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Cytoduction Preserves Genetic Diversity Following Plasmid Transfer Into Pooled Yeast Libraries

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ABSTRACT

Introducing plasmids into yeast is a critical step for many phenotypic assays and genetic engineering applications. However, it is often challenging for applications that involve large pools of variants because the population structure can be easily altered by traditional methods such as chemical transformation. In this study, we introduce drug-marked plasmids into a heterogeneous yeast population using both transformation and cytoduction (mating without nuclear fusion). Using a highly diverse barcoded yeast collection, we quantify the efficiency of both methods. We demonstrate that for cytoduction, but not transformation, nearly all the genotypes in the initial pool were detected in the final pool, with a high correlation to their initial frequencies. Finally, we map QTL that impact both cytoduction and transformation. Overall, we demonstrate the efficiency of cytoduction as a means of introducing plasmids into yeast. This is significant because it provides a means of manipulating diverse yeast populations, such as pools constructed for bulk segregant analysis, deep mutational scanning, large-scale gene editing, or populations from long-term evolution experiments.

1 | Introduction

Much of our understanding of functional genomics derives from insights gained from large strain libraries including the yeast deletion collection, the GFP and TAP-tagged libraries, QTL mapping populations, among others (Arita et al. 2021; Ghaemmaghami et al. 2003; Mnaimneh et al. 2004; Sopko et al. 2006; Winzeler et al. 1999). A limitation of these libraries is that it is not easy to introduce reporters or make genetic perturbations to all strains in these collections. Tools such as Synthetic Genetic Arrays allow for the genetic manipulation of these libraries but are labor intensive and require specialized equipment for high throughput pinning (Kuzmin et al. 2014). Manipulating a diverse library *en mass* without losing diversity remains challenging. Ultimately, this limitation stems from the inefficiency of transformation, which is the standard method for genetic manipulation in yeast.

We test the efficacy of cytoduction (mating without nuclear fusion [Georgieva and Rothstein 2002]) as a method to transfer plasmids directionally from a "Donor" to a diverse pool of "Recipient" strains. The mating process in *Saccharomyces cerevisiae* involves the fusion of haploid yeast cells of opposite mating types (*MAT***a** and *MAT*α) to form a *MAT***a**/α diploid. If one of the mating partners carries the *kar1Δ15* mutation, mating will result in cell fusion but not nuclear fusion (Rose and Fink 1987; Vallen et al. 1992). This process (cytoduction) is an efficient method for transferring plasmids (Georgieva and Rothstein 2002; Spencer et al. 1994), mitochondria (Lancashire and Mattoon 1979; Matsuoka et al. 1982), cytoplasmic viruses

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Summary

- Cytoduction is significantly more efficient than transformation at introducing plasmids.
- Cytoduction makes it possible to genetically manipulate pooled yeast libraries.
- Multiple QTL effect cytoduction and transformation efficiencies.

(Seki et al. 1985; Buskirk et al. 2020), prions (Dorweiler et al. 2020; Manogaran et al. 2010, 2011; Masison et al. 1997), and rarely, individual chromosomes (Torres et al. 2007). Because cytoduction is a modification of the mating process, it has the potential to achieve much higher efficiency at introducing plasmids than transformation. Here we quantify the efficiency of cytoduction and show that it enables the introduction of plasmids into high-diversity libraries with minimal impact on population diversity.

2 | Materials and Methods

2.1 | Donor and Recipient Strains

We used a pooled library of 4,401 barcoded segregants from a cross between BY4742 and RM11-1a strains (Nguyen Ba et al. 2022). The library has the genotype *MAT***a** *ura3*Δ *can1*Δ*::pSTE2-SpHIS5 ho*Δ*::URA3,* and the barcodes are within an artificial intron in *URA3*. We propagated the population and archived multiple aliquots at cell density 10^8 cells/ml. A single aliquot was thawed and 200 µl was used to inoculate 10 ml YPD (Yeast Extract, Peptone, Dextrose), which was grown for 10 h at 30°C. The culture was then diluted 1:20 in YPD, incubated for 3 h at 30°C, and the resulting log-phase cells were used for the transformation and cytoduction experiments described below. Throughout the growth, selection, and genomic and library preparations, population bottlenecks were at least eight-fold larger than the library diversity. The starting population lineage frequencies spanned four orders of magnitude (Supporting Information: Supplemental Figure 1).

The Donor strains for cytoduction were built upon yGIL1940, an S288c derivative with the genotype *MAT*α *HAP1 leu2*Δ *his3*Δ *met17*Δ *nej1*Δ *ura3*Δ *CAN1 kar1*Δ*15*. yGIL1940 was transformed with four plasmids: pRSII416-KanMX, pRSII416-NatMX, pRSII426-KanMX, and pRSII426-NatMX. All four plasmids carry the *URA3* marker. These plasmids have the four possible combinations of drug marker (KanMX and NatMX) and base (2-micron and CEN/ARS).

2.2 | Chemical Transformation

The Recipient yeast population was transformed using the lithium acetate protocol (Gietz and Schiestl 2007) with either of four plasmids—pRSII416-KanMX, pRSII416-NatMX, pRSII426-KanMX, and pRSII426-NatMX. For the transformation we used ~10^7 mid-log phase Recipient cells and 1 µg of plasmid DNA. Heat shock at 42°C was done for 25 min. Before selection on 150 mm agar plates, cells were recovered in YPD at 30°C for an hour to allow for drug resistance expression. SD-HIS + G418 plates were used for pRSII416-KanMX and pRSII426-KanMX, and SD-his+ClonNAT plates were used for pRSII416-NatMX and pRSII426-NatMX, respectively. Final antibiotic concentrations were 200 µg/ml for G418 and 100 µg/ml for ClonNAT. Approximately 10^5 colonies were harvested by scraping after incubation at 30°C for 2 days and subjected to genomic DNA extraction.

2.3 | Plasmid Transfer by Cytoduction

We performed cytoduction by mixing ~10^7 mid-log phase cells of the yeast library with ~5×10^7 mid-log phase cells of each of the Donor strains in 10 ml final volume of YPD. The mixtures were then concentrated on filter paper using a vacuum manifold. The filters were transferred to YPD plates and incubated at 30°C for 6 h to allow for ~1 round of mating. Mating mixtures were then washed off in 1 ml of sterile distilled water and spread on selection plates. The same selective medium was used for both the transformation and cytoduction to minimize the influence of differential growth rates across media. SD-his-arg+Can+G418 plates were used for pRSII416-KanMX and pRSII426-KanMX, and SD-his-arg+Can+ClonNAT plates were used for pRSII416-NatMX and pRSII426-NatMX, respectively. G418 and ClonNAT were used to select for cells containing the plasmids, while canavanine was used to select against the donor and diploid cells. Cells were harvested by scraping after incubation at 30°C for 2 days and subjected to genomic DNA extraction. The number of colonies far exceeded the number of colonies yielded by transformation, as cytoduction is much more efficient (Supporting Information: Supplemental Figure 2).

2.4 | Barcode Sequencing

The libraries were prepared as previously described (Aggeli et al. 2022). Genomic DNA extraction was done as described previously using 10^8 cells per sample and quantified by Qubit. Briefly, 1 µg of DNA was used in a 2-step PCR protocol to amplify the barcoded locus and introduce Unique Molecular Identifiers (UMIs). DNA from all libraries was pooled isostoichiometrically based on DNA concentrations estimated by Nanodrop. A 350-bp band was gel purified with the QIAGEN gel extraction kit (QIAGEN, Germantown, MD). Final sample quantification was done by Qubit. Final pools were analyzed by BioAnalyzer on a High-Sensitivity DNA Chip (BioAnalyzer 2100, Agilent) before Illumina sequencing on a NovaSeq with 150×150 bp paired-end reads at the Sequencing Core Facility within the Lewis-Sigler Institute for Integrative Genomics at Princeton University. Each library had at least 5×10^5 reads (Figure 1B). Barcodes were extracted from raw reads using UMI-tools, which uses network-based methods to account for errors (Smith et al. 2017). A list of known barcodes was given, and a pattern that allows up to 3 mismatches in each of the 2 invariable regions around the barcodes was used for regex search of the barcodes. As a result, $92.3 \pm 0.8\%$ of the reads matched barcode sequences. Estimates for the required number of reads for recovering barcodes after cytoduction and transformation were obtained by downsampling. We quantified

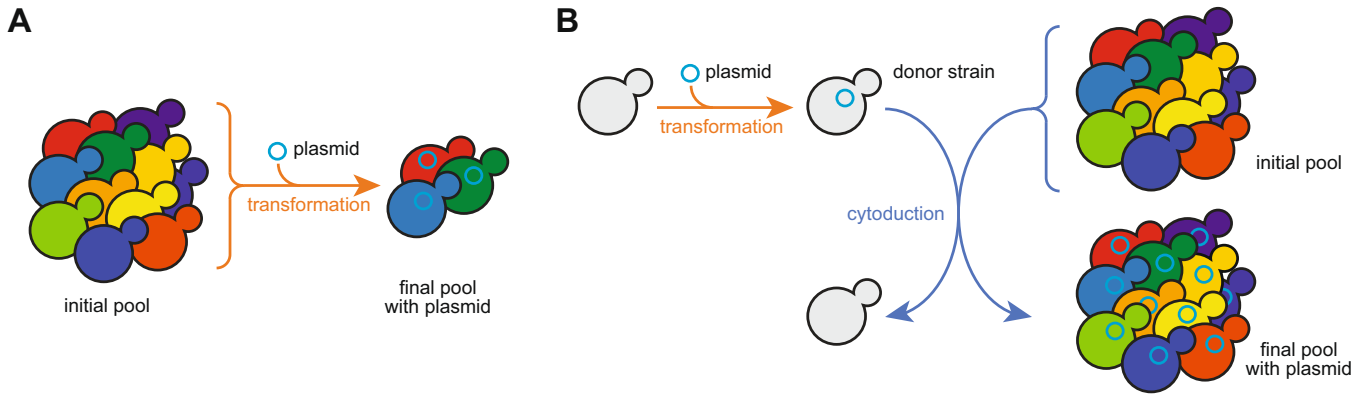

FIGURE 1 | Schematic of transformation and cytoduction procedures. Two alternative methods for introducing a plasmid into a diverse pool of yeast strains. In principle, cytoduction should be more efficient than direct transformation, maintaining the genetic diversity present in the initial pool. (A) Direct transformation of the plasmid into a diverse pool will result in a reduction of genetic diversity because only a small fraction of cells will take up the plasmid. (B) Cytoduction first requires transformation of the plasmid into a donor strain carrying a mutation that causes a karyogamy defect. This strain is then mated to the diverse pool of strains, and the plasmid is transferred by cytoduction.

barcode diversity using Shannon entropy, a common metric for assessing population diversity (Shannon 1948).

2.5 | Estimation of Bottleneck Size

To estimate the number of required sequencing reads for recovering the barcodes after either cytoduction or transformation, we performed in silico bootstrapping. The bootstrapping was done with the random generator tools in NumPy using Python (Harris et al. 2020). Lineage frequencies in each library were used as probability distributions for the sampling. The processes were repeated 100 times, and the mean number of unique values was used. We determined bottleneck sizes through a simulation-based approach involving the recovery of barcodes from the initial population until the observed numbers of barcodes in the final pools were attained. We used the barcode frequencies in the initial pool as the probability for each lineage to be picked. Genetic differences were assumed to have no influence on the probability. The requisite retrieval counts were then calculated as the average across 100 simulations.

2.6 | QTL Mapping

We performed a genome-wide scan for the QTL using Pearson correlation. First, we used inferred probabilistic genotype values and inferred fitness values with a likelihood model for time-dependent barcode frequencies (Nguyen Ba et al. 2022). As the genotype values are probabilities and not definitive, we next calculated the Pearson correlation coefficient between the inferred phenotype and genotype values at each locus using a linear regression model. Standard error of correlation coefficients across the genome and experiments was used to find significant calls. Correlation values greater than one standard error were considered significant. Annotations are candidate genes based on previously reported QTL that colocalize with peaks in the correlation values (Nguyen Ba et al. 2022). The prominent QTL on Chromosome IV is due to the barcode locus (Nguyen Ba et al. 2022). KanMX and NatMX were used to mark the barcode locus in BY and RM, respectively. Though the markers should have been removed during construction, it is likely that some segregants retained the markers. Selections on G418 and ClonNat enriched for segregants carrying BY or RM parentage, respectively, around the barcode locus.

3 | Results and Discussion

In this study, we set up a direct comparison between transformation and cytoduction as a means of introducing plasmids into yeast libraries (Figure 1). For this test we used a barcoded library of segregants from a cross between BY4742 and RM-11a (Nguyen Ba et al. 2022). This library contains 4,401 genotypes, each carrying a unique barcode. In addition, this library differs from other commercially available libraries in that it contains a *can1* mutation, which we used to select against the Donor strain and diploids during cytoduction. For transformation, we used the high-efficiency lithium acetate method, which is the gold standard in the field of yeast genetics (Gietz and Woods 2002). For cytoduction, we first introduce the plasmid into a Donor (*kar1Δ15* mutant) strain. We then mate the Donor strain to the Recipient library. Following mating, we select for the Recipient library and the transferred plasmid, and we counter-select against the Donor strain and Donor/Recipient diploids.

We conducted parallel cytoduction and transformation experiments using four different plasmids. These plasmids were either CEN/ARS-based (pRSII416) or 2-micron-based (pRSII426) and included a selectable marker, either KanMX or NatMX. The plasmids were introduced to the Recipient library through either transformation or cytoduction. We conducted the transformation and cytoduction experiments using an equal division (~10^7 cells each) of the same initial population. Transformations were performed using the standard lithium acetate protocol with 1 μg of plasmid. Cytoductions were performed by mixing the *MAT***a** Recipient library with a five-fold excess of *MATα* Donor cells, followed by 6 h of mating on YPD before selective plating. A five-fold excess was chosen to increase the likelihood that all *MAT***a** Recipient cells will have access to a mating partner. We collected all the colonies from the selection plates, with approximately 10^5 colonies from each transformation and a lawn of cells (> 10^7

colonies) from each cytoduction experiment, reflecting the higher efficiency of cytoduction (Supporting Information: Supplemental Figure 2). The number of colonies collected in each case was at least two orders of magnitude greater than the diversity of the library. We then amplified barcodes for Illumina sequencing, using ~10^8 cells per experiment, which were then sequenced to a depth of ~5×10^5 reads per sample.

Following cytoduction, most barcodes from the initial pool were recovered (97% on average), and these exhibited a strong correlation with the initial pool (0.88 on average, Figure 2A and Supporting Information: Supplemental Figure 3A). In contrast, after transformation, only 43% of the barcodes were recovered in the final pools on average, and these exhibited a lower correlation with the initial pool (0.49 on average). As a result of preserved barcode frequencies, fewer sequencing reads are needed to recover a greater number of barcodes from cytoduction compared to transformation experiments (Figure 2B).

The high recovery of barcodes in cytoduction is not due to having more reads. Across all the experiments, we used comparable numbers of sequencing reads for the final pools generated through cytoduction and transformation (Table 1). After sequencing read deduplication, we also observed a similar count of unique reads for both methods (Table 1). These findings indicate that the improved recovery and correlation in the final pools following cytoduction cannot be attributed to variations in sequencing depth. The skewed distribution of lineages after transformation could result from bottleneck effect. To test this hypothesis, we estimated the bottleneck size for each library, assuming no genetic effects. Cytoduction exhibited a bottleneck size of 140,285 ± 23,627, whereas transformation had a much smaller bottleneck size of only 3,224 ± 1,104 (Table 2). The bottleneck size in transformation is more than 40 times smaller than that in cytoduction. It is also approximately 30 times smaller than the number of collected transformants in our experiments, strongly suggesting transformation biases, potentially originating from genotype biases.

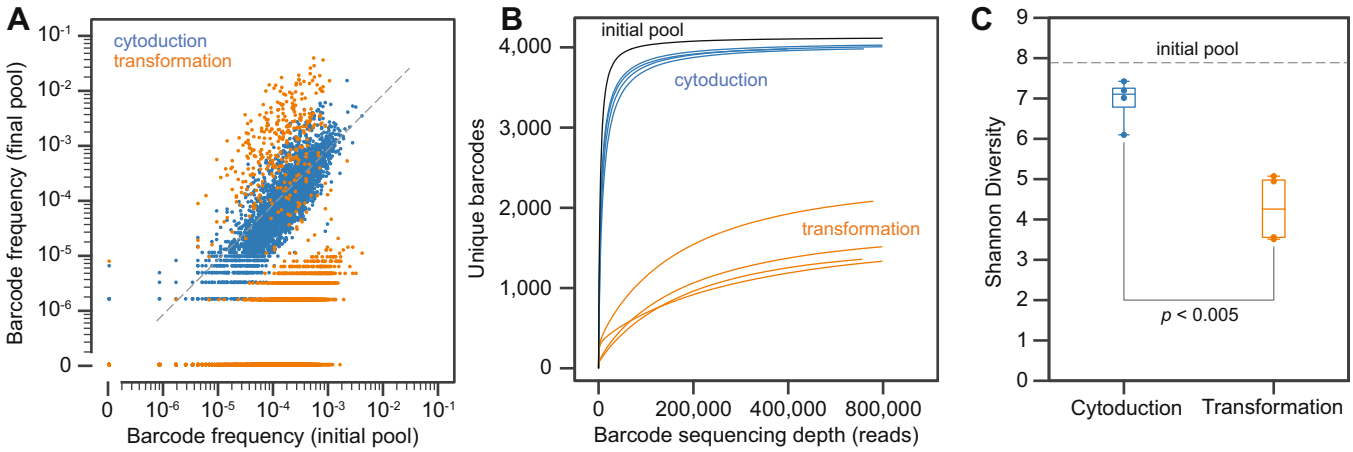

FIGURE 2 | Cytoduction is highly efficient at transferring plasmids while maintaining diversity in the Recipient population. We introduced four plasmids representing all combinations of 2-micron or CEN/ARS plasmids with NatMX or KanMX markers into ~10^7 cells from a library containing 4,401 unique barcodes. A total of 4,149 barcodes were identified in the input library. (A) The distribution of barcode frequencies shows that cytoduction maintains relative abundance. The data shown are from the cytoduction and transformation experiments using the KanMX-marked CEN/ARS plasmid. The barcode recovery and correlation coefficients are 98%/0.88% and 38%/0.41 for cytoduction and transformation, respectively. The other three scatter plots are shown in Supporting Information: Supplemental Figure 1A. (B) Rarefaction curves show that cytoduction recovers significantly more barcodes compared to transformation, especially with fewer than 100k reads. Even with > 400k reads, ~60% of the barcodes are missing from the transformation, but only ~2.5% from the cytoduction. (C) Shannon Diversity is higher in the cytoduction population compared to transformation across all four experiments ($p < 0.005$, paired t-test).

TABLE 1 | Barcode recovery from cytoduction and transformation experiments.

Plasmid	Method	# reads	# UMIs	# barcodes	% barcodes	Correlation
Initial pool	—	1,626,685	1,166,312	4,132	100.0	1.0
416-KanMX	Cytoduction	811,698	605,154	4,049	97.99	0.88
416-KanMX	Transformation	921,516	623,451	1,568	37.95	0.41
416-NatMX	Cytoduction	531,869	380,754	4,011	97.07	0.89
416-NatMX	Transformation	968,914	477,210	1,677	40.59	0.51
426-KanMX	Cytoduction	845,801	598,098	4,035	97.65	0.85
426-KanMX	Transformation	774,405	490,826	2,289	55.40	0.55
426-NatMX	Cytoduction	747,787	498,539	4,010	97.05	0.88
426-NatMX	Transformation	743,372	400,489	1,498	36.25	0.49

To better assess the changes in the diversity of the yeast libraries after manipulation, we quantified barcode diversity using Shannon entropy, a standard metric used to assess diversity (Shannon 1948). Shannon entropy was the highest for the input pool with only a minor loss of diversity following cytoduction (Figure 2C). In contrast, we observed a substantial loss of diversity following transformation (Figure 2C, $p < 0.005$, paired t-test), indicating that transformation has comparatively low efficiency. We do not find significant differences in Shannon entropy when comparing experiments with different plasmid types, indicating that efficiency is not strongly influenced by the selective marker or the type of replication origin on the plasmid (Supporting Information: Supplemental Figure 3B, $p = 0.041$ and $p = 0.275$, paired t-tests).

To test the extent to which genetic factors influence the observed changes in barcode frequencies, we performed a QTL analysis using the known genotypes associated with each barcode. Fitness values were estimated based on changes in barcode frequencies as described previously (Nguyen Ba et al. 2022). We then calculated the correlation between fitness and the genotype at each of the 41,595 polymorphic sites (Figure 3 and Supporting Information: Supplemental Table 1). For cytoduction, we observed enrichment for loci commonly associated with growth but not mating efficiency. This suggests that the five-fold excess of Donor strain mitigated selection on mating but that the 6 h outgrowth may impose modest selection for loci effecting growth rate. For transformation, in contrast, we observed few enriched loci, suggesting that sampling is largely stochastic and not strongly influenced by genetic variation represented in our library.

TABLE 2 | Estimates of bottleneck sizes.

Plasmid	Cytoduction	Transformation
416-KanMX	174,842	2,556
416-NatMX	119,003	2,851
426-KanMX	149,424	5,114
426-NatMX	117,873	2,376
Average	140,285	3,224

This study demonstrates the efficacy of cytoduction as a tool to introduce plasmids into yeast libraries while preserving the diversity and the relative abundance of individual genotypes. We were able to manipulate 97% of the variants, while also achieving good population representation (correlation with the initial pool is 0.88). As a comparison, only 43% of the variants were retained after chemical transformation with correlation being only 0.49. At a minimum the method requires a haploid Recipient library with a functional mating pathway, a *kar1Δ15* Donor strain, and appropriate genetic markers to select for the haploid Recipient library and/or against diploid and Donor cells. In the present study, we used the dominant drug-susceptible *CAN1*. In the absence of native markers, an inducible lethal gene could be engineered into the donor strain. Another potential caveat is rare aneuploids that might be generated by cytoduction. With a low rate, whole chromosomes may transfer from the Donor to the Recipient. These events are rare (frequency of 10^{-4} to 10^{-7}), with smaller chromosomes more prone to transfer (Torres et al. 2007; Dutcher 1981). In this case, the Universal Donor Strain, in which each centromere can be inactivated by a galactose-inducible promoter, can be used to reduce the likelihood of aneuploidy (Manogaran et al. 2010; Reid et al. 2011). Our approach excels at introducing a single plasmid into a diverse yeast library. Our method could be extended to introduce a diverse plasmid library into a Recipient yeast library; however, this application would be limited by

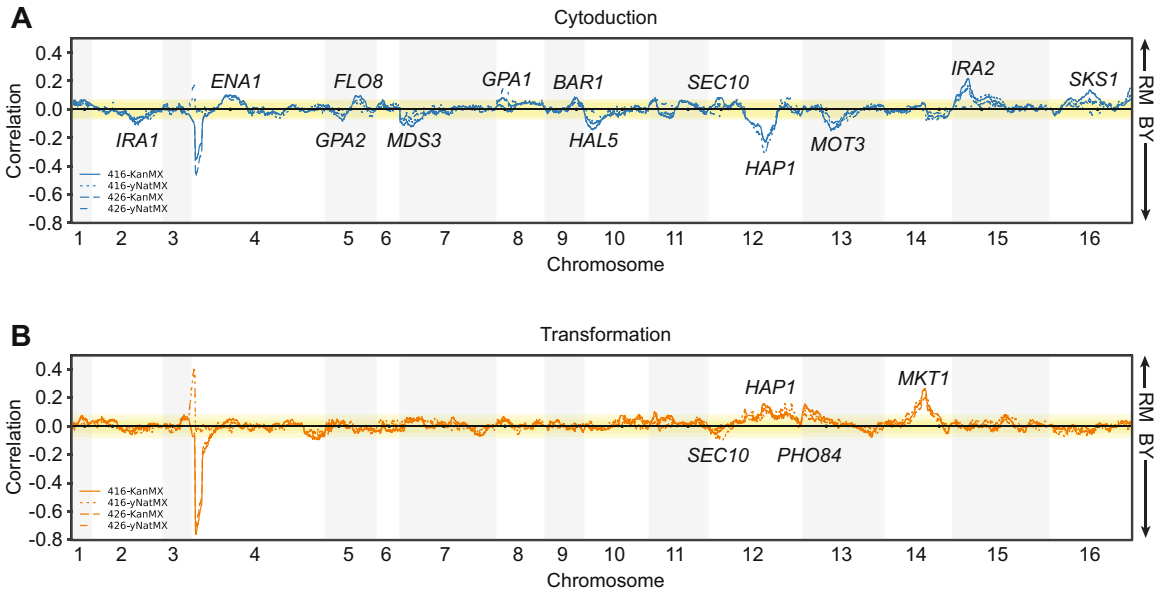

FIGURE 3 | Changes of lineage frequencies in cytoduction correlate with loci associated with growth while few loci were correlated in transformation. (A) Correlation between individual polymorphic sites and changes of lineage frequencies in cytoduction. Positions of the peaks co-localize with genes associated with growth in Nguyen Ba et al. (2022). (B) Correlation between individual polymorphic sites and changes of lineage frequencies in transformation. Fewer QTL are enriched in the transformation experiments, suggesting random sampling as the predominant factor for the changes in barcode frequencies.

the efficiency of transformation of the plasmid library into the Donor strain. Finally, our method could be combined with CRISPR/Cas9 editing, further expanding the potential for modifying the genetic background of diverse yeast libraries.

Author Contributions

Han-Ying Jhuang, Dimitra Aggeli, and Gregory I. Lang designed the study. Han-Ying Jhuang and Dimitra Aggeli performed the experiment. Han-Ying Jhuang, Dimitra Aggeli, and Gregory I. Lang analyzed the data. Han-Ying Jhuang and Gregory I. Lang wrote the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.