

Development of a high-frequency *in vivo* transposon mutagenesis system for cyanobacteria
and establishment of the forward genetic analysis of the Chl *d*-dominated cyanobacterium,

Acaryochloris marina by use of the system

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Abbreviations

<i>aadA</i>	streptomycin/spectinomycin resistance gene
ADP	adenosine-5'-diphosphate
ATP	adenosine-5'-triphosphate
A ₀	PS I primary electron acceptor
A ₁	PS I secondary electron acceptor
CAO	chlorophyllide <i>a</i> oxygenase
Chl	chlorophyll
ChlG	chlorophyll synthase
Chlide	chlorophyllide
Cm	chloramphenicol
CP43'	membrane-embedded Chls binding antenna
DNA	deoxyribonucleic acid
DVR	3,8-divinyl protochlorophyllide <i>a</i> 8-vinyl reductase
Em	erythromycin
Fd	ferredoxin
HPLC	high performance liquid chromatography
IE	inside end
IPTG	isopropyl- β -D-1-thiogalactopyranoside
Km	kanamycin
ME	mosaic end
MoaA	molybdenum cofactor biosynthesis protein A
NADP ⁺	nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NCBI	National Center for Biotechnology Information

OE	outside end
P680	special pair of PS II
P700	special pair of PS I
PBS	phycobilisome
PC	plastocyanin
PCR	polymerase chain reaction
Phe	pheophytin
PS	photosystem
P _{<i>psbA2</i>}	upstream region (170 bp) of the <i>psbA2</i> gene of <i>Synechocystis</i> (<i>slr1311</i>)
P _{<i>tac</i>}	<i>tac</i> promoter
Q _A	primary electron acceptor plastoquinone molecule
Q _B	secondary electron acceptor plastoquinone molecule
RC	reaction center
Sm	streptomycin
Sp	spectinomycin
Tnp	transposase
T _{<i>psbA2::tonB</i>}	tandemly-arrayed terminator [downstream region (180 bp) of <i>slr1311</i> and <i>tonB</i> terminator]
T _{<i>rrnB</i>}	<i>rrnB</i> terminator
T _{<i>soxR</i>}	<i>soxR</i> terminator
UV	ultraviolet
WT	wild type strain

Chapter 1

General introduction

1-1 Significance of the investigation of cyanobacteria

Cyanobacteria, the origin of chloroplast, are the only prokaryotes that have ability of oxygenic photosynthesis, and cyanobacteria and photosynthetic eukaryote caused an increase in oxygen levels in the long history of the earth. Most of organisms including human directly or indirectly use photosynthetic products as energy sources. Additionally, fossil fuels that are considered to be formed from the sediment of ancient organisms especially photosynthetic organisms such as cyanobacteria are essential energy sources for production activities on human. Hence, it is not too much to say that oxygenic photosynthesis sustains activities of human and other heterotrophic organisms.

During the course of evolution, cyanobacteria that had thrived 2.7 billion years ago have acquired various and unique characteristics; differentiation into specialized cell that functions in nitrogen-fixation in filamentous cyanobacteria (heterocyst), utilization of unique photosynthetic pigments [chlorophyll (Chl) *d* and Chl *f*] that enable to use far-red light for photosynthesis, and others. The great capacity for environmental adaptability based on the characteristics made cyanobacteria to be the most widely-distributed photosynthetic organisms.

Human beings face many problems such as food issues, depletion of fossil fuels and environmental disruption. To resolve the problems, we require a better understanding about photosynthesis that plays a key role in energy production and material cycle. For this purpose, cyanobacteria are one of the ideal model organisms because oxygenic photosynthetic eukaryotes are more complex than cyanobacteria. Moreover, it is expected that the introduction of unique properties of cyanobacteria into other photosynthetic organisms may lead to an increase in photosynthetic production. Therefore, the investigation of cyanobacteria is highly significant from the perspective of not only basic science but also application.

1-2 Influence of cyanobacteria on the global environment and the evolution of life

Oxygenic photosynthesis by cyanobacteria changed a reducing atmosphere to an oxidizing one, and promoted complication of organisms. Photosynthetic prokaryotes contain "photosynthetic bacteria" [purple photosynthetic bacteria, green sulfur bacteria, green non-sulfur bacteria, heliobacteria and a recently-reported bacterium, *Candidatus Chloracidobacterium thermophilum* (Bryant et al. 2007)] and "cyanobacteria". Photosynthetic bacteria that cannot evolve oxygen utilize sulfide or organic acid as electron donors whose supply is limited. In contrast, cyanobacteria use water, ubiquitous molecule, as electron donor, and evolve oxygen as by-product. Therefore, cyanobacteria contribute heavily to increase oxygen concentration in the ocean and atmosphere. The increase of oxygen level prompted evolution of aerobic organisms whose terminal electron acceptor in respiration is oxygen. In particular, acquirement of mitochondrion, which is an organelle for aerobic respiration, contributes enlargement and complication of eukaryotes because energy efficiency of aerobic respiration is higher than that of anaerobic respiration. Moreover, ozone layer created by ultraviolet (UV) light breaking oxygen in atmosphere decreased UV light reaching the earth's surface, which enabled organisms to crawl on land. As described above, oxygenic photosynthesis by cyanobacteria triggered a huge change in the composition of atmosphere and the evolution of aerobic organisms.

1-3 Characteristics of cyanobacteria

1-3-1 Oxygenic photosynthesis of cyanobacteria

Oxygenic photosynthetic reaction is divided into light reaction that converts light energy into chemical energy and dark reaction that performs carbon dioxide fixation using the energy. In this section, I will explain the light reaction using a diagram of electron transfer in thylakoid of typical cyanobacteria (Fig. 1).

Firstly, light energy is absorbed by photosynthetic pigments in peripheral antenna system [phycobilisome (PBS)] or core antenna system of photosystem (PS) II (CP43 and CP47). The absorbed energy is transferred from the antennas to dimer of Chl *a*, which is named special pair, in the reaction center (RC) of PS II (P680), through the pigments. Excited P680 transfers an electron to pheophytin (Phe) *a*, which calls charge separation. The electron is finally transferred to secondary electron acceptor plastoquinone molecule (Q_B) via primary electron acceptor plastoquinone molecule (Q_A). The Q_B reduced by receiving two electrons is released into thylakoid membrane. On the other hand, $P680^+$, the oxidized form of P680, is reduced by electron derived from the oxidation of water, and oxygen and protons produced from the water are released into lumen, simultaneously. The reduced plastoquinone from PS II gives the electrons to the Rieske iron-sulfur center in cytochrome *b₆f* complex, and protons are released into lumen. The electron reduces plastocyanin (PC) localized in lumen side of thylakoid through cytochrome *f*. Similar to PS II, light energy captured by antenna system excites special pair of PS I (P700), and the electron of P700 is transferred to PSI primary electron acceptor (A_0) (charge separation). The electron migrates to ferredoxin (Fd) via PSI secondary electron acceptor (A_1) and three kinds of iron-sulfur center (F_A , F_B , F_X). Reduced Fd reduces nicotinamide adenine dinucleotide phosphate (oxidized form) ($NADP^+$) to nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH) by the function of Fd: $NADPH^+$ oxidoreductase. Meanwhile, $P700^+$, the oxidized form of P700, is reduced by the electron transferred by the reduced PC. Moreover, proton gradient between lumen and stroma generated by the electron transfer promotes adenosine-5'-triphosphate (ATP) synthesis from adenosine-5'-diphosphate (ADP) by ATP synthase.

In the light reaction, three types of photosynthetic pigment (phycobilin, carotenoid and Chl) assume important roles. Phycobilins that bind covalently to apoprotein of PBS capture the light energy, and transfer the energy to PSs. In addition to collecting the light energy,

carotenoids included in PSs serve to prevent photoinhibition caused by excess light. Chls take a role as not only capturing the light energy but also charge separation. For oxygenic photosynthetic organisms, seven natural Chls (Chl *a*, Chl *b*, Chl *c*, Chl *d*, Chl *f*, [8-vinyl]-Chl *a*, [8-vinyl]-Chl *b*) are confirmed until now, and only Chl *a*, Chl *d* and [8-vinyl]-Chl *a* work for charge separation. A basic skeleton of all Chls except for Chl *c* is chlorin (Fig. 2), a porphyrin derivative whose C-17/C-18 double bond is reduced. Moreover, difference of functional groups at C-2, C-3, C-7 and C-8 positions of chlorin changes absorption spectra (the structure of Chls is explained later).

1-3-2 Diversity of cyanobacteria

Cyanobacteria can inhabit severe environments (hypersaline lake, hot spring, terrestrial rock surface and its inside, desert and polar region) where many of photosynthetic eukaryotes may be inhibited or excluded. The widespread distribution is achieved by highly-diverse characteristics of cyanobacteria. There are multicellular cyanobacteria in addition to unicellular cyanobacteria. Moreover, some cyanobacteria have a potential of the nitrogen fixation under nitrogen deficient condition. Because nitrogenase, an enzyme responsible for nitrogen fixation, is inactivated by oxygen, unicellular nitrogen-fixing cyanobacteria perform the nitrogen fixation at night. On the other hand, a portion of cells of multicellular nitrogen-fixing cyanobacteria differentiates into heterocyst that possesses nitrogen-fixation ability (Haselkorn 1978).

In cyanobacteria, photosynthetic pigments are rich in diversity, and six species of Chl (Chl *a*, Chl *b*, Chl *d*, Chl *f*, [8-vinyl]-Chl *a*, [8-vinyl]-Chl *b*) are confirmed, whereas there are only three species of Chl (Chl *a*, Chl *b*, Chl *c*) in photosynthetic eukaryote. Three genera of cyanobacteria called prochlorophyta (*Prochlorothrix*, *Prochloron* and *Prochlorococcus*) have chlorophyllide *a* oxygenase (CAO), which is responsible for Chl *b* biosynthesis (Tanaka et al.

1998, Tomitani et al. 1999, Satoh and Tanaka 2006). Therefore, *Prochlorothrix* and *Prochloron* use Chl *b* (Fig. 2) which has formyl group at C-7 position in addition to Chl *a* for photosynthesis (Lewin and Withers 1975, Burger-Wiersma et al. 1986). On the other hand, molecular phylogenetic analysis indicated CAO of *Prochlorococcus* was distantly related to ones of green algae and other prochlorophyta (Satoh and Tanaka 2006). Additionally, *Prochlorococcus* lost 3,8-divinyl protochlorophyllide *a* 8-vinyl reductase (DVR) which reduces vinyl group at C-8 position of 3,8-divinyl protochlorophyllide *a*, a intermediate of Chl *a* biosynthesis (Nagata et al. 2005). Thus, *Prochlorococcus* has Chls which maintain vinyl group at C-8 position ([8-vinyl]-Chl *a* and [8-vinyl]-Chl *b*) (Fig. 2) (Chisholm et al. 1992), and the former functions in not only energy transfer but also charge separation. If other photosynthetic organisms live in upper side, *Prochlorococcus* can grow in the under side because absorption maximum wavelength of Soret band of [8-vinyl]-Chl *a* and [8-vinyl]-Chl *b* are approximately 10 nm longer than those of Chl *a* and Chl *b*, respectively. Moreover, some cyanobacteria have Chls that can absorb longer wavelength light than other Chls. Chl *d* (Fig. 2), a major Chl of *Acaryochloris* spp. (Miyashita et al. 1996), can absorb approximately 30 nm longer wavelength light (far-red light) than Chl *a* because of formyl group at the C-3 position of chlorin. Therefore, *Acaryochloris* spp. can live under light environment where most oxygenic photosynthetic organisms cannot grow. As described above, Chl *d* is utilized in not only light energy harvesting but also charge separation (Hu et al. 1998, Tomo et al. 2007, Tomo et al. 2008). Recently, Chl *f* (Fig. 2) which has formyl group at C-2 position was discovered from cyanobacterium inhabiting the stromatolite in Shark Bay (Chen et al. 2010). The absorption maximum of Chl *f* at long wavelength side is approximately 10 nm red-shifted compared to Chl *d* (Chen et al. 2010, Willows et al. 2013) and the biosynthesis of the pigment is induced by far-red light (Chen et al. 2012). Hence, it is expected that cyanobacteria acquired unique Chls to adapt varied light environments during

longtime evolution.

1-4 Molecular genetics of cyanobacteria

1-4-1 Research methodologies of cyanobacteria

Oxygenic photosynthesis and unique characters of cyanobacteria are attractive research objects. As with investigations of other organisms, study of cyanobacteria employs physiological, biochemical and molecular biological techniques. Moreover, spectroscopic analysis is applied to photosynthetic research because many proteins responsible for the light reaction contain photosynthetic pigments. Proteins involving photosynthesis have been targets of structural biology since the determination of crystal structure of RC from a photosynthetic bacterium, *Rhodospseudomonas viridis* (Deisenhofer et al. 1985). The high-resolution structures of PS I and PS II of cyanobacteria were recently reported (Jordan et al. 2001, Umena et al. 2011). According to the data from National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>), genome sequencing of over 90 species of cyanobacteria have been completed since *Synechocystis* sp. PCC 6803 (hereafter referred to as *Synechocystis*) became the first photosynthetic organism whose whole genome was sequenced (Kaneko et al. 1996). With the increase in sequence information, bioinformatics and postgenomic analyses have proceeded. In the research of cyanobacteria, complex approaches that combine molecular genetic tool with other techniques have been increasing.

1-4-2 The current situation of molecular genetic analysis of cyanobacteria

Molecular genetics, a research area that elucidates functions of genes at a molecular level, is one of the most important categories of cyanobacteria study. There are three types of transformation procedures for molecular genetic analysis, natural transformation, conjugation

and electroporation (Fig. 3). As to the natural transformation, some cyanobacteria such as *Synechocystis* and *Synechococcus elongatus* PCC 7942 (hereafter referred to as *Synechococcus*) naturally take up exogenous deoxyribonucleic acid (DNA) and are transformed by the DNA (Fig. 3A). For the conjugation, vectors are transferred from *Escherichia coli* to cyanobacteria by biological function (Fig. 3B). In electroporation, an electric field is applied to cyanobacteria in order to make holes in the cell membrane, and DNA is introduced into cyanobacteria thorough the holes (Fig. 3C). For many species of cyanobacteria, conjugal gene transfer and electroporation have been applied, whereas natural transformation can be applied to limited cyanobacteria.

Natural transformation popularized gene targeting for *Synechocystis*, and this technique advanced reverse genetics of *Synechocystis* in combination with the use of genome information. In reverse genetic analysis, a particular gene is disrupted or modified, and then the function of the gene is clarified by phenotype of the mutant. Conversely, in forward genetic analysis, phenotype of a mutant generated by random mutation elucidates the function of the causative gene, and the methodology and technique of forward genetics have been applied to various cyanobacteria. In early days of forward genetic analysis of cyanobacteria, mutants were produced by physical [e.g. UV irradiation (Singh and Tiwari 1969)] or chemical [e.g. exposure to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Herdman and Carr 1972)] treatments of the cells. However, if a mutation introduced by the treatments creates disadvantage for growth, the mutation can be removed during cycle of cell division because many cyanobacteria have multiple copies of chromosomes (Hu et al. 2007, Griesse et al. 2011). For instance, about 12 copies of chromosomes are present in *Synechocystis* (Labarre et al. 1989). Therefore, another technique, transposon mutagenesis is often applied to random mutagenesis of cyanobacteria because selective pressure of the introduced mutation is maintained by antibiotic (Wolk et al. 1991).

1-4-3 Transposon mutagenesis system for cyanobacteria

In transposon mutagenesis, mutants are generated by random insertion of transposable element, transposon, into genomic DNA. Tn901 (Tandeau de Marsac et al. 1982) and Tn5 (Cohen et al. 1998) were utilized in cyanobacterial transposon mutagenesis. However, now derivatives of Tn5 are mainly employed because investigation of Tn5 enables a frequent transposition higher than Tn901. The molecular mechanism of *in vivo* transposon mutagenesis system is briefly summarized as follows and the diagram (Fig. 4). (i) A transposon delivery vector is introduced into cyanobacterial cell by conjugal gene transfer. (ii) The gene encoding transposase (Tnp) on the vector is expressed. (iii) Tnp recognizes and interacts with 19 bp sequences (IE, inside end; OE, outside end) situated at either end of transposon. (iv) Tnp catalyzes the DNA cleavage that releases DNA between IE and OE as a Tnp-transposon complex from the vector. (v) The transposon is inserted into genome DNA with low sequence specificity at the insertion site. As a consequence of insertion, 9 bp of the target sequence is duplicated on both ends of the inserted Tn5. Results of recent research on Tn5 established *in vitro* transposition system (Goryshin and Reznikoff 1998). Therefore, *in vitro* transposon mutagenesis system has been utilized for cyanobacteria with high efficiency in natural transformation such as *Synechocystis* and *Synechococcus* (Bhaya et al. 2001, Kato et al. 2010, Taniguchi et al. 2007, Tanaka et al. 2012). Nevertheless, *in vivo* transposon mutagenesis system is also an important tool because the system can be applied to a wide range of cyanobacteria.

1-5 Characteristics of model cyanobacterium, *Synechocystis* sp. PCC 6803

Synechocystis is a unicellular cyanobacterium, which was isolated from freshwater in California by Kunisawa (Stanier et al. 1997), and is one of the most studied cyanobacteria because it has two advantageous characteristics for investigators, natural transformation

(Grigorieva and Shestakov 1982) and light-activated heterotrophic growth (Anderson and McIntosh 1991). To alter the *Synechocystis* genome, natural transformation is usually applied to gene targeting because natural transformation is an easier technique than other transformation procedures, such as conjugation and electroporation. Furthermore, *Synechocystis* can grow under heterotrophic conditions using glucose as a carbon source when cells are irradiated with light for 5-15 min per day. These characteristics are suitable for producing *Synechocystis* mutants that could not grow under photoautotrophic conditions due to the disruption of essential genes for photosynthesis. Therefore, these beneficial features for molecular genetic analysis of photosynthesis made *Synechocystis* become model organism of photosynthesis study. Moreover, whole genome of *Synechocystis* was sequenced as the first photosynthetic autotroph (Kaneko et al. 1996). The genome information prompted reverse genetic analysis, and function of many genes containing photosynthesis genes were analyzed. In addition to reverse genetic analysis, entire genome sequence of *Synechocystis* encouraged post-genomic analyses, for instance, transcriptome analysis (Hihara et al. 2001, Suzuki et al. 2001), proteome analysis (Sazuka and Ohara 1997, Simon et al. 2002) and two-hybrid assay (Sato et al. 2007).

1-6 Characteristics of Chl *d* dominated cyanobacteria, *Acaryochloris* spp.

Acaryochloris marina MBIC 11017, which possesses Chl *d* as a major Chl, was discovered from a suspension of algae squeezed out of a colonial ascidian in the Palau islands by Miyashita et al. (1996). Although Chl *d* was reported as the minor Chl in some species of red algae (Manning and Strain 1943) before isolation of *A. marina*, Chl *d* was suspected to be artifact in the extraction process. However, Murakami et al. demonstrated that true producer of Chl *d* detected in red algae is periphytic *Acaryochloris* (2004). Chl *d*, the characteristic photosynthetic pigment of *Acaryochloris*, can absorb far-red light which is not available for

almost all oxygenic photosynthesis using Chl *a* as a major Chl. Therefore, adaptation to far-red light enables *Acaryochloris* spp. to grow in the non-competitive light environment. In fact, *Acaryochloris* that cohabits with a colonial ascidian lives in a condition rich in far-red light because *Prochloron* absorbs visible radiation in the upper side (Kühl et al. 2005).

Five species of *Acaryochloris* have been reported to be isolated from the colonial ascidian (Miyashita et al. 2012), red algae (Murakami et al. 2004, Larkum et al. 2012), the microbial mat in a eutrophic hypersaline lake (Miller et al. 2005) and a dead coral skeleton (Mohr et al. 2010) respectively. Analyses of environmental DNA and Chl *d* derivatives indicated that *Acaryochloris* spp. are distributed in not only the ocean but also freshwater area and land (McNamara et al. 2006, de los Ríos et al. 2007, Kashiyaama et al. 2008). Entire genomes of two species of *Acaryochloris* [*Acaryochloris marina* MBIC 11017 (Swingley et al 2008), *Acaryochloris* strain CCMEE 5410 (Miller et al. 2011)] were determined and the genome sizes [8.36 Mb (*Acaryochloris marina* MBIC 11017), 7.88 Mb (*Acaryochloris* strain CCMEE 5410)] were larger than other cyanobacteria [e.g. 3.96 Mb (*Synechocystis*)].

Acaryochloris marina MBIC 11017 is a type strain of *Acaryochloris* (Miyashita et al. 2003) and has intensively been studied. In *A. marina*, Chl *d* accounted for more than 92% of total Chl in cell (Miyashita et al. 1997), and special pair in addition to many accessory Chls are Chl *d* (Hu et al. 1998, Tomo et al. 2007, Tomo et al. 2008). To compensate for approximately 100 mv lower excited energy of Chl *d* than that of Chl *a*, the redox potential of Phe *a* and Q_A in PS II of *A. marina* is more positive by 124 and 66 mv respectively compared with them of *Synechocystis* (Allakhverdiev et al. 2010, Allakhverdiev et al. 2011). These results proposed that utilization of Chl *d* in *A. marina* accelerated the evolution of apoproteins composing PS II for positive shift of redox potential of electron acceptors such as Phe *a* and Q_A. However, PSs always contain a small amount of Chl *a* (Hu et al. 1998, Chen et al. 2002, Tomo et al. 2007, Tomo et al. 2008). Although a portion of Chl *d* was replaced with

novel Chl ([7-formyl]-Chl *d*) (Fig. 2) in PS II of the transformant carrying the *CAO* gene, an amount of Chl *a* was maintained constant (Tsuchiya et al. 2012a). Therefore, Chl *a* must be significant pigment for PSs of *A. marina*. *A. marina* uses the membrane-embedded Chls binding antenna (CP43') in addition to PBS as peripheral antenna. CP43', which is a member of gene family of CP43, a core antenna of PS II, exists in *A. marina* and prochlorophyta (La Roche et al. 1996, Chen et al. 2008). Electron micrographs of the PSs from *A. marina* showed that PSs were surrounded by CP43' (Chen et al. 2005).

The elucidation of Chl *d* biosynthesis is required for understanding of an adaptation mechanism for far-red light and evolution of apoproteins of PSs. In addition, the introduction of Chl *d* biosynthesis ability into other photosynthetic organisms such as plant may contribute to improve efficiency of photosynthesis because light energy corresponding to a wider range of wavelength would be available for photosynthesis. However, the gene responsible for Chl *d* biosynthesis has not been identified through biochemical or bioinformatics approaches. Thus, establishment of molecular genetic analysis seemed to be needed to identify the gene, and Tsuchiya et al. developed transformation system for *A. marina* by conjugal gene transfer with expression vectors (2012b). Introduction of the *CAO* gene using expression vector enabled the transformant to synthesized novel Chl, [7-formyl]-Chl *d* (Fig. 2) (Tsuchiya et al. 2012b). This result indicated that molecular genetic analysis for *A. marina* was effective for study on Chl biosynthesis in *A. marina*.

1-7 Major issues of cyanobacteria study

Despite drastic development of molecular genetic analysis of *Synechocystis* after 1996, investigation attached weight to reverse genetic analysis delayed analyses of genes with uncertain functions. Therefore, the rate of genes of *Synechocystis* annotated as 'hypothetical protein' or 'unknown protein' remains at about 50% according to database of Cyanobase

(<http://genome.microbedb.jp/cyanobase/>). These genes would contain important genes for photosynthesis and other physiological reaction, and forward genetic analysis is suitable for the elucidation of the function of such genes. However, popular *in vitro* transposon mutagenesis system used for *Synechocystis*, has two problems as follows: (i) tortuous procedure composed of the preparation of genomic DNA and *in vitro* transposon insertion into the DNA; (ii) Tnp is expensive to purchase.

On the other hand, high throughput genome analysis using next-generation DNA sequencer dramatically increased the number of organisms whose entire genomes were sequenced, and genome sequences of over 90 species of cyanobacteria are registered at database of NCBI. Because some cyanobacteria among them possess unique characteristics such as Chl *d*, it is expected that forward genetic analysis for cyanobacteria will be required to identify causative genes of the unique characteristics. However, natural transformation is unavailable for many cyanobacteria, and transformation of them employs conjugation or electroporation in many instances. Therefore, *in vivo* transposon mutagenesis system is preferable to producing mutants for forward genetic analysis.

It is expected to increase the demand for forward genetic analysis of both model cyanobacteria and cyanobacteria possessing unique characters. However, the repeated transposition sometimes occurs after transposon insertion in popularized *in vivo* transposon mutagenesis system because coding region of *tnp* exists within transposon (Fig. 4) (Koksharova and Wolk 2002a). In addition, a frequency of transposition of the system was not so high in model cyanobacteria that have potential for highly efficient transformation. Thus, it was expected that the frequency would be decreased for other cyanobacteria. In this way, the unoptimized *in vivo* transposon mutagenesis hindered the progress of forward genetic analysis of cyanobacteria.

1-8 Purpose of this research

To streamline forward genetic analysis of model cyanobacteria such as *Synechocystis*, a high-frequent and user-friendly transposon mutagenesis system will be required for selection of mutants under various conditions in each laboratory. Moreover, forward genetic analysis is one of ideal means of the identification of genes responsible for unique characters such as Chl *d* biosynthesis. Therefore, establishment of *in vivo* transposon mutagenesis system available for a wide range of cyanobacteria is desired. In this study, I aimed to develop *in vivo* transposon mutagenesis system for forward genetic analysis of not only model cyanobacteria but also other cyanobacteria.

Chapter 2

**Development of a high-frequency *in vivo* transposon mutagenesis system for
Synechocystis sp. PCC 6803 and *Synechococcus elongatus* PCC 7942**

Summary

Synechocystis is the first sequenced photosynthetic organism and has two advantages: natural transformation and light-activated heterotrophic growth. Such characteristics have mainly promoted reverse genetic analysis in this organism, however, to date approximately 50% of genes are still annotated as ‘unknown protein’ or ‘hypothetical protein’. Therefore, forward genetic analysis is required for the identification of significant genes responsible for photosynthesis and other physiological phenomena among the genes of unknown function. The *in vivo* transposon mutagenesis system is one of the major methods for random mutagenesis. However, present *in vivo* transposon mutagenesis systems for cyanobacteria face problems such as relatively low frequency of transposition and repeated transposition in the host cells. In this chapter, transposon delivery vectors were constructed based on a mini-Tn5-derived vector that was designed to prevent repeated transposition. These vectors carry a hyperactive Tnp and optimized recognition sequence of Tnp, which were reported to enhance frequency of transposition. Highly frequent transposition (9×10^{-3} per recipient cell) in *Synechocystis* was achieved by the use of one of these vectors. Transposon insertion sites of 10 randomly selected mutants indicated that the insertion sites spread throughout the genome with low sequence dependency. Furthermore, one of the 10 mutants exhibited the slow-growing phenotype, and the mutant was functionally complemented by using the expression vector. This system also worked with another model cyanobacterium, *Synechococcus*, with high frequency. These results indicate that the developed system can be applied to the forward genetic analysis of a broad range of cyanobacteria.

2-1 Introduction

Photosynthesis has a key role in primary production on the earth, and it is one of the most important biological reactions. Moreover, plants, algae and cyanobacteria release molecular

oxygen, which is required for aerobic respiration, as the product resulting from oxygenic photosynthesis. Therefore, oxygenic photosynthesis has been investigated intensively. Cyanobacteria have been utilized as model organisms for photosynthesis research because cyanobacteria share similar photosynthetic properties with other oxygenic photosynthetic organisms.

The intensive investigation of model cyanobacteria is important to understand the mechanisms common to all oxygenic photosynthetic organisms because basic experimental techniques have already been established and widely used for such cyanobacteria. A model cyanobacterium, *Synechocystis* is one of the most studied cyanobacteria because it has two advantageous characteristics for investigators, natural transformation (Grigorieva and Shestakov 1982) and light-activated heterotrophic growth (Anderson and McIntosh 1991). These features enable the production of *Synechocystis* mutants that could not grow under photoautotrophic conditions due to the disruption of essential genes for photosynthesis. Therefore, *Synechocystis* was recognized as a candidate for a model cyanobacterium and became the first photosynthetic organism whose entire genome was sequenced (Kaneko et al. 1996). Based on the genome information, reverse genetic analyses of *Synechocystis* rapidly progressed, and the functions of many genes including photosynthetic genes were analyzed. Whole-genome sequencing of *Synechocystis* also promoted post-genomic analyses as described in chapter 1. In spite of progress in post-genomic analyses, approximately 50% of genes in the genome are still annotated as ‘hypothetical protein’ or ‘unknown protein’ according to the data from CyanoBase. Among them, many functionally important genes might exist. To identify those genes, forward genetic analysis is an efficient approach. However, there are a limited number of reports that describe forward genetic analysis of *Synechocystis*, compared with reverse genetic analysis. Thus, systematic forward genetic analysis is still needed for further investigation of *Synechocystis*.

For forward genetic analysis of prokaryotes, mutants can be generated by physical [e.g. UV irradiation (Singh and Tiwari 1969)], chemical [e.g. exposure to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Herdman and Carr 1972)] or biological [e.g. transposon mutagenesis (Wolk et al. 1991)] treatments of the cells. Because the antibiotic resistance gene within the transposon that is inserted into the genome maintains the mutation, transposon mutagenesis is preferable among the above methods. If the introduced mutations cause severe defects, they will be removed during repeated cell division without selection pressure on the maintenance of the mutations because of the presence of multiple copies of the chromosome per cell in many cyanobacteria including *Synechocystis* (Labarre et al. 1989, Hu et al. 2007, Griesse et al. 2011). In the case of transposon mutagenesis, the antibiotic resistance gene present in the transposon promotes the segregation of the mutated chromosome by increasing the concentration of antibiotic. Furthermore, the transposon inserted into genomic DNA can be used as a tag, which facilitates the identification of the transposon insertion site. Because of these advantages, transposon mutagenesis is considered to be the most effective method for forward genetic analysis of cyanobacteria.

Now, almost all transposon mutagenesis systems for cyanobacteria employ derivatives of Tn5, and the molecular mechanism of its transposition is described in chapter 1. Recent investigations on Tn5 enabled researchers to use Tn5 for *in vitro* transposition mutagenesis (Goryshin and Reznikoff 1998). For the mutagenesis of *Synechocystis* (Bhaya et al. 2001, Kato et al. 2010) and *Synechococcus* (Taniguchi et al. 2007, Tanaka et al. 2012) that have the ability for natural transformation, *in vitro* transposon mutagenesis is an effective tool. In this system, a transposon that contains an antibiotic resistance gene is inserted into genomic DNA or a genomic DNA library *in vitro*. Then, the mutagenized DNA is used for natural transformation of *Synechocystis* and *Synechococcus*. However, this system requires time, effort and cost to prepare the mutagenized DNA. Therefore, an *in vivo* transposon

mutagenesis system is another effective tool even for the naturally transformable cyanobacteria because only *E. coli* harboring proper plasmids is needed for the conjugal transfer of a transposon delivery vector. The *in vivo* transposon mutagenesis of cyanobacteria has been developed mainly for *Anabaena*, and many efforts to increase the frequency of transposition through improvement of transposon delivery vectors have been made (Koksharova and Wolk 2002a). Furthermore, the improved *in vivo* transposon mutagenesis system was also applied to a model cyanobacterium, *Synechococcus* (Katayama et al. 1999, Koksharova and Wolk 2002b, Miyagishima et al. 2005) that can be transformed by *in vitro* transposon mutagenesis. However, the transposon delivery vectors used in these works contain *tnp* inside the transposon like wild-type Tn5, thereby repeated transposition may sometimes occur by the function of Tnp after the insertion of the transposon into genomic DNA (Koksharova and Wolk 2002a). Therefore, a stable *in vivo* transposon mutagenesis system with high frequency would be an ideal tool for forward genetic analysis of *Synechocystis* and other cyanobacteria.

In this chapter, I first constructed transposon delivery vectors that has four important features: (i) the prevention of repeated transposition; (ii) the hyperactivation of Tnp; (iii) the regulation of *tnp* by the promoter and terminator; and (iv) the optimization of the end sequences recognized by Tnp. Using one of the vectors, highly frequent transposition was observed in the case of *Synechocystis* and *Synechococcus*. Restriction fragments that contain a transposon were cloned to a cloning vector, and the transposon insertion site of each exconjugant was determined. As a pilot experiment, a transposon-tagged mutant with slower growth was isolated, and the gene responsible was confirmed by complementation using the expression vector reported previously (Tsuchiya et al. 2012b).

2-2 Materials and methods

2-2-1 Culture of cyanobacteria

Synechocystis sp. PCC 6803 and *Synechococcus elongatus* PCC 7942 were grown in BG11 medium (Allen 1968) under photoautotrophic conditions; the light intensity was 20 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ and the temperature was 25°C. For transposon-tagged mutants of *Synechocystis* and the WT (pKUT1121), 5 $\mu\text{g ml}^{-1}$ spectinomycin (Sp) (and 5 $\mu\text{g ml}^{-1}$ kanamycin (Km) for transposon-tagged mutants harboring the pKUT2121 derivative) was added to the medium.

2-2-2 Construction of transposon delivery vectors

Oligonucleotides used in the construction of vectors are summarized in Table 1. The *tnp* gene was amplified by polymerase chain reaction (PCR) using primers (TnpFw and TnpRv), and the PCR product was subcloned into pZErO-2 (Invitrogen). The cloned *tnp* was mutagenized by the megaprimer method (Sarkar and Sommer 1990) using TnpMutRv in addition to TnpFw and TnpRv, and the site-directed mutant *tnp* with restriction sites (KpnI and SacI) at each end was used as a hyperactive *tnp*. All regions of pUTmini-Tn5 Sm/Sp except for *tnp* were amplified by PCR using primers (pUTFw1 and pUTRv1). The PCR product and the hyperactive *tnp* excised by restriction enzyme digestion were fused using In-Fusion Advantage PCR Cloning Kit (Clontech) according to the manufacturer's instructions. The resultant vector was digested with *SacI*, and a tandemly arrayed terminator [*rrnB* terminator (*T_{rrnB}*) and *soxR* terminator (*T_{soxR}*)] that was produced by PCR using TrnBFw and TrnBRv was introduced into the vector. The vector with the terminator downstream of hyperactive *tnp* was further modified by introducing the *tac* promoter (*P_{tac}*) from pKUT1121 (Tsuchiya et al. 2012b) upstream of the hyperactive *tnp*. All regions of the resultant vector except for inside of the transposon were amplified by PCR using primers (pUTFw2 and pUTRv2). The PCR

product had *Mlu*I sites at both ends, and IE and OE were replaced by mosaic end (ME). The PCR product and a Km resistance gene cassette excised from pBSL97 (Alexeyev et al. 1995) by *Sma*I treatment were ligated. To remove major restriction sites, the resultant vector was digested with *Mlu*I, and the Km resistance gene cassette was replaced by a streptomycin (Sm)/Sp resistance gene (*aadA*) cassette from pBSL130 (Alexeyev et al. 1995) to yield pKUT-Tn5-Sm/Sp. From pKUT-Tn5-Sm/Sp, pKUT-Tn5-Km and pKUT-Tn5-Cm were constructed by replacing the Sm/Sp resistance gene cassette with a Km resistance gene cassette and a chloramphenicol (Cm) resistance gene cassette (Alexeyev et al. 1995), respectively.

2-2-3 Transposon mutagenesis

Conjugal gene transfer of pUTmini-Tn5 Sm/Sp or pKUT-Tn5-Sm/Sp was performed by diparental mating as described by Elhai and Wolk (1988) with slight modification. The helper plasmid, pRK2013 (Figurski and Helinski 1979), prepared from HB101 (pRK2013) was introduced into *E. coli* strain JM109 λ pir, and pUTmini-Tn5 Sm/Sp or pKUT-Tn5-Sm/Sp was further introduced into JM109 λ pir (pRK2013). JM109 λ pir (pRK2013, pUTmini-Tn5 Sm/Sp) and JM109 λ pir (pRK2013, pKUT-Tn5-Sm/Sp) at the logarithmic growth phase were harvested and washed with BG11 medium twice. The optical density (600 nm, 1 cm path length) of each cell suspension was adjusted to 10. *Synechocystis* and *Synechococcus* grew to the growth phase with an optical density at 730 nm of approximately 1.0, and the culture was diluted with BG11 medium to 1.0×10^5 cells ml⁻¹. An equal amount of *E. coli* [JM109 λ pir (pRK2013, pUTmini-Tn5 Sm/Sp) or JM109 λ pir (pRK2013, pKUT-Tn5-Sm/Sp)] cells and *Synechocystis* cells were mixed. Aliquots of the mixture (20 μ l) were spotted onto nitrocellulose membranes on solidified BG11 agar medium. Following 24 h incubation under light, the membranes were transferred to BG11 solid medium containing 5 μ g ml⁻¹ Sp.

Sp-resistant colonies appeared after a few days. For *Synechococcus*, a similar procedure was applied except for the selection of exconjugants by 20 $\mu\text{g ml}^{-1}$ Sm. The expression vector pKUT1121 constructed by Tsuchiya et al. (2012b) was introduced into *Synechocystis* by the same procedure to yield the WT (pKUT1121) as a control transformant.

2-2-4 Southern blotting

Genomic DNA was prepared from *Synechocystis* as described by Wilson (1997). Southern blotting was performed using the AlkPhos Direct Labelling and Detection System (GE Healthcare) according to the manufacturer's instructions. Genomic DNA (10 μg) and pKUT-Tn5-Sm/Sp, a positive control plasmid, were digested with *Hind*III (37°C, overnight), and then subjected to agarose gel electrophoresis. After staining with ethidium bromide, the gel was blotted onto nylon membrane (Hybond-N⁺; GE Healthcare). The transferred DNA fragments were fixed to the membrane by a UV cross-linker (UVC500; Hoefer Inc.). The DNA fragment corresponding to the coding region of *aadA* was used as probe, which was chemically labeled with thermostable alkaline phosphatase. The DNA fragments fixed to the membrane were hybridized with the probe overnight. After washing the membrane, CDP-Star Detection Reagent (GE Healthcare) was added to the membrane, and the chemiluminescent signal was detected by a luminescent image analyzer (LAS-3000 UVmini; Fujifilm).

2-2-5 Cloning of the restriction fragment that contains the transposon

Genomic DNA (3 μg) from transposon-tagged mutants of *Synechocystis* and pZErO-2 (1 μg) were digested with the respective restriction enzymes (*Bam*HI, *Eco*RI, *Hind*III, *Kpn*I and *Xba*I), and the enzymes were inactivated by heat treatment. The DNA concentrations of both digested genomic DNA and digested pZErO-2 were adjusted to 100 and 10 $\text{ng } \mu\text{l}^{-1}$, respectively. The restriction fragments (4.98 μl) prepared from the genomic DNA were

ligated to pZErO-2 digested with the same restriction enzyme (1 μ l), and *E. coli* strain OmniMAX 2-T1^R (Invitrogen) was transformed with the ligation. Transformants were screened on LB solid medium containing 20 μ g ml⁻¹ Sm, 25 μ g ml⁻¹ Km and 100 μ M isopropyl- β -D-1-thiogalactopyranoside (IPTG). The plasmids prepared from the obtained *E. coli* transformants were subjected to sequencing using outward-directed transposon-specific primers (Tn5-Seq1 and Tn5-Seq2). The transposon insertion site of each mutant was determined by comparison of transposon flanking sequences with the genomic sequence of *Synechocystis* using the Blast algorithm (Altschul et al. 1990).

2-2-6 Complementation of the transposon-tagged mutant

The expression vector pKUT2121 for genetic functional complementation originated from pKUT1121, which was constructed by Tsuchiya et al. (2012b). pKUT1121 was digested with *Mlu*I, and the Sm/Sp resistance gene cassette in the vector was replaced by a Km resistance gene cassette from pBSL98 (Alexeyev et al. 1995) to yield pKUT2121. *Slr1979* and *slr1980* were amplified by PCR using primers (for *slr1979*, slr1979Fw and slr1979Rv; for *slr1980*, slr1980Fw and slr1980Rv), and the PCR products were subcloned into pZErO-2. *Slr1979* and *slr1980* with restriction sites (an *Nde*I site at the 5' end and an *Xho*I site at the 3' end) were excised from the vectors by digestion with *Nde*I and *Xho*I, and were subcloned into pKUT2121 digested with the same enzymes. The resultant expression vectors (pKUT2121-*slr1979* and pKUT2121-*slr1980*) and pKUT2121 were introduced into the ST4 mutant by conjugation. Transformants were selected and cultured in BG11 solid medium containing 5 μ g ml⁻¹ Sp and 5 μ g ml⁻¹ Km.

2-3 Results

2-3-1 Construction of transposon delivery vectors

I first attempted transposon mutagenesis of *Synechocystis* using a mini-Tn5 transposon delivery vector, pUTmini-Tn5 Sm/Sp (de Lorenzo et al. 1990) (Fig. 5A). Because the vector carries the replication origin, *oriR6K*, which requires π protein encoded by the *pir* gene for replication (Inuzuka and Helinski 1978), the vector is expected not to be replicated in bacteria without the *pir* gene. Similarly, vectors derived from pBR322 that contains a ColE1 origin have been used for cyanobacteria as suicide vectors. However, the above vectors cannot be applied to bacteria that can replicate plasmids containing the ColE1 origin such as *E. coli*. Therefore, the vector that contains *oriR6K*, was employed considering the future applications of the vector. In pUTmini-Tn5 Sm/Sp, a *tnp* gene is located outside the transposon. This leads to the loss of the transposon delivery vector that contains the *tnp* gene during cell growth. These features prevent the inserted transposon from repeating transposition in the recipient cells, resulting in stable transposition. Thus, I first tried to introduce pUTmini-Tn5 Sm/Sp into *Synechocystis* by conjugation. However, no antibiotic-resistant exconjugants appeared after antibiotic selection (Fig. 6).

I therefore altered the pUTmini-Tn5 Sm/Sp to develop the *in vivo* transposon mutagenesis system based on recent progress in Tn5 research (Goryshin and Reznikoff 1998). At first, mutations were introduced into *tnp* to increase the frequency of Tn5 transposition (Fig. 5B). The resultant gene encodes hyperactive Tnp that contains two mutations: E54K enhances the OE binding activity of Tnp (Zhou and Reznikoff 1997) and M56A prevents expression of the inhibitor protein, which is translated from M56 as a truncated Tnp (Wiegand and Reznikoff 1992). Then, both recognition sequences for Tnp, the IE and OE, were replaced by their hybrid recognition sequence (ME) that was optimized for the maximal binding of hyperactive Tnp (Zhou et al. 1998) (Fig. 5B). In addition to the above

improvements previously reported for *in vitro* transposition, the vector was further altered. A promoter and terminator were added to the hyperactive *tnp*, and major restriction sites inside the transposon were removed from the vector (Fig. 5B). I utilized P_{lac} (de Boer et al. 1983) as the promoter and a DNA fragment that contains a ρ factor-dependent terminator (T_{rrnB}) (Brosius et al. 1981) tandemly fused with a factor-independent terminator (T_{soxR}) (Wu and Weiss 1991) as the terminator of the hyperactive *tnp*. Although almost all major restriction sites (*Bam*HI, *Eco*RI, *Hind*III, *Sal*I, *Sfi*I and *Sma*I) were removed, a *Not*I site remained in the transposon as the unique restriction site. The improved transposon delivery vector (pKUT-Tn5-Sm/Sp, DDBJ under accession number: AB971580) (Fig. 5B) contains a *aadA* cassette that can be excised from the transposon region of the vector by treatment with the restriction enzyme *Mlu*I. Therefore, the Sm/Sp resistance gene cassette of pKUT-Tn5-Sm/Sp was replaced by the Km resistance gene cassette or the Cm resistance gene cassette to yield pKUT-Tn5-Km (DDBJ under accession number: AB971581) and pKUT-Tn5-Cm (DDBJ under accession number: AB971582), respectively. In the following study, pKUT-Tn5-Sm/Sp was used for *in vivo* transposon mutagenesis of *Synechocystis*.

2-3-2 Production of transposon-tagged mutants by the developed transposon mutagenesis system

The transposon delivery vector, pKUT-Tn5-Sm/Sp, was introduced into *Synechocystis* by conjugal gene transfer whose efficiency is higher than that of natural transformation (see below), and, for typical experiments, 9 ± 3 Sp-resistant exconjugants per 1000 recipient cells appeared after Sp selection. Among them, 10 exconjugants (ST1–ST10 mutants) were selected randomly for further analyses. Total DNA prepared from each mutant was used as template for PCR to amplify *aadA* using specific primers (*aadA*Fw and *aadA*Rv). The pKUT-Tn5-Sm/Sp and total DNA prepared from the wild type strain (WT) harboring a

plasmid, pKUT1121 [WT (pKUT1121)] (Tsuchiya et al. 2012b) were utilized as positive controls. Consequently, DNA fragments corresponding to *aadA* were amplified from all mutants, whereas no corresponding fragment was amplified from the WT (Fig. 7A). This result indicated that the ST1–ST10 mutants were transformants that harbor *aadA*. To confirm the loss of pKUT-Tn5-Sm/Sp including hyperactive *tnp* in the ST1–ST10 mutants, PCR was performed to amplify hyperactive *tnp* using specific primers (TnpFw and TnpRv). As a result, hyperactive *tnp* was amplified only from pKUT-Tn5-Sm/Sp, a control plasmid (Fig. 7B). In contrast, no amplification of hyperactive *tnp* was observed in all transformants including the ST1–ST10 mutants, as in the WT (Fig. 7B). These results showed that *aadA* detected in the ST1–ST10 mutants did not originate from the introduced pKUT-Tn5-Sm/Sp, which might be lost during cell growth.

The transposon insertion into the chromosome of each mutant was confirmed by Southern blotting using a probe corresponding to the coding region of *aadA*. Consequently, one band in the range of 2.4–21.0 kb was detected in the ST1–ST10 mutants, and the positions of bands were different from each other, whereas no band was detected in the lane of the WT, a negative control (Fig. 8). Moreover, no band exhibited an identical migration pattern to that of pKUT-Tn5-Sm/Sp (2.2 kb), indicating the insertion of the transposon into the chromosome of the ST1–ST10 mutants.

2-3-3 Determination of transposon insertion sites

To determine transposon insertion sites, restriction fragments with the transposon from genomic DNA of the ST1–ST10 mutants were cloned, and the flanking regions of the transposon insertion site of each cloned restriction fragment were sequenced. Sequence analysis confirmed that the transposon was inserted into the chromosome at a different position in each clone (Fig. 9, 10). Furthermore, all transposons in the chromosomes were

flanked by a 9-bp duplication at each end, which is characteristic of Tn5 insertion. Compared with the result of Southern blot analysis, the length of the estimated restriction fragments that contain the transposon of each transformant was reasonable. Thus, it was concluded that the transposon on pKUT-Tn5-Sm/Sp was introduced into the chromosome of the ST1–ST10 mutants by the function of hyperactive Tnp. In most mutants, the transposon was inserted into a predicted gene, whereas the insertion of the transposon into an intergenic region was found in the case of the ST2 and ST3 mutants (Fig. 9). The genomic map that presents the respective insertion positions revealed that transposition was observed in a broad range of positions of the genome (Fig. 10). To examine the progress of segregation of the transposon-inserted chromosome, the genomic region that contains the transposon of each mutant was amplified (Fig. 11). Specific primers (Table 2) were used for PCR. Compared with the control using WT genomic DNA as template, no band originating from the wild-type chromosome was found in any mutant, except for the ST4 mutant. Furthermore, all mutants possessed a transposon-inserted chromosome (Fig. 11). These results demonstrated that nine of 10 mutants exhibit complete segregation of the transposon-inserted chromosome at the level of PCR detection. Only the ST4 mutant exhibited incomplete segregation. In the case of the ST7 amplification, three bands were detected for the WT. These bands are caused by the highly conserved tandem repeats (888 bp each) to which the forward primer for ST7 can bind (Fig. 12).

Because 10 exconjugants randomly selected from Sp-resistant colonies that were obtained by transposon mutagenesis possessed the transposon in their chromosome, it was concluded that virtually all Sp-resistant colonies were transposon-tagged mutants. According to this hypothesis, the frequency of transposition was calculated to be 9×10^{-3} per recipient cell. Moreover, the transposon mutagenesis system was applied to another model cyanobacterium, *Synechococcus*. As a result, transposon-tagged mutants were obtained at the

frequency of 5×10^{-3} per recipient cell. Furthermore, I tried to apply natural transformation to the introduction of pKUT-Tn5-Sm/Sp into *Synechocystis*. Although transposon-tagged mutants were obtained by natural transformation, the frequency of transposition was approximately 600 times lower than that by conjugal gene transfer. Therefore, it was concluded that conjugal gene transfer is the most efficient method to obtain transposon-tagged mutants.

2-3-4 Analysis of the slow-growing ST4 mutant

I further checked whether the transposon mutagenesis system developed in this chapter was sufficient to generate mutants with an apparent phenotype. Annotation of genes around the insertion sites is summarized in Table 3. Five of these genes were categorized as genes for ‘unknown protein’ or ‘hypothetical protein’. Therefore, the phenotype of transposon-tagged mutants was not predictable. To determine the phenotypic difference, ST1–ST10 mutants were cultured on BG11 solid medium (Fig. 13A). Clearly, only the ST4 mutant grew more slowly than the other mutants, and the ST4 mutant was analyzed in detail. For the ST4 mutant, the transposon was inserted near the 3′ end of *slr1979* (Fig. 9). Downstream of *slr1979*, *slr1980* was aligned in the same direction. To identify the gene responsible for the phenotype of the ST4 mutant, *slr1979* and *slr1980* were introduced into the ST4 mutant using expression vectors (pKUT2121-*slr1979* and pKUT2121-*slr1980*, respectively). As a consequence, ST4 (pKUT2121-*slr1979*) grew more rapidly than ST4 (pKUT2121-*slr1980*), with the latter exhibiting a similar growth speed to that of the control mutant, ST4 (pKUT2121) (Fig. 13B). The growth speed of ST4 (pKUT2121-*slr1979*) was similar to that of the WT (pKUT1121) (Fig. 13A, B). Thus, it was concluded that *slr1979* was the causative gene for the decreased growth rate of the ST4 mutant. As shown in Table 3, *slr1979* was annotated as *trpE* encoding anthranilate synthase component I that is responsible for

tryptophan biosynthesis. Therefore, insertion of the transposon into *slr1979* might have reduced tryptophan biosynthetic activity in the ST4 mutant, which affected cell growth.

2-4 Discussion

Fundamental research on the increase in frequency of Tn5 transposition *in vitro* was reported 16 years ago (Goryshin and Reznikoff 1998, Zhou et al. 1998), and *in vitro* transposon mutagenesis has been applied to the mutagenesis of cyanobacteria (Bhaya et al. 2001, Zhang et al. 2004, Taniguchi et al. 2007, Tanaka et al. 2012). For *in vivo* transposon mutagenesis of cyanobacteria, a series of transposon delivery vectors was developed (Cohen et al. 1998), and a later vector, pRL692, was improved by modifications, i.e. hyperactivation of Tnp and optimization of recognition sequences (Koksharova and Wolk 2002b). However, repeated transposition sometimes occurs in the transposon-tagged mutant (Koksharova and Wolk 2002a) because the vector contains hyperactive *tnp* within the transposon, which can be inserted into the chromosome. Therefore, I first attempted to use a mini-Tn5 transposon delivery vector, pUTmini-Tn5 Sm/Sp (de Lorenzo et al. 1990), for the transposon mutagenesis of *Synechocystis*. However, no antibiotic-resistant exconjugants appeared (Fig. 6). Since the vector had the wild-type *tnp* and the IE and OE, I constructed pKUT-Tn5-Sm/Sp that carries hyperactive *tnp* and two MEs like pRL692 by improving pUTmini-Tn5 Sm/Sp. Unlike pRL692, hyperactive *tnp* was placed under the control of P_{tac} , a hybrid promoter derived from *trp* and *lacUV5* promoters (de Boer et al. 1983) (Fig. 5). The expression of the hyperactive *tnp* must be tightly repressed in *E. coli* to avoid the transposition of the transposon into the *E. coli* genome prior to conjugal transfer of the vector into *Synechocystis*. P_{tac} is repressed by LacI, a *lac* repressor; therefore, *E. coli* strains that possess the *lacI^q* genotype which causes overexpression of LacI (Calos 1978) are ideal hosts for pKUT-Tn5-Sm/Sp. Conversely, P_{tac} was reported to be a strong and light-independent

promoter in cyanobacteria because of the lack of *lacI* (Marraccini et al. 1993). Thus, it is expected that P_{tac} increases transcription of hyperactive *tnp* in *Synechocystis*, which probably causes a high frequency of transposition. In addition, the influence of the strong promoter activity on the downstream genes can be reduced by terminators (T_{rrnB} and T_{soxR}). Additionally, the major restriction sites were removed from the transposon, except for a *NotI* site (Fig. 5). This elimination allowed for the utilization of the above restriction enzymes for the cloning of restriction fragments containing the transposon. This feature is also advantageous for the inverse PCR method, another method to determine the transposon insertion site. Furthermore, similar to pUTmini-Tn5 Sm/Sp, pKUT-Tn5-Sm/Sp has two characteristics to avoid repeated transposition; (i) (hyperactive) *tnp* is located outside the transposon; and (ii) the replication origin is *oriR6K_γ*, that is used for suicide vectors. *E. coli* used for conjugation requires π protein encoded by *pir* (Kolter et al. 1978) for replication of pKUT-Tn5-Sm/Sp. Therefore, I selected JM109 λ *pir* that has both *pir* and *lacI^q*, and the introduction of a helper plasmid, pRK2013 (Figurski and Helinski 1979), into the strain allowed for diparental mating.

Determination of the transposon insertion sites of the ST1–ST10 mutants demonstrated that each transposon was flanked by the 9-bp duplication that is characteristic of Tn5 transposition (Reznikoff 2008). Furthermore, the transposon-inserted positions of these transposon-tagged mutants were spread throughout genome with low sequence dependency (Fig. 10), which is also a characteristic of Tn5 transposition (Reznikoff 2008). These results confirm that the ST1–ST10 mutants were produced by the function of mini-Tn5 in pKUT-Tn5-Sm/Sp. Using pKUT-Tn5-Sm/Sp, the frequency of transposition in *Synechocystis* was 9×10^{-3} per recipient cell and that in *Synechococcus* was 5×10^{-3} per recipient cell. In a previous report, the frequency of transposition in *Synechococcus* for pRL692 was $3\text{--}6 \times 10^{-5}$ per recipient cell (Koksharova and Wolk 2002b). Comparing the frequency of transposition,

the frequency of the system developed in this study was approximately 80- to 170-fold higher than that of the system using pRL692. pRL692 has already been improved by the hyperactivation of Tnp and the optimization of the recognition sequence, and an approximately 100-fold increase of the transposition rate in comparison with employment of a previous vector was observed due to this improvement (Koksharova and Wolk 2002a). Therefore, it was speculated that other reasons for the difference in transposition frequency between pKUT-Tn5-Sm/Sp and pRL692 exist. One possible reason is the use of P_{tac} for hyperactive *tnp* expression. The other possible reason is the difference in the length of the transposon. The length of the transposon of pRL692 (6.9 kb) that contains a replication origin, two antibiotic resistance genes and the hyperactive *tnp* is 3.5-fold longer than that of pKUT-Tn5-Sm/Sp (2.0 kb). Because pKUT-Tn5-Sm/Sp bears a minimal size of the transposon with *aadA*, shortening the size of the transposon may be significant for an increase in transposition frequency. For this reason, a replication origin was not introduced inside the transposon for fear of reduction of the transposition frequency. However, such a modification allows for the recovery of the transposon and its flanking regions by plasmid rescue. Therefore, the introduction of a replication origin into the transposon will be a further improvement of pKUT-Tn5-Sm/Sp.

The transposon mutagenesis system is suitable for construction of a mutant library. Ozaki et al. (2007) reported that about 15% of genes in the genome of *Synechocystis* were disrupted by an *in vitro* transposon mutagenesis system. In their study, the transposon was inserted into cosmid clones containing *Synechocystis* genome fragments, and gene disruptions were performed by natural transformation using the cosmids whose transposon insertion sites were determined previously. On the other hand, transposon-tagged mutants that cover the disruption of almost all genes will be easily obtained using the *in vivo* transposon mutagenesis system in this study. There is one gene per 1.1 kb in the

Synechocystis genome (Kaneko et al. 1996). When the equation $N = \ln(1 - P) / \ln(1 - f)$ (Alexander et al. 2004) was applied to *Synechocystis* [N , the number of mutants required; P , the probability desired; f , frequency of insertions within the genome (1.1 kb/3,600 kb)], approximately 15,000 mutants are required for a confidence level of 99%. Because 2×10^7 cells are required to obtain 15,000 mutants in the routine experiment, approximately 0.2 ml of culture whose cell density is 1×10^8 cells ml^{-1} is enough to produce 15,000 transposon-tagged mutants. I therefore conclude that the system in this study would be suitable for the comprehensive screening of mutants under various conditions.

The ST4 mutant grew significantly more slowly than the control and other transposon-tagged mutants (Fig. 13A). In the mutant, the transposon was inserted into the coding region of *slr1979* (Fig. 9). The functional complementation of the ST4 mutant using the expression vector confirmed *slr1979* as a causative gene. However, the result of PCR indicated that the transposon-inserted chromosome was not completely segregated in the ST4 mutant, and some chromosomes with wild-type *slr1979* remained (Fig. 11). This result indicated that mutants without complete segregation of the mutated gene would be obtained using the system in this study. *Slr1979* is annotated as a gene for anthranilate synthase component I that is related to tryptophan biosynthesis. Therefore, a decrease in tryptophan biosynthesis is a possible cause of the slow growth of the ST4 mutant. However, another gene (*slr0738*) annotated as anthranilate synthase component I was also found in the genome of *Synechocystis*. For this reason, the relationship between *slr1979* and tryptophan biosynthesis is not confirmed; thus, further study will be required to resolve the function of *slr1979*. Even in this pilot experiment, one transposon-tagged mutant with an apparent growth phenotype was isolated, and the expression vector (Tsuchiya et al. 2012b) enabled genetic functional complementation of the mutant. A large-scale experiment using the transposon mutagenesis system developed in this study and a functional complementation

system using an expression vector will help to identify the function of genes for any ‘unknown protein’ or ‘hypothetical protein’ by screening mutants under various conditions.

Using the *in vivo* transposon mutagenesis system developed in this chapter, transposition with a significantly high frequency was observed for *Synechocystis* and *Synechococcus*. The system has two advantages. One is the application of natural transformation for the *in vivo* transposon mutagenesis system. The efficiency of producing transposon-tagged mutants by natural transformation was lower than that by conjugal gene transfer. However, this result will promote the use of pKUT-Tn5-Sm/Sp by researchers who usually study *Synechocystis* or *Synechococcus* by molecular genetic analysis. The other advantage is the application of this system to other cyanobacteria with unique properties. For example, Tsuchiya et al. and Araki et al. recently reported the development of a transformation system for two unique cyanobacteria, *A. marina* (Tsuchiya et al. 2012b) and *Gloeobacter violaceus* PCC 7421 (Araki et al. 2013), respectively. However, the frequencies of transformation of these cyanobacteria were much lower than that of *Synechocystis*. Therefore, the highly frequent *in vivo* transposon mutagenesis system established in this study is ideal to produce transposon-tagged mutants for these cyanobacteria. Moreover, conjugal gene transfer is applicable to other Gram-negative bacteria. Therefore, the transposon mutagenesis system will contribute to the molecular genetic analysis not only of various cyanobacteria but also of a broad range of other Gram-negative bacteria including photosynthetic bacteria.

Chapter 3

Establishment of the forward genetic analysis of the chlorophyll *d*-dominated cyanobacterium *Acaryochloris marina* MBIC 11017 by applying *in vivo* transposon mutagenesis system

Summary

Acaryochloris marina MBIC 11017 possesses Chl *d* as a major Chl, which enables this organism to utilize far-red light for photosynthesis. Thus, the adaptation mechanism of far-red light utilization, including Chl *d* biosynthesis, has received much attention, though a limited number of reports on this subject have been published. To identify genes responsible for Chl *d* biosynthesis and adaptation to far-red light, molecular genetic analysis of *A. marina* was required. Tsuchiya et al. (2012b) developed a transformation system for *A. marina* and introduced expression vectors into *A. marina*. In this chapter, the high-frequency *in vivo* transposon mutagenesis system established in chapter 2 was applied to *A. marina*. As a result, mutants with the transposon in their genomic DNA at various positions were obtained. By screening transposon-tagged mutants, a mutant (Y1 mutant) that formed a yellow colony on agar medium was isolated. In the Y1 mutant, the transposon was inserted into the gene encoding molybdenum cofactor biosynthesis protein A (MoaA). The Y1 mutant was functionally complemented by introducing the *moaA* gene or increasing the ammonium ion in the medium. These results indicate that the mutation of the *moaA* gene reduced nitrate reductase activity, which requires molybdenum cofactor, in the Y1 mutant. This is the first successful forward genetic analysis of *A. marina*, which will lead to the identification of genes responsible for adaptation to far-red light.

3-1 Introduction

Cyanobacteria that thrived 2.7 billion years ago have diversified their composition of photosynthetic pigments to adapt to various photoenvironments throughout evolution. Some cyanobacteria utilize specific Chl species that have not been discovered in photosynthetic eukaryotes. In photosynthetic eukaryotes, Chl *a* functions in both light energy harvesting and charge separation, and some groups of organisms employ Chl *b* or Chl *c* as an accessory

pigment for light energy harvesting. Similarly, all cyanobacteria except for *Prochlorococcus*, which possesses [8-vinyl]-Chl *a* instead of Chl *a* (Chisholm et al. 1992), also use Chl *a*, and some species utilize other Chl species in addition to Chl *a*. *Prochloron* (Lewin and Withers 1975) and *Prochlorothrix* (Burger-Wiersma et al. 1986), categorized into Prochlorophyta, have the ability to synthesize Chl *b* like plants and green algae, and the other Prochlorophyta, *Prochlorococcus*, accumulates [8-vinyl]-Chl *b* (Chisholm et al. 1992). Other specific Chl species found among cyanobacteria, Chl *d* (Miyashita et al. 1996) and Chl *f* (Chen et al. 2010), share a characteristic; absorption maximum of Q_y bands of the Chls is located at longer wavelengths than that of Chl *a*. The function of Chl *d* in photosynthesis has been intensively investigated using *Acaryochloris* spp. (see chapter 1), whereas a limited number of studies on the recently discovered Chl *f* have been reported (Chen et al. 2012, Li et al. 2014, Tomo et al. 2014).

Because Chl *d* can absorb approximately 30 nm longer wavelength light than Chl *a*, PSs in *Acaryochloris* spp. can be driven by far-red light, which cannot drive PSs in most photosynthetic organisms (Miyashita et al. 1996). The characteristic of *Acaryochloris* spp. prompted investigations of this organism, and novel knowledge has been revealed, as described in chapter 1. Moreover, as the type strain of the only genus that synthesizes Chl *d*, entire genome of *A. marina* was sequenced in 2008 (Swingley et al. 2008). In spite of biochemical analysis after the genome sequencing (Schliep et al. 2010), the gene(s) responsible for Chl *d* biosynthesis and the intermediate product of Chl *d* have not been identified.

To identify the genes responsible for adaptation to far-red light, including Chl *d* biosynthesis in *A. marina*, the development of molecular genetic techniques for *A. marina* was required. Tsuchiya et al. (2012b) recently succeeded in introducing expression vectors into *A. marina* by conjugal gene transfer. Additionally, the Chl biosynthetic pathway of *A.*

marina was altered by introducing the *CAO* gene responsible for Chl *b* biosynthesis (Tanaka et al. 1998) using the transformation system (Tsuchiya et al. 2012b). To apply this system to *A. marina*, I developed a high-frequency *in vivo* transposon mutagenesis system for two model cyanobacteria, *Synechocystis* and *Synechococcus*. Because the frequency of transposition in the system was approximately 80- to 170-fold higher than that in the commonly used system, it was expected that the system would be applied to cyanobacteria whose transformation efficiency is much lower than those of model cyanobacteria. Therefore, the production of the mutants required in forward genetic analysis of *A. marina* should be achieved by the combination of the transposon mutagenesis system and the transformation system for *A. marina*.

In this chapter, I applied the high-frequency *in vivo* transposon mutagenesis system established in chapter 2 to *A. marina* and succeeded in producing transposon-tagged mutants. In the mutants, the transposon was inserted into various positions in the genome and an endogenous plasmid. Moreover, a mutant that displayed yellow color was isolated and was functionally complemented by introducing the gene that had been disrupted by the transposon insertion.

3-2 Materials and methods

3-2-1 Culture of *A. marina*

Acaryochloris marina MBIC 11017 was grown in IMK medium (Nihon Pharmaceutical Co., Ltd.) under continuous incandescent light ($10 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) at 25°C. For transposon-tagged mutants and the WT harboring pKUT7123 [WT (pKUT7123)], $1 \mu\text{g ml}^{-1}$ erythromycin (Em) [and $5 \mu\text{g ml}^{-1}$ Sp for transposon-tagged mutants harboring the pKUT1321 derivative] was added to the medium.

3-2-2 Construction of vectors and transposon mutagenesis of *A. marina*

The oligonucleotides used in the construction of vectors are summarized in Table 4. The upstream region (170 bp, P_{psbA2}) of the *psbA2* gene of *Synechocystis* (*slr1311*) was amplified by PCR using primers (PSypsbA2Fw and PSypsbA2Rv), and the PCR product was subcloned into a cloning vector (pZErO-2) to yield pZErO- P_{psbA2} . A tandem-arrayed terminator [downstream region (180 bp) of *slr1311* and *tonB* terminator, $T_{psbA2::tonB}$] was produced by PCR using primers (TSypsbA2Fw and TSypsbA2Rv), and the PCR product was subcloned into pZErO-2 (pZErO- $T_{psbA2::tonB}$). The nucleotide sequences of cloned PCR products were confirmed by sequencing. The cloned terminator with restriction sites (*SalI* site at 5' end and *PstI* site at 3' end) was excised from pZErO- $T_{psbA2::tonB}$ by digestion with *SalI* and *PstI* and was subcloned into pZErO- P_{psbA2} digested with the same enzymes to yield pZErO- P_{psbA2} - $T_{psbA2::tonB}$. An Em resistance gene with *XhoI* sites at both ends was generated by PCR using primers (EmrFw and EmrRv), and the gene excised from the vector by *XhoI* treatment was subcloned into pZErO- P_{psbA2} - $T_{psbA2::tonB}$ digested with *SalI* (Em resistance gene cassette). To construct the expression vector, pKUT7123, an Sm/Sp resistance gene cassette of pKUT1121 (Tsuchiya et al. 2012b) was replaced with the Em resistance gene cassette. A transposon delivery vector, pKUT-Tn5-Em, was similarly constructed by replacing its Sm/Sp resistance gene cassette of pKUT-Tn5-Sm/Sp with the Em resistance gene cassette.

Conjugal gene transfer of pKUT-Tn5-Em and pKUT7123 was performed by diparental mating as previously reported (Tsuchiya et al. 2012b) with slight modifications. Each vector was introduced into JM109 λpir (pRK2013). Equal amounts of *E. coli* [JM109 λpir (pRK2013, pKUT-Tn5-Em) or JM109 λpir (pRK2013, pKUT7123)] cells and *A. marina* cells were mixed. Aliquots of the mixture were spotted onto nitrocellulose membranes on solidified IMK agar medium. Following 48 h incubation under continuous light, the membrane was transferred to IMK solid medium containing 5 $\mu\text{g ml}^{-1}$ Em. Em-resistant

colonies appeared after a few weeks.

3-2-3 Cloning of the restriction fragment that contained the transposon

Genomic DNA was prepared from the transposon-tagged mutants of *A. marina* as described by Wilson (1997). A restriction fragment containing the transposon was cloned into pZErO-2 as described in chapter 2 with slight modifications. The concentrations of the genomic DNA and pZErO-2 digested with the respective restriction enzymes (*EcoRI*, *KpnI*, *BamHI*) were adjusted to 100 and 20 ng μl^{-1} , respectively. The restriction fragments (4.98 μl) prepared from the genomic DNA were ligated to the pZErO-2 digested with the same restriction enzyme (1 μl), and *E. coli* (OmniMAX 2-T1^R) was transformed with the ligation product. Transformants were screened on LB solid medium containing 100 $\mu\text{g ml}^{-1}$ Em, 25 $\mu\text{g ml}^{-1}$ Km, and 100 μM IPTG. The plasmids prepared from the obtained transformants were subjected to sequencing using outward-directed transposon-specific primers (PSypsbA2Rv and TSypsbA2Fw). Transposon insertion site of each clone was determined by comparing the transposon flanking sequences with the genomic sequence of *A. marina* using the Blast algorithm (Altschul et al. 1990).

3-2-4 Inverse PCR

According to the procedure described by Sambrook and Russell (2001), inverse PCR was performed to amplify the fragments that contained the transposon. Genomic DNA from the transposon-tagged mutant was digested with *HindIII* or *XbaI*, and the enzyme was inactivated by heat treatment. The concentration of the digested genomic DNA was adjusted to 150 ng μl^{-1} . Self-ligation of the restriction fragment (1 μl) was used as a template for inverse PCR using specific primers (PSypsbA2Rv and Emr-Seq). For the PCR product, nested PCR was performed using specific primers (PSypsbA2Rv and TSypsbA2Fw). The resultant PCR

product was purified and subjected to sequencing using outward-directed transposon-specific primers (PSypsbA2Rv and TSypsbA2Fw), and transposon insertion site of the mutant was determined by the same procedure described above.

3-2-5 Analysis of pigment composition

The pigment compositions of cells were analyzed by high performance liquid chromatography (HPLC) as described by Tsuchiya et al. (2012a, b) based on Zapata et al. (2000). The pigments extracted with cold methanol from cells were immediately analyzed by HPLC (Prominence series; Shimadzu) with a Symmetry C8 column (135 × 1.0 mm, 3.5 μm particle size; Waters). The elution profile was monitored with a photodiode array detector (SPD-M20A; Shimadzu).

3-2-6 Complementation of the transposon-tagged mutant with yellow-colored phenotype

The genomic DNA of *A. marina* was used as a template for PCR using primers (AM1_5516Fw and AM1_5516Rv) to amplify *AMI_5516* with an *Nde*I site at the 5' end and a *Xho*I site at the 3' end, and the PCR product was subcloned into pZErO-2. After confirmation of the nucleotide sequence, the cloned *AMI_5516* with restriction sites (an *Nde*I site and an *Xho*I site) was excised from the vector by digestion with *Nde*I and *Xho*I and was subcloned into pKUT1321 (Tsuchiya et al. 2012b) digested with the same enzymes. The resultant expression vector (pKUT1321-*AMI_5516*) and pKUT1321 were introduced into the transposon-tagged mutant with yellow-colored phenotype by conjugation to generate transformants Y1 (pKUT1321) and Y1 (pKUT1321-*AMI_5516*), respectively. Each transformant was selected on IMK solid medium containing 2.35 mM NH₄Cl, 2 μg ml⁻¹ Em, and 40 μg ml⁻¹ Sp.

3-3 Results

3-3-1 The application of the high-frequency *in vivo* transposon mutagenesis system to *A. marina*

To establish the techniques for forward genetic analysis of *A. marina*, two kinds of antibiotics for transformation were required: one was for the screening of transposon-tagged mutants, and the other was for the functional complementation of the transposon-tagged mutant by introducing an expression vector with the causal gene. Because Sp had been already utilized to introduce the expression vectors (Tsuchiya et al. 2012b), I searched for another effective antibiotic for the screening of mutants. *A. marina* was sensitive to Em at lower concentration (5 $\mu\text{g ml}^{-1}$) than Sp. Thus, I determined the proper concentration of Em for screening of transformant by introducing an expression vector containing an Em resistance gene. Because *A. marina* exhibited sensitivity to Em at low concentration, an Em resistance gene cassette that contained the promoter region of the *Synechocystis psbA2* gene (Mohamed et al. 1993) was constructed. Additionally, to repress the influence of the transcription of the Em resistance gene on the downstream genes, a terminator was added to the downstream of Em resistance gene. The terminator consisted of the downstream region of the *Synechocystis psbA2* gene tandem-fused with a factor-independent terminator, *tonB* terminator (Postle and Good 1985). The Sm/Sp resistance gene cassette of pKUT1121 (Tsuchiya et al. 2012b) was replaced with the Em resistance gene cassette to yield an expression vector (pKUT7123), and the vector was introduced into *A. marina* by conjugation. WT harboring pKUT7123 [WT (pKUT7123)] was obtained by screening exconjugants at the concentration of 5 $\mu\text{g ml}^{-1}$ Em. This result revealed that Em was effective for the screening of *A. marina* transformant. Therefore, the Sm/Sp resistance gene cassette of the transposon delivery vector (pKUT-Tn5-Sm/Sp) was replaced by the Em resistance gene cassette. The resultant transposon delivery vector, pKUT-Tn5-Em (Fig. 14), was introduced into *A. marina* by

conjugal gene transfer. Consequently, Em-resistant exconjugants appeared after Em selection. Among them, 10 exconjugants (AT1–AT10 mutants) were selected randomly to confirm successful transposon mutagenesis. Total DNA prepared from each mutant was used as template for PCR using specific primers (EmrFw and EmrRv) to amplify the Em resistance gene. The plasmid pKUT-Tn5-Em and total DNA prepared from WT (pKUT7123) were used as positive controls. Consequently, DNA fragments corresponding to the Em resistance gene were amplified from all mutants similarly to positive controls, whereas no corresponding DNA fragment was amplified from WT, like the negative control (Fig. 15A). This result demonstrated that the AT1–AT10 mutants were the transformants carrying the Em resistance gene. To confirm the loss of pKUT-Tn5-Em in the mutants, PCR using specific primers (TnpFw and TnpRv) was performed to amplify the hyperactive *tnp*. Hyperactive *tnp* was amplified only from pKUT-Tn5-Em, a control plasmid (Fig. 15B). In contrast, no amplification of hyperactive *tnp* was observed in any transformant, including the AT1–AT10 mutants, like WT (Fig. 15B). These results indicate that the transposon was successfully inserted into the genome of each mutant, because the Em resistance gene amplified from the AT1–AT10 mutants did not originate from the introduced pKUT-Tn5-Em, which might be lost during cell growth.

3-3-2 Analysis of transposon insertion sites of the mutants

To identify the transposon insertion sites, restriction fragments with the inserted transposon from genomic DNA of the AT1–AT10 mutants were cloned, and the flanking regions of transposons in the restriction fragment were sequenced. The results showed that the transposon was inserted into the genome at different positions in each mutant (Fig. 16 and Table 5). Furthermore, all transposons in the genome were flanked by a 9-bp duplication of the inserted site at each end, which is characteristic of Tn5 insertion. These results

demonstrated that transposon was inserted into the genomic DNA of each mutant by the action of Tn5 Tnp. In all mutants, transposon insertion sites were within predicted genes (Fig. 16 and Table 5). Moreover, in the AT3 mutant, the transposon was inserted into one of nine endogenous plasmids, pREB3 (Fig. 17). The genomic map that presented the respective insertion positions revealed that the transposon was inserted into a broad range of not only the chromosome but also the endogenous plasmid (Fig. 17). To determine the progress of segregation of transposon-inserted chromosomes or endogenous plasmids, the genomic region that contained the transposon of each mutant was amplified by PCR using specific primers (Table 6). Compared to the control using WT genomic DNA as template, the PCR product of each mutant was longer, and no band that originated from WT genomic DNA was found in any mutant (Fig. 18). This result indicated the complete segregation of the transposon-inserted chromosome or endogenous plasmid in all mutants. However, the AT1–AT10 mutants expressed no apparent phenotype compared to WT.

Because all 10 mutants randomly selected from Em-resistant colonies that were obtained by transposon mutagenesis had the transposon in their genomic DNA, it was presumed that all Em-resistant colonies obtained after Em selection were transposon-tagged mutants. According to this hypothesis, the frequency of transposition in *A. marina* was calculated to be 2×10^{-8} per recipient cell.

3-3-3 Isolation and analysis of the yellow-colored mutant

More than 100 mutants of *A. marina* were produced by the *in vivo* transposon mutagenesis system. Among them, a mutant that displayed yellow color on agar medium was isolated and was named the Y1 mutant (Fig. 19A). The pigment composition of the Y1 mutant was confirmed by reverse-phase HPLC (Fig. 20). Like WT, the Y1 mutant possessed zeaxanthin (peak 1 in Fig. 20), Chl *d* (peak 2) and α -carotene (peak 3) as major pigments and Chl *a* as a

minor pigment. The molar proportions of the major pigments were Chl *d*:zeaxanthin: α -carotene = 2.5:1.1:1.0 for the Y1 mutant and Chl *d*:zeaxanthin: α -carotene = 5.5:1.1:1.0 for WT. Therefore, the relative amount of Chl *d* to major carotenoids in the Y1 mutant was decreased to 45% of that in WT. The ratio of zeaxanthin to α -carotene was similar between strains. To determine the transposon insertion site of the Y1 mutant, I tried to clone the restriction fragment containing the transposon from genomic DNA of the mutant. However, the fragment could not be cloned, unlike the case of the AT1–AT10 mutants. Therefore, DNA fragment with the transposon in the Y1 mutant was amplified by inverse PCR, and the transposon insertion site in the mutant was determined. For the Y1 mutant, the transposon was inserted into *AMI_5516* (Fig. 19B), which is annotated as the *moaA* gene encoding molybdenum cofactor biosynthesis protein A responsible for molybdenum cofactor biosynthesis. In the Y1 mutant, no fragment originating from the WT chromosome was detected when the genomic region containing the transposon was amplified by PCR (Fig. 19C). This result indicated the complete segregation of the transposon-inserted chromosome, similar to the AT1–AT10 mutants (Fig. 18). Because the molybdenum cofactor is a constituent of nitrate reductase, which catalyzes reduction of nitrate to nitrite, the disruption of *moaA* caused a deficiency of nitrate reduction in *Synechococcus* (Rubio et al. 1998). Therefore, it was speculated that the transposon insertion in the Y1 mutant resulted in the disruption of *moaA*, which might lead to the reduction of nitrate reductase activity. In cyanobacteria, nitrate taken from outside of the cell is utilized as nitrogen source after conversion into ammonium via nitrite (Flores et al. 2005). Nitrate reductase catalyzes the first step of this pathway. Therefore, the Y1 mutant was assumed to be deficient in nitrogen source on IMK agar medium, because the concentration of NH_4Cl (50 μM) was 47 times lower than that of NaNO_3 (2,350 μM) in the medium. The growth of the Y1 mutant was compared on three agar media with different nitrogen source compositions: IMK medium,

IMK medium containing NaNO_3 as sole nitrogen source ($\text{IMK}_0\text{NO}_3^-$), and IMK medium containing NH_4Cl as sole nitrogen source ($\text{IMK}_0\text{NH}_4^+$). The concentration of nitrogen source in each medium was equal. The Y1 mutant cultured on the $\text{IMK}_0\text{NH}_4^+$ turned green, whereas the mutants cultured on IMK or $\text{IMK}_0\text{NO}_3^-$ were yellow (Fig. 19D). In contrast, WT (pKUT7123) was green in all media (Fig. 19D). These results indicate that the Y1 mutant utilized not nitrate but ammonium as nitrogen source. Based on these results, the Y1 mutant was maintained in the IMK liquid medium containing 2,350 μM NH_4Cl because the Y1 mutant could not grow in the IMK liquid medium.

To identify the gene responsible for the phenotype of the Y1 mutant, *AMI_5516* was introduced into the Y1 mutant using the expression vector pKUT1321-*AMI_5516* to yield Y1 (pKUT1321-*AMI_5516*). As a control mutant, the Y1 mutant harboring pKUT1321, Y1 (pKUT1321), was also produced. Successful introduction of the vectors into each transformant was confirmed by PCR (Fig. 21A). The genomic region that contained *AMI_5516* and inserted transposon was amplified from the DNA of all transformants. The Sm/Sp resistance gene, *aadA* was amplified from the DNA of the transformants harboring the expression vector [Y1 (pKUT1321) and Y1 (pKUT1321-*AMI_5516*)]. Moreover, a PCR product for the tandem arrangement of the *AMI_5516* and *T_{rrnB}* originating from pKUT1321-*AMI_5516* was detected only in the Y1 (pKUT1321-*AMI_5516*). These results demonstrated that the plasmid introduced into Y1 mutant was retained in each transformant. Thus, the growth of the Y1 (pKUT1321-*AMI_5516*) was compared to that of the Y1 (pKUT1321) on the three media described above. The Y1 (pKUT1321) was yellow on IMK and $\text{IMK}_0\text{NO}_3^-$ and turned green on $\text{IMK}_0\text{NH}_4^+$ (Fig. 21B), like the Y1 mutant (Fig. 19D). In contrast, the color of the Y1 (pKUT1321-*AMI_5516*) became green on the all media (Fig. 21B). These results indicate that introduction of *AMI_5516* enabled the Y1 mutant to utilize NaNO_3 as a nitrogen source. It was concluded that *AMI_5516* was the causative gene of the

Y1 mutant.

3-4 Discussion

A. marina, which utilizes the PSs driven by far-red light, is in the only known genus that possesses Chl *d* as a major Chl. Although the isolation and analysis of the PSs were performed to investigate the unique properties, molecular genetic analysis was required to understand them further. For model cyanobacteria such as *Synechocystis* and *Synechococcus*, a technique of gene disruption by gene targeting was established. This promoted reverse genetic analysis of the model cyanobacteria in combination with genomic information. Although the entire genome of *A. marina* was sequenced in 2008 (Swingley et al. 2008), the lack of gene targeting technique prevented many researchers from studying this organism by reverse genetic analysis. To develop a transformation system for *A. marina*, Tsuchiya et al. (2012b) first tried to introduce expression vectors and succeeded in obtaining the transformant carrying an exogenous gene. However, specific genes in the genome of *A. marina* cannot be disrupted because no gene targeting system for *A. marina* has been developed. To identify the genes involved in adaptation to far-red light in *A. marina* exhaustively, forward genetic analysis is preferable to reverse genetic analysis. Thus, the transposon mutagenesis system, whose transposition frequency was extremely high for model cyanobacteria in chapter 2, was applied to *A. marina*, and mutants that possessed transposon-inserted chromosomes were obtained. All ten Em-resistant exconjugants selected randomly contained the transposon in the genome (Fig. 16, 17), indicating the high reliability of the system. Furthermore, all transposons in the genome of the AT1–AT10 mutants were flanked by a 9-bp duplication of the target site at each end, which is characteristic of Tn5 insertion. This demonstrated that the AT1–AT10 mutants were produced by the action of Tn5 Tnp. As in model cyanobacteria, transposon insertion sites of *A. marina* were spread

throughout the genome (Fig. 17). These results indicate that the transposon mutagenesis system developed in the present study is an effective way to produce mutants whose transposon is inserted into various positions of the genome. However, the frequency of transposition in *A. marina* (2×10^{-8}) was considerably lower than that in model cyanobacteria (*Synechocystis*, 9×10^{-3} ; *Synechococcus*, 5×10^{-3}). There is a significant difference in the transformation efficiency of the expression vector between *A. marina* and model cyanobacteria. The low transposition frequency in *A. marina* might reflect this difference. In other words, the use of the *in vivo* transposon mutagenesis system with high frequency in model cyanobacteria might be a breakthrough to develop a transposon mutagenesis system for *A. marina*. Transposon-inserted chromosome in *A. marina* was completely segregated in all mutants analyzed in this study (AT1–AT10 mutants and Y1 mutant) (Fig. 18). This result implies that gene disruption by transposon insertion in *A. marina* is easier than other cyanobacteria. Although the transposon was inserted into open reading frames, including four hypothetical proteins, in the AT1–AT10 mutants (Table 5), there was no mutant with an apparent phenotype among them. Because the number of open reading frames in the genome, including nine endogenous plasmids, is 8,528 in *A. marina* (Swingley et al. 2008), more mutants should be produced to obtain mutants with interesting phenotypes.

The Y1 mutant that exhibited yellow color was isolated by screening more than 100 transposon-tagged mutants (Fig. 19A). Additionally, the relative amount of Chl *d* to major carotenoids in the Y1 mutant was lower than that in WT (Fig. 20). The molar ratios of Chl *a* were 1.4% of the total Chl for WT and 2.5% for the Y1 mutant (data not shown). These values fall within the range of variable Chl *a* ratio. In general, cyanobacteria under nitrogen deficiency utilize PBS as nitrogen source and stop the synthesis of Chl, and they turn yellow. Therefore, the yellow-colored phenotype of the Y1 mutant indicated nitrogen deficiency. In the Y1 mutant, the transposon was inserted into the gene *AMI_5516*, which is one of the

genes responsible for molybdenum cofactor biosynthesis (*moaA*) (Fig. 19B). Introduction of the expression vector carrying *A. marina moaA* revealed that *moaA* was the causative gene of the Y1 mutant (Fig. 21B). MoaA, which was first identified in *E. coli*, is involved in molybdenum cofactor biosynthesis (Leimkühler et al. 2011). The molybdenum cofactor is required for the activity of several oxidoreductases, including nitrate reductase (Leimkühler et al. 2011). In cyanobacteria, disruption of the *moaA* gene was only reported in *Synechococcus* (Rubio et al. 1998). Similar to the cultivation of the Y1 mutant in the $\text{IMK}_0\text{NO}_3^-$ liquid medium, the *moaA* mutant of *Synechococcus* did not grow in liquid medium containing nitrate as sole nitrogen source, whereas it grew in the medium containing ammonium (Rubio et al. 1998). Additionally, the mutants, whose nitrate reductase activity had been reduced, were generated by chemical (exposure to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) or biological treatments (transposon mutagenesis using *Tn901*) (Kuhlemeier et al. 1984, Madueño et al. 1988). These mutants grown on the BG11 medium (which contains no ammonium salt) were selected by color; the yellow color is an indicator of nitrogen deficiency. For the Y1 mutant, yellow color in $\text{IMK}_0\text{NO}_3^-$ and green color in $\text{IMK}_0\text{NH}_4^+$ were observed (Fig. 19D), like the *moaA* mutant of *Synechococcus*. These results indicate that the *A. marina moaA* is also responsible for the biosynthesis of molybdenum cofactor in *A. marina*. Thus, this is the first case in which the function of a gene in *A. marina* was identified by forward genetic analysis. Depending on the screening procedure, various mutants with curious phenotypes will be isolated in the future. In particular, the transposon mutagenesis and functional complementation system in this study will be applied to the investigation of the adaptation mechanism to far-red light, including Chl *d* biosynthesis.

Cyanobacteria have various and unique features such as nitrogen fixation and chromatic adaptation. To understanding the characteristics on the molecular level, molecular genetic

analysis is the effective way. Forward genetic analysis, one of genetic approaches, requires mutants with random mutation, and the transposon mutagenesis is the effective method for cyanobacteria. To date, the production of mutants by transposon mutagenesis, the screening of the mutants, and the identification of the causative genes were reported on some cyanobacteria. For *Synechocystis* (Bhaya et al. 2001, Kato et al. 2010) and *Synechococcus* (Taniguchi et al. 2007, Tanaka et al. 2012), *in vitro* transposon mutagenesis system was often utilized. In contrast, *in vivo* transposon mutagenesis system was applied to *Anabaena* (Koksharova and Wolk 2002) and *Synechococcus* sp. strain WH8102 (McCarren and Brahamsha 2005), which can be transformed by conjugal gene transfer. Additionally, a transposon mutagenesis system was developed for *Prochlorococcus* strain MIT9313, but no mutant with an apparent phenotype was isolated (Tolonen et al. 2006). As described above, transposon mutagenesis system was developed for a few species of cyanobacteria.

In this chapter, I succeeded in transposon mutagenesis and functional complementation in *A. marina*. This achievement is the first case of forward genetic analysis of *A. marina* and is a major breakthrough of molecular genetic analysis of *A. marina*. Thus, the system could contribute to the understanding of the adaptation to far-red light in *A. marina*, especially the identification of the gene responsible for Chl *d* biosynthesis. Moreover, this result strongly suggests that the technique developed in this study could be applied to a wide range of cyanobacteria. Therefore, if transformation systems for different cyanobacteria can be established, the technique could contribute to the study of cyanobacteria with unique photosynthetic systems, such as Chl *f*-containing cyanobacterium (Chen et al. 2010) and recently reported Chl *d*- and Chl *f*-containing cyanobacteria (Airs et al. 2014, Gan et al. 2014). Hence, the forward genetic technique established in this study will assist in understanding the genetics and evolution of photosynthesis in cyanobacteria.

Chapter 4

Summary and perspectives

In chapter 2, the high-frequency transposon mutagenesis system was developed to accelerate forward genetic analysis of model cyanobacteria. Although forward genetic analysis of model cyanobacteria is an effective strategy for obtaining fundamental knowledge of oxygenic photosynthesis, it has not been spread compared with reverse genetic analysis. In the future, a familiar forward genetic tool will be required to progress molecular genetic analysis of model cyanobacteria.

In forward genetic analysis, *in vivo* transposon mutagenesis system has been applied to a wide range of cyanobacteria (Katayama et al. 1999, Koksharova and Wolk 2002b, Miyagishima et al. 2005). However, the *in vivo* transposon mutagenesis system for *Anabaena* and *Synechococcus* faced two problems, relatively low frequency of transposition and repeated transposition in the host cells. (Koksharova and Wolk 2002a). To solve the problems, based on the plasmid (pUTmini-Tn5 Sm/Sp) (Fig. 5A) whose *tnp* gene is located outside the transposon (de Lorenzo et al. 1990), I constructed the transposon delivery vector (pKUT-Tn5-Sm/Sp) (Fig. 5B) which has three features as follows: (i) the hyperactivation of Tnp and the optimization of the end sequences recognized by Tnp for high frequency of transposition (Goryshin and Reznikoff 1998); (ii) the utilization of the suicide vector (de Lorenzo et al. 1990) and *tnp* located outside of the transposon for the prevention of repeated transposition (de Lorenzo et al. 1990); (iii) the control of *tnp* gene expression by the *tac* promoter (de Boer et al. 1983). Additionally, the pKUT-Tn5-Sm/Sp carries three major restriction sites inside the transposon, two *Mlu*I sites for replacement of antibiotic resistant gene cassette and unique *Not*I site for improvement by insertion of DNA fragments in the future. Transposon-tagged mutants of *Synechocystis* were obtained at high frequency (9×10^{-3} per recipient cell) with the use of pKUT-Tn5-Sm/Sp, and the transposon insertion sites of each mutant spread into a broad range of the chromosome (Fig. 10). In *Synechococcus*, one of the other model cyanobacteria, the frequency of the transposon mutagenesis system in

this study (5×10^{-3} per recipient cell) was approximately 80- to 170- fold higher than that of the popularized system using pRL692 ($3\text{-}6 \times 10^{-5}$ per recipient cell) (Koksharova and Wolk 2002b). Moreover, it was estimated that 0.2 ml of culture of *Synechocystis* (1×10^8 cells ml⁻¹) is required to obtain transposon-tagged mutants (15,000 mutants) that cover the disruption of all genes at the 99% level of confidence. Therefore, the system would encourage many researchers to perform comprehensive screening of mutants under various conditions. In fact, the transposon-tagged mutant with slower growth was obtained and was functionally complemented by the introduction of the expression vector (Tsuchiya et al. 2012b) carrying the causative gene (Fig. 13).

In chapter 2, the effective forward genetic approach for model cyanobacteria was established. Furthermore, it is expected that the system developed in this study will be applied to not only cyanobacteria but also a large variety of Gram-negative bacteria that can be transformed by conjugal gene transfer.

In chapter 3, I discussed the application of *in vivo* transposon mutagenesis to the forward genetic analysis of *A. marina*. Molecular genetic analysis of *A. marina* was required to identify the gene responsible for Chl *d* biosynthesis. Therefore, the *in vivo* transposon mutagenesis system established in chapter 2 was applied to *A. marina*, and the transposon insertion sites of each resultant mutant were widely spread in the genome including the endogenous plasmid (Fig. 17). Because segregation of chromosome containing the transposon was confirmed in all mutants, the gene disruption of *A. marina* might be easy (Fig. 18). Additionally, the mutant that displayed yellow color (Y1 mutant) was isolated by screening transposon-tagged mutants (Fig. 19A). In the Y1 mutant, the transposon was inserted into the gene (*moaA*) responsible for biosynthesis of molybdenum cofactor that is a constituent of nitrate reductase. The Y1 mutant was yellow on medium containing NaNO₃ as

sole nitrogen source and turned green on medium containing NH_4Cl as sole nitrogen source (Fig. 19D). As with the *moaA* mutant of *Synechococcus* (Rubio et al. 1998), the Y1 mutant utilized not nitrate but ammonium as nitrogen source. Furthermore, the Y1 mutant was functionally complemented by introduction of the expression vector (Tsuchiya et al. 2012b) carrying the *A. marina moaA* gene (Fig. 21). Thus, it was indicated that *A. marina moaA* gene is responsible for molybdenum cofactor biosynthesis, and the activity of MoaA would be repressed in the Y1 mutant.

In chapter 3, I reported the first case of forward genetic analysis of *A. marina*. These results indicated that this forward genetic tool could be employed to reveal the adaptation mechanism of *A. marina* to the far-red light, containing Chl *d* biosynthesis. Moreover, the approach was available for *A. marina* in addition to model cyanobacteria, which was strongly support that the system has applicability to a wide range of cyanobacteria.

Reverse genetic analysis is the mainstream of molecular genetic analysis of *Synechocystis*. However, it is hard to perform exhaustive analysis of gene functions by reverse genetic analysis alone. Therefore, the high-frequency transposon mutagenesis system was developed for the purpose of promoting forward genetic analysis in *Synechocystis*. This system would give many biologists easy way to produce the mutants which cover the disruption of almost all genes and would facilitate screening under various conditions based on interest of each researcher.

Some further modifications of the *in vivo* transposon mutagenesis system are suggested hereafter. In addition to the disruption of genes by transposon insertion (loss-of-function), if a strong expression promoter [e.g. P_{tac} (Marraccini et al. 1993) and P_{psbA2} (Mohamed et al. 1993)] is introduced into *NotI* site of the transposon in outward direction, it is expected that the downstream gene of transposon insertion site will be activated (gain-of-function).

Moreover, if a reporter gene (e.g. luciferase gene) without promoter is cloned into *NotI* site of the transposon in inward direction, it will be available for the analysis of expression patterns of upstream genes of transposon insertion site (gene trapping). Thus, these techniques will contribute to obtain much knowledge of *Synechocystis* and other cyanobacteria for understanding of physiologically important reactions such as photosynthesis.

In chapter 3, forward genetic analysis of *A. marina* was established, and this technology has potential for the identification of the gene(s) responsible for Chl *d* biosynthesis. The intermediates and biosynthetic pathway of Chl *d* in *A. marina* remain unclear. However, it is assumed that biosynthetic pathways of all Chl species branch from the Chl *a* biosynthetic pathway. Therefore, the intermediates of Chl *d* may be synthesized from chlorophyllide (Chlide) *a*, which is the precursor of Chl *a* (Gomez Maqueo Chew and Bryant 2007). On the other hand, ¹⁸O labeling experiment indicated that a oxygen atom in the formyl group of Chl *d* came from molecular oxygen, thus Schliep et al. proposed that Chl *d* produced from Chl *a* (2010). In the Chl *d* biosynthesis, the vinyl group at C-3 position of Chl *a* is converted into the formyl group whether the branching point of Chl *d* biosynthesis is Chlide *a* or Chl *a*. In addition to Chl *d*, Chl *b* and Chl *f* also have the formyl group, and are the formylation of the methyl group at the C-7 and C-2 positions of Chl *a*, respectively (Fig. 2). The enzyme for Chl *b* biosynthesis (CAO) was already identified, and *in vitro* experiment demonstrated that CAO catalyzed two successive hydroxylation of the methyl group at C-7 position of Chlide *a* (Fig. 22) (Tanaka et al. 1998, Oster et al. 2000). Subsequently, the product spontaneously loses water and forms Chlide *b* (Oster et al. 2000) (Fig. 22). A phytyl group is added to Chlide *b* by chlorophyll synthase (ChlG) to yield Chl *b* (Fig. 22). Regarding Chl *f* biosynthesis, it is possible that the reaction is similar to biosynthesis of Chl *b*. In contrast, it is expected that the biosynthesis pathway of Chl *d* differ from that of Chl *b*, because breaking of the carbon double bond of the vinyl group at C-3 position of Chl *a* is required in the Chl *d* biosynthesis.

Chen et al. suggested that P450 oxygenase catalyzed the conversion of Chl *a* to Chl *d* (Fig. 23) (Chen and Blankenship 2011). However, the report that refers to the experimental results is unpublished. Therefore, I cannot discuss it. Although the biosynthetic pathway of Chl *d* is under discussion, I suggest one possible pathway as follows. A reductase catalyzes reduction of the vinyl group at C-3 position of Chlide *a*, and yields [3-ethyl]-Chlide *a* (Fig. 23). Subsequently, [3-ethyl]-Chlide *a* is converted into Chlide *d* by an oxidase catalyzing addition of molecular oxygen (Fig. 23). A phytyl group is added into Chlide *d* by ChlG to yield Chl *d* (Fig. 23). As mentioned above, there is a possibility that more than one enzyme may be involved in the Chl *d* biosynthesis. Additionally, few operons exist in cyanobacterial genome in contrast to other bacteria. Therefore, it is difficult to produce transformants of *Synechocystis* accumulating Chl *d* or the intermediates of it by introduction of cosmids containing *A. marina* genome fragments. The suitable mean of identification of the gene(s) responsible for Chl *d* biosynthesis is forward genetic analysis of *A. marina* because the mutation in one of genes related to Chl *d* biosynthesis can cause the accumulation of the intermediate of Chl *d* biosynthesis or the change of Chl *a*/Chl *d* ratio in the cell. Therefore, I would like to perform the screening of transposon-tagged mutants of *A. marina* by HPLC for selection of the mutants with high Chl *a*/Chl *d* ratio or the mutants accumulating the intermediates of Chl *d* biosynthesis. The analyses of such mutants will reveal the genes responsible for the Chl *d* biosynthesis.

A. marina has attractive research issues other than the Chl *d* biosynthesis. For example, Chl *d* dominated PS II would be used as an ideal control for PS II containing only Chl *a*. However, PS II prepared from wild type *A. marina* lost oxygen evolution activity and CP43, the core antenna of PS II (Tomo et al. 2007). On the other hand, in *Synechocystis*, a histidine tag was fused to C-terminal of CP47, another core antenna of PS II, by gene targeting. Therefore, PS II could be easily prepared by affinity chromatography, and the PS II had

oxygen evolution activity and kept CP43 (Bricker et al. 1998). Reverse genetic tool for *A. marina* is required to produce the transformant similar to the mutant of *Synechocystis*, however, the technique is not established. Therefore, for further investigation of PS II, I would like to develop gene targeting system of *A. marina*.

Because the *in vivo* transposon mutagenesis system employs conjugation, the system developed in this study would be applied to a wide range of Gram-negative bacteria containing cyanobacteria. Moreover, the expression vector utilized in the functional complementation is also available for many Gram-negative bacteria, because the vector originated from a broad-host-range plasmid, RSF1010 (Guerry et al. 1974, Scholz et al. 1989, Tsuchiya et al. 2012b). Moreover, *Mlu*I sites of the transposon delivery vector and the expression vector facilitate the replacement of the antibiotic resistance gene. In the other hand, the forward genetic tool developed in this study can be easily customized for target organisms. Therefore, if the system can be applied to *Halomicronema hongdechloris*, Chl *f* containing cyanobacterium (Chen et al. 2010, Chen et al. 2012), or recently-reported cyanobacteria carrying Chl *d* and Chl *f* in addition to Chl *a* (Aris et al. 2014, Gan et al. 2014), it would contribute to not only the identification of the gene responsible for Chl *f* biosynthesis but also understanding of inductive mechanism of Chl *d* and Chl *f* biosynthesis by far-red light. In conclusion, I established the forward genetic analytical method that would be available for a wide range of Gram-negative bacteria, thus the system must contribute the progress of bacteriology.

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Table 1 Oligonucleotides used in the construction of vectors, the confirmation of the production of *Synechocystis* transformants and the determination of the transposon insertion sites

Name	Sequence ¹
aadAFw	5'-ATGCGCTCACGCAACTGG-3'
aadARv	5'-TTATTTGCCGACTACCTTGGTGAT-3'
TnpFw	5'-GGGTACCGGGCATATGATAACTTCTGCTCTTCATCGTG-3'
TnpRv	5'-GGAGCTCTCAGATCTTGATCCCCTGCGCCAT-3'
TnpMutRv ²	5'-CCTTCCTGggcGGCTTtACTACCCTC-3'
pUTFw1	5'-GTTATCATATGCCCGGTACCGAACGTTACCATGTTAGGAGGT-3'
pUTRv1	5'-GGATCAAGATCTGAGAGCTCTCGGTCGACCTGCAGAT-3'
TrnBFw	5'-GGAGCTCGCTGTTTTGGCGGATGAGA-3'
TrnBRv ³	5'-GGAGCTCGAAACAACTAAAGCGCCCTTGTGGCGCTTTAGT TTTGTTAGAGTTTGTAGAAACGCAAAAAGGC-3'
pUTFw2	5'-ACGCGTGCGGCCGCAGATGTGTATAAGAGACAGAA-3'
pUTRv2	5'-ACGCGTAGATGTGTATAAGAGACAGTCTAGAAGAAGCTTGG-3'
Tn5-Seq1	5'-GCCCTACACAAATTGGGAGA-3'
Tn5-Seq2	5'-GGATCTGGATTTCGATCACG-3'
slr1979Fw	5'-GCATATGGCATCAATTGCGCATTGTTTCGGGC-3'
slr1979Rv	5'-GCTCGAGTTATAGTCCCTCCAATGCCGCCAA-3'
slr1980Fw	5'-GCATATGGTTAGAATCATTGCAAAAAGCCAAA-3'
slr1980Rv	5'-GCTCGAGTTAATTCGCCCCCTTGTTTACCTCC-3'

¹Sequences corresponding to the introduced restriction sites are underlined.

²Letters represented in lower case are bases for mutagenesis.

³Blue characters correspond to the sequence of *T_{soxR}*.

Table 2 Oligonucleotides used in the confirmation of the segregation of transposon-inserted chromosome in the ST1–ST10 mutants

Name ¹	Sequence
ST1Fw	5'-GTTGCCCTTCAATCTCCCGA-3'
ST1Rv	5'-GGTGACGGTAATGGGACTGG-3'
ST2Fw	5'-TCGGTTTTAGTCCGCCCTTC-3'
ST2Rv	5'-CCCCTTCTACCAGGCCTTTG-3'
ST3Fw	5'-AAACCCAACAGGGGTTGCTG-3'
ST3Rv	5'-GGCATATAGGCCCGCGTTAC-3'
ST4Fw	5'-CGAAACCATGGTCAATCAAGCGA-3'
ST4Rv	5'-GGAGTACCAAATGCAGGCAGAAG-3'
ST5Fw	5'-GGTTGAGGGTACGGACAGTG-3'
ST5Rv	5'-ATCGTATCACCACCCATCGC-3'
ST6Fw	5'-GATCGGACTGCTCGGACCAA-3'
ST6Rv	5'-ATTAACCGCCCTCTCCGTCG-3'
ST7Fw	5'-GCACCCTCCTGGCAGATG-3'
ST7Rv	5'-TCGGTTGCAACGATGGTGTA-3'
ST8Fw	5'-ATTTGACGAGTAGCGCAGGC-3'
ST8Rv	5'-CCATTGGCTGCCGACGTATC-3'
ST9Fw	5'-ATGTGCTCGCTCGCTATGGA-3'
ST9Rv	5'-CCTGGCGAAGATAGGCGGAA-3'
ST10Fw	5'-CAAGCGAGTTCCTTTGCCAC-3'
ST10Rv	5'-GAGGCTATCGTCACAGTCGG-3'

¹Oligonucleotides are named after their target transposon-tagged mutant.

Table 3 Summary of the transposon-inserted genes or flanking genes in the ST1–ST10 mutants

Mutant	Position ¹	Gene	Annotation
ST1	chr: 2162474	<i>slr0408</i>	unknown protein
ST2	chr: 2210219	intergenic region between <i>ssl0241</i> and <i>ssr0256</i>	hypothetical protein (Ssl0241) putative transposase (Ssr0256)
ST3	chr: 470962	intergenic region between <i>slr1603</i> and <i>slr1604</i>	hypothetical protein (Slr1603) cell division protein, FtsH (Slr1604)
ST4	chr: 1801740	<i>slr1979</i>	anthranilate synthase component I, TrpE
ST5	chr: 2419702	<i>sll0751</i>	hypothetical protein YCF22
ST6	chr: 3078289	<i>sll0779</i>	unknown protein
ST7	chr: 2359448	<i>slr0364</i>	hypothetical protein
ST8	chr: 526721	<i>slr1753</i>	hypothetical protein
ST9	chr: 497610	<i>slr1047</i>	hypothetical protein
ST10	chr: 2513593	<i>sll0207</i>	glucose-1-phosphate thymidyltransferase

¹Each nucleotide number indicates the right-side positions of transposon.

Table 4 Oligonucleotides used in the construction of vectors, the confirmation of the production of *A. marina* transformants and the determination of the transposon insertion sites

Name	Sequence ¹
PSypsbA2Fw	5'- <u>GACGCGT</u> TACCCATGGAAAAACGACAATTA-3'
PSypsbA2Rv	5'- <u>GGTCGACT</u> TGGTTATAATTCCTTATGTATTTGTCG-3'
TSypsbA2Fw	5'- <u>GGTCGACT</u> TCCTTGGTGTAATGCCAACTGAATAA-3'
TSypsbA2Rv ²	5'- <u>GCTGCAGACGCGT</u> <u>AGTCAAAGCCTCCGGTCGGAGGCTTT</u> <u>GACTTTCTGCAATTCCTCTATCGAAGATGGATTAAT</u> -3'
EmrFw	5'- <u>GCTCGAGAT</u> GAAACGAGAAAAATATAAACACAG-3'
EmrRv	5'- <u>GCTCGAGTT</u> ACTTATTAAATAATTTATAGCTATTGAAA-3'
Emr-Seq	5'-AAACATGCAGGAATTGACGA-3'
AM1_5516Fw	5'- <u>GCATATGA</u> ATGCAGTTGATTATCTTCGA-3'
AM1_5516Rv	5'- <u>GCTCGAGTT</u> ATCCGCCAATTTGAGACA-3'
TnpFw	5'-GGGTACCGGGCATATGATAACTTCTGCTCTTCATCGTG-3'
TnpRv	5'-GGAGCTCTCAGATCTTGATCCCCTGCGCCAT-3'
aadAFw	5'-ATGCGCTCACGCAACTGG-3'
aadARv	5'-TTATTTGCCGACTACCTTGGTGAT-3'
TrrnBRv	5'-CTGCAGAGAGTTTGTAGAAACGCAAAAAG-3'

¹Sequences corresponding to the introduced restriction sites are underlined.

²Blue characters correspond to the sequence of *tonB* terminator.

Table 5 Summary of the transposon inserted genes in the AT1–AT10 mutants

Mutant	Position ¹	Gene	Annotation
AT1	chr: 3538036	<i>AM1_3498</i>	diguanylate cyclase (GGDEF) domain protein
AT2	chr: 54528	<i>AM1_0054</i>	conserved hypothetical protein
AT3	pREB3: 17580	<i>AM1_C0018</i>	efflux transporter, RND family, MFP subunit
AT4	chr: 2992976	<i>AM1_2963</i>	hypothetical protein
AT5	chr: 5207132	<i>AM1_5145</i>	two-component sensor histidine kinase, putative
AT6	chr: 963355	<i>AM1_0990</i>	hypothetical protein
AT7	chr: 5871280	<i>AM1_5788</i>	hydroxyacylglutathione hydrolase, putative
AT8	chr: 46316	<i>AM1_0046</i>	ATP binding protein, putative
AT9	chr: 5908043	<i>AM1_5834</i>	hypothetical protein
AT10	chr: 912578	<i>AM1_0944</i>	peptide ABC transporter, permease subunit, putative

¹Each nucleotide number indicates the right-side positions of transposon.

Table 6 Oligonucleotides used in the confirmation of the segregation of transposon-inserted chromosome or endogenous plasmid in the AT1–AT10 mutants and the Y1 mutant

Name ¹	Sequence
AT1Fw	5'-AAGAACAGCTGCCCTTGAGC-3'
AT1Rv	5'-GTTCTGCTTCCGGATTTTGC-3'
AT2Fw	5'-CCCTTCTCGGATCTGAATCG-3'
AT2Rv	5'-CCAAGTCTTTGGGGCAACTC-3'
AT3Fw	5'-AACGTTGAACAAGCCCGTCT-3'
AT3Rv	5'-GTCCCAACTCTCGGTTTCAGG-3'
AT4Fw	5'-GCCATGCGAATCACACCTTA-3'
AT4Rv	5'-GATGCACAACGGACCACAGT-3'
AT5Fw	5'-TATCCACCTGCATCCACAGC-3'
AT5Rv	5'-CCCTTAGCCCTGCAAGAATG-3'
AT6Fw	5'-GAGCCTTCAGCCACCATAACC-3'
AT6Rv	5'-TGCCTTGCAGTTGAGGCTTA-3'
AT7Fw	5'-ACCTCGCGAACAGAAAGCTC-3'
AT7Rv	5'-ACCGGCCTTTTCCGATAACT-3'
AT8Fw	5'-GGGATTAGGGAAGGGACTCG-3'
AT8Rv	5'-ACAGATTCGGCGACAAAGGT-3'
AT9Fw	5'-CACACCCAATGGATTGATGG-3'
AT9Rv	5'-GCCCCGCTCGATAGACAATTC-3'
AT10Fw	5'-CCAAAAATTTGCAGGGGAAT-3'
AT10Rv	5'-CCGCCCTAGAGGTAATCCAG-3'
Y1Fw	5'-TTGTGGCTTGTGGCTAATGG-3'
Y1Rv	5'-GCAAACCACCGCTTATGTCA-3'

¹Oligonucleotides are named after their target transposon-tagged mutant.

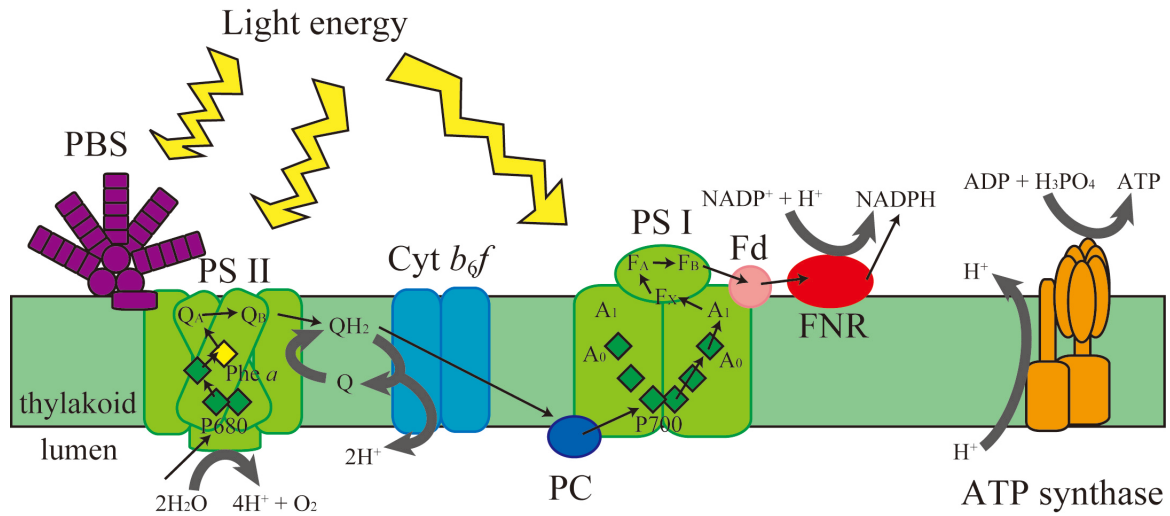


Fig. 1 Diagram of electron transfer in thylakoid of typical cyanobacteria. The electron transfer is explained above. Black arrows, green diamonds and a yellow diamond indicate electron flow, Chl *a* and Phe *a*, respectively. PBS, phycobilisome; PS II, photosystem II; Cyt *b₆f*, cytochrome *b₆f* complex; PC, plastocyanin; PS I, photosystem I; Fd, ferredoxin; FNR, Fd:NADPH⁺ oxidoreductase; P680, special pair of PS II; Q_A, primary electron acceptor plastoquinone molecule; Q_B, secondary electron acceptor plastoquinone molecule; QH₂, reduced plastoquinone; Q, oxidized plastoquinone; P700, special pair of PS I; A₀, PS I primary electron acceptor; A₁, PS I secondary electron acceptor; F_A, iron-sulfur center; F_B, iron-sulfur center; F_X, iron-sulfur center.

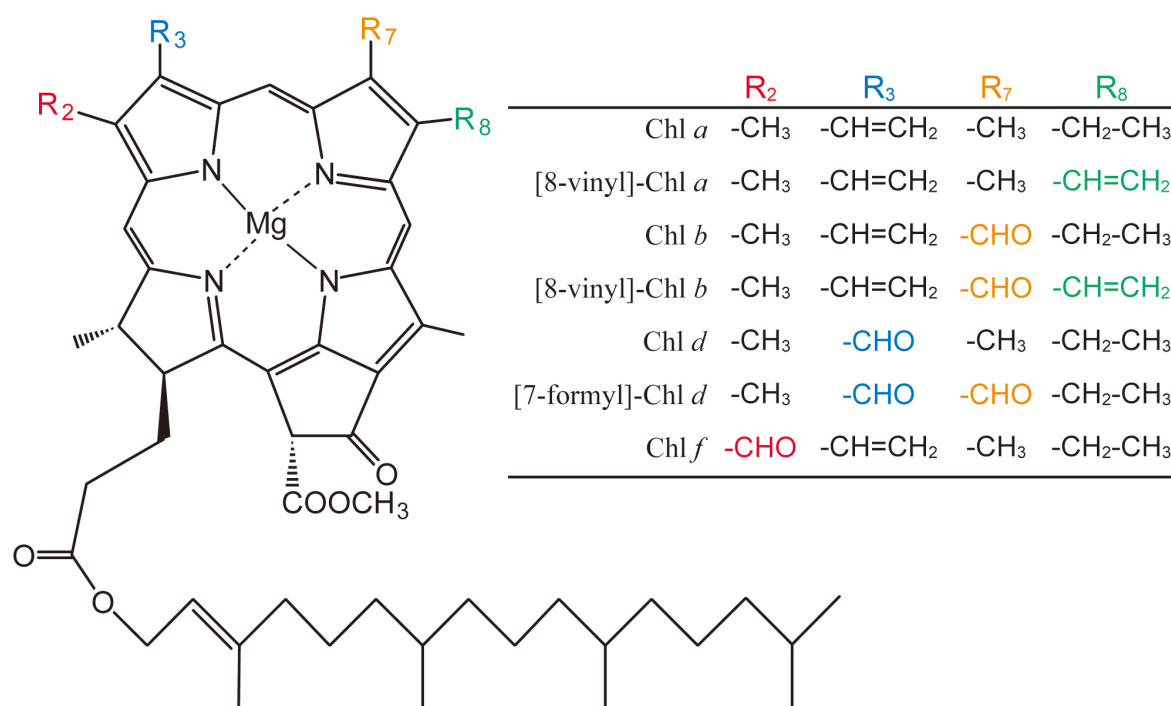


Fig. 2 Chemical structures of chlorin-type Chls. R₂, R₃, R₇ and R₈ of Chl *a* are methyl group, vinyl group, methyl group and ethyl group, respectively. R₂, R₃, R₇ and R₈ of [8-vinyl]-Chl *a* are methyl group, vinyl group, methyl group and vinyl group, respectively. R₂, R₃, R₇ and R₈ of Chl *b* are methyl group, vinyl group, formyl group and ethyl group, respectively. R₂, R₃, R₇ and R₈ of [8-vinyl]-Chl *b* are methyl group, vinyl group, formyl group and vinyl group, respectively. R₂, R₃, R₇ and R₈ of Chl *d* are methyl group, formyl group, methyl group and ethyl group, respectively. R₂, R₃, R₇ and R₈ of [7-formyl]-Chl *d* are methyl group, formyl group, formyl group and ethyl group, respectively. This Chl was artificially produced by metabolic engineering of *A. marina*. R₂, R₃, R₇ and R₈ of Chl *f* are formyl group, vinyl group, methyl group and ethyl group, respectively.

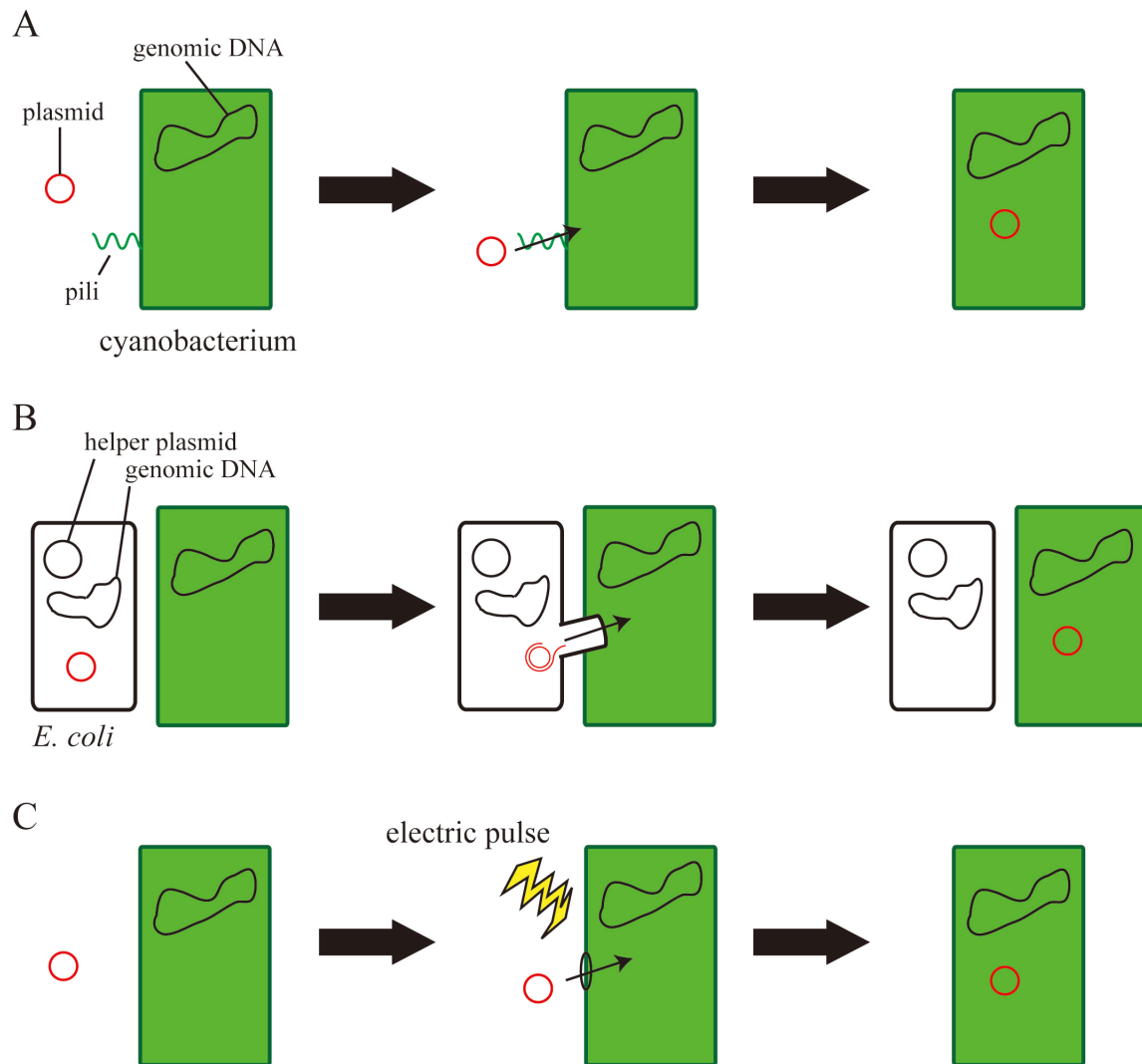


Fig. 3 Diagram of transformation methods of cyanobacteria. (A) Natural transformation. Some cyanobacteria naturally take up exogenous DNA including plasmid by pili when cyanobacteria and plasmids mixed. Consequently, cyanobacteria are transformed by the plasmid. (B) Conjugation. The plasmid is transferred from *E. coli* to cyanobacteria by conjugal gene transfer. *E. coli* harbors the plasmids and the helper plasmids. (C) Electroporation. An electric pulse is applied to cyanobacteria in order to make temporary pores in the cell membrane, and the plasmid can be transferred into cyanobacteria thorough the holes.

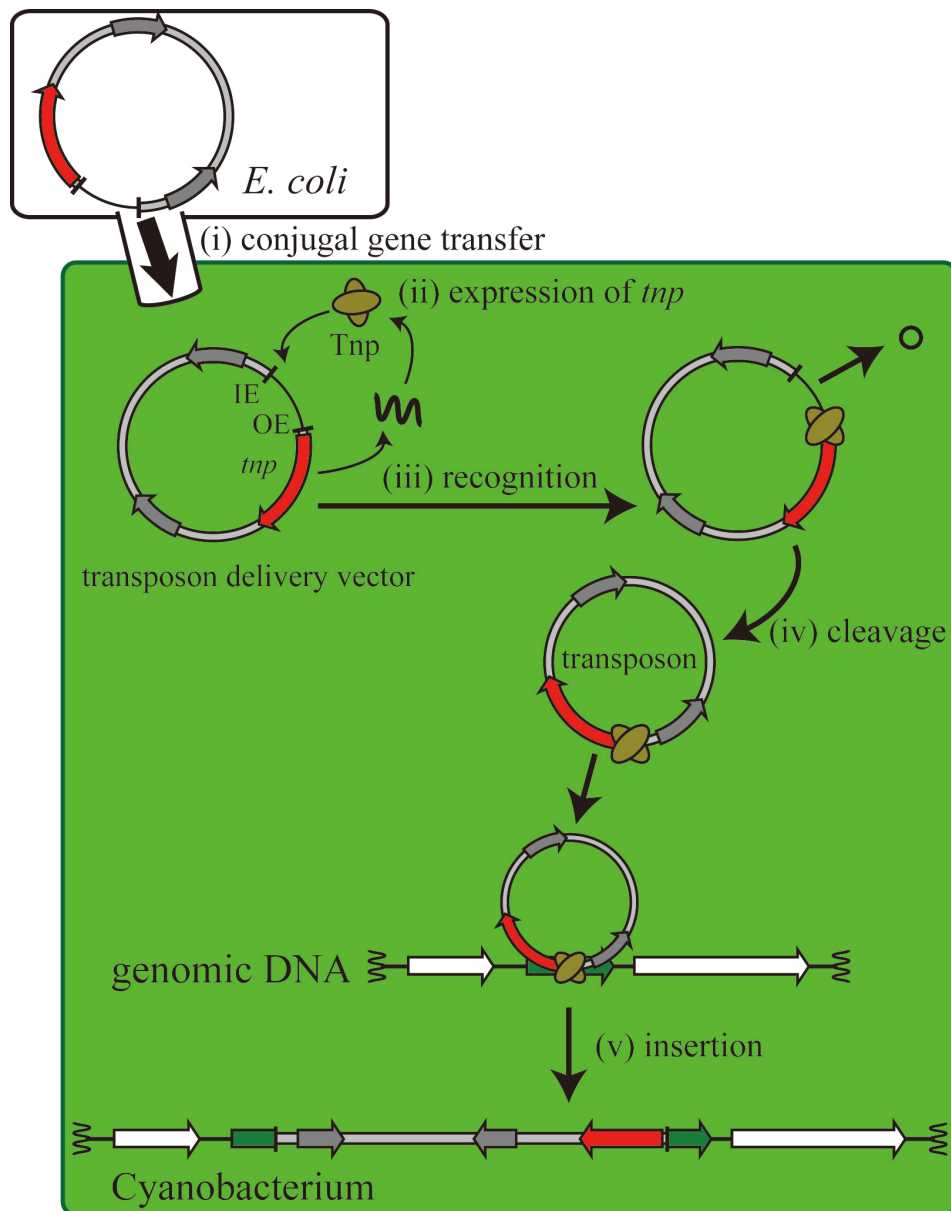


Fig. 4 Diagram of *in vivo* transposon mutagenesis in cyanobacteria. A transposon delivery vector is introduced into cyanobacterial cell by conjugal gene transfer. The transposase gene (*tnp*) in the vector is expressed. Transposase (Tnp) recognizes and interacts with two of 19-bp sequences (IE, inside end; OE, outside end) situated at either end of transposon, and cut out DNA between IE and OE as a Tnp-transposon complex from vector. The transposon is inserted into genome DNA without high sequence specificity at the insertion site.

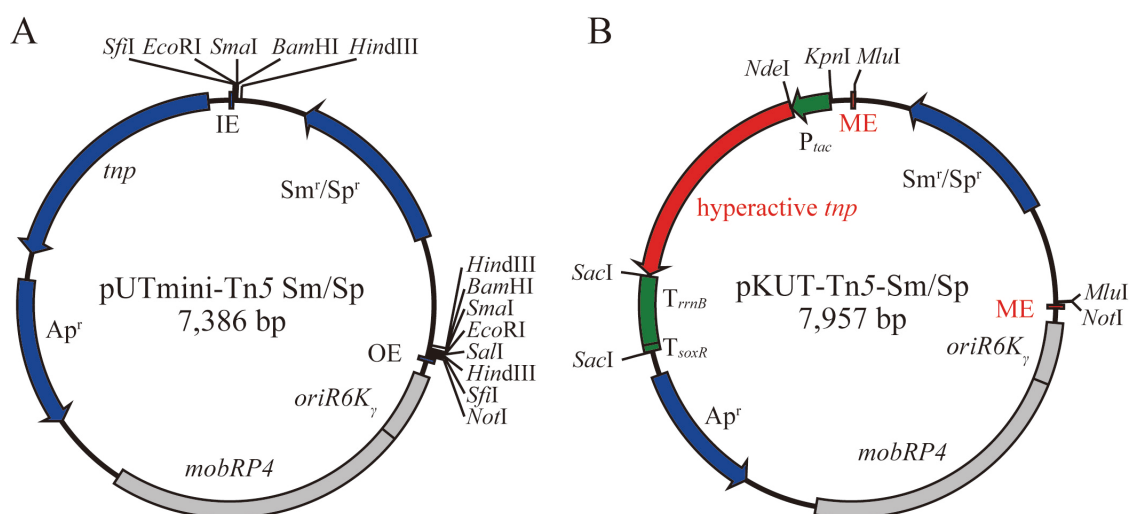
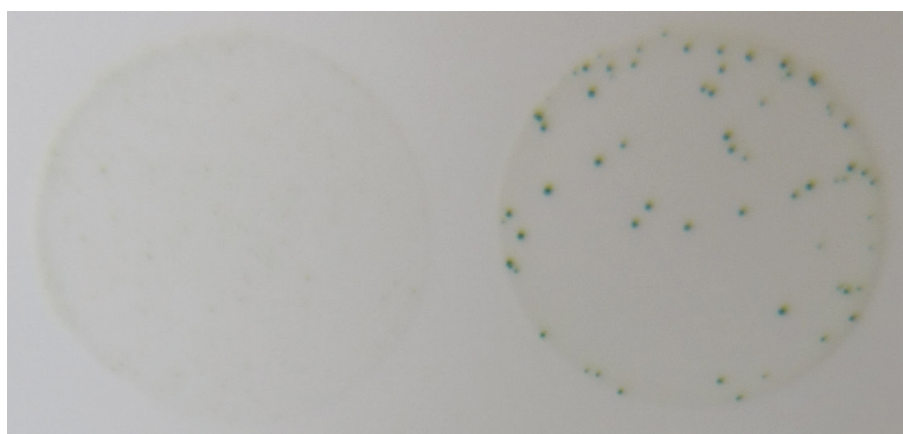


Fig. 5 Transposon delivery vectors before and after improvement. (A) Original transposon delivery vector, pUTmini-Tn5 Sm/Sp. (B) Improved transposon delivery vector, pKUT-Tn5-Sm/Sp. Sm^r/Sp^r, gene for streptomycin and spectinomycin resistance; Ap^r, gene for ampicillin resistance; *oriR6K_γ*, origin of vegetative DNA replication; *mobRP4*, origin of conjugal DNA transfer; *tnp*, Tn5 transposase gene; IE, inside end; OE, outside end; P_{tac}, *tac* promoter; hyperactive *tnp*, hyperactive Tn5 transposase gene; T_{rrnB}, *rrnB* terminator; T_{soxR}, *soxR* terminator; ME, mosaic end. Red characters indicate the improved sequence to enhance the transposition of Tn5.



pUTmini-Tn5 Sm/Sp

pKUT-Tn5-Sm/Sp

Fig. 6 Difference in the formation of Sp-resistant colonies after conjugation using transposon delivery vectors before and after improvement. Transposon delivery vectors, pUTmini-Tn5 Sm/Sp and pKUT-Tn5-Sm/Sp, were independently introduced into *Synechocystis* by conjugal gene transfer under the same conditions. In the case of transformation with pUTmini-Tn5 Sm/Sp, no Sp-resistant colony appeared (left). In contrast, using pKUT-Tn5-Sm/Sp, Sp-resistant colonies were obtained (right).

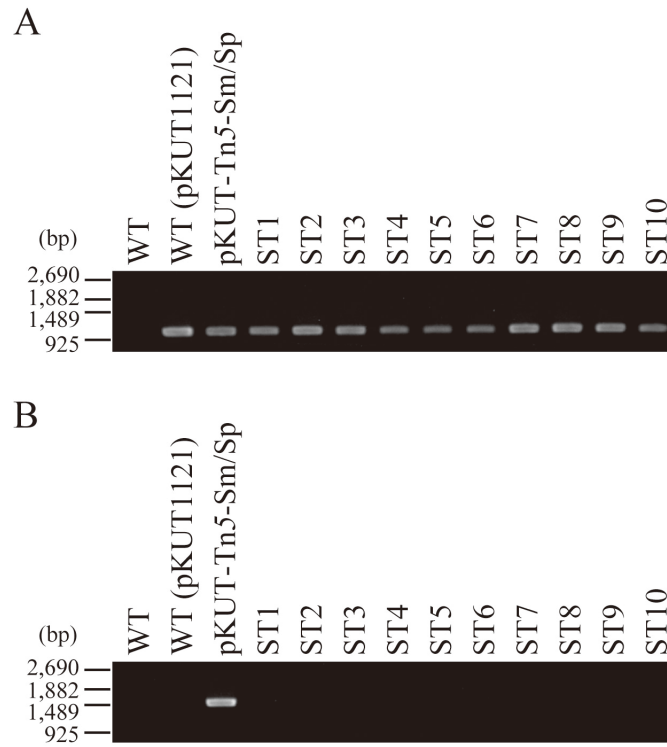


Fig. 7 Confirmation of the production of *Synechocystis* transformants. Total DNA was prepared from WT, WT (pKUT1121) and Sp-resistant exconjugants (ST1–ST10 mutants) obtained by conjugation using pKUT-Tn5-Sm/Sp. pKUT-Tn5-Sm/Sp prepared from *E. coli* was utilized as a positive control. The coding regions of two genes [*aadA* (A) and hyperactive *tnp* (B)] were amplified by PCR using total DNA and the vector as templates.

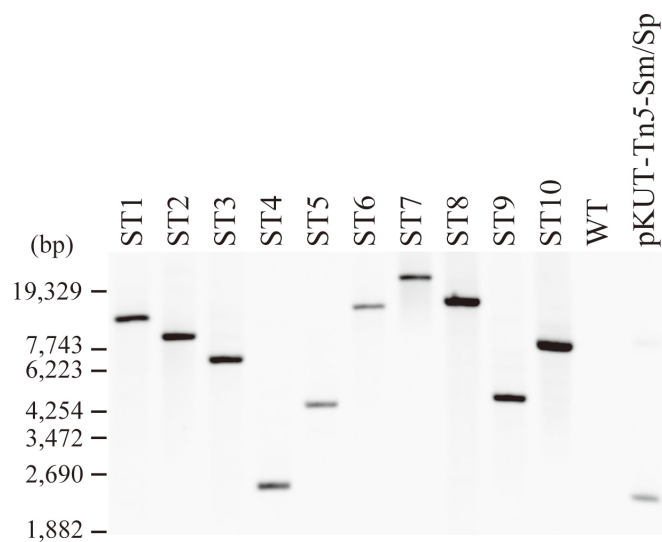


Fig. 8 Southern blot analysis of the ST1–ST10 mutants. Total DNA was prepared from the WT and the ST1–ST10 mutants. pKUT-Tn5-Sm/Sp was utilized as a positive control. DNA was digested with *Hind*III, and restriction fragments containing the transposon were detected by a DNA probe corresponding to the coding region of *aadA*.

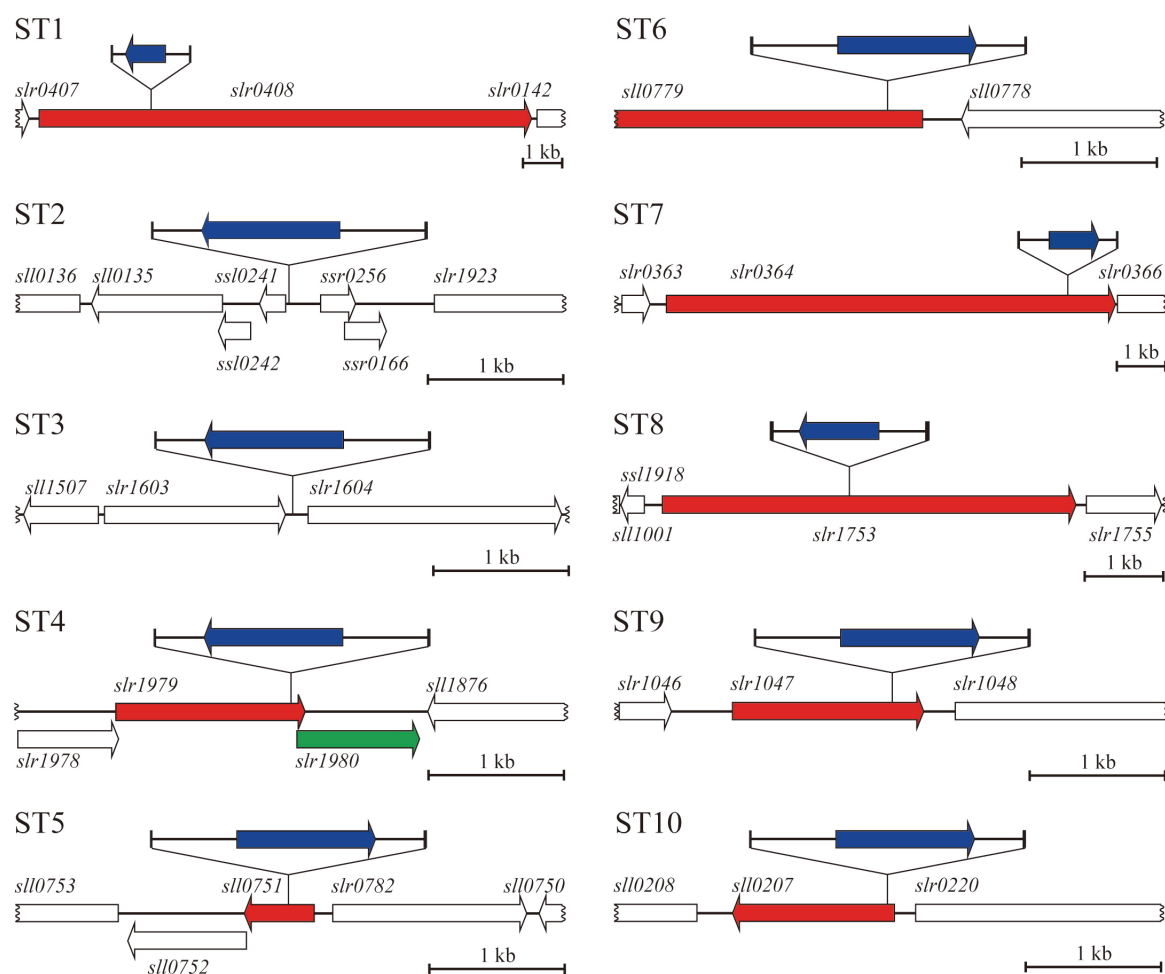


Fig. 9 Detailed transposon insertion sites of the ST1–ST10 mutants. The mosaic end and *aadA* are represented by black bars and blue arrows, respectively. Red arrows represent genes into which transposon is inserted. In the ST4 mutant, green arrows indicate *slr1980*, which is downstream of *slr1979*.

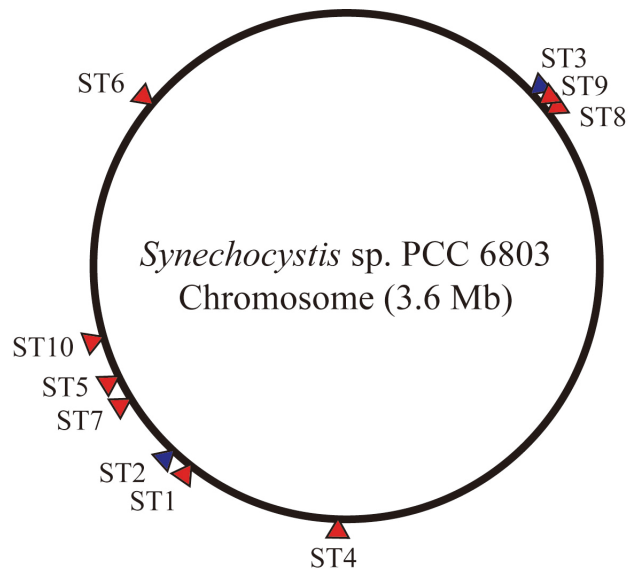


Fig. 10 Position of the transposon insertion sites in the chromosome of the ST1–ST10 mutants. The map of the *Synechocystis* chromosome with transposon insertion sites. Transposon insertion sites within predicted genes and intergenic regions are indicated by red and blue triangles, respectively.

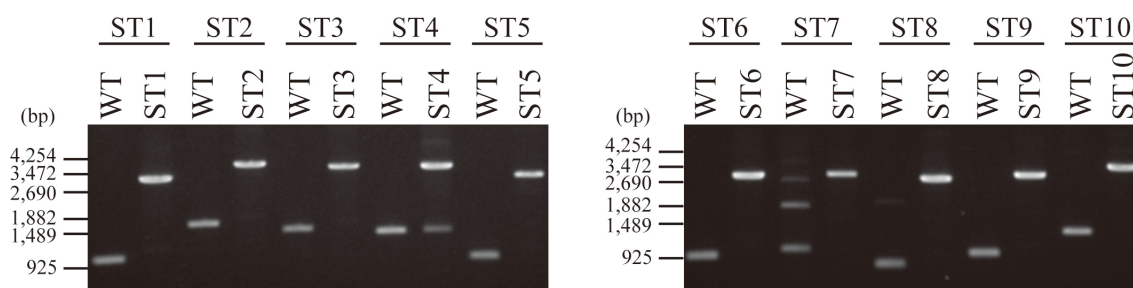


Fig. 11 Confirmation of the segregation of the transposon-inserted chromosome in the ST1–ST10 mutants. The genomic region that contained the transposon in each mutant was amplified by PCR. Three bands observed in the WT (ST7 amplification) are caused by the highly conserved tandem repeats to which the forward primer for ST7 can bind.

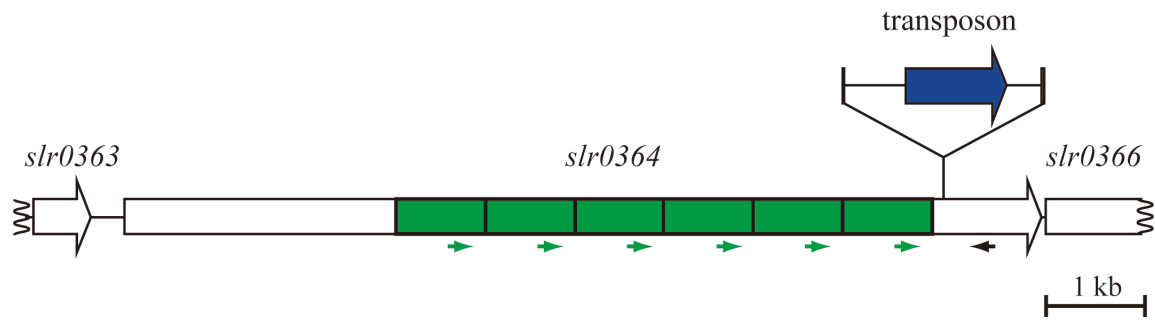


Fig. 12 Transposon insertion site and its upstream repeats in the ST7 mutant. Six green boxes represent tandem repeats with high conservation. Green arrows and a black arrow are the potential primer binding sites of forward primer (ST7Fw) and reverse primer (ST7Rv), respectively.

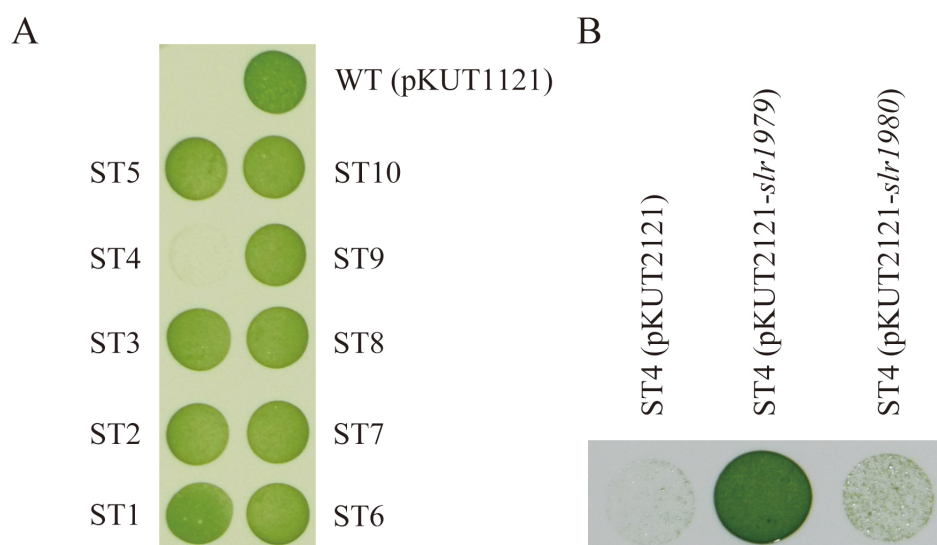


Fig. 13 Analysis of the ST4 mutant. (A) Comparison of photoautotrophic growth among transposon-tagged mutants. Liquid culture (10 μ l each, adjusted to the same optical density at 730 nm) of the transposon-tagged mutants (ST1–ST10 mutants) and the WT (pKUT1121) was spotted onto a nitrocellulose membrane on BG11 solid medium containing 5 μ g ml⁻¹ Sp. Cells were grown under photoautotrophic conditions; the light intensity was 25 μ mol photons m⁻² s⁻¹. (B) Genetic complementation of the ST4 mutant. Liquid culture (10 μ l each, adjusted to the same optical density at 730 nm) of transformants [ST4 (pKUT2121), ST4 (pKUT2121-*slr1979*) and ST4 (pKUT2121-*slr1980*)] were spotted onto a nitrocellulose membrane on BG11 solid medium containing 5 μ g ml⁻¹ Sp and 5 μ g ml⁻¹ Km. Cells were grown under photoautotrophic conditions; the light intensity was 25 μ mol photons m⁻² s⁻¹.

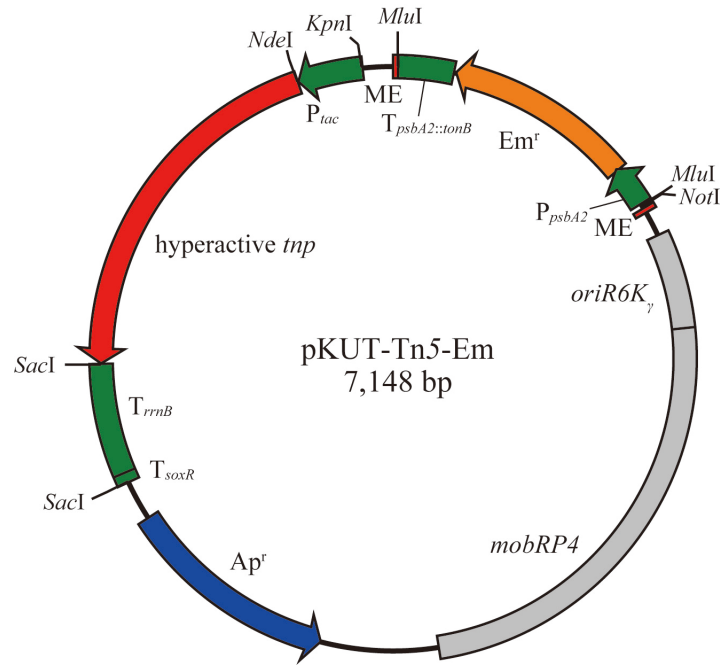


Fig. 14 Transposon delivery vectors that were introduced into *A. marina*. *Em^r*, gene for erythromycin resistance; *Ap^r*, gene for ampicillin resistance; *oriR6K_γ*, origin of vegetative DNA replication; *mobRP4*, origin of conjugal DNA transfer; *P_{tac}*, *tac* promoter; *P_{psbA2}*, upstream region (170 bp) of the *psbA2* gene of *Synechocystis* (*slr1311*); hyperactive *tnp*, hyperactive Tn5 transposase gene; *T_{rrnB}*, *rrnB* terminator; *T_{soxR}*, *soxR* terminator; *T_{psbA2::tonB}*, tandemly-arrayed terminator [downstream region (180 bp) of *slr1311* and *tonB* terminator]; ME, mosaic end.

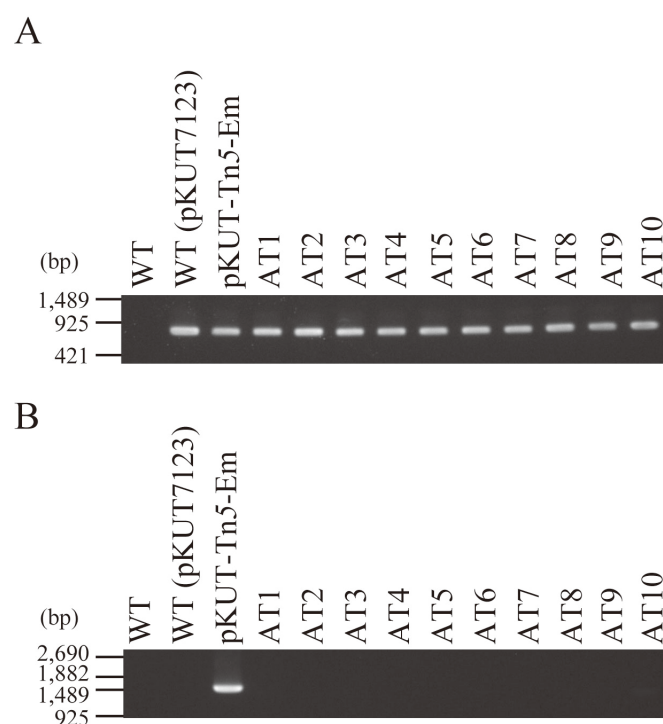


Fig. 15 Confirmation of the production of *A. marina* transformants. Total DNA was prepared from WT, WT (pKUT7123) and Em-resistant exconjugants (AT1–AT10 mutants) obtained by conjugation using pKUT-Tn5-Em. The pKUT-Tn5-Em prepared from *E. coli* was the positive control. Coding regions of two genes, the Em resistance gene (A) and hyperactive *tnp* (B), were amplified by PCR using total DNA and the plasmid as templates.

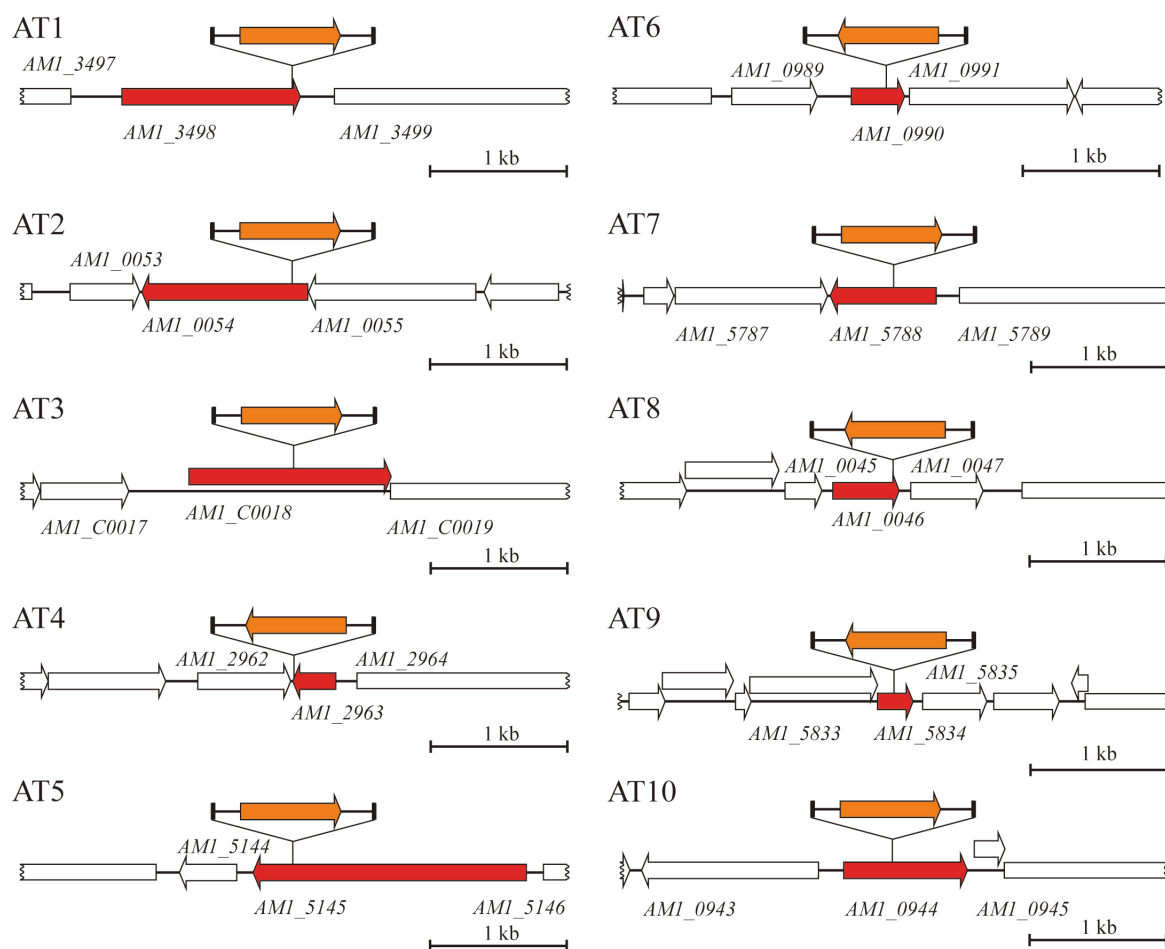


Fig. 16 Detailed transposon insertion sites of the AT1–AT10 mutants. The mosaic end and Em resistance gene inside the transposon are shown by black bars and orange arrows, respectively. Red arrows represent genes into which transposon is inserted.

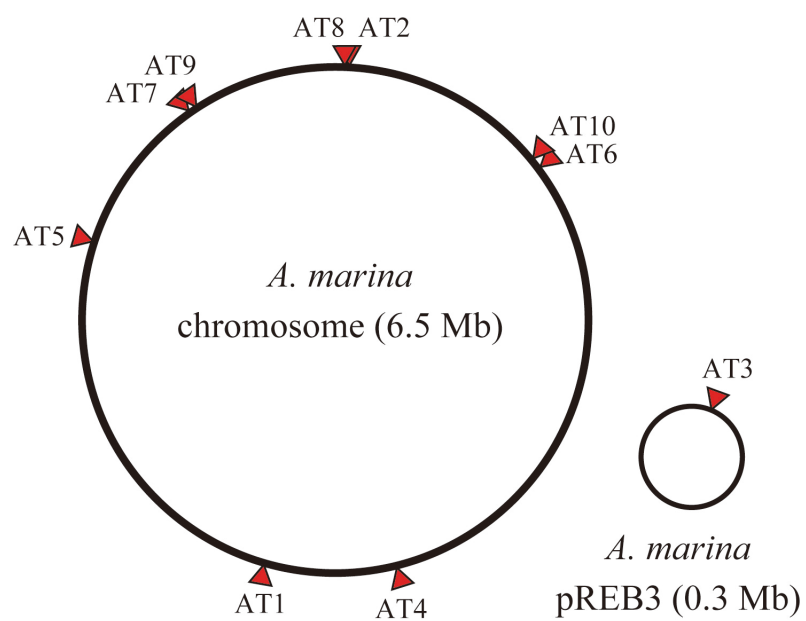


Fig. 17 Position of the transposon insertion site in the genome of the AT1–AT10 mutant. The map of *A. marina* genome with transposon insertion sites. Transposon insertion sites are indicated by red triangles. The pREB3 is one of the nine endogenous plasmids of *A. marina*.

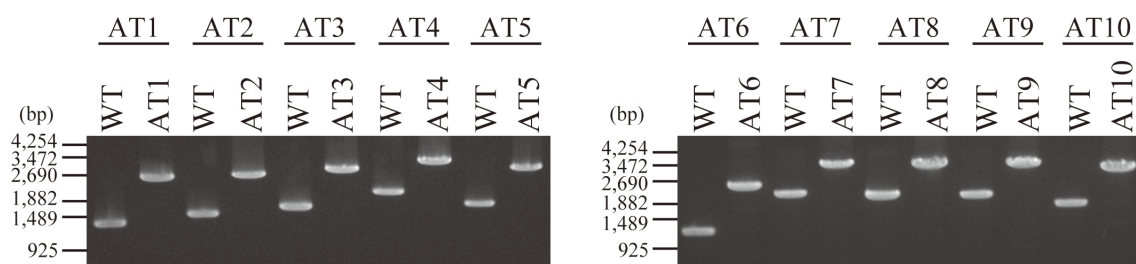


Fig. 18 Confirmation of the segregation of transposon-inserted chromosome in the AT1–AT10 mutants. The genomic region that contained transposon of each mutant was amplified by PCR.

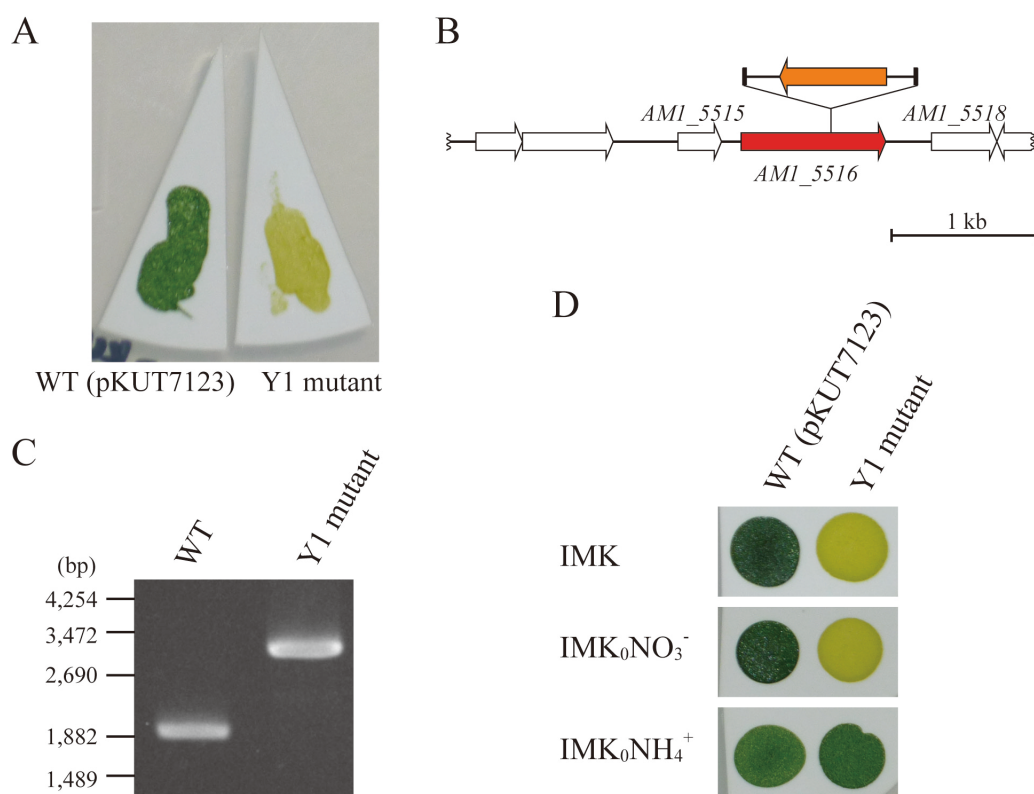


Fig. 19 Analysis of the Y1 mutant. (A) Comparison of growth between the Y1 mutant and WT (pKUT7123). The mutants were grown on a nitrocellulose membrane on IMK solid medium containing $1 \mu\text{g ml}^{-1}$ Em. (B) Position of the transposon insertion site in the chromosome of the Y1 mutant. The mosaic end and Em resistance gene inside the transposon are shown by black bars and orange arrow, respectively. Red arrow shows *AMI_5516* into which the transposon is inserted. (C) Confirmation of the segregation of transposon-inserted chromosome in the Y1 mutant. The genomic region containing the transposon was amplified by PCR. (D) Comparison of photoautotrophic growth between the Y1 mutant and WT (pKUT7123). Liquid cultures ($15 \mu\text{l}$ each, adjusted to the same optical density at 800 nm) of transformants were spotted onto a nitrocellulose membrane on three media (IMK, IMK₀NO₃⁻, IMK₀NH₄⁺) containing $1 \mu\text{g ml}^{-1}$ Em. The transformants were photosynthetically cultured under continuous illumination of incandescent light ($5 \mu\text{mol photons m}^{-2} \text{s}^{-1}$).

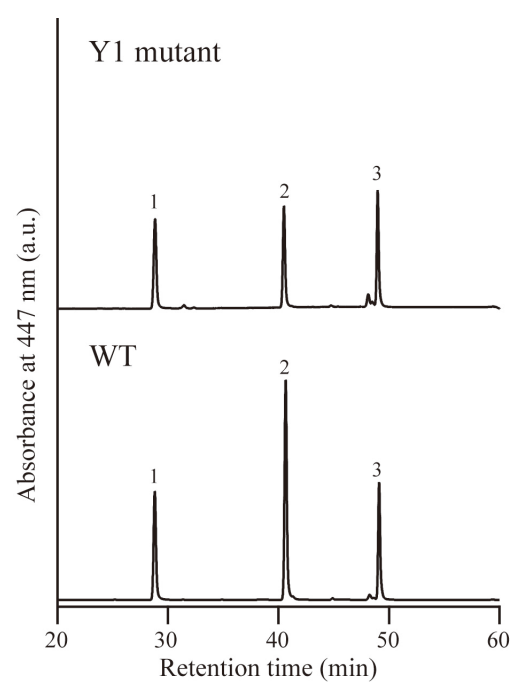


Fig. 20 Analyses of pigment compositions by HPLC. HPLC chromatogram of pigment extracts from the Y1 mutant and WT monitored at 447 nm, which is the maximum-absorption wavelength of α -carotene. 1, zeaxanthin; 2, Chl *d*; 3, α -carotene. a.u. arbitrary unit.

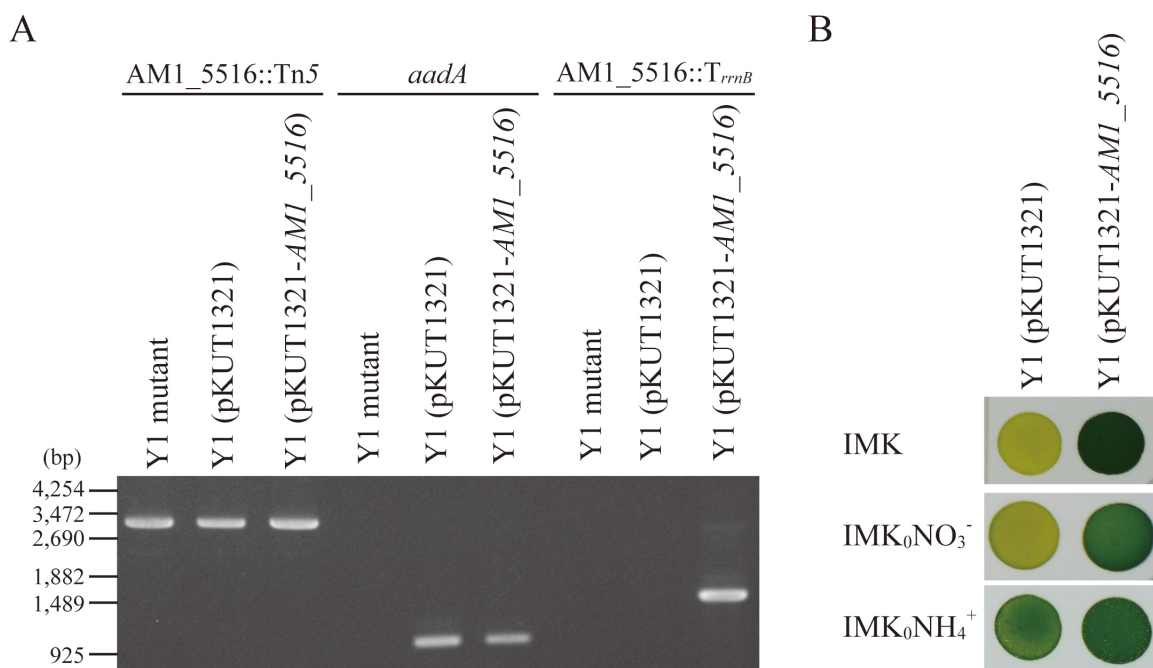


Fig. 21 Genetic complementation of the Y1 mutant. (A) Confirmation of the production of the Y1 mutant harboring the expression vectors. Three fragments, the genomic region containing the transposon (*AM1_5516::Tn5*), Sm/Sp resistance gene (*aadA*) and *AM1_5516* tandem-fused with *T_{rrnB}* (*AM1_5516::T_{rrnB}*) were amplified by PCR. (B) Comparison of photoautotrophic growth between the Y1 (pKUT1321-*AM1_5516*) and the Y1 (pKUT1321). Liquid cultures (15 μ l each, adjusted to the same optical density at 800 nm) of transformants were spotted onto a nitrocellulose membrane on three media (IMK, IMK₀NO₃⁻, IMK₀NH₄⁺) containing 1 μ g ml⁻¹ Em and 5 μ g ml⁻¹ Sp. The transformants were photosynthetically cultured under continuous illumination of incandescent light (5 μ mol photons m⁻² s⁻¹).

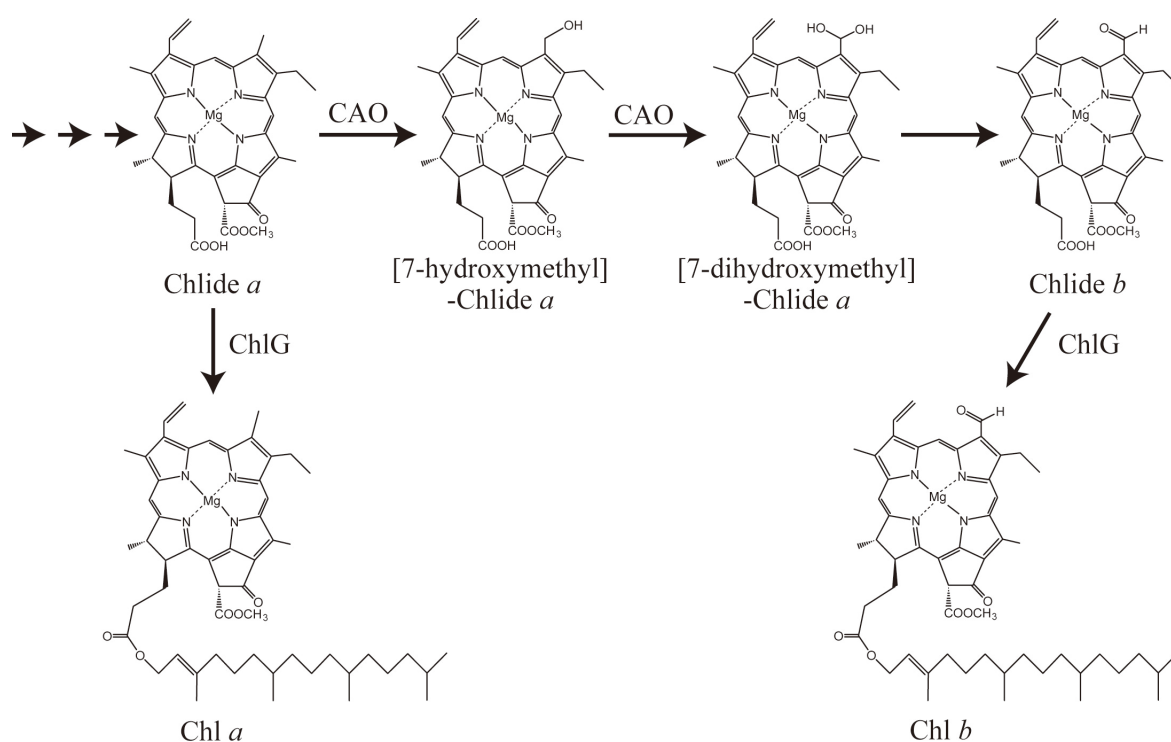


Fig. 22 Biosynthesis pathway of Chl *a* and Chl *b*. Chl *a* is synthesized by addition of a phytyl group to Chlide *a* catalyzed by chlorophyll synthase (ChlG). In Chl *b* biosynthesis, Chlide *a* is converted into Chlide *b* by two successive hydroxylations catalyzed by CAO, and spontaneous dehydration of the dihydroxymethyl group at C-7 position occurs. A phytyl group is added to Chlide *b* by ChlG to yield Chl *b*.

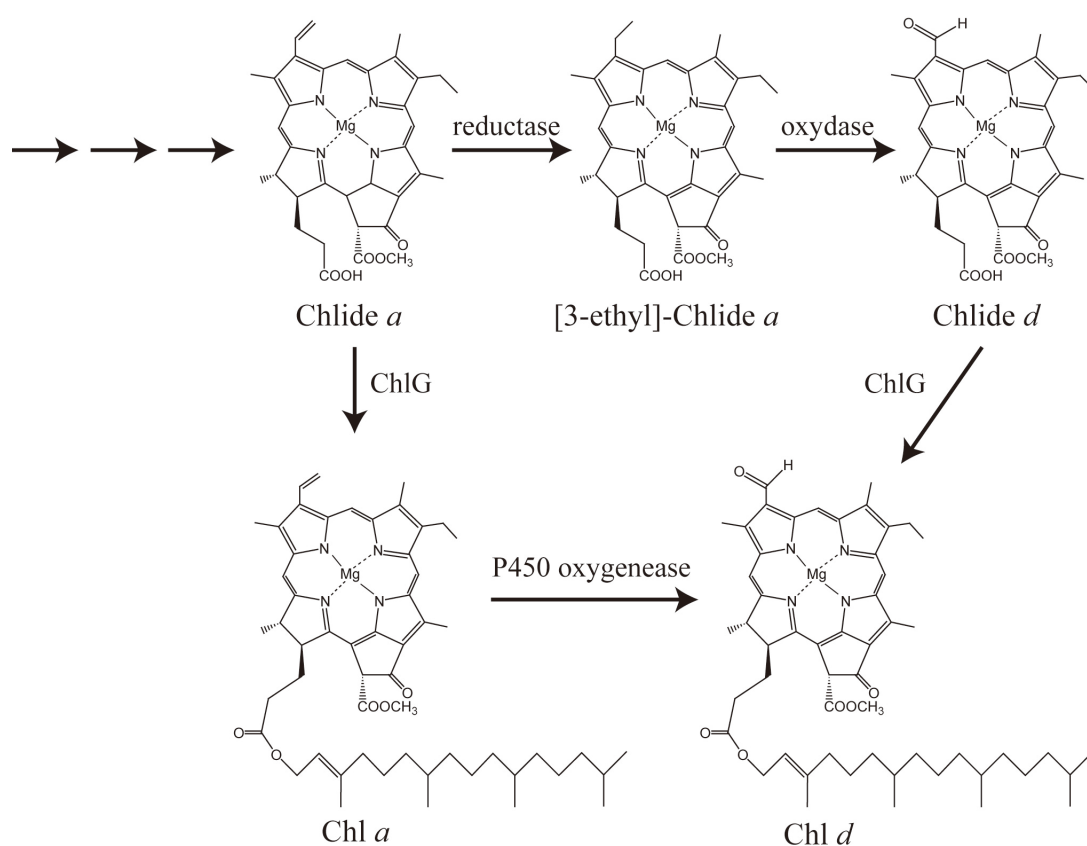


Fig. 23 Prospective biosynthesis pathway of Chl *d*. Chen et al. proposed that P450 oxygenase catalyzed the conversion of Chl *a* to Chl *d*. On the other hand, I suggest another possible pathway. The vinyl group at C-3 position of Chlide *a* is reduced by a reductase, and yields [3-ethyl]-Chlide *a*. [3-ethyl]-Chlide *a* is converted into Chlide *d* by an oxydase catalyzing addition of molecular oxygen. A phytyl group is added to Chlide *d* by ChlG to yield Chl *d*.