in sphR deletion mutants

To determine the activation of SphR under acidic conditions, we performed qRT-PCR analysis using phoA, sphX, and pstS2 primers. The phosphate-specific transport (Pst) systems consist of two sets of operons. The Pst1 system includes 6 ORFs: sphX-pstS1-pstC1-pstA1-pstB1-pstB1', whereas the Pst2 system includes four ORFs: pstS2-pstC2-pstA2-pstB2. phoA for alkaline phosphatase is involved in increasing the availability of phosphate in the extracellular environment. Importantly, these genes are all known to be upregulated by SphR under phosphate-limiting conditions [21,28].

The expression of pstS2, phoA, and sphX increased (5.8-, 5.31-, and 2.26-, respectively) in the wild-type cells after acid stress treatment for 150 min (Fig. 2A–C, black bars). pstS2 expression did not increase (0.71-fold) in the sphR cells after acid stress for 150 min (Fig. 2A, white bars), whereas the phoA and sphX mRNA levels increased (2.83- and 1.95-fold, respectively; Fig. 2B and C, white bars). These results suggest that SphR is activated in the wild-type cells after acid
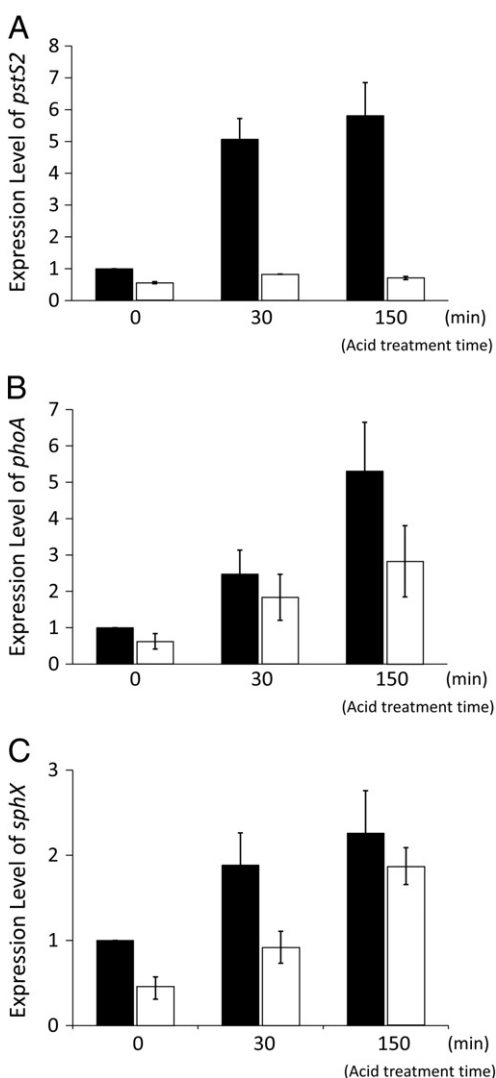
treatment. In addition, the results demonstrate that the expression levels of phoA and sphX were regulated in a SphR-independent manner because their expression increased in both the wild-type and sphR mutant cells. These data indicate the presence of an additional pathway that upregulates phoA and sphX RNA levels after acid treatment. This novel pathway may be linked with stress sigma factors (sigB or sigD) because sigma factors are upregulated under acid stress conditions [7].

3.3. ICP-AES analysis of the SphR deletion mutant

Next, we performed ICP-AES analysis to determine whether acid stress treatment caused phosphate starvation. The emission intensity of inorganic phosphate in wild-type Synechocystis 6803 cells under low pH (pH 6.0) for 7 days was more decreased than that in cells subjected to normal pH (pH 8.0) for 7 days (Fig. 3). The results in Fig. 3 suggest that acid stress treatment may cause phosphate starvation in the wild-type cells. Low pH treatment induced severe phosphate starvation in the sphR deletion mutant cells, suggesting that phosphate starvation is a possible explanation for cell death in these cells after acid stress treatment. Therefore, phosphate starvation may have led to lethality after acid stress treatment of the sphR deletion mutant cells.

Recently, Burnt-Archanai et al. [29] reported that Synechocystis 6803 cells exhibited similar orthophosphate (Pi) uptake activity under a broad range of alkaline conditions ranging from pH 7 to 10, whereas Pi uptake was inhibited under acidic conditions. These results match those in the current study, which showed that acid stress-induced signals are involved in phosphate starvation. This may be due to the greater abundance of monovalent species ($H_2PO_4^-$) compared with the divalent form (HPO_4^{2-}) with an external pH of 7, which is lower than the pK_2 of phosphoric acid.

Interestingly, the phosphate levels did not differ in the wild type (pH 6.0) and sphR mutant (pH 8.0) (Fig. 3), although sphR deletion mutant (pH 8.0) grew more rapidly than the wild-type cells after acid treatment (Fig. 1). This suggests that other factors in addition to phosphate starvation play a role in acid stress-induced growth inhibition.

3.4. DNA microarray analysis of the sphR deletion mutant

Our results showed that the sphR deletion mutant induced phosphate starvation, which was partially responsible for lethality under acid stress conditions. We then examined the genome-wide expression of genes in the sphR deletion mutant using DNA microarrays to determine whether sphR was involved in the expression of other acid stress responsive genes. We compared acid stress response genes that were expressed three-fold more in the wild-type strain after acid stress for 30 min with those that were not induced in ΔsphR cells after acid stress treatment (Table 2). Thus, we identified 15 genes that were involved in acid stress
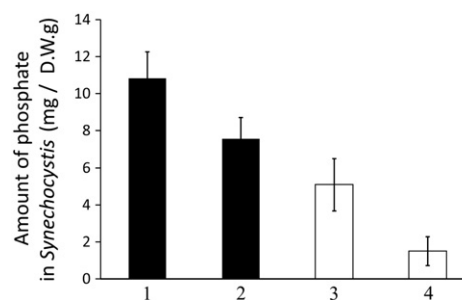

Fig. 2. qRT-PCR analysis of expression levels of pstS2, phoA, and sphX in wild-type and ΔsphR mutant cells after acid treatment (pH 3.0). Expression of Pho regulon genes (pstS2 (A), phoA (B), and sphX (C)) under acid stress conditions (pH 3.0). Bar heights indicate absolute transcript abundance per 25 ng of cDNA used in each qRT-PCR. Black bars represent the wild-type cells, and white bars represent ΔsphR mutant cells. Error bars indicate the maximum standard deviations observed.

Fig. 3. Effects of acid stress on wild-type and ΔsphR mutant cells by ICP-AES. Black bars indicate the wild-type cells (1, 2), and white bars indicate ΔsphR mutant cells (3, 4). Cells were grown at 30 °C in liquid BG-11 (pH 8.0) medium (1, 3) and liquid BG-11 (pH 6.0) medium (2, 4). The error bar indicates the standard deviation.

in a SphR-dependent manner, including *slr1675* (*hypA*), which encodes the hydrogenase expression/formation factor; *ssr2542* (*hilA*) and *ssr2595* (*hliB*), which encode the high light-inducible proteins; *slr1214*, which encodes a two-component response regulator in the PatA subfamily; *slr1204*, which encodes a serine protease HtrA; *sll0306* (*sigB*) and *sll2012* (*sigD*), which encode group 2 sigma factors; and eight genes for proteins with unknown functions. It is possible that some genes induced by SphR under acid conditions were necessary for growth.

To determine the effects of the *sphR* deletion mutation on acid tolerance in *Synechocystis* 6803 cells, we constructed deletion mutants for each gene identified by microarray analysis. Plasmids carrying genes that had been interrupted with Km-resistance gene were used for transformation of *Synechocystis* 6803. At pH 8.0 and 6.0, the growth of most mutant cells was similar to that of the wild-type cells (data not shown). *slr0967* and *sll0939* mutants exhibited similar photoautotrophic doubling times and similar cell viabilities in normal BG-11 medium at pH 8.0 (Fig. 4A and C). However, the *slr0967* and *sll0939* mutants were more sensitive to acid stress than the wild-type cells (Fig. 4B and C), indicating that *slr0967* and *sll0939* were involved in the acid tolerance of *Synechocystis* 6803 cells.

slr0967 and *sll0939*, comprising 453 nt and 378 nt, respectively, encode proteins of unknown functions. According to SOSUI analysis, the Slr0967 protein was predicted to be soluble, whereas Sll0939 was predicted to be a membrane protein with two transmembrane regions. Interestingly, these two genes are adjacent in the *Synechocystis* 6803 genome.

3.5. Transcription analysis of slr0967 and sll0939 in the sphR deletion mutants

Based on the results of the DNA microarray analysis of the *sphR* mutant under acid stress conditions and phenotypic analysis of each gene, the expression of *slr0967* and *sll0939* was found to be essential for growth under acid stress conditions. We then performed qRT-PCR analysis of the diachronic expression of these genes in the *sphR* deletion mutants to elucidate the relationship between *sphR* and *slr0967* and *sll0939* under acid stress conditions (Fig. 5A and B). In the wild-type cells subjected to acid stress, the transcription levels of *slr0967* and *sll0939* increased 9.04- and 53.85-fold, respectively
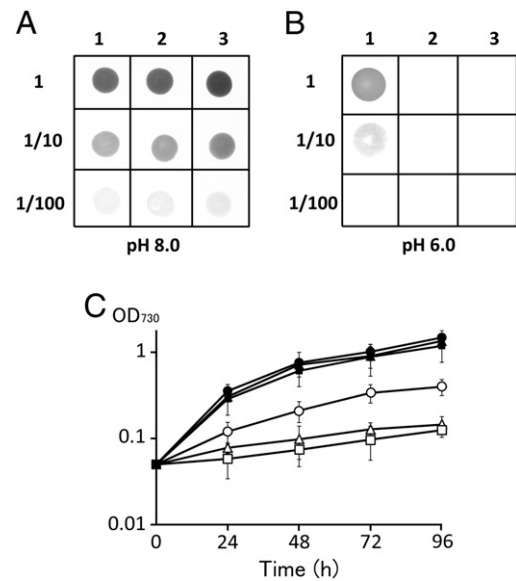

Fig. 4. Effects of acid stress on the growth of wild-type and mutant strains. Typical growth of wild type (1), Δ*slr0967* mutant (2), and Δ*sll0939* mutant (3) cultivated on solid BG11 media. Plates were photographed and examined after 7 days of incubation at 30 °C at pH (A) 8.0 and (B) 6.0. (C) Photoautotrophic growth of strains was measured as the optical density at 730 nm in BG-11 (black symbols) or acidic BG-11 (white symbols); wild type (circles); Δ*slr0967* mutant (triangles); and Δ*sll0939* mutant (squares).

(Fig. 5A, black bars). In contrast, the expression of *slr0967* in *sphR* mutant cells did not increase (1.11-fold) after acid stress treatment (Fig. 5A and B white bars). The expression of *sll0939* in *sphR* mutant cells increased slightly after acid stress treatment. These results suggest that SphR upregulated the transcription of *slr0967* and *sll0939*, which was consistent with the DNA microarray analysis results under acid stress conditions. In addition, the slight increase of *sll0939* expression in *sphR* mutant cells may suggest that expression is regulated by a factor other than SphR under acid stress conditions.

To obtain conclusive evidence that SphR upregulated the transcription of *slr0967* and *sll0939* genes, we performed a northern blot analysis of *sphR* deletion mutant cells under acid stress conditions (Fig. 5C). In the wild-type cells under acid stress conditions, the expression levels of *slr0967* and *sll0939* increased, which was also demonstrated by qRT-PCR. In SphR mutant cells, the expression of these genes did not increase after acid stress treatment. These results support our previous findings that SphR induced the upregulation of *slr0967* and *sll0939*.

The *Synechocystis* 6803 genome map indicated that *slr0967* was located immediately downstream of *sll0939* on the opposite strand of DNA. Interestingly, it was previously reported that these two genes are induced by high salt stress and hyperosmotic stress [30,31]. Under salt stress and hyperosmotic stress, these genes were controlled by the Hik16 (Slr1805)-Hik41 (Sll1229)-Rre17 (Sll1708) phosphorelay signal transduction cascade. Our results demonstrated a novel pathway in the transcriptional regulation of *slr0967* and *sll0939*, which was controlled through SphR under acidic conditions.

3.6. Expression of slr0967 and sll0939 is not induced by phosphate limitation

We performed qRT-PCR analysis of *slr0967* and *sll0939* expression after phosphate limitation to determine whether *slr0967* and *sll0939* were upregulated as part of the Pho regulon under phosphate-limiting conditions. The expression of *slr0967* and *sll0939* was not significantly increased in the wild-type cells (1.12- and 1.18-fold, respectively) after phosphate limitation (Fig. 6A and B). Similarly, the transcription levels of these genes did not increase in *sphR* deletion
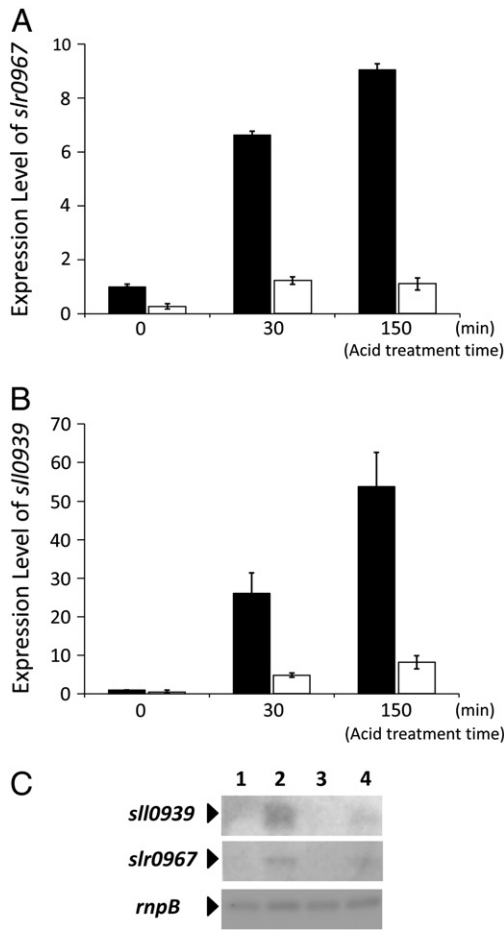
Table 2
Genes with acid-inducible expression regulated by SphR. Each value indicates the ratio of the relative level of expression of the indicated gene in stressed cells to that in unstressed cells. The numbering of ORFs and the annotation of gene products correspond to that in the CyanoBase (http://www.kazusa.or.jp/cyanobase/). Based on a result of DNA microarray analysis performed before (5), this table lists the acid-inducible genes with induction factors ≥3.0 in the wild-type cells. Values are averages ± error ranges from three independent experiments.

ORF number	Gene product	Levels of induction after 30 min incubation under pH3.0	
		Wild type	ΔSphR
ssr2016	Hypothetical protein	46.38 ± 12.88	0.85 ± 0.24
sll0528	Hypothetical protein	36.82 ± 6.51	1.02 ± 0.12
ssr2595	High light inducible protein HliB	34.78 ± 9.53	1.21 ± 0.13
slr1544	Unknown protein	24.46 ± 10.47	1.14 ± 0.15
slr1674	Hypothetical protein	22.07 ± 2.74	1.15 ± 0.06
sll0846	Hypothetical protein	18.76 ± 7.31	1.03 ± 0.13
slr1687	Hypothetical protein	16.57 ± 3.03	1.18 ± 0.11
slr1675	Hydrogenase expression/formation protein HypA	13.51 ± 3.37	1.31 ± 0.07
sll0306	Group 2 RNA polymerase sigma factor SigB	10.71 ± 8.58	1.12 ± 0.33
ssl2542	high light-inducible polypeptide HliA	10.04 ± 3.12	1.17 ± 0.11
slr1214	Two-component response regulator PatA subfamily	9.85 ± 3.61	0.97 ± 0.15
slr1204	serine protease HtrA	8.92 ± 2.58	1.31 ± 0.21
sll2012	Group 2 RNA polymerase sigma factor SigD	7.77 ± 1.04	1.22 ± 0.23
sll0939	Hypothetical protein	7.75 ± 1.89	0.95 ± 0.06
slr0967	Hypothetical protein	3.60 ± 0.63	0.95 ± 0.05

Fig. 5. qRT-PCR and northern blot analyses of the expression levels of *slr0967* and *sll0939* in the wild-type cells and Δ*sphR* mutant cells after acid treatment (pH 3.0). Expression of *slr0967* (A) and *sll0939* (B) genes under acid stress conditions (pH 3.0). Bar height indicates absolute transcript abundance per 25 ng of cDNA used in each qRT-PCR. Black bars represent the wild-type cells, and white bars represent Δ*sphR* mutant cells. Error bars indicate the maximum standard deviations observed. (C) Northern blot analysis of the stress-inducible expression of *slr0967* and *sll0939*. Total RNA (10 μg) was denatured, separated by electrophoresis on a 1.5% formaldehyde gel, blotted, and hybridized with probes for *slr0967* and *sll0939*. Lane 1 is untreated wild type; lane 2 is wild type after acid treatment (150 min); lane 3 is untreated Δ*sphR*; lane 4 is Δ*sphR* after acid treatment (150 min). The filters were stripped and hybridized with an *rnpB* probe as a loading control. Three independent experiments were performed.

mutants (Fig. 6A and B). Interestingly, this result differed from the response of *phoA* and *pst*. The transcription levels of *phoA* and *pst* increased in the wild-type cells after phosphate limitation, whereas the transcription level of the Pho regulon gene *phoA* did not increase in *sphR* mutant cells after phosphate limitation (Fig. 6C) [20]. These results suggest that a transcriptional factor other than SphR was activated under acid stress conditions, which was essential for the upregulation of *slr0967* and *sll0939* expression (Fig. 7). SphR and the acid-inducible transcription factor may form a heterodimer, such as GadE/RcsB in *E. coli* [32], regulating the transcription level of *slr0967* and *sll0939*. This novel pathway may be involved in Hik16-Hik41-Rre17 because the system controlled the transcription of *slr0967* and *sll0939* under conditions of salt stress and hyperosmotic stress.

4. Conclusions

We conclude that SphR has an important role in the growth of *Synechocystis* 6803 under acid stress conditions, and we suggest that the activation of SphR in stress conditions is due to phosphate limitation. As a result, the RNA expression levels of *phoA*, *pstS2*, *sphX*,
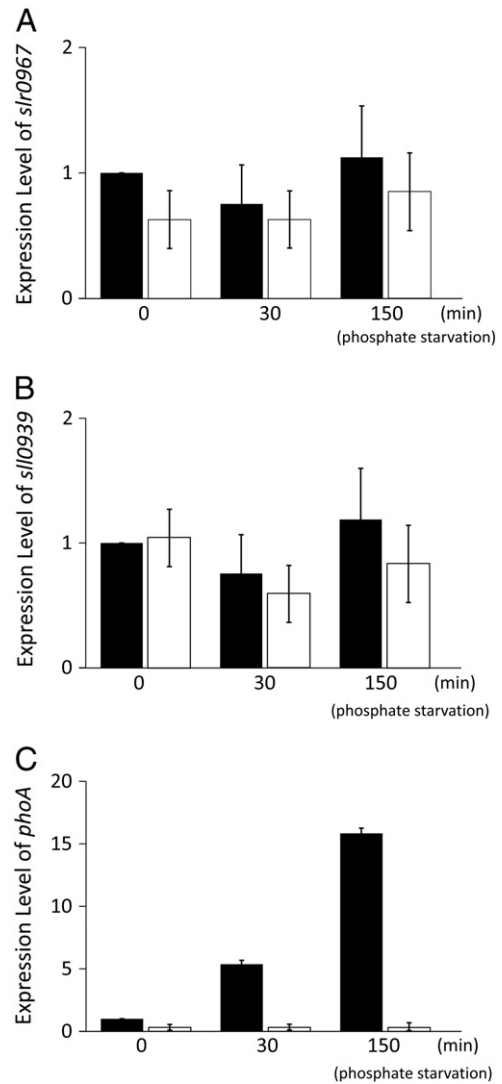

Fig. 6. qRT-PCR analysis of *slr0967*, *sll0939*, and *phoA* expression levels in wild-type and Δ*sphR* mutant cells after phosphate starvation. Expression of acid response genes (*slr0967* (A) and *sll0939* (B)) and the Pho regulon gene (*phoA* (C)) under phosphate starvation conditions. Bar height indicates absolute transcript abundance per 25 ng of cDNA used in each qRT-PCR. Black bars represent the wild-type cells, and white bars represent Δ*sphR* mutant cells. Error bars indicate the maximum standard deviations observed.

slr0967, and *sll0939* were increased by acid stress. Interestingly, the increased expression levels of *phoA* and *sphX* after acid stress were regulated by an acid-inducible regulation factor similar to *sigB* or *sigD* (X), which differed from SphR (Fig. 7). SphR and an acid-inducible unknown transcription factor, Y, are essential for upregulating the transcription of *slr0967* and *sll0939* (Fig. 7). Slr0967 and Sll0939 proteins were shown to contribute to cell growth in conditions of acid stress and their induction by SphR may be essential for growth under acid stress conditions. In future studies, it will be important to elucidate the acid-tolerance regulatory mechanisms of cyanobacteria and the identities of unknown transcriptional factors to clarify the roles of Slr0967 and Sll0939 in the acid stress response. In addition, clarification of the identity of unknown acid-inducible transcription factors will play a crucial role in improving our understanding of the acquisition of acid resistance in cyanobacteria.
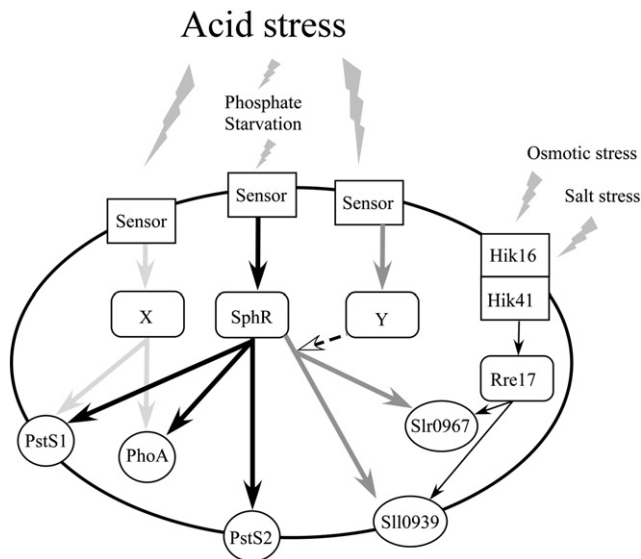
Acid stress

Fig. 7. Cascade model of the acid stress response. X and Y show an unknown transcriptional factor which is activated under acid stress. The dash arrow means that it functions as a transcription assistance factor.

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