

# Application of the FLP/FRT recombination system in cyanobacteria for construction of markerless mutants

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**Abstract** Due to efficient photosynthetic capability, robust growth, and clear genetic background, cyanobacteria are recently used for production of different biofuel and biochemical molecules by genetic engineering and showed great potentials as the next-generation microbial cell factory. For improving the production of bio-products, a number of genetic modifications are important for cyanobacteria. However, the system-level genetic modification of cyanobacteria is limited by the lack of efficient method for marker recycling. In this investigation, we introduced the self-replicable shutter vectors harboring the flipase (FLP) gene from *Saccharomyces cerevisiae* into two mutants of *Synechocystis* sp. PCC6803 and *Synechococcus elongatus* PCC7942 whose genomes were inserted by a kanamycin resistance gene with flipase recombination target (FRT) flanking, respectively. Transcriptional analysis by reverse transcription polymerase chain reaction showed that FLP gene was transcribed in both the two cyanobacterial strains. Genotyping analysis indicated that FLP performed its function in vivo in both two cyanobacterial strains, and the following DNA sequencing analysis on the targeted loci further confirmed that FLP exactly excised and ligated the two FRT sites between which a kanamycin resistance gene is located. The homozygous mutants free of the kanamycin resistance gene cassette were obtained by conditional expression of FLP and further

dilution plating. The shuttle vectors carrying the FLP gene were then lost in these mutants by growing in the absence of antibiotics and the further single colony separation. These results demonstrate that FLP/FRT recombination system is able to be applied to the construction of markerless mutant in both *Synechocystis* sp. PCC6803 and *S. elongatus* PCC7942.

**Keywords** FLP/FRT · Cyanobacteria · Markerless mutant · Marker recycling · Genetic engineering · Biofuels

## Introduction

With advantages in photosynthetic capability, fast growth and established genetic modification system, cyanobacteria, specifically *Synechocystis* sp. PCC6803 (hereafter Syn6803) and *Synechococcus elongatus* PCC7942 (hereafter Syn7942), were recently engineered to produce diverse biofuel and biochemical molecules, such as ethylene (Sakai et al. 1997; Ungerer et al. 2012), isoprene (Lindberg et al. 2009), acetone (Zhou et al. 2012), ethanol (Dexter and Fu 2009; Gao et al. 2012), 1-butanol (Lan and Liao 2011; Lan and Liao 2012), isobutanol (Varman et al. 2013), isobutyraldehyde (Atsumi et al. 2009), 2-methyl-1-butanol (Shen and Liao 2012), 2,3-butanediol (Oliver et al. 2013), fatty alcohol (Tan et al. 2011), fatty acid (Liu et al. 2011), lactic acid (Angermayr et al. 2012), and sucrose (Ducat et al. 2012; Niederholtmeyer et al. 2010). These researches showed great potentials in industrial applications. However, so far, most of these strategies for cyanobacterial biofuel and biochemical production are still under development and not economically feasible for large-scale production (Quintana et al. 2011) because of the poor yield of bio-products. The systematically genetic modification with the purpose of maximally redirecting carbon flux to the product-forming pathways is essential to achieve economically applicable titers of bio-products.

Traditionally, cyanobacteria can be genetically modified by inserting an antibiotic resistance cassette into the targeted

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locus of the genome or replacing the part of targeted DNA fragments with an antibiotic resistance marker (Golden et al. 1987; Williams 1988). Although the traditional method for genetic modification works well in the researches focusing on the effect evaluation of deletion of single, double, or triple genes, it cannot afford for the future researches on large-scale genetic engineering of cyanobacteria. It is because the traditional modification method depends on the selectable markers, and the genetic engineering of multiple gene loci will be restricted by the limited numbers of the available markers. Moreover, culturing of the biofuel-producing cyanobacteria with selectable markers using antibiotics in the industrial scale may also raise biosafety concerns from the public and increase the cost of the whole process. Thus, eliminating the selectable markers from the cyanobacterial mutants which could make the selectable marker recycling will be necessary for the large-scale genetic modification and the future industrial application of cyanobacteria.

Mutant complementation-based systems, including *recA* mutant-based in *Synechococcus* sp. PCC7002 (Akiyama et al. 2011) and *rps12* mutant-based in Syn7942 (Matsuoka et al. 2001), have been proven to be feasible for constructing cyanobacterial mutants without antibiotic resistance marker. However, *recA* mutant-based method is not suitable for marker recycling since *recA* gene marker also could not be used again after the first complementation of *recA* mutant. As to the *rps12* mutant-based method in Syn7942, tremendous efforts are needed to screen and obtain the streptomycin-resistant *rps12* gene mutant first.

The *sacB*-based negative selection system (Vermaas 1996), by using an antibiotic resistance gene as the positive selection marker and the *sacB* gene as the negative selection marker (Fig. 1a), has been applied to the construction of the marker-free mutant strains for the production of ethanol (Dexter and Fu 2009) or free fatty acids (Liu et al. 2011) in Syn6803. Although its successful application in the glucose tolerance (GT) strain of Syn6803 (Dexter and Fu 2009; Liu et al. 2011), *sacB*-based system can't be used to engineer some other sub-strains of Syn6803 that are sucrose-sensitive and are unable to grow in the presence of sucrose as indicated by Cheah et al. (2013). Very recently, a novel negative selection method based on *mazF* from *Escherichia coli*, which is the same to *sacB*-based one, except for the differences in negative selection gene and selection methods after secondary transformation, was developed for markerless genetic modification in Syn6803 (Cheah et al. 2013), and *mazF*-based method might be suitable for more cyanobacterial strains since the gene product of *mazF* that cleaves mRNA at the ACA triplet sequence (Zhang et al. 2003) acts as a general inhibitor to protein synthesis.

Different from the negative selection systems which depends on the sensitivity of host to negative selection genes, site-specific recombinase, such as FLP from *Saccharomyces*

*cerevisiae* or Cre from *E. coli* bacteriophage P1, et al., could actively catalyze recombination between two site-specific recombinase recognition sites, such as FLP recognition target (FRT) or locus of crossover in phage P1 (*loxP*) (Fig. 1b). Site-specific recombination systems could also be used for eliminating the selectable markers and have already been successfully applied in bacteria (Schweizer 2003), fungi (Kopke et al. 2010), plant (Nguyen et al. 2012), and animal (Metzger and Feil 1999). Cre-loxP system has specially been used in a filamentous cyanobacterium *Anabaena* sp. PCC7120 for generating the unmarked mutant (Zhang et al. 2007). However, the FLP/FRT recombination system has not yet been used in cyanobacteria until now. Here, we present the first application of FLP/FRT system for construction of marker-free mutants in two typical cyanobacterial strains, Syn6803 and Syn7942, which were frequently used for biofuel and biochemical production in the recent years (Machado and Atsumi 2012; Quintana et al. 2011).

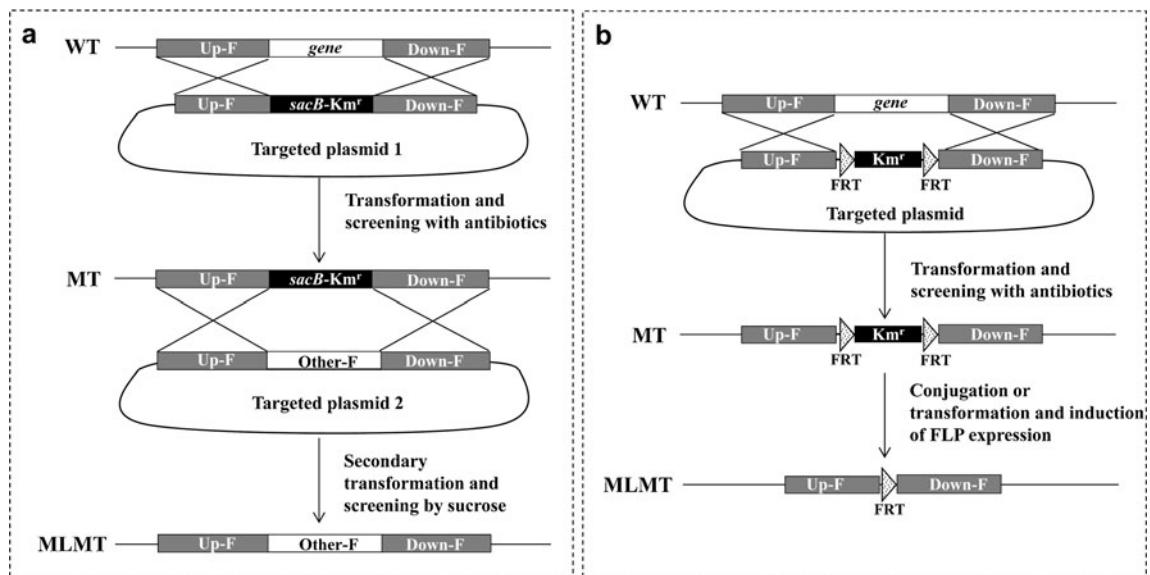
## Materials and methods

### Chemicals and reagents

All chemicals were from Ameresco (USA) or Sigma-Aldrich (USA) unless specified otherwise. Oligonucleotides synthesis and DNA sequencing were carried out by Invitrogen (Beijing, China) or Sunny (Shanghai, China). All restriction endonucleases and other tool enzymes were purchased from Fermentas (Canada) or Takara (Japan). The kits used for molecular cloning were from Omega (USA) or Takara (Japan).

### Bacteria, media, and growth conditions

*E. coli* DH5 $\alpha$  was used for molecular cloning and *E. coli* HB101 was used for conjugal transfer of plasmid DNA to cyanobacteria. All *E. coli* strains were cultured in Luria–Bertani media (Sambrook and Russell 2001) containing antibiotics at standard concentrations at 37 °C. Unless noted otherwise, Syn6803, Syn7942, and their derived strains were cultured under normal culture condition: at 30 °C under constant light at an intensity of 30–50  $\mu\text{E m}^{-2} \text{s}^{-1}$  in BG11 medium (Rippka et al. 1979) (liquid or solid) with antibiotics when needed. To induce FLP's expression in Syn6803-derived strain, cyanobacterial cells were grown under copper-limited culture condition which is the same as normal condition except that the used BG11 medium lacked copper as a microelement. To induce FLP's expression in Syn7942-derived strain, cyanobacterial cells were grown under isopropylthio- $\beta$ -galactoside (IPTG)-induced condition which is the same as normal condition except the addition of 1 mM IPTG into BG11 medium.



**Fig. 1** Schematic representation of the strategies for construction of a markerless mutant in cyanobacteria by *sacB*-based negative selection system (a) or FLP/FRT-based recombination system (b). *WT*, the wild type; *MT*, the mutant type inactivated by the DNA fragments containing FRT-flanked antibiotic resistance gene cassette; *MLMT*, the markerless mutant inactivated by the DNA fragments without drug

marker; *gene*, the targeted gene locus; *Up-F*, the upstream fragment of the targeted gene; *Down-F*, the downstream fragment of the targeted gene; *other-F*, the DNA fragments without selectable marker; *Km'*, the kanamycin-resistant gene cassette; *sacB-Km'*, the DNA fragments harboring both *sacB* and kanamycin resistance gene cassettes

### Strains and plasmids construction

Strains and plasmids used in this study are shown in Table 1, and the details are described as follows.

#### Construction of pXT206 used for *phaAB* deletion in Syn6803

The upstream and downstream fragments of *phaAB* genes encoding  $\beta$ -ketothiolase and acetoacetyl-CoA reductase with length of about 1 kb were amplified from the genome of Syn6803 by polymerase chain reaction (PCR) using primers *phaAB*-1/*phaAB*-2 and *phaAB*-3/*phaAB*-4 (Table 2) and cloned into pMD18-T vector (Takara, Japan), resulting in pKC102 and pKC103, respectively. The cloned DNA fragments were excised from pKC102 with *Nco*I and *Bam*HI and inserted into the same sites of pKC103, resulting in pKC104. The FRT-flanked kanamycin resistance gene cassette was cut from pKD4 (Datsenko and Wanner 2000) with *Nde*I and *Cla*I and blunted with T4 DNA polymerase and ligated with the blunted *Nco*I site of pKC104, resulting in pXT206.

#### Construction of pXT212 used for deletion of NS1 in Syn7942

A 2.1-kb DNA fragment containing the NS1 site was cloned from the genome of Syn7942 into pMD18-T by PCR using primers 7942-NS1-F/7942-NS1-R (Table 2), resulting in pXT176. The FRT-flanked kanamycin resistance gene

cassette was cut from pKD4 with *Nde*I and *Cla*I and blunted with T4 DNA polymerase and then ligated with the blunted *Xho*I site of pXT176, resulting in pXT212.

#### Construction of pXT218a and pXT218b used for FLP expressing in Syn7942 and Syn6803

A 0.5-kb DNA fragment containing the promoter of *petJ* and a 1.3-kb DNA fragment containing FLP gene were amplified from the genome of Syn6803 and plasmid pCP20 (Cherepanov and Wackernagel 1995) by PCR using primers PpetJ-F/PpetJ-R and Flp-F/Flp-R (Table 2), respectively. Two DNA fragments were fused by in-fusion PCR and cloned into pMD18-T, resulting in pXT208. Omega cassette was excised from pRL57 (Elhai and Wolk 1988b) with *Dra*I and inserted into the blunted *Xba*I site or *Sal*I site of pXT208, resulting in pXT217a and pXT217b, respectively. The DNA fragments containing Omega cassette and *P<sub>petJ</sub>-flp* fragments were cut from pXT217a and pXT217b with *Bam*HI and *Pst*I, blunted and inserted into the *Sma*I site of the RSF1010-derived plasmid pRL59EH (Black and Wolk 1994), resulting to pXT218a and pXT218b (Fig. 2), respectively.

#### Construction of cyanobacterial mutant strains

*phaAB* deletion mutant was constructed by recombination of pXT206 at *phaAB* locus of Syn6803 and named as 6803-XT206. NS1 insertion mutant was constructed by recombination of pXT212 at NS1 site of Syn7942 and named as

**Table 1** Strains and plasmids used in this work

Strains, plasmids		Genotype or relevant characteristics <sup>a</sup>	Source or reference
<b>Strains</b>			
Syn6803	WT	Wild type	Prof. X. Xu
	6803-XT206	$\Delta phaAB::(\text{FRT-Km}^r\text{-FRT})$	This study
	6803-XT206b1	$\Delta phaAB::(\text{FRT-Km}^r\text{-FRT}), \Omega\text{-P}_{\text{petJ}}\text{-}flp\text{-}T_{\text{rrnB}}$	This study
	6803-XT206b2	$\Delta phaAB::\text{FRT}, \Omega\text{-P}_{\text{petJ}}\text{-}flp\text{-}T_{\text{rrnB}}$	This study
	6803-XT206b3	$\Delta phaAB::\text{FRT}$	This study
Syn7942	WT	Wild type	Prof. X. Xu
	7942-XT212	$\text{NS1}::(\text{FRT-Km}^r\text{-FRT})$	This study
	7942-XT212a1	$\text{NS1}::(\text{FRT-Km}^r\text{-FRT}), \text{P}_{\text{tac}}\text{-P}_{\text{petJ}}\text{-}flp\text{-}\Omega\text{-}T_{\text{rrnB}}$	This study
	7942-XT212a2	$\text{NS1}::\text{FRT}, \text{P}_{\text{tac}}\text{-P}_{\text{petJ}}\text{-}flp\text{-}\Omega\text{-}T_{\text{rrnB}}$	This study
	7942-XT212a3	$\text{NS1}::\text{FRT}$	This study
<b>Plasmids</b>			
	pMD18-T	Cloning vector	Takara
	pKD4	$\text{Km}^r$ , source of FRT-flanked kanamycin resistance cassette	Datsenko and Wanner (2000)
	pCP20	$\text{Ap}^r$ , $\text{Cm}^r$ , source of FLP gene	Cherepanov and Wackernagel (1995)
	pRL59EH	$\text{Ap}^r$ , RSF1010 derivative, $\text{P}_{\text{tac}}$ , shuttle vector	Black and Wolk (1994)
	pRL57	$\text{Ap}^r$ , $\text{Sp}^r$ , source of Omega ( $\Omega$ ) cassette	Elhai and Wolk (1988b)
	pRL443	$\text{Ap}^r$ , $\text{Tc}^r$ , RP4 derivative, conjugal plasmid	Elhai et al. (1997)
	pRL623	$\text{Cm}^r$ , <i>M.AvaI</i> , <i>M.Eco47II</i> , <i>M.EcoT221</i> , helper plasmid	Elhai et al. (1997)
	pKC102	$\text{Ap}^r$ , 1 kb upstream fragment of <i>phaAB</i> cloned in pMD18-T	This study
	pKC103	$\text{Ap}^r$ , 1 kb downstream fragment of <i>phaAB</i> cloned in pMD18-T	This study
	pKC104	$\text{Ap}^r$ , 1 kb <i>BamHI-NcoI</i> fragment from pKC103 ligated into the same sites of pKC102, <i>phaAB</i> targeting vector	This study
	pXT176	$\text{Ap}^r$ , 2.1 kb <i>NS1</i> fragment amplified from genome of Syn7942 cloned in pMD18-T, <i>NS1</i> targeting vector	This study
	pXT206	$\text{Ap}^r$ , $\text{Km}^r$ , 1.8 kb blunted <i>ClaI-NdeI</i> fragment from pKD4 ligated into the blunted <i>NcoI</i> of pKC104, <i>phaAB</i> targeting vector	This study
	pXT208	$\text{Ap}^r$ , $\text{Km}^r$ , 1.7 kb fragment from in-fusion PCR cloned in pMD18-T	This study
	pXT212	$\text{Ap}^r$ , $\text{Km}^r$ , 1.8 kb blunted <i>ClaI-NdeI</i> fragment from pKD4 ligated into the blunted <i>XhoI</i> of pXT176, <i>NS1</i> targeting vector	This study
	pXT217a	$\text{Ap}^r$ , $\text{Sp}^r$ , 1.9 kb <i>DraI</i> fragment from pRL57 cloned into the blunted <i>XbaI</i> of pXT208	This study
	pXT217b	$\text{Ap}^r$ , $\text{Sp}^r$ , 1.9 kb <i>DraI</i> fragment from pRL57 cloned into the blunted <i>SalI</i> of pXT208	This study
	pXT218a	$\text{Ap}^r$ , $\text{Sp}^r$ , 3.6 kb blunt-ended <i>BamHI-PstI</i> fragment from pXT217a cloned into the <i>SmaI</i> site of pRL59EH, shuttle vector	This study
	pXT218b	$\text{Ap}^r$ , $\text{Sp}^r$ , 3.6 kb blunt-ended <i>BamHI-PstI</i> fragment from pXT217b cloned into the <i>SmaI</i> site of pRL59EH, shuttle vector	This study

<sup>a</sup> *Ap*, ampicillin; *Cm*, chloramphenicol; *Km*, kanamycin; *Sp*, spectinomycin; *Tc*, Tetracycline;  $\Omega$ , Omega cassette;  $\text{P}_{\text{tac}}$ , *tac* promoter;  $\text{P}_{\text{petJ}}$ , 0.5 kb fragment containing *petJ* promoter;  $T_{\text{rrnB}}$ , *rrnB* terminators T1 and T2

7942-XT212. 6803-XT206b1 and 7942-XT212a1 were constructed by conjugal transfer of pXT218b and pXT218a into the completely segregated 6803-XT206 and 7942-XT212, respectively.

Transformation of cyanobacteria and conjugal transfer of plasmid DNA to cyanobacteria

All cyanobacterial cells were grown to the exponential phase, collected by centrifugation, washed by fresh BG11

twice, and re-suspended in BG11 again to a cell density of about  $1 \times 10^9$  cells  $\text{mL}^{-1}$  for the following transformation or conjugation. The transformations of Syn6803 and Syn7942 with plasmids were performed respectively as described previously (Golden et al. 1987; Williams 1988) with modification. The cyanobacterial cell suspension was mixed with plasmid DNA to a final concentration of 10–20  $\mu\text{g mL}^{-1}$  and incubated for 4–5 h at 30 °C under light ( $30\text{--}50 \mu\text{E m}^{-2} \text{s}^{-1}$ , for Syn6803) or dark (for Syn7942). For transformation of Syn6803, the DNA–cell mixture was

**Table 2** Sequences of primers used in this work

Primers	Sequences (5'–3')	Specificity
phaAB-1	GGGGACCATCTGACTACACGG	Upstream fragment of <i>phaAB</i>
phaAB-2	TTCCAATGCCATGGGTGGGAT	Upstream fragment of <i>phaAB</i>
phaAB-3	GTAGCCATGGTAGCTATGTCACCG	Downstream fragment of <i>phaAB</i>
phaAB-4	TGTTGATGGTGGGTATCGTGGTG	Downstream fragment of <i>phaAB</i>
7942-NS1-F	TGGATGTGATCGGAACCTGA	NS1
7942-NS1-R	TCATCACTGCCACTGTCCTGC	NS1
PpetJ-F	CATCGGGGGCTGTGTTGGC	P <sub>petJ</sub>
PpetJ-R	GTGTTTTACATAATATACCAAATTGTGGCATATGTTCTCTTTCAAGGATAAAGT	P <sub>petJ</sub>
Flp-F	ACTTTATCCTTGAAAGGAGAACATATGCCACAATTTGGTATATTATGTAAAACAC	<i>flp</i>
Flp-R	TTATATGCGTCTATTTATGTAGGATGAAAGG	<i>flp</i>
Pha-c1	GGGGGGATTGTTTATTGTTGTCA	<i>phaAB</i>
Pha-c2	CCCCATTTACCCGTAATACTTCGCC	<i>phaAB</i>
7942-NS1-seq-1	GTTATCTCTCGGCTAGTGGAC	NS1
7942-NS1-seq-2	GTAGGGATTTCGCCAGATCAATG	NS1
flp-rt-F	TGTGCTGCTGAACTAACC	<i>flp</i>
flp-rt-R	GGCTTCCAGAAATTGTTGC	<i>flp</i>
6803-16S-rt-F	GCGTCCGTAGGTGGTTATG	16S rDNA
6803-16S-rt-R	GTCCCTCAGTGTCAAGTTTCAGC	16S rDNA
7942-16S-rt-F	CGCGTGAGAATCTGCCTACA	16S rDNA
7942-16S-rt-R	AGCTACTGATCGTCGCCTTG	16S rDNA

spread onto membrane filters (0.45  $\mu\text{m}$  pore size, Shanghai Xinya), resting on BG11 agar plates without antibiotic and incubated under normal culture condition (30 °C and 30–50  $\mu\text{E m}^{-2} \text{s}^{-1}$  constant light) for 18–24 h. Then, the filters were shift to the BG11 plates with 25  $\mu\text{g mL}^{-1}$  kanamycin and incubated under the same condition until the transformants appeared (about 1–2 weeks). For transformation of Syn7942, the DNA–cell mixture was spread directly onto the BG11 plates with 25  $\mu\text{g mL}^{-1}$  kanamycin and incubated further for about 1–2 weeks.

For conjugation, cargo plasmid, pXT218b or pXT218a, was firstly transformed into the *E. coli* HB101 harboring the conjugal plasmid pRL443 (Elhai et al. 1997) and the helper plasmid pRL623 (Elhai et al. 1997). Overnight culture (10 mL) of the *E. coli* strain harboring three plasmids were collected by centrifugation, washed by fresh LB medium without any antibiotics, and re-suspended in 0.5 mL of LB medium for the further conjugation. The conjugal transfer between *E. coli* and cyanobacteria was carried out according to the published procedure (Elhai and Wolk 1988a) with modification. The cell suspension of cyanobacteria were mixed with the equal volume of *E. coli* cell suspension and spread on the sterile filters (0.45  $\mu\text{m}$  pore size, Shanghai Xinya) which was layed on the solid BG11 plates without antibiotic. After incubation under normal culture condition (30 °C and 30–50  $\mu\text{E m}^{-2} \text{s}^{-1}$  constant light) for 24 h, the filters were then transferred to the selective BG11

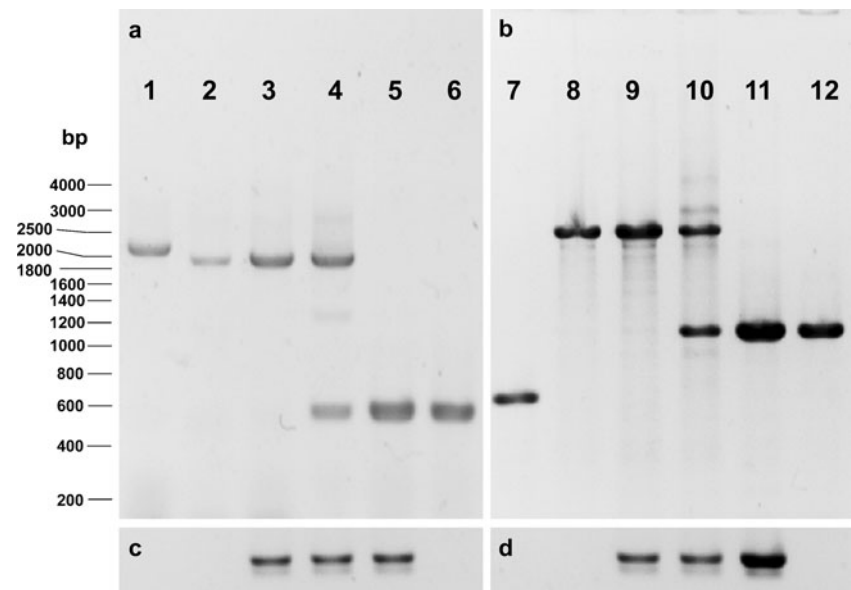
agar plates containing 20  $\mu\text{g mL}^{-1}$  spectinomycin (for Syn6803 derived strain) or 2  $\mu\text{g mL}^{-1}$  streptomycin (for Syn7942 derived strain).

After transformation or conjugal transfer, the emerging single colonies (transformants or transconjugates) were picked up and restreaked twice on the same BG11 plates. Then, the cells were inoculated into the liquid BG11 media with the corresponding antibiotics. After about 1 week of culturing, the liquid culture was reinoculated into the same liquid BG11 media at a starting OD<sub>730</sub> of 0.05. Aliquots of the liquid cultures were plated on LB agar plates to detect the contamination of *E. coli* donor or other unknown bacteria. The further dilution plating would be carried out if the cyanobacterial culture was proved to be contaminated. Only the culture proved to not to be contaminated would be used for the further DNA isolation and PCR analysis.

#### DNA isolation and genotyping of cyanobacterial strains by PCR

Liquid culture (5 mL) were centrifuged, re-suspended in 200  $\mu\text{L}$  of TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA), mixed with 50 mg of 150–212  $\mu\text{m}$  glass beads (Sigma, USA) and 200  $\mu\text{L}$  of phenol:chloroform:isoprenol (25:24:1), vortexed for 5 min, and centrifuged again. The upper phase was recovered, mixed with 1/5 volume of 5 M sodium chloride and 2 volumes of ethanol, and incubated in

**Fig. 2** Genotype analyses of a series of cyanobacterial strains. The DNA templates from the wild type of Syn6803 (lane 1), 6803-XT206 (lane 2), 6803-XT206b1 cultured under normal condition (lane 3) or copper-limited condition (lane 4), 6803-XT206b2 (lane 5), 6803-XT206b3 (lane 6), the wild type of Syn7942 (lane 7), 7942-XT212 (lane 8), 7942-XT212a1 cultured under normal condition (lane 9) or IPTG-induced condition (lane 10), 7942-XT212a2 (lane 11) and 7942-XT212a3 (lane 12) were analyzed by PCR with *phaAB* (a), NS1 (b), or *flp* (c and d) specific primers



–20 °C for more than 1 h. The total DNA was isolated by the following centrifugation, washing twice with 70 % ethanol and dissolving in 20 µL of sterile water. The isolated DNA was used as templates for genotyping of the colonies by PCR with gene-specific primers. Pha-c1/Pha-c2 (Table 2) as well as 7942-NS1-seq-1/7942-NS1-seq-2 were used as primers for examining the genotype of *phaAB* and NS1 loci, respectively. Flp-F/Flp-R (Table 2) were used for testing the existence of cargo plasmids. For genotyping of 6803-XT206b1 or 7942-XT212a1, the total DNA isolated from transconjugants together with the DNA from 6803-XT206 or 7942-XT212 were used to transform *E. coli* DH5α, and the plasmids recovered from the transformants of *E. coli* were analyzed by DNA sequencing.

RNA isolation and reverse transcription polymerase chain reaction (RT-PCR) analyses of FLP expression in cyanobacteria

For transcriptional analyses, the wild-type Syn6803 and derived 6803-XT206b1 were grown under copper-limited condition, whereas the wild-type Syn7942 and derived 7942-XT212a1 were under IPTG-induced condition. Total RNA was extracted from 50 mL cyanobacterial culture using TRIzol reagent (Invitrogen) according to manufacturer's instruction, treated with RNase-free DNase I (Takara, Japan) to eliminate contaminating chromosomal DNA, and examined by PCR using RNA as templates with primers specific to 16S rDNA. The same volume of RNA from different sample was used for reverse transcription with M-MuLV reverse transcriptase (Fermentas), followed by the normal PCR with primers specific to FLP gene (flp-rt-F/flp-rt-R) or 16S rDNA (6803-16S-rt-F/6803-16S-rt-R, 7942-16S-rt-F/7942-16S-rt-R).

Selection, segregation, and genotyping of marker-free mutants

To obtain the markerless mutants of cyanobacteria, the isolated transconjugants were firstly transferred from the normal BG11 plates to the solid BG11 medium without copper (for 6803-XT206b1) or the solid BG11 medium with 1 mM IPTG (for 7942-XT212a1) and grown for about 1 week. Then, the cells were re-suspended in the sterile deionized water, plated onto the corresponding BG11 plates, and grown for 10–14 days to obtain single colonies. Finally, the isolated single colonies were picked up and restreaked on BG11 plate with kanamycin as well as BG11 plate with spectinomycin (for 6803-XT206b1) or streptomycin (for 7942-XT212a1). The colonies which were sensitive to kanamycin but not to spectinomycin or streptomycin were positive candidates for further genotyping by PCR and further DNA sequencing. The confirmed markerless colonies were named as 6803-XT206b2 and 7942-XT212a2 and used for the following plasmid loss experiment.

Plasmid loss in cyanobacteria

6803-XT206b2 and 7942-XT212a2 cells were re-suspended in the sterile deionized water and spread onto normal BG11 plates for isolation of single colonies. The isolated colonies were streaked on BG11 plate containing kanamycin, spectinomycin (or streptomycin) as well as BG11 plates without antibiotics. The colonies sensitive to both kanamycin and spectinomycin (or streptomycin) were positive candidates for further genotyping by PCR. The confirmed markerless colonies without FLP-expressing plasmid were named as 6803-XT206b3 and 7942-XT212a3.

## Results

### Construction of starting strains and FLP-expressing plasmids

To investigate whether the FLP/FRT recombination system could function in cyanobacteria, the *phaAB* locus (Cheah et al. 2013; Gao et al. 2012) of Syn6803 and the NS1 site (Atsumi et al. 2009; Shen and Liao 2012) of Syn7942 were chosen as the targeted loci because they have been proven to be completely disrupted and usually used as the neutral sites for genetic modifications. The FRT-flanked kanamycin resistance cassette from pKD4 (Datsenko and Wanner 2000) which was also used for inactivation of genes in *E. coli* was firstly targeted into the *phaAB* locus and the NS1 site, resulting in 6803-XT206 and 7942-XT212 as the starting strains, respectively. As shown in Fig. 2, the expected PCR bands were amplified from the genomes of both 6803-XT206 and 7942-XT212 (lane 2 in panel A, lane 8 in panel B), whereas there were no PCR bands, which has the same length as the bands amplified from the wild type of Syn6803 and Syn7942, amplified from the same templates. It was indicated that both *phaAB* and NS1 loci were successfully targeted, and two mutants were segregated completely.

FLP gene (*flp*) was cloned from the temperature sensitive pCP20 (Cherepanov and Wackernagel 1995), which was also used for expression of FLP in *E. coli*. To express FLP gene in two cyanobacterial mutant strains, two shuttle vectors, pXT218b and pXT218a, were constructed as shown in Fig. S1 (supplementary material). For 6803-XT206, promoter of *petJ* gene from Syn6803 which is induced by copper limitation (Tous et al. 2001) was fused in the front of promoter-less *flp* gene, and Omega cassette (Elhai and Wolk 1988b; Prentki et al. 1991), which consists of two flanking loop-stem structures, was designed in the front of P<sub>petJ</sub> for selection and stopping the driving activities from P<sub>tac</sub> promoter, which is often used in genetic engineering of *E. coli* and has already been proven to be good at expressing heterologous genes in Syn7942 (Atsumi et al. 2009), was chosen for FLP's expression in 7942-XT212, since there was no evidence showing that P<sub>petJ</sub> from Syn6803 could drive gene expression in Syn7942. According to the published reports (Asayama 2012; Huang et al. 2010), some plasmids harboring RSF1010 origin could be used as shuttle vectors for gene expression and be successfully transferred into Syn6803 by conjugal transfer. pRL59EH (Black and Wolk 1994), containing a RSF1010 origin which makes plasmids autonomously replicating in a wide range of hosts and a bom (oriT) site which is necessary for conjugal transfer, was utilized as the shuttle vector for FLP expression in both Syn6803 and Syn7942.

### Conjugal transfer of plasmid into mutant strains

Conjugation between cyanobacteria and *E. coli* harboring pXT218b or pXT218a were performed in this work as the procedures described above. About 1–2 weeks after conjugation, transconjugates appeared on the plates. Total DNA of the spectinomycin- or streptomycin-resistant colonies were isolated and analyzed by PCR and transformation of *E. coli*. By PCR with FLP gene-specific primers (Flp-F/Flp-R), it was proven that the FLP gene was successfully transferred into two cyanobacterial mutant strains, respectively (lane 3 in panel c, lane 9 in panel d of Fig. 2). By transformation of *E. coli* with the extracted total DNA from transconjugants, ampicillin- and spectinomycin-resistant colonies of *E. coli* appeared on LB plates. The plasmids recovered from the *E. coli* transformants were partially sequenced by DNA sequencing (Fig. S2 and S3 in the supplementary material), showing the same sequences with the pXT218b or pXT218a. As controls, no colony appeared in the transformation of *E. coli* with the total DNA from the starting strains, 6803-XT206 and 7942-XT212. These results indicated that pXT218b and pXT218a were existing as the autonomously replicating plasmids in their host strains, respectively.

### Induction of *flp* gene expression in Syn6803 and Syn7942

The sterile water, cDNA from the wild-type strain, total RNA from the FLP-expressing mutant strain, and the cDNA synthesized from the total RNA were used as templates for PCR with *flp*-specific and 16S rDNA-specific primers. As blank control, there was no PCR band amplified from sterile water using either *flp*-specific or 16S rDNA-specific primers (lanes 1 and 5 in Fig. 3), showing no template contamination in the PCR reaction. Also, there was no PCR band appearing using the total RNA either from 6803-XT206b1 or 7942-XT212a1 grown under induction conditions (lanes 3 and 7 in Fig. 3), indicating that the total RNA used for cDNA preparation was not contaminated by the chromosome DNA. The *flp*-specific PCR band was only amplified from the cDNA of 6803-XT206b1 or 7942-XT212a1 cultured under induced conditions (lanes 4 and 8 in Fig. 3) and not from the cDNA from the wild-type strains under induced conditions (lanes 2 and 6 in Fig. 3). These results from RT-PCR proved that the foreign FLP was transcriptionally expressed in both 6803-XT206b1 and 7942-XT212a1 under induced conditions.

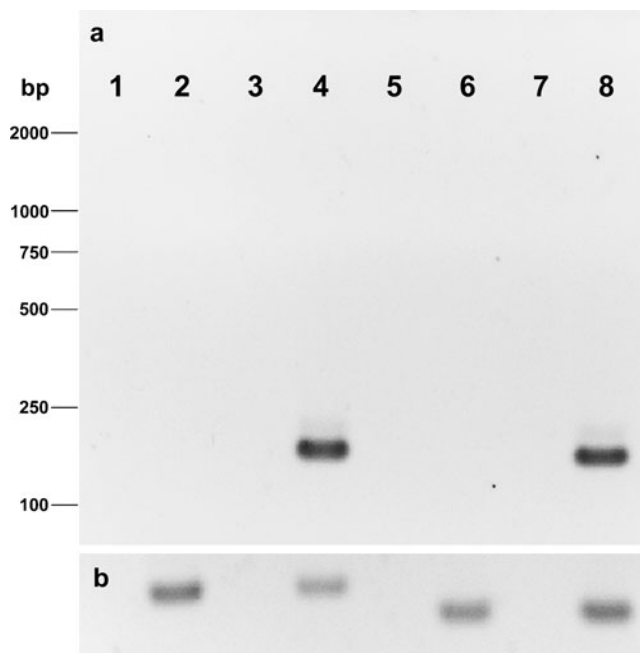
As shown in Fig. 2, the genotype of *phaAB* locus in 6803-XT206b1 was the same with that of its starting strain 6803-XT206 when both of them were grown under normal condition. However, when 6803-XT206b1 cells were transferred from normal BG11 with 0.32  $\mu$ M copper to the BG11 medium without copper, another PCR band, about 600 bp in

length, appeared besides the PCR band (MT-specific band) with the same length as the product amplified from 6803-XT206. The difference in size between the MT-specific band and the additional band is about 1,400 bp, which is close to the size of kanamycin resistance gene located between two FRT sites. The further DNA sequencing analysis (Fig. S4 in the supplementary material) of the additional PCR band showed that two FRT sites were exactly cut and ligated together, just the same as FLP-mediated recombination in *E. coli* (Datsenko and Wanner 2000). In the case of FLP expression in Syn7942, an additional experiment was performed to confirm which promoter was functional in the host cells since there are two promoters,  $P_{tac}$  and  $P_{petJ}$ , located in the front of *flp* gene (Fig. S1a in the supplementary material). By comparing the changes in genotype of NS1 locus, it was found that an expected PCR band at the length of about 1 kb appeared only when 7942-XT212a1 cells were transferred from normal BG11 to the BG11 medium with 1 mM IPTG but did not appear when cells were transferred to the BG11 medium without copper (Fig. S5 in the supplementary material and Fig. 2). It was indicated that  $P_{tac}$  worked well in Syn7942 and  $P_{petJ}$  promoter existing between  $P_{tac}$  and *flp* gene of pXT218a did not work in contrast. The further DNA sequencing analysis (Fig. S6

in the supplementary material) of this 1 kb fragment confirmed that two FRT sites in the genome of 7942-XT212a1 were also exactly edited. These results demonstrated that FLP gene was successfully driven by  $P_{petJ}$  or  $P_{tac}$  by culturing under induction conditions, and FLP could play its role to exactly eliminate the FRT-flanked kanamycin resistance gene in both 6803-XT206b1 and 7942-XT212a1 strains.

Separation of complete segregated mutant and controllable loss of shuttle plasmid

Unfortunately, the genotype of 6803-XT206b1 and 7942-XT212a1 was still heterozygous even when they have been cultured under the induction conditions (deprivation of copper or addition of IPTG) for 3–5 culturing cycles. For obtaining the completely segregated mutants, cells with heterozygous genotype were spread and selected on the BG11 plates without copper (for 6803-XT206b1) or with 1 mM IPTG (for 7942-XT212a1), respectively. One of the 12 tested single colonies was found to be sensitive to kanamycin and resistant to spectinomycin or streptomycin in the antibiotic sensitivity tests for 6803-XT206b1 cells, while one of eight was found for 7942-XT212a1 cells (Fig. S7 in the supplementary material). The PCR analyses of the selected kanamycin-sensitive colonies showed that there was only the shorted PCR band appearing (lane 5 in Fig. 2a, lane 11 in Fig. 2b), and these colonies were named as 6803-XT206b2 and 7942-XT212a2. The 6803-XT206b2 and 7942-XT212a2 cells were then cultured and spread on the normal BG11 plate without any antibiotics for separation of single colonies again. Four of twenty single colonies, sensitive to any tested antibiotics, were selected as positive candidates from the antibiotic sensitivity tests for 6803-XT206b2 cells, whereas six were selected for 7942-XT212a2 cells. As shown in Fig. 2c, d, there was no *flp*-specific PCR band amplified from the selected colonies sensitive to both kanamycin and spectinomycin or streptomycin, indicating that the shuttle vectors were completely lost in these cells. Finally, the resultant mutants, 6803-XT206b3 and 7942-XT212a3, do not contain any selectable markers.



**Fig. 3** Transcriptional analyses of expression of FLP (a) and 16S rDNA (b) in cyanobacteria strains by RT-PCR. The PCR products, amplified from sterile water (lanes 1 and 5), the cDNA from wild-type strain of Syn6803 (lane 2), the total RNA (lane 3), the cDNA (lane 4) from 6803-XT206b1, the cDNA from wild-type strain of Syn7942 (lane 6), the total RNA (lane 7), and the cDNA (lane 8) from 7942-XT212a1, respectively, were electrophoresed on agar gels and stained with ethidium bromide

## Discussion

Although natural transformation and further homologous recombination is the routine method for genetic modification in unicellular cyanobacteria that possess the high efficiency of both transformation and the homologous recombination, conjugation of the replicable plasmid carrying FLP gene into cyanobacteria was also found to be suitable for conditional expression of FLP and marker recycling. In addition, Huang et al. (2010) reported that

cargo plasmid could be transferred into Syn6803 in the absence of the helper plasmid pRL623, carrying the *M. Aval* methylase which will methylate *Aval*, II, and III target sites of cargo plasmid in order to protect cargo plasmid from the corresponding restriction enzymes' attack. However, in this work, pXT218b and pXT218a could also be conjugated and exist as the automatically replicating plasmid in Syn6803 and Syn7942, respectively, in the presence of pRL623, indicating that the helper plasmid is not fatal to the conjugation of unicellular cyanobacteria at least.

It was expected that if the FLP was functionally expressed, the heterozygous mutants would be purified under the active cutting action of FLP. However, as described above, the homozygous mutants without the kanamycin cassette could not be directly obtained by simply growing the transconjugates under the induction conditions (deprivation of copper or addition of IPTG) in the absence of kanamycin for 3–5 culturing cycles but could be obtained by the further single colony separation procedure. There might be some reasons as follows. Firstly, the expression level of FLP gene was not as high as enough to excise all the chromosomes containing kanamycin resistance gene, although FLP gene was transcriptionally expressed in both two strains under induction condition. Secondly, the chromosome substrate in the single cell is too much because most of cyanobacterial strains are polyploidy (Griese et al. 2011). Specifically, the genome copy numbers of Syn6803 (motile wild-type strain) at the exponential phase is as high as  $218.0 \pm 7.6$ , whereas that of Syn7942 is  $4.0 \pm 0.3$  (Griese et al. 2011). Thirdly, the heterozygous cells with kanamycin marker face the same selection pressure as the homozygous cells without kanamycin marker when the mixture of them was grown in the absence of kanamycin. Anyway, the results shown here indicates that the homozygous markerless mutants can be successfully separated by dilution plating, leaving the room for further improvement of FLP's expression in cyanobacteria.

In conclusion, the classic method for genetic modification that lies on the available marker genes is not suitable for large-scale genetic engineering in cyanobacteria. In this work, FLP gene from *S. cerevisiae* was transferred into the mutants of Syn6803 and Syn7942 by conjugal transfer, functionally expressed under the induction conditions, and finally removed from the marker-free mutants by plasmid loss. By doing the whole procedure, the selectable marker could be recycled and used for next round of genetic operation at another locus. The successful application of FLP/FRT recombination system in cyanobacteria for construction of markerless mutants demonstrates feasibility and potential of large-scale genetic engineering at multiple chromosome sites with multiple target genes, which is useful for building cyanobacterial cell factory to produce biofuels and biochemical with high efficiency.

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