# The Role of *sll1558* and *sll1496* Genes under Acid Stress Conditions in the Cyanobacterium *Synechocystis* sp. PCC 6803

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**Abstract:** The molecular mechanisms underlying plant sensitivity to acid stress are still unclear. Therefore, we intend to elucidate the mechanism of acid stress acclimation. The *sll1558* gene in *Synechocystis* sp. PCC 6803 was identified as an up-regulated gene by DNA microarray analysis in a short-time acid treatment (Ohta *et al*., 2005). This gene encodes mannose-1-phosphate guanylyltransferase, which catalyzes the reaction to GDP-D-mannose involved in N-glycan biosynthesis. The *sll1496* gene encodes the same enzyme. In this study, deletion mutants of these genes were constructed and phenotypes were analyzed. Both genes were found to be dispensable under normal growth conditions at pH 8.0. However, the *sll1558* deletion mutant was highly sensitive to acid stress conditions at pH 6.0. In contrast, the *sll1496* gene was found to be dispensable, as the *sll1496* deletion mutant was slightly sensitive to acid stress. Furthermore, the *At2g39770* (*cyt1*) gene in *Arabidopsis thaliana* has an orthologous relationship with *sll1558*. The *cyt1* mutant was more sensitive to acid stress conditions when compared with wild-type (WT) cells in the roots. The results of this study indicate that N-glycan biosynthesis contributes to acid stress and that only the *sll1558* gene plays an important role in acid stress in *Synechocystis*.

**Keywords:** Acid stress; Cyanobacteria; Mannose-1-phosphate guanylyltransferase; N-glycan

## Introduction

Acid rain is a serious environmental problem that can have harmful effects on plants and animals through the processes of wet and soil deposition. In particular, plants, being unable to escape from harmful environments, are prone to acid stress-induced growth inhibition. In soil acidified by acid rain, toxicity induced by elution of aluminum ions has a negative influence on the growth of crops. Therefore, plants capable of growing under acid stress conditions would be expected to grow all over the world. However, the molecular mechanisms underlying plant sensitivity to acid stress are still unclear. Therefore, we intend to elucidate the mechanism of acid stress acclimation in plants.

In a previous study, we surveyed time-dependent gene expression in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 as affected by transfer acid conditions. This organism is well suited for such study because the entire genomic sequence has been determined (Kaneko *et al*., 1996), and DNA microarrays representing all open reading frames (ORFs) are now available. In the previous study, we found that the expression of the *sll1558* gene was up-regulated within 1 hour after being shifted to acid conditions. The *sll1558* gene encodes mannose-1-phosphate guanylyltransferase (EC 2.7.7.13), which catalyzes the reaction to GDP-D-mannose involved in N-glycan biosynthesis. The *sll1496* gene encodes the same enzyme.

In this study, to understand the effect of *sll1558* and s*ll1496* on acid stress, we performed quantitative real-time RT-PCR (qRT-PCR) analysis of the transcripts of the 2 genes. In addition, we examined the physiological function of cyanobacteria genes using mutant cells in which each gene was disrupted by a kanamycin and chloramphenicol resistance gene

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cassette (*Km*$^r$ and *Cm*$^r$). Based on phenotypes, the effect of acid stress on *sll1558* and *sll1496* deletion mutants was compared with that on WT cells. Furthermore, growth analyses were performed under acid stress conditions using the *cyt1* mutant in *Arabidopsis*, because the *cyt1* gene in *Arabidopsis thaliana* has an orthologous relationship with *sll1558*.

## Materials and Methods

### *Strains and culture conditions*

A glucose-tolerant WT strain of *Synechocystis* sp. PCC 6803 and disrupted mutants of the *sll1558* and *sll1496* genes prepared by inserting *Km*$^r$ and *Cm*$^r$, were grown at 30 °C in BG-11 medium (Stanier *et al*., 1971) with 10 mmol TES–NaOH (pH 8.0) under continuous illumination provided by fluorescent lamps. Cells were grown in volumes of 50 ml in test tubes and bubbled with 3% $CO_2$-containing air. Cell density was measured at 730 nm using a spectrophotometer (Ultrospec 4,000 UV/Visible Spectrophotometer; Amersham Pharmacia Biotech, Uppsala, Sweden). Acid condition experiments were performed by transferring cells at the exponential growth phase ($OD_{730}$ = 0.1–0.2) to acid condition BG-11 plates with 10 mmol MES-NaOH (pH 6.0).

### *Construction of mutants*

A standard mutagenesis protocol for *Synechocystis* was used. The coding sequences of *sll1558* and *sll1496* were substituted into the antibiotic resistance genes *Km*$^r$ and *Cm*$^r$, respectively, by homologous recombination. First, the *Cm*$^r$ sequences and the *sll1558*/*sll1496* neighboring sequences were amplified by PCR. Approximately 2 or 3 kb of PCR products were cloned into pUC19 (Toyobo, Osaka, Japan). The primers for amplification were designed using the complete genome sequence of *Synechocystis*. Sequences that contained appropriate restriction sites were selected to improve cloning of fragments. The *Km*$^r$ isolated from plasmid pUC4K (Amersham Pharmacia Biotech, Uppsala, Sweden) was inserted into unique restriction sites of the encoding sequences. Transformants were initially selected on a medium containing 10 μg mL$^{-1}$ kanamycin (Km) and chloramphenicol (Cm) (Wako Pure Chemical, Osaka, Japan), whereas the segregation of clones was performed by restreaking (at least 3 transfers) of primary clones on plates supplemented with 50 μg mL$^{-1}$ Km and 30 μg mL$^{-1}$ Cm. During the cultivation of mutants, Km and Cm were added to the liquid media.

### *RNA isolation and quantitative real-time RT-PCR*

Total RNA was isolated using the RNeasy Mini kit (Qiagen, Hilden, Germany), as described by (Hihar *et al*., 2001). For the reverse transcriptase (RT) reaction, 100 ng RNA was incubated with a mixture of PCR reverse primers for 10 min at 70 °C before addition of 100 U Superscript II RT (Gibco-BRL, Carlsbad, CA, USA). The RT reaction was performed at 42 °C for 1 h and terminated by incubating the cells at 72 °C for 10 min. A Perfect Real Time kit (Takara Bio, Shiga, Japan) was used according to the manufacturer's instructions.

### *Plant materials and growth condition of Arabidopsis*

The plant materials used in this study included WT *A. thaliana* (Col-0 ecotypes) and genetic mutants derived from Col-0 ecotype. Seed germination and seedling growth were accomplished through the use of modified MS (Murashige-Skoog) media. The media was supplemented with 0.5% (w/v) sucrose and 20 mmol MES-KOH, adjusted to pH 5.7, and solidified with 1.5% purified agar (Nacalai Tesque Inc, Kyoto, Japan). In the acid condition experiments, we used the MS media with 20 mmol $CH_3COONa$–$CH_3COOH$ (pH 4.5). *Arabidopsis* growth occurred in a controlled environment incubator (EYELATRON FLI-160; Eyela, Tokyo, Japan), preset with a 16-h light/8-h dark photoperiod, light intensity of 3,000 Lux, and a constant temperature of 22 °C. These growth conditions are referred to in Qin *et al*. (2008).

## Results and Discussion

### *Characterization of the sll1558 and sll1496 deletion mutants*

We first investigated mRNA levels of the genes in detail. The *sll1558* and *sll1496* expressions were analyzed using qRT-PCR to estimate the changes in transcript amounts in cells grown under acid stress conditions (pH 3.0). The *sll1558* gene showed clearly increased transcript levels. However, in the case of *sll1496,* we were not able to confirm a significant increase (data not shown). Next, we constructed the deletion mutants. The WT *Synechocystis* was transformed with *sll1558* and *sll1496* that had been interrupted with a gene cassette conferring the *Km*$^r$

and *Cm*$^r$. To examine the *segregation* of these genes within the *Synechocystis* genome, we performed PCR analysis using DNA obtained from WT, *Δsll1558*, *Δsll1496*, and double mutant cells. PCR with the chromosomal DNA of WT cells as a template was used to amplify respective DNA fragments for *sll1558* and *Δsll1496*, whereas PCR with DNA from *Δsll1558*, *Δsll1496,* and the double mutant cells yielded a fragment of resistance-cassette length (Fig. 1). This result indicated that the *sll1558* and *sll1496* genes in *Δsll1558*, *Δsll1496,* and the double mutant cells had been disrupted by the substitution of the *Km*$^r$ and *Cm*$^r$ genes. Subsequently, we performed phenotype analyses to identify the role of *sll1558* and *sll1496*. In normal BG-11 medium at pH 8.0, all strains exhibited a similar photoautotrophic doubling time, suggesting that deletion of these genes did not affect their growth under normal conditions (Fig. 2A). In contrast, in the acid stress conditions at pH 6.0, the growth of all mutant cells was slightly or significantly inhibited compared with that of WT cells. The *Δsll1558* and double mutants (*Δsll1558* and *Δsll1496*) were more sensitive to acid stress than the *Δsll1496* cells (Fig. 2B). Interestingly, *sll1558* and *sll1496* encode an enzyme with the same function. However, this result indicated that *sll1558* was involved more strongly in acid tolerance of *Synechocystis* cells as compared with *sll1496*.

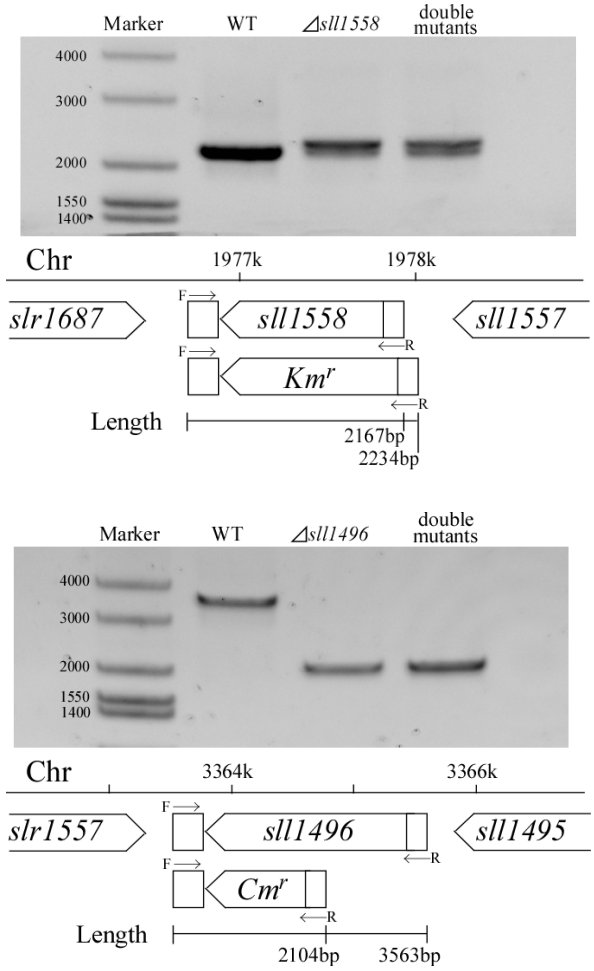


**Fig. 1** Deficit confirmation of the *sll1558* and *sll1496.* Schematic drawing showed the gene organization of the chromosomal site where the *sll1558* and *sll1496* coding region and the *Km*$^r$ and *Cm*$^r$ were inserted. The PCR analyses using chromosomal DNA of the WT and the mutant (*Δsll1558*, *Δsll1496* and the double mutant) as a template. The primers used of specific for the *sll1558* and *sll1496* genes in order to verify disruption in the chromosomal DNA of the mutant. The marker used of Wide-Range (TaKaRa Bio, Shiga, Japan).

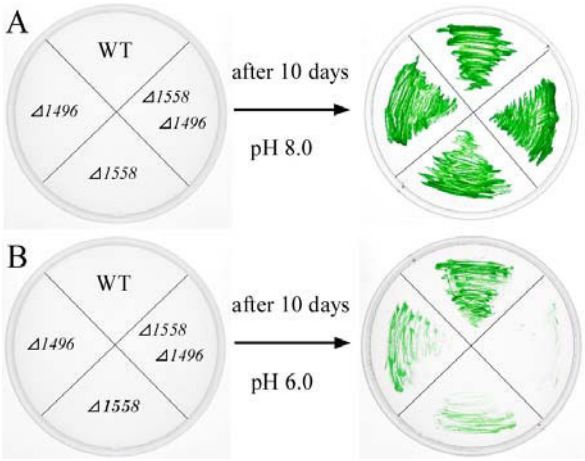


**Fig. 2** Phenotypical confirmation of deletion mutants in *Cyanobacteria.*
Saturated dilutions of cultures of WT cells and deletion mutants (*Δsll1496*, *Δsll1558,* and double mutant) were streaked onto BG-11 plate (A: buffered with 10 mmol TES-NaOH [pH 8.0], B: buffered with 10 mmol MES-NaOH [pH 6.0]) and cultured for 10 days at 30 °C under 3,000 Lux of continuous cool white fluorescent light.

### *Morphological characterization of cyt1 mutant in Arabidopsis*

To test the importance of N-glycan biosynthesis in plant growth under acid stress conditions, the level of growth in *Arabidopsis cyt1* mutant was analyzed under acid stress conditions (pH 4.5). The growth of both WT cells and *cyt1* mutants reduced under acid stress conditions as compared with normal conditions. Moreover, the level of growth retardation of *cyt1* mutants, in both aerial and root organs, also increased under acid stress conditions (Fig. 3). These results suggest that N-glycan biosynthesis contributes to the acid stress tolerance of *Synechocystis* and *Arabidopsis*.

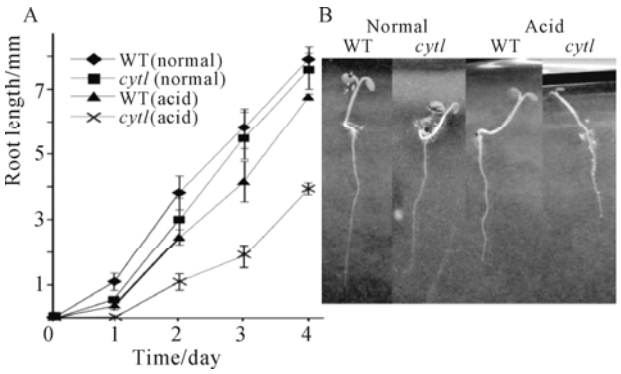


**Fig. 3** Phenotypes of *cyt1* mutants in *Arabidopsis*.A: Root lengths on each of 4 days (WT-normal is a closed diamond, WT-acid is a closed square, *cyt1* mutants-normal is a closed triangle, and *cyt1* mutants-acid is a cross). B: The state of the roots 4 days after germination. The WT and *cyt1* mutants were seeded on 1/2 MS plates with 20 mmol MES-KOH adjusted to pH 5.7 (normal). In the acid condition experiments, we used MS media with 20 mmol $CH_3COONa$–$CH_3COOH$ adjusted to pH 4.5 (acid).

## Conclusion

We found significant differences between *sll1558* and *sll1496*, including their transcript level under acid stress conditions and sensitivity to deletion mutants. These results suggest that *sll1558* is more strongly involved in acid tolerance of *Synechocystis* cells than is *sll1496.* In addition, a positive regulation may act on only *sll1558* under acid stress conditions. To further elucidate the important differences between *sll1558* and *sll1496* under acid stress conditions, it is necessary to investigate the mechanism underlying transcriptional control.

Furthermore, our results suggest that N-glycan biosynthesis contributes to acid stress tolerance of *Synechocystis* and *Arabidopsis*. This may be the cause of the damage to the cell outer layer, such as the cell wall when sensitivity to acid stress is increased, because N-glycan is important for cell wall biosynthesis. Therefore, it is important to determine the factors that cause damage to the cell outer layer of WT cells and mutants. We are currently researching this subject through electron microscopy and analysis of the extracellular polysaccharide and cell outer layer of WT cells and mutants.

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