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gRNA-transient expression system for simplified gRNA delivery in CRISPR/Cas9 genome editing

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The clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (CRISPR/Cas9) system is one of the most powerful tools for genome engineering. However, some of the steps are laborious, reducing its usability. In this study, we have developed a simplified method, called the guide RNA-transient expression system (gRNA-TES), to deliver gRNA in yeast. In gRNA-TES, a DNA fragment containing the promoter and gRNA is prepared by two simple PCR steps and co-transformed with a DNA module into the host strain; all steps including PCR steps and yeast transformation are completed within 5–6 h in a single day, in contrast to conventional plasmid-based gRNA delivery systems, which require at least 3–4 days to construct and verify the gRNA-expressing plasmids. The performance of gRNA-TES was evaluated by the replacement of 150-kb, 200-kb, 300-kb, 400-kb, and 500-kb regions of yeast chromosome 4 with a DNA module. Increased numbers of transformants with a high frequency of expected replacement of even the 500-kb region were obtained with gRNA-TES as compared with transformation without gRNA-TES. In addition, the integrity of the replaced region was verified in 67%–100% of transformants tested by colony PCR. We believe that gRNA-TES will vastly increase the accessibility of CRISPR/Cas9 technology to biologists and biotechnologists by offering a simple, fast, and cost-effective tool to deliver gRNA in genome engineering. Furthermore, it might be applied to plant and animal systems if appropriate gene promoters are incorporated in the technology.

[**Key words:** CRISPR-Cas9; Genome engineering; Guide RNA; PCR-based; Yeast]

The ability to edit the genome is essential for many state-of-the-art experimental methods. Genome editing by the clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) system has been shown to be functional in *Saccharomyces cerevisiae* (1). In the CRISPR/Cas9 system for yeast genome engineering, guide RNAs (gRNAs) direct the plasmid-expressed Cas endonuclease to introduce a double-strand break (DSB) at a specific genome site, which is then repaired by the cellular repair machinery. Depending on the gRNA, the genome can be edited during the repair process.

To date, various strategies for expressing the gRNAs have been developed. In most methods developed for yeast, gRNA components are expressed from a DNA cassette (1–3). For example, DiCarlo et al. (1) identified an effective yeast RNA polymerase III promoter and terminator pair (*SNR52*p and *SUP4*t) for driving gRNA expression and used them in place of the mammalian U6 promoter cassette. Their design achieved nearly 100% marker-less integration at a target locus when the appropriate donor DNA was included. Yeast RNA polymerase II promoters can also drive expression of gRNA constructs that are fused to a ribozyme that can cleave the interfering 30 UTR sequence (2). Using this method, Ryan and Cate (2) increased the level of gRNA expression, leading to a higher rate of integration of linear DNA modules. In addition, Bao et al. (4) reported highly efficient gRNA expression in yeast via using the natural, two-component crisprRNA (crRNA) and trans-activating crRNA (tracrRNA) format. With respect to gRNA delivery, these multi-step methods result in similarly high engineering efficacy.

More recently, Horwitz et al. (5) reported an efficient technique in which yeast's ability to repair gaps was used to construct a gRNA-expressing vector inside the yeast cell. In their approach, linear DNA fragments bearing an origin of replication and a selectable marker cassette were co-transformed with multiple DNA fragments containing gRNA cassettes with short flanking regions sharing homology with the linear vector plasmid. Proper recombination *in vivo* then reconstituted a functional circular vector within the host cell. In addition, Hao et al. (6) synthesized a 114-bp crRNA component containing two gRNAs and partial direct repeats, which was digested by *Bsa*I, and then cloned into the pCRCT plasmid for gRNA expression. By contrast, Sasano et al. (7) constructed a gRNA expression plasmid to target a specific genomic locus by the SLIC technique (8) with some modifications.

However, all of the above-mentioned methods are plasmid-based. Constructing plasmids for expressing gRNA can be laborious, costly, and time-consuming. To overcome these drawbacks, we have developed a simpler PCR-based system for producing the gRNA, which we call the gRNA–transient expression system (gRNA-TES). In the present study, we describe the gRNA-TES procedure and evaluate its performance. The results of our analysis show that gRNA-TES technology is efficient and is completed within 5–6 h, including the transformation process. It therefore facilitates easy, rapid, and effective genome engineering, which will be beneficial to scientists applying the CRISPR/Cas9 system in the yeast genome.

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TABLE 1. Strains and plasmids used in this study.

Strain or plasmid	Description	Remarks and reference
Strain		
SJY5	*MATa ura3-52 his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63*	10
SJY6	*MATα ura3-52 his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63*	10
SJY267	Leu$^+$ transformant of SJY5 with pSJ14 (YEp + *LEU2*)	This study
SJY269	His$^+$ transformant of SJY6 with pSJ12 (YEp + *HIS3*)	This study
SJY270	SJY267 x SJY269	This study
SJY344	His$^-$ Leu$^-$ clone from SJY270 (Diploid between SJY267 and SJY269)	This study
SJY347	Trp$^+$ transformant of SJY344 with pSJ30	This study
Plasmid		
pSJ12	pRS303 (*HIS3*) + 2 μ DNA origin	11
pSJ14	pRS305 (*LEU2*) + 2 μ DNA origin	11
pSJ30	p414-TEF1p-Cas9-CYC1t (=YCp + CEN6/ARSH4 + TRP1)	1
pSJ32	p426-SNR52-gRNA.CAN1.Y-SUP4t	1
pSJ69	*loxP* site-deleted p3008	Our stock
p3008	A derivative of pUG6 carrying *loxP-CgLEU2-loxP*	9

MATERIALS AND METHODS

Strains, plasmids, primers, and media The strains and plasmids used in this study are listed in Table 1 (1,9–11). Strain SJY344 was constructed as follows: *S. cerevisiae* strain SJY270 was cultivated in YPDA without selection pressure for plasmid maintenance and then plated on YPDA plate after appropriate dilution. Colonies appeared were replica-plated on to -Leu, -His, and YPDA plate. We found colonies showing Leu$^-$ and His$^-$ at frequency of approximately 10% which cannot grow on -Leu and -His plate but can grow on YPDA plate. We selected one of such clones (Leu$^-$ His$^-$) and named SJY344. Strains SJY347 (Trp$^+$ transformant of SJY344 with p414-TEF1p-Cas9-CYC1t [cat. no. 43802, Addgene repository, http://www.addgene.org]) (1) and SJY344 were used as host strains for the gRNA-TES experiment. For liquid cultivation, yeast strains were grown at 30°C in YPDA medium (2% peptone, 2% glucose, 1% yeast extract and 0.004% adenine sulfate). Synthetic complete (SC) medium (0.67% yeast nitrogen base without amino acids [Difco, Sparks, MD, USA], 0.2% drop out mix for each amino acid or nucleic acid base, and 2% glucose) lacking specific amino acids was used to select transformants and to examine auxotrophic phenotype. For plate assays, 2% agar was added to solidify the medium.

We used a loxP site-deleted plasmid pSJ69 derived from p3008 (9) to avoid undesired site-specific recombination that might delete loxP-flanked chromosomal DNA sequences. pSJ69 was constructed as follows: p3008 was digested with *Sal*I and *Eco*RV, and the larger fragment was purified by agarose gel electrophoresis. Separately, a *CgLEU2* fragment was prepared by PCR using p3008 as a template, and SJP430 (5′-TACGCTGCAGGTCGACCAATTCTGTGTTTCCCGG-3′) and SJP431 (5′-ACTAGTGGATCTGATTTACGTAAAGTTCGTTTGCC-3′) as the respective forward and reverse primers. These two fragments were combined by In-Fusion cloning (Takara, Shiga, Japan). pSJ69 was used as the template for all DNA module (Fig. 1 and Table 1). All primers are described in Tables S1–S3. Plasmid p414-TEF1p-Cas9-CYC1t (cat. no. 43802) expressing *Streptococcus pyogenes* Cas9, and plasmid p426-SNR52p-gRNA.CAN1.Y-SUP4t (cat. no. 43803) expressing gRNA for the *CAN1* gene were purchased from the Addgene repository (1).

Preparation of DNA modules DNA modules to replace the target regions were prepared by PCR. Plasmid pSJ69 harboring the selective marker *Candida glabrata LEU2* (*CgLEU2*) was used as a common template to amplify all donor DNAs using appropriate forward and reverse primers (Table S1) depending on the individual PCR experiment. The forward primer was designed as the first 30-bp sequence of the target region using *Saccharomyces* Genome Database (SGD: www.yeastgenome.org/) and an additional 20-bp sequence homologous to the 5′-GGCCGCCAGCTGAAGCTTCG-3′ sequence (nt 7–26) of plasmid p3008 (9). The reverse primer was designed as the last 30-bp reverse sequence of the respective target region using the same database and an additional 20-bp reverse sequence homologous to the 5′-AGGCCACTAGTGGATCTGAT-3′ sequence (nt 1602–1621) of plasmid p3008.

PCR conditions for generating PCR fragments for gRNA-TES Two rounds of PCR were used for gRNA-TES. The first-round PCR contained 0.25 μl of Ex Taq DNA polymerase (Takara, Shiga, Japan), 5 μl of 10x buffer for *Ex Taq* DNA polymerase, 4 μl of 2.5 mM dNTPs, 1.5 μl (15 pmol) of forward and reverse primers (Table S2), and 1 μl (approximately 50 ng) of template plasmid in a volume of 50 μl. The PCR conditions were 94°C for 2 min, followed by 30 cycles of 98°C for 10 s, 55°C for 30 s, and an extension step at 68°C for an appropriate time. First-round PCR products were diluted 100-fold and used as a template for the second-round PCR with the same reaction mixture in a final volume of 50 μl. The second-round PCR conditions were 94°C for 2 min, followed by 30 cycles of 98°C for 10 s, 58°C for 30 s, and an extension step at 68°C for 3 min.

Yeast transformation Yeast transformation was carried out according to Gietz and Schiestl (12). YPDA broth was used for both main and preculture. For the selection of transformants, cells were plated on appropriate SC medium and incubated at 30°C for 3–4 days.

Yeast colony PCR Colony PCR was used to determine whether expected replacement of the chromosomal target region had occurred in transformants. A small amount of cells from each colony was suspended in 10 μl of 0.02 M NaOH solution, heated at 98°C for 10 min, and then transferred to ice. Next, we added 12.5 μl of Prime Star Max Premix 2x (Takara, Shiga, Japan); 0.5 μl of genomic DNA; control primers to amplify the *CNE1* region as an internal control (forward primer, SJP121, 7.5 pmol; reverse primer, SJP242, 7.5 pmol; resulting in 670-bp band); and forward primer SJP238 (7.5 pmol) and the appropriate reverse primer (7.5 pmol) for the targeted chromosomal region (Table S3), resulting in a 1975-bp PCR product if expected replacement of the target region had occurred. Water was added to a final volume of 25 μl. The PCR conditions were 30 cycles of 98°C for 10 s, 55°C for 5 s, and an extension step at 72°C for 5 s. All PCR amplifications were carried out on an Astec PC-320 Program Temp Control System.

RESULTS

Development of a PCR-based gRNA expression system To simplify the expression of gRNA in the *S. cerevisiae* host (diploid strain SJY347), we developed a system based on only PCR without plasmid-based cloning. Our basic idea was to develop a simple gRNA expression system that expresses gRNA from a PCR fragment (called fragment C) (Fig. 1A, B). Fragment C comprised the *SNR52* promoter, targeted genome sequence as a guiding sequence, and gRNA scaffold, and was prepared by overlap PCR using fragments A and B (Fig. 1A).

First, we prepared fragments A and B in separate PCR reactions by the first-round PCR. For preparation of fragment A left, forward primer SJP180 and for preparation of fragment A right, forward primers SJP342, SJP343, SJP355, SJP356 or SJP357 were designed to include a 35-nt sequence from part of the *SNR52* promoter region (nt 3855–3889), which comes from the template plasmid p426-SNR52p-gRNA.CAN1.Y-SUP4t (full sequence available at Addgene repository, https://www.addgene.org/43803/sequences/), followed by a 20-nt guiding sequence specific to the targeted genome sequence, and a further 23-nt sequence encoding the 5′ part of the gRNA scaffold from plasmid p426-SNR52p-gRNA.CAN1.Y-SUP4t (nt 3910–3932). The software CRISPRdirect (https://crispr.dbcls.jp/) was used to select the correct target sequence for any input DNA sequence. The 3′ end of the DNA target sequence contained a protospacer adjacent motif (PAM) sequence (5′-NGG-3′). The 20 nt upstream of the PAM sequence was the targeting sequence, and the Cas9 nuclease cleave target sequence was 3 nt upstream of the PAM. The reverse primer sequence SJP115 was also taken from the template plasmid (nt 5001–5020 in reverse sequence) (Table S2).

For PCR preparation of fragment B (Fig. 1A), the forward primer SJP120 consisted of a 20-nt sequence from plasmid p426-SNR52p-gRNA.CAN1.Y-SUP4t (nt 3301–3320), and the reverse primer SJP117 consisted of a 30-nt sequence from the same plasmid (nt 3855–3884 in reverse sequence). The complementary sequence of these 30 nt in fragment B was the same as the first 30 nt of fragment A in order to anneal fragments A and B in the second-round overlap PCR.

Next, to prepare two kinds of fragment C (left and right), an overlap PCR system (Fig. 1A) was adopted to incorporate the whole *SNR52* promoter region from plasmid p426-SNR52p-gRNA.CAN1.Y-SUP4t in order to ensure proper and efficient gRNA expression. A 100-fold dilution (x1/100) of fragment A was mixed with the same dilution of fragment B and used as a template for PCR to generate fragment C with forward primer SJP120 (used to generate fragment B) and reverse primer SJP115 (used to generate fragment A) (Fig. 1A). Example results of PCR for the preparation of fragments A,

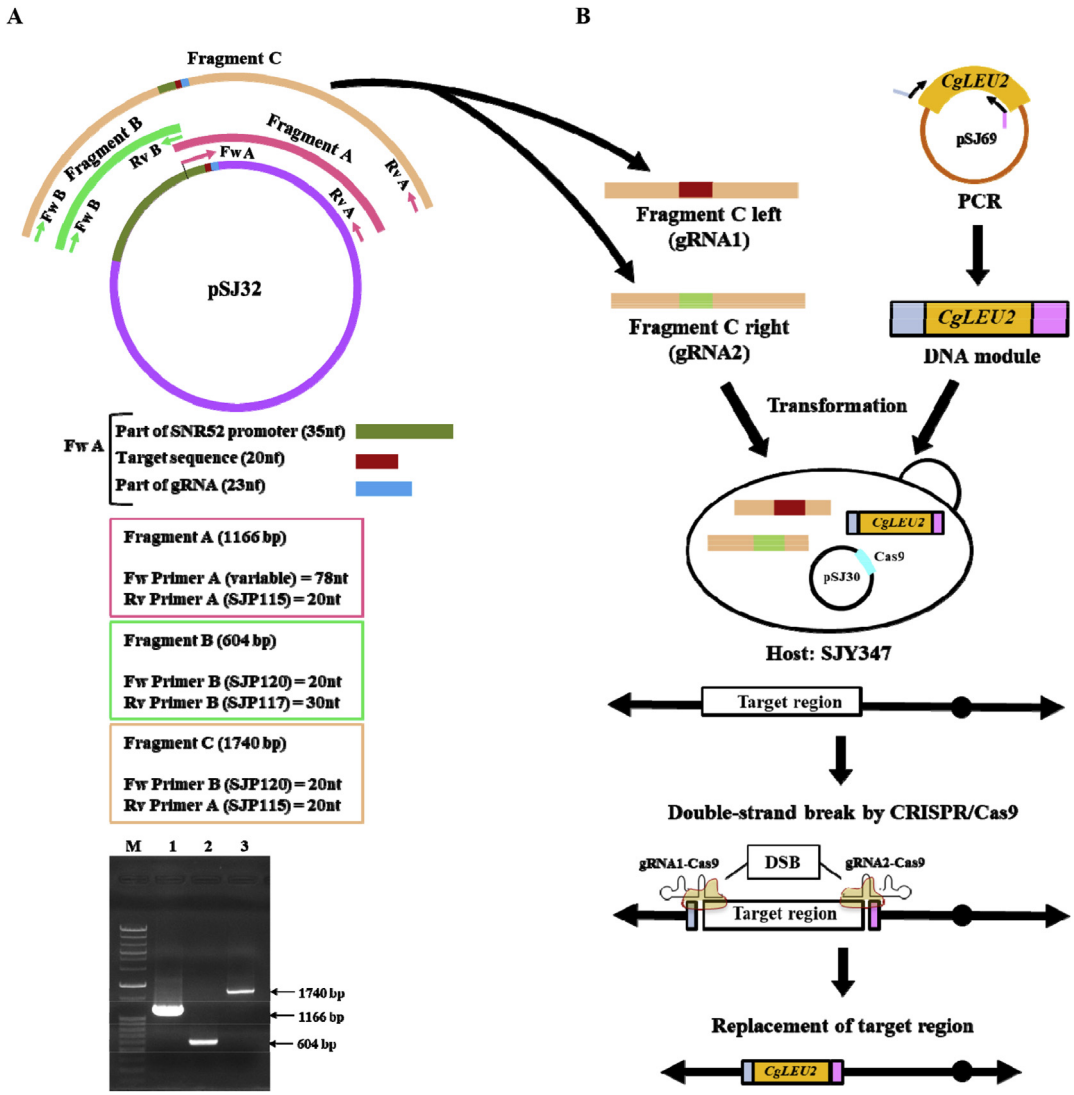

FIG. 1. Construction and generation of gRNA-expressing PCR fragments and introduction of these fragments to replace targeted chromosomal regions. (A) The three types of fragments A, B, and C used for gRNA-TES. Fragments A and B were prepared by PCR using plasmid pSJ32 (p426-SNR52p-gRNA.CAN1.Y-SUP4t) as a common template. Agarose gel electrophoresis image shows one example of the generation of fragments A, B, and C by PCR. M represents markers (Gene Ladder Wide 2, Nippon Gene, Toyama, Japan); lanes 1, 2, and 3 show fragments A (1166 bp), B (604 bp), and C (1740 bp), respectively. (B) Overview of gRNA-TES. After preparing the gRNA-expressing PCR fragments (to cause DSB for the left and right edge of target chromosomal region to be replaced) and DNA module, they were introduced together into an *S. cerevisiae* strain expressing Cas9 (SJY347). Cas9 introduces DSB at both ends of the target site, leading to increased frequency of homologous recombination at their flanking regions. The target region is then thought to be efficiently replaced by the DNA module (*CgLEU2*) via homologous recombination.

B, and C for a test target sequence are shown in Fig. 1A. Fragments A, B, and C were well amplified by PCR, showing the expected size without additional bands. Similar results were obtained for other target sequences (data not shown). Although overlap PCR might generate insertion or deletion in fragment C which may not cause much influence, we sequenced approximately 800 bp region including the junction region between fragment A and fragment B and its flanking region in two arbitrarily selected fragment C. Sequencing was performed in both forward and reverse orientation. The result of sequencing showed that the sequence of the junction region and its flanking region was exactly the expected one in these two fragment C. Therefore, it would be no problem if PCR reaction mixture containing fragment C is used directly for yeast transformation without DNA sequencing.

Optimization of amount of fragment C in transformation Because the above experiments showed that the gRNA-TES method works well, we next investigated the amount of fragment C needed for optimal performance to elaborate this method. In these experiments, the Chr4-200 region was taken for replacement under the assumption that the optimal amount of fragment C would not differ drastically from other regions. For this optimization experiment, we pooled PCR product from 5 tubes (each has 50 µl PCR-reaction mixture) and it was subjected to ethanol precipitation and finally dissolved in 50 µl water. Then, DNA concentration was measured at 260 nm using spectrophotometer. We found that the concentration of DNA after ethanol precipitation was 0.94 µg/µl. Since we have known from preliminary transformation experiment that 11 µl (approximately 10 µg) of fragment C gave us dozens of transformants, we decided to use 11 µl of each fragment C (left and right) in this optimization experiment (Table 2). In order to add less amount of fragment C such as 0.5 µg, 2.5 µg, 5.0 µg, 7.5 µg for each fragment C (left and right) in yeast transformation mixture, the DNA solution of 0.94 µg/µl concentration was diluted with appropriate amount of water to adjust DNA concentration so that 11 µl of each DNA solution corresponds to respective amount of DNA (Table 2).

Next, various amount of fragment C (left and right) was used for yeast transformation. Performance of gRNA-TES using various amounts of the gRNA-expressing fragment C was compared in terms of the number of transformants obtained (Table 2). Results revealed that as the amount of fragment C is increased, the number of transformants seems to increase; however, more than 10 μg of fragment C did not result in further rise in transformant numbers. By contrast, the number of transformants was very small without gRNA-TES. On the basis of these findings, we concluded that 10 μg of fragment C (in total) is optimized amount of fragment C to achieve the best performance of gRNA-TES.

However, since this work was originally to provide a new simple method for preparing fragment C expressing gRNA, the protocol was set up without measuring DNA concentration every time PCR reaction was done. By this reason, we expressed the amount of DNA as μl of reaction mixture (footnote for Table 2) but not μg and always used 11 μl of PCR reaction mixture for other routinely performed yeast transformation because we obtained a sufficient number of transformants using such amount (11 μl + 11 μl = 22 μl) of PCR reaction mixture containing each left and right fragment C. We suggest that there would be no problem to use PCR reaction mixture directly containing fragment C for yeast transformation without purification.

Performance of gRNA-TES and upper size limits of replacement by gRNA-TES The performance of gRNA-TES was evaluated by an experiment in which 150-kb, 200-kb, 300-kb, 400-kb, and 500-kb regions of chromosome 4 were replaced by a small-sized DNA module both with gRNA-TES and without gRNA-TES (i.e., transformation of the DNA module without fragment C) (Figs. 2A–C and S1A–F), and the number of transformants was compared (Table 3). Strain SJY347, which harbors the Cas9-expressing plasmid p414-TEF1p-Cas9-CYC1t, was used as the host, and the coordinates of the Chr4-150, Chr4-200, Chr4-300, Chr-400, and Chr4-500 regions were nt 494271–644270, 494271–694270, 494271–794270, 494271–894270, and 494271–994270, respectively.

As a starting point, 11 μl of each fragment C (left and right) was used in the yeast transformation mixture. The frequency of transformants varied by target region; considering the Chr4-150 region as an example, 263 transformants were obtained by using gRNA-TES, whereas only 9 transformants were obtained without gRNA-TES (Table 3). Similarly, 287 transformants were obtained by using gRNA-TES for the Chr4-200 region, whereas only 9 transformants were obtained without gRNA-TES. Thus, the frequency of transformants was much higher when gRNA-TES was used. These observations indicate that gRNA-TES performs well because a higher number of transformants was obtained with the gRNA-TES method than without gRNA-TES.

We also investigated the upper size limit of the chromosomal region that can be replaced by the gRNA-TES method. Again, the diploid strain SJY347 was used as the host, and replacement of an increasing region from 150 kb to 500 kb was attempted. The primers were designed to amplify a 1975-bp PCR product when the expected replacement occurred (Fig. 2A–C). The expected 1975-bp band was obtained with a frequency of 100% (5/5) for replacement of the 500-kb region by the gRNA-TES method (Fig. 2C, lanes 7–9 and Table 3); by contrast, the expected 1975-bp band was not observed for replacement of the 500-kb region without gRNA-TES (Fig. 2C, lanes 14–15 and Table 3). In a control experiment using the strain SJY344 lacking the Cas9-expressing plasmid, the expected band was not observed (data not shown). These observations indicate that the efficiency of sequence replacement is much higher with gRNA-TES than without gRNA-TES (Fig. 2A–C and Table 3). Taken together, our observations strongly suggest that gRNA-TES works effectively and can successfully achieve replacement of 500-kb or possibly even longer genome regions.

Structural analysis of the homologous chromosome in the transformants Because a diploid strain was used in this study, the target region is present in both homologous chromosomes. Therefore, gRNA expressed from fragment C allows Cas9 to introduce a double-strand break in both target regions with equal probability. If the target region in both homologous chromosomes is replaced with the DNA module, however, the cell will die because this region (150–500 kb) of chromosome 4 contains essential genes whose deletion leads to cell death. To distinguish the presence of a chromosome with or without replacement, we used colony PCR to detect a 1500-bp region of the target sequence in the homologous chromosome. We reasoned that if the 1500-bp region in the homologous chromosome remains, we would observe both the 1500-bp band and the 1000-bp band (representing the DNA module containing *CgLEU2* marker) by colony PCR (Fig. S1G). By contrast, if the homologous chromosome in the transformant has a structural alteration that does not lead to cell death, we would not observe the 1500-bp band.

Transformants obtained with gRNA-TES and without gRNA-TES for the Chr4-150, Chr4-200, Chr4-300, Chr4-400, and Chr4-500 regions were tested by colony PCR using appropriate primers (Table S3), and the expected 1500-bp band were observed in all transformants examined (Fig. S1H, lanes 1–18; Fig. S1I, lanes 1–15). Thus, the frequency of the expected band (1500 bp) was 100% for all regions (Table 3). These results suggest that in the viable diploid transformants one of the homologous chromosomes had no replacement of at least this 1500-bp region, indicating that gRNA-TES is effective for obtaining viable transformants with replacement of the desired targeted region of one chromosome without causing unexpected structural alteration of the homologous chromosome in the diploid host.

DISCUSSION

In this study, we have developed gRNA-TES, a very simple PCR-based method for delivering gRNA in the CRISPR/Cas9 system of genome editing. gRNA-TES can be completed within 5–6 h including the transformation process, whereas conventional a plasmid-based method requires at least 3–4 days to construct and verify the plasmid that delivers gRNA. Furthermore, we have evaluated the performance of gRNA-TES in terms of (i) the number of transformants, (ii) frequency (percentage) of transformants with

TABLE 2. Approximate amount of fragment C used in transformation and number of transformants obtained.

Target region[a]	Approximate amount of fragment C left, μg[b]	Approximate amount of fragment C right, μg[b]	Total approximate amount of fragment C, μg[b]	Number of transformants (with gRNA)	Number of transformants (without gRNA)
Chr4-200	0.5	0.5	1.0	70	0
	2.5	2.5	5.0	150	3
	5.0	5.0	10.0	270	4
	7.5	7.5	15.0	100	7
	10.0	10.0	20.0	40	5

[a] The coordinate and length of the target region were 494271–694270 and 200 kb, respectively.
[b] The total volume of the DNA mixture was 34 μl (transformation mixture, 360 μl): 11 μl for fragment C left, 11 μl for fragment C right, and 12 μl for DNA module.

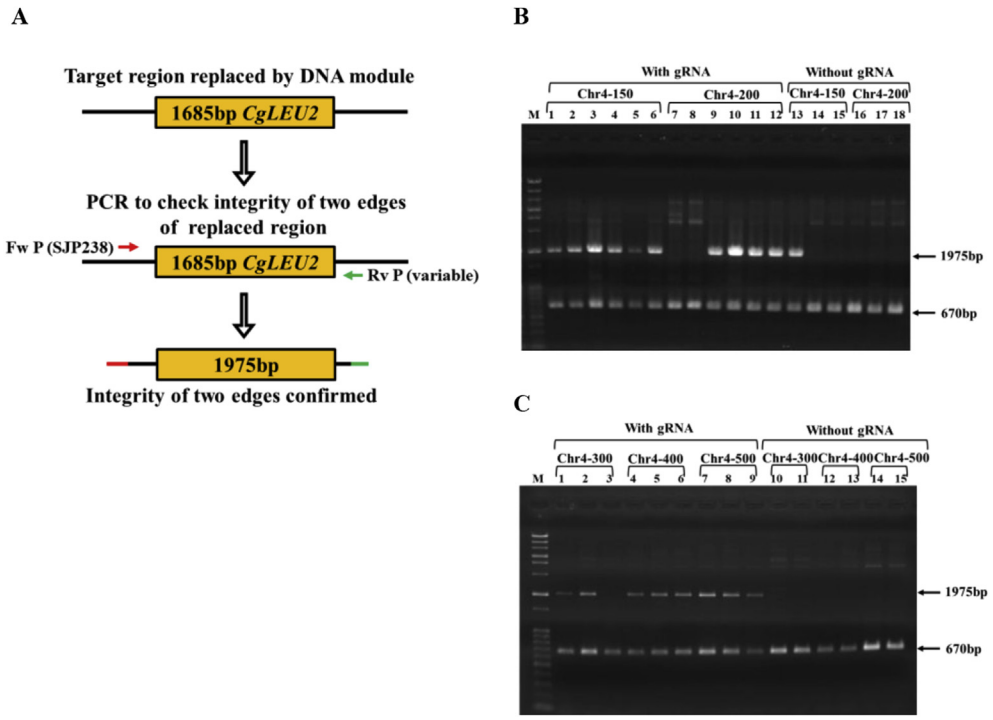

FIG. 2. Analysis of chromosome alteration by yeast colony PCR. (A) Principle of colony PCR for verification of the integrity of two edges of the target region. The forward primer SJP238 was used with an appropriate reverse primer to generate a 1975-bp band. (B) Colony PCR to verify replacement of the Chr4-150 and Chr4-200 regions. Primers SJP238 and SJP350 were used for the 150-kb region (lanes 1–6), and SJP238 and SJP351 for the 200-kb region (lanes 7–12) in transformants obtained with gRNA-TES. Lanes 13–18 represent results from transformants obtained without gRNA-TES. (C) Colony PCR to verify replacement of the Chr4-300, Chr4-400, and Chr4-500 regions. Primers SJP238 and SJP364 were used for the 300-kb region (lanes 1–3), SJP238 and SJP365 for the 400-kb region (lanes 4–6), and SJP238 and SJP366 for the 500-kb region (lanes 7–9). Lanes 10–15 represent results from transformants obtained without gRNA-TES.

expected replacement, and (iii) efficient replacement of very long target regions of the genome (<500 kb). Our results demonstrate that higher numbers of transformants overall, and a high frequency of transformants with the expected replacement are obtained with gRNA-TES as compared with transformation without gRNA-TES.

In addition to the simplicity of the methodology, gRNA-TES has another advantage. In previous CRISPR/Cas9 systems, gRNA is expressed continuously from an autonomously replicating plasmid, meaning that the target is continuously attacked by Cas9 because the autonomously replicating plasmid continues to deliver gRNA during mitotic growth. In gRNA-TES, by contrast, gRNA is transiently expressed and likely to be gradually lost during colony formation because it is supplied from a non-replicating PCR fragment (fragment C). This situation may contribute to increased numbers of transformants in cases where the target sequence remains present after the desired genome manipulation has been completed, for example, in applications of PCDup technology (13) in which the target site remains in the intact chromosome.

During this study, we noted that some of the DNA polymerases produced multiple unexpected bands in PCR. Therefore, to refine gRNA-TES and also make it more user-friendly, different kinds of DNA polymerase were tested in the PCR steps. Except for Ex Taq DNA polymerase, DNA polymerases including KOD Plus Neo, KOD Fx, KOD Fx Neo, and Prime STAR HS were found to frequently produce multiple unexpected bands, especially during the overlap PCR used to prepare fragment C (data not shown). Although the reason for this is not known at present, we recommend using Ex Taq DNA polymerase for gRNA-TES.

To assess the chromosomal changes in transformants, we adopted a modified colony PCR method in which genomic DNA was prepared within 15 min (NaOH protocol) and we used PrimeSTAR Max Premix (2x), which requires an extension time of only 5 s/kb

TABLE 3. Transformants obtained by gRNA-TES.

Deletion region[a]	Method	Number of transformants						Frequency of expected replacement (%)	Presence of homologous chromosome (%)
		Transformation			Average	Analyzed	Expected replacement		
		1st	2nd	3rd					
Chr4-150	+gRNA-TES	202	300	287	263	6	6	100	6/6 (100)
	−gRNA-TES	10	8	9	9	6	1	16.6	6/6 (100)
Chr4-200	+gRNA-TES	256	257	350	287	6	4	66.6	6/6 (100)
	−gRNA-TES	12	7	8	9	3	0	0	3/3 (100)
Chr4-300	+gRNA-TES	49	42	63	51	5	4	80	5/5 (100)
	−gRNA-TES	6	5	12	7	4	0	0	4/4 (100)
Chr4-400	+gRNA-TES	38	39	54	43	5	5	100	5/5 (100)
	−gRNA-TES	5	6	11	7	3	0	0	3/3 (100)
Chr4-500	+gRNA-TES	90	119	102	103	5	5	100	5/5 (100)
	−gRNA-TES	7	5	5	5	4	0	0	4/4 (100)

[a] The length of the Chr4-150, Chr4-200, Chr4-300, Chr4-400, and Chr4-500 regions was 150 kb, 200 kb, 300 kb, 400 kb, and 500 kb (chromosome 4), respectively. These regions were replaced in the diploid host strain SJY347. The experiment was carried out three times, and the number of transformants is given.

colony PCR. By employing these modifications, the analysis of chromosomal structure alteration was also completed in a very short time.

gRNA-TES enabled the replacement of a very long chromosomal region of up to 500 kb (Fig. 2C) with a frequency of 100%. In addition, replacement of the desired target region of one chromosome occurred without causing structural changes to the homologous chromosome (Fig. S1H, I). These observations suggest that gRNA-TES can be used to effectively obtain transformants harboring replacement of a desired targeted region of one chromosome without triggering unexpected structural alterations of the homologous chromosome in a diploid host.

In our study, we occasionally obtained a transformant with only left or right edge replacement. For example, among six transformants tested for replacement of the Chr4-200 region, one did not show the expected 1000-bp band in left edge replacement analysis (Fig. S1B, lane 7), but showed the expected band in right edge replacement analysis (Fig. S1E, lane 7). It is possible that homologous recombination occurred only at the right edge of the target region, while erroneous recombination with the DNA module might have occurred at the left. Nevertheless, this event was observed infrequently among the transformants.

Taking all our observations together, we conclude that gRNA-TES provides a simple, fast, cost-effective and highly efficient tool for genome engineering, and is likely to make CRISPR/Cas9 technology accessible to more biologists and biotechnologists.

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