Construction of Marker-Free Transgenic Strains of *Chlamydomonas reinhardtii* Using a Cre/loxP-Mediated Recombinase System

Yuki Kasai*, Shigeaki Harayama

Department of Biological Sciences, Faculty of Science and Engineering, Chuo University, Bunkyo-ku, Tokyo, Japan

* ykasai@kc.chuo-u.ac.jp

Abstract

The *Escherichia coli* bacteriophage P1 encodes a site-specific recombinase called Cre and two 34-bp target sites of Cre recombinase called *loxP*. The Cre/loxP system has been used to achieve targeted insertion and precise deletion in many animal and plant genomes. The Cre/loxP system has particularly been used for the removal of selectable marker genes to create marker-free transgenic organisms. For the first time, we applied the Cre/loxP-mediated site-specific recombination system to *Chlamydomonas reinhardtii* to construct marker-free transgenic strains. Specifically, *C. reinhardtii* strains cc4350 and cc124 carrying an aphVIII expression cassette flanked by two direct repeats of *loxP* were constructed. Separately, a synthetic Cre recombinase gene (*CrCRE*), the codons of which were optimized for expression in *C. reinhardtii*, was synthesized, and a *CrCRE* expression cassette was introduced into strain cc4350 carrying a single copy of the *loxP*-flanked *aphVIII* expression cassette. Among 46 transformants carrying the *CrCRE* expression cassette stably, the excision of *aphVIII* by CrCre recombinase was observed only in one transformant. We then constructed an expression cassette of an in-frame fusion of *ble* to *CrCRE* via a short linker peptide. The product of *ble* (Ble) is a bleomycin-binding protein that confers resistance to bleomycin-related antibiotics such as Zeocin and localizes in the nucleus. Therefore, the *ble*-(*linker*)-*CrCRE* fusion protein is expected to localize in the nucleus. When the *ble*-(*linker*)-*CrCRE* expression cassette was integrated into the genome of strain cc4350 carrying a single copy of the *loxP*-flanked *aphVIII* expression cassette, CrCre recombinase-mediated excision of the *aphVIII* expression cassette was observed at a frequency higher than that in stable transformants of the *CrCRE* expression cassette. Similarly, from strain cc124 carrying a single *loxP*-flanked *aphVIII* expression cassette, the *aphVIII* expression cassette was successfully excised after introduction of the *ble*-(*linker*)-*CrCRE* expression cassette. The *ble*-(*linker*)-*CrCRE* expression cassette remained in the genome after excision of the *aphVIII* expression cassette, and it was subsequently removed by crossing with the wild-type strain. This precise Cre-mediated deletion method applicable to transgenic *C. reinhardtii* could further increase the potential of this organism for use in basic and applied research.
Introduction

The green unicellular alga *Chlamydomonas reinhardtii* has been widely used as a model system for studying the genetic and molecular mechanisms of biological processes such as photosynthesis and flagellar motility [1, 2, 3]. Recently, this alga has also been used to manipulate metabolic pathways involved in biofuel and hydrogen production using the range of genetic manipulation tools available to this organism [4, 5, 6, 7]. However, the number of selectable marker genes used in *C. reinhardtii* is limited even though availability of multiple selectable markers is necessary for the sequential introduction of transgenes.

*C. reinhardtii* is considered to be a model organism for basic research and an industrial biotechnology host [8]. For the large-scale deployment of transgenic *C. reinhardtii* for various industrial applications, there are public concerns regarding the spread of marker genes in the environment.

Therefore, efficient methods for the removal of marker genes from transgenic *C. reinhardtii* are highly anticipated. Sexual crossing is a powerful tool for this purpose. However, this technique cannot be used if the linkage between a marker gene and a co-introduced transgene is tight. Such tight linkage between a marker gene and co-introduced transgenes was observed in transgenic rice and soybean generated by biolistic bombardment, in which most of the transgenes were co-integrated together with a marker gene at one or multiple loci [9, 10, 11]. Co-transformation of plants by *Agrobacterium tumefaciens*-mediated transformation using multiple plasmids also resulted in the integration of multiple T-DNAs at the same locus on plant chromosomes [12, 13, 14]. Thus, although the fate of multiple-plasmid co-transformation in *C. reinhardtii* was not examined systematically, we presume that multiple plasmids are frequently integrated at the same locus, leading to a tight genetic linkage between marker genes and co-introduced transgenes in transgenic *C. reinhardtii*.

One strategy to increase co-transformation frequency is the use of a marker gene physically linked to a gene of interest [15]. Several vectors systems were developed for this purpose [16, 17], including those enabling sustained expression of transgenes in recipients [18]. In cases in which transgenes were obtained using such vectors, the marker gene and transgene are genetically linked and usually inherited together.

The genomic sequence of *C. reinhardtii* includes numerous functionally uncharacterized genes [19]. Reverse genetics is a robust method for revealing the functions of such genes. Because *C. reinhardtii* displays an extremely low efficiency of homologous recombination [20, 21, 22], insertional random mutagenesis using selectable markers in *C. reinhardtii* was identified as a valuable tool for investigating diverse biological functions [23, 24, 25, 26, 27, 28, 29]. Although the removal of selectable markers from insertional mutants without the loss of mutant phenotypes is desired for further genetic manipulation or industrial application, marker rescue from insertional mutants using sexual crossing is not possible.

To overcome the limitations of sexual crossing, several strategies have been developed to remove selectable markers from transgenic eukaryotic cells [30], including the use of site-specific DNA excision systems such as Cre/loxP from bacteriophage P1 [31, 32, 33, 34], Flp/frt from *Saccharomyces cerevisiae* [35, 36], R/RS from *Zygosaccharomyces rouxii* [37, 38], and Gin/gix from bacteriophage Mu [39]. In the bacteriophage P1 bipartite Cre/loxP-mediated site-specific DNA excision system, Cre recombinase specifically recognizes the loxP sequence of 34 bp in length and excises a DNA segment flanked by two direct repeats of loxP, leaving a single copy of loxP [40, 41, 42]. This system has been proven to be a powerful marker rescue tool in eukaryotes [43, 44, 45].

Curiously, no research on the use of Cre/loxP-mediated system in *C. reinhardtii* has been published. In this study, we discuss the exact excision of a marker gene from the nuclear...
genome of *C. reinhardtii* via Cre/loxP-mediated site-specific recombination. This report expands the list of available genetics tools in this organism.

**Materials and Methods**

**Plasmid Construction**

PCR for plasmid construction was performed using PrimeSTAR Max DNA polymerase (Takara) and appropriate primers, the sequences of which are listed in Table 1. *aphVIII* from *Streptomyces rimosus* encodes aminoglycoside 3′-phosphotransferase type VIII and confers resistance to paromomycin. The pSI103 plasmid carries the *aphVIII* expression cassette consisting of the *C. reinhardtii* HSP70_RBCS2 promoter, *aphVIII*, and the RBCS2 terminator [46]. For PCR amplification of the *aphVIII* expression cassette flanked by two direct repeats of loxP (*loxP-P-aphVIII-T-loxP*), the pSI103 plasmid was used as a template, and loxPphsp70_F1 and loxPtrbcS_R1 were employed as primers. The amplified fragment was digested using *Sma*I and *Xba*I and inserted between the *Sma*I and *Xba*I sites of the pBluescript II SK (+) plasmid to construct the ploxP-aphVIII plasmid (Fig 1).

The codons of the Cre recombinase gene were optimized on the basis of the nuclear codon usage of *C. reinhardtii* stored in the codon usage database at Kazusa DNA Research Institute (http://www.kazusa.or.jp/codon/). Codon optimization was performed using the Optimum-Gene™ algorithm, and the optimized gene (*CrCRE*) was synthesized by GenScript (New Jersey, USA). The *CrCRE* sequence was cloned into the pUC57 plasmid to create the pUCrcre plasmid. The pCrcre plasmid (Fig 1) carrying the *CrCRE* sequence flanked by the HSP70-RBCS2 promoter and RBCS2 terminator (hereafter referred as “the *CrCRE* expression cassette”) was constructed using an overlapping PCR method as follows. In the first step, three DNA fragments were amplified separately using PCR: the 0.7-kb *HSP70-RBCS2* promoter sequence was amplified using phsp70_F2 and prbcS_R1 as primers and the pSI103 plasmid as a template; the 0.3-kb RBCS2 terminator sequence was amplified using trbcS_F1 and trbcS_R2 as primers and the pSI103 plasmid as a template; and the 1.0-kb *CrCRE* sequence was amplified using Crcre_F1 and Crcre_R1 as primers and the pUCrcre plasmid as a template. In the second step, the three fragments amplified in the first step were assembled into a single fragment by PCR using the three fragments as templates and phsp70_F2 and trbcS_R2 as primers. The amplified product was purified using a PCR purification kit (Qiagen), digested with *Sma*I and *Xba*I, and cloned between the *Sma*I and *Xba*I sites of the pBluescript II SK (+) plasmid.

To facilitate the nuclear localization of CrCre recombinase, *CrCRE* was fused in frame to ble from *Streptoalloteicus hindustanus*, conferring bleomycin/Zeocin resistance [47], to generate ble-CrCRE expression cassette I as follows. A 1.2-kb fragment containing the *HSP70-RBCS2* promoter fused to *ble* was amplified using phsp70_F3 and ble_R as the primers and the pMF59 plasmid [47] as a template. A 1.3-kb fragment containing *CrCRE* fused to the RBCS2 terminator was amplified using Crcre_F2 and trbcS_R2 as primers and the pSI103 plasmid as a template; and the 1.0-kb *CrCRE* sequence was amplified using Crcre_F1 and Crcre_R1 as primers and the pUCrcre plasmid as a template. In the second step, the three fragments amplified in the first step were assembled into a single fragment by PCR using the three fragments as templates and phsp70_F2 and trbcS_R2 as primers. The amplified product was purified using a PCR purification kit (Qiagen), digested with *Sma*I and *Xba*I, and cloned between the *Sma*I and *Xba*I sites of the pBluescript II SK (+) plasmid.

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C. reinhardtii Strains and Growth Conditions

*C. reinhardtii* strains cc124 (mt−) and cc4350 (cw15 arg7-8 mt+, Chlamydomonas Resource Center) were used as recipients of the ploxP-aphVIII plasmid, whereas strain cc125 (mt+) was used in backcross experiments. Cells were cultivated mixotrophically at 25°C in Tris acetate phosphate (TAP) medium \[49\] supplemented with 10 μg ml\(^{-1}\) arginine if necessary under white fluorescent light (100 μmol photons m\(^{-2}\) s\(^{-1}\)) with gentle shaking or on solid medium supplemented with 1.5% Bacto agar (BD Difco).

### Table 1. Primers used in this study.

| Primer | Sequence (5' to 3') | Underlined sequence |
|--------|----------------------|---------------------|
| **Primers for plasmid construction** | | |
| phsp70_F1 | TCCCCGGGATAACTTCGTATAGCATACATTATACGAAGTTATGAGCTCGCTGAGGCTTGACA | Smal site |
| trbcS_R1 | CTCCTAGATATACCTCGTATAATGAGCTATATCGAAGTTATGAGCTCGCTGAGGCTTGACA | Xbal site |
| phsp70_F2 | TCCCCGGGGAAGCTCGCTGAGGCTTGACA | Smal site |
| prbcS_R1 | TACAGCAAGTGGCTCATGCTCAGAACAAATCTTCAGCACCCGG | Underlined sequence is complementary to the underlined sequence of Crcre_F1 |
| trbcS_F1 | CGGAGCCGCGGAATCTTATTGCTCGGCCTCCACAGACGGCG | Underlined sequence is complementary to the underlined sequence of Crcre_R1 |
| trbcS_R2 | CACCTAGACCTCAGAAATACGCGACCCGG | Xbal site |
| Crcre_F1 | GAAGAATTTCAGAAGTCAGACGGTGAGCTCGCTGAGGCTTGACA | Underlined sequence is complementary to the underlined sequence of prbcS_R1 |
| Crcre_R1 | CGGAGCCGCGGAATCTTATTGCTCGGCCTCCACAGACGGCG | Underlined sequence is complementary to the underlined sequence of trbcS_F1 |
| phsp70_F3 | CACAGAATGGCGCGGATCGCTGGA | HindIII site |
| ble_R | TTCTGGTGACGTCCTACTGCTCCTCCTGGCCACAGACGGCG | Underlined sequence is complementary to the underlined sequence of Crcre_F2 |
| Crcre_F2 | GCCGAGGAGGACCACCGAATCTGCTGAGGCTCGCTGAGGCTTGACA | Underlined sequence is complementary to the underlined sequence of ble_R |
| Crcre_F3 | GCCGAGGACTCTGGGCCTGCGCGCATGAGACACCTGCTGACCTGACCCACCA | Underlined sequence encodes a linker peptide, and is complementary to the underlined sequence of Crcre_F3 |

**Primers used for the detection of specific sequences**

| Primer | Sequence (5' to 3') | Underlined sequence |
|--------|----------------------|---------------------|
| aphVIII_F | ATGGACGATGCGTTGCGT | |
| aphVIII_R | TCAAGAAGATCTCGCTCCAAC | |
| loxP_F | AGCCCGGATAACTTCGTA | |
| loxP_R | GCCGCTCTAGAATAACTTCGT | |
| Crcre_F4 | GAGAACACCTGGAAGATGCT | |
| Crcre_R2 | CAGGTAGTGGTTGGGCTG | |
| trbcS_inv_F1 | GCCGAGGATGGAAGATCTGCTCCTC | |
| aphVIII_F2 | CGACTTGGAGGATCTCGAC | |
| phsp70_inv_F2 | CCGCCAAATCATCGCTGAGCTTCA | |
| trbcS_inv_F2 | AGTTTTCGATTTTGTGGTTTGT | |
| trbcS_inv_R | GGGCAAGGCTCAGATCAAC | |
| LPm1_F2 | TCTGATTTTACTGATTTTTCGAGG | |
| LPm1_R4 | GACACGATTCGAGTAGGC | |
| LPm19_F | AGCCACCTGACCACACTGCTGCGGA | |
| LPm19_R | GCCCTGCGGACTCAATTAAATGCTGCG | |

doi:10.1371/journal.pone.0161733.t001
Genetic Transformation of *C. reinhardtii* Strains

Nuclear transformation was performed using electroporation as described previously [50]. Briefly, the cells were grown for approximately 24 h until the cell densities reached $1 \times 10^6$–$2 \times 10^6$ cells ml$^{-1}$ in TAP medium. Cells were harvested by centrifugation at 800 \times g for 5 min and washed with EP solution (30 mM HEPES, 5 mM MgSO$_4$, 50 mM potassium acetate, 1 mM calcium acetate, 60 mM sucrose, pH 7.4), and suspend in EP solution to a final density of $1 \times 10^8$–$3 \times 10^8$ cells ml$^{-1}$. Then, 4 \text{μl} of 500 \text{μg/ml} DNA were added to 121 \text{μl} of the cell suspension. The cell suspension was placed into an electroporation cuvette with a 2-mm gap (Bio-Rad) and incubated at 15°C for 2 min. An exponential electric pulse of 2000 V/cm was applied to the suspension of strain cc124 using a GenePulser XCell™ (Bio-Rad) electroporation apparatus. The capacitance was set at 25 \text{μF}, and no shunt resistor was used. For strain cc4350, an exponential electric pulse of 700 V/cm at a capacitance of 600 \text{μF} was applied. After electroporation, cells were incubated at 15°C for 1 h and transferred to 10 ml of fresh TAP medium containing 40 mM sucrose. After incubation for 18 h at 25°C under dim light, the cells were collected by centrifugation at 800 \times g for 5 min and selected on TAP agar plates supplemented with 20 \text{μg/ml} paromomycin (Wako) or 10 \text{μg/ml} Zeocin (Invitrogen). Each single colony developed on the agar plates was screened by PCR to identify gene-positive clones as described previously [51, 52] with some modifications. Each paromomycin-resistant (Pmr) clone was suspended in 10 \text{μl} of distilled water, into which the same volume of ethanol and 100 \text{μl} of 50% Chelex-100 (Bio-Rad, USA) were added. After incubation at 100°C for 10 min, cell debris was removed by centrifugation at 6000 rpm for 10 min. PCR was then performed using the supernatant as a template and aphVIII_F and aphVIII_R as primers to detect a partial aphVIII sequence or loxP_F and loxP_R as primers to detect the loxP-P-aphVIII-T-loxP sequence. In addition, PCR was performed to detect a partial CrCRE sequence using the primers Crcre_F4 and Crcre_R2, whereas detection of the full-length sequence of the CrCRE expression cassette,
ble-CrCRE expression cassette I, or ble-CrCRE expression cassette II on the pCrcre, pbleCrcre, or pbleLCrcre plasmid was performed by two PCR amplifications using two primer sets: phsp70_F2 plus Crcre_R2 and Crcre_F4 plus trbcS_R2. The sequences of the primers used for the detection of transgenes are listed in Table 1.

Southern Blot Analysis to Detect aphVIII Insertions

C. reinhardtii genomic DNA was extracted using a standard phenol-chloroform protocol [53]. Five micrograms of genomic DNA were digested with BamHI, separated on 0.8% (w/v) agarose gel, and blotted onto a Hybond-N+ membrane (GE Healthcare, UK) by a standard capillary transfer method using 20 × SSC as a transfer buffer. The blotted membrane was then baked at 80°C for 2 h. An aphVIII fragment prepared by PCR using aphVIII_F and aphVIII_R as primers and the pSI103 plasmid as a template was labeled using a DIG High Prime DNA labeling and detection kit (Roche Applied Science). Hybridization and signal detection were performed according to the manufacturer’s instructions.

Isolation of the Flanking Region of loxP-P-aphVIII-T-loxP Insertions

DNA regions flanking the loxP-P-aphVIII-T-loxP insertion were determined using inverse PCR as follows. Genomic DNA (0.5 μg) of transformants carrying a single copy of the loxP-P-aphVIII-T-loxP sequence was digested with BamHI or PvuII (Takara), both enzymes being single cutters of the ploxP-aphVIII plasmid (Fig 1). After inactivation of the restriction enzymes using phenol, digested DNA was ethanol-precipitated and dissolved in TE buffer. To amplify the 5′-flanking region of the loxP-P-aphVIII-T-loxP insertion, PvuII-digested DNA was self-ligated and used as a template for an inverse PCR using trbcS_inv_F and phsp70_inv_R as primers. Similarly, to amplify the 3′-flanking region of the loxP-P-aphVIII-T-loxP insertion, BamHI-digested DNA was self-ligated and used as a template for an inverse PCR using trbcS_inv_F and trbcS_inv_R as primers. The PCR reactions were conducted using Advantage-GC Genomic PCR mix (Clontech) using the step-down PCR protocol according to the manufacturer’s instruction. The resulting amplified fragments were purified using a QIAquick Gel Extraction kit (Qiagen) and cloned into the pGEMT-Easy plasmid (Promega). The nucleotide sequences of the fragments were then determined using dideoxy chain termination via a commercial service provided by Macrogen Japan Corp. The nucleotide sequences thus obtained were compared with the genome sequence of Chlamydomonas at a Joint Genome Institute site (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Creinhardtii).

To verify excision of the loxP-P-aphVIII-T-loxP sequence integrated in the genomes of C. reinhardtii strains cc124_LPm1 and cc4350_LPm19 by Cre/loxP-mediated recombination, the insertion/excision regions were PCR-amplified using the primers designed from the sequences outside the loxP-P-aphVIII-T-loxP cassette sequence, namely primers LPm1_F and LPm1_R for the derivatives of strain cc124_LPm1 and primers LPm19_F and LPm19_R for the derivative of strain cc4350_LPm19. The nucleotide sequences of the PCR-amplified fragments were then determined as described previously.

Reverse Transcription (RT)-PCR for the Detection of CrCRE Expression

Total RNA was extracted from cells grown in TAP medium to an OD750 of 2.0 using a TRIzol® plus RNA purification kit (Ambion), and the remaining DNA was digested using a TURBO DNA-free kit (Ambion) according to the manufacturer’s instructions. First-strand cDNA was synthesized using a PrimeScript™ RT reagent kit with gDNA Eraser (Perfect Real Time, TaKaRa) and an RT primer mix containing oligo (dT)18 and random hexamers. PCR to confirm the expression of CrCRE was performed using primers Crcre_F4 and Crcre_R2.
Backcrossing and Segregation Analysis

Strain BLCP30, a derivative of cc124_LPm1 containing a single copy of loxP after Cre-mediated excision of the loxP-P-aphVIII-T-loxP sequence, was backcrossed to cc125 (mt+) to remove the CrCRE expression cassette. Mating was performed as described previously [54]. The resulting Zeocin-sensitive progenies were tested for the presence of the loxP sequence and the absence of the CrCRE expression cassette by PCR using primer sets LPm1_F2/LPm1_R4 and Crcre_F4/Crcre_R2.

Accession Numbers

Sequence data from this study can be found in the DDBJ/NCBI data libraries under the accession numbers LC150884 (pCrcre), LC150885 (pbleCrcre), and LC150883 (pbleLCrcre).

Results and Discussion

Construction and Characterization of C. reinhardtii Transformants Carrying a Single loxP-P-aphVIII-T-loxP Insertion

The EcoRI-linearized ploxP-aphVIII plasmid was introduced in strains cc124 and cc4350, and 16 and 79 Pmr transformants, respectively, were isolated. The sequences of the loxP-flanked aphVIII expression cassettes (loxP-P-aphVIII-T-loxP) integrated in the genomes of these transformants were analyzed by PCR with primers loxP_F and loxP_R, and the integration of the whole loxP-P-aphVIII-T-loxP sequence was confirmed in 6 cc124-derived and 13 cc4350-derived Pmr transformants (Fig 2). Southern blot analyses were performed to detect the aphVIII sequence in BamHI-digested DNAs isolated from 4 cc124-derived and 13 cc4350-derived transformants.
transformants carrying the whole loxP-P-aphVIII-T-loxP sequence. The BamHI restriction endonuclease cuts ploxP-aphVIII plasmid once at the 3'-end of aphVIII; therefore, the number of bands revealed by the probe corresponds to the number of aphVIII insertions in the host genomes. The top band in each lane was thought to be non-specific signals as the band was also observed in the lanes for the wild type strains, cc124 and cc4350. The analyses thus revealed that most transformants contained a single aphVIII insertion, whereas the remainder carried two (Fig 3). The sizes of the majority of the bands were different from each other, indicating that most of the loxP-P-aphVIII-T-loxP insertions were located at different loci on the C. reinhardtii chromosomes. Two transformants, cc124_LPm1 and cc4350_LPm19, each carrying a single copy of the loxP-P-aphVIII-T-loxP insertion, were selected for further studies to demonstrate the excision of the loxP-P-aphVIII-T-loxP insertion by CrCre recombinase.

To map the insertion sites of the loxP-P-aphVIII-T-loxP sequence in strains cc124_LPm1 and cc4350_LPm19, flanking DNA regions were amplified using inverse PCR and sequenced as described in the Materials and Methods. The nucleotide sequences of the flanking regions were then aligned to the C. reinhardtii genome sequence [gene model version JGI 5.5 (Phytozome 10), Joint Genome Institute: http://www.phytozome.net/chlamy]. In strain cc124_LPm1, the loxP-P-aphVIII-T-loxP sequence was inserted in a gene of unknown function (Cre08. g362400, 1,099,596 . . .1,102,295 on chromosome 8) at location 1,099,903, whereas the insertion site in the cc4350_LPm19 genome was mapped to multiple locations in the genome, which could not be determined unequivocally (Table 2).
Demonstration of CrCre Recombinase-Mediated Site-Specific Recombination in *C. reinhardtii*

To examine excision of the *loxP*-P-*aphVIII*-T-*loxP* sequence by CrCre recombinase, the pCrcre plasmid carrying the *CrCRE* expression cassette was introduced into strain cc4350_LPm19 via co-transformation with the pMF59 plasmid carrying *ble* conferring Zeocin resistance (*Zeor*), and *Zeor* transformants were screened on TAP agar plates containing Zeocin. The existence of the *CrCRE* expression cassette in 226 *Zeor* transformants was then examined by PCR with two primer sets: phsp70_F2 plus Crcre_R2 and Crcre_F4 plus trbcS_R2 (Fig 4A). The entire *CrCRE* cassette sequence was detected in 46 *Zeor* transformants. We first expected that all transformants carrying the intact *CrCRE* expression cassette would be Pm-sensitive (Pms), as the *loxP*-P-*aphVIII*-T-*loxP* sequence might have been excised by CrCre recombinase. However, only 1 of the 46 transformants was Pms, and excision of the *aphVIII* sequence in the Pms transformant was confirmed by PCR (Fig 4B).

This unexpectedly low excision rate of the *loxP*-P-*aphVIII*-T-*loxP* sequence in the pCrcre transformants may be due to several reasons. The first possibility was the low expression of
CrCRE from the CrCRE expression cassette. Then, CrCRE expression was examined in nine randomly selected pCrcre transformants by RT-PCR using PCR primers Crcre_F4 and Crcre_R2. CrCRE expression was detected in seven of nine strains, whereas aphVIII expression was detected in all strains (Fig 4C).

To overcome potential problems including malfunction of translation and/or inefficient nuclear translocation of the CrCre protein, CrCRE was fused to ble to construct the pbleCrcre plasmid (Fig 1). There were two reasons for the construction of the Ble-CrCre fusion proteins: (i) As the level of resistance to Zeocin is proportional to the protein expression level of Ble [55], transformants expressing the Ble-CrCre fusion protein at high levels could readily be isolated by selecting for ZeoR at higher levels. (ii) Ble is a bleomycin-binding protein that localizes in the nucleus [47]; thus, fusion with the Ble protein would further facilitate the nuclear translocation of the CrCre recombinase.
When the pbleCrcre plasmid carrying ble-CrCRE expression cassette I was introduced into strain cc4350_LPm19 by selecting Zeor transformants, excision of the loxP-P-aphVIII-T-loxP sequence was not detected. We expect that the CrCre recombinase directly fused to the Ble protein was not functional in the Zeor transformants probably because two domains in the bifunctional fusion protein were not effectively separated each other [56, 57], or that the fusion protein had a high chance of misfolding [58]. A fusion gene encoding the Ble protein fused to CrCre recombinase via a flexible linker of six amino acids was then designed. The pbleLCrcre plasmid harboring ble-CrCRE expression cassette II ([the HSP-RBCS promoter]–[the ble-linker-CrCRE fusion protein gene]–[the RBCS terminator]) (Fig 1) was introduced into strain cc4350_LPm19, and Zeor transformants were screened on TAP agar plates containing Zeocin. Seventy-four Zeor transformants were obtained, and the existence of the ble-CrCRE expression cassette II sequence in the transformants was examined by PCR as described previously (Fig 5A). In the genomes of 12 of 74 transformants, the intact ble-CrCRE expression cassette II was integrated (Fig 5B). The absence of the aphVIII sequence in the genome of 12 transformants was next examined by PCR using primers aphVIII-F and aphVIII-R. The aphVIII sequence was not detected in four of the transformants (Fig 5B). These four aphVIII-free transformants were Pm⁺, whereas the remaining eight transformants were Pm⁻. The four aphVIII-free transformants were named strains BLCP1, BLCP6, BLCP15, and BLCP17. From the remaining
eight Pm\(^r\) transformants, aphVIII-free descendants were isolated after single-colony isolation repeated 2–6 times, indicating that CrCre recombinase-mediated site-specific recombination could be delayed, requiring many generations to elapse before recombination.

Genomic DNA was extracted from strains BLCP6, BLCP15, and BLCP17, and Southern blotting with a probe specific for the aphVIII sequence was performed (Fig 6). The aphVIII signal was not detected in these three strains. The DNA sequences of strains BLCP6, BLCP15, and BLCP17 corresponding to the "loxP-P-aphVIII-T-loxP" integration site in their parental strain, cc4350_LPm19, were analyzed by PCR using primers LPm19_F and LPm19_R. A 2.7-kb fragment was amplified from strain cc4350_LPm19, whereas a 0.9-kb fragment was amplified from BLCP6, BLCP15, and BLCP17 (Fig 7). The nucleotide sequence of the 0.9-kb fragment revealed that the "loxP-P-aphVIII-T-loxP" sequence was accurately excised by Cre/loxP-mediated recombination, leaving a single copy of loxP.
ble-CrCRE expression cassette II was also introduced into strain cc124_LPm1. In the genomes of 5 of the 163 Zeor transformants, the entire cassette was integrated, and one of the five Zeor transformants was Pms. This strain was named BLCP30, and the \textit{aphVIII} sequence was absent in its genome (Fig 8A). The DNA sequence of strain BLCP30 corresponding to the \textit{loxP-\textit{aphVIII-T-loxP}} insertion site in its parental strain, cc124_LPm1, was analyzed by PCR using primers LPm1\_F2 and LPm1\_R4. The 2.9-kb fragment was amplified from strain cc124_LPm1, whereas a 1.1-kb fragment was amplified from strain BLCP30 (Fig 8B). The accurate excision of \textit{loxP-\textit{aphVIII-T-loxP}} leaving a single copy of \textit{loxP} was confirmed by nucleotide sequencing of the 1.1-kb fragment.

**Removal of ble-CrCRE Expression Cassette II via Backcross to a Wild-Type Strain**

ble-CrCRE expression cassette II remained in the genomes of \textit{aphVIII}-cured derivatives. The presence of ble-CrCRE expression cassette II in a host genome hinders the subsequent introduction of a \textit{loxP}-flanked marker gene into the host. Furthermore, constitutive expression of CrCre
recombinase may induce DNA damage at off-target sites [59, 60, 61, 62]. To remove ble-CrCRE expression cassette II from strain BLCP30, which is a descendent of strain cc124 (mt−), this strain was crossed to the wild-type strain cc125 (mt+). Seven tetrads were dissected, and 25 recombinant progenies were isolated. They were grown on TAP plates for 72 h and tested for their phenotypes. In total, 12 of 25 progeny were sensitive to Zeocin, and four carried the loxP sequence. The absence of CrCRE in the genomes of the four progeny was also confirmed by PCR (Fig 9).

**Conclusion**

In this study, we developed a method to obtain marker-free transgenic strains in *C. reinhardtii*, and the steps of the method are outlined in Fig 10. The Cre/loxP-mediated precise marker excision method applicable to transgenic *C. reinhardtii* could further increase the potential of this organism for use in basic and applied research.
**Fig 9.** Removal of ble-CrCRE expression cassette II sequence by backcross. Agarose gel electrophoresis of PCR-amplified loxP and CrCRE sequences from the genomes of four progeny obtained by backcross between strains BLCP30 and cc125. Lane M, DNA size marker (λ-EcoT14 I digest) with molecular size in kb. Template DNAs were extracted from the following: lane 1, strain cc124; lane 2, strain BLCP30; lanes 3–6, progeny; lane N, no template.

doi:10.1371/journal.pone.0161733.g009

**Fig 10.** Summary of the method to obtain marker-free transgenic strains in *C. reinhardtii*.

doi:10.1371/journal.pone.0161733.g010
Acknowledgments

We thank Takako Minoura and Ritsu Kamiya for advice concerning the *Chlamydomonas* mating experiments and for the kind gifts of the pSI103 plasmid and strains cc124 (mt−) and cc125 (mt+). We also thank Jun Abe for the kind gift of the pMF59 plasmid. This work was supported by the New Energy and Industrial Technology Development Organization (NEDO, P11502725-0).

Author Contributions

Conceptualization: SH.

Data curation: YK SH.

Funding acquisition: SH.

Investigation: YK.

Methodology: YK.

Project administration: SH.

Resources: SH.

Software: YK.

Supervision: SH.

Validation: YK.

Writing – original draft: YK.

Writing – review & editing: SH.

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