



Title	Identification of a gene essential for protoporphyrinogen IX oxidase activity in the cyanobacterium <i>Synechocystis</i> sp. PCC6803
Author(s)	Kato, K.; Tanaka, R.; Sano, S.; Tanaka, A.; Hosaka, H.
Citation	Proceedings of the National Academy of Sciences, 107(38), 16649-16654 https://doi.org/10.1073/pnas.1000771107
Issue Date	2010-09-21
Doc URL	http://hdl.handle.net/2115/48275
Type	article (author version)
Additional Information	There are other files related to this item in HUSCAP. Check the above URL.
File Information	PNAS-2010_107_38_tanaka.pdf (本文)



[Instructions for use](#)

Classification:

Major category: BIOLOGICAL SCIENCES

Minor category: Microbiology

Title:

Identification of a gene essential for protoporphyrinogen IX oxidase activity in the cyanobacterium

Synechocystis sp. PCC6803

Author affiliation:

Kazushige Kato^{1*}, Ryouichi Tanaka¹, Shinsuke Sano^{*}, Ayumi Tanaka¹, Hideo Hosaka^{*}

^{*}Odawara research center, Nippon Soda Co., Ltd., Odawara 250-0280, Japan; and ¹Institute of Low

Temperature Science, Hokkaido University, Sapporo 060-0819, Japan

Author contributions: K.K., R.T., S. S., A.T., and H.H. designed research; K.K. performed research;

K.K. and R.T. analyzed data; and K.K. and R.T. wrote the paper.

Corresponding author:

Name: Kazushige Kato

Address: *Odawara Research Center, Nippon Soda Co. Ltd., Takada 345, Odawara, Kanagawa
250-0280, Japan*

Phone: +81-465-42-3511. Fax: +81-465-42-4377.

E-mail: k.katou@nippon-soda.co.jp

Manuscript information:

Twenty-nine text pages, two figures, and two tables.

Two supplementary tables and four supplementary figures.

Abstract

Protoporphyrinogen oxidase (Protox) catalyses the oxidation of protoporphyrinogen IX to protoporphyrin IX during the synthesis of tetrapyrrole molecules. Protox is encoded by the *hemY* gene in eukaryotes and by the *hemG* gene in many γ -proteobacteria, including *Escherichia coli*. It has been suggested that other bacteria possess a yet unidentified type of Protox. To identify a novel bacterial gene encoding Protox, we first introduced the *Arabidopsis hemY* gene into the genome of the cyanobacterium, *Synechocystis* sp. PCC6803. We subsequently mutagenized the cells by transposon tagging and screened the tagged lines for mutants that were sensitive to acifluorfen, which is a specific inhibitor of the *hemY*-type Protox. Several cell lines containing the tagged *slr1790* locus exhibited acifluorfen sensitivity. The *slr1790* gene encodes a putative membrane-spanning protein that is distantly related to the M subunit of NADH dehydrogenase complex I. We attempted to disrupt this gene in the wild-type background of *Synechocystis*, but we were only able to obtain heteroplasmic disruptants. These cells accumulated a substantial amount of protoporphyrin IX, suggesting that the *slr1790* gene is essential for growth and Protox activity of cells. We found that most cyanobacteria and many other bacteria possess *slr1790* homologues. We overexpressed an *slr1790* homologue of *Rhodobacter sphaeroides* in *Escherichia coli* and found that this recombinant protein possesses Protox activity *in vitro*. These results collectively demonstrate that *slr1790* encodes a novel Protox enzyme and we propose to name the *slr1790* gene as *hemJ*.

¥body

INTRODUCTION

Tetrapyrroles, such as chlorophyll, heme and bilins, play vital roles in various biological processes including photosynthesis and respiration. The early steps of tetrapyrrole biosynthesis, which convert 5-aminolevulinate acid (ALA) into protoporphyrin IX (Proto IX), are present in both chlorophyll and heme biosynthesis in nearly all organisms (1). Protoporphyrinogen oxidase (Protox, EC 1.3.3.4) catalyses the oxidation of protoporphyrinogen IX (Proto IX) to Proto IX, which is the last common step toward chlorophyll and heme synthesis, respectively. Based on the solved crystal structures of Protox (2) and ferrochelatase (3), which catalyzes the subsequent enzymatic step of Protox, it has been suggested that both enzymes form a complex to enable efficient channeling of Proto IX (2). Because Protox transfers Proto IX to Mg-chelatase and ferrochelatase, which catalyze the committed steps in chlorophyll and heme biosynthesis, respectively, it is plausible that a protein-protein interaction between Protox and Mg-chelatase or ferrochelatase may regulate the substrate channeling between the heme and the chlorophyll branches (2).

In most eukaryotes and many aerobic or facultative bacteria such as Firmicutes, Protox is encoded by the *hemY* gene (1). This type of Protox is a FAD-containing oxidase of approximately 55 kDa (4, 5). In contrast, a number of γ -proteobacteria, including *Escherichia coli* (6), *Salmonella typhimurium* (7) and several other bacteria, have another type of Protox encoded by the *hemG* gene. The molecular

weight of the *hemG*-type Protox is approximately 21 kDa. Interestingly, homologues of *hemY* nor *hemG* are lacking from the genomes of most archaea and many other bacteria (1, 8). In particular, the absence of *hemY* in the majority of cyanobacteria is puzzling, as it is now generally believed that the first chloroplast resulted from endosymbiosis between a cyanobacterium and a nonphotosynthetic eukaryote (9). Since the oxidation of the Protox is a prerequisite step for the synthesis of heme and chlorophylls, several researchers have suggested that these organisms have a yet unidentified type of Protox (1, 8, 10). Identification of the cyanobacterial Protox would aid in our understanding of the evolution of tetrapyrrole biosynthesis in bacteria. Moreover, phylogenetic analysis of Protox genes may provide molecular insights into the early events of evolution of photosynthesis (11).

The study of Protox genes is also of interest, because Protox is a target of commonly used herbicides such as diphenyl-ether herbicides (12). These herbicides act as competitive inhibitors of the *hemY*-encoded Protox (13), resulting in the accumulation of Protox within the chloroplast.

Protox leaks out of the chloroplast into the cytoplasm where it is rapidly oxidized to Proto IX by non-specific peroxidases and/or auto-oxidation (14). Proto IX is a potent photosensitizer, thus causing rapid oxidation of various molecules in cells, ultimately leading to cell death (5, 13). Because cyanobacterial Protox is probably not related to the *hemY*-type Protox, it is hypothesized that transformation of plants with the cyanobacterial Protox gene may confer resistibility of plants, which may result in the generation of herbicide-resistant crops. Cloning of the cyanobacterial Protox gene

would enable the manipulation of herbicide sensitivity of plants that target the *hemY*-type Protox.

Using *in vitro* transposon mutagenesis in conjunction with functional complementation with the *Arabidopsis hemY*-type Protox, we report here that the *slr1790* gene is essential for Protox function in the cyanobacterium *Synechocystis* sp. PCC6803. The *slr1790* gene encodes a protein that is distantly related to a subunit of NADH dehydrogenase complex I. We found that a number of bacteria, including most cyanobacteria and the majority of proteobacteria, contain a homologue of *slr1790*. We overexpressed a *Rhodobacter* homologue of *slr1790* in *E. coli* and detected Protox activity of the recombinant protein. Collectively, these results demonstrate that *slr1790* encodes a novel Protox enzyme.

RESULTS

Isolation of mutants sensitive to diphenyl-ether herbicides

In order to identify the gene encoding Protox in the cyanobacterium *Synechocystis* sp. PCC6803, we screened a mutant pool in which the cyanobacterial genes were randomly disrupted by insertion of the kanamycin resistance gene (15). Since the oxidation of Protox is an obligatory step in tetrapyrrole synthesis, the disruption of the gene encoding Protox was predicted to be lethal, unless redundant genes encode Protox in *Synechocystis* sp. PCC6803. Therefore, prior to screening, we first introduced the *hemY* gene of *Arabidopsis thaliana* (D83139) into the wild-type (WT) strain of

Synechocystis sp. PCC6803. Consequently, this transgenic cyanobacterium (designated as the AT strain) had both *Arabidopsis hemY*-type Prottox and the endogenous cyanobacterial Prottox. We then introduced mutations by transposon-mediated random insertion of the kanamycin-resistance cassette into the chromosome of the AT strain. We predicted that disruption of the cyanobacterial Prottox gene would not cause severe growth retardation of the cell, because the gene disruption should be complemented by the already-introduced *Arabidopsis hemY* gene. Therefore, we hypothesized that disruption of the endogenous Prottox gene would render the cells sensitive to diphenyl-ether because the cells were predicted to become dependent on the *Arabidopsis* Prottox that is sensitive to these herbicides. To the best of our knowledge, the use of a specific inhibitor with genetic pre-complementation is a novel screening strategy for essential enzymes. This strategy may therefore be applicable to the identification of a variety of genes encoding essential enzymes, as long as another enzyme with altered sensitivity to the selection marker is available.

We screened 6,686 transformants and successfully isolated nine mutant lines that were sensitive to the diphenyl-ether herbicide, acifluorfen. We determined the insertion sites of the kanamycin resistance cassette in these lines by inverse PCR. We identified seven lines containing the kanamycin-resistance cassette at 21 bp upstream of the start of the *slr1790* gene open reading frame (BAA16808.1), suggesting that these lines were clonal. Among all the lines isolated, these exhibited the strongest herbicide sensitivity. The other lines, which showed sensitivity to the herbicide,

contained the kanamycin-resistant cassette in the *slr0844* locus, encoding NADH dehydrogenase I chain L (BAA10530.1). They were characterized by slower growth in the presence of acifluorfen, but unlike the lines with a mutation upstream of the *slr1790* gene, they survived in the presence of acifluorfen.

Targeted disruption of the *slr1790* gene

To investigate the function of the protein encoded by the *slr1790* gene, we disrupted the *slr1790* gene in the WT and AT strains by targeted insertion of the kanamycin-resistance cassette. We designated these disruptants as WTSK and ATSK, respectively (Supporting information Fig. S1A). Since *Synechocystis* is polyploid, we assessed the segregation of the mutant genome copy in the mutant strains by PCR after several rounds of selection on kanamycin-containing agar plates. Genomic DNA was extracted from cells and amplified by PCR, using primer pairs that hybridized to the upstream and downstream regions of the *slr1790* gene. Amplification of genomic DNA extracted from the WT and the AT strains generated a PCR fragment of 1.9 kbp, which corresponds to the size of the intact *slr1790* gene (Fig. S1B). In contrast, PCR using genomic DNA isolated from the ATSK strain yielded a 3.2-kbp PCR fragment, which corresponds to the size of the WT fragment (1.9 kb) plus the inserted kanamycin-resistance cassette (1.3 kb). Our results demonstrate that the disrupted *slr1790* gene segregated in the ATSK strain. On the other hand, PCR using genomic DNA isolated

from the WTSK strain resulted in two fragments of 1.9 kbp and 3.2 kbp, suggesting that segregation of the tagged *slr1790* gene was incomplete. It is likely that the *slr1790* gene is essential for the growth of the cells and that disruption of this gene is lethal.

Effect of herbicides on cell growth

Supporting our hypothesis that the *slr1790* gene encodes an essential protein, the WTSK strain with heteroplasmic DNA (see Fig. S1B) showed retarded growth compared to the WT strain (Fig. 1A). In contrast, the ATSK strain, in which the *slr1790* gene appeared to be completely knocked-out, survives like the WT and AT strains (Fig. 1A). These results indicate that the *Arabidopsis hemY* gene can compensate the loss of the *slr1790* gene and can restore the growth of the mutant to the level comparable to that of WT (Fig. 1A).

At the present stage, a possibility cannot be excluded that the *slr1790* gene has an unidentified role other than being involved in Protox activity and that the *Arabidopsis hemY* gene coincidentally compensated this unidentified function. To clarify this point, we fed acifluorfen to the mutant strains to inhibit Protox activity of the *hemY* gene product. In the presence of 100 μ M acifluorfen, the growth of the ATSK strain was significantly retarded compared to the WT, AT and the WTSK strains (Fig. 1B). These results demonstrate that Protox activity of the *hemY* gene product is necessary to compensate the loss of the *slr1790* gene.

To further substantiate that acifluorfen specifically inhibited Protox activity in the ATSK strain, we tested the effects of two other classes of Protox inhibitors, pyraflufen-ethyl and flumioxazin (Table 1). While the AT strain was insensitive to the diphenylether herbicides when used in a concentration of up to 10^{-4} M, growth of the ATSK strain was severely affected with I_{50} values of $10^{-4.5}$ to $10^{-6.7}$ M (Table 1). These results are consistent with our hypothesis that the ATSK strain is dependent on the *HemY*-encoded Protox due to the lack of the cyanobacterial-type Protox. However, we could not rule out a possibility that the *slr1790* gene is involved in scavenging ROS and that the disruption of the *slr1790* locus conferred sensitivity to ROS, which might be generated by a small amount of Proto IX or Protogen IX accumulation. Therefore, we tested the effect on cell growth in the presence of diquat, a generator of ROS in cells. In contrast to diphenylether herbicides, there was no difference in the growth of the AT strain and the ATSK strain on diquat-containing cultures. Therefore, our data support the hypothesis that the disruption of the *slr1790* gene abolished the endogenous Protox activity, resulting in growth retardation of the WTSK strain and the ATSK strain in the presence of the diphenylether herbicide acifluorfen.

Analysis of pigments by HPLC

We examined the levels of chlorophyll *a* and intermediates of chlorophyll biosynthesis in WT, AT and the insertional mutants by HPLC (Table 2) in the presence and absence of acifluorfen. Although

the WTSK strain was heteroplasmic, this strain accumulated a substantial amount of Proto IX, while the WT strain failed to accumulate detectable levels of Proto IX. It should be noted that we could not detect Protogen IX in the cell extracts of the WTSK strain. One might expect that inhibition of Protox activity may lead to accumulation of Protogen IX, the substrate of the Protox enzyme. However, it has been reported that inhibition of Protox usually results in the detection of only Proto IX, as Protogen IX is readily oxidized by oxygen or by other non-specific cellular activities within the cells, or during the procedure of pigment analysis (13, 16-18). Thus, we assumed that the level of Proto IX detected by our HPLC system most likely represented that of Protogen IX plus Proto IX within the cells.

In the WTSK strain, the levels of chlorophyll *a* substantially reduced to nearly 1/2 of the WT strain both in the absence and presence of acifluorfen (Table 2). These data are in accordance with the increase in the Proto IX level in this strain. Similarly, the ATSK strain accumulated a higher level of Proto IX (890 pmol/10⁸ cells) than any other strains tested in the presence of acifluorfen, which indicates that metabolism of Proto(gen) IX is severely impaired in this strain. It was rather unexpected that the same strain accumulated a moderate amount of Proto(gen) IX (76 pmol/10⁸ cells) in the absence of acifluorfen. We are speculating that tetrapyrrole synthesis is partially stalled due to the presence of HemY in the ATSK strain. Slr1790 may be in tight contact with the subsequent enzymes such as ferrochelatase or Mg-chelatase, but HemY may not have such a

contact with subsequent enzymes. As a result, efficient channeling of Proto IX might have been compromised in the ATSK strain. The level of chlorophyll *a* in the ATSK strain was close to that of WT. In the AT strain, we observed a substantial accumulation of Proto IX (197 pmol/10⁸ cells) and reduction in the chlorophyll contents in the presence of acifluorfen. This observation can be explained by assuming that the inhibited HemY is competing with endogenous HemJ for access to docking sites on Mg-chelatase and ferrochelatase. Collectively, we conclude that the *slr1790* disruptants are defective in their native Protox activity.

Phylogenetic analysis of the Slr1790 sequences

The *slr1790* gene encodes a protein of 193 amino acid residues with five putative transmembrane helices. The amino acid sequence shares homology to the NuoM (ND4) subunit of the NADH dehydrogenase complex I from various organisms, including *E. coli* (CAA48372), human (ABX40271), and *Synechocystis* sp. PCC6803 (K05575)(Fig. S2). This subunit is proposed to be involved in proton translocation across membranes (19). However, the Slr1790 sequences are about half of the NuoM sequences in length, and a few conserved regions of the NuoM sequences are missing in the Slr1790 sequences including the essential residues for the function of the NuoM protein (19)(see Fig. S2). Therefore, it is unlikely that the protein encoded by *slr1790* possesses the same function as NuoM.

The *slr1790* homologues are highly conserved in most cyanobacteria (Fig. S3). Among 30 cyanobacterial genomes that have been sequenced at the time of writing this manuscript, 23 cyanobacterial species contain *slr1790* homologues, but no *hemG* or *hemY* homologues are present (Table S1). In contrast, two *Prochlorococcus* species (*Prochlorococcus marinus* str. MIT 9215 and *Prochlorococcus marinus* str. MIT 9515) only contain *hemG* homologues, while four other cyanobacteria (*Synechococcus* sp. JA-2-3B'a(2-13), *Synechococcus* sp. JA-3-3Ab, *Thermosynechococcus elongatus* BP-1 and *Trichodesmium erythraeum* IMS101) only have *hemY* homologues. *Gloeobacter violaceus* PCC 7421 is an exception, because it contains both *slr1790* and *hemY* homologues. It is noteworthy that except for this organism, distribution of *slr1790*, *hemG* and *hemY* homologues in cyanobacteria was exclusive to each other (Table S1). We examined whether the distribution of any other genes in cyanobacteria was mutually exclusive to that of *hemG* and *hemY* using the genome comparison tool CCCT (20). We found that *slr1790* is the only gene that shows such an exclusive distribution pattern. Thus, these genome analyses also support our genetic data, suggesting that *hemG*, *hemY* and *slr1790* encode proteins that share the same function.

slr1790 homologues were found in a variety of bacterial species, including Bacteroidetes, α -, β -, ϵ -, and δ -proteobacteria (Fig. S4). In proteobacteria, except for γ -proteobacteria, *slr1790* homologues seemed to dominate over the other two genes. In contrast, many Chloroflexi species contain *hemY* homologues. *hemG* homologues were mainly found in γ -proteobacteria, in which

distributions of *slr1790* and *hemG* homologues were mutually exclusive. Likewise, distribution of *slr1790* and *hemY* homologues in the Bacteroidetes/Chlorobi was mutually exclusive (Table S2). Within this group of bacteria, those which have more than one *hemY*, *hemG*, and *slr1790* homologue, seem to be an exception. For example, only *Chlorobium phaeobacteroides* DSM 266 contains both *hemG* and *hemY* homologues among the Bacteroidetes/Chlorobi group (Table S2). Out of 17 sequenced Bacteroidetes/Chlorobi genomes, seven are lacking *slr1790*, *hemG* or *hemY* homologues (Table S2), indicating that there is at least one yet unidentified gene encoding Protox in these species.

Protox activity in recombinant *Rhodobacter* protein similar to the cyanobacterial Slr1790 proteins

To examine whether Slr1790 protein possess Protox activity in vitro, we attempted to obtain recombinant Slr1790 protein by expressing the *Synechocystis slr1790* gene in *E. coli* or using the Baculovirus expression system in insect cells. However, the protein did not accumulate to a detectable level in these systems by unidentified reasons. Therefore, we attempted to express an *slr1790* homologue from another organism to examine if it encodes a protein with Protox activity. Because Gomelsky and Kaplan had reported that they successfully overexpressed a *Rhodobacter sphaeroides* homologue of *slr1790* (hereafter referred to as the *Rs-slr1790* gene) in *E. coli* (21), we

began with this gene to examine Protox activity. The *E. coli* transformant harboring the *Rhodobacter* homologue successfully produced a chimeric recombinant protein fused with the maltose-binding protein (MBP) tag (hereafter referred to as Rs-Slr1790 protein). It should be noted that Rs-Slr1790 was solubilized in the presence of 0.04% (w/v) Tween 20 when the protein was fused with the MBP tag. Similar observations were reported with other putative membrane-spanning proteins (e.g. (22, 23)). When we added this protein into a reaction mixture containing Protogen IX, the pigment was converted to Proto IX and emitted strong fluorescence at 633 nm (Fig. 2). In contrast, addition of the MBP tag alone to the reaction mixture did not significantly increase the rate of the Protogen IX-to-Proto IX conversion when it was compared to the auto-oxidation rate of Protogen IX (Fig. 2). These results clearly demonstrate that Rs-Slr1790 possess Protox activity.

Discussion

We applied a new screening strategy to isolate a cyanobacterial mutant defective in Protox activity. By introducing the *hemY* gene into cyanobacterial cells prior to screening, we obtained viable mutants. The major advantage of our screening method is that it automatically guaranteed mutant complementation with the *hemY* gene, suggesting that the mutant phenotypes were primarily due to the loss of the endogenous Protox activity. Our newly developed strategy may be applied to

screening for additional types of mutants. One important limitation of our strategy, however, is that it can only be used when the protein of interest shares the same function with a different type of known protein from other species. Our strategy also requires a specific inhibitor such as acifluorfen for the known counterpart of the protein. Nevertheless, a number of enzymatic reactions are catalyzed by two or more types of enzymes (20, 24). Thus, this screening strategy might be a desirable option for researchers attempting to identify novel proteins through genetic screening.

In this study, we showed that the *slr1790* gene is essential in Protox activity in *Synechocystis* PCC6803, by genetic complementation with the *Arabidopsis hemY* gene. In addition, we overexpressed *Rs-slr1790* in *E. coli* to demonstrate that the gene product has Protox activity. These results suggest that *slr1790/Rs-slr1790* encodes a novel type of Protox. This conclusion is further supported by the results of comparative genome analysis which reveals mutually exclusive distribution (except for *Gloeobacter*) of *slr1790*, *hemY* and *hemG* homologues in cyanobacteria and other classes of bacteria. Accordingly, we propose to name the *slr1790* gene *hemJ*, following the bacterial nomenclature convention (25).

The predicted structure of the protein encoded by *slr1790* is very different from those deduced for the *HemY*-type and the *HemG*-type Protox, both of which do not contain transmembrane regions (26). Accordingly, the *slr1790*-encoded protein presumably has a different reaction mechanism compared to HemY or HemG proteins. HemY protein likely transfers electrons from Proto IX to oxygen

through its flavin cofactor (2). HemG protein also uses a flavin cofactor to transfer electrons from Protogen IX to the electron transfer chain (6). On the other hand, a search for a motif database (27) did not identify any known co-factor binding motifs on the *slr1790* sequence. One possibility is that the *slr1790*-encoded protein may use heme for its reaction, since the *slr1790* gene shows weak homology to the *NuoM* gene encoding a subunit of NADH dehydrogenase complex I, which also contains heme as a cofactor. However, at the present stage, it is not clear whether the NuoM subunit and the Slr1790 protein bind heme.

In our assay system, the recombinant Rs-*slr1790* protein showed Protox activity without any electron acceptors added to the reaction mixture. Thus, a possible candidate for an electron acceptor of the Protox reaction is oxygen. Even though we performed the assay under a micro-aerobic conditions as described in Materials and Methods, it was evident from the auto-oxidation of Protogen IX that some molecular oxygen was present in the reaction mixture. It is not clear at this stage what molecule accepts electrons from Protogen IX *in vivo*.

A survey for *slr1790*, *HemY*, and *HemG* homologues in the Genbank database did not identify any homologues in the majority of the Archaea and several bacterial phyla, including Acidobacteria, Actinobacteria, Deinococcus, Fusobacteria, Spirochaetes, and Thermotogae. It is plausible that these organisms contain yet unidentified types of Protox genes. It is intriguing that more than three unrelated Protox genes have occurred in the evolutionary history of tetrapyrrole biosynthesis. Such

occurrence of multiple enzymes is not rare in the tetrapyrrole biosynthetic pathway (24). For example, reduction of protochlorophyllide is catalyzed by two distinct enzymes, POR and the Chl BLN complex, in cyanobacteria (28). In this case, POR operates under both aerobic and micro-oxic conditions, but it requires light for its reaction. On the other hand, the Chl BLN complex catalyzes the same reaction only under micro-oxic conditions regardless of illumination (28). In contrast, in the case of *slr1790*, *HemY*, and *HemG* homologues, it is difficult to assume any reasons for the presence of *slr1790*, *HemY* or *HemG* in the bacterial kingdom, as these genes coexist in the same class of bacteria. It is probably necessary to characterize the enzymatic properties of each type of Protox to unravel the evolution of Protox encoding genes. For example, oxygen-sensitivity, substrate affinity, and interaction with enzymes involved in the preceding (coproporphyrinogen oxidation) or the following steps (metal chelation to Proto IX) of Proto IX oxidation may provide a plausible explanation to the mosaic distribution of Protox-encoding genes.

In summary, our data suggest that the *slr1790/Rs-slr1790* gene encodes a novel type of Protox, which is mainly distributed in cyanobacteria, Bacteroidetes and proteobacteria. Our phylogenetic analysis also suggests that there is at least one yet-unknown type of Protox present in bacteria and archaea. Identification of such an enzyme would aid in our understanding of the evolutionary history of tetrapyrrole biosynthesis. Our findings may also lead to future investigations of the enzymatic activity of Protox, which will provide additional information on the branch-point regulation between

the heme/bilin and chlorophyll branches in cyanobacteria and other bacteria. Furthermore, results presented in this study may be useful for the development of novel antibacterial agents and transgenic plants expressing the cyanobacterial Protox, making them resistant to diphenyl-ether herbicides.

Materials and methods

Culture and growth conditions

Escherichia coli strains JM109, XL1-Blue MRF' and SOLR were grown in Luria-Bertani medium with 100 µg/ml ampicilin or 25 - 50 µg/ml kanamycin at 37°C. A glucose-tolerant wild-type (WT) strain of *Synechocystis* sp. PCC 6803 (29) and mutants were grown at 30°C in BG-11 medium buffered with 10mM TES-KOH (pH 7.5) under continuous illumination (10-20 µmol photons m⁻² s⁻¹). When appropriate, antibiotics were included in the medium at a final concentration of 25 µg/ml chloramphenicol or 25 µg/ml kanamycin. Herbicides were dissolved in dimethyl sulfoxide and were added to the culture medium just before subculturing, giving a final dimethyl sulfoxide concentration of 5 ml litre⁻¹. Cell density in liquid culture was determined by measuring the optical density at 730 nm (OD₇₃₀) or 620nm (OD₆₂₀).

Transformation of cyanobacteria

For transformation of cyanobacteria, we used the plasmid vector pFS10 (a kind gift of Prof. Ikeuchi, Tokyo Univ.) which carries a promoter and terminator of *psbA2* encoding the D1 protein of the cyanobacterium *Synechocystis* sp. PCC 6803 (30) and a chloramphenicol-resistance marker. A DNA fragment of approximately 1.6 kbp encoding the *Arabidopsis thaliana hemY* gene (accession no. D83139) (4) was amplified by PCR using the following primers, ATHPPOX. *AseI* fr.

(5'-GGGGATTAATGGAGTTATCTCTTCTCCGT-3') and ATHPPOX. rev.

(5'-TTACTTGTAAGCGTACCGTG-3'). The PCR fragment was cut with *AseI* and inserted into the *NdeI* and *HincII* sites of the multiple cloning region of the pFS10 vector. The plasmid was then transformed into the *Synechocystis* WT strain according to the method of Williams (31). This transgenic cyanobacterium containing the introduced *Arabidopsis thaliana hemY* gene was designated the AT strain.

Genomic library preparation and transposon mutagenesis

To isolate genomic DNA, *Synechocystis* WT strain cells were pelleted and resuspended in 50 mM Tris-HCl (pH8.0) and 20 mM EDTA. Cells were lysed with 0.5% (w/v) SDS at room temperature. After several rounds of phenol-chloroform treatment, DNA was precipitated with ethanol.

For construction of a genomic library, *Synechocystis* genomic DNA was digested with *Tsp5091* and ligated into the Lamda-ZAP II vector (Stratagene). The Lamda-ZAP II *Synechocystis* genomic library contained approximately 10^5 clones with an average insert size of 2.0 kbp. The library was amplified and pBluescript was excised from the phage DNA. The pBluescript *Synechocystis* genomic library was subjected to *in vitro* transposon mutagenesis (protocol of Epicentre) and then transformed into cells of the *Synechocystis* AT strain. Transformed cells were spread on 1.5% BG-11 agar plates containing 25 µg/ml kanamycin and plates were incubated at 30°C under continuous

illumination ($20 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$).

Screening for mutants

The disruption mutants to be tested for sensitivity to diphenyl-ether herbicides were grown under continuous illumination. Each mutant was collected and resuspended individually in fresh BG-11 medium at a cell density of $\text{OD}_{730} = 0.01, 0.1$ and 0.3 with precultures. Three microliters of the cell suspensions were spotted onto BG-11 agar plates with or without $500 \mu\text{M}$ acifluorfen. Plates were incubated at 30°C under continuous illumination ($20 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) and evaluated for cell growth at 7, 10, 15 and 25 days.

Construction of the *slr1790* gene disruption mutants

A DNA fragment containing the *slr1790* ORF, a 600-bp upstream and a 700-bp downstream region (Fig. S1A) was amplified by PCR using the following primers: *slr1790* km *EcoRI* fr. (5'-GGGGAATTCTGCTTGCATCAATATGGTGGC-3') and *slr1790* km *HindIII* rev. (5'-GGGAAGCTTACCCTGGAGATCCACTGGTT-3'). The resulting 1.9 kbp fragment was cloned into the pGEM-T Easy vector. The resulting plasmid pSlr1790 contains a unique *NheI* site in the ORF *slr1790*. To disrupt the *slr1790* ORF, the plasmid pSslr1790 was linearized via *NheI* and ligated to a kanamycin-resistance cassette. This gene disruption vector was transformed into the

Synechocystis WT and AT strains. Following transformation, cells were spread on 1.5% BG-11 agar plates containing 25 µg/ml kanamycin and plates were incubated at 30°C under continuous illumination (20 µmol photons m⁻² s⁻¹).

Pigment analysis by HPLC

The WT strain, AT strain and the insertional mutants were incubated for 3 days in the light. At the end of incubation, Proto IX and chlorophyll *a* were extracted from these strains with 80% acetone and analyzed by HPLC on a Symmetry C₈ column (150 mm long, 4.6mm i.d.; Waters, Tokyo, Japan), according to the method of Zapata et al. (32). Proto IX was monitored by a fluorescence detector (RF-10AXL, Shimadzu Co., Kyoto, Japan) at an excitation wavelength of 405 nm and an emission wavelength of 633 nm, while chlorophyll *a* was monitored by absorbance at 663 nm. Commercial standards of Proto IX (Sigma) and chlorophyll *a* (Juntec Co.) were used.

Protox enzyme assay

The *Rs-slr1790* gene was amplified by PCR from *Rhodobacter sphaeroides* DNA (a kind gift of Prof. Shinji Masuda, Tokyo Institute of Technology). The amplicon was cloned into pMAL-c2E (New England Biolab), which encodes an MBP tag. The construct was introduced into the *E. coli* strain JM109 and inoculated with LB supplemented with ampicillin. Expression of the *Rs-slr1790* gene

was induced by the addition of IPTG to a final concentration of 0.4 mM. The cells were harvested by centrifugation and resuspended in 50 mM Tris-Cl (pH 8.0) containing 50 mM NaCl, 0.04% Tween 20, protease inhibitor cocktail (P8465, Sigma) and were subsequently disrupted by sonication. The supernatant was collected after centrifugation at 40,000 x g for 20 min. The Rs-slr1790 protein was partially purified with amylose resin (New England Biolab). An aliquot equivalent to 20 ug protein was subjected to a Prottox assay according to the method of Jacobs and Jacobs (33). Readers are encouraged to view the supporting information section for a detailed protocol describing the Prottox assay. Briefly, a reaction mixture contained a protein aliquot, 10 uM Protogen IX, 50 mM Tris-Cl (pH 8.0) and 50 mM NaCl 0.04% Tween 20, 5U glucose oxidase (from *Aspergillus niger*, Wako Chemicals), 40 mM glucose and 10U catalase (Sigma). A reaction mixture with MBP fused with or without protein was prepared as a negative control. The reaction was initiated by adding Protogen IX to the mixture and the reaction proceeded at room temperature in darkness for 30 min. The Proto IX production was monitored by fluorescence with the excitation at 405 nm and the emission at 633 nm using a calibration curve constructed with a serial dilution of the standard Proto IX.

Acknowledgements

We are grateful to Profs. Hachiro Inokuchi (National Institute of Technology and Evaluation, and

Kyoto Univ.), Shigeru Taketani (Kyoto Institute of Technology), Yuichi Fujita (Nagoya Univ.) and Nobuo Takakusa (Nippon Soda) for helpful suggestions, and Takako Fukagawa (Nippon Soda), Motoaki Sato (Nippon Soda), Masumi Hirashima (Hokkaido Univ.) and Sachiko Tanaka (Hokkaido Univ.) for their excellent technical assistance. We thank Prof. Shinji Masuda (Tokyo Inst. Tech.) for providing us with *Rhodobacter* DNA. Financial support was provided through a Grant-in-Aid for Scientific Research (17770027 to R.T.) and Grant-in-Aid for Creative Scientific Research (no. 17GS0314 to A.T.) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

1. Oborník M & Green BR (2005) Mosaic origin of the heme biosynthesis pathway in photosynthetic eukaryotes. *Mol Biol Evol* 22:2343-2353.
2. Koch M, *et al.* (2004) Crystal structure of protoporphyrinogen IX oxidase: a key enzyme in haem and chlorophyll biosynthesis. *EMBO J* 23:1720-1728.
3. Wu CK, *et al.* (2001) The 2.0 Å structure of human ferrochelatase, the terminal enzyme of heme biosynthesis. *Nat Struct Biol* 8:156-160.
4. Narita S, *et al.* (1996) Molecular cloning and characterization of a cDNA that encodes protoporphyrinogen oxidase of *Arabidopsis thaliana*. *Gene* 182:169-175.
5. Lermontova I, Kruse E, Mock HP, & Grimm B (1997) Cloning and characterization of a plastidal and a mitochondrial isoform of tobacco protoporphyrinogen IX oxidase. *Proc Natl Acad Sci USA* 94:8895-8900.
6. Boynton TO, Daugherty LE, Dailey TA, & Dailey HA (2009) Identification of *Escherichia coli* HemG as a novel, menadione-dependent flavodoxin with protoporphyrinogen oxidase activity. *Biochemistry* 48:6705-6711.
7. Xu K, Delling J, & Elliott T (1992) The genes required for heme synthesis in *Salmonella typhimurium* include those encoding alternative functions for aerobic and anaerobic coproporphyrinogen oxidation. *J Bacteriol* 174:3953-3963.
8. Panek H & O'Brian MR (2002) A whole genome view of prokaryotic haem biosynthesis.

- Microbiol* 148:2273-2282.
9. McFadden GI (2001) Chloroplast origin and integration. *Plant Physiol* 125:50-53.
 10. Vavilin DV & Vermaas WF (2002) Regulation of the tetrapyrrole biosynthetic pathway leading to heme and chlorophyll in plants and cyanobacteria. *Physiol Plant* 115:9-24.
 11. Xiong J, Fischer WM, Inoue K, Nakahara M, & Bauer CE (2000) Molecular evidence for the early evolution of photosynthesis. *Science* 289:1724-1730.
 12. Hirai K (1999) in *Peroxidizing herbicides*, eds Böger P & Wakabayashi K (Springer-Verlag, Berlin Heidelberg), pp 15-71.
 13. Matringe M, Camadro JM, Labbe P, & Scalla R (1989) Protoporphyrinogen oxidase as a molecular target for diphenyl ether herbicides. *Biochem J* 260:231-235.
 14. Murata S, Kimura Y, Mabuchi T, & Miura Y (2004) Accumulation of Protoporphyrinogen IX prior to Protoporphyrin IX in intact plants treated with protoporphyrinogen IX oxidase-inhibiting herbicides. *J Pestic Sci* 29:195-199.
 15. Bhaya D, Takahashi A, & Grossman AR (2001) Light regulation of type IV pilus-dependent motility by chemosensor-like elements in *Synechocystis* PCC6803. *Proc Natl Acad Sci USA* 98:7540-7545.
 16. Deybach JC, de Verneuil H, & Nordmann Y (1981) The inherited enzymatic defect in porphyria variegata. *Hum Genet* 58:425-428.
 17. Lermontova I & Grimm B (2000) Overexpression of plastidic protoporphyrinogen IX oxidase leads to resistance to the diphenyl-ether herbicide acifluorfen. *Plant Physiol* 122:75-83.
 18. Lermontova I & Grimm B (2006) Reduced activity of plastid protoporphyrinogen oxidase causes attenuated photodynamic damage during high-light compared to low-light exposure. *Plant J* 48:499-510.
 19. Torres-Bacete J, Nakamaru-Ogiso E, Matsuno-Yagi A, & Yagi T (2007) Characterization of the NuoM (ND4) subunit in *Escherichia coli* NDH-1: CONSERVED CHARGED RESIDUES ESSENTIAL FOR ENERGY-COUPLED ACTIVITIES. *J Biol Chem* 282:36914-36922.
 20. Ito H, Yokono M, Tanaka R, & Tanaka A (2008) Identification of a novel vinyl reductase gene essential for the biosynthesis of monovinyl chlorophyll in *Synechocystis* sp. PCC6803. *J Biol Chem* 283:9002-9011.
 21. Gomelsky M & Kaplan S (1996) The *Rhodobacter sphaeroides* 2.4.1 rho gene: expression and genetic analysis of structure and function. *J Bacteriol* 178:1946-1954.
 22. Hampe W, *et al.* (2000) Engineering of a proteolytically stable human beta 2-adrenergic receptor/maltose-binding protein fusion and production of the chimeric protein in *Escherichia coli* and baculovirus-infected insect cells. *J Biotechnol* 77:219-234.

23. Korepanova A, *et al.* (2007) Expression of membrane proteins from *Mycobacterium tuberculosis* in *Escherichia coli* as fusions with maltose binding protein. *Protein Expr Purif* 53:24-30.
24. Tanaka R & Tanaka A (2007) Tetrapyrrole biosynthesis in higher plants. *Annu Rev Plant Biol* 58:321-346.
25. Demerec M, Adelberg EA, Clark AJ, & Hartman PE (1966) A proposal for a uniform nomenclature in bacterial genetics. *Genetics* 54:61-76.
26. Camadro JM (1999) *Peroxidizing herbicides*, (Springer-Verlag, Berlin Heidelberg) pp 245-277.
27. Marchler-Bauer A, *et al.* (2009) CDD: specific functional annotation with the Conserved Domain Database. *Nucleic Acids Res* 37:D205-210.
28. Yamazaki S, Nomata J, & Fujita Y (2006) Differential operation of dual protochlorophyllide reductases for chlorophyll biosynthesis in response to environmental oxygen levels in the cyanobacterium *Leptolyngbya boryana*. *Plant Physiol* 142:911-922.
29. Ikeuchi M & Tabata S (2001) *Synechocystis* sp. PCC 6803 - a useful tool in the study of the genetics of cyanobacteria. *Photosynth Res* 70:73-83.
30. Jansson C, Salih G, Eriksson J, & Wiklund R (1998) Use of *Synechocystis* 6803 to study expression of a *psbA* gene family. *Methods Enzymol* 297:166-182.
31. Williams J (1988) Construction of specific mutations in photosystem II photosynthetic reaction center by genetic engineering methods in *Synechocystis* 6803. *Methods Enzymol* 167:766-778.
32. Zapata M, Rodriguez F, & Garrido JL (2000) Separation of chlorophylls and carotenoids from marine phytoplankton: a new HPLC method using a reversed phase C-8 column and pyridine-containing mobile phases. *Mar Ecol: Prog Ser* 195:29-45.
33. Jacobs JM & Jacobs NJ (1999) in *Current Protocols in Toxicology*, eds Costa LG, Hodgson E, Lawrence DA, & Reed DJ (John Wiley & Sons, Inc., Hoboken, NJ).

Figure legends

Fig. 1 Growth of the *slr1790* insertional mutants in the presence and absence of the herbicide

The WT, AT, WTSK and ATSK lines were grown in liquid media in the absence (A) and presence (B) of 100 μM acifluorfen under continuous illumination (20 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$).

Fig. 2 Protox activity of Rs-slr1790 recombinant protein

Protox assays are shown for purified recombinant Rs-slr1790 protein fused with an MBP tag (open squares), purified MBP fused with the α subunit of β -galactosidase (crosses), and a buffer control without protein (filled squares). Each reaction mixture was incubated at 25°C as described in Materials and Methods. The production of Proto IX was monitored by fluorescence with a fluorometric plate reader equipped with 405 nm (excitation) and 633 nm (emission) bandpass filters. Error bars indicate SD of duplicate measurements.

Table Legends

Table 1 Effect of peroxidizing herbicides

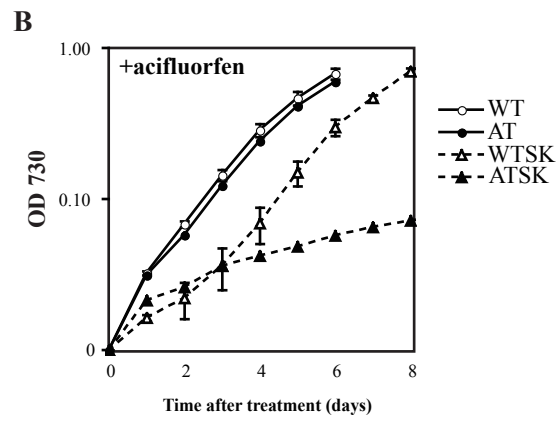
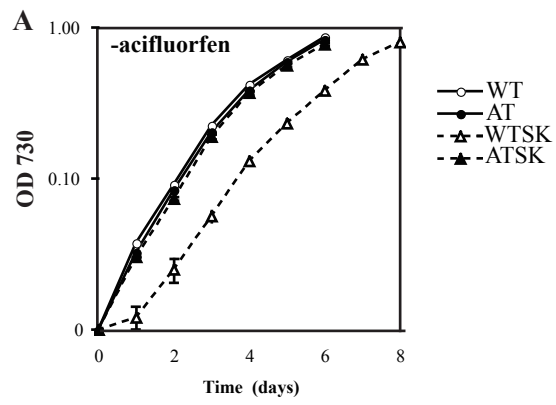
a) The negative logarithm of the concentration (M) of herbicides for 50% inhibition of cell growth

(I_{50} values). The cells were grown until the OD_{620} reached around 0.4.

b) Effects of the herbicides were not observed at the highest concentration (10^{-4} M) used in this experiment.

Table 2 Accumulation of Proto IX and chlorophyll in the absence and presence of acifluorfen

a) The number of cells was estimated from the following assumption: $1.0 \text{ (OD730)} = 1 \times 10^8$ cells/ml. b) $10^{-12} \text{ mol}/10^8 \text{ cells}$. c) not detected. (The detection limit of our HPLC system for Proto IX is 0.05 pmol per analysis, which corresponds to $1.0\text{-}1.2 \times 10^{-12} \text{ mol}/10^8 \text{ cells}$ in the WT and AT strains.)



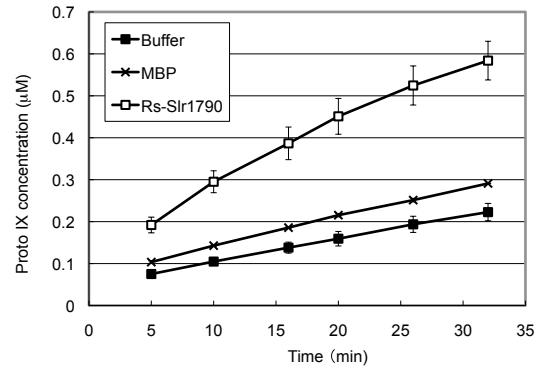


Table 1. Effect of peroxidizing herbicides

Name	-log (I ₅₀) ^{a)}	
	AT strain	ATSK strain
Acifluorfen (Diphenyl-ether)	<4 ^{b)}	4.5
Pyraflufen-ethyl (Phenylpyrazole)	<4 ^{b)}	6.6
Flumioxazin (<i>N</i> -phenylphthalimide)	<4 ^{b)}	6.7
Diquat (Bipyridylum)	5.2	5.0

Table 2. Accumulation of Proto IX and chlorophyll in the absence and presence of acifluorfen

Strain	OD ₇₃₀ ^{a)}	Proto IX ^{b)}	Chlorophyll <i>a</i> ^{b)}
Without acifluorfen			
WT	0.66	n.d. ^{c)} (<1.2)	3450
AT	0.67	n.d. ^{c)} (<1.2)	4120
WTSK	0.59	42	1660
ATSK	0.64	76	3120
With acifluorfen			
WT	0.69	n.d. ^{c)} (<1.2)	3500
AT	0.65	197	2610
WTSK	0.59	14	1960
ATSK	0.32	891	1140