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Research Article

Efficient assembly of a large fragment of monkeypox virus genome as a qPCR template using dual-selection based transformation-associated recombination

Lei Yang [a,b], Lingqian Tian [a,b], Leshan Li [a,b], Qiuhong Liu [a,b], Xiang Guo [a,b], Yuan Zhou [a], Rongjuan Pei [a], Xinwen Chen [a,*], Yun Wang [a,*]

[a] State Key Laboratory of Virology, Wuhan Institute of Virology, Center for Biosafety Mega-Science, Chinese Academy of Sciences, Wuhan, 430071, China
[b] University of Chinese Academy of Sciences, Beijing, 100049, China

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ABSTRACT

Transformation-associated recombination (TAR) has been widely used to assemble large DNA constructs. One of the significant obstacles hindering assembly efficiency is the presence of error-prone DNA repair pathways in yeast, which results in vector backbone recircularization or illegitimate recombination products. To increase TAR assembly efficiency, we prepared a dual-selective TAR vector, pGFCS, by adding a P_{ADH1}-URA3 cassette to a previously described yeast-bacteria shuttle vector, pGF, harboring a P_{HIS3}–HIS3 cassette as a positive selection marker. This new cassette works as a negative selection marker to ensure that yeast harboring a recircularized vector cannot propagate in the presence of 5-fluoroorotic acid. To prevent pGFCS bearing *ura3* from recombining with endogenous *ura3-52* in the yeast genome, a highly transformable *Saccharomyces cerevisiae* strain, VL6-48B, was prepared by chromosomal substitution of *ura3-52* with a transgene conferring resistance to blasticidin. A 55-kb genomic fragment of monkeypox virus encompassing primary detection targets for quantitative PCR was assembled by TAR using pGFCS in VL6-48B. The pGFCS-mediated TAR assembly showed a zero rate of vector recircularization and an average correct assembly yield of 79% indicating that the dual-selection strategy provides an efficient approach to optimizing TAR assembly.

1. Introduction

One of the characteristic features of yeast is that exogenous DNA fragments can be efficiently taken up and recombined. Typically, two linearized DNA fragments with 60 base pairs (bp) of overlapping sequences can be readily recombined and ligated by homologous recombination (HR) in yeast (Noskov et al., 2001). Based on this feature, transformation-associated recombination (TAR) was developed. TAR has shown great value in the isolation of chromosomal fragments from the genomic DNA pool (TAR cloning), as well as in the assembly of multiple DNA fragments (TAR assembly) into a single yeast or bacterial artificial chromosome (YAC or BAC) [reviewed in (Kouprina and Larionov 2016)]. The basic approach of TAR is to use a linearized vector to capture DNA of interest by "hook" sequences through HR after they have been cotransformed into yeast cells. However, the efficiency of TAR can be severely hampered by error-prone DNA repair pathways, including but not limited to the nonhomologous end joining (NHEJ) or microhomology-mediated end joining (MMEJ) [reviewed in (Lewis and Resnick, 2000)]. It was estimated that at least 10%–80% of yeast transformants contain false TAR products, and a considerable fraction is attributed to vector recircularization (Kuijpers et al., 2013).

To enhance the efficiency of TAR cloning, Vladimir and Natalay et al. designed a dual-selection system (Noskov et al., 2003). In addition to an autonomously replicating sequence (ARS) that supports plasmid replication in yeast, two auxotrophic selection markers were introduced into the TAR vector. A HIS3 expression cassette serves as a positive selection marker to ensure that recombinants survive in synthetic defined media without histidine (SD/-His). The "hook" sequences are inserted between an ADH1 promoter (P_{ADH1}) and a URA3 coding sequence. The interspace between P_{ADH1} and URA3 can tolerate DNA insertions of up to 130 bp in length (Furter-Graves and Hall, 1990; Miret et al., 1998), providing enough space for two 60-bp "hooks" to capture DNA. When the linearized TAR vector is recircularized through NHEJ or MMEJ, URA3 directly reconnects to P_{ADH1} without insertion. Thus, the P_{ADH1}-controlled expression of URA3 converts 5-fluoroorotic acid (5-FOA) in the culture medium into the toxic product 5-fluorouracil and kills yeast bearing the

* Corresponding authors.
E-mail addresses: chenxw@wh.iov.cn (X. Chen), wangyun@wh.iov.cn (Y. Wang).

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recircularized TAR vector. In contrast, if the TAR vector captures DNA using its hook through HR, then the insertion between P_{ADH1} and URA3 is larger than 130 bp in length, and URA3 expression is abolished. The resulting yeast bearing legitimate recombination products can propagate in the presence of 5-FOA. Thus, P_{ADH1}-URA3 serves as a negative selection marker to abolish vector recircularization. For TAR cloning, the dual-selective system could lead to a greater than tenfold increase in the number of legitimate cloning products (Noskov et al., 2003).

Although the dual-selection system has proven beneficial for TAR cloning, it has never been applied to TAR assembly. A previously described yeast-bacteria shuttle vector pGF, which harbors a P_{HIS3}–HIS3 cassette as a positive selection marker, has been successfully applied to assemble large microbial genomes (Hou et al., 2016; Shang et al., 2017). In this study, we added the P_{ADH1}-URA3 cassette to pGF as a negative selection marker and explored whether the dual-selection system could be applied in TAR assembly.

Monkey poxviruses (MPXVs) are large DNA viruses with reported genome sizes ranging from 190,083 to 206,372 bp in length. As a member of the *Orthopoxvirus* genus in the family *Poxviridae*, MPXV is subdivided into the West African and Congo Basin clades. The latter is more pathogenic and has been reported to infect humans in various parts of the world [reviewed in (Brown and Leggat, 2016)]. Quantitative polymerase chain reaction (qPCR) is the gold standard for the detection of orthopoxvirus (including MPXV). For pan-orthopoxviruses detection, the *E9L* (DNA polymerase) gene has been shown to be an excellent target for qPCR assays (Kulesh et al., 2004). For MPXV detection, Li et al. reported that the *C3L* (complement-binding protein) gene could be used as the qPCR target for the MPXV Congo Basin strain (Li et al., 2010). Since MPXV infection has never been associated with an outbreak in China, the viral genomic material required for qPCR detection is unavailable. In this report, we employed dual-selective TAR to assemble a 55-kb MPXV genomic fragment that encompasses *E9L* and *C3L*, two valuable qPCR targets for detecting MPXV or other orthopoxviruses. The dual-selective TAR assembly showed a zero rate of vector recircularization and an average legitimate assembly yield of 79%, demonstrating that P_{ADH1}-URA3, serving as a negative selective marker, can optimize TAR assembly by abolishing vector recircularization.

2. Material and methods

2.1. Microbial strains and plasmid information

Saccharomyces cerevisiae strain VL6-48 (*MATα, his3-delta200, trpl-delta1, ura3-52, ade2-101, lys2, psi+cir°*) (Larionov et al., 1994), which has been extensively employed in TAR cloning and assembly, was chosen for genetic modification. *Escherichia coli* strain EPI300 (Lucigen), which supports high copy number amplification of plasmids bearing *ori*V from plasmid RK2 (Wild and Szybalski, 2004), was selected as the bacterial host for YAC/BAC amplification. The yeast-bacteria shuttle vector pGF harboring an *oriV* and a P_{HIS3}–HIS3 expression cassette was previously described (Hou et al., 2016).

2.2. Yeast transformation and gene disruption

To disrupt *ura3-52* in the VL6-48 genome, the blasticidin S deaminase (BSD) gene (514 bp in length) was amplified by polymerase chain reaction (PCR) with the primer pair URA3-BSD-S/A (Supplementary Table S1). The upstream and downstream regions of *ura3-52* were incorporated into the PCR product at the 5′- and 3′-termini (Fig. 1A), respectively, serving as homologous arms for chromosomal substitution of *ura3-52* with *bsd* by HR. The PCR product was transformed into VL6-48 using a Gene Pulser Xcell Electroporation System (Bio-Rad) following a previously published protocol (DiCarlo et al., 2013). After transformation, the yeast cells were spread onto a YPD agar plate containing blasticidin (300 μg/mL) and incubated at 30 °C for 48–72 h until colonies appeared. The genomic content of the transformed yeast was extracted and subjected to genotyping. Primers for genotyping (BSD-S/Ter, Supplementary Table S1) were located at the start codon and 184 bp downstream of the stop codon of *ura3-52* (Fig. 1A). The yeast strain with *ura3-52* replaced by *bsd* was designated VL6-48B. Sequence information of all the primers and probes used in this study is listed in Supplementary Table S1.

2.3. Plasmid construction

An expression cassette of P_{ADH1}-URA3 was cloned from pCAP-BAC by PCR (Bauman et al., 2019). The P_{ADH1}-URA3 cassette was then inserted into the pGF backbone between the M13 forward primer site and the SP6 promotor using the ClonExpress Ultra One Step Cloning Kit according to the manufacturer's protocol (Vazyme). The resulting vector was designated as pGFCS (pGF with a negative selection marker, Fig. 2A; full sequence is attached in supplementary materials).

2.4. Viral sequence

The 5′-end terminus (1–55543 bp) of the MPXV strain Congo_2003_358 (GenBank accession DQ011154.1) genome was chemically synthesized. The synthesized viral genomic DNA includes four continuous segments. Each viral segment was approximately 15 kb in length (Fig. 3A), with 100 bp of overlap between adjacent segments.

2.5. TAR assembly

The TAR vector pGFCS was linearized by two PCRs using the primer pairs ARS-HIS3/60-ADH1 and HIS3-ARS/55483-URA3 (diagrammed in Fig. 3A). The resulting two PCR fragments overlapped at one end between ARS and P_{HIS3}, and the other ends overlapped with 1–60 bp or 55483–55543 bp of MPXV (Fig. 3A). Then, equal molar amounts (0.05 pmol) of pGFCS amplicons and viral fragments were mixed and transformed into competent VL6-48B by electroporation as described in the previous section. Transformed yeasts were cultured on SD/-His agar plates supplemented with 5-FOA (1 mg/mL). At 72 hpt, 16 yeast colonies from pGFCS transformants were picked, and the DNA contents were extracted and subjected to multiplex PCR assay.

2.6. Multiplex PCR

Multiplex PCR was performed by adding the indicated primer pairs (0.1 μmol/L of each primer) and 1 μL of each assembly product into 2 × multiplex buffer of the Multiplex PCR kit (Vazyme). The multiplex PCR reaction program was as follows: DNA denaturation at 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 60 °C for 90 s, 72 °C for 45 s; and 72 °C for 10 min as a final extension.

2.7. Quantitative PCR

The primers and probes for the detection of pan-orthopoxviruses (E9L) and MPXV Congo Basin strains (C3L) by qPCR have been described previously (Kulesh et al., 2004; Li et al., 2010). The assembled product was serially diluted to 1×10^1–10^9 copies/μL, and one μL of each dilution was subjected to qPCR assay as the template. The qPCR reaction was prepared by mixing the required components (primers, probe, and template) with Taq Pro HS Universal Probe Master mix (Vazyme). The copy numbers of MPXV genomic fragments were measured using an ABI QuantStudio 6 Flex real-time PCR system.

3. Results

3.1. Chromosomal substitution of ura3-52

Since *ura3* was added to the vector backbone as a negative selection marker, the *ura3-52* sequence in the VL6-48 genome must be removed to prevent the vector from recombining with the yeast chromosome.

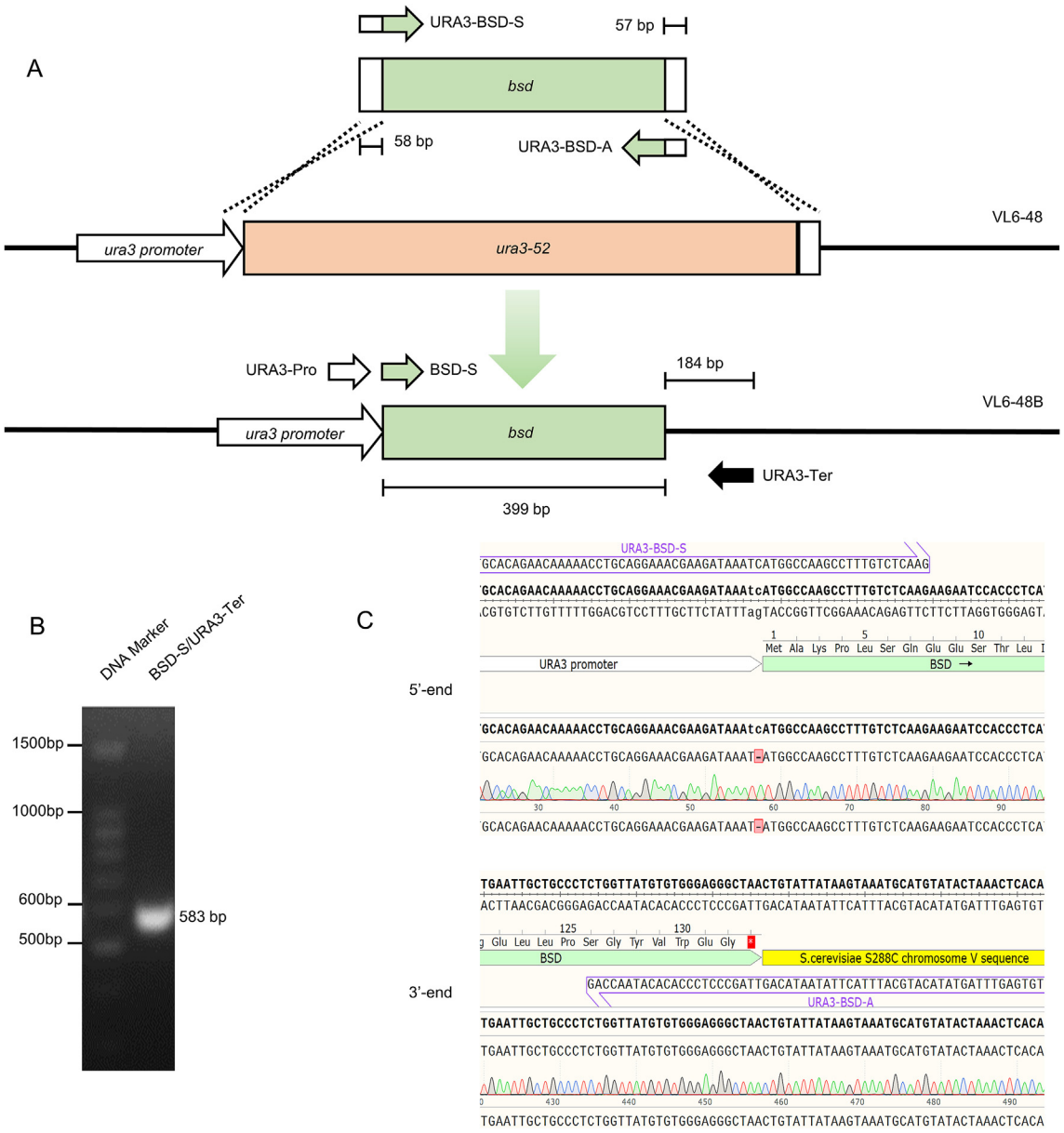

Fig. 1. Development of the *ura3-52*-defective strain VL6-48B. **A** A diagram of the *ura3-52* locus in VL6-48 and primer pairs for amplifying the *bsd* gene (URA3-BSD-S/A), genotyping (BSD-S/URA3-Ter), and sequencing (URA3-Pro) of the transgene. The PCR amplicon of *bsd* contains 58- and 57-bp homologous sequences surrounding the *ura3-52* open reading frame (ORF) at its 5′- and 3′-termini. The primers for genotyping targeted the start codon and 184 bp downstream of the *ura3-52* ORF. **B** The genomic content of transformed yeast was extracted and served as the template for genotyping PCR. The amplicon was then subjected to gel electrophoresis, and a 583-bp band was seen by ethidium bromide staining. **C** The genomic content of transformed yeast was extracted and subjected to sequencing using the primer URA3-Pro. The sequenced region consists of *bsd* at the center region and the *ura3* surrounding sequence at the 5′- and 3′-ends.

Genotyping of the blasticidin-resistant yeast showed that a 583-bp PCR product was amplified from the transformed yeast, identical to the calculated length of the *bsd* insertion (Fig. 1B). Sequencing of the yeast's genomic DNA confirmed that a *bsd* gene had been inserted into the coding region of *ura3-52* (Fig. 1C). Therefore, the transformed yeast was designated VL6-48B.

3.2. Construction of a TAR vector with dual-selection markers

To prepare pGFCS, a P_{ADH1}-URA3 expression cassette was inserted into the pGF vector backbone (Fig. 2A). The assembled plasmid was validated by *Kpn*I digestion, and the restriction pattern was identical to the computer-predicted pattern (Fig. 2B). To test whether pGFCS could be used in dual selection, pGF or pGFCS was transformed into VL6-48B cells cultured in SD/-His agar plates supplemented with 5-FOA (1 mg/mL). As expected, VL6-48B itself failed to form any colonies on SD/-His agar plates (Fig. 2C). Transformation of pGF into VL6-48B led to the formation of yeast colonies on SD/-His agar plates at 48 hpt (Fig. 2C), indicating that the P_{HIS3}–HIS3 expression cassette provides a positive selection marker to support VL6-48B growth in histidine-free medium. In contrast, the pGFCS transformant failed to form colonies even at 72 hpt in the presence of 5-FOA (Fig. 2C), suggesting that the P_{ADH1}-URA3 cassette of pGFCS serves as a negative selection marker to stop yeast growth by converting 5-FOA to cytotoxic 5-fluorouracil. After a 15-kb DNA fragment was inserted into pGFCS between P_{ADH1} and URA3, the resulting construct was transformed into VL6-48B. At 48 hpt, yeast colonies could be seen in the SD/-His agar plate supplemented with 5-FOA (Fig. 2C), indicating that URA3 expression was abolished by DNA insertion. Taken

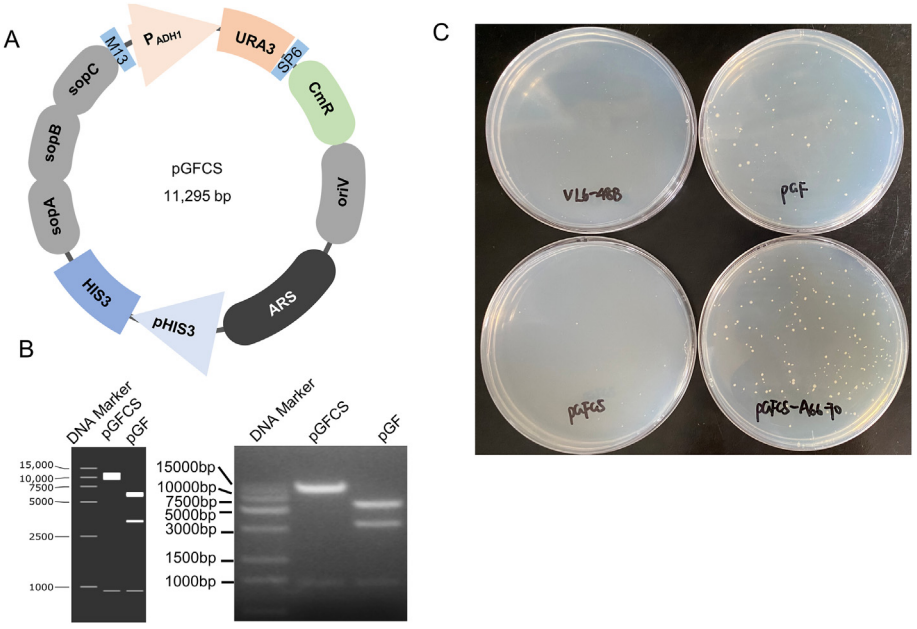

Fig. 2. Preparing pGFCS with dual selection capability in VL6-48B. **A** Plasmid map of pGFCS. The yeast-bacteria shuttle plasmid pGFCS was generated by the insertion of a P_{ADH1}-URA3 expression cassette into pGF. **B** Restriction pattern of pGF and pGFCS. A *Kpn*I restriction digestion was performed to validate the construction of the pGFCS. The pGF and pGFCS digestion products were separated by gel electrophoresis and were compared to the computer-predicted *Kpn*I restriction pattern (left panel). **C** Dual selection in VL6-48B. Three shuttle plasmids, pGF, pGFCS, and pGFCS-A66-70 (pGFCS with a 15-kb DNA fragment insertion), were transformed into VL6-48B cells. The transformed and untransformed yeast were cultured on SD/-His agar plates supplemented with 5-FOA.

together, the transformation of pGFCS to VL6-48B enables dual selection for screening yeast transformants.

3.3. Assembly of an MPXV genomic fragment by TAR

To prepare a qPCR detection template for MPXV, pGFCS was used as a TAR vector to capture and assemble the four synthesized MPXV segments into one large fragment in VL6-48B. Multiplex PCR was performed to screen the candidate assembly products. Five primer pairs (Supplementary Table S1), which anneal to the upstream and downstream regions of each TAR recombination site (Fig. 3A), were used to validate the assembly products. A legitimate assembly product should generate five multiplex amplicons, sized 582 bp (Primer pair: ADH1/411), 291 bp (Primer pair: 14136/14426), 411 bp (Primer pair: 26501/26911), 348 bp (Primer pair: 40873/41220), and 665 bp (Primer pair: 55377/URA3) in length. If vector recircularization had ever occurred, then a single amplicon of 653 bp in length should be generated by the primer pair ADH1/URA3 included in the primer mixture. Multiplex PCR assays of three independent TAR assembly experiments showed that on average, 79% of pGFCS-transformants contained legitimate assembly products (Fig. 3B). Notably, none of these transformants generated a single amplicon of 653 bp, indicating that vector recircularization had been efficiently abolished (Fig. 3B). To further validate the assembled product, one of the pGFCS transformants with a correct multiplex amplicon pattern was transformed to EPI300 for high-efficiency plasmid amplification. Amplified plasmids were subjected to restriction enzyme digestion by *Bam*HI, and the restriction pattern was identical to the computer-predicted pattern (Fig. 3C). The assembly product was confirmed by Sanger sequencing (Supplementary material).

3.4. Quantification of the assembly product by qPCR

The primary purpose of assembling a fragment of the MPXV genome is to provide a nucleotide template for MPXV detection. By plotting the Ct values against the number of copies in each dilution, linear regression lines were drawn. The coefficient of determination (R2) of E9L was 0.9968, and that of C3L was 0.9981, indicating that either qPCR showed a strong linear relationship between the mean Ct values and the logarithm of the template DNA concentration (Fig. 4).

4. Discussion

HR occurs in all cell types as an essential tool to repair DNA with double-stranded breaks (DSBs). Through the activity of *Rad52* [reviewed in (Paques and Haber, 1999)], HR-mediated DNA repair is a high-fidelity process. In contrast to HR, *Ku*-dependent NHEJ provides an alternative repair pathway for DNA breaks [reviewed in (Critchlow and Jackson, 1998)], featuring direct ligation of two break ends with no homology template required. Recently, MMEJ was defined as a new type of DNA repair in mammalian cells and yeast cells (Ma et al., 2003). MMEJ is dependent on *Mre11*, *Rad50*, and *Rad1* (Ma et al., 2003) and repairs DNA lesions in an error-prone manner. To assemble legitimate products by TAR, HR rather than NHEJ or MMEJ is required because the latter two usually result in vector recircularization or illegitimate recombination products due to their error-prone nature.

In this study, we demonstrated that the introduction of P_{ADH1}-URA3 to the vector backbone can effectively abolish vector recircularization during TAR assembly through a negative selection procedure. However, a legitimate TAR assembly product is obtained through multiple HR events, depending on the number of DNA fragments to be assembled. Although the current dual-selection system can effectively guarantee that DNA fragments (*e.g.,* vector backbone fragments in this study) bearing negative selection markers recombine with homologous sequences through HR, it is unable to prevent other DNA fragments without a negative selection marker (*e.g.,* MPXV segments in this study) from recombining with nonhomologous sequences through error-prone DNA repair pathways such as NHEJ or MMEJ. This limitation may contribute to the illegitimate assembly products presented in our TAR assembly using a dual-selection system.

Several strategies based on modifying TAR vectors or yeast strains have been proposed to improve TAR assembly efficiency. Kuijpers et al. designed a set of standardized 60-bp synthetic recombination sequences nonhomologous with the yeast genome (Kuijpers et al., 2013). These sequences were introduced at each flank of the assembly fragments as "capture hooks" to recombine with adjacent fragments by HR. Although excellent assembly efficiency could be achieved using standardized capture hooks, the final assembly product is interrupted by these hooks and is unsuitable for seamless assembly. Mila Vrancic et al. proposed preparing a mutant yeast strain with its major NHEJ components disrupted (Vrančić

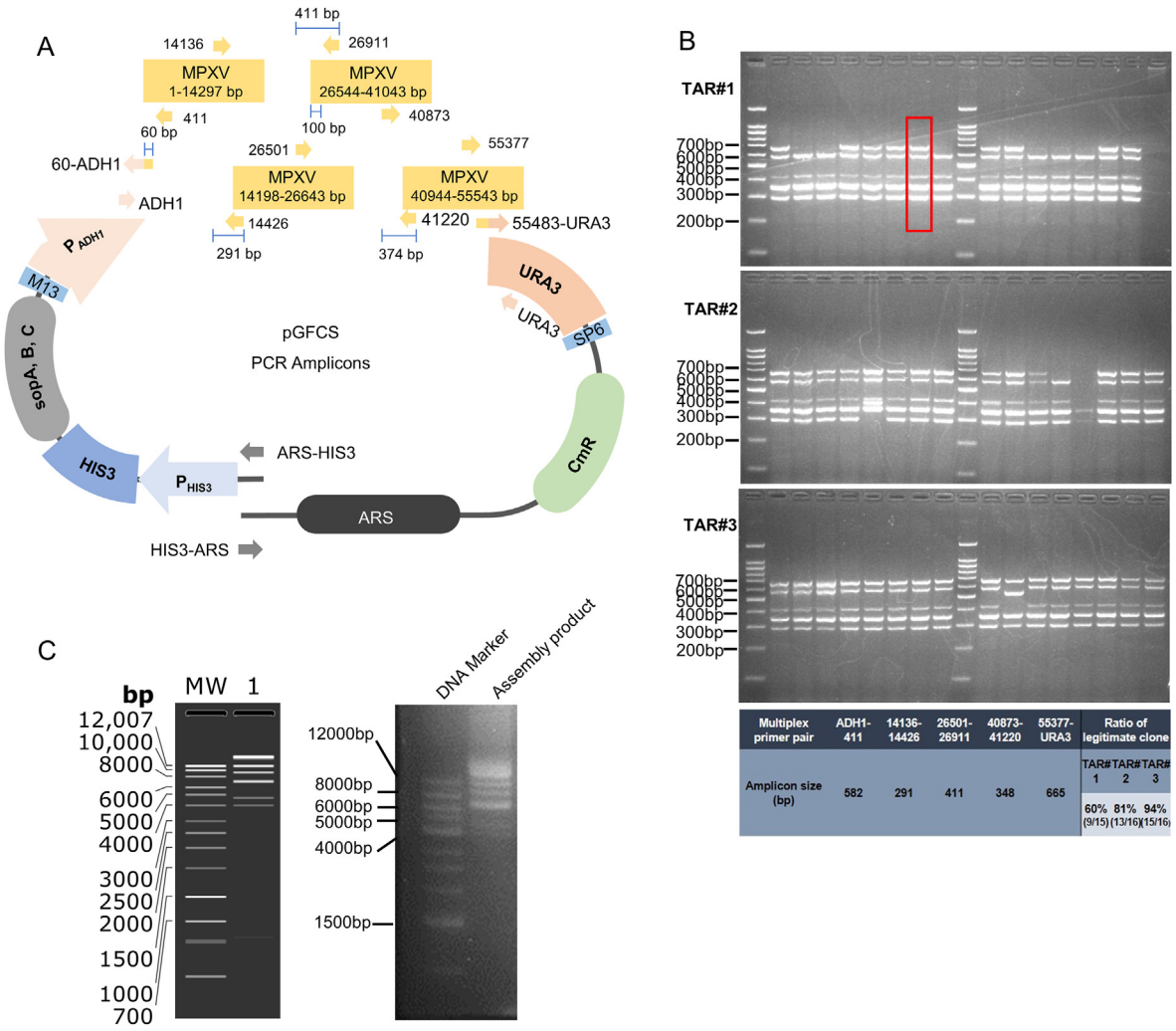

Fig. 3. Assembly of an MPXV fragment by TAR. **A** Diagram of the DNA fragments for TAR assembly. The TAR vector pGFCS was linearized into two PCR amplicons with the primer pairs 60-ADH1/ARS-HIS3 and HIS3-ARS/55483-URA3. The resulting vector parts and four continuous MPXV segments (yellow rectangle) partially overlapped with each other and formed a circular YAC/BAC. The relative location of each primer is indicated in the diagram. **B** Validation of assembly products by multiplex PCR. Sixteen yeast colonies from pGFCS-transformants were picked, and DNA contents were extracted as the template for multiplex PCR. Five primer pairs annealed to each TAR recombination site's upstream and downstream regions (diagrammed in **A**) were used to validate the TAR assembly products. The PCR amplicons were subjected to gel electrophoresis. A legitimate assembly product should generate five amplicons by multiplex PCR, sized 582 bp (primer pair: ADH1/411), 291 bp (primer pair: 14136/14426), 411 bp (primer pair: 26501/26911), 348 bp (primer pair: 40873/41220), and 665 bp (primer pair: 55377/URA3) in length. A recircularized vector should generate a single amplicon 653 bp in length. A typical lane containing five correct amplicons is indicated in the red box. The table at the bottom shows the size of each amplicon and the ratio of legitimate assembly products from three independent TAR assembly experiments (TAR#1, #2, and #3). **C** Genotyping of the assembly product. Restriction pattern of the assembly product. One of the candidate assembly products was extracted from VL6-48B and transformed to EPI300 for high-efficiency amplification. The resulting DNA was extracted and digested by *Bam*HI. The restriction fragments were separated by gel electrophoresis and were compared to the computer-predicted *Bam*HI restriction pattern (left panel).

et al., 2008). Thus, illegitimate ligation by NHEJ would be suppressed, and TAR efficiency is subsequently enhanced in this particular NHEJ-defective yeast strain. However, at least two error-prone DNA repair pathways may contribute to vector recircularization or illegitimate assembly products. Furthermore, it is unclear which pathway, NHEJ or MMEJ, plays a major role in the above situation. Future efforts to optimize TAR should disable more targets involved in both NHEJ and MMEJ.

As an efficient tool for assembling large DNA fragments up to 592 kb in length (Gibson et al., 2008), TAR assembly has become essential for preparing infectious clones of large DNA/RNA viruses. Using pGF as a TAR vector, Shang et al. assembled a synthetic baculovirus with a genome length of more than 145 kb (Shang et al., 2017). To establish a reverse genetics system for SARS-CoV-2, a joint team successfully assembled the full-length cDNA clone of SARS-CoV-2 in less than a week using TAR assembly (Thi Nhu Thao et al., 2020). However, this DNA assembly tool applied in virological research could also raise potential security concerns, especially when the assembled product contains a full set of genetic material that can be recovered into a contagious pathogen. Recently, a group of scientists was funded by a biotech company to synthesize a full-length horsepox virus genome and recover it into an infectious virus (Noyce et al., 2018). Not surprisingly, such a controversial achievement has received enormous attention and raised global debate on its biosecurity implications (DiEuliis et al., 2017; Koblentz, 2017, 2018; DiEuliis and Gronvall, 2018). In this study, although a full-length viral genome would be the ideal reference template for detecting MPXV by qPCR, we only sought to assemble a 55-kb viral fragment, less than one-third of the MPXV genome. This assembly product is fail-safe by virtually eliminating any risk of recovering into an infectious virus while providing multiple qPCR targets for detecting MPXV or other *Orthopoxviruses* (Li et al., 2010).

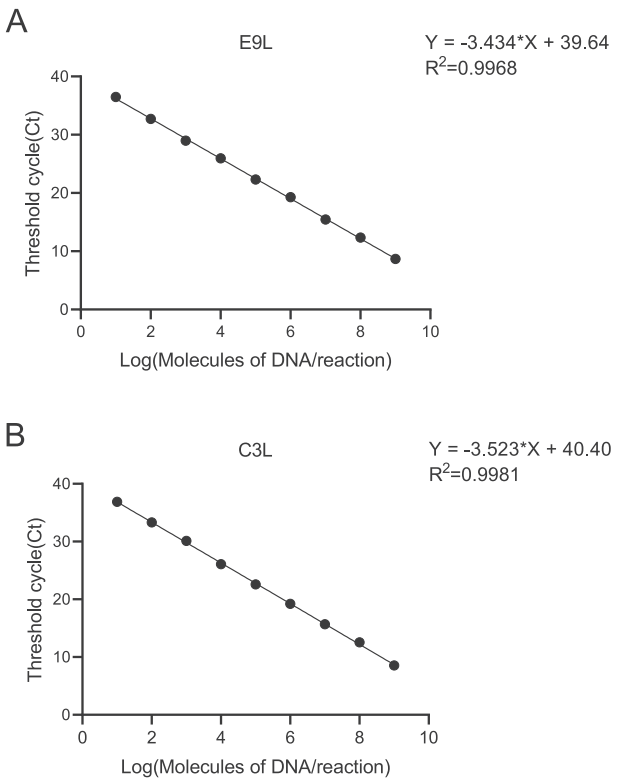

Fig. 4. Standard curves from qPCR assays. Tenfold dilutions of the assembled MPXV fragment ranging from 1×10^1–10^9 copies per reaction were measured using qPCR. The calculated Ct represents the average of 3 different assays. A best-fit line and the coefficients of determination (R^2) of E9L (**A**) and C3L (**B**) are presented.

5. Conclusions

In summary, our study demonstrated that a P_{ADH1}-URA3 cassette can effectively abolish vector recirculation during TAR assembly, promoting the potential use of TAR in genomics and reverse genetics. Nevertheless, more researches are still needed to be done to further optimize TAR assembly efficiency and to refine the administrative rules regarding the biosecurity issues related to DNA assembly.

Data availability

All the data generated during the current study are included in the manuscript.

Ethics statement

Animal and Human Rights Statement: This article does not contain any studies with human or animal subjects performed by any of the authors.

Author contributions

Lei Yang: conceptualization, formal analysis, investigation, methodology, visualization, writing – original draft. Lingqian Tian: data curation, investigation, methodology. Leshan Li: investigation. Qiuhong Liu: investigation. Xiang Guo: investigation. Yuan Zhou: project administration. Rongjuan Pei: formal analysis, validation. Xinwen Chen: conceptualization, data curation, formal analysis, supervision, validation. Yun Wang: conceptualization, data curation, formal analysis, funding acquisition, methodology, supervision, validation, writing – review & editing.

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2022.02.009.

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