The Fate of Linear DNA in *Saccharomyces cerevisiae* and *Candida glabrata*: The Role of Homologous and Non-Homologous End Joining

Mary W. Corrigan, Christine L. Kerwin-Iosue, Alexander S. Kuczmarski, Kunj B. Amin, Dennis D. Wykoff*

Department of Biology, Villanova University, Villanova, Pennsylvania, United States

Abstract

In *vivo* assembly of plasmids has become an increasingly used process, as high throughput studies in molecular biology seek to examine gene function. In this study, we investigated the plasmid construction technique called gap repair cloning (GRC) in two closely related species of yeast – *Saccharomyces cerevisiae* and *Candida glabrata*. GRC utilizes homologous recombination (HR) activity to join a linear vector and a linear piece of DNA that contains base pair homology. We demonstrate that a minimum of 20 bp of homology on each side of the linear DNA is required for GRC to occur with at least 10% efficiency. Between the two species, we determine that *S. cerevisiae* is slightly more efficient at performing GRC. GRC is less efficient in *rad52* deletion mutants, which are defective in HR in both species. In *dnl4* deletion mutants, which perform less non-homologous end joining (NHEJ), the frequency of GRC increases in *C. glabrata*, whereas GRC frequency only minimally increases in *S. cerevisiae*, suggesting that NHEJ is more prevalent in *C. glabrata*. Our studies allow for a model of the fate of linear DNA when transformed into yeast cells. This model is not the same for both species. Most significantly, during GRC, *C. glabrata* performs NHEJ activity at a detectable rate (>5%), while *S. cerevisiae* does not. Our model suggests that *S. cerevisiae* is more efficient at HR because NHEJ is less prevalent than in *C. glabrata*. This work demonstrates the determinants for GRC and that while *C. glabrata* has a lower efficiency of GRC, this species still provides a viable option for GRC.

Introduction

Plasmid construction is an essential technique in molecular biology. Assembly of plasmids, containing specific DNA fragments, can be carried out either *in vitro* or *in vivo*. *In vitro* cloning, which requires restriction enzymes and DNA ligase, can be costly and inefficient for high throughput methods. *In vivo* cloning, such as gap repair cloning (GRC), utilizes homologous recombination (HR) activity and can be cheaper and more efficient [1,2]. GRC uses available homologous ends of a linearized vector and a DNA fragment (usually generated by PCR) to fuse the two, creating a circular plasmid (Figure 1). Yeast species are appealing for GRC as many species appear to perform HR efficiently [3,4,5,6,7]. By understanding the minimal requirements for GRC, costs can be minimized.

Several studies have examined GRC in the budding yeast, *Saccharomyces cerevisiae*, and demonstrated this species is capable of efficient GRC [1,8,9,10]. These analyses specify that 30 base pairs of homology is sufficient for DNA integration into a linearized vector, but it may not be the minimum requirement [9,11]. Recently, a similar study indicated that GRC is a viable cloning technique in *Schizosaccharomyces pombe* [4]. Additionally, our laboratory routinely uses GRC in *C. glabrata*, but the specific requirements for efficient GRC are unknown.

Our studies with *C. glabrata* suggest that HR appears to be less efficient than in *S. cerevisiae*, as deletion of genes using HR is less efficient in *C. glabrata* [5](data not shown). We expected that a detailed analysis of GRC in the two species would elucidate the role of HR, the mechanism by which this technique takes place, and non-homologous end joining (NHEJ). Additionally, *C. glabrata* is closely related to *S. cerevisiae* and pathogenic to mammals, allowing for comparisons over evolutionary time. We examined the role of two genes involved in HR and NHEJ – *RAD52* and *DNL4*. *RAD52* has previously been identified as a gene involved in DNA double-strand break repair and it facilitates HR in *S. cerevisiae* and *S. pombe* [12]. When *RAD52* is deleted, HR should be decreased and we would expect that GRC will either not take place or will be dramatically reduced. *DNL4* is required for NHEJ, which is the repairing of double stranded DNA breaks without homologous ends via its ligase activity [13,14]. Mutations in *dnl4* in *S. pombe* lead to increased frequency of HR, and we predicted that loss of *DNL4* should lead to higher frequency of GRC in *S. cerevisiae* and *C. glabrata*, as NHEJ would be reduced.

The goal of this study was to define the determinants of GRC in both *S. cerevisiae* and *C. glabrata*, with regard to the amount of homology and ratios of DNA concentrations. Additionally, we aimed to examine the role of HR in GRC and how NHEJ influences the frequency of GRC. Finally, we incorporated our
data into a model of the fate of linear DNA when transformed into the two species.

Materials and Methods

Yeast Strains and Growth Conditions

Wild-type S. cerevisiae and wild-type C. glabrata were used as the host strains for GRC transformations, both containing ura3Δ his3Δ (See Table 1 for strains). Deletions of RAD52 and DNL4 in both S. cerevisiae and C. glabrata were generated using antibiotic resistance genes KANMX5 and NATMX6 (confering resistance to G-418 and nourseothricin, respectively) and homologous recombination to delete the ORFs [5,15,16], which was confirmed by PCR. For transformations, yeast strains were grown in YEPD medium at 30°C until logarithmic growth phase (OD 0.2–0.5). To select for plasmids, strains were grown in synthetic dextrose (SD) medium with CSM lacking the appropriate amino acids (either histidine or uracil) (Sunrise Science, San Diego, CA, USA). Transformations were performed using a standard lithium acetate protocol. We were successful in isolating plasmid DNA from yeast preparations of linearized vector, pRS313 ([19,20]. It is unclear whether CEN6 is functional in both species and C. glabrata strains. Based on the size of these primers were used. pRS313 (HIS3) was linearized with Smal or with EcoRV and Smal. PCR products and linear vectors were subjected to gel electrophoresis, purified using a GeneClean II Kit (M.P. Biomedicals, CA, USA), and quantified with a NanoDrop 2000. The CgURA3 PCR products and linearized vectors were co-transformed at different molar ratios (0, 0.02, 0.2, and 1.0) into S. cerevisiae and C. glabrata strains. Based on the size of fragments, we estimated that equal amounts of DNA in nanograms were a molar ratio of 0.2, as the plasmid was ~5× the size of the insert. For all transformations, we used 50 ng of linearized vector. All cells from S. cerevisiae transformations and 20% of cells from C. glabrata were plated onto SD medium lacking histidine, so that individual colonies per plate were <500 cfu. After 3 days of growth, these HIS3+ colonies were then replica plated to SD medium lacking uracil.

Sequencing

To sequence plasmids created by GRC, cells were grown overnight in selective medium and yeast plasmids were purified utilizing an ammonium acetate procedure [19]. Yeast plasmid preparations were transformed into chemi-competent XL1-Blue Escherichia coli cells and plasmids were isolated by an alkaline lysis protocol. We were successful in isolating plasmid DNA from yeast >90% of the time. Plasmids were sequenced (GENEWIZ, NJ, USA) with T7 and T3 primers to determine the sequence on each side of the insertion.

Results

Investigation of GRC Efficiency in S. cerevisiae and C. glabrata

To compare the ability of S. cerevisiae and C. glabrata to carry out GRC, we performed transformation reactions using the same preparations of linearized vector, pRS313 (HIS3), and the same C. glabrata gene, CgURA3 with promoter, in both species. pRS313 contains an autonomously replicating sequence (ARS) that is functional in both species and CEN6 from S. cerevisiae [19,20]. It is unclear whether CEN6 is functional in C. glabrata, but it is unlikely as there are very specific sequence requirements for centromeric sequences in C. glabrata [21,22] and we have observed relatively quick plasmid loss under non-selective conditions in C. glabrata.
(data not shown). Each CgURA3 product was amplified to include 40 bp of vector homology on each side, which is sufficient homology for GRC. Using different molar ratios of CgURA3 to digested pRS313 (0, 0.02, 0.2, and 1.0), we titrated the concentration of DNA required for effective GRC. We measured GRC efficiency as the percentage of HIS3+ colonies (from linearized pRS313) that became URA3+ (Figure 2). Not surprisingly, the higher the molar ratio of insert DNA (CgURA3 with homology) to digested vector, the better the GRC efficiency. We determined that an insert to vector molar ratio of 1.0 produced an average 55% DNA insertions in S. cerevisiae and an average 49% DNA insertion in C. glabrata, and adding 5-fold molar excess of insert DNA allowed GRC to approach 90% in both species (data not shown). We concluded that S. cerevisiae and C. glabrata both efficiently engage in GRC, and that GRC can occur even when there is a relatively low amount of insert. It is not surprising that equal molar amounts are not required for efficient GRC, as it is likely that multiple DNA fragments enter the cell during transformation, and all that is required for our assessment of GRC is for one linear DNA vector molecule to be repaired by one insert with homologous ends.

It is worth noting that an alternative reason for observing URA3+ HIS3+ colonies could be integration of either piece of DNA into the genome or URA3+ integration coupled with re-circularization of the pRS313. To confirm that we were observing GRC plasmids, we grew 92 Ura"His" colonies from S. cerevisiae and 115 colonies from C. glabrata on non-selective (YEPD) medium overnight to allow for plasmid loss and then replica-plated to medium containing 5-FOA, which selects against cells that are expressing the URA3 gene (Figure S1 and Figure S2). In C. glabrata 115/115 colonies resulted in FOA-R colonies, and in S. cerevisiae 91/92 colonies, suggesting almost all Ura+ colonies were a consequence of URA3 incorporation into a plasmid as opposed to stable integration into the genome. Importantly, all of the FOA-R colonies except for one C. glabrata colony resulted in a His+

![Figure 2. Average insertion percentage for CgURA3 with 40 bp homology in S. cerevisiae and C. glabrata.](image)

Effects of Inactivating Homologous Recombination or Non-homologous Recombination Pathways on the Frequency of GRC

To examine the role of HR and NHEJ on the rate of GRC, we deleted RAD52 and DNL4 in both S. cerevisiae and C. glabrata and repeated the transformation experiments with 40/40 bp and 40/20 bp of homology. RAD52 is a key gene in genetic recombination and DNA repair, and is required for most forms of HR, but deleting RAD52 does not completely eliminate HR [7,12,23,24]. DNL4 also mediates DNA repair, but through non-homologous repair mechanisms [14,25].

Decreasing HR activity through deletion of RAD52 considerably reduces the frequency of GRC in both species compared with wild-type strains (Figure 5A and 5G – note difference in scale). In S. cerevisiae, the data support that deleting RAD52 does decrease the frequency of GRC, but does not completely eliminate it. Likewise,
in the Cgrad52Δ strain, the efficiency of GRC is dramatically decreased but not completely eliminated.

Inactivating the pathway responsible for NHEJ activity has different effects on S. cerevisiae and C. glabrata (Figure 5B and 5C). Notably, in Scdnl4Δ cells, the percent of DNA insertion into the plasmid is relatively unaffected compared with transformations in the wild-type - i.e. loss of NHEJ does not dramatically increase GRC efficiency. However, in C. glabrata, GRC increases in a Cgdnl4Δ strain, suggesting that NHEJ is more active, and loss of NHEJ drives the equilibrium of re-circularization towards HR.

Determination of Proportion of Resealed Vectors through HR or NHEJ in S. cerevisiae

In some transformations, such as those in a rad52Δ strain or where PCR product with low bp homology was used, the likelihood of acquiring plasmids with the URA3 insertion was low. To determine whether these rare URA3⁺ transformants were a consequence of HR or NHEJ, we rescued the plasmids in E. coli and sequenced the plasmids that resulted from the transformations. Not surprisingly, plasmids rescued from a S. cerevisiae wild-type transformation with 40 bp of homology on each side exhibited re-circularization via HR (data not shown); however, even with 10 bp of homology on each side, S. cerevisiae appeared to only re-circularize via HR (Figure 6). Likewise, in a Scrad52Δ, which has decreased HR (Figure 5A), transformations with 40 bp of homology on each side yielded plasmids that re-circularized via HR, indicating that loss of RAD52 does not completely eliminate GRC. We sequenced re-circularized S. cerevisiae plasmids that did not contain URA3 (HIS3⁺ only), and found that all appeared to not have been digested at all with SmaI. Knowing that restriction enzyme digestion may not proceed to completion, we hypothesized that these few HIS3⁺ URA3⁺ plasmids could be a consequence of incomplete digestion. To test this hypothesis, we doubly-digested pRS313 with two blunt cutting enzymes, EcoRV and SmaI, expecting that if re-circularization is caused by NHEJ and not a consequence of partial restriction enzyme digestion, we should observe the loss of 13 bp in the plasmid sequence (Figure 1).
We only observed completely undigested plasmid in *S. cerevisiae* plasmids, suggesting either we are unable to observe NHEJ in *S. cerevisiae* in this experiment, or that a singly digested plasmid ligated with no change in sequence. Supporting the lack of detectable NHEJ in this GRC assay, we were unable to recover *URA3* + *HIS3* + plasmids in *S. cerevisiae* strains where *RAD52* was deleted and the homology was decreased to 10 bp on each side.

**Determination of Proportion of Resealed Vectors through HR or NHEJ in *C. glabrata***

In *C. glabrata* we also rescued the plasmids from the rare *URA3* + *HIS3* + transformants using DNA inserts with 10 bp of homology on each side or from Cgrad52Δ transformations. The sequencing data from these plasmids indicated that DNA was inserted via HR in *C. glabrata* wild-type (with 10 bp of homology) and in Cgrad52Δ (with 40 bp of homology), strengthening the conclusion that HR is the preferred mechanism in *C. glabrata* as well (Figure 6). We then transformed the Cgrad52Δ strain using linear vector and CgURA3 with 10 bp of homology, and noted a difference between *S. cerevisiae* and *C. glabrata*. Whereas we never identified a *URA3* + *HIS3* + colony in *Scrad52*Δ with 10 bp homology, we identified colonies that were both *URA3* + and *HIS3* + in *C. glabrata*. We purified these plasmids and confirmed that 60% of the time the linear PCR product was inserted into the plasmid via NHEJ, because there was a 10 bp duplication of sequence flanking both sides of the *URA3* gene.

Following this experiment, plasmids were rescued from a transformation using a doubly digested plasmid and no insert. In *S. cerevisiae*, 100% of the sequences from these double-digested plasmids indicated that the vector was likely never fully digested. In *C. glabrata*, 100% of the sequences demonstrated that the rescued vectors were digested and re-circularized, lacking the 13 bp, and thus were a consequence of NHEJ. These data reveal that *C. glabrata* is more capable of performing NHEJ relative to *S. cerevisiae*.

Our results may appear to conflict with other published studies – i.e. our observation of no NHEJ activity in *S. cerevisiae*, however, it is worth pointing out four major differences between this study and the others. First, we used at least 40× less plasmid DNA (50 ng vs. 2 μg) than another study using plasmids making it unlikely that we have saturated the HR machinery in the cell and skewing results towards HR [13]. We chose these low concentrations to see subtle differences between the two species in a titration of different variables, but these conditions minimize NHEJ activity (Figure 2), as previous experiments have demonstrated less than 5 transformants/μg of non-homologous DNA [26]. Second, most NHEJ studies examine events in the chromosome, not in plasmids [5,26,27]. Third, many studies indicate high levels of HR in yeast species and so it is not surprising given our conditions that we are not observing relatively rare NHEJ events [10,13,24]. Finally, previous studies have indicated that direct ligation may be a mode of repair, but we have also examined the role of partial plasmid digestion (see below). Because we digest with two blunt end restriction enzymes, and observe ligation events in *C. glabrata*, but not in *S. cerevisiae*, we can conclude that this ligation/NHEJ activity...
is higher in *C. glabrata* relative to *S. cerevisiae*. Therefore, in our conditions we do not observe NHEJ in *S. cerevisiae*, but there are likely rare NHEJ events that we are not observing. In fact, in other studies even with higher concentrations of plasmid in *S. cerevisiae* the vast majority of “re-circularization events” were actually incomplete digestion events [10].
Assessment of the Impact of Chromosomal Integration on GRC

A possible complication to our work is that URA3+ could be integrated into the chromosomal genome via HR or NHEJ at the same time that vector re-circularizes, overestimating the frequency of GRC. To determine whether integration into the genome of the URA3+ gene could cause a Ura+ phenotype independent of plasmid re-circularization, we performed a reciprocal experiment. We transformed both species as before, but selected for URA3+ first and measured the frequency of these colonies that were HIS3+. In S. cerevisiae, we did not observe any Ura+ colonies in the absence of linearized vector and we only observed Ura+ His+ colonies during co-transformation with both PCR product and linearized vector, suggesting little/no NHEJ.

In C. glabrata, we observed Ura+ colonies with PCR product alone; however, the frequency of these colonies was ~5% of the maximal number of colonies observed when linearized vector was present. Additionally, during co-transformation, we observed ~50% of colonies that were Ura+ but not His+. Initially, this might suggest that NHEJ and chromosomal integration is very high; however, the CgURA3 PCR product is derived from C. glabrata, and we hypothesized that HR between the PCR product and the endogenous Cgura3::NATMX6 locus might be occurring in these transformations. Because we deleted CgURA3 in the genome with the NATMX6 cassette, we expected loss of the NATMX6 cassette if CgURA3 is integrated into the genome through HR. Importantly, all Ura+ His+ colonies were resistant to nourseothricin and all Ura+ His- colonies were sensitive, indicating that HR is far more prevalent than NHEJ in C. glabrata.

Discussion

The goal of this project was to characterize GRC in S. cerevisiae and C. glabrata. Our results suggest that GRC can be effectively carried out in both yeast species. This has previously been demonstrated in S. cerevisiae but not in C. glabrata. We were also able to uncover the determinants of GRC for both species. Our data indicate that 20 bp of homology on each side of the PCR product is required for detectable GRC to occur. An insert/vector molar ratio of 1.0 was shown to be sufficient for obtaining insertion of the PCR product at ~50%, while increasing this ratio to 5.0 drives GRC to almost 100%. We also found that S. cerevisiae is slightly more efficient at performing GRC. This result was supported by the GRC data we obtained using the rad52Δ mutants. The S. cerevisiae rad52Δ mutant was only able to perform GRC at a very low frequency and required 40 bp of homology on each side, indicating that this species is very dependent on HR for re-circularization. The C. glabrata rad52Δ mutant produced re-circularized plasmids that contained URA3 at a low frequency as well. However, the sequencing data suggests that NHEJ, in addition to HR, is an important factor for GRC. The presence of URA3 into the vector (Figure 6). The presence of NHEJ activity in C. glabrata may be an effect of slower DNA degradation in this species, although we have not tested that hypothesis in this work. Interestingly, sequencing from a separate experiment (data not shown) indicated that C. glabrata displayed a strong preference for performing HR. In this experiment, a sequence contained a fragment of salmon sperm DNA that had been inserted into the vector by GRC, with only 11 bp of homology on one side of the salmon sperm DNA fragment. This fortuitous finding suggests DNA may persist longer in C. glabrata, allowing more time for NHEJ or extremely rare HR events to occur, even with unintended targets, such as small fragments of carrier salmon sperm DNA.

The S. cerevisiae dnl1Δ mutant shows a similar rate of GRC as the wild-type strain. Here again, our data suggest that S. cerevisiae may not be capable of performing NHEJ at a detectable rate in this GRC assay, indicating that HR is the only means to re-circularize DNA through insertion. In contrast to S. cerevisiae, the Cgdnl4Δ strain shows an increased frequency of GRC compared to wild-type, suggesting that NHEJ activity is detectable in this GRC assay and that C. glabrata has more NHEJ activity in general.

Based on results from all of the experiments that were performed, we generated a model for the fate of linear DNA in S. cerevisiae and C. glabrata (Figure 7). In S. cerevisiae, it appears that the linear DNA (both pRS313 and PCR product) takes two out of three avenues in the majority of instances: HR or vector degradation (the eventual fate of transformed linear DNA in cells if vector is not re-circularized). Removing NHEJ activity in S. cerevisiae, through deletion of DNL1, has little effect on GRC, because there is little NHEJ to begin with. On the other hand, the fate of linear DNA in C. glabrata is influenced by HR, NHEJ, or degradation. When NHEJ activity is deleted in C. glabrata, there is an increase in HR. Although both species are capable of performing GRC at a high level, this model suggests that S. cerevisiae is the preferred species in which to perform this technique. Our data indicate that NHEJ is more prevalent in C. glabrata; however we cannot fully determine whether this is because NHEJ is inherently more active in this species, or whether linear DNA is degraded more slowly in C. glabrata and the NHEJ machinery is able to work for a longer period.

Supporting Information

Figure S1 S. cerevisiae Ura+His+ cells are primarily a consequence of GRC. Using 40 bp homology on each side, colonies that were Ura+His+ were picked to a YEPD plate and then replica-plated to 5-FOA plates and colonies were scored for growth. Then, the colonies were replica-plated to medium lacking uracil or histidine to assess whether the two markers were coupled (EPS).

Figure S2 C. glabrata Ura+His+ cells are primarily a consequence of GRC. The same experiment was performed as described in Figure S1. One colony out of the 115 was identified as His+ and Ura+ and is potentially a consequence of plasmid integration into the genome. (EPS)

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Author Contributions

Conceived and designed the experiments: MWC CLK DDW. Performed the experiments: MWC CLK ASK KBA DDW. Analyzed the data: MWC CLK ASK DDW. Contributed reagents/materials/analysis tools: MWC CLK ASK DDW. Wrote the paper: MWC CLK DDW.
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