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Simple and Effective Gap-Repair Cloning Using Short Tracts of Flanking Homology in Fission Yeast

Author(s)

Yuzy MATSUO, Hayafumi KISHIMOTO, Tomitaka HORIUCHI, Katsuhiro TANAE, Makoto KAWAMUKAI

Journal

Bioscience, Biotechnology, and Biochemistry 2010 年 74 巻 3 号 p. 685-689

Published

2010/03/23

URL

https://doi.org/10.1271/bbb.90967

Communication

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Simple and Effective Gap-repair Cloning Using Short Tracts of Flanking Homology in Fission Yeast

Yuzy Matsuo, Hayafumi Kishimoto, Tomitaka Horiuchi, Katsuhiro Tanae, and Makoto Kawamukai[+]

[1]*Department of Applied Bioscience and Biotechnology, Faculty of Life and Environmental Science, Shimane University, Matsue, 690-8504, Japan*

+To whom correspondence should be addressed.
Tel:+81-852-32-6587;Fax:+81-852-32-6092;E-mail: kawamuka@life.shimane-u.ac.jp

Abbreviations: GRC, gap-repair cloning; AbA, aureobasidin A; GFP, green fluorescent protein; KanMX6, kanamycin-resistance marker; P_{nmt1}, *nmt1* promoter; T_{nmt1}, *nmt1* terminator; T_{adh1}, *adh1* terminator; P_{TEF}, promoter sequences of the *Ashbya gossypii* translation elongation factor 1a gene; T_{TEF} , terminator sequences of the *Ashbya gossypii* translation elongation factor 1a gene

Abstract

Gap-repair cloning for plasmid construction in budding yeast is very effective and often used. In contrast, the same method is not widely used in fission yeast, because of its shortage of appropriate information. Here, we described a simple and effective gap-repair cloning for plasmid construction using short tracts of flanking homology. In this method, we have combined concentrated DNA fragments with short (20bp) tracts of flanking homology with the marker gene or the pre-existing gene module. In addition, we showed that this method can be applied for one-step cloning of multiple DNA fragments to construct fusion gene.

Key words: Fission yeast; Gap-repair cloning; short tracts of flanking homology; one-step cloning of multiple DNA fragments

Plasmid construction is one of the most commonly and frequently used molecular biological technique in many laboratories. Generally, restriction enzymes and DNA ligase have been used for cloning of specific gene onto plasmid.[1)] However, this traditional and conventional cloning method is laborious and often inefficient. In budding yeast *Saccharomyces cerevisiae*, the gap-repair cloning has been widely used for plasmid construction.[2)] This method depends on the ability of yeast to repair gapped DNA sequences *in vivo* by homologous recombination. In this method, DNA fragments with flanking homology to the target plasmid can be directly cloned into a linearized plasmid by homologous recombination without the need for an *in vitro* DNA ligation. The gap-repair cloning is simple and convenient because short (20bp) tracts of flanking homology are sufficient for effective gap-repair cloning in budding yeast.[3)] In particular, this method is very effective and powerful when suitable restriction sites are not available.[4,5)]

In contrast to budding yeast, the gap-repair cloning is not widely used for plasmid construction in fission yeast *Schizosaccharomyces pombe* even though the method has been adapted to construct plasmids[6)] and to obtain novel alleles of a specific gene.[7)] The reason why the gap-repair cloning is not widely used in fission yeast appears to be involved in the requirement for longer region of flanking homology. It was shown that when homologous recombination mediated-gene targeting in fission yeast was performed, homologous integration efficiencies of 1~3% were obtained with about 40bp of flanking homology,[8)] that of >29% were obtained with about 80bp of flanking homology[9)], and that of >50% were obtained with >500bp of

flanking homology.[10,11] On the basis of these results, we may suspect that a long tracts of flanking homology are necessary for effective gap-repair cloning in fission yeast. However, it has not been systematically examined how long the flanking homology are necessary and sufficient for effective gap-repair cloning in fission yeast. Here, we described a simple and effective gap-repair cloning using short tracts of flanking homology in fission yeast. Table1 Fig.1

To examine the effects of the length of flanking homology on the efficiency of gap-repair cloning, we performed the following experiments. At first, we performed PCR amplification of the *aur1*^{R} (aureobasidin A resistant) gene[12], with short (20bp) and long (40bp, 80bp, 250bp, 500bp) tracts of flanking homology with the *nmt1* promoter and the *nmt1* terminator (Fig.1A). PCR was generally done by 30 cycles of 94 degree for 30s, 48 degree for 30s, and 68 degree for 1 – 3 min using KOD-plus and linearized pREP1-*aur1*^{R} (Kominami unpublished) as template. All primers used in this study are listed in Table1. Then, a fission yeast strain PR110 (*h*$^{+}$, *leu1-32, ura4-D18*) was co-transformed with these PCR fragments and a *Bam* HI digested pREP1 vector[13] (molar ratio of mixed DNA of a gapped pREP1 and P_{nmt1}-*aur1*^{R}-T_{nmt1} is 1 to 20). For transformation, a protocol based on lithium acetate method was used[9]. Transformants were screened by EMM plates[14] which lack leucine and thiamine, and the number of total transformants was counted (Table S1). Next, transformants were replicated onto EMM plates containing 0.5mg/L of AbA with or without 2mM thiamine and the number of AbA-resistant transformants was counted. Only when the accurate homologous recombination occurs between the gapped vector and the inserts, transformants will express the *aur1*^{R} gene in a *nmt1* promoter-dependent manner.

Therefore, the proportion of total transformants that were resistant to AbA indicates the proportion of colonies resulting from proper gap-repair cloning. Expectedly, most transformants were resistant to AbA in a *nmt1* promoter-dependent manner (Fig. 2A). We found that the proportion of colonies harboring proper gap-repaired plasmid was over 80% when using long (80bp, 250bp, 500bp) tracts of flanking homology (Fig. 2B). This is consistent with previous results that large flanking homology is required for effective homologous recombination[9,10] and effective gap-repair cloning[7] in fission yeast. To confirm the structure of the constructed plasmids, we rescued the plasmids from AbA-resistant transformants. All transformants were collected and disrupted by acid washed glass beads (425–600μm; Sigma Co. Ltd). After then, the plasmids were isolated from the disrupted cells by QIAprep Spin Miniprep kit (QIAGEN) and introduced into *E. coli* DH5α. After purification of plasmids from *E. coli* DH5α, the sequences of the ligation junction of constructed plasmids were confirmed by sequencing analysis using primer, P_{nmt1} 80bp F or T_{nmt1} 80bp R. We found that 40~100% of *E. coli* transformants carried the constructed plasmids containing the $aur1^R$ gene. We next examined whether the efficiency of gap-repair cloning with short (20bp) tracts of flanking homology could be improved by different molar ratio of mixed DNA. We found that the efficiency of gap-repair cloning was about 20% when the molar ratio of mixed DNA of gapped pREP1 and P_{nmt1}-$aur1^R$-T_{nmt1} was over 1 to 50 (Fig.2C). These data suggest that gap-repair cloning with short (20bp) tracts of flanking homology is indeed feasible in fission yeast. Fig.2

To further demonstrate the effectiveness of gap-repair cloning using short (20bp) tracts of flanking homology, we performed PCR

amplification of the GFP(S65T)-kanMX6 gene with short (20bp) tracts of flanking homology with the vector (Fig. 1B). PCR was performed by using the two primers, P_{nmt1}-pFA6a uni F and pFA6a-T_{nmt1} uni R, and pFA6a-GFP(S65T)-kanMX6[9] as template. After then, PR110 were co-transformed with the PCR fragment and a *Bam* HI digested pREP1 vector (molar ratio of mixed DNA of gapped pREP1 and P_{nmt1}-GFP(S65T)-kanMX6-T_{nmt1} is 1 to 50). Homologous recombination between the gapped vector and GFP(S65T)-kanMX6 *in vivo* will enable transformants to express GFP(S65T) in a *nmt1* promoter-dependent manner and grow on YES plate[14]containing 100mg/L of G418 (Fig. 1B). To examine the expression of GFP(S65T) in a *nmt1* promoter-dependent manner, we observed the GFP fluorescence by a BX51 microscope (Olympus) and illumination at 485nm. Furthermore, we performed rapid protein extraction as described previously[15] and western blot analysis to detect GFP(S65T) by an anti-GFP antibody (Roche Diagnostics). Tubulin was also detected as a loading control by an anti-tubulin antibody (Sigma Co. Ltd). As expected, we found that transformants could grow on YES plate containing G418 and expressed GFP(S65T) in a *nmt1* promoter-dependent manner (Fig. 3A and B). After isolation of plasmids from fission yeast, we transformed *E. coli* DH5α with gap-repaired plasmids and selected transformants on LB plates containing 50mg/L of kanamycin. Plasmids were purified from *E. coli* DH5α, and the sequences of the ligation junction of constructed plasmids were confirmed by sequencing analysis using primer, P_{nmt1} 80bp F or T_{nmt1} 80bp R. We found that most *E. coli* transformants carried the constructed plasmids containing the GFP(S65T)-kanMX6. These results demonstrate that gap-repair cloning using short (20bp)

tracts of flanking homology is simple and effective method for plasmid construction in fission yeast.

We next tested the feasibility of the cloning of multiple DNA fragments by gap-repair cloning, which has not been tested in fission yeast. To this end, we first performed PCR amplification of the *cia1* gene[16] which encodes a histone H3/H4 chaperone and the GFP(S65T)-kanMX6 gene with short (20bp) tracts of flanking homology (Fig.1C). To amplify these DNA fragments, the two primers, P_{nmt1}-*cia1* F and *cia1*-pFA6a R, were used to amplify the *cia1* gene with 20bp tracts of flanking homology from fission yeast genomic DNA and the two primers, pFA6a uni F and pFA6a-T_{nmt1} uni R, were used to amplify the GFP(S65T)-kanMX6 gene with 20bp tracts of flanking homology from pFA6a-GFP(S65T)-kanMX6. After then, fission yeast strain PR110 were co-transformed with these two PCR fragments and a *Bam* HI digested pREP41 vector[17] (molar ratio of mixed DNA of gapped pREP41 and P_{nmt1}-*cia1* and GFP(S65T)-kanMX6-T_{nmt1} is 1 to 20 to 20). Homologous recombination between the gapped vector and the *cia1* gene and GFP(S65T)-kanMX6 *in vivo* will enable transformants to express Cia1-GFP(S65T) protein that localizes in the nucleus and grow on YES plates containing G418. The proportion of total transformants that were resistant to G418 and expressed Cia1-GFP(S65T) protein gives the proportion of colonies resulting from proper gap-repair cloning. In contrast to gap-repair cloning with two DNA fragments, the efficiency of gap-repair cloning with three DNA fragments was low (about 5%). However, we found that transformants harboring proper gap-repaired plasmid could grow on YES plates containing G418 and expressed Cia1-GFP(S65T) proteins in a *nmt41* promoter-dependent manner (Fig.

3C and D). Localization of the Cia1-GFP(S65T) protein in the nucleus was verified by staining the same cells with DAPI (data not shown). To confirm structure of the constructed plasmids, the sequences of the ligation junction were confirmed by sequencing analysis using primer, P_{nmt1} 80bp F or T_{nmt1} 80bp R after purification of plasmids from *E. coli* DH5α. We found that most *E. coli* transformants carried the constructed plasmids containing the *cia1*-GFP(S65T)-kanMX6. Therefore, these data suggest that gap-repair cloning of multiple DNA fragments using short (20bp) tracts of flanking homology is feasible in fission yeast even though the efficiency is not high. Fig.3

In this study, we demonstrated that short (20bp) tracts of flanking homology are sufficient for effective gap-repair cloning for plasmid construction and that gap-repair cloning of multiple DNA fragments with short (20bp) tracts of flanking homology is feasible in fission yeast. This simple and effective gap-repair cloning using shorts tracts of flanking homology should be convenient and time-saving when suitable restriction sites are not available and cloning of multiple DNA fragments or large DNA fragments is needed.

Acknowledgments

Y. Matsuo is a research fellow of the Japan Society for the Promotion of Science. We thank K. Kitamura, K. Kominami and T. Toda for providing materials used in this study. We also thank H. Katoh , T. Nakagawa and S. Katayama for helpful suggestion and discussion, and Saori Ueda for technical assistant. This work was supported by JSPS research fellowships for young scientists.

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Figure legends

Fig.1. Schematic overview of the gap-repair cloning in this study. (A) We amplified the *aur1*^{R} gene with short (20bp) and long (40bp, 80bp, 250bp, 500bp) tracts of flanking homology with a vector. Fission yeast was co-transformed with these PCR products and a linearized pREP1 vector. Only when the accurate homologous recombination occurs *in vivo*, the *aur1*^{R} gene will be expressed from the *nmt1* promoter and cells will be resistant to AbA. (B) We amplified the GFP(S65T)-kanMX6 gene with short (20bp) tracts of flanking homology with a vector. Fission yeast was co-transformed with this PCR product and a linearized pREP1 vector. Homologous recombination *in vivo* results in a repaired plasmid that expresses GFP(S65T) from the *nmt1* promoter and confers cells the resistance to G418. (C) Schematic overview of the gap-repair cloning of multiple DNA fragments in fission yeast. For our pilot experiment, we amplified the *cia1* gene and GFP(S65T)-kanMX6 gene with short (20bp) tracts of flanking homology. Fission yeast was co-transformed with these PCR products and a linearized pREP41 vector. Homologous recombination *in vivo* results in a repaired plasmid that expresses Cia1-GFP(S65T) protein that localizes in the nucleus.

Fig.2. Short (20bp) tracts of flanking homology are sufficient for gap-repair cloning in fission yeast.
(A) Most transformants displayed the resistance to AbA in a *nmt1* promoter-dependent manner. Transformants resulting from propegap-repair cloning can grow on EMM plates containing 0.5mg/L of AbA without thiamine. N; Negative control (PR110 harboring pREP1), P; Positive control (PR110 harboring pREP1-*aur1*^{R}). (B) The

efficiency of gap-repair cloning when using different length of flanking homology. The efficiency of gap-repair cloning was calculated from the percentage of AbA-resistant cells in the total transformants harboring the gap-repaired plasmid. The molar ratio of mixed DNA of gapped pREP1 and P_{nmt1}-*aur1*^{R}-T_{nmt1} is 1 to 20. (C) The efficiency of gap-repair cloning when using short (20bp) tracts of flanking homology under different molar ratio of mixed DNA. The molar ratio of mixed DNA of gapped pREP1 and P_{nmt1}-*aur1*^{R}-T_{nmt1} is shown.

Fig.3. Gap-repair cloning using short (20bp) tracts of flanking homology is simple and effective for plasmid construction. (A) GFP(S65T) fluorescence in living cells was monitored by fluorescence microscopy. Cells harboring pREP1-GFP(S65T)-kanMX6 were grown on EMM plate with or without thiamine. (B) Western blot analysis of GFP(S65T). Cells harboring pREP1-GFP(S65T)-kanMX6 were grown on EMM plate with or without thiamine. GFP(S65T) were detected with an anti-GFP antibody (upper panel) and tubulin was detected as a loading control (lower panel). (C) Subcellular localization of Cia1-GFP(S65T) protein. Cells harboring pREP41-*cia1*-GFP(S65T)-kanMX6 were grown on EMM plate with or without thiamine. (D) Western blot analysis of Cia1-GFP(S65T) protein. Cells harboring pREP41-*cia1*-GFP(S65T)-kanMX6 were grown on EMM plate with or without thiamine. Cia1-GFP(S65T) protein was detected with an anti-GFP antibody (upper panel) and tubulin was detected as a loading control (lower panel).

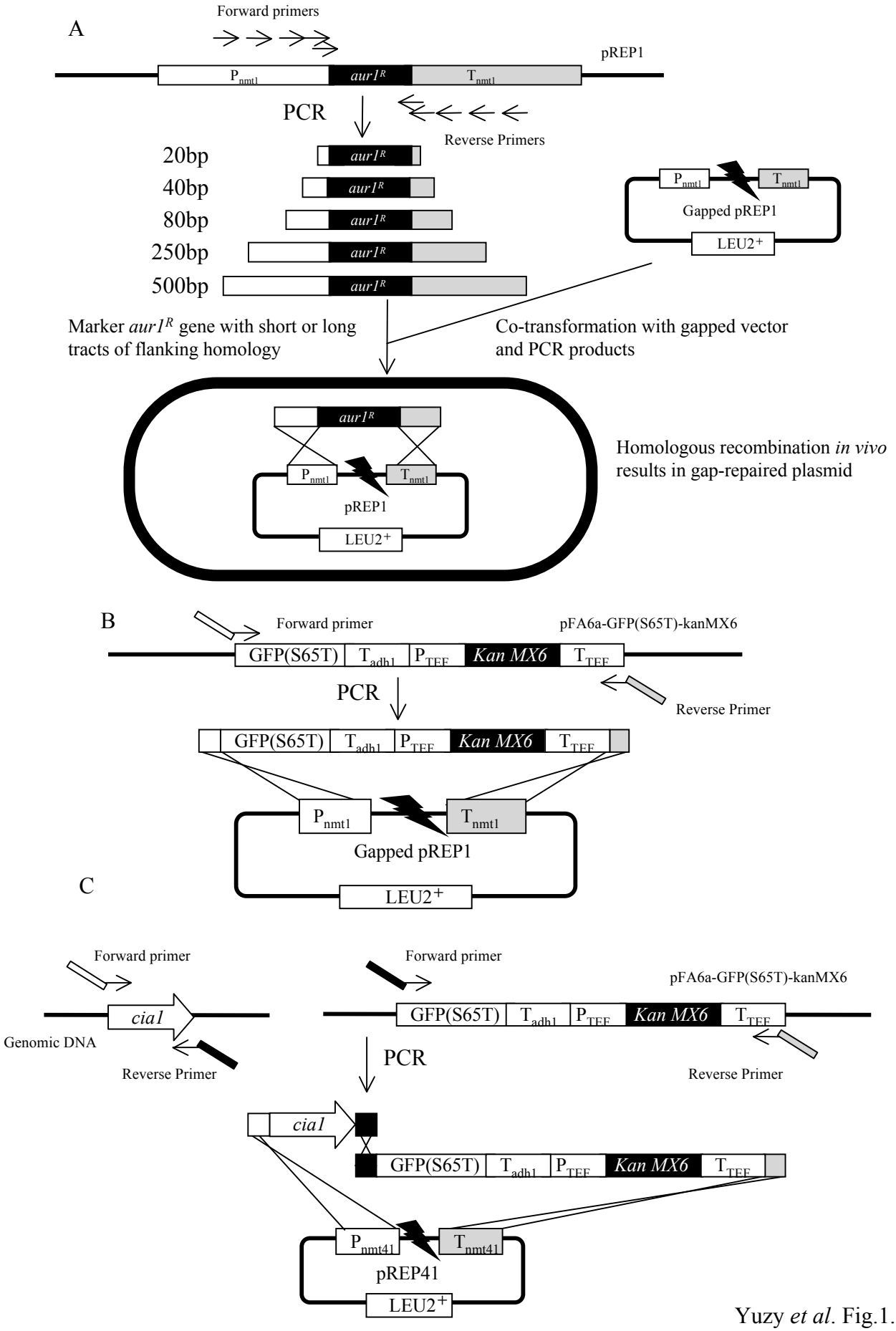
Table1. Oligonucleotide primers used in this study

Primers	Sequence 5'to 3'
P_{nmt1} 20bp F	TCGCTTTGTTAAATCATATG
T_{nmt1} 20bp R	CAAGGGAGACATTCCTTTTA
P_{nmt1} 40bp F	TCTCACTTTCTGACTTATAG
P_{nmt1} 40bp R	AAAAACCCTAGCAGTACTGG
P_{nmt1} 80bp F	GGCATATCATCAATTGAATA
T_{nmt1} 80bp R	TAATATGCAGCTTGAATGGG
P_{nmt1} 250bp F	AACGTAACTCTCGGCTACTG
T_{nmt1} 250bp R	ATTCTTAACTACACCACTCG
P_{nmt1} 500bp F	GATTGTTAGAAGAAAAGAGC
T_{nmt1} 500bp R	TTTATGGCATTTCCATGTTG
P_{nmt1}-*aur1* F	TCGCTTTGTTAAATCATATGTCTGCTCTTTCGACCTT
aur1-T_{nmt1} R	CAAGGGAGACTTCCTTTTATTAAGGAAGATGACTTGCAT
P_{nmt1}-pFA6a uni F	TCGCTTTGTTAAATCATATGTCGTACGCTGCAGGTCGACG
pFA6a-T_{nmt1}uni R	CAAGGGAGACATTCCTTTTACATCGATGAATTCGAGCTCG
P_{nmt1}-*cia1* F	TCGCTTTGTTAAATCATATG TCAATCG TGAATATCCT
cia1-pFA6a R	TCGACCTGCAGCGTACGACTGAGACGTCTCTGGTTTCT
pFA6a uni F	TCGTACGCTGCAGGTCGACG
kanMX6 chk F	GAACTGCCTCGGTGAGTTTT

Table S1. The number of total transformants in Fig.2B

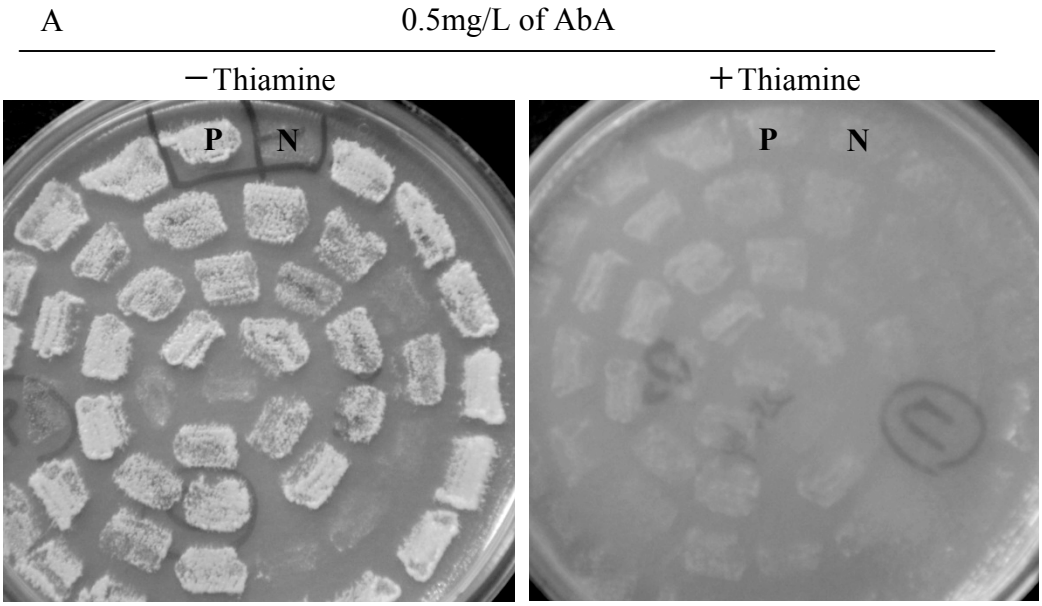
Length (bp) of flanking homology	Number of cells
none	64
20	127
40	148
80	236
250	290
500	403

The molar ratio of mixed DNA of gapped pREP1 and P_{nmt1}-*aur1*^{R}-T_{nmt1} with different length of flanking homology is 1 to 20. Each experiment was repeated three times, and the average is shown.



Yuzy *et al*. Fig.1.

A



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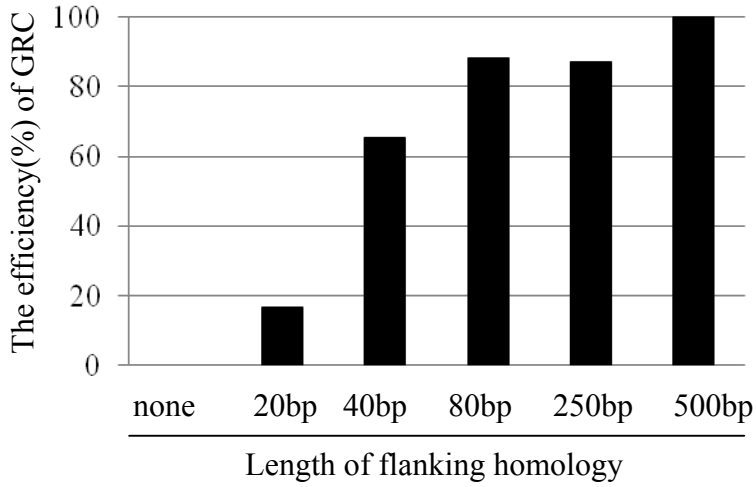

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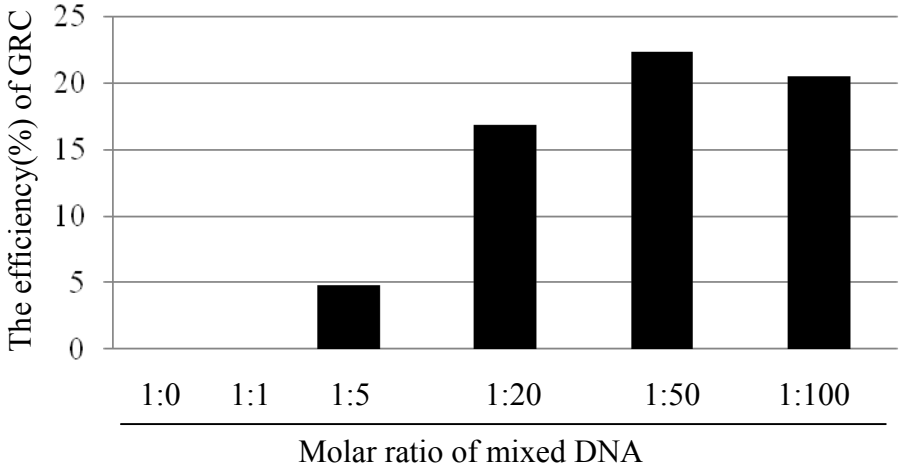
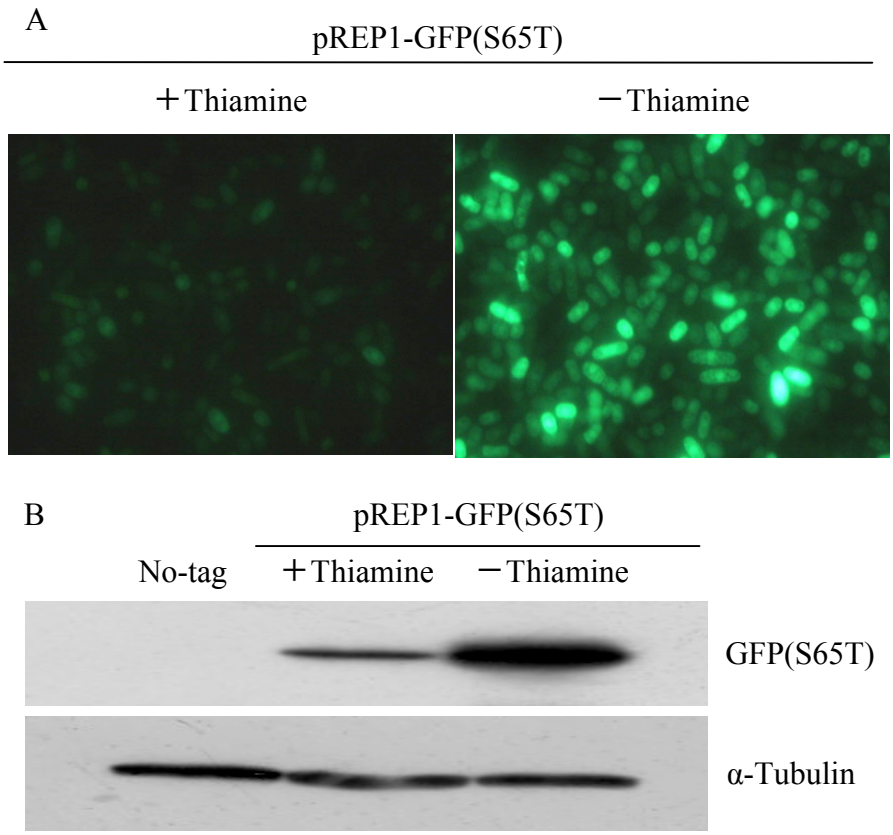


Yuzy *et al*. Fig.2.



Yuzy *et al*. Fig.3.