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論　文　要　旨

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論文題名

Development of rapid and versatile genome editing technologies toward creation of genome diversity in yeast

（ゲノムの多様性創出を目指した出芽酵母ゲノム工学技術の開発）

論文の要旨

Creating genome diversity is a very important issue to understanding genome function and breeding. For this purpose, clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) system is one of the most powerful tools which was recently developed. Genome engineering by the CRISPR/Cas9 system has been shown to be functional in *Saccharomyces cerevisiae*. In this system, 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 intrinsic cellular repair machinery. Depending on the gRNA and designed donor DNA, the genome can be edited at any desired site or region during the repair process. To date, several different approaches for gRNA expression have been reported. However, by reviewing various gRNA expression and delivery system in Chapter 1, I realized that all methods employ plasmid-based gRNA expression system which is laborious and time-consuming since it requires cloning of respective target sequence.

To overcome these drawbacks, in Chapter 2, I have developed a simpler PCR-based system for producing the gRNA in a short period of time, which I called the gRNA-Transient Expression System (gRNA-TES). It is based on only PCR without plasmid-based cloning. My basic idea was to develop a simple gRNA expression system that expresses gRNA from a PCR fragment. The PCR fragment comprised the *SNR52* promoter, targeted genome sequence as a guiding sequence, and gRNA scaffold, and was prepared by simple PCR. This PCR fragment is co-transformed with DNA modules into *S. cerevisiae* host strain to replace a targeted chromosomal region. All steps including PCR and yeast transformation are completed within 5-6 hours in a single day, whereas conventional plasmid-based gRNA delivery system requires at least 3-4 days to construct and verify the gRNA-expression plasmids. The performance of gRNA-TES was evaluated by colony PCR in terms of following parameters, i) the number of transformants, ii) frequency of transformants with expected replacement, iii) the length of chromosomal regions to be replaced and iv) the integrity of the replaced region. Although the frequency of transformants varied by target region, we usually obtained 50 to 300 transformants, by using gRNA-TES whereas only several transformants were obtained without gRNA-TES. Thus, the frequency of transformants was much higher when gRNA-TES was used. Structural analysis of targeted chromosomal region by colony PCR revealed that 67%-100% of transformants showed expected single replacement of even the 500 kb region by gRNA-TES.

In addition to the simplicity of the methodology, I have discussed another important advantage of gRNA-TES. In previous CRISPR/Cas9 systems, gRNA was expressed from an autonomously replicating plasmid, meaning that the target is continuously attacked by Cas9 nuclease. This is because the autonomously replicating plasmid continues to deliver gRNA during mitotic growth and therefore, target site is continuously exposed to Cas9 attack. In gRNA-TES, by contrast, gRNA is transiently expressed and likely to be lost during colony formation because it is supplied from a non-replicating PCR fragment. This situation may contribute to increasing numbers of transformants even in cases that the target sequence remains after the desired genome manipulation has been completed. All of the observations demonstrated that a high frequency of

transformants with the expected replacement were obtained with gRNA-TES as compared with transformation without gRNA-TES. I concluded that gRNA-TES should 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.

For the analysis of genome function, the deletion or replacement of desired chromosomal regions, is very important. Because of low homologous recombination activity even in *S. cerevisiae* host, however, current methods are limited to manipulation of only one chromosomal region in a single transformation, making the simultaneous deletion or replacement of multiple chromosomal regions difficult. Therefore, in Chapter 3, I developed two highly efficient and versatile genome engineering technologies by integrating the CRISPR/Cas9 genome editing system with our deletion and replacement technology, named CRISPR-PCD for simultaneous chromosomal deletion and CRISPR-PCRep for simultaneous chromosomal replacement in *S. cerevisiae*. In CRISPR-PCD, for a deletion of segmental chromosomal region, two deletion modules are introduced into a yeast cell together with two gRNAs to obtain a deletion strain. One deletion module contains a selective marker (*CgLEU2* or *CgHIS3*), while the other contains *CEN4* as a centromere. In transformed cells, DSBs are induced near the target site by CRISPR/Cas9, and the specific chromosomal region is deleted by CRISPR-PCD. As a result of deletion of the targeted region, two new split chromosomes are generated in the transformants. I attempted to delete two terminal regions, Chr.8-L (22 kb) and Chr.11-L (25 kb), and two internal regions, Chr. 9-5 (22 kb) and Chr. 15-11 (26 kb), located on different chromosomes simultaneously. Double selection was used for transformation, resulting in 1,096 and 47 His^+ Leu^+ transformants by CRISPR-PCD for the deletion of two terminal regions and two internal regions, respectively. Results demonstrated that simultaneous double deletion of terminal as well as internal regions can be achieved in a one-step transformation using CRISPR-PCD.

To replace multiple chromosomal regions simultaneously, I combined CRISPR/Cas9 with PCRep technology. In this approach, a donor DNA module containing a *CgLEU2, CgHIS3*, or *ScURA3* marker gene is introduced into a yeast cell, together with two PCR fragments expressing gRNAs. After transformation, the resultant chromosome remains as a single independent chromosome because the targeted chromosomal region is simply replaced by the donor DNA module. I attempted to replace four sets of three internal chromosomal regions [set 1: Chr. 3-2 (15 kb), Chr. 4-10 (14 kb), and Chr. 9-5 (22 kb); set 2: Chr. 4-10 (14 kb), Chr. 7-4 (19 kb), and Chr. 12-2 (14 kb); set 3: Chr. 3-2 (15 kb), Chr. 7-4 (19 kb), and Chr. 15-11 (26 kb); and set 4: Chr. 3-2 (15 kb), Chr. 4-10 (14 kb), and Chr. 7-4 (19 kb)], simultaneously via a replacement experiment using CRISPR-PCRep, by one-step transformation. After triple selection for transformants with *CgLEU2*, *CgHIS3* and *ScURA3* markers, 83, 25, 14, and 7 Leu^+ His^+ Ura^+ transformants were obtained for sets 1, 2, 3, and 4, respectively. By contrast, no transformants were obtained by conventional PCRep for any set. Colony PCR analysis of structural alterations showed that triple replacement of four different sets of chromosomal regions was successful in 83%-100% of transformants analyzed. I also obtained the simultaneous replacement of up to three chromosomal regions by using two selection markers. Based upon these observations I would suggest that it is possible to obtain triple replacement simultaneously using two selection markers (partly marker-free) in a one-step transformation although simultaneous quadruple replacement was not observed using two selection markers. In conventional PCD, an experiment for a single deletion takes at least 11 days including 6 days for verification by PFGE followed by Southern blot analysis. Therefore, if the deletion of two, three, and four chromosomal regions is conducted sequentially by conventional PCD, it will take 22, 33, and 44 days, respectively. By contrast, the CRISPR-PCD and CRISPR-PCRep described in this study will take only 7 to 10 days. This considerable reduction in experimental time for generating multiple deletions and replacements greatly enhances the usability and applicability of CRISPR-PCD and CRISPR-PCRep for genome engineering.

In Chapter 4, I summarized all the observations about three newly developed genome engineering methods i.e., gRNA-TES for simple gRNA expression, CRISPR-PCD and CRISPR-PCRep for simultaneous multiple segmental chromosomal deletion and replacement. I also discussed the importance and significance of my Ph D thesis in genome engineering field. By using these technologies, genome manipulation, especially deletion or replacement of chromosomal regions, will be much faster and easier. Based upon this general discussion, finally, I concluded that these newly developed genome engineering technologies offer a more rapid means for manipulating multiple chromosomal regions toward creation of genome diversity both for breeding and for revealing genome function in yeast. It should also be emphasized that these powerful technologies are applicable to not only yeasts, but also a broad range of organisms.