Title

Rapid phosphate uptake via an ABC transporter induced by sulfate deficiency in *Synechocystis* sp. PCC 6803

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Abstract

Transport systems, which perform a pivotal function in regulating the import and export of various substances via cellular membranes, are important machinery in all living organisms. The ATP-binding cassette (ABC) transporter, one of such transporters, has a crucial role in the high-affinity uptake of various nutrients in bacteria. In the cyanobacterium *Synechocystis* sp. PCC 6803, a model cyanobacterium, its response to phosphate (Pi)-depleted conditions and the consequential regulation of the Pi transport system have been well-studied. However, its responses and the Pi uptake under Pi-repleted conditions are not comprehended yet. This study observed the peculiar stimulation of the Pi uptake following severe chlorosis in the *Synechocystis* cells grown under Pi-repleted conditions. The *Synechocystis* cells grown under high Pi conditions (approximately 4 mM) showed the rapid Pi uptake after 3 d of cultivation, and significantly severe degradation of chlorophyll was observed. Remarkably, the *Synechocystis* cells showed more than 16-fold higher Pi uptake at that time. Furthermore, we revealed that sulfate (S) deficiency-induced Pi uptake and that magnesium deficiency caused chlorosis and released the intracellularly accumulated Pi. Moreover, reverse genetics revealed that the rapid Pi uptake occurred via an ABC transporter induced by S deficiency, namely, a putative sulfate transporter, SbpA-CysTWA. Our findings provide a new perspective on the Pi uptake under high Pi conditions in cyanobacteria and bacteria.

Keywords

chlorosis, cyanobacteria, excess phosphate, magnesium deficiency, polyphosphate, sulfate transporter

Highlights

S deficiency in *Synechocystis* induced the rapid Pi uptake.

Mg deficiency stimulated consequent severe chlorosis.

The rapid Pi uptake was induced by a putative sulfate transporter.

1. Introduction

The transport of compounds via cellular membranes is one of the most critical functions in all living organisms, whose cells and organelles are separated by membranes composed of the lipid bilayer. The import and export of various substances against the concentration gradient are conducted by transporters embedded in the membranes. The ATP-binding cassette (ABC) transporters constitute one of the largest superfamilies of transporters and are ubiquitous in all kingdoms of life and participate in many physiological and pathological processes. One of the essential roles of the ABC transporter in bacteria is the high-affinity uptake of various nutrients [1], such as phosphorus (P) and sulfur (S).

P is an essential element for all living organisms and is involved in various roles in cells and bodies, *e.g.*, a backbone of nucleic acids, energetic metabolism, regulation of protein activity by phosphorylation, a constituent of membrane lipids, and material of biominerals, such as endoskeleton and teeth in higher animals. In nature, most of the P input into aquatic systems is insoluble mineral phosphate, which originates from runoff from land. Since those insoluble phosphates are unavailable for living organisms,

orthophosphate (hereafter inorganic phosphate, Pi) is preferred as the primary source of P. Thus, living organisms, especially those living in freshwater environments, developed efficient Pi acquisition systems [2-4]. In addition, it was suggested that the surplus of Pi to aquatic systems, which originated from human activity, is one of the main reasons for eutrophication. It is a common understanding that eutrophication causes the intensive growth of alga, which might be bothersome. Although algal blooming caused by eutrophication has received attention, knowledge about its response to excessive Pi remains limited.

Synechocystis sp. PCC 6803 (hereafter *Synechocystis*) is a unicellular, freshwater cyanobacterium. Although *Synechocystis* is the most extensively studied cyanobacteria, understanding many physiological and biochemical key features is still limited compared to other model microorganisms, e.g., *Escherichia coli* and *Bacillus subtilis* [5]. The *Synechocystis* cells have two ABC transporters for the high-affinity Pi uptake: phosphate-specific transport 1 (Pst1) and 2 (Pst2). These are the primary Pi uptake systems in this organism [6-7]. Moreover, it was well-reported that expression of the transporter gene is regulated depending on Pi availability [8]. Under insufficient Pi conditions, a Pi-deficient sensor, SphS, activates autophosphorylation activity and transfers the phosphoryl group to the cognate response regulator, SphR [9]. The phosphorylated SphR enhances its function as a transcriptional activator and upregulates the expression of genes related to the Pi acquisition system, including Pst1, Pst2 and alkaline phosphatase. Cells store the incorporated surplus Pi as a polymer of Pi of various lengths, i.e., polyphosphate (poly-P). Over the past five decades, numerous roles of poly-P were revealed [10-13]; however, this multifunctional biopolymer remains underexplored. In poly-P, 700–800 orthophosphates are linearly connected, and there are numerous anionic charges in it [14].

Thus, poly-P chelates plenty of metal cations, such as Mg^{2+}, Ca^{2+}, Na^{+}, and K^{+}, and it was reported that the ratio of those metal contents in poly-P is diverse depending on organisms and environmental conditions [15]. Furthermore, it has been reported that the growth of *Chlorella vulgaris* under excessively high Pi levels (>150 mg P L^{-1}, equivalent to 4.8 mM) was hindered [16], implying potential toxicity of excess P, which might have originated from poly-P. In contrast, there is no report on the toxicity of excess P in cyanobacteria [17].

S, another essential nutrient, plays important roles as a constituent of S-containing amino acids (cysteine and methionine) and various cellular cofactors, such as biotin, coenzyme A, *S*-adenosylmethionine, thiamin, glutathione, lipoic acid, and iron–S clusters [18]. In the aerobic environment, sulfate is the primary S source; it is also a target for ABC transporter in microorganisms. However, in *Synechocystis*, its transport is poorly understood. Although five genes (*sll0834*, *slr0096*, *slr1776*, *slr1453*, and *slr1454*) are annotated as sulfate permeases according to the CYORF database (http://cyano.genome.jp), the actual functions of these proteins are not yet confirmed, except *sll0834* for BicA, which belongs to the SulP transporter family (TC 2.A.53) and is identified as a HCO_3^- transporter [19]. The *slr1453* and *slr1454* genes are parts of an operon consisting of five genes: *slr1452-ssr2439-slr1453-slr1454-slr1455*. The *slr1452* encodes a sulfate-binding protein (*sbpA*), a substrate-binding protein for the ABC transporter. The *slr1453* and *slr1454* encode membrane-bound subunits, which are similar to CysT and CysW; *slr1455* is a gene for an ATP-binding subunit, CysA, and *ssr2439* encodes a small protein of unknown function and is particularly conserved in homologous operons from cyanobacteria. When the homologous ABC transporter was deleted in the relevant cyanobacterium, *Synechococcus elongatus* PCC 7942, the cells did not grow

under the medium containing a standard concentration of sulfate as a sole S source [20]. In addition, the expression of the operon in *Synechocystis* was significantly induced under the limitation of sulfate, indicating that the operon encodes a high-affinity uptake system for sulfate in *Synechocystis* [21]. However, as we mentioned above, there is no direct evidence to support the capability of the *sbpA-cysTWA* complex to transport sulfate in *Synechocystis*.

In this study, we confirmed the rapid Pi uptake following severe chlorosis in *Synechocystis* cultivated under 4 mM Pi conditions, which is an exceptionally high concentration compared with that of the natural freshwater environment. We also revealed the mechanisms that caused the rapid Pi uptake and chlorosis.

2. Materials and methods

2.1. Organisms and culture conditions

A glucose-tolerant strain of *Synechocystis* sp. PCC 6803 [22] was used as a wild-type strain in this study. The *Synechocystis* cells were grown in a modified BG-11 medium [23] buffered with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-NaOH (pH 7.5) at 34°C under continuous illumination (70 μmole photons·m^{-2}·s^{-1}) by incandescent lamps and aerated with 1% (v/v) CO_2-enriched air [24]. High Pi (4 mM) conditions were obtained by supplementing K_2HPO_4 to the BG-11 medium. Each of BG-11 ingredient, e.g., NO_3^-, $MgSO_4$, Ca^{2+}, CO_3^{2-}, Fe^{2+}, and microelements, was limited by removing each stock solution shown in Table S1. $MgSO_4$ was replaced by K_2SO_4 and $MgCl_2$ for Mg- or S-depleted conditions, respectively.

2.2. Measurement of optical density and chlorophyll *a*

Cell growth was confirmed by measuring optical density at 730 nm (OD_{730}) and Chlorophyll *a* (Chl*a*) concentration every 24 h until 7 d. Chl*a* was extracted using 90% methanol, and the concentration was calculated from the absorbance of the extract at 665 nm [25]. OD_{730} and absorbance of Chl*a* were determined by an ultraviolet-visible spectrophotometer, UV-1900i (Shimadzu, Kyoto, Japan).

2.3. Estimation of Pi uptake

The Pi uptake of the *Synechocystis* cells was evaluated by measuring Pi concentration in the medium. The Pi uptake under the 4 mM Pi condition was calculated using the following formulas (1) or (2).

$$\text{Pi uptake rate} = \Delta Pi \cdot \Delta t^{-1}\ (\mu M/d) \quad \cdots\cdots (1)$$

$$\text{Pi uptake rate} = \Delta Pi \cdot \text{Chl}a\ \text{concentration}^{-1} \cdot \Delta t^{-1}\ (\mu mole/mg\ \text{Chl}a/d) \quad \cdots\cdots (2)$$

ΔPi represents a change in Pi concentration in the medium (P_{t0}–P_{t1}), and Δt represents a change in time (t_1–t_0). Formula (1) was used when the cells grew during the period, and formula (2) was used when the cell growth can be ignored because the time is insufficient or under the nutrient limitation. Pi in the medium was determined using the molybdenum blue method [26, 27]. Furthermore, the Pi uptake rate was estimated by measuring the incorporated radiolabeled ^{32}P-phosphate (PerkinElmer, Waltham, MA, USA) into the cells under 0.3 mM Pi in the static culture conditions. The cells were collected every 1 h until 4 h by centrifugation with tube filters (Ultrafree-MC, Millipore, Burlington, MA, USA). And the cells trapped on the filter were washed twice with 2 mM phosphate buffer (pH 7.5) and the filter was transferred into a vial containing 2 mL of a liquid scintillation cocktail (Hionic-Fluor, PerkinElmer). The number of ^{32}P-decay events per minute was then determined using a liquid scintillator (LSC-6100, Hitachi Aloka

Medical, Tokyo, Japan).

2.4. Cell observation by optical microscopy

The *Synechocystis* cells grown for 5 d in the BG-11 medium or BG-11 medium containing 4 mM Pi were observed using a light microscope (BX53, Olympus, Tokyo, Japan). The cell diameters were determined by microscopic observation and a particle counter (CDA-1000, Sysmex, Kobe, Japan).

2.5. Measurement of expression level of the *pstS1*, *pstS2* and *sbpA* genes

The relative expression of the *pstS1*, *pstS2*, and *sbpA* genes were determined by real-time qPCR. These genes encode a substrate-binding protein for phosphate- and sulfate-uptake systems in *Synechocystis* cells and locate at the most or the secondly upstream in each gene cluster. The cells were grown in the 4 mM Pi and 2 mM $MgSO_4$-containinig BG-11 and washed with BG-11 medium without Pi and $MgSO_4$ for 3-times. The cells were inoculated into the media with or without 4 mM Pi and with or without 2 mM $MgSO_4$ and grown under the standard culture conditions. The cultures were withdrawn at 0 time and after 12 h and 24 h to harvest the cells. The extraction of total RNAs and quantitative PCR were performed as previously described [28]. The total RNAs were extracted by Invitrogen TRIzol Max Bacterial RNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA) and recovered by precipitation with isopropanol. One μg of the purified total RNAs was used to obtain complementary DNAs (cDNAs) with PrimeScript RT reagent Kit (Takara Bio, Kusatsu, Japan). The quantitative RT-PCR was performed with GOTaq qPCR Master Mix (Promega, Madison, WI, USA) and a PikoReal 96 Real-Time PCR system (Thermo Fisher Scientific). The *rnpB* gene for the RNA subunit of RNase P was used as a reference gene, and the primer sets used were listed in

Table S2. The threshold cycle (CT) values were determined using the PikoReal software version 2.2 (Thermo Fisher Scientific), and the relative expression levels of the genes were calculated from the CT values.

2.6. Plasmid construction and transformation

To obtain the *sbpA-cysTWA* operon-deleted strain, we substituted the entire coding regions of the operon in the chromosome of *Synechocystis* with the chloramphenicol resistance gene cassette by double homologous recombination (Fig. S1). The chloramphenicol resistance gene cassette was amplified by PCR using the plasmid pTCHT2031v [29] as a template and primers, Cm^r_up and Cm^r_down (Table S2). Approximately 1-kbp fragments of the upstream and downstream of the operon were also obtained by PCR, the genomic DNA of the *Synechocystis,* and primer sets SulT_KO_up and SulT_KO_inf_up_R, and SulT_KO_inf_down_F and SulT_KO_down (Table S2), respectively. The resultant three fragments were confirmed by agarose gel electrophoresis and connected by PCR. We used PrimeSTAR (Takara Bio) as a high-fidelity thermostable DNA polymerase. The connected fragments were cloned into a T-vector pMD19 simple vector (Takara Bio). The DNA sequence of the plasmid was confirmed using SeqStudio Genetic Analyzer (Thermo Fisher Scientific). The resulting plasmid was introduced into the competent cells of *E. coli* JM109 (TaKaRa Bio) using heat shock, and the transformed *E. coli* cells were selected on an agar-solidified LB medium [30] containing 50 μg/mL chloramphenicol (Fujifilm Wako Pure Chemicals, Osaka, Japan). The plasmid was recovered from the transformant of *E. coli* and introduced into the *Synechocystis* cells by their natural competency [22]. For the screening of transformants and maintaining of the *Synechocystis* cells, we used the agar-solidified BG-11 medium or BG-11 medium containing 3 mM of SO_4^{2-} supplemented with 25 μg/mL chloramphenicol. The complete

segregation of deletion mutants was confirmed by genomic PCR using the primer set SulT_KO_up and SulT_KO_down (Table S2).

3. Results and discussion

3.1. Cultivation of *Synechocystis* sp. PCC 6803 with BG-11 containing 4 mM Pi

Synechocystis cells were cultivated with the BG-11 medium containing a high Pi concentration (4 mM); Pi concentration in the standard BG-11 medium is 180 μM. The cell growth assayed by OD_{730} indicated that the cells similarly increase in the high Pi and standard Pi conditions until 5 d, after that the culture in the high Pi decreased quickly due to chlorosis (Fig. 1a). On the other hand, Chl*a* concentration in both cultures increased similar manner until 3 d, it held after that only in the standard Pi conditions (Fig. 1b). The Chl*a* concentration in the high Pi conditions was linearly increased until 4 d (Fig. 1b) and then suddenly dropped thereafter 4 d, one day before the decrease in OD_{730} (Fig. 1b). The *Synechocystis* culture consumed Pi in the standard medium totally within 2 d (Fig. 1c). However, Pi deprivation in the medium did not immediately retard cell growth. The culture maintained the growth until 5 d and 3 d determined by OD_{730} and Chl*a*, respectively (Fig. 1a,b), suggesting that certain factor(s) might be limited in the culture at least after 3 d. In addition, it held the maximum levels of Chl*a*, *i.e.*, stationary phase, until 7 d. Moreover, the Pi uptake rates under both conditions were similar (94 ± 6 μM/d for 0.18 mM Pi condition and 80 ± 33 μM/d for 4 mM Pi condition) until day 3, despite being more than 20 times different in Pi concentration (Fig. 1c,d). Surprisingly, the Pi uptake rate was stimulated in the high Pi conditions after 3 d and continued until 5 d. The Pi uptake rate at the period was 1,330 ± 45 μM/d, which was 16-fold more elevated than the

Pi uptake rate before 3 d. Pi in the medium was rapidly increased after 5 d with a decrease in the Chl*a* concentration. The appearance of the cultures after 7 d is shown in Fig. 1e. The culture under the high Pi conditions entirely caused chlorosis. Drastic Pi uptake and consequently rapid cell bleaching were observed under the high Pi conditions (Fig. 1).

As we mentioned, the transport system of Pi in *Synechocystis* was previously well-described; however, with that knowledge, several questions on our results remained. Two Pi-specific transport systems (Pst1 and Pst2) were induced by Pi shortage. However, the rapid Pi uptake occurred with the BG-11 medium containing 4 mM Pi, which maintained the Pi-repleted conditions (Fig. 1c). These results indicate an unknown transport system for Pi, which could be activated under P-repleted conditions, not by P-depletion, in the *Synechocystis* cells.

In addition, several reports highlight the toxicity of excess Pi concentration in the medium [16] or the importance of the homeostasis of intracellular Pi [31]. However, these studies do not interpret our results. Unlike *Chlorella regularis*, whose growth is suppressed under high Pi concentrations [16], the *Synechocystis* cells under the high Pi conditions showed the same growth rate in the culture with the standard Pi conditions until 3 to 5 d (Fig. 1a,b). The rates of OD_{730} increment, Chl*a* accumulation and Pi uptake by the *Synechocystis* cells were the same under both high and low Pi conditions until 3 d (Fig. 1a,b,c,d), suggesting that the same Pi transport system, whose activity is well-regulated, might function until 3 d and another type of Pi transport system might be activated after that. The Pi uptake rate after 3–5 d was more than 16 times faster than before (Fig. 1d). Thereafter, the Pi concentration in the medium was rapidly recovered, and the Chl*a* content was decreased after 4 d. We could not imagine the mechanisms that discharged the Pi absorbed by the cells, except Na^+ and heavy metal ions, which were

simultaneously induced by the loss of Chl*a* content. Thus, we speculated that the cell structures were damaged under these conditions, and it caused the release of the accumulated Pi during the rapid Pi uptake phase. Observation by optical microscopy agreed well with the hypothesis (Fig. S2). The diameters of the cells grown under the standard and high Pi conditions for 5 d were 2.48 ± 0.02 and 2.70 ± 0.04 μm, respectively. In addition, some cells grown under the high Pi conditions ruptured during the observation (Fig. S2b and Movie. 1). These observations demonstrated that cells under high Pi conditions tended to break after the rapid Pi uptake, thereby leading to growth arrest and release of Pi.

3.2. Determination of key factor(s) that induced the rapid Pi uptake and chlorosis

We noted that the arrest of Chl*a* accumulation under the standard Pi condition and rapid Pi uptake under high Pi conditions simultaneously began after 3 d (Fig. 1a,b). We hypothesized that certain nutrient limitations might induce the rapid Pi uptake and chlorosis. To identify the factors, we restricted each BG-11 medium ingredient, except Pi, and cultivated the *Synechocystis* cells under each condition (Fig. S3). Among the medium ingredients, when only $MgSO_4$ was eliminated, severe cell chlorosis was induced under the high Pi conditions (Fig. S3). Furthermore, we investigated the effect of each element (P, Mg, and S) regarding the phenomenon (Fig. S4). The cell growths were inhibited by the limitation of one of these essential elements. Still, the reappearance of the phenomenon was observed only under the high Pi (4 mM) and concomitantly Mg- and S-depleted conditions. Interestingly, the symptom was relieved under the Pi-depleted conditions, implying that P has a pivotal role in this peculiar phenomenon (Fig. S4).

The rapid Pi uptake was observed under the S-deficient conditions in *Synechocystis*

cultures (Fig. 2). Under this condition, the growth of the *Synechocystis* cells immediately ceased (Fig. 2a), and the Pi concentration in the medium decreased very drastically (Fig. 2b). These results clearly indicated that S deficiency caused the Pi uptake. As show in Fig. 2, when Mg^{2+} is repleted, chlorosis and Pi-release were not observed.

These results indicated that Pi uptake was stimulated through unknown transporters in *Synechocystis*, which might be induced under the S-depleted condition. To confirm the canonical phosphate-transporter in *Synechocystis* was not induced under high Pi and S-depleted conditions, we determined the expression levels of the *pstS1* and *pstS2* genes (Fig. 3a,b). As previously characterized, the *pstS1* and *pstS2* genes were highly transcribed under the Pi-deficient conditions (Fig. 3a,b), but not under the S-deficient conditions. Although high expression level of the *pstS1* and *pstS2* was maintained until 24 h in P-deplete, S-replete (-P, +S) conditions, that in P-deplete, S-deplete (-P, -S) conditions was found in 12 h only, but not at 24 h. These results might be interpreted that the S-limitation declined the general cellular activity to maintain the transcriptional activity until 24 h. And as expected, the gene for sulfate-transporter subunit, *sbpA*, expression was induced under the S-depleted condition regardless of the Pi-availability. These results clearly indicated that the Pi-transporter functioning in the high Pi conditions were not the Pst1 and Pst2, and uncharacterized transporter might be involved in the rapid Pi uptake under the S-depleted conditions.

Interestingly, the rapid Pi uptake under S deficiency was also observed in another cyanobacterium, *Synechococcus elongatus* PCC 7942 (Fig. S5), indicating that it was not a unique phenomenon observed in *Synechocystis*.

Although several previous publications reported that intracellular poly-P increases

under S deficiency [32, 33], these findings do not straightforwardly coincide with our results. For instance, in the green alga *Parachlorella kessleri*, it was reported that total P accumulates under S-depleted conditions through a sodium/phosphate symporter, which is a phosphate inorganic transport (Pit) system [33]. However, in *Synechocystis*, we could not find other Pi transporters, namely, the Pit system, except Pst1 and Pst2, which are not induced by S deficiency. In addition, the increase in Pi uptake under the S-deficient conditions was also reported in a model land plant, *Arabidopsis thaliana* [34]. However, the clear mechanism has not been interpreted yet.

Notably, *Synechocystis* cell chlorosis under the high Pi conditions was significantly alleviated by adding adequate Mg^{2+} (Fig. 4). We cultivated the *Synechocystis* cells with S-depleted (0 mM), P-repleted (4 mM), and various concentrations of Mg^{2+} conditions (0–1 mM). Interestingly, under 0.3 mM Mg^{2+} conditions (same as the standard BG-11), Pi concentrations in the medium recovered along with the decrease in the Chl*a* concentrations after 2-d cultivation (Fig. 4a,b). These phenomena were not observed under high Mg^{2+} conditions (*e.g.*, 1 mM), indicating that the limitation of Mg^{2+} coupled with Pi uptake was a key in promoting chlorosis and Pi release.

Since Mg^{2+} is indispensable for Chl*a* synthesis and SO_4^{2-} is also essential for the synthesis of S-containing amino acids (Met, Cys), a membrane lipid (sulfoquinovosyl diacylglycerol), vitamins, and cofactors (coenzyme A, thiamin, biotin, etc.), it was not surprising that the restriction of these essential elements severely affected basal metabolism. However, it was unexpected that intracellular Pi, probably stored as a poly-P, was fatal to the Chl*a* or *Synechocystis* cells. The intracellular poly-P is thought of as a necessary molecule for survival, considering its various and important roles [13]. Although it was reported that the addition of poly-P to the medium induces cell lysis in

Bacillus cereus by sequestering divalent metal cations, such as Mg^{2+} and Ca^{2+} [35], and overexpression of the *ppk* gene encoding poly-P kinase suppresses the growth of *E. coli* [36], no study has described a relationship between intracellular poly-P and chlorosis in cyanobacteria. We speculated the mechanisms of the phenomenon as follows: (i) under the S-depleted and high Pi conditions, the Pi uptake was stimulated; (ii) the incorporated Pi into the *Synechocystis* cells might be stored as poly-P; (iii) under Mg^{2+} depletion, the accumulated poly-P might be unstable because of the limitation of counterions and the continuation of the Pi uptake increased the intracellular Pi concentration; (iv) the cell enlargement occurred because of the increase in the osmotic pressure by the high Pi concentration in the cells; and (v) cell lysis finally occurred, and the poly-P and incorporated Pi in the cells were released back to the media; then, the thylakoid membrane might be fragmented and dispersed into the media, and chlorophyll might be degraded to cause chlorosis.

3.3. The rapid Pi uptake was diminished in the deletion strain of the S depletion-inducible transporter

The rapid Pi uptake was induced under the S-depleted conditions. Under this condition, it is demonstrated that the expression of an operon consisting of the five genes, *sbpA*(*slr1452*)-*ssr2439*-*cysT*(*slr1453*)-*cysW*(*slr1454*)-*cysA*(*slr1455*), encoding an ABC transporter was intensely induced in *Synechocystis* [21]. And it was also confirmed in Fig. 3c. To investigate the relationship between the function of this transporter and the rapid Pi uptake, we deleted the entire operon from the chromosome of *Synechocystis* by substituting it with the chloramphenicol resistance gene cassette (Fig. S1). The fully segregated deletion strains were easily obtained on the standard BG-11 containing 25 μg/mL chloramphenicol, indicating that the transporter was not essential and did not

function under the standard sulfate concentration (*i.e.*, 0.3 mM) conditions.

Interestingly, the rapid Pi uptake observed under the S-depleted conditions was significantly decreased in this deletion strain (Fig. 5). We assessed the uptake rate by measuring the uptake of ^{32}P (Fig. 5b) and Pi concentrations in the medium (Fig. 5a). These results clearly showed that the rapid Pi uptake was primarily implemented by the transporter induced by the S-depleted conditions. Furthermore, from the incomplete reduction of the Pi uptake in the deletion strain, we hypothesized that other transporter(s) induced under the S-depleted conditions might also be involved in the Pi uptake.

Only one previous publication hypothesized that one of the sulfate permeases in *Bacillus subtilis*, CysP, which is related to the Pit family, might be able to transport Pi [37]. However, this was not experimentally confirmed. Thus, our results are the first to reveal the capability of an ABC transporter annotated as a sulfate transporter involved in the transfer of Pi in cyanobacteria. We thought that the membrane-bound transporter might have very high specificity to the substrate. This common-sense was not always correct.

3.4. The rapid Pi uptake was inhibited by the presence of sulfate

Under low sulfate concentration (*e.g.*, 30 μM, two orders less than 4 mM Pi), the Pi uptake via the sulfate deficiency-inducible transporter(s) was clearly inhibited, as well as at 0.3 mM sulfate, a standard concentration in BG-11 medium (Fig. 6). These results indicated that the main transporter for Pi under S deficiency, *i.e.*, SbpA-CysTWA, did not transfer Pi under S-repleted conditions. In addition, chloramphenicol, which suppresses protein synthesis, could not fully repress the Pi uptake than the addition of sulfate. This result indicated that the supply of sulfate did not inhibit the Pi uptake by blocking the

expression of the SbpA-CysTWA transporter.

The specificity and activity of the bacterial ABC transporter are highly dependent on a substrate-binding protein located in the periplasmic space. It was well-studied that the substrate-binding protein (CysP) of a sulfate ABC transporter in *Salmonella typhimurium* is very specifically associated with sulfate but not with phosphate [38]. The dissociation constants K_d for sulfate and phosphate in the purified CysP from *S. typhimurium* are 0.12 μM and 60 mM, respectively. The identity of the primary sequences of the CysP from *S. typhimurium* and the SbpA from *Synechocystis* is approximately 40%. Especially, the sulfate-binding site in the CysP was highly conserved in the SbpA, implying that the SbpA protein might also have a high affinity for sulfate [38]. Therefore, it might be assumed that the specific transport of sulfate via SbpA-CysTWA is highly dependent on SbpA. However, under 3 μM sulfate conditions, a sulfate concentration that may be sufficient for the saturation of the binding site in CysP, the Pi uptake was still observed (Fig. 6), implying that SbpA might not contribute to the Pi uptake under S-repleted (μM order) conditions.

Overall, it was suggested that there is an unknown regulatory system inhibiting Pi transport via membrane proteins, CysT and CysW, under the S-repleted conditions.

4. Conclusion

This study revealed a rapid Pi uptake and chlorosis under high Pi conditions in *Synechocystis*. In addition, we implicated the key factors that stimulated each phenomenon. Finally, we revealed that the Pi uptake occurred via an S depletion-inducible transporter, SbpA-CysTWA and that Mg^{2+} ion might need to stabilize poly-P in

cells under unexpectedly excess Pi conditions to avoid chlorosis.

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Author's contributions

Jinwoong Lee: Conceptualization, formal analysis, investigation, methodology, validation, visualization, and writing (original draft).

Yasushi Iwata, Yuji Suzuki: Resources and supervision.

Iwane Suzuki: Conceptualization, funding acquisition, methodology, project admini stration, resources, supervision, visualization, and writing (review and editing).

Conflict of interest statement

The authors declare no conflict of interest.

Statement of informed consent, human/animal rights

No conflicts, informed consent, and human or animal rights are applicable.

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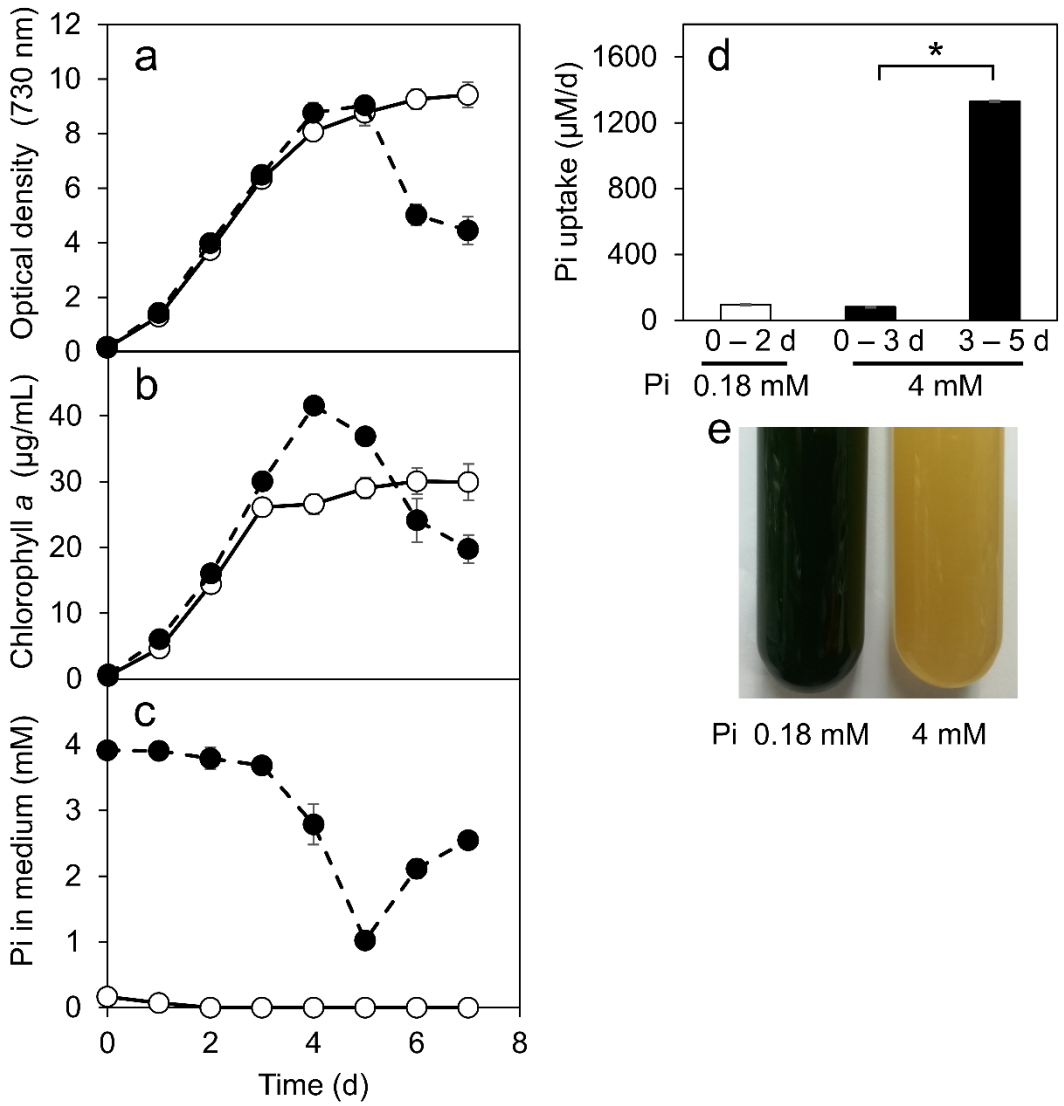

Fig. 1. Growth of the *Synechocystis* cells in 4 mM phosphate (Pi)-containing BG-11 medium. (a) Changes in optical density at 730 nm (OD_{730}) of the culture. (b) Chlorophyll *a* (Chl*a*) concentration in the culture. (c) Changes in Pi concentration in the medium. The closed and open circles indicate 4 mM Pi-containing BG-11 and standard BG-11, respectively. (d) Comparison of the Pi uptake rate. The closed bars indicate activities under 4 mM Pi conditions, and the open bar indicates activity under the standard BG-11. Each symbol and error bar indicates average values and standard deviations with triplicated results. (e) Seven-day-old cultures in the standard and 4 mM Pi-containing BG-11. Significance was evaluated by *t*-test. * = $P < 0.0001$

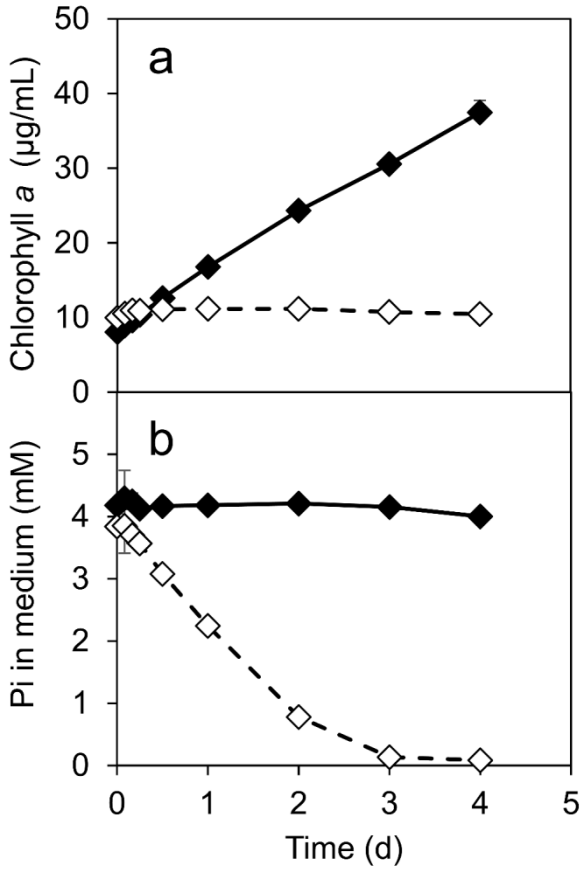

Fig. 2. Growth of the *Synechocystis* cells in sulfate (S)-depleted conditions. (a) Chlorophyll *a* concentration in the culture. The closed diamond indicates phosphate (Pi) (4 mM)-, Mg (2 mM)-, and S (2 mM)-repleted conditions. The open diamond indicates Pi (4 mM)- and Mg (2 mM)-repleted and S-depleted conditions. (b) Changes in Pi concentration in the media. The representation of each symbol is the same as above. Each symbol and error bar indicates average values and standard deviations with triplicated results.

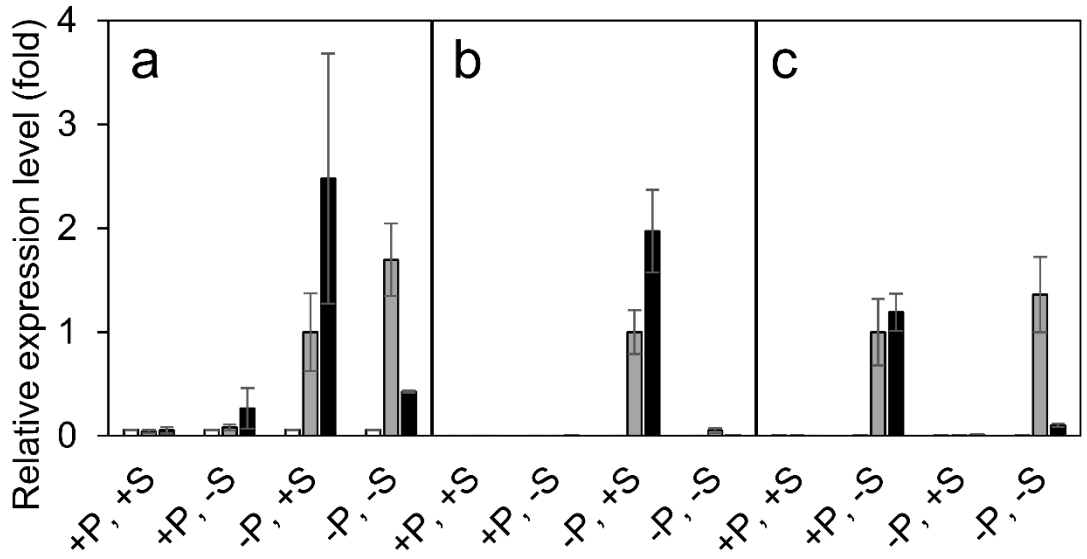

Fig. 3. Relative expression levels of the *pstS1*, *pstS2* and *sbpA* genes under the specific nutrient(s) deficient conditions. (a) *pstS1*, (b) *pstS2*, and (c) *sbpA* genes. The open, gray, and black bars indicate the relative expression level of each gene at 0, 12, and 24 h, respectively. The expression levels of the *pstS1* and *pstS2* genes were standardized by the values at 12 h under the Pi-deplete conditions (-P, +S), and the expression levels of the *sbpA* gene were standardized by the value at 12 h under the S-deplete conditions (+P, -S). The +P and +S indicate cultures in the media containing 4 mM Pi and 2 mM SO_4^{2+} conditions. Both of -P and -S indicate cultures without each nutrient. Mg^{2+} was supplied as $MgCl_2$ at 2 mM. The error bar indicates standard deviations with triplicated results.

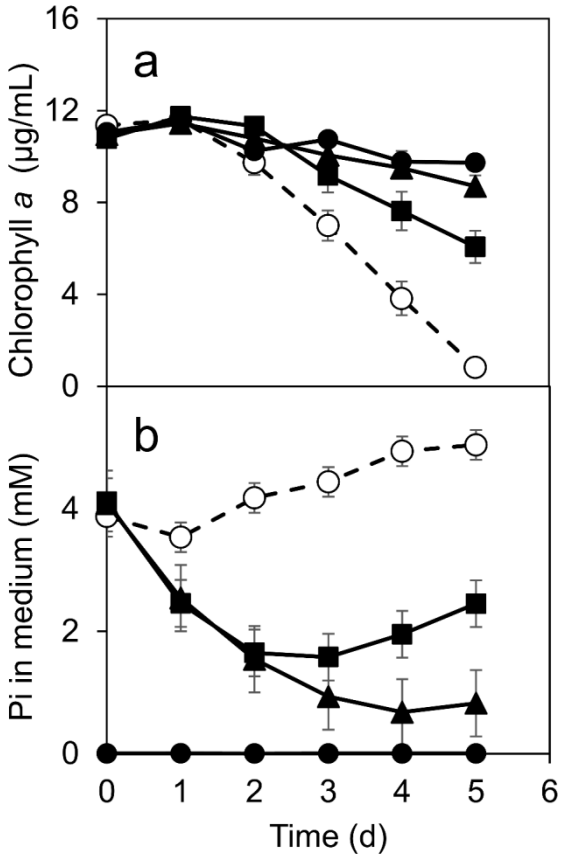

Fig. 4. Growth of the *Synechocystis* cells in various concentrations of Mg^{2+}. (a) Chlorophyll *a* concentration in the culture. Open circle, BG-11 without $MgSO_4$; closed square, 0.3 mM Mg^{2+}; closed triangle, 1 mM Mg^{2+}; closed circle, cultured under $MgSO_4$-depleted and phosphate (Pi)-depleted conditions. (b) Change in Pi concentration in the medium. The representation of each symbol is the same as above. Each symbol and error bar indicates average values and standard deviations with triplicated results.

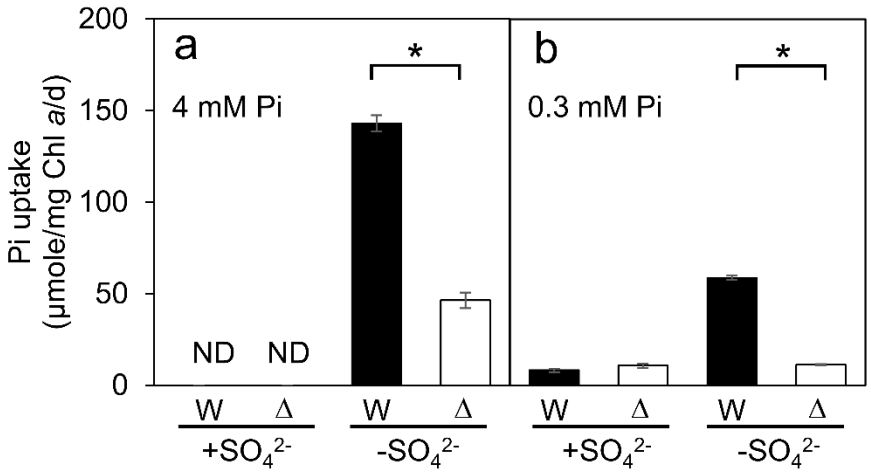

Fig. 5. Phosphate (Pi) uptake rate of the wild-type strain (W) and *sbpA-cysTWA* deletion strain (Δ) with (2 mM) or without (0 mM) sulfate (S) conditions. (a) 4 mM Pi-containing BG-11; ND, not detectable. The Pi uptake rate under 4 mM Pi condition was determined as described in Method (2.3.). (b) ^{32}Pi-containing BG-11 condition (0.3 mM Pi + 28.612 fmole ^{32}P in 5 mL of medium). Significancy was evaluated by *t*-test. * = $P < 0.0001$.

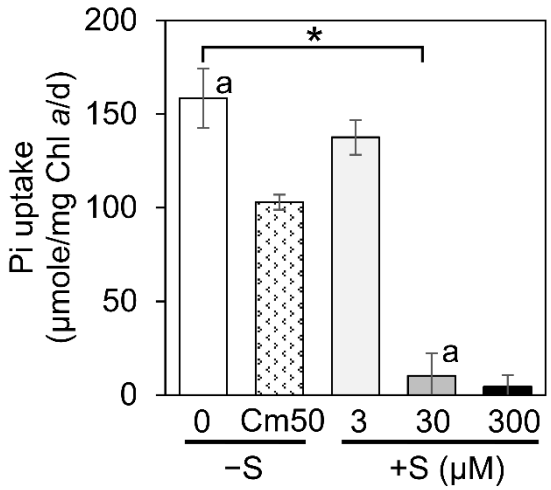

Fig. 6. Inhibition of phosphate (Pi) uptake with the presence of sulfate. The experiment was performed for 6 h. "Cm50" represents the addition of 50 μg/mL chloramphenicol. Significance was evaluated by *t*-test. * = $P < 0.0001$