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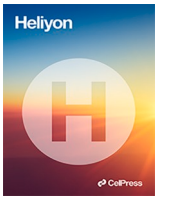

Markerless bacterial artificial chromosome manipulation method by red proteins of phage λ mediated homologous recombination utilizing fluorescent proteins for both positive and counter selection

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ABSTRACT

Manipulating viral genomes is an essential technique in reverse genetics and recombinant vaccine development. A strategy for manipulating large viral genomes involves introducing their entire genome into bacterial artificial chromosomes and employing *Escherichia coli* genetic tools. For sequence manipulation on bacterial artificial chromosomes (bacterial artificial chromosomes recombineering), a well-established method that relies on the *Escherichia coli* strain GS1783, and the template plasmid, pEPKan-S, is often used. This method, known as markerless DNA manipulation, allows for the generation of a recombinant bacterial artificial chromosome that does not retain the selection markers used during recombination. Although this method is highly innovative, there remains room for improvement as the plasmid is currently only available for positive selection. Additionally, differentiating true recombinants from false negatives often proves time-consuming. Consequently, an improved method for bacterial artificial chromosomes recombineering, which utilizes fluorescent proteins, has been developed. This method's core comprises three plasmids containing the I-SceI recognition site, antibiotic resistance genes (ampicillin, kanamycin, and zeocin), and fluorescent genes (YPet, mOrange, and mScarlet). The success or failure of Red recombination can be confirmed via fluorescent signals. To validate this method, the Lassa virus genes were introduced into the bacterial artificial chromosomes, containing the entire genome of the vaccinia virus strain LC16m8. Consequently, the expression of fluorescent protein genes contributed to positive selection, such as blue-white screening and counter-selection during the first and second Red recombination.

1. Introduction

Manipulating viral genomes is an essential technique in reverse genetic approaches and in the insertion of foreign genes during the

Abbreviations: BAC, bacterial artificial chromosome; Red, Red proteins of phage λ; Red recombination, Red-mediated homologous recombination; NP, nucleoprotein; Z, matrix protein; GPC, glycoprotein precursor; LASV, Lassa virus; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS polyacrylamide gel electrophoresis; GOI, gene of interest.

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development of recombinant vaccine. Various viral families, including *Poxviridae*, *Herpesviridae*, and *Coronaviridae*, present challenges for genetic engineering owing to their sizable genomic composition. A solution to this issue involves cloning the entire viral genome into a bacterial artificial chromosome (BAC) [1–3]. BAC is a plasmid with the unique ability to incorporate more than 300 kilobase pairs (kb) of DNA [4]. Once the viral genome is cloned into a BAC, it can be manipulated using *Escherichia coli* (*E. coli*) genetic tools through BAC recombineering [5]. BAC recombineering frequently employs Red proteins of phage λ (Red)-mediated homologous recombination (Red recombination) for sequence and gene insertion and deletion [6,7].

Previously, Tischer et al. devised a method for BAC recombineering using an *E. coli* strain GS1783, in which the chromosome encodes inducible genes, including Red and the homing endonuclease I-SceI expression [8]. The template plasmid pEPKan-S also plays a crucial role as a polymerase chain reaction (PCR) template for constructing a transfer construct [8,9]. This plasmid carries two selection markers: the kanamycin resistance gene *aphAI* (Kan^R) and I-SceI recognition sites. In this method, the first Red recombination introduces the transfer construct flanked by approximately 50 bp homology to the insertion region in a BAC *in vivo*. GS1783 containing the successful recombinant BAC is positively selected on an LB agar plate under kanamycin stress. Subsequently, the I-SceI site in the introduced construct is cleaved *in vivo* by inducing I-SceI, followed by second Red recombination to remove the I-SceI site and Kan^R flanked by the homologous sequences. As a result, the final recombinant BAC does not contain the I-SceI site and KanR, hence it is termed markerless.

Despite the innovative nature of this method, there is still room for improvement, particularly in minimizing time loss. The template plasmid is limited to kanamycin selection; hence it cannot be used if GS1783 already contains a BAC with Kan^R. Additionally, false-positive GS1783 colonies occasionally appear under kanamycin stress during the first Red recombination. This issue can be

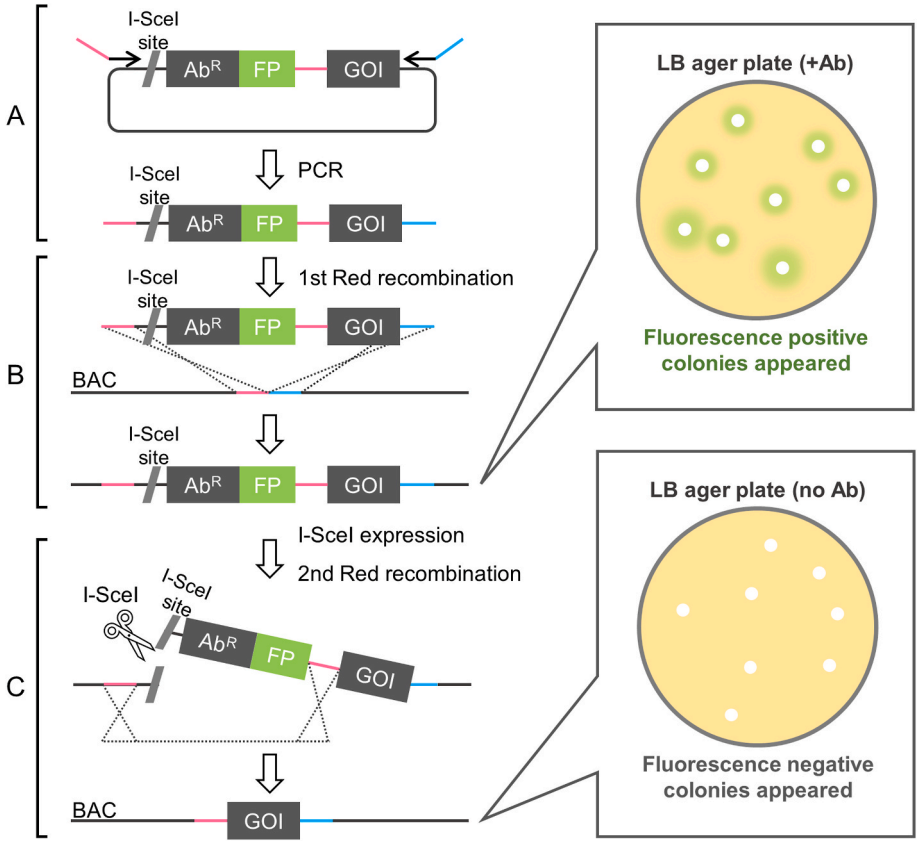

Fig. 1. Schematic diagram of BAC recombineering using the Red recombination method.
This diagram illustrates the insertion of a gene of interest (GOI) into the BAC. The template plasmid contains a restriction endonuclease I-SceI recognition site, an antibiotic-resistant gene (Ab^R), and a fluorescent protein gene (FP). The GOI was cloned into the template plasmid before generating the transfer construct. Initially, a transfer construct was generated using PCR (A). The specific PCR primers contained approximately 50 bp of sequences homologous to the desired region in the BAC. Subsequently, the transfer construct was electroporated into *E. coli* strain GS1783, which retains the BAC. The first Red recombination occurs between the duplicated sequences in GS1783 by the induction of the chromosome-encoded Red gene (B). If recombination occurs successfully, the GS1783 containing the mutant BAC grows on an LB agar plate with antibiotics. Successful recombination can also be visualized by the fluorescence of these GS1783 colonies on LB agar with antibiotics on an LED *trans*-illuminator. Finally, the second Red recombination occurred in GS1783 by the induction of chromosome-encoded Red and I-SceI genes. If recombination was successful, the Ab^R and FP were self-excised. Therefore, colonies of GS1783 containing a successfully mutated BAC were fluorescence-negative on an LB agar plate without antibiotics on the LED transilluminator.

addressed using an additional selection technique, such as blue-white selection. Moreover, this method does not employ a counter-selection marker [9]. Therefore, the colonies that emerge on an LB agar plate during the second Red recombination must be further plated on both LB plates with and without kanamycin, a process that can be time-consuming.

Hence, this study developed an improved method for BAC recombineering utilizing fluorescent proteins for both positive and counter-selection. These fluorescent protein genes have been confirmed to function as positive selection indicators, such as blue-white selection and counter-selection markers in the Red recombination.

2. Results

2.1. Summary of the BAC manipulation method

In the BAC recombineering method established in this study, the fluorescent protein functioned as a marker for both positive (Fig. 1A and B) and counter-selection (Fig. 1C). This method can be applied to the insertion, deletion, and substitution of sequences and genes. For instance, when a gene of interest (GOI) is introduced into the BAC, it is cloned into the template plasmid, as shown in Fig. 1. Three template plasmids, pAmp-9mScarlet, pKan-9YPet, and pZeo-9mOrange, which contain a restriction endonuclease I-SceI recognition site, an antibiotic-resistant gene, and a fluorescent protein gene, played pivotal roles (Fig. 2). During the first Red recombination, both the antibiotic-resistant gene product and the fluorescent protein functioned as positive selection markers, indicating successful homologous recombination. During the second Red recombination, the fluorescent protein functioned as a counter-selection marker. The successful removal of the selection markers was indicated by the quenching of the fluorescence in *E. coli* colonies. The desired recombinant BAC was obtained by picking up and culturing fluorescent-negative *E. coli* colonies.

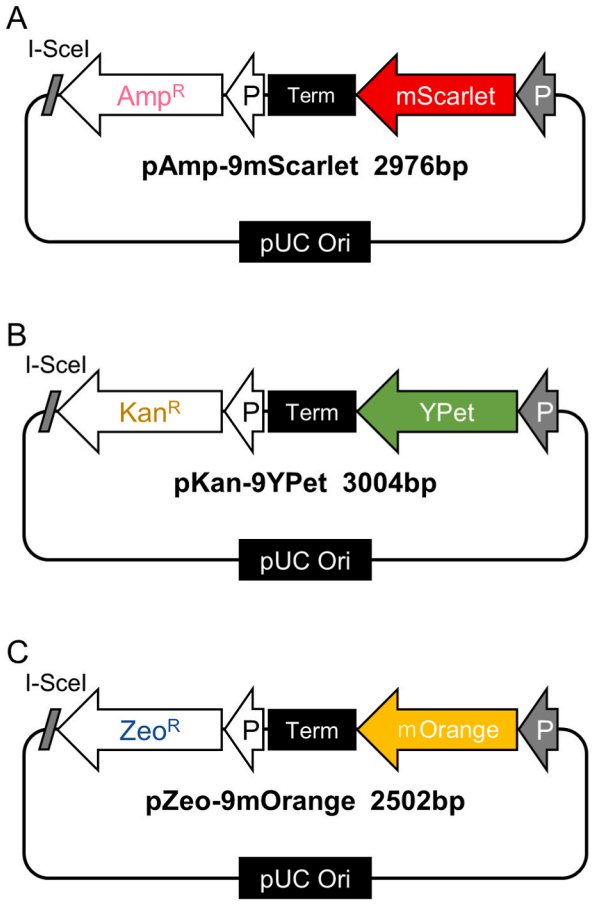

Fig. 2. Template plasmids for BAC recombination.
A template plasmid pAmp-9mScarlet was constructed with the I-SceI recognition site, Amp^R driven by its original promoter (P in white arrow), and a fluorescent protein mScarlet gene driven by the promoter BBa_J23119 (P in grey arrow) with a terminator rrnB T1 (Term). In addition to pAmp-9mScarlet, pKan-9Ypet (B) and pZeo-9mOrange (C) contain I-SceI recognition sites, KanR, and ZeoR. The fluorescent protein genes are driven by the promoter Bba_J23119 (P in grey arrow) with the terminator rrnB T1.

2.2. Construction of template plasmids for BAC recombination

Three template plasmids were constructed based on the pUC19 plasmid with an I-SceI site and different antibiotic-resistant genes: ampicillin-resistant gene (Amp^R) (Fig. 2A), Kan^R (Fig. 2B), or zeocin-resistant gene (Zeo^R) (Fig. 2C), along with fluorescent protein genes (mScarlet, YPet, and mOrange) (Fig. 2A–C and Table 1). These fluorescent protein genes were driven by the most potent constitutive promoter, BBa_J23119, with a transcription terminator. The terminator was confirmed to be indispensable for gene expression intensity and plasmid yield (Fig. 3). The pAmp-9mScarlet plasmid was constructed without the rrnB T1 terminator (Fig. 3A). *E. coli* was transformed with pAmp-9mScarlet with or without the terminator and cultivated with ampicillin at 37 °C overnight. The fluorescence intensity of mScarlet was stronger in cells transformed with pAmp-9mScarlet with the terminator than in those transformed without the terminator (Fig. 3B). Plasmid production was significantly more efficient in pAmp-9mScarlet with the terminator than in the absence of the terminator (Fig. 3C). Additionally, mScarlet, YPet, and mOrange were chosen from a total of six fluorescent protein genes (Table 1) based on their toxicity to *E. coli* (Fig. 3D and E). *E. coli* HST16CR or HST08 cells were transformed with plasmids containing one of the six fluorescent protein genes to evaluate toxicity (Fig. 3D). HTS16CR is a derivative of the HST08 strain that reduces the copy number of ColE1-like plasmids (e.g., pUC plasmid) even at 37 °C, similar to those retained in general *E. coli* strains at 30 °C. The cells were streaked onto an LB plate containing ampicillin and grown overnight at either 30 °C or 37 °C (Fig. 3E). HST16CR transformed with any fluorescent protein grew well. Additionally, HST08 transformed with mClover3 expression plasmid grew well at 30 °C and 37 °C. However, HST08 cells, transformed with mKO2 expression plasmid, did not grow at either 30 °C or 37 °C. HST08 cells transformed with YPet, mOrange, and mScarlet expression plasmids grew well at 30 °C, but their growth capacities were typically reduced at 37 °C. Therefore, they were selected as template plasmids because their genes did not show toxicity at 30 °C and their fluorescence was more intense than that of enhanced green fluorescent protein (EGFP) (Table 1). As with other characteristics, these three plasmids were small (approximately 3 kb), and the promoters driving the antibiotic-resistant gene and fluorescent protein gene expression were in the same direction. Therefore, they are expected to facilitate the insertion of toxic foreign genes in the opposite promoter orientation to avoid leaky expression.

2.3. Efficacy of the established BAC manipulation method

To validate the efficacy of the established BAC manipulation method, the Lassa virus (LASV) genes were introduced into the desired locations in the BAC, pLC16m8.8S-BAC, which contains the entire genome of the highly attenuated vaccinia virus strain LC16m8 (m8) [10]. To introduce LASV genes into the pLC16m8.8S-BAC without interfering with the original viral promoters and genes, we identified sites where the open reading frames were oriented to each other (Fig. 4A–C). At least three sites, B16R and B17L, A40R and A41L, and A46R and A47L, fulfilled this criterion. Consequently, based on the basic template plasmids, pKan-9YPet-B16, pZeo-9mOrange-A40, and pAmp-9mScerlet-A46 were constructed. These plasmids contained a synthetic vaccinia virus early/late promoter with the restriction enzyme *Age*I site flanked by 50 bp homologous sequences of B16R and B17L, A40R and A41L, and A46R and A47L (Fig. 4D–F).

LASV nucleoprotein (NP), matrix protein (Z), and glycoprotein precursor (GPC) genes were inserted at the *Age*I site of pKan-9YPet-B16, pZeo-9mOrange-A40, and pAmp-9mScerlet-A46, respectively. Transfer constructs were prepared from the template plasmids using PCR amplification. Subsequently, GS1783 cells containing pLC16m8.8S-BAC were electroporated with transfer constructs to perform the first Red recombination. After overnight cultivation with chloramphenicol and either kanamycin, Zeocin, or ampicillin, antibiotic-resistant colonies were assessed for their fluorescent protein expression on an LED transilluminator (Fig. 5A–D). All the colonies on the agar plates displayed fluorescence. Consequently, nine colonies on each plate were tested for successful insertion using colony PCR and Sanger sequencing (Fig. 5E–G). The PCR amplicon size was approximately as anticipated. Moreover, the sequence of the PCR amplicon confirmed that most of the colonies (colony numbers 2–8, 1–8 and 1–8 in Fig. 5E–G, respectively) contained successful intermediate recombinant pLC16m8.8S-BAC, except for some colonies. This result illustrates that fluorescent signals can indicate the insertion of constructs. Interestingly, colony number nine in Fig. 5E, whose *E. coli* clone did not retain the insert, demonstrated a tendency of slightly more intense fluorescence signal compared to the others (Fig. 5A). This difference in fluorescence intensity may be a way to identify false positives. Subsequently, a second Red recombination with I-SceI digestion was performed to remove the expression cassettes of the selection markers from the intermediate recombinant pLC16m8.8S-BAC. The LASV gene arrangement in the final recombinant pLC16m8.8S-BAC is shown in Fig. 6A–C. After overnight cultivation with chloramphenicol on agar plates, the colonies did not express fluorescent proteins (Fig. 6D–F). Confirmation of successful removal of the selection marker genes was performed through colony PCR for the six colonies on each plate (Fig. 6G–I). More than 50% of the colonies contained

Table 1
Characteristics of fluorescent proteins used in this study.

Fluorophore	Color	Excitation (nm)	Emission (nm)	Relative brightness
EGFP	Green	488	507	1.0
mClover3	Green	506	518	2.5
YPet	Yellow	517	530	2.4
mOrange	Orange	548	562	1.4
mKO2	Orange	551	565	1.2
mScarlet	Red	569	594	2.1

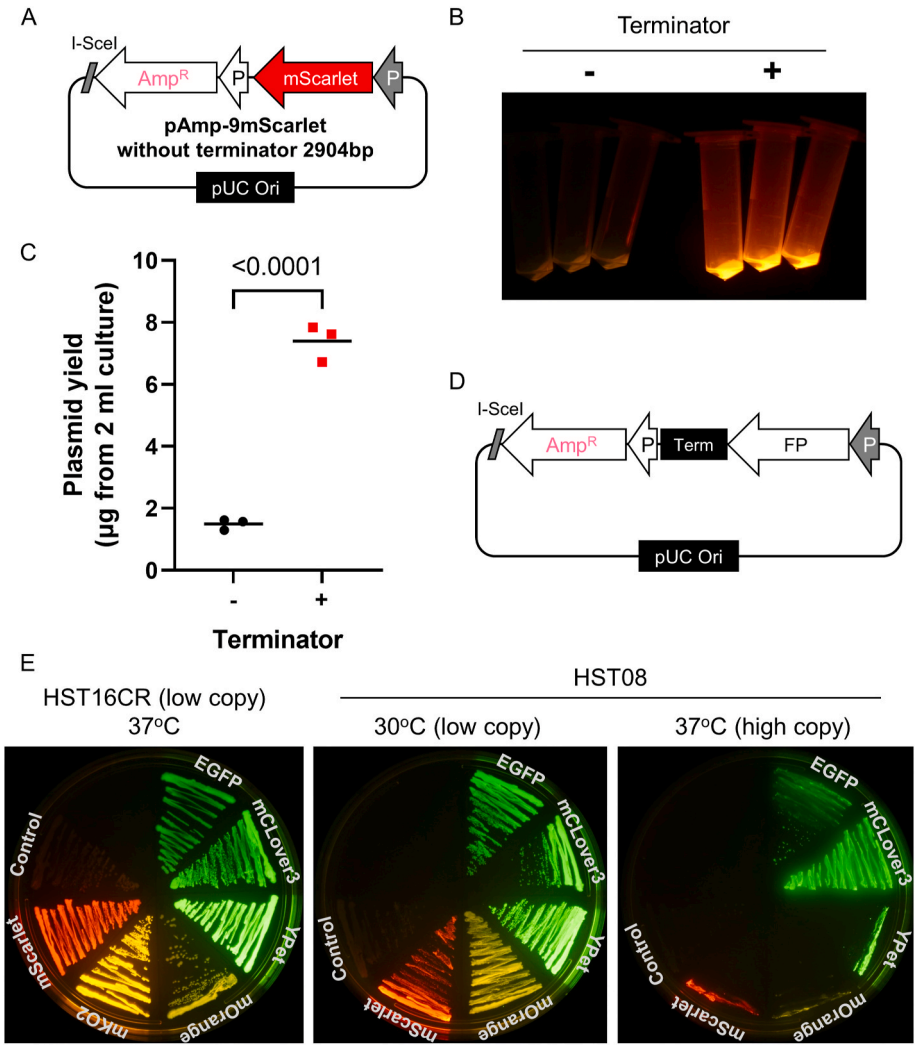

Fig. 3. Optimization of template plasmids.
pAmp-9mScarlet without the terminator rrnB T1 was constructed (A). mScarlet protein expression in the pelleted HTS16CR cells was observed on an LED transilluminator after the cells were transformed with pAmp-9mScarlet (+) or pAmp-9mScarlet without a terminator (−) following overnight cultivation in 2 mL of LB medium at 37 °C (B) (Supplementary Fig. 3B). Additionally, the plasmid yields of 2 mL of HST16CR cultures transformed with pAmp-9mScarlet (+) (with terminator) and pAmp-9mScarlet (−) (without terminator) were compared (C). The fluorescent protein gene (FP), EGFP, mClover3, YPet, mOrange, mKO2, or mScarlet were ligated into a plasmid (D), which has the same backbone as pAmp-9mScarlet, except for the fluorescent gene, as shown in Fig. 1. *E. coli*, HST16CR, or HST08 were transformed with these plasmids or pAmp-S as the negative control (control) and then cultivated on LB agar plates at either 30 °C or 37 °C overnight under ampicillin selective pressure (E) (Supplementary fig. 3E-1, -2 and -3). Note that HST08 cells transformed with mKO2 did not grow on the plates either at 30 °C or 37 °C. An unpaired *t*-test was used to determine the statistical significance. The calculated p values are shown above the compared groups.

successful final recombinant pLC16m8.8S-BAC, and the removal efficacy was sufficient for practical use. The final BACs containing LASV NP, Z, and GPC were named pLC16m8.8S–B16-LASV_NP, pLC16m8.8S-A40-LASV_Z, and pLC16m8.8S-A46-LASV_GPC, respectively.

2.4. Validation of recombinant pLC16m8.8S-BAC expressing LASV genes

The growth kinetics and LASV gene expression of the infectious virus recovered from the BAC plasmids were validated. Growth kinetics were determined using RK13 cells infected with either m8 recovered from pLC16m8.8S-BAC, pLC16m8.8S–B16-LASV_NP, pLC16m8.8S-A40-LASV_Z, or pLC16m8.8S-A46-LASV_GPC at a multiplicity of infection (MOI) of 0.01 (Fig. 7A). The growth efficiency of the recombinant m8s varied depending on the introduced LASV gene. LASV Z did not affect the growth of recombinant m8; however, NP and GPC adversely affected it. Additionally, LASV gene expression in RK13 cells infected with recombinant m8 was validated using western blotting (Fig. 7B). As expected, these genes were expressed in the infected RK13 cells.

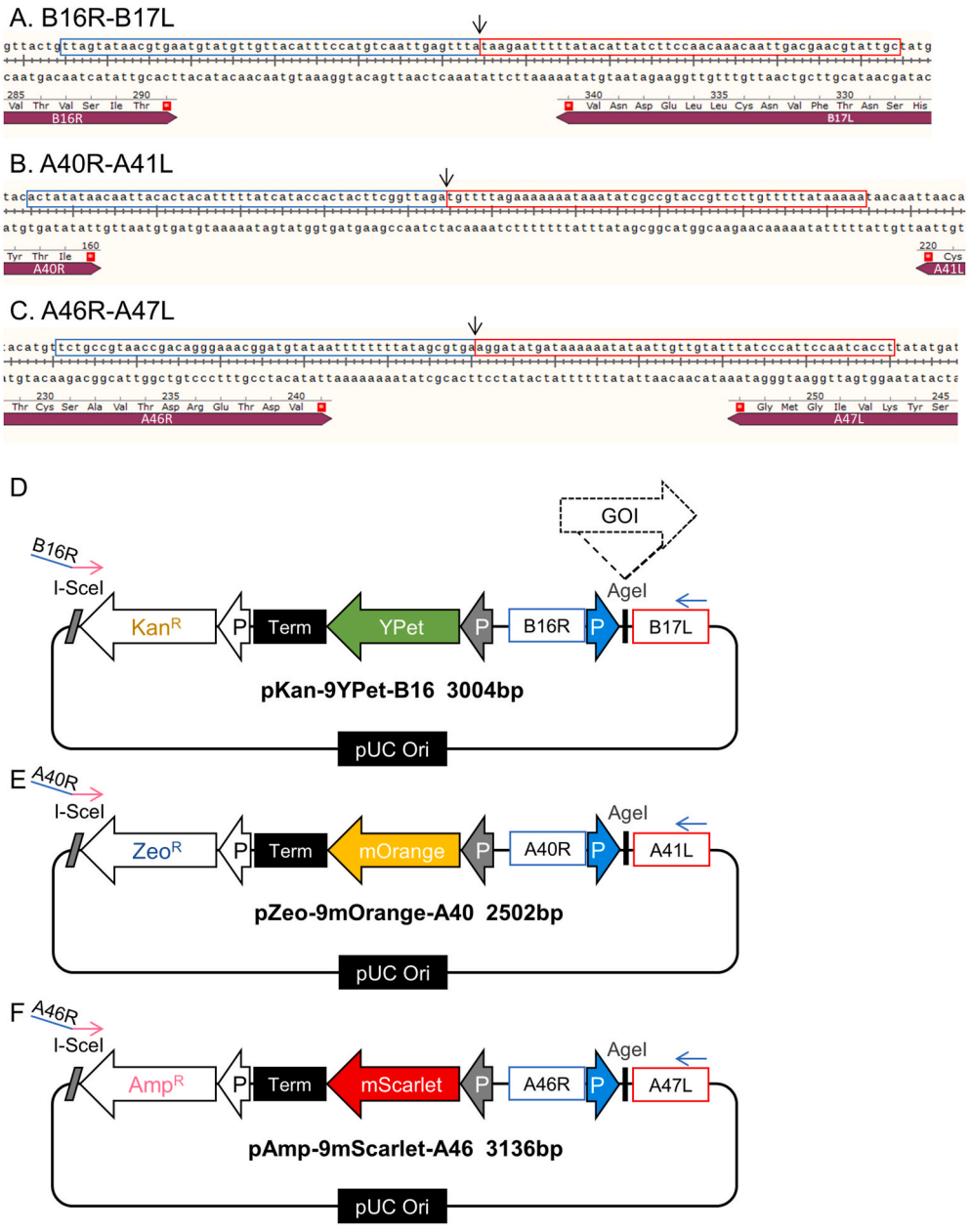

Fig. 4. Selected regions in the m8 genome for introducing expression cassettes of the gene of interest.
The site was sandwiched between B16R and B17L (A), A40R and A41L (B), or A46R and A47L (C) facing each other. The 50 bp of up- (blue box) and downstream (red box) sequences homologous to the transfer constructs are shown. Black arrows indicate the exact sites where expression cassettes were introduced. pKan-9YPet-B16 (D), pZeo-9mOrange-A40 (E), and pAmp-9mScerlet-A46 (F), which contain a vaccinia virus early/late promoter (P in blue arrow) with the *Age*I site flanked by 50 bp homologous sequences (blue or red line boxes) between B16R and B17L, A40R and A41L, and A46R and A47L in the m8 genome. The forward (red arrow) and reverse (blue arrow) primers for preparing the transfer constructs are indicated, and the forward primers contain 50-bp homologous sequences.

3. Discussions

This study has established an improved method for markerless DNA manipulation. The core of this method involves a template plasmid containing expression cassettes for fluorescent genes and antibiotic-resistance genes. This method has the following advantages compared to its predecessor (Table 2): First, it provides a broader range of options for antibiotic-resistance genes (i.e., Kan^R, Amp^R, and Zeo^R). For instance, if Kan^R has already been introduced, the BAC can still be manipulated by Amp^R or Zeo^R. Second, utilizing fluorescent protein genes as selection markers alongside the antibiotic resistance gene during the initial Red recombination

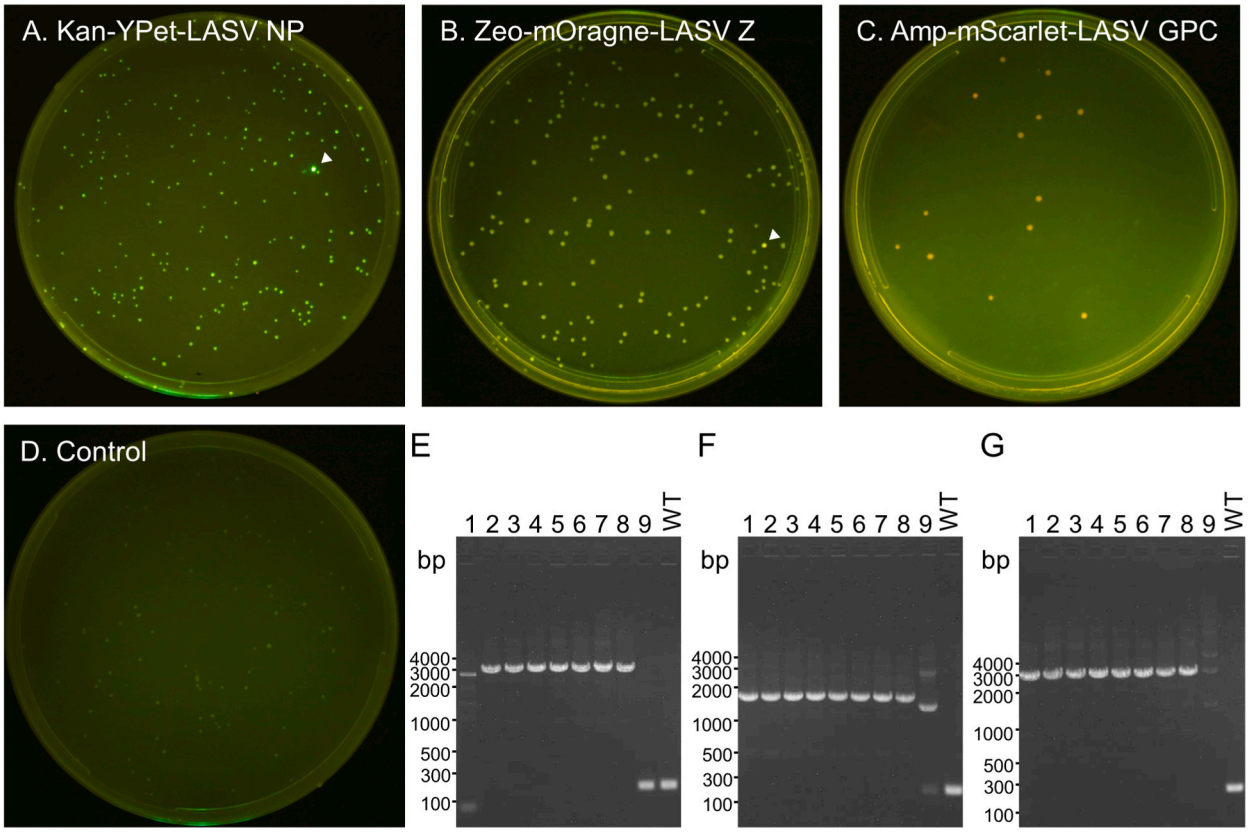

Fig. 5. Introduction of the transfer constructs by first Red recombination.
The GS1783 colonies grew under selective pressure with chloramphenicol and kanamycin, Zeocin, or ampicillin. Fluorescent protein expression as a consequence of the introduction of the transfer constructs, Kan-YPet-LASV NP (A) (Supplementary Fig. 5A), Zeo-mOrange-LASV Z (B) (Supplementary Fig. 5B), and Amp-mScarlet-LASV GPC (C) (Supplementary Fig. 5C) into pLC16m8.8S-BAC was confirmed using an LED transilluminator. A colony with intense fluorescence signals on the agar plates (A) is indicated using an arrowhead. Colonies of GS1783 cells containing pLC16m8.8S-BAC (D) (Supplementary Fig. 5D) were used as negative controls. The insertion of the transfer constructs of Kan-YPet-LASV NP (E) (Supplementary Fig. 5E), Zeo-mOrange-LASV Z (F) (Supplementary Fig. 5F), and Amp-mScarlet-LASV GPC (G) (Supplementary Fig. 5G) was confirmed by colony PCR with gel electrophoresis by picking up nine colonies, and that of the wild-type (WT, i.e., GS1783 cells containing pLC16m8.8S-BAC) as the negative control. The sizes of a DNA ladder marker are indicated on the left. The PCR primers flanked the immediately outside region of homologous recombination, as shown in Fig. 4. The expected size of the PCR amplicons from Kan-YPet-LASV NP, Zeo-mOrange-LASV Z, and Amp-mScarlet-LASV GPC was 3.7 kb (WT; 179 bp), 1.9 kb (WT; 279 bp), and 3.5 kb (WT; 292 bp), respectively.

enhances the likelihood of identifying true positive *E. coli* clones, surpassing the method that relies solely on an antibiotic resistance gene as a selection marker. Third, employing the fluorescent protein gene as a counter-selection marker at the second recombination allows for visual confirmation of BAC retention in *E. coli* clones and the successful removal of selection markers. This confirmation is unachievable using antibiotic-resistance genes alone. Further, none of the GS1783 colonies showed fluorescent signals after the second Red recombination (Fig. 6D–F). Therefore, fluorescent proteins may pose challenges for removing the selection marker cassettes during the second Red recombination.

The colony PCR result to verify the selection cassette removal showed that some GS1783 cells contained neither LASV genes nor a genomic region of m8 (Fig. 6G–I). Therefore, these cells likely contained only a mini-F cassette region with a chloramphenicol resistance gene, and the m8 genomic region in pLC16m8.8S was spontaneously excised during the second Red recombination.

This study adopted fluorescent proteins as positive- and counter-selection markers for achieving "scarless" recombineering. Several genes have been reported as counter-selection markers. The *ccdB* gene, which codes for the toxic protein *CcdB,* is commonly used for counter-selection systems, including BAC recombineering [11]. Expression of *CcdB* is toxic to *E. coli* in the absence of the *ccdA* gene product, which is the antitoxin protein *CcdA*. Therefore, the primary selection is achieved by general antibiotic selection in the presence of *CcdA*, and the removal of *CcdA* expression initiates counter-selection. *CcdA* is generally supplied from an expression plasmid encoding *ccdA* onto the temperature-sensitive pSC101 enabling counterselection through the elimination of the expression plasmid via a standard temperature shift. Therefore, it is impossible to adopt the counter section with *CcdB* for BAC recombineering using GS1793 cells because the temperature shift procedure to remove the *CcdA* expression plasmid conflicts with the induction of the Red recombination system. Alternatively, BAC needs to be transferred between the *CcdB*-resistant and *E. coli*-susceptible strains during recombineering. Another gene, *sacB*, with a gene product that converts sucrose to a toxic metabolite, is also popularly used [12]. Thus,

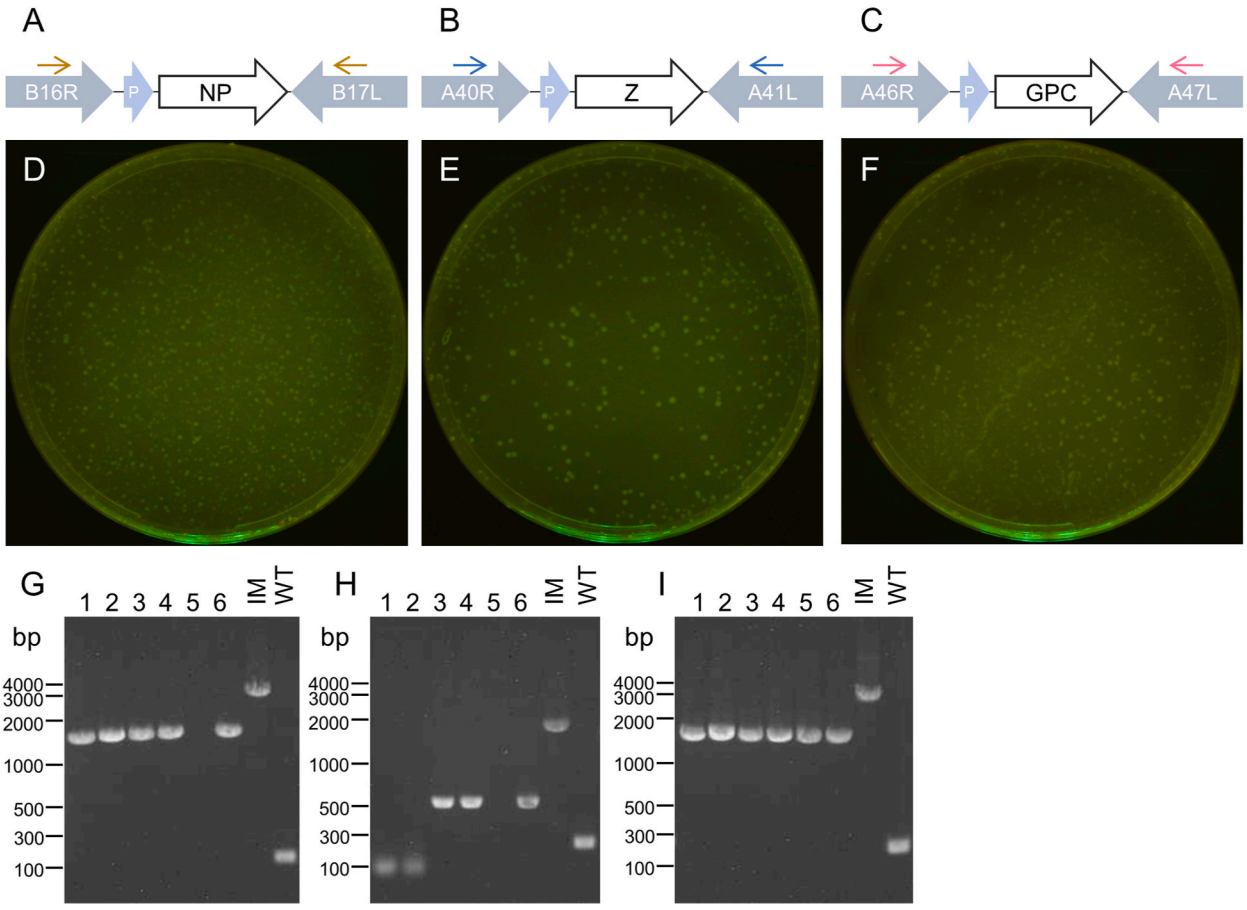

Fig. 6. Removal of the expression cassettes of selection markers by second Red recombination.
Expected schematic arrangements of LASV NP (A), Z (B), and GPC (C) in the final recombinant LC16m8.8S–B16-LASV_NP, pLC16m8.8S-A40-LASV_Z, and pLC16m8.8S-A46-LASV_GPC, respectively, are shown. The removal of the selection markers Kan-YPet (D) (Supplementary Fig. 6D), Zeo-mOrange (E) (Supplementary Fig. 6E), and Amp-mScarlet (F) (Supplementary Fig. 6F) was confirmed by the loss of fluorescence expression in the colonies. In addition, colony PCR with gel electrophoresis was performed to verify the removal of selection markers, Kan-YPet (G), Zeo-mOrange (H), and Amp-mScarlet (I) (Supplementary Figs. 6G–I), in six colonies with the intermediate recombinant obtained at the first Red recombination (IM), as shown in Fig. 5, and that of WT as the negative control. The sizes of a DNA ladder marker were indicated on the left. The expected size of the PCR amplicons from pLC16m8.8S–B16-LASV_NP, pLC16m8.8S-A40-LASV_Z, and pLC16m8.8S-A46-LASV_GPC was 1.9 kb (intermediate; 3.7 kb and WT: 179 bp), 0.6 kb (intermediate; 1.9 kb and WT: 279 bp) and 1.8 kb (intermediate; 3.5 kb and WT: 292 bp), respectively.

counter-selection can be achieved by the expression of *SacB* with the addition of sucrose in the culture media. However, this counter system requires considerable optimization since the counter-selection by the *sacB* gene leads to a high false-positive rate due to the high spontaneous inactivation of *SacB* [13–15]. Therefore, fluorescent genes have sufficient advantages over others for counter-selection.

Finally, the recent outbreak of COVID-19 has reminded us of the importance of basic virological research employing recombinant viruses and rapid vaccine development utilizing various vaccine platforms [16]. Additionally, poxviruses, especially vaccinia viruses, are expected to serve as recombinant vaccine vectors for other infectious diseases, including COVID-19 [17–21]. Fortunately, BACs that contain whole viral genomes have been reported for various viruses, such as coronaviruses, including SARS-CoV-2 [22,23], poxviruses including vaccinia viruses [10,24–26], and herpesviruses [2,27]. Therefore, although further validation is required beyond the BAC containing the m8 genome, the method established in this study is expected to facilitate the generation of recombinant viruses by BAC recombineering and further accelerate research on these viruses and vaccine development.

4. Materials and methods

4.1. *E. coli* strains

The *E. coli* strain GS1783, gifted by Dr. Gregory A Smith from Northwestern University, USA encodes inducible Red- and I-SceI expression in chromosomes [8]. The *E. coli* strain HST16CR (Takara-bio, Shiga, Japan), used for plasmid preparation, is derived from

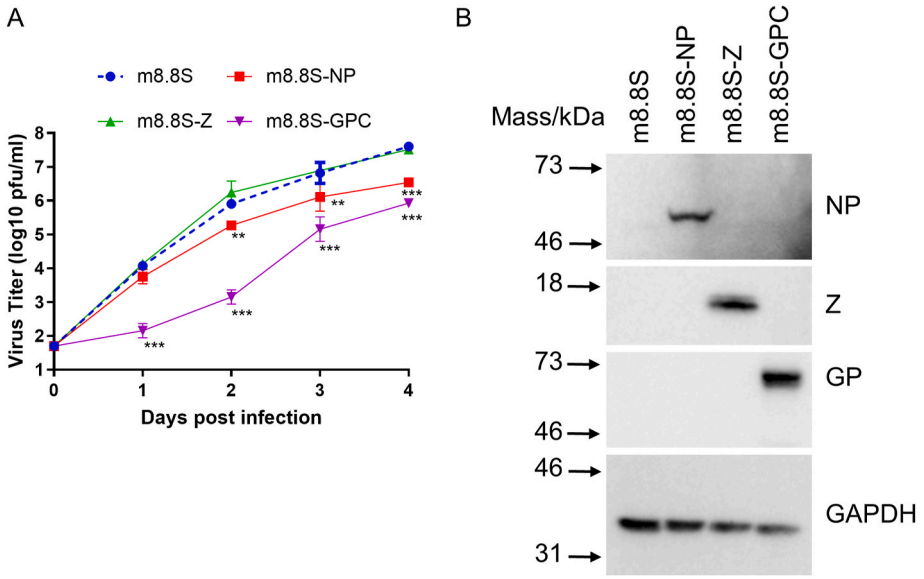

Fig. 7. Growth kinetics (A) and foreign genes expression (B) of the recovered viruses from pLC16m8.8S–B16-LASV_NP, pLC16m8.8S-A40-LASV_Z, and pLC16m8.8S-A46-LASV_GPC.

To measure viral growth, RK13 cells were infected with m8, m8-LASV_NP, m8-LASV_Z, or m8-LASV_GPC at an MOI of 0.01. Cells infected with the culture media were collected and freeze-thawed at the indicated time points. The amount of virus present was determined using a standard plaque assay. To validate LASV gene expression, RK13 cells were infected with m8, m8-LASV_NP, m8-LASV_Z, or m8-LASV_GPC at an MOI of 0.1. The infected cells were lysed 2 days post-infection, resolved by SDS-PAGE, and analyzed by western blotting using rabbit anti-LASV NP, Z, GPC, or anti-GAPDH polyclonal antibody (Supplementary Fig. 7B). The sizes of a protein molecular weight marker are indicated on the left. A two-way ANOVA with Dunnett's multiple comparisons test was used to determine the statistical significance on each day of post infection. Asterisks (** or ***) are shown in the figure only when the calculated p-value is less than 0.1 or 0.001, respectively, when comparing m8 with the others.

Table 2
Advantages of the improved method in this study.

Method	Selection marker		Advantages
	Previous report	This study	
Positive selection	Kan^R	Amp^R + mScarlet	Broader choice of antibiotic-resistance genes
		Kan^R + YPet	Enhanced identification of true positive *E. Coli* clones by employing a fluorescent protein gene
		Zeo^R + mOrange	
Counter selection	None	mScarlet YPet mOrange	Visual confirmation of retention and successful removal

the HST08 strain (Takara-bio) by removing the *pcnB* gene to reduce the copy number of ColE1-like plasmids, including pUC. The genotype of HST16, which is F^-, *endA1*, *supE44*, *thi-1*, *recA1*, *relA1*, *gyrA96*, *phoA*, Φ80d*lacZ*Δ*M15*, Δ(*lacZYA-argF*)*U169*, Δ(*mrr-hsdRMS-mcrBC*), Δ*mcrA*, Δ*pcnB*, λ^-, is identical to that of HST08 except for Δ*pcnB*.

4.2. Plasmids

The BAC plasmid, pLC16m8.8S-BAC (accession no. LC315596), contains the full genome of the vaccinia virus strain LC16m8 with self-excisable EGFP and mini-F cassettes [10]. Basic template plasmids pAmp-S, pKan-S, and pZeo-S were constructed from pUC19. These plasmids contain an I-SceI recognition site and antibiotic resistance genes: Amp^R, driven by its original (beta-lactamase) promoter from pUC19, Kan^R from pACYC177, or Zeo^R, which was synthesized and codon-optimized for *E. coli* (Integrated DNA Technologies, Inc., Coralville, IA, USA) driven by the beta-lactamase promoter. The characteristics of fluorescent proteins used in this study [28–31] are summarized in Table 1, and these protein genes were obtained from the following plasmids. The mClover3 gene was obtained from pNCS-mClover3 gifted by Michael Lin (#74236; Addgene, Watertown, MA, USA) [30]. The YPet gene was obtained from pCEP4YPet-MAMM, gifted by Patrick Daugherty (#14032, Addgene) [32], and was later synthesized and codon-optimized for *E. coli* (Integrated DNA Technologies). The mOrange gene was obtained from mOrange-pBAD, gifted by Michael Davidson (#54751, Addgene). The mKO2 gene was obtained from mKO2-pBAD gifted by Michael Davidson and Atsushi Miyawaki (#54555, Addgene) [33]. The mScarlet gene was obtained from pmScarlet_C1, which was a gift from Dorus Gadella (#85042, Addgene) [29] and was later synthesized and codon-optimized for *E. coli* (Integrated DNA Technologies). The fluorescent protein genes were ligated with a constitutive promoter (BBa_J23119) in *E. coli* from the Anderson promoter collection in the Registry of Standard Biological Parts

(http://parts.igem.org/Promoters/Catalog/Anderson), with or without the rrnB T1 terminator, into *Bam*HI-digested pAmp-S. The plasmid containing the pAmp-S mScarlet expression cassette was named as pAmp-9mScarlet. Similarly, YPet or mOrange was ligated with Bba_J23119 and rrnB T1 terminator into *Bam*HI-digested pKan-S or pZeo-S, and these were named pKan-9Ypet or pZeo-9mOrange, respectively. Synthesized DNA fragments containing a synthetic vaccinia virus early/late promoter [34], flanked by 50 bp of untranslated regions between B16R (sequence positions 191184 to 191233 in pLC16m8.8S-BAC) and B17L (191234–191283), A40R (156625–156674) and A41L (156675–156724), or A46R (161061–161110) and A47L (161111–161160) (Integrated DNA Technologies) were ligated into *Bam*HI-digested pKan-9YPet, pZeo-9mOrange, or pAmp-9mScarlet. The plasmids were named pKan-9YPet-B16, pZeo-9mOrange-A40, and pAmp-9mScarlet-A46, respectively. The total lengths of the NP, Z, and GPC genes of LASV strain Josiah (accession no. HQ688672 and HQ688674) were synthesized (Integrated DNA Technologies) and ligated into *Age*I digested pKan-9Ypet-B16, pZeo-9mOrange-A40, and pAmp-9mScarlet-A46. The plasmids were named pKan-9YPet-B16-LASV_NP, pZeo-9mOrange-A40-LASV_Z, and pAmp-9mScarlet-A46-LASV_GPC, respectively.

4.3. Cells and viruses

293FT (Thermo Fisher Scientific, Waltham, MA) and RK13 cells, which was kindly gifted from Chiba Serum Research Institute, Chiba, Japan, were grown in Dulbecco's Modified Eagle's Medium (DMEM; WAKO, Osaka, Japan) supplemented with 5% heat-inactivated fetal bovine serum (FBS). The fowlpox virus strain Beaudette was propagated and titrated on chicken embryo fibroblast cells. Virus titration was based on the standard plaque assay and visualization of the plaques with 100-fold diluted chicken anti-fowlpox antibody (MyBioSource, Vancouver, Canada), followed by incubation with 1.5 μg/mL Pierce FITC-conjugated rabbit anti-chicken IgY (Thermo Fisher Scientific).

4.4. Preparation of electrocompetent cell

Electrocompetent cells were prepared from GS1783 cells containing pLC16m8.8S by inducing the Red recombination system. Briefly, the GS1783 cells were cultivated in 200 mL of LB broth Lennox (Nacalai Tesque, Kyoto, Japan) with 12.5 μg/mL chloramphenicol at 32 °C until OD600nm of approximately 0.5 was obtained. Next, the cultured cells were incubated at 42 °C for 15 min to induce Red recombination and then chilled in ice-cold water for 15 min. After Red induction, the cells were washed twice with 100 mL of H_2O and twice with 10% glycerol in H_2O (v/v). Finally, the cells were aliquoted and stored at −80 °C until needed.

4.5. Preparation of transfer constructs

Transfer constructs were prepared from the template plasmids pKan-9Ypet-B16-LASV_NP, pZeo-9mOrange-A40-LASV_Z, and pAmp-9mScarlet-A46-LASV_GPC using PCR amplification. Briefly, 100 ng/μL plasmid was added to a 30-μL reaction mixture, which contained 15 μL of 2x PrimeSTAR Max DNA Polymerase premix (Takara-bio), 300 nM each specific primer, and H_2O. The primers used for PCR amplification were Red-B16-F (TTAGTATAACGTGAATGTATGTTGTTACATTTCCATGTCAATTGAGTTTA GCCGCCAGTGT-GATGGATAT), Red-B16-R (GCAATACGTTCGTCAATTGTTTGTT), Red-A40-F (ACTATATAACAATTACACTACATTTTTATCA-TACCACTACTTCGGTTAGA TTTTCTACGGGGTCTGACGC), Red-A40-R (TTTTTATAAAAACAAGAACGGTACGGCGATATTTA), Red-A46-F (TCTGCCGTAACCGACAGGGAAACGGATGTATAATTTTTTTTATAGCGTGA AGTGTGATGGATATCTGCAGAATTC), and Red-A46-R (AGGTGATTGGAATGGGATAAATACAAC). These primers contained specific sequences of the plasmids with 50 bp homologous sequences in the m8 genome, indicated in italics. A total of 40 amplification cycles were performed under the following conditions: denaturing at 98 °C for 10 s, annealing at 60 °C for 5 s, and extension at 72 °C for 5 s/kb. This was followed by a 1 °C decrease in the annealing temperature for the first five cycles and maintenance of the annealing temperature at 55 °C for the rest of the cycles. The reaction mixtures were then incubated with 1 μL of the restriction enzyme, *Dpn*I, at 37 °C for 1.5 h to digest the template plasmids. The transfer constructs were purified from the reaction mixtures with 20 μL elution buffer using the NucleoSpin Gel and PCR Clean-up kit (Takara Bio), according to the manufacturer's protocol.

4.6. Red recombination

The transfer constructs were inserted into the pLC16m8.8S-BAC using the first Red recombination as follows. Electrocompetent GS1783 cells containing pLC16m8.8S-BAC were electroporated with approximately 100 ng transfer constructs at 1800V using an Eporator (Eppendorf, Hamburg, Germany). The cells were precultured in 1 mL of LB broth Lennox at 32 °C for 70 min. Subsequently, they were cultivated on LB agar plates at 32 °C for 1–2 days with 12.5 μg/mL of chloramphenicol and either 50 μg/mL ampicillin, 25 μg/mL kanamycin, or 25 μg/mL Zeocin selective pressure. Antibiotic-resistant colonies were further examined for fluorescent protein expression using visualization on an LED transilluminator (GELmieru, WAKO) at a wavelength of 500 nm. The colonies exhibiting intermediate but not strong-fluorescence intensity were selected and streaked onto a fresh LB agar plate at 32 °C for 1 day under appropriate antibiotic selective pressure. Standard colony PCR was performed to confirm successful recombination. Briefly, the 10-μL reaction mixture contained a selected colony, 5 μL of KOD, one PCR master mix (TOYOBO, Osaka, Japan), 300 nM of each specific primer, and H_2O. A total of 45 amplification cycles were conducted under the following conditions: denaturation at 98 °C for 10 s, annealing at 65 °C for 5 s, and extension at 68 °C for 5 s/kb. This was followed by a 1 °C decrease in the annealing temperature for the first five cycles, and maintenance of the annealing temperature at 60 °C for the remaining cycles. The primers Insert-B16R–B17L-F and –R (CGTTTACGTGTATGGCGTTT and ATGCATAGTATCGTGGGAGT), Insert-A40R-A41L-F and –R (TACTCCCAAACTGCATTCGT and

TTGCGTAAACGATGTGACTG), and Insert-A46R-A47L-F and –R (ATGTAAGTATCTACGCGGGC and TGCGGGTCTAACCATAAAGT), which flanked the recombination sites, were used for PCR amplification. The primers contained specific sequences of the plasmids and 50 bp homologous sequences in the m8 genome (italic characters). After electrophoresis on 1% agarose gels, PCR amplicons with the expected sizes were visualized by staining with UltraPower DNA Safedye (Gellex International, Tokyo, Japan). The PCR amplicons were also used for sequence confirmation using Sanger sequencing with a 3500 Genetic Analyzer (Thermo Fisher Scientific). The second Red recombination was performed to remove the expression cassettes of antibiotic-resistant and fluorescent genes with I-SceI digestion to generate a final recombinant. A single colony on the streak plate was transferred and incubated in 100 μL of LB broth with 12.5 μg/mL chloramphenicol at 32 °C for 1 h. Next, 5 μL of 20% (w/v) L-arabinose (Tokyo Chemical Industry, Tokyo, Japan) was added to 100 μL of the culture to a final concentration of 1%, and further incubated at 32 °C for 1 h for the induction of the I-SceI digestion system. The culture was then incubated at 42 °C for 30 min and cultivated on LB agar plates with 12.5 μg/mL chloramphenicol selective pressure at 32 °C for 1–2 days. Chloramphenicol-resistant colonies were further checked for the loss of fluorescent gene expression. Standard colony PCR was performed using the primer sets described above, and the sequencing of the PCR amplicons.

4.7. Recovery of recombinant m8 from BAC

Infectious recombinant m8s were recovered from pLC16m8.8S-BAC, as previously described [10]. Briefly, 2.5×10^5 293FT cells were transfected with 0.5 μg of a purified pLC16m8.8S-BAC containing a LASV gene and 1.5 μL of X-tremeGENE 9 (Roche Applied Science, Penzberg, Germany). The 293FT cells were immediately infected with the fowlpox virus, commonly used as a helper virus to provide transcriptional machinery for the recovery of infectious vaccinia viruses from BACs containing the virus genome [1,10,25], at an MOI of 1. The cells were collected by freeze-thawing three times to prepare a recovered virus stock when EGFP-positive plaques were confirmed on the cell monolayer by fluorescent microscopy 4–6 days post-infection. The final recombinant m8, from which the EGFP and mini-F cassettes were removed from the viral genome by self-excision, was plaque-purified from the recovered virus stocks in the absence of EGFP expression as an indicator using RK13 cells. The final recombinant m8 was expanded twice (i.e., passage number 2) in RK13 cells to create a working stock.

4.8. Confirmation of LASV genes expression

RK13 cells were infected with recombinant m8s expressing LASV at an MOI of 0.1. The cells were lysed with sodium dodecyl sulfate (SDS) sample buffer two days post-infection. The cell lysates were fractionated using SDS polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to western blotting. The membrane was reacted with an in-house rabbit anti-LASV NP polyclonal antibody, rabbit anti-LASV Z polyclonal antibody (number 0307-002, IBT Bioservices, Northbrook, IL, USA), rabbit anti-LASV glycoprotein G2 polyclonal antibody (number GTX134883, GeneTex, Irvine, CA, USA), or rabbit anti-GAPDH polyclonal antibody (number 10494-1-AP, Proteintech, Rosemont, IL, USA), followed by reaction with horseradish peroxidase-conjugated goat anti-rabbit IgG (H + L) antibody (KPL, Gaithersburg, MD, USA). Immunoreactive proteins were visualized using LAS-3000 (Fujifilm, Tokyo, Japan) with SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific).

4.9. Statistical analysis

An unpaired *t*-test and a two-way ANOVA with Dunnett's multiple comparisons test were used to determine the statistical significance. Statistical analysis was performed using GraphPad Prism 9.3.0 software (GraphPad Software, La Jolla, CA). Statistical significance was set at $p < 0.05$.

Declarations

Author contribution statement

1 - Conceived and designed the experiments;
2 - Performed the experiments;
3 - Analyzed and interpreted the data;
4 - Contributed reagents, materials, analysis tools or data;
5 - Wrote the paper.

Data availability statement

Data will be made available on request.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e18983.

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