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Pioneering precision in markerless strain development for *Synechococcus* sp. PCC 7002

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Abstract

Marine cyanobacteria such as *Picosynechococcus* sp. (formerly called *Synechococcus* sp.) PCC 7002 are promising chassis for photosynthetic production of commodity chemicals with low environmental burdens. Genetic engineering of cyanobacteria conventionally employs antibiotic resistance markers. However, limited availability of antibiotic-resistant markers is a problem for highly multigenic strain engineering. Although several markerless genetic manipulation methods have been developed for PCC 7002, they often lack versatility due to the requirement of gene disruption in the host strain. To achieve markerless transformation in *Synechococcus* sp. with no requirements for the host strain, this study developed a method in which temporarily introduces a mutated phenylalanyl-tRNA synthetase gene (*pheS*) into the genome for counter selection. Amino acid substitutions in the PheS that cause high susceptibility of PCC 7002 to the phenylalanine analog *p*-chlorophenylalanine were examined, and the combination of T261A and A303G was determined as the most suitable mutation. The mutated PheS-based selection was utilized for the markerless knockout of the *nblA* gene in PCC 7002. In addition, the genetic construct containing the *lldD* and *lldP* genes from *Escherichia coli* was introduced into the *ldhA* gene site using the counter selection strategy, resulting in a markerless recombinant strain. The repeatability of this method was demonstrated by the double markerless knockin recombinant strain, suggesting it will be a powerful tool for multigenic strain engineering of cyanobacteria.

Keywords Markerless, *Synechococcus*, Phenylalanyl-tRNA synthetase, Counter selection

Introduction

Cyanobacteria are promising photoautotrophic chassis for sustainable chemical production, and they can convert CO_2 directly into the desired compounds. Research on cyanobacteria for the production of alcohols, diols, fatty acids, terpenoids, and organic acids, which are utilized in biofuels and pharmaceuticals, has been actively pursued [1–3]. Understanding and engineering the cyanobacterial metabolism will promote the efficiency of the CO_2 conversion, remove bottlenecks, and introduce the synthetic metabolic pathways to produce chemicals using CO_2 as the sole carbon source. For example, the production of organic acids such as succinate and D-lactate were improved in *Synechocystis* sp. PCC 6803 (PCC 6803) by

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the metabolomics-based genetic engineering approach [4, 5]. The heterologous expression of the limonene synthase gene allows for the production of monoterpene limonene in *Synechococcus* sp. PCC 7002 (PCC 7002), which naturally lacks the ability to produce limonene [6]. PCC 7002 is an ideal host to develop as chassis for chemical production due to its salt, thermo, and extreme high-light tolerances [7, 8]. As a marine strain, PCC 7002 doesn't need freshwater resources, which is advantageous for mass production of low-value commodities like biofuels. These tolerances of PCC 7002 enable it to grow in open pond systems and photobioreactors even in a hot environment of about 40°C [8]. Despite these valuable properties, its potential as a chassis for engineering applications is hindered by the lack of available genetic tools. Cyanobacterial strain development is typically achieved through natural transformation, as well as conjugation and electroporation methods. Regardless of the transformation method employed, all require antibiotic resistance genes for selecting recombinants. The limited number of the available selective markers restricts the genetic manipulation and the constitutive expression of marker genes may hinder growth and production. To address these issues, markerless recombination tools have been developed. As for markerless gene recombination, the SacB-based system was reported in PCC 6803 [9], and the CRISPR-based markerless tools were also reported in PCC 6803, *Synechococcus* UTEX 2973 (UTEX 2973), and *Anabaena* sp. PCC 7120 [10–13]. In PCC 7002, Begemann MB *et al.* reported the markerless gene deletions and insertions by disrupting the *acsA* gene encoding acetyl-CoA ligase and acrylate counter selection a decade ago [14]. Additionally, Kojima K *et al.* reported the markerless counter selection using lauric acid by disrupting the *aas* gene encoding acyl-acyl carrier protein synthetase [15]. These methods require disruption of the native *acsA* and *aas* genes to select mutants and lack versatility.

To establish the chassis strain, a markerless tool is needed where gene integration sites can be freely selected without disrupting specific native genes. Because the SacB-based system, utilizing sucrose counter selection, cannot be introduced to PCC 7002 [14], and the CRISPR-based system is more complex than current markerless tools for PCC 7002, we focused on PheS as a new markerless recombination system in cyanobacteria. Our aim is to establish a straightforward and effective markerless tool. The PheS-based markerless systems have been reported in various bacteria, including *Thermus thermophilus* [16], *Bacillus* [17, 18], lactic acid bacteria [19], and *Streptococcus* species [20–22]. Its initial report was in *Burkholderia* species [23], and markerless systems using mutated PheS have been developed over the last decade. The *pheS* gene encodes the alpha subunit of phenylalanyl-tRNA synthetase, which plays a crucial role in catalyzing the aminoacylation of tRNA and the charging of $tRNA^{Phe}$ with phenylalanine. This process is essential for peptide bond formation. In *Escherichia coli*, an amino acid substitution at position A294G in the PheS enzyme enables the misincorporation of *p*-chlorophenylalanine (PCPA), a phenylalanine analog [24]. This leads to protein misfolding and either growth arrest or reduction. Miyazaki reported that mutations at positions T251A/A294G in *E. coli* PheS resulted in increased lethality in the presence of PCPA [25]. In this study, our objective was to establish a universal and straightforward markerless transformation method in PCC 7002 using mutated PheS. We aimed to delete the *nblA* gene, which encodes nonbleaching protein A, and performed markerless gene introduction at the *ldhA* gene site, which encodes lactate dehydrogenase. The newly developed markerless gene knockout and knockin method will not only help uncover the functions of unknown genes and metabolic regulations but also expedite the process, facilitating high-throughput modifications and rapid advancements in both research and production strategies.

Method

Strains and culture conditions

Cyanobacterium *Synechococcus* sp. PCC 7002 and its recombinants were cultured in MA2 medium (1.49 g/L $NaNO_3$, 50 mg/L KH_2PO_4, 18 g/L NaCl, 5 g/L $MgSO_4{\cdot}7H_2O$, 0.37 g/L $CaCl_2{\cdot}2H_2O$, 0.6 g/L KCl, 32 mg/L $Na_2EDTA{\cdot}2H_2O$, 8 mg/L $FeCl_3{\cdot}6H_2O$, 34 mg/L H_3BO_3, 4.3 mg/L $MnCl_2{\cdot}4H_2O$, 0.32 mg/L $ZnCl_2$, 50 µg/L $Na_2MoO_4{\cdot}2H_2O$, 3.0 µg/L $CuSO_4{\cdot}5H_2O$, 12 µg/L $CoCl_2{\cdot}6H_2O$, 4.0 µg/L cobalamin, and 1 g/L tris(hydroxymethyl)aminomethane) with or without antibiotics or *p*-chlorophenylalanine (PCPA) (FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan). The MA2 agar plate contained 1.5% agar. Cyanobacterial cells were cultured in 70 mL of MA2 in closed double-deck flasks with 2% (v/v) CO_2 at 30°C under a white fluorescence with continuous irradiation at 100 µmol/m^2/s under 100 rpm agitation as previously described [26]. For confirmation of transformation and segregation, transformants were cultured in MA2 medium with or without antibiotics at 30°C in cell culture plate with 2% (v/v) CO_2 at 30°C under a white fluorescence with continuous irradiation at 100 µmol/m^2/s under 100 rpm agitation. The optical density at 750 nm (OD_{750}) was measured with a UV mini spectrophotometer (Shimadzu, Kyoto, Japan), and the cell density in the medium was also determined as dry cell weight (DCW). The cyanobacterial recombinants used in this study were listed in Table 1.

Escherichia coli strain DH5α was used for gene cloning and plasmid construction. *E. coli* was grown in LB

Table 1 Recombinants of *Synechococcus* sp. PCC 7002 used in this study

Strain	Genotype	Description
Syn001	Δ*nblA*::*KmR*-wt*PheS*	Replacement of the *nblA* gene with genes for kanamycin resistance and native PheS
Syn002	Δ*nblA*::*KmR*-*PheS*^{T261A}	Replacement of the *nblA* gene with genes for kanamycin resistance and mutated PheS (T261A)
Syn003	Δ*nblA*::*KmR*-*PheS*^{A303G}	Replacement of the *nblA* gene with genes for kanamycin resistance and mutated PheS (A303G)
Syn004	Δ*nblA*::*KmR*-*PheS*$^{T261A/A303G}$	Replacement of the *nblA* gene with genes for kanamycin resistance and mutated PheS (T261A/A303G)
Syn005	Δ*nblA*	Markerless replacement of the *nblA* gene with a small fragment containing a *Nde*I site
Syn006	Δ*ldhA*::*lldD*-*lldP*	Markerless replacement of the *ldhA* gene with the *lldD* and *lldP* genes
Syn007	Δ*ldhA*::*lldD*-*lldP*, Δ*nblA*::*mNG*	Derivative strain of Syn006 with markerless replacement of the *nblA* gene with the gene for mNeonGreen

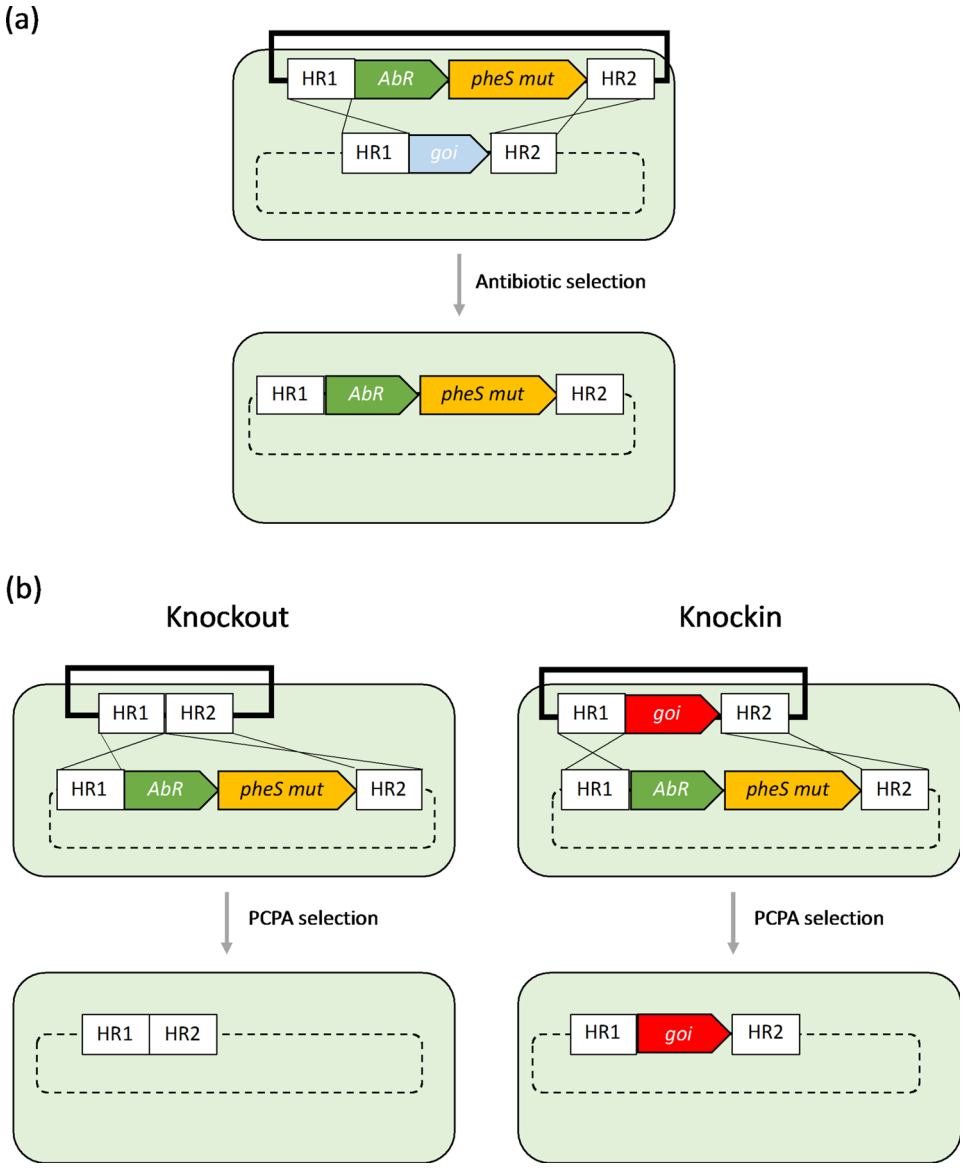

Fig. 1 Scheme for markerless gene knockout and knockin. This method consists of two double crossover steps: (**a**) First-step recombination: The DNA fragment containing the antibiotic resistant gene (*AbR*) and the mutated *pheS* (*pheS mut*) gene is integrated, deleting the gene of interest (*goi*, shown in pale-blue). The recombinants are selected with antibiotics and then subjected to the second-step recombination (**b**). (**b**) Second-step recombination: — For markerless knockout (left): The recombinants are re-transformed using the plasmids containing homologous arms (HR1 and HR2). — For markerless knockin (right): The transformants are re-transformed using plasmids containing homologous arms and *goi* (shown in red). Recombination occurs under the selection pressure of PCPA, and final recombinants are obtained through PCPA selection. During the second-step recombination, *AbR* and *pheS mut* are excised from the genome, resulting in recombinants without these markers. The recombinants still harboring *AbR* and *pheS mut* are eliminated by PCPA selection. In the diagram, rounded rectangles represent PCC 7002 cells, dotted lines indicate the native genome of PCC 7002, and bold lines represent the plasmid regions

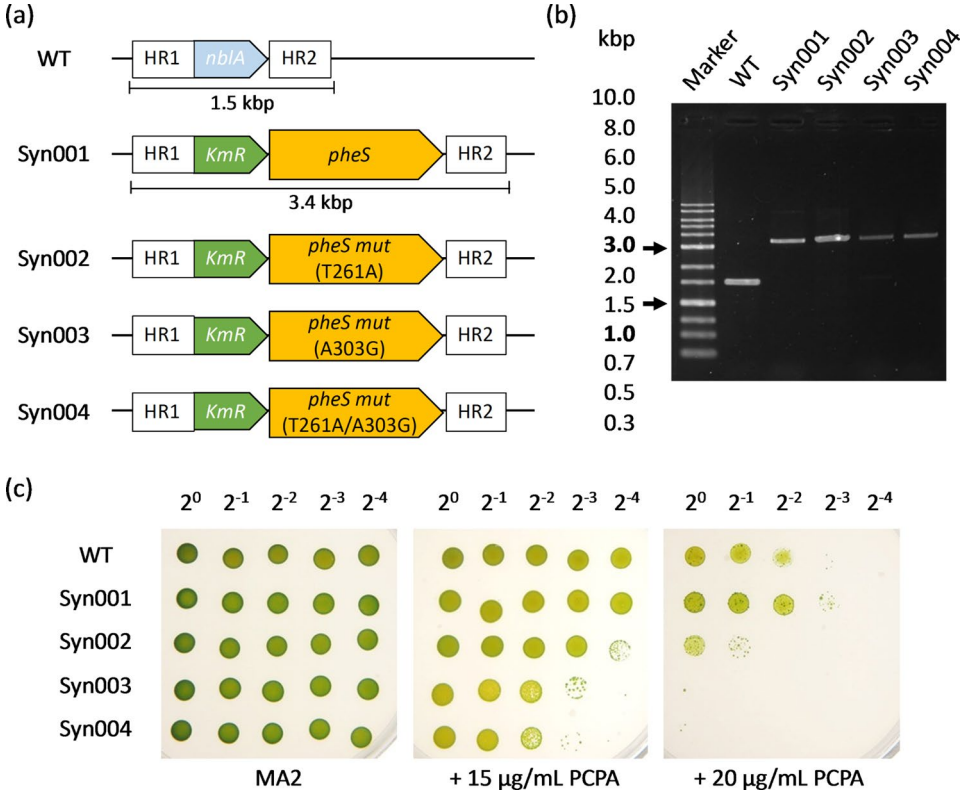

Fig. 2 PCPA tolerance of the recombinant strain. (**a**) Gene constitutions of wild-type strain (WT) and recombinant strains: The amino acid residues of threonine 261 and alanine 303 in PheS from PCC 7002 were replaced with alanine and glycine, respectively. The strains produced to investigate the suitable mutation in PheS include WT (control), Syn001 (Δ*nblA*::*KmR-wtPheS*), Syn002 (Δ*nblA*::*KmR-PheS*^{T261A}), Syn003 (Δ*nblA*::*KmR-PheS*^{A303G}), and Syn004 (Δ*nblA*::*KmR-PheS*$^{T261A/A303G}$). (**b**) Confirmation of complete segregation and deletion of the marker genes: The region from the 5′- to 3′-flanking regions of the *nblA* gene (HR1 to HR2) were amplified by PCR with the primer pairs, nblA_seq1 and nblA_seq2, using genomic DNA as templates. The PCR products were analyzed by agarose gel electrophoresis (Marker: DNA ladder). Black arrows indicate the markers shown in the bold type. The lengths of the PCR products were 1.5 kbp for wild-type (WT) and 3.4 kbp for the others, Syn001-Syn004. (**c**) Determination of the suitable mutation and proper PCPA concentration: The WT and recombinant strains were cultivated in MA2 medium. PCPA tolerance was assayed by spot culture. Cell cultures in a log phase were adjusted to OD_{750} = 1.0, and serially diluted by half until 2^{-4} dilutions. The culture of OD_{750} = 1.0 and half-dilution series were spotted onto agar plates with or without PCPA and incubated under white fluorescent lamps at 100 µmol photons/m^2/s and 30°C. Since PCPA-susceptible strains cannot grow under the presence of PCPA and therefore the spot will not appear, the incubation time was extended to 11 days to investigate the concentration at which cells cannot grow

medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) at 37°C under 200 rpm agitation.

Chemicals, enzymes, and molecular biology kits

PCPA, kanamycin monosulfate, and gentamicin sulfate (Nacalai Tesque, Kyoto, Japan) were used for mutant selection, DNA polymerase for polymerase chain reaction (PCR) and In-Fusion Snap Assembly Master Mix were purchased from TOYOBO CO., LTD. (Osaka, Japan) and TaKaRa Bio Inc. (Shiga, Japan), respectively. Restriction enzymes were purchased from New England Biolabs (Ipswich, MA, USA). DNA purification was performed using Wizard® Plus SV Miniprep DNA Purification Systems and Wizard® SV Gel and PCR Clean-Up Systems (Promega Corporation, Madison, WI, USA).

Plasmid construction

The plasmid pUC118-KmR-PheS was constructed by assembling the linearized pUC118 by double digestion of *Eco*RI and *Sma*I, the kanamycin resistance gene, a J23119 promoter (http://parts.igem.org/Part:BBa_J23119), the *pheS* gene, and the upstream and downstream regions of the *nblA* gene (Gene ID: SYNPCC7002_A1821) via In-Fusion cloning. The mutated *pheS* genes, encoding the α-subunit of phenylalanyl-tRNA synthetase (PheST261A, PheSA303G, PheST261A/A303G), were generated by PCR from pUC118-KmR-PheS. The PCR products amplified with PheS_T261A_F and PheS_T261A_R, and PheS_A303G_F and PheS_A303G_R were self-ligated by In-Fusion cloning, resulted in pUC118-KmR-PheS_T261A and pUC118-KmR-PheS_A303G, respectively. The plasmid, pUC118-KmR-PheS_T261A/A303G, was constructed by self-ligation of the PCR product amplified from pUC118-KmR-PheS_T261A using primers

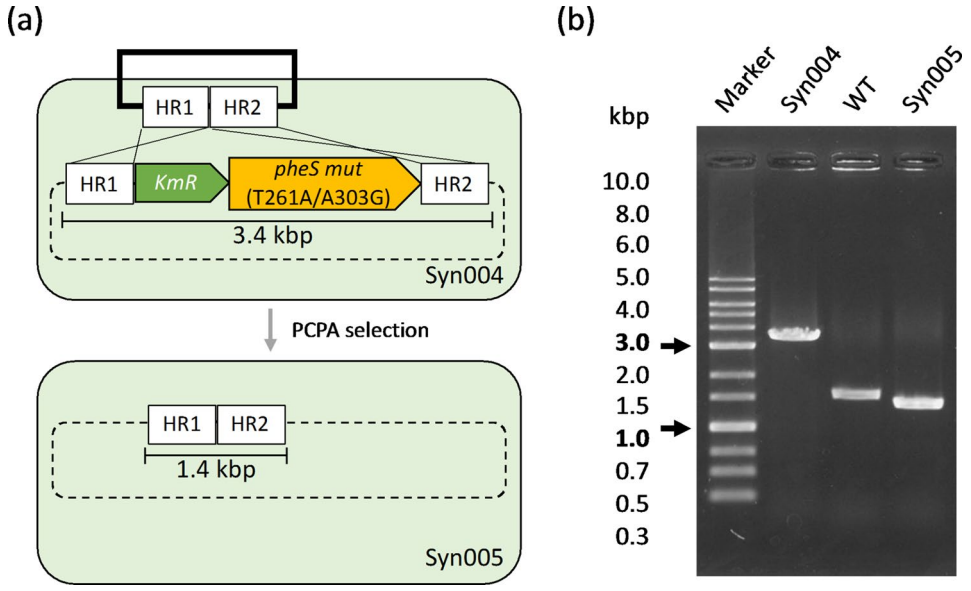

Fig. 3 Markerless *nblA* gene knockout. (**a**) Recombination scheme for Syn004 with pUC1118-nblA-KO: In the presence of PCPA, recombination occurs, leading to the elimination of the marker genes. (**b**) Confirmation of complete segregation and deletion of the marker genes: The region from the 5′- to 3′-flanking regions of the *nblA* gene (HR1 to HR2) were amplified by PCR with the primer pairs, nblA_seq1 and nblA_seq2, using genomic DNA as templates. The PCR products were analyzed by agarose gel electrophoresis (Marker: DNA ladder). Black arrows indicate the markers shown in the bold type. The lengths of the PCR products were 3.4 kbp for Syn004, 1.5 kbp for wild-type (WT), and 1.4 kbp for Syn005

PheS_A303G_F and PheS_A303G_R via In-Fusion cloning. Primers and principal DNA sequences used in this study were listed in Supplemental Table S1 and S2, respectively.

To construct the markerless knockout plasmid pUC118-nblA-KO, the linearized pUC118 was combined with the upstream and downstream regions of the *nblA* gene. An *Nde*I site was inserted between these upstream and downstream regions.

The plasmid pUC118-KmR-pheSmut was constructed by assembling replication *ori*, the kanamycin resistant gene, and the mutated *pheS* gene cording PheST261A/A303G.

The plasmid pUC118-GmR-PheSmut was constructed by replacing the kanamycin resistant gene in pUC118-KmR-PheSmut with a gentamicin resistant gene. To create the knockin plasmid for deleting the *ldhA* gene and introducing the *lldD* (NCBI reference sequence: NP_418062.1) and *lldP* (NCBI reference sequence: NP_418060.1) genes, which encode NAD-independent L-lactate dehydrogenase and lactate permease from *Escherichia coli*, respectively, the following components were assembled: the linearized pUC118-GmR-PheSmut (digested with *Sac*I and *Xba*I), the upstream and downstream regions of the *ldhA* gene, the *lldD* and *lldP* genes, and a 400 bp homologous region of the *lldP* gene, resulting in pUC118-ldhA-KO-lldD-lldP.

Another knockin plasmid, pUC118-nblA-KO-mNG, was constructed for the deletion of the *nblA* gene and the introduction of the gene for mNeonGreen. This was achieved by inserting the mNeonGreen gene, the gentamicin resistance gene, the mutated *pheS* gene and a 400 bp homologous region of the mNeonGreen gene into the *Nde*I-digested pUC118-nblA-KO.

Construction of conventional recombinant strains

Cells were cultivated until reaching an OD_{750} of approximately 1.0. Subsequently, 100 µL of the culture was mixed with plasmid DNA containing the recombination fragment. The mixed culture was incubated on a rotator for 24 h under shading. After the shading, the culture was spread on an immobilon nitrocellulose membrane (HATF08250, Merck KGaA, Darmstadt, Germany) on the agar plate and incubated at 30°C for approximately 2 days. The membrane was transferred to the agar plate containing 100 µg/mL kanamycin or 40 µg/mL gentamicin and incubated for 4–7 days until colonies appeared. The transformation and segregation were confirmed by PCR using the culture as a template. The PCR was conducted following manufacturer's instructions. Briefly, denaturing at 98°C for 10 s., annealing at 60°C for 5 s., extension at 68°C for 5 s./ 1 kbp, and these steps were continued for 30 cycles. When incomplete segregation was observed, the transformants were sub-cultured until the complete segregation was confirmed.

Construction of markerless gene knockout strain

To obtain a markerless *nblA*-deficient strain, the recombinant strain Syn004 was cultured until reaching an OD_{750} of approximately 1.0. Subsequently, 100 µL of

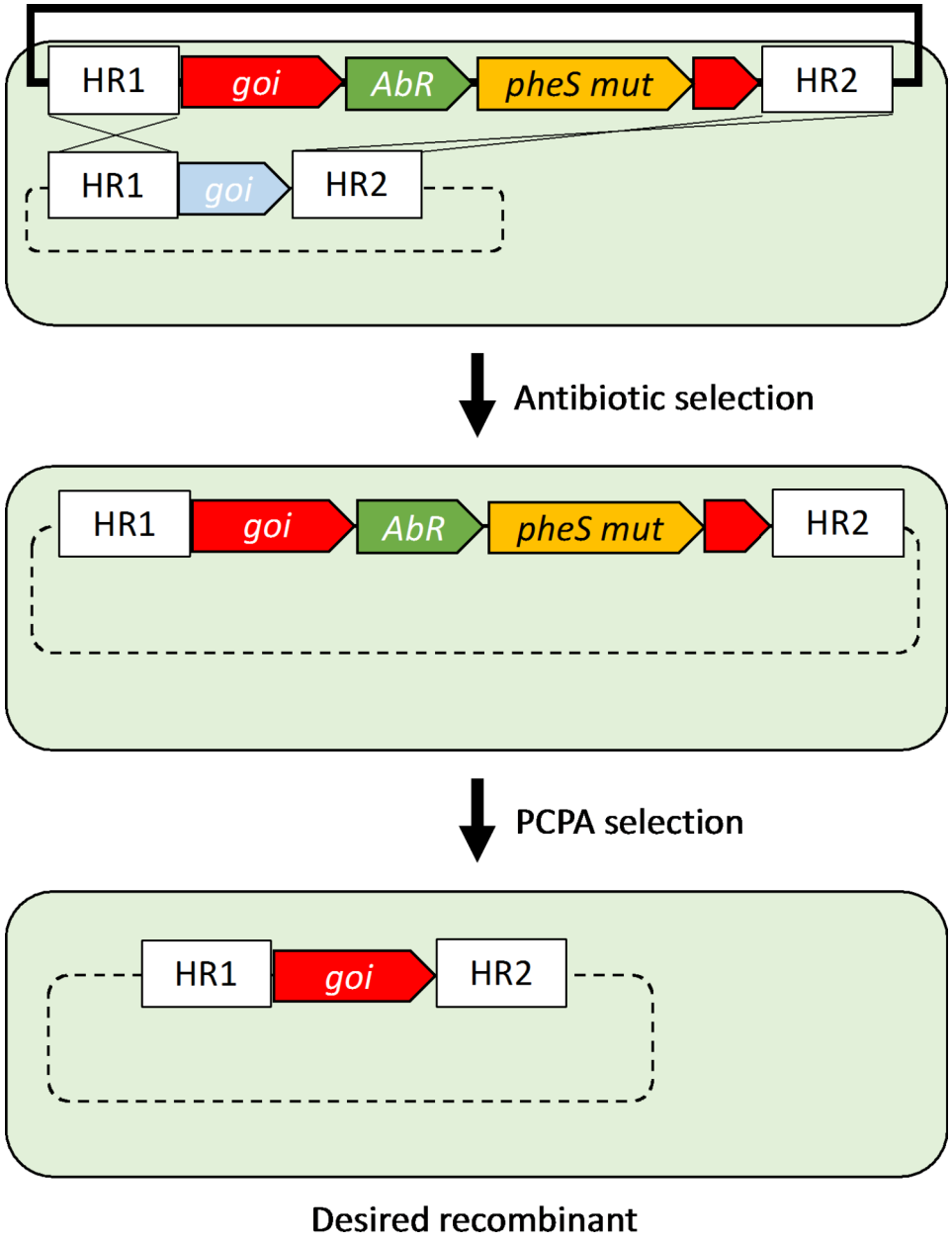

Fig. 4 Improved markerless knockin system. The scheme of markerless gene knockin involves two recombination steps. The first-step recombination follows the protocol described in Fig. 1, the conventional transformation method using antibiotic screening. The DNA fragment containing the gene of interest (*goi* in red, adjacent to HR1 and *AbR*), the antibiotic resistance gene (*AbR*), the mutated *pheS* (*pheS mut*) gene, the partial region of the *goi* (red) (400 bp of 3′-terminal regions in red, adjacent to *pheSmut* and HR2) is integrated, deleting the *goi* shown in pale-blue. In the second-step recombination, the homologous recombination occurs between the *goi* (red) and its partial region under the selection pressure of PCPA. This process results in the construction of the desired recombinant strain by eliminating the unnecessary marker genes, *AbR* and *pheSmut*

the culture was mixed with the plasmid pUC118-nblA-KO. The mixed culture was incubated on a rotator for 24 h under shading. After the shading, the mixture was spread on agar plate containing 20 µg/mL PCPA and incubated for 1 week until colonies appeared. The resulting colonies were then streaked onto another agar plate with 20 µg/mL PCPA and incubated for 3–4 days. The re-streaked colonies were cultured in MA2 medium for approximately 3 days, and transformation, elimination of the marker genes, and complete segregation were confirmed by PCR using the culture as a template.

Construction of markerless gene knockin strains

For markerless knockin mutants, the transformation was conducted following the conventional methods. After antibiotic selection, the transformation and complete

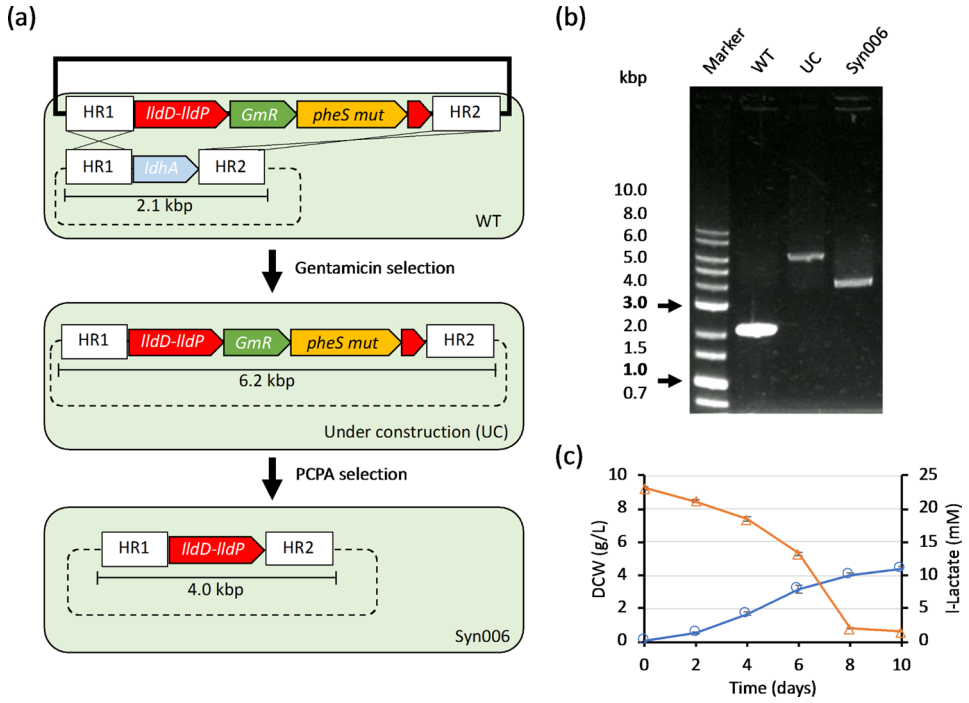

Fig. 5 Markerless gene knockin at the *ldhA* gene site. (**a**) Markerless gene knockin scheme using the pUC118-ldhA-KO-lldD-lldP plasmid: This scheme employs the gentamicin resistance gene (*GmR*). The DNA fragment containing codon-optimized genes for NAD-independent L-lactate dehydrogenase (*lldD*) and lactate permease (*lldP*) from *E. coli* was inserted into the intrinsic pAQ7 plasmid at the *ldhA* gene site, which encodes D-lactate dehydrogenase. The *lldD* and *lldP* genes were constitutively expressed under the control of the *trc* promoter. (**b**) Confirmation of the complete segregation and deletion of the intergenic region of the plasmid: The region from the 5′- to 3′-flanking regions of the *ldhA* gene (HR1 to HR2) was amplified by PCR using the wild-type, the recombinant at antibiotic screening (Under construction) and Syn006 genomes as templates, with primers, G0164_FW and G0164_RV. The PCR products were analyzed by agarose gel electrophoresis (Marker: DNA ladder). The lengths of the PCR products of the wild-type (WT), Under construction (UC), and Syn006 are 2.1, 6.2, and 4.0 kbp, respectively. (**c**) Growth curve (circle) and L-lactate consumption (triangle) of Syn006: Syn006 was cultivated at 30°C. The growth curve was obtained by measuring dry cell weight (DCW)-based biomass concentrations. L-Lactate concentration in the media was measured by HPLC. Error bars indicate the standard deviation of three replicate experiments

segregation were confirmed by PCR. The transformants which passed antibiotic selection and PCR confirmation were cultivated in antibiotic-free MA2 medium until reaching an OD_{750} of approximately 1.0. Subsequently, 200–300 µL of the culture was spread on the agar plate containing 20 µg/mL PCPA and incubated for 1 week until colonies appeared. These colonies were then streaked onto an agar plate containing 20 µg/mL PCPA and incubated for 3–4 days. The elimination of the marker genes and complete segregation were confirmed by PCR.

Measurement of lactate

Measurement of lactate was performed using high performance liquid chromatography (HPLC) as previously described [26]. Briefly, cultures were centrifuged at 8,000 ×g for 10 min, and supernatants were subjected to HPLC. L-Lactic acid (FUJIFILM Wako Pure Chemical Corporation) was used as the quantitative standard to determine the lactate concentration using a calibration curve. The HPLC conditions were as follows: column, Aminex® HPX-87H column (Bio-Rad, Hercules, CA); column temperature, 50 °C; mobile phase, 5 mM sulfuric acid; flow rate, 0.6 mL min^{-1}; detector, refractive index detector.

Result

Selection of the mutated PheS and PCPA titration

The scheme of markerless transformation method is shown in Fig. 1. At the first step, the plasmid containing an antibiotic resistance gene and mutated *pheS* gene is integrated by replacing the target gene for disruption in the genome via conventional double crossover method at the upstream and downstream region of the target gene. The recombinants are selected with antibiotic tolerance. Then, the recombinants are re-transformed and selected with PCPA. To achieve markerless transformation in PCC 7002, both PCPA susceptibility and a functional mutated PheS specific to this strain are essential. We sought a suitable mutation in PheS to confer high PCPA susceptibility to PCC 7002. Based on previous reports by Kast *et al.* [27] and Miyazaki [25], amino acid substitutions T261A and A303G were identified as potential mutations for enhancing PCPA susceptibility in *E. coli*. Considering

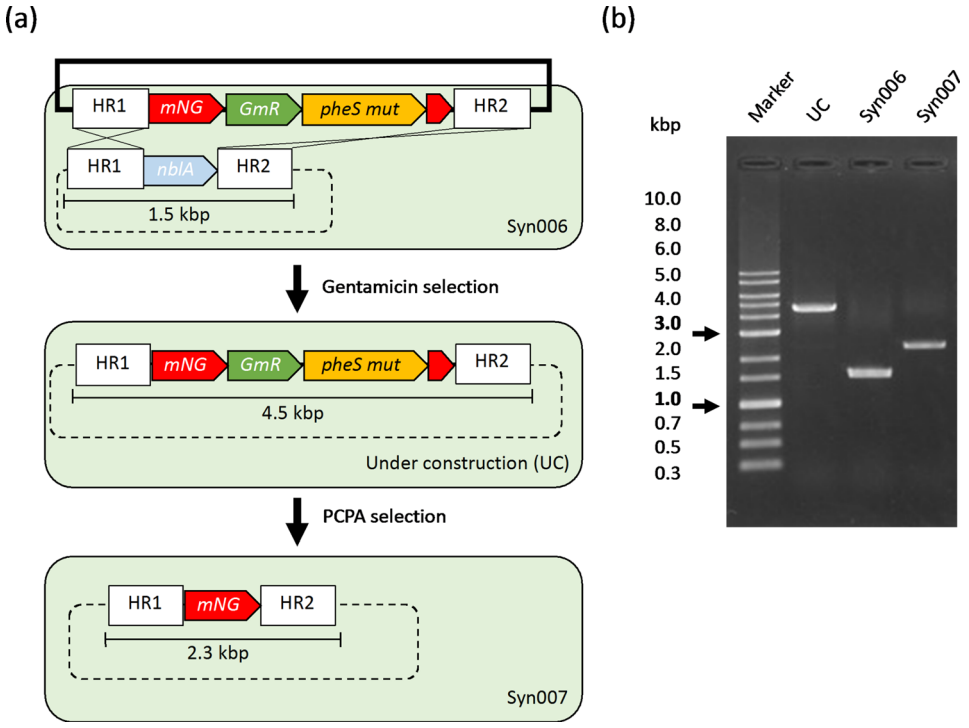

Fig. 6 Demonstration of repeatability of the markerless gene knockin system. (**a**) Second round markerless gene knockin in strain Syn006 using the pUC118-nblA-KO-mNG plasmid: The DNA fragment containing the codon-optimized gene for mNeonGreen (*mNG*) was inserted at the *nblA* gene site. The *mNG* gene was constitutively expressed under the control of the *trc* promoter. (**b**) Confirmation of the complete segregation and deletion of the intergenic region of the plasmid: The region from the 5′- to 3′-flanking regions of the *nblA* gene (HR1 to HR2) was amplified by PCR using the genomes of the recombinant strains as templates with primers nblA_seq1 and nblA_seq2. The PCR products were analyzed by agarose gel electrophoresis (Marker: DNA ladder). The lengths of the PCR products of Under construction (UC), Syn006, and Syn007 were 4.5 kbp, 1.5 kbp, and 2.3 kbp, respectively

the high amino acid sequence similarity between PheS in *E. coli* and PCC 7002 (69% similarity, as shown in Supplemental Fig. S1), we anticipated that these mutations would render PCC 7002 PheS susceptible to PCPA. To examine these candidate mutations, the mutated *pheS* genes were introduced into PCC 7002 cells with the kanamycin resistance marker. The *nblA* gene site was selected for markerless gene disruption since the phenotype of its disruption recombinant was studied in other cyanobacteria and it was not lethal [28, 29]. We obtained four recombinants: Syn001 (Δ*nblA*::*KmR-wtPheS*), Syn002(Δ*nblA*::*KmR-PheS*^{T261A}), Syn003 (Δ*nblA*::*KmR-PheS*^{A303G}), and Syn004 (Δ*nblA*::*KmR-PheS*$^{T261A/A303G}$), and confirmed complete segregation (Fig. 2a). Subsequently, these recombinants were spotted on agar plates containing PCPA to assess their susceptibility. The wild-type strain and Syn001 grew in the presence of 20 µg/mL PCPA (Fig. 2b). Syn002 exhibited greater susceptibility to PCPA than Syn001 but was more tolerant than Syn003 and Syn004, as both recombinants did not grow with 20 µg/mL PCPA. The appropriate PCPA concentration was 20 µg/mL. At 15 µg/mL PCPA, the susceptibility was highest in Syn004, followed by Syn003 and Syn002. Based on their growth in the presence of 15 µg/mL PCPA, the double amino acid substitutions of T261A and A303G were identified as suitable mutations that confer high susceptibility to PCPA in PCC 7002.

Establishment of the markerless knockout system

To establish the markerless knockout system, we targeted the *nblA* gene, which encodes the NblA (nonbleaching protein A) protein responsible for degradating phycobilisome under nitrogen- or sulfur-deprived conditions [27]. In some cyanobacteria such as UTEX 2973, deletion of the *nblA* gene strain results in a non-bleaching phenotype upon sulfur depletion [29] and decreased cell growth [28, 29]. We constructed a pUC118-nblA-KO plasmid designed specifically for markerless deletion of the *nblA* gene. This plasmid introduced an *Nde*I site into the *nblA* gene, simplifying the confirmation of transformation. With the pUC118-nblA-KO plasmid, Syn004 was transformed (Fig. 3a), and the Δ*nblA* recombinant which lost marker genes in the genome was obtained. The elimination of the marker genes and complete segregation were confirmed by PCR (Fig. 3b). As a result, the markerless Δ*nblA* recombinant, Syn005, was obtained, and the markerless knockout system was successfully established.

Establishment of the markerless knockin system

Since no colony appeared at PCPA selection using our original markerless knockin scheme shown in Fig. 1b, we developed an improved markerless knockin system shown in Fig. 4. In this new system, the marker genes are flanked by 400 bp homologous regions derived from the target gene for introduction. The cells were transformed via double crossover recombination, and the recombinants were selected based on antibiotic tolerance. The culture of the recombinants was then spread on the agar plate containing 20 μg/mL PCPA. Under the selection pressure of PCPA, homologous recombination occurred at the 400 bp homologous regions, resulting in recombinants where the marker genes were eliminated from the genome. To demonstrate our markerless knockin system, the genetic construct containing the *lldD* and *lldP* genes from *Escherichia coli*, which confers L-lactate assimilation ability to PCC 7002 [26], was introduced at the *ldhA* gene site as the model (Fig. 5a).

The pUC118-ldhA-KO-lldD-lldP plasmid was constructed for the markerless transformation. After introducing the pUC118-ldhA-KO-lldD-lldP plasmid into PCC 7002 by natural transformation, the candidates of the first-step recombination were selected with gentamicin, and then the integration and complete segregation were confirmed by PCR. The recombinant was subjected to the second-step recombination with PCPA selection. The complete deletion of the marker genes, gentamicin resistant gene and the mutated *pheS* gene, was confirmed by PCR (Fig. 5b). The phenotype of L-lactate consumption was confirmed by culturing in the presence of L-lactate (Fig. 5c). Then, the markerless L-lactate-consuming recombinant, Syn006, was obtained, and our markerless transformation system developed in this study was also shown to be available for gene knockin.

Verification of the repeatability of the markerless knockin system

To demonstrate the usefulness of our markerless system developed in this study, the repeatability of the markerless knockin system was attempted to be proved. The markerless L-lactate-consuming recombinant, Syn006, was transformed using the plasmid, pUC118-nblA-KO-mNG (Fig. 6a). The recombinants in which the fragment containing the full-length mNeonGreen gene, gentamicin resistant gene, the mutated *pheS* gene, and the 3′ region of the mNeonGreen gene was introduced by disrupting the *nblA* gene were obtained at gentamicin selection. After PCPA selection, the complete deletion of the marker genes, gentamicin resistant gene and the mutated *pheS* gene, was confirmed by PCR (Fig. 6b). The fluorescence of mNeonGreen was also observed in the recombinants (Supplemental Fig. S2). Therefore, the double markerless recombinant, Syn007, was successfully obtained, and our system enabled to transform PCC 7002 and its recombinants repeatedly.

Discussion

In this study, we established a markerless transformation method in PCC 7002 using mutated PheS-based counter selection. This approach doesn't require the disruption of specific genes and allows for unrestricted gene manipulation without relying on a limited number of available antibiotic resistance genes. Previous markerless methods for PCC 7002 required disrupting native *acsA* and *aas* genes. However, deleting the *acsA* gene increases acetate secretion [14], wasting carbon resources, while the *aas* gene is vital for acclimating *Synechococcus elongatus* strain PCC 7942 (PCC 7942) to high-light conditions [30]. Disrupting these genes may hinder PCC 7002 from becoming a production chassis strain, but the method developed in this study circumvents these issues. Unlike previous tools using acrylate or lauric acid counter selection, which involve two transformation steps with two plasmids for gene knockout and gene knockin at multiple gene sites, our tool for gene knockin achieves this with a single transformation step and plasmid. This simplicity is advantageous for faster and more simple recombination when establishing various recombinants in PCC 7002. Compared to the conventional markerless methods, the markerless method developed in this study requires only about 2 additional weeks for the eliminate process of the marker genes by PCPA counter selection.

We employed the *pheS* gene as a counter-selection marker for the first time in cyanobacteria. The *pheS* gene of PCC 7002 was utilized to obtain mutated phenylalanyl-tRNA synthetase gene, which confers PCPA susceptibility to the cyanobacterium. There was a concern regarding the replacement of the native *pheS* gene, as there was a possibility that the desired recombinants might not be obtained. Indeed, Xie *et al.* mentioned the high rate of recombination between the mutated *pheS* and native *pheS* genes in *Streptococcus mutans* [20]. In various bacteria, mutated and synthetic *pheS* genes with coding sequences comprised of synonymous codons have been used to reduce homology, i.e., to minimize recombination events [16, 20, 23, 31]. However, in this study, this was not a problem, as the recombinants in which native PheS was replaced by the mutated PheS could not survive under the present PCPA selection condition, and the desired recombinants were obtained satisfactorily.

Our gene knockin method developed in this study would not theoretically differ from the traditional double crossover method in terms of the segregation efficiency, maximum size of *goi* and the potential for the reversion to the wild-type genome though we didn't confirm it in detail. In Syn006 strain, the reversion to the wild-type genome was not observed in several times sub-culturing

and PCPA selection in the construction of Syn007, and the genotype acquired in our method was stable. The maximum size of *goi* in our method is about 1.5 kbp smaller than the conventional one due to the temporary introduction of the mutated *pheS* gene (1.1 kbp) and homologous region of *goi* (0.4 kbp). However, this can be overcome by separately introducing genes at multiple sites via our markerless method. Furthermore, by introducing multiple DNA fragments at the same site several times markerlessly to connect the fragments, it may eventually be possible to introduce the gene which couldn't be introduced in the conventional method. Therefore, we believe that our method doesn't limit the construction of multigenic strains, but rather expands the possibilities of genetic engineering.

Generally, false positives often appear in the transformations of PCC 7002. In our markerless transformation scheme, we theoretically should obtain only the desired recombinants through PCPA selection. In this study, we also obtained 3 types of false positives. One was a recombinant heterozygously harboring the mutated *pheS* gene. It probably acquired PCPA-resistance due to an insufficient toxicity of the mutated *pheS* gene or through unintended mutations into the other genomic region before second recombination step. The frequency of this type of the false positives in this study was less than 25%. Therefore, we concluded that it was not a major problem. The others were a wild-type revertant and a heterozygous one with the wild-type and the desired genomic regions. It probably resulted from the incomplete segregation. Since false positives are excluded by PCR, the occurrence of such undesired recombinants is not a significant issue. It may be effective to use an additional pair of primers specific to the deleted regions of the targeted genomic locus to confirm the antibiotic-resistant mutants are homozygous. In cyanobacteria, the SacB-based markerless system was previously reported only in PCC 6803 [9], and its usability depended on bacterium. However, the PheS-based system shows potential for widespread use due to its applicability in bacteria that cannot utilize sucrose counter selection. Recognizing the importance of robust expression of the mutated *pheS* gene for PCPA susceptibility [19], we employed the endogenous PheS and strong promoter. In *E. coli* PheS, threonine 251 plays a role in substrate recognition [32], while the substitution of alanine 294 with glycine enlarges the cavity for the para position of the aromatic ring, thereby increasing aminoacylation of tRNA using PCPA as the substrate [24]. In this study, we examined amino acid substitutions in PheS in PCC 7002. Our findings suggest that the A303G substitution is crucial for PCPA susceptibility, with the additional amino acid substitution of T261A enhancing susceptibility.

In conclusion, this study successfully established a novel markerless transformation method in PCC 7002 using the mutated *pheS* gene derived from PCC 7002, which was demonstrated to be effective for both gene knockout and knockin. This method does not require the disruption of specific genes, thus allowing for markerless transformation without limitations on the host PCC 7002. Moreover, our tool can be easily applied to other cyanobacteria, such as PCC 6803 and PCC 7942, which are naturally transformable like PCC 7002. The markerless transformation method is crucial as a foundational technology for developing a chassis strain, as it enables genome engineering without being constrained by marker genes. Though it is necessary for application of our tool to verify its effectiveness and limitations by examining the efficiency of each target gene in the future, particularly in the situations requiring gene introduction at multiple sites, our tool will prove to be an invaluable asset.

Supplementary Information

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Supplementary Material 1

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Author contributions
A.T. designed the research, performed experiments, wrote the original draft. K.I. designed the research and performed experiments. R.H. designed the research and edited manuscript. Y.K and L.D edited the manuscript. T.H. and A.K. supervised the project. All authors reviewed and consented to the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All authors approved the manuscript.

Competing interests
The authors declare no competing interests.

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