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A vector set for systematic metabolic engineering in *Saccharomyces cerevisiae*

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Abstract

A set of shuttle vectors was constructed to facilitate expression of genes for metabolic engineering in *Saccharomyces cerevisiae*. Selectable markers include the *URA3, TRP1, MET15, LEU2-d8, HIS3* and *CAN1* genes. Differential expression of genes can be achieved as each marker is available on both *CEN/ARS*- and 2 μ-containing plasmids. Unique restriction sites downstream of *TEF1*, *PGK1* or *HXT7-391* promoters and upstream of the *CYC1* terminator allow insertion of open-reading frame cassettes for expression. Furthermore, a fragment appropriate for integration into the genome via homologous recombination can be readily generated in a polymerase chain reaction. Vector marker genes are flanked by *loxP* recognition sites for the CreA recombinase to allow efficient site-specific marker deletion and recycling. Expression and copy number were characterized for representative high- and low-copy vectors carrying the different marker and promoter sequences. Metabolic engineering typically requires the stable introduction of multiple genes and genomic integration is often preferred. This requires an expanded number of stable expression sites relative to standard gene expression studies. This study demonstrated the practicality of polymerase chain reaction amplification of an expression cassette and genetic marker, and subsequent replacement of endogenous retrotransposons by homologous recombination with flanking sequences. Such reporters were expressed comparably to those inserted at standard integration loci. This expands the number of available characterized integration sites and demonstrates that such sites provide a virtually inexhaustible pool of integration targets for stable expression of multiple genes. Together these vectors and expression loci will facilitate combinatorial gene expression for metabolic engineering.

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Supporting Information
Supporting information may be found in the online version of this article.
Methods - strain and plasmid construction
S1. Sequence for codon-optimized creA
S2. Oligonucleotide primers used in this study
S3. Sequences for promoters, genetic markers and copy-number control
S4. Four strongly expressed and three weakly expressed Ty1 elements in *S. cerevisiae* S288C

Keywords

Saccharomyces cerevisiae; vectors; integrants; markers; Cre recombinase; chromosomal sites; metabolic engineering

Introduction

Saccharomyces cerevisiae is an important organism for metabolic engineering. This yeast has been studied extensively, molecular and genetic tools are available for its manipulation and it is a successful industrial microorganism. Sets of low-and high-copy plasmid vectors and integrating vectors have found extensive use in gene function studies. These include plasmid sets that contain multiple cloning sites and are genetically marked with *TRP1, HIS3, LEU2, URA3* (Christianson *et al*., 1992; Gietz and Sugino, 1988; Sikorski and Hieter, 1989), *MET15* and *ADE2* (Brachmann *et al*., 1998). These plasmids are ideal for studies of individual genes under their native promoters. Expression vectors include promoters and terminators flanking the cloning site. Examples include the pYES2.0 galactose-inducible *URA3*-marked vector (Invitrogen, Carlsbad, CA, USA), the *tet*-on/off vectors (Gari *et al*., 1997) and a set of vectors with *ADH1, TEF1* and *GPD1* promoters (Mumberg *et al*., 1995).

Although plasmid vectors present a useful platform for many individual gene expression studies, high copy plasmids are relatively unstable (Futcher and Carbon, 1986; Mead *et al*., 1986) and CEN/ARS plasmids can also become unstable in combination (Futcher and Carbon, 1986). Depending on the application, stable maintenance of sequences can be accomplished with genomic integration of the cloned sequences. In *S. cerevisiae*, this was originally achieved by tagging the gene of interest with a selectable marker and incorporating sequences homologous to the integration target site to allow targeted integration of plasmids (Gietz and Sugino, 1988; Scherer and Davis, 1979; Sikorski and Hieter, 1989). This strategy was later modified by flanking the *URA3* marker gene with direct repeats of the 1 kb bacterial *hisG* sequence from bacteria (‘*URA3* blaster’) to facilitate gene knockouts (Alani *et al*., 1987) or insertions (Lee and Da Silva, 1997) and subsequent marker recycling by homologous recombination between the direct repeats. However, the relatively long *hisG* sequences used to mediate homologous recombination in that strategy left a significant footprint and thereby risked off-target homologous recombination during subsequent transformations. This complication limited the potential for marker recycling. Site-specific recombination systems were introduced to address this problem. These utilized much shorter direct repeats that could be efficiently recombined upon expression of the appropriate recombinase (Johansson and Hahn-Hagerdal, 2002; Prein *et al*., 2000; Radhakrishnan and Srivastava, 2005). One example of such a strategy is the CreA–*loxP* system (Gueldener *et al*., 2002; Güldener *et al*., 1996; Sauer, 1994). In addition to vector-based integration, polymerase chain reactions (PCRs) have been used to generate fragments for genomic insertion (Lorenz *et al*., 1995).

Current vector and PCR-based integration cassettes are particularly well-suited to manipulation of individual genes. Metabolic engineering differs from this undertaking in its specific requirement for testing of multiple genes in order to optimize pathway expression and function. Combinations of plasmids are sometimes useful for initial testing. However, because of issues of plasmid instability, as discussed above, it is ultimately desirable to stabilize pathways by integration of multiple expression cassettes and the number of characterized genomic loci for such integrations is limited.

In order to facilitate metabolic engineering in *S. cerevisiae*, we have constructed and characterized a toolkit of 28 expression vectors (pXP). This pXP vector set includes high-

and low-copy plasmids marked with six standard genetic markers. Cloning sites are flanked by a choice of three promoters and the *CYC1* terminator (T_{CYC}). These are adjacent to selectable markers in the vector plasmids, so that the cloned sequence of interest and selectable marker can be amplified together to obtain a fragment suitable for genomic integration. Markers are flanked by *loxP* sites to allow recycling. Expression of luciferase reporters (McNabb *et al*., 2005) carried on vectors was compared to expression of the same reporters from previously characterized and novel chromosomal loci. These vector- and genome-based metabolic engineering tools enable plasmid-based testing followed seamlessly by genomic integration for pathway optimization.

Materials and methods

Yeast and bacterial strains and culture conditions

Yeast and bacterial culture methods were standard (Amberg *et al*., 2005; Ausubel, 2008) except where noted. *Escherichia coli* strain DH5*α* [F^- *φ* 80*lacZM15Δ* (*lacZYA-argF*) *U169 deoR recA1 endA1 hsdR17* (r_K^- m_K^+) *phoA supE44 thi-1 gyrA96 relA1* λ^-] (Invitrogen) was used for plasmid preparations. *S. cerevisiae* BY4741 (*MAT* **a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) (Open Biosystems, Huntsville, AL, USA) is related to the sequenced strain S288C (Goffeau *et al*., 1996). Yeast strain yBF1587 was derived from strain BY4741 by deletions of the *ADH2, GRE3* and *TRP1* open reading frames (ORFs). When using vectors in the *trp1 Δ* background, it should be kept in mind that the *trp1 Δ* ORF allele may confer undersirable side-effects, depending upon the experimental conditions (Gonzalez *et al*., 2008). Strains are described in Table 1. Derivation of reporter strains is described below and derivation of yBF1587 is described in the Materials section of the Supporting information. Yeast were cultured in synthetic minimal medium (SD) with dextrose (2%) as a carbon source and lacking uracil (SD-ura) or specific amino acids (Amberg *et al*., 2005).

pXP series plasmid constructions

Recombinant manipulations were conducted using standard molecular techniques (Ausubel, 2008). Sequences of fragments amplified in PCR were verified by DNA sequence analysis (data not shown) (GeneWiz, South Plainfield, NJ, USA). Strains with genomic integrations were identified as prototrophs based on marker gene activity and were verified by PCR generation of diagnostic fragments of appropriate size using primers within the integrated sequence and within flanking DNA (data not shown). Details of constructions, oligonucleotide primer sequences, and sequences of plasmids are provided in the Supporting information. Plasmid constructs and source DNAs are described in Table 2.

Plasmid pUC18 (Yanisch-Perron *et al*., 1985) provided the backbone for the pXP vector series. The pUC18 plasmid is 2686 bp in length and contains a multiple cloning site (MCS), the pMB1 replication origin mutated to ensure high-copy number in bacteria and the *bla* ampicillin resistance marker to enable selection. Two different promoters ($P_{PGK\ 1}$ and P_{TEF1}) coupled with $T_{CYC\ 1}$ for transcription termination were inserted into the unique *Ssp*I site. Promoter and terminator sequences were separated by unique *Spe*I and *Xho*I sites to allow directional cloning of ORFs to be expressed. *CEN6/ARS4* or 2 μ sequences were inserted into the *Eco*RI site of the MCS and six different marker genes were individually inserted into the *Sma*I site of the MCS in the respective high-and low-copy vectors. Altogether these manipulations yielded a set of 24 plasmids (Figure 1A, Table 2). The 391 bp *HXT7* promoter ($P_{HXT\ 7–391}$) coupled with $T_{CYC\ 1}$ was inserted at the unique *Nde*I and *Hin*dIII sites in the MCS in high- and low-copy plasmids with *URA3* and *LEU2-d8* marker genes to create an additional four plasmids for a final total of 28 plasmids (Figure 1B, Table 2). The *LEU2-d8* allele encodes *LEU2* truncated by 8 bp and complements *LEU2Δ*.

Individual fragments for the construction of these vectors were derived by PCR amplification of genomic DNA or plasmid templates, using *Pfu* Ultra II polymerase (Stratagene, La Jolla, CA, USA) or KOD polymerase (Novagen, Gibbstown, NJ, USA) as suggested by the manufacturer. Each fragment was cloned into the pCR Blunt II TOPO cloning vector (Invitrogen).

CreA plasmid construction

The gene encoding bacteriophage P1 CreA recombinase was recoded for expression in *S. cerevisiae* using computationally optimized DNA assembly (CODA) (Hatfield and Roth, 2007; Larsen *et al*., 2008) and the optimized *creA* sequence (see Supporting information S1). The assembled fragment was amplified using PCR and primers containing *Nco*I and *Sma*I sites at the 5′ and 3′ ends, respectively. The *creA* fragment was ligated into the pCR-Blunt II TOPO cloning vector. Plasmid pKN2736 is similar to pYES2.0, but is marked with *LEU2* and contains a *Sma*I restriction site in the MCS (K. Nguyen, personal commmunication). The *creA Nco*I–*Sma*I fragment was cloned into the *Nco*I and *Sma*I sites of plasmid pKN2736 to create plasmid pBF3038. In order to express CreA from a *URA3*-marked vector, the *Sca*I fragment containing *LEU2* was replaced with a fragment containing *URA3* to produce plasmid pBF3060 (Figure 1C). Recoded CreA recombinase was confirmed to be active based on the deletion of marker genes flanked by direct repeats of the *loxP* recognition sequence. In a typical experiment ~200 cells were plated onto YPD, allowed to grow into colonies and replica-plated onto SD-ura or -leu. Typically 10–20% of cells had lost the marker even in the absence of galactose induction of the CreA promoter. Appropriate auxotrophs were then screened by PCR for loss of the marker between the loxP sites using primers in the flanking genomic target DNA. Typically four colonies were tested and all four had the expected deletion. Up to five sequential rounds of marker deletion have been performed in the same strain (data not shown).

Luciferase reporter plasmid construction

Gene expression from vectors and integration sites was measured as activity of *Renilla* luciferase (RLuc) relative to activity of an integrated copy of Firefly luciferase (FLuc) (described below). The *Rluc* gene templated from phRL-null (Promega, Madison, WI, USA) was amplified with flanking *Spe*I and *Xho*I sites and cloned under the control of $P_{PGK\ 1}$ and $T_{CYC\ 1}$ in the *Spe*I and *Xho*I sites of low-copy pXP100 and high-copy pXP200 derivatives containing *MET15, TRP1, LEU2-d8, HIS3* and *URA3* genetic markers. This fragment was similarly cloned under control of P_{TEF1} and $P_{HXT\ 7–391}$ and $T_{CYC\ 1}$ in *URA3*-marked low- and high-copy vectors.

Construction of yeast reporter strains

In order to monitor expression from a subset of vectors and from test chromosomal sites, luciferase assays were used. RLuc reporter activity was normalized to FLuc activity expressed from an integrated copy of *Fluc*. *Fluc* was expressed under control of the human thymidine kinase promoter (P_{TK}) and $T_{CYC\ 1}$ (P_{TK} -*Fluc*-$T_{CYC\ 1}$). P_{TK} was amplified in a PCR using pRL-TK (Promega) as a template and primers with *Nde*I and *Spe*I sites. This fragment was used to replace $P_{HXT\ 7–391}$ in pXP522. The *Fluc* ORF was amplified in a PCR using pGL3-Basic (Invitrogen) as a template and primers to incorporate a *Spe*I restriction site followed by A_6 and a *Xho*I site at the 5′ and 3′ ends, respectively. It was then inserted downstream of P_{TK} between the unique *Spe*I and *Xho*I sites to give plasmid pBF3187.

P_{TK} -*Fluc*-$T_{CYC\ 1}$ was introduced into yeast strain yBF1587 by two-fragment transformation (Nielsen *et al*., 2007). PCR was used to generate fragments containing P_{TK} -*Fluc*-$T_{CYC\ 1}$ and *loxP-LEU2-d8-loxP* with 50 bp of overlapping sequence at the 3′ end of the former and the 5′ end of the latter and 50 bp on the non-overlapping ends of each with homology to

genomic sequences flanking *HIS3*. The two PCR fragments were co-transformed (Gietz *et al*., 1995) into yeast strain yBF1587. The *LEU2-d8* marker was deleted by introduction of plasmid pBF3060 from which CreA was expressed. The resulting strain was designated yFF1683.

To compare gene expression from different chromosomal sites, the $P_{PGK\ 1}$-*Rluc-loxP-URA3-loxP* cassette was introduced at test loci in strain yFF1683 containing the integrated P_{TK} -*Fluc*-$T_{CYC\ 1}$ reporter. This was accomplished by amplification of $P_{PGK\ 1}$-*Rluc*-$T_{CYC\ 1}$ and *loxP-URA3-loxP* fragments and co-transformation as described for *Fluc*. Integrations into chromosomal loci were confirmed by PCR analysis using primers that annealed upstream and downstream of these chromosomal sites.

Dual luciferase assay

Luciferase activity was assayed as previously described (McNabb *et al*., 2005), using the dual luciferase reporter (DLR) assay (Sherf *et al*., 1996; Promega) in which activity of the test reporter, Rluc, was normalized to activity of the integrated control reporter, Fluc. Luminescence measurements were performed with a Sirius Single Tube Luminometer (Berthold Detection Systems GmbH, Pforzheim, Germany). Data are expressed as the ratio of RLuc to FLuc activity (Rluc/Fluc) within the linear range. Cells were sampled in the range $OD_{600\ nm}$ = 0.8–1.0.

Copy-number determination

Low- and high-copy plasmids were transformed into yeast strain yFF1683 containing the integrated P_{TK} - *Fluc*-$T_{CYC\ 1}$ reporter. Cells were grown to exponential phase in the appropriate dropout medium at 30 °C, suspended in breaking buffer (10 m_M Tris–HCl, pH 8.0, 1 m_M EDTA, 100 m_M NaCl, 2% Triton X-100, 1% SDS) and vortexed vigorously with glass beads and phenol : chloroform. Extracted nucleic acid was precipitated in ethanol, redissolved and incubated with RNase (Hoffman and Winston, 1987). DNA was digested with *Hin*dIII and subjected to Southern blot analysis using standard procedures (Ausubel, 2008). Plasmid copy number was determined based on hybridization of a ^{32}P-radiolabelled $P_{PGK\ 1}$-specific probe. Intensity of hybridization signals was determined using Quantity One software (Bio-Rad, Richmond, CA, USA). Intensity of the band from the vector-based promoter $P_{PGK\ 1}$ signal was normalized to the intensity of the chromosomal promoter $P_{PGK\ 1}$ signal.

Results and discussion

Vector design strategy

Genetic engineering of metabolic pathways often involves expression of multiple genes. In this study, a shuttle vector series based on pUC18 was constructed for use in *E. coli* and *S. cerevisiae*. Six markers that can be selected in commonly-used strains of *S. cerevisiae* (*CAN1, MET15, TRP1, URA3, LEU2-d8* and *HIS3*) are represented in the series. In addition, each marker was cloned in vectors with either 2 μ or *CEN/ARS* sequences to allow high- or low-copy level expression, respectively. Plasmids were constructed with three *S. cerevisiae* promoters including, for all six markers, two strong promoters ($P_{PGK\ 1}$ and P_{TEF1}) (Figure 1A) and, in addition, $P_{HXT\ 7–391}$, for *URA3* and *LEU2-d8* (Figure 1B). Should a particular marker not be available as an ORF deletion allele in the background strain of choice, it is possible to amplify a deletion cassette from any of the plasmid markers. The vector markers are flanked by *loxP* sites so that marker and flanking *loxP* sites can be amplified and used to replace chromosomal sequences, resulting in generation of deletion alleles. The marker gene can then be removed following CreA expression and recombination of the *loxP* repeats.

Although plasmids provide a useful testbed for the initial characterization of gene expression, pathway construction and optimization typically requires stable expression and therefore chromosomal integration of expression cassettes. To facilitate this process, the pXP vectors were designed not only to include a wide choice of marker genes but also with compact arrangement of expression cassette and marker gene to allow straightforward amplification of a PCR fragment for homology-targeted chromosomal integration. Thus, the expression cassette and flanking marker can be amplified for transformation using primers whose outside ends have ~50 bp of homology to the target genomic sequence. Introduction of a pathway typically involves integration of multiple genes. An advantage of the current collection of markers is that genes can be integrated for preliminary testing without need for time-consuming sequential deletion of markers for re-use. However, most applications ultimately require deletion of markers to allow recycling or other strain manipulation. One method of marker gene elimination is to flank the marker with direct repeats of sufficient length to insure gene loss via homologous recombination (Alani *et al*., 1987; Wilson *et al*., 2000). Alternatively, a site-specific recombinase can be employed, such as the bacteriophage *loxP*– CreA (Hoess and Abremski, 1984) or the yeast *FRT*–FLP (Radhakrishnan and Srivastava, 2005) systems. For marker deletion to allow recycling, we chose CreA-mediated site-specific recombination. Marker genes were flanked with direct repeats of *loxP*, the 34 bp recognition sequence of the CreA recombinase, which is active in yeast (Sauer, 1987). In the direct repeat configuration of *loxP* sites, CreA-mediated recombination deletes the marker gene and leaves one copy of *loxP* (Güldener *et al*., 1996; Sauer, 1994). Advantages of this approach include the small size of the recognition sequence (avoiding inappropriate targeting of subsequent integrations), the efficiency of the marker excision and the ability to remove multiple markers simultaneously (Delneri *et al*., 2000) or sequentially (Güldener *et al*., 1996). CreA was expressed from P_{GAL1} on high-copy plasmids marked with *URA3* or *LEU2* (Figure 1C). Even uninduced expression of the recombinase resulted in sufficient recombination to allow cells from which the marker is lost to be readily identified on appropriate drop-out media in previous studies (Gueldener *et al*., 2002) and in our study involving different CreA expression vectors (data not shown).

Comparison of plasmid copy number

Plasmid copy number is an important basal determinant of cloned gene expression level. Copy number was determined for the $P_{PGK\ 1}$ vectors, marked with *MET15* and *URA3*, for both the empty and *Rluc*-expression derivatives. DNA was isolated from cells and Southern blot analysis was performed using hybridization of vector and chromosomal DNA to a $P_{PGK\ 1}$-specific probe. As shown in Figure 2 and Table 3, this ratio for empty low-copy *CEN/ARS* vectors with either marker was 1.5. The empty 2 μ vector showed differences between the *MET15*- and *URA3*-marked versions with ratios of 7.5 and 11.6, respectively, resulting in ratios of high- to low-copy empty vectors marked with *MET15* and *URA3* of 5.2 and 7.8, respectively. A caveat in these experiments is that they measure average copy number and the 2 μ plasmid in particular is known to be relatively unstable (Mead *et al*., 1986).

Expression of cloned genes can affect plasmid copy number. In order to explore this variable, DNA from cells containing low- and high-copy vectors carrying the $P_{PGK\ 1}$-*RLuc*-$T_{CYC\ 1}$ expression cassette was hybridized to the $P_{PGK\ 1}$ probe. The copy numbers of the low-copy Rluc reporter plasmids were 1.9 and 1.6 for the *MET15*- and *URA3*-marked vectors, respectively; these values were similar to the empty *CEN/ARS* vectors. In contrast, the copy numbers of the high-copy $P_{PGK\ 1}$-*RLuc*-$T_{CYC\ 1}$ reporter plasmids were lower than those for the empty vectors, dropping from 7.5 to 4.2 and from 11.6 to 5.6 for the plasmids marked with *MET15* and *URA3*, respectively. The copy-number ratios of 2 μ to CEN/ARS for *MET15*-and *URA3*-marked plasmids actively expressing the reporter under the *PGK1*

promoter were therefore 2.2 and 3.4, respectively. Overall, these ratios are lower than what might have been anticipated based on published reports of vector copy number and suggest that effects of cloned gene expression on copy number should be a consideration in planning vector-based experiments. These findings also underscore the potential advantage of integrating multiple copies of genes rather than relying on vector-based strategies.

Luciferase assays for evaluation of vector-based gene expression

Because expression level is critical for metabolic engineering, but is affected by multiple variables, vectors were also directly compared using reporter assays. Luciferase reporter systems offer the advantages of a rapid and quantitative assay. Reporters configured so that activity can be measured as the ratio of Rluc test activity to Fluc control activity have the advantage of being internally controlled. P_{TK} displays weak activity in *S. cerevisiae* (Moriyoshi, 2009) and was therefore used to express *Fluc* at a useful baseline level for normalization. P_{TK} -*Fluc*-$T_{CYC\ 1}$ was integrated into yeast strain yBF1587 to create strain yFF1683. The *Rluc* ORF was cloned under $P_{PGK\ 1}$ in the high-and low-copy *MET15*-, *TRP1*-, *HIS3*-, *LEU2-d8*-, and *URA3*-marked vectors. RLuc reporter plasmid transformants were grown in logarithmic phase in selective medium for several doublings and the dual luciferase assay (McNabb *et al*., 2005; Sherf *et al*., 1996) was performed as described in Materials and methods. To determine the linear range of the assay, RLuc/Fluc was determined in cells expressing the *URA3*-marked high- and low-copy plasmids with amounts of lysate representing 500–10 000 cells ($R^2 = 0.99$). Within this range, the mean RLuc : FLuc ratio in cells expressing the low-copy plasmid was 33 (±3) × 10^3 (Figure 3A). Similarly, activity was relatively independent of cell number for measurements from cells containing the high-copy plasmid between 500 and 10 000 cells, with some loss of linearity between 5000 and 10 000. With <5000 cells, the mean Rluc : Fluc ratio was 141 (±6) × 10^3 (Figure 3A). Thus, Rluc activity in cells expressing the high-copy *URA3* reporter was about four-fold greater than activity in cells expressing the low-copy *URA3* reporter. This is consistent with the 3.4 ratio of high- to low-copy plasmid number observed for the *URA3* reporter (Figure 2B).

RLuc/FLuc activity ratios were also determined for cells transformed with low- and high-copy vectors carrying five different selectable markers and $P_{PGK\ 1}$-*Rluc*-$T_{CYC\ 1}$. As observed for cells containing the *URA3*-marked vectors, cells containing vectors carrying other marker genes showed three- to five-fold greater activity for high-copy than low-copy versions (Figure 3B). The *MET15* marker showed the least and the *TRP1* and *LEU2-d8* marked plasmids the greatest differential between high- and low-copy vector transformants.

Effect of promoter on gene expression

In addition to copy number, promoter activity is a major determinant of expression level. Therefore, it was of interest to determine the RLuc : FLuc ratios for plasmids carrying different promoters but a common marker. $P_{PGK\ 1}$, P_{TEF1} and $P_{HXT\ 7–391}$ (Lai *et al*., 2007) were chosen because they are relatively strong promoters under most growth conditions. Expression from these promoters on *URA3*-marked low- and high-copy plasmids in logarithmic growth was compared using the luciferase assay (Figure 3C). In cells containing low-copy plasmids, the Rluc : Fluc ratio was 41 (±1) × 10^3 with $P_{PGK\ 1}$ and 52 (±4) × 10^3 with P_{TEF1}. This suggests that P_{TEF1} may be slightly stronger than $P_{PGK\ 1}$. This would be consistent with previous reports (Nacken *et al*., 1996). In cells expressing RLuc from the high-copy plasmids, the RLuc/FLuc ratios for $P_{PGK\ 1}$ and P_{TEF1} were similar [163 (±4) × 10^3 vs. 155 (±19) × 10^3**]**. However, cells expressing RLuc from these promoters had significantly higher RLuc : FLuc ratios than cells expressing RLuc from $P_{HXT\ 7–391}$. The RLuc : FLuc ratios in cells with vectors utilizing the $P_{HXT\ 7–391}$ promoter were 12 (±1) × 10^3 for the low-copy plasmid and 92 (±6) × 10^3 for the high-copy plasmid.

The difference in RLuc expression among $P_{PGK\ 1}$ and P_{TEF1} and $P_{HXT\ 7–391}$ indicated that $P_{HXT\ 7–391}$ is the weakest promoter in the context tested. In the case of $P_{PGK\ 1}$-*Rluc*-$T_{CYC\ 1}$, on *URA3*-marked plasmids there is an approximately four-fold difference in activity between high- and low-copy number plasmids. Similarly, in the case of P_{TEF1}-*Rluc*-$T_{CYC\ 1}$ the ratio between high- and low-copy plasmids is about 3. In contrast, for $P_{HXT\ 7–391}$-Rluc, this ratio is about 8 (Figure 3C). These results suggest that in the case of the 2 μ plasmid, a weaker promoter suppresses copy number less than a strong promoter. Mumberg *et al*. (1995) compared the effect of promoter strength on relative activity of beta-galactosidase in cells carrying a *lacZ* reporter on high- or low-copy pRS vectors. These investigators found that the ratio of *β*-gal between cells with 2 μ and CEN/ARS vectors was three-fold when *lacZ* was driven by the strong P_{GPD1} promoter but 30-fold when it was expressed under the weaker $P_{ADH\ 1}$. Different versions of the P_{GAL1} showed similar patterns. Four-fold higher *β*-gal activity was observed in cells expressing the reporter from P_{GAL1} on a high-copy plasmid compared to a low-copy plasmid, but if the UAS was truncated the ratio increased to 30-fold (Mumberg *et al*., 1994). These data are consistent with our results.

Comparison of expression from marker and Ty genomic loci

For the introduction of multiple pathway genes, chromosomal integration of expression cassettes is often desired to both control and stabilize expression levels. However, there are relatively few quantitative comparisons of expression from different chromosomal integration sites. Furthermore, the number of frequently used sites is limited so that in the case of pathway engineering it may be necessary to integrate multiple genes into one site or into uncharacterized sites. In order to compare expression from various genomic loci, the expression cassette $P_{PGK\ 1}$-*Rluc*-$T_{CYC\ 1}$-*loxP-URA3-loxP* was amplified using PCR, and integrated at several frequently used loci in the yFF1683 P_{TK} -*Fluc*-$T_{CYC\ 1}$ reporter strain. The amplification used primers with outside ends of 50 bp of sequence flanking the target promoter and downstream end of the ORF (see Supporting information S2), so that $P_{PGK\ 1}$-*Rluc*- $T_{CYC\ 1}$-*loxP-URA3-loxP* replaced the target loci (Figure 4A). Replacement integrations targeted the *URA3, MET15, LEU2* and *TRP1* loci using the homologous sequences upstream of promoter and downstream of terminator regions. Comparison of RLuc : FLuc activity ratios in strains with different integration sites showed that they varied from 17 (±1) × 10^3 to 25 (±3) × 10^3, but overall were similar (Figure 4B). This included expression at the *TRP1* locus, which is peri-centromeric (*CENIV*). Comparison of activity of integrated reporters to activity in a strain transformed with pXP100, (*CEN/ARS, URA3*-marked vector) carrying $P_{PGK\ 1}$-*Rluc*-$T_{CYC\ 1}$ grown under selection for the plasmid, showed about 1.5-fold higher activity in the strain expressing the low-copy vector $P_{PGK\ 1}$-*Rluc*-$T_{CYC\ 1}$ (Figure 4B). This is consistent with the copy number slightly greater than one for the *CEN/ARS* plasmids determined in this study and previously reported (Amberg *et al*., 2005; Singh and Weil, 2002).

Current *S. cerevisiae* strain engineering typically targets genes to well-characterized chromosomal loci, such as the ones described above. However, this strategy can be ultimately limited by availability of appropriate replacement sites, as relatively few such sites have been thoroughly characterized. An approach that potentially addresses this limitation is replacement of endogenous retrotransposons with heterologous sequences. Ty retrotransposons exist in multiple copies in most strains and ones that are represented in current genomes are likely well tolerated (Sikorski and Boeke, 1991). Ty1 and Ty3 long terminal repeat (LTR) retrotransposons in *S. cerevisiae* are present in 50 and two copies, respectively, in the sequenced genome. Ty1 LTRs or *δ* elements are present in several hundred copies and individual Ty3 LTRs are present in approximately 40 copies (Kim *et al*., 1998). Ty1 and Ty3 elements insert preferentially into the region upstream of RNA polymerase III-transcribed genes (Chalker and Sandmeyer, 1990; Devine and Boeke, 1996).

tRNA genes have been associated with reduced expression of associated RNA polymerase II promoters (Hull *et al*., 1994; Ji *et al*., 1993; Kinsey and Sandmeyer, 1991). Nonetheless, expression of marker genes associated with Ty1 and Ty3 elements can be readily detected at levels expected for single gene copies (Chalker and Sandmeyer, 1990; Lee and Da Silva, 1996; Wang and Da Silva, 1996). Cumulatively, Ty1 RNAs have been shown to account for approximately 10% of the total poly(A) RNA in the yeast cells and the individual expression of 31 Ty1 elements has been characterized using *lacZ* reporter fusions (Lesage and Todeschini, 2005). A δ sequence fused to a linearized *URA3* blaster cassette (Alani *et al*., 1987) has been successfully used to reiteratively target expression cassettes to multiple Ty1 LTRs in the genome (Lee and Da Silva, 1997). Indeed, expression from such insertions is very consistent and nearly linearly correlated with gene copy number (Lee and Dasilva, 2006). Nonetheless, because of the large number of potential targets, this strategy is somewhat unpredictable. Furthermore, stable gene insertion via double-crossover integration is limited; typically only one copy of the same gene can be inserted as gene replacements are obtained rather than new targeted integrations. The feasibility of completely replacing Ty1 and Ty3 elements with expression cassettes was therefore explored.

In order to test the utility of a Ty1 replacement strategy, Ty1 elements previously shown to have different levels of expression (Lesage and Todeschini, 2005) were completely replaced by the $P_{PGK\ 1}$-*Rluc*-$T_{CYC\ 1}$ reporter in the P_{TK} -*Fluc*-$T_{CYC\ 1}$ reporter strain yFF1683. The $P_{PGK\ 1}$-*Rluc*-$T_{CYC\ 1}$ and *loxP-URA3-loxP* were amplified as two overlapping fragments with collective outside ends homologous to target genome sequences flanking selected Ty1 elements (for details, see Supporting information S4). Seven Ty1 elements, including four loci that showed high Ty1–*lacZ* fusion expression and three that represented relatively low Ty1–*lacZ* fusion expression (Lesage and Todeschini, 2005), and one Ty3 (YILWTy3-1) element were replaced with the $P_{PGK\ 1}$-*Rluc*-$T_{CYC\ 1}$ expression cassette (Figure 4A). Successful integration demonstrated that the PCR-based strategy could be used to exchange sequences of about 3 kb for genomic sequences of 5–6 kb. In order to test the effect of flanking tRNA genes on reporter expression, the YMLWTy1-2 and the YMLWTy1-1 loci were replaced in both orientations, with $P_{PGK\ 1}$ either proximal or distal to the tRNA genes.

Three isolates with $P_{PGK\ 1}$-*Rluc*-$T_{CYC\ 1}$-*loxP-URA3-loxP* integrated into each locus were grown to logarithmic phase and Rluc : Fluc ratios determined. Protein expression levels from all loci were similar and similar to that of the four standard loci tested (Figure 4B). Thus, differences in expression among these Ty1 elements previously seen by Lasage and Todeschini (2005) most likely reflect differences in promoter strength among individual elements, rather than influences of flanking chromatin structure on Ty1 expression. Furthermore, in the YMLWTy1-1 locus, $P_{PGK\ 1}$ is located only 201 bp from a tRNA, and in the YMLWTy1-2 locus $P_{PGK\ 1}$ is only 161 bp away from the adjacent tRNA. Nonetheless, in contrast to the prediction that tRNA gene proximity might interfere with expression, $P_{PGK\ 1}$-*Rluc*-$T_{CYC\ 1}$ activity was similar whether $P_{PGK\ 1}$ was proximal or distal to the tRNA gene at both the YMLWTy1-2 and YMLWTy1-1 loci (Figure 4B). The relative insensitivity of the expression cassette to tRNA gene-based repression could be due to the strength of the *PGK1* promoter.

Summary

The system described here is designed to facilitate metabolic engineering in *S. cerevisiae*. The first element of this system is a set of plasmids with alternative strong promoters present on low-and high-copy plasmids to allow initial testing of pathway genes. Alternative promoters can be easily inserted via unique flanking restriction sites; currently the plasmid set is being expanded to include the *GAL1*, *CUP1* and *ADH2* promoters. The second element of the system is ease of PCR amplification of the expression cassette flanked by the

marker gene embedded in the plasmid for integration into genomic sites of choice. Multiple marked expression cassettes can be thus be readily integrated to optimize pathway functions. The third element of the system is demonstration that it is practical to replace loci including redundant Ty1 elements with expression-marker amplicons and to subsequently delete the associated markers using CreA recombinase. Together these steps constitute a practical and rapid method for assembly of metabolic pathways in *S. cerevisiae*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Schematic representation of pXP plasmid series and *creA* plasmids. (A) The pXP plasmids contain *CEN/ARS* or 2 micron sequences, unique *Spe*I–*Xho*I restriction sites separating $P_{PGK1/TEF1}$ and T_{CYC1} located at the unique *Ssp*I site (note that P_{PGK1}-T_{CYC1} cassette is in the negative orientation, opposite to that of P_{TEF1}-T_{CYC1}), and six alternative markers flanked by *loxP* sites cloned into the unique *Sma*I site. (B) The pXP plasmids contain *CEN/ARS* or 2 micron sequences, unique *Spe*I–*Xho*I restriction sites separating $P_{HXT7–391}$ and T_{CYC1} cloned into the unique *Nde*I and *Hin*dIII sites in the multiple cloning site (MCS), and *URA3*- or *LEU2-d8* markers as described in (A). (C) The pYES2.0-based *creA* expression plasmid marked with *URA3* or *LEU2* marker

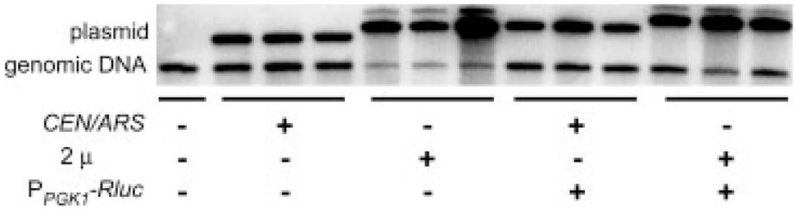

Figure 2.

CEN/ARS and 2 μ plasmid copy number. Southern blot analysis of cells containing *URA3*-marked plasmids. DNA was prepared from logarithmic phase cultures containing low- and high-copy *URA3*-marked vectors and restricted with *Hin*dIII. Triplicate samples of 20 μg total DNA for low-copy plasmids and 2 μg total DNA for high-copy plasmids were analysed by Southern blot analysis using a probe specific for P_{PGK1}, as described in Materials and methods. Band intensity was quantitated in the linear range for plasmid and chromosomal fragment hybridization using Quantity One Software (Bio-Rad)

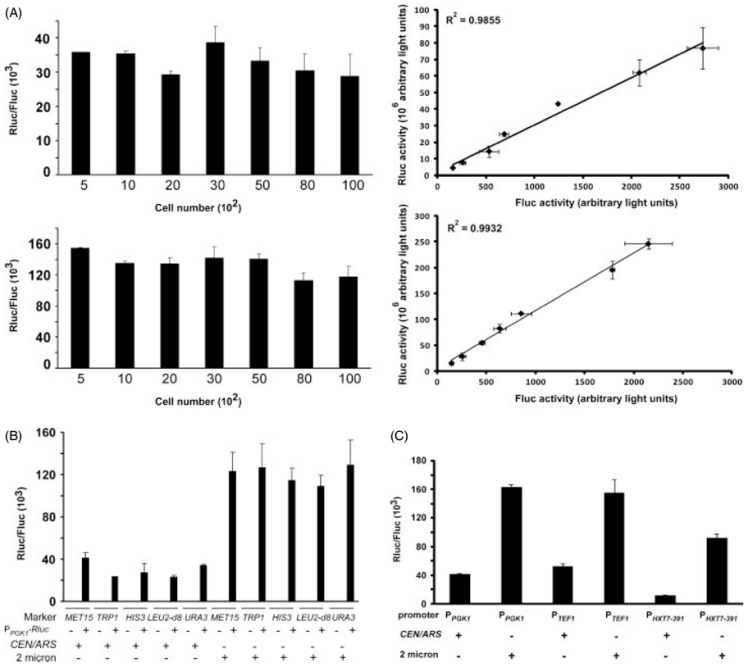

Figure 3.

Analysis of vector expression by dual luciferase assay (DLR). (A) Luciferase assay for cells containing *CEN/ARS* and 2 μ *URA3*-marked plasmids. P_{PGK1}-*Rluc* reporter in plasmids pXP118 and pXP218 were transformed into yeast strain yFF1683 and cells were grown in SD – ura medium to logarithmic phase. Cell number was determined by OD at A_{600} and the indicated number of cells was used to assay plasmid-borne *Rluc* and chromosomal *Fluc* activities. Data represent the mean of three independent assays and the bars show one standard deviation (SD). The plasmids without the *Rluc* reporter served as negative controls. Upper panel, cells contain pXP118-*Rluc* (*CEN/ARS* plasmid with *URA3* marker). Lower panel, cells contain pXP218-*Rluc* (2 μ plasmid with *URA3* marker). Left panels, RLuc : FLuc ratios; right panels, scatter plots of Rluc vs. Fluc for the same number of cells as shown in left panels. (B) Comparison of expression from differently marked pXP vectors. P_{PGK1}-*Rluc*-T_{CYC1} reporters in the *CEN/ARS* and 2 μ plasmids with *MET15*, *TRP1*, *HIS3*, *LEU2-d8* and *URA3* were transformed individually into yeast strain yFF1683. Activities were analysed as described in (A). (C) Comparison of expression from pXP *CEN/ARS* and 2 μ vectors using different promoters. Cells containing *URA3*-marked vectors with P_{PGK1}, P_{TEF1} and $P_{HXT7–391}$ controlling expression of *Rluc* were analysed as described in (A)

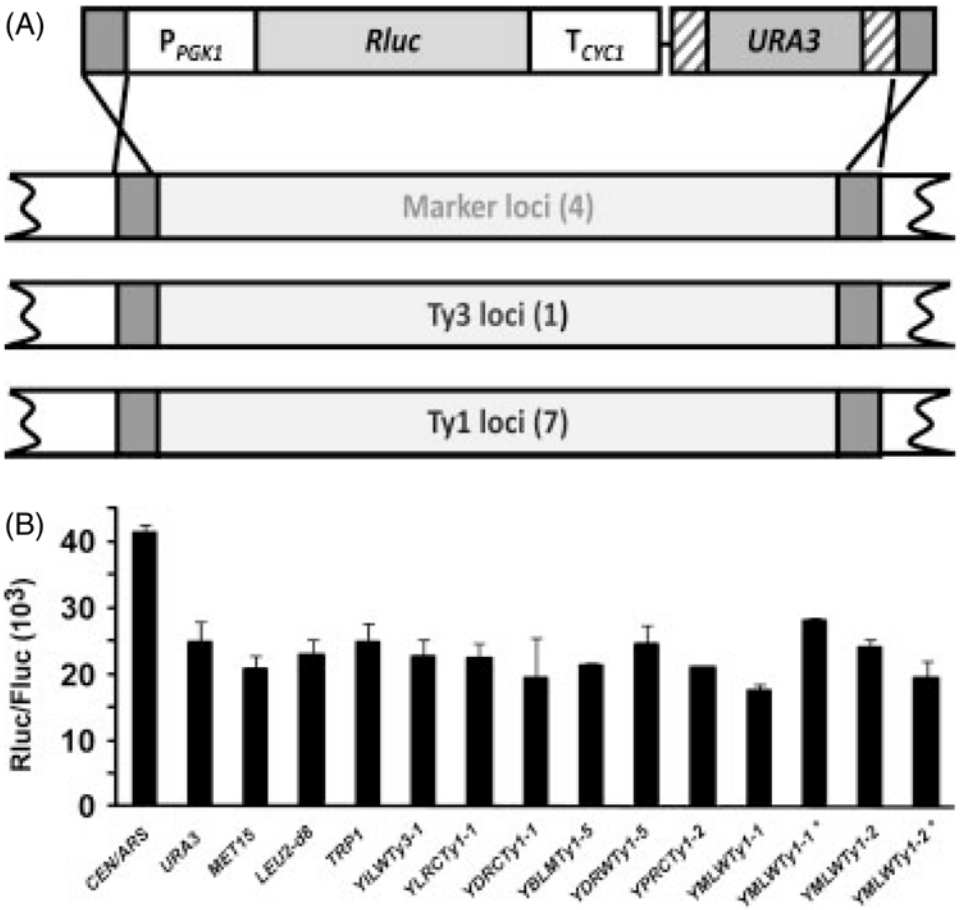

Figure 4.

Comparison of P_{PGK1}-*Rluc*-T_{CYC1} expression from different chromosomal loci. (A) Schematic representation of integration strategy. P_{PGK1}-*Rluc*-T_{CYC1} and *URA3* marker cassette were used to replace genomic loci in strain yFF1683. (B) Rluc/Fluc is shown for P_{PGK1}-*Rluc*-T_{CYC1} expression from a *CEN/ARS* vector (pXP118) and from genomic sites in strain yFF1683. Assays were as described in Figure 3 legend. Asterisks indicate that the P_{PGK1}-Rluc-T_{CYC1} promoter is proximal to the tRNA in the Ty replacement

Table 1

Yeast strains constructed in this study

Strain*	Genomic integration
yFF1743	*ura3::*P_{PGK1}*-Rluc-loxP-URA3-loxP*
yFF1744	*met15::* P_{PGK1}*-Rluc-loxP-URA3-loxP*
yFF1745	*leu2::* P_{PGK1}*-Rluc-loxP-URA3-loxP*
yFF1746	*trp1::* P_{PGK1}*-Rluc-loxP-URA3-loxP*
yFF1748	*yilmty3-1::* P_{PGK1}*-Rluc-loxP-URA3-loxP*
yFF1749	*ylrcty1-1::* P_{PGK1}*-Rluc-loxP-URA3-loxP*
yFF1750	*ymlwty1-1::*P_{PGK1}*-Rluc-loxP-URA3-loxP*
yFF1751	*ydrcty1-2::* P_{PGK1}*-Rluc-loxP-URA3-loxP*
yFF1752	*yblwty1-1::*P_{PGK1}*-Rluc-loxP-URA3-loxP*
yFF1753	*ydrwty1-5::*P_{PGK1}*-Rluc-loxP-URA3-loxP*
yFF1754	*ymlwty1-2::*P_{PGK1}*-Rluc-loxP-URA3-loxP*
yFF1755	*yprcty1-2::*P_{PGK1}*-Rluc-loxP-URA3-loxP*
yFF1756	*ymlwty1-1::*P_{PGK1}*-Rluc-loxP-URA3-loxP* (P_{PGK1} is proximal to tRNA region)
yFF1757	*ymlwty1-2::*P_{PGK1}*-Rluc-loxP-URA3-loxP* (P_{PGK1} is proximal to tRNA gene)
yBF1587	*MAT***a** *his3 Δ 1 leu2 Δ 0 met15 Δ 0 ura3 Δ 0 adh2 Δ 0 gre3 Δ 0 trp1 Δ 0 his3*
yFF1683	*MAT***a** *his3 Δ 1 leu2 Δ 0 met15 Δ 0 ura3 Δ 0 adh2 Δ 0 gre3 Δ 0 trp1 Δ 0 his3::*P_{TK}*-Fluc-loxP*

*Strains yFF1743–yFF1757 are derivatives of yFF1683. yBF1587 and yFF1683 are derived from BY4741.

Table 2

List of plasmids created in this study

Plasmid name	Base vector	Description
pXP1	pCR Blunt II	P_{PGK1} with 5^3 *Ssp*I and 3^3 *Spe*I
pXP2	pCR Blunt II	T_{CYC1} with 5^3 *Spe*I-N_{12}-*XhoI* and 3^3 *Ssp*I
pXP3	pCR Blunt II	2 μ with *Eco*RI flanking sites
pXP4	pCR Blunt II	CEN/ARS *ori* with *Eco*RI flanking sites
pXP5	pCR Blunt II	*TRP1* marker with *loxP* repeats and *Sma*I flanking sites
pXP6	pCR Blunt II	*URA3* marker with *loxP* repeats and *Sma*I flanking sites
pXP7	pCR Blunt II	*CAN1* marker with *loxP* repeats and *Sma*I flanking sites
pXP8	pCR Blunt II	*MET15*marker with *loxP* repeats and *Sma*I flanking sites
pXP9	pCR Blunt II	P_{PGK1}-T_{CYC1} *Ssp*I cassette
pXP13	pUC18	P_{PGK1}-T_{CYC1} *Ssp*I cassette
pXP16	pCR Blunt II	P_{TEF1} with 53 *Ssp*I and 3^3 *Spe*I
pXP17	pUC18	P_{TEF1}-T_{CYC1} *Ssp*I cassette
pXP100	pXP13	*CEN/ARS*, P_{PGK1}
pXP200	pXP13	2 μ, P_{PGK1}
pXP300	pXP17	*CEN/ARS*, P_{TEF1}
pXP400	pXP17	2 μ, P_{TEF1}
pXP112	pXP100	$P_{PGK1}{}^{-}$, *CAN1*$^{+}$, *CEN/ARS*$^{-}$
pXP212	pXP200	$P_{PGK1}{}^{-}$, *CAN1*$^{+}$, 2 μ^{-}
pXP312	pXP300	$P_{TEF1}{}^{+}$, *CAN1*$^{-}$, *CEN/ARS*$^{+}$
pXP412	pXP400	$P_{TEF1}{}^{+}$, *CAN1*$^{+}$, 2 μ^{-}
pXP114	pXP100	$P_{PGK1}{}^{-}$, *MET15*$^{+}$, *CEN/ARS*$^{-}$
pXP214	pXP200	$P_{PGK1}{}^{-}$, *MET15*$^{+}$, 2 μ^{-}
pXP314	pXP300	$P_{TEF1}{}^{+}$, *MET15*$^{-}$, *CEN/ARS*$^{+}$
pXP414	pXP400	$P_{TEF1}{}^{+}$, *MET15*$^{-}$, 2 μ^{+}
pXP116	pXP100	$P_{PGK1}{}^{-}$, *TRP1*$^{+}$, *CEN/ARS*$^{-}$
pXP216	pXP200	$P_{PGK1}{}^{-}$, *TRP1*$^{+}$, 2 μ^{-}
pXP316	pXP300	$P_{TEF1}{}^{+}$, *TRP1*$^{+}$, *CEN/ARS*$^{+}$
pXP416	pXP400	$P_{TEF1}{}^{+}$, *TRP1*$^{+}$, 2 μ^{+}
pXP118	pXP100	$P_{PGK1}{}^{-}$, *URA3*$^{-}$, *CEN/ARS*$^{-}$
pXP218	pXP200	$P_{PGK1}{}^{-}$, *URA3*$^{-}$, 2 μ^{-}
pXP318	pXP300	P_{TEF1}+, *URA3*$^{+}$, *CEN/ARS*$^{+}$
pXP418	pXP400	P_{TEF1}+, *URA3*$^{+}$, 2 μ^{+}
pXP120	pXP100	$P_{PGK1}{}^{-}$, *HIS3*$^{-}$, *CEN/ARS*$^{-}$
pXP220	pXP200	$P_{PGK1}{}^{-}$, *HIS3*$^{+}$, 2 μ^{-}

Plasmid name	Base vector	Description
pXP320	pXP300	P_{TEF1}+, *HIS3*$^+$, *CEN/ARS*$^+$
pXP420	pXP400	$P_{TEF1}$$^+$, *HIS3*$^+$, 2 μ$^-$
pXP122	pXP100	$P_{PGK1}$$^-$, *LEU2-d8*$^-$, *CEN/ARS*$^-$
pXP222	pXP200	$P_{PGK1}$$^-$, *LEU2-d8*$^-$, 2 μ$^-$
pXP322	pXP300	$P_{TEF1}$$^+$, *LEU2-d8*$^+$, *CEN/ARS*$^+$
pXP422	pXP400	$P_{TEF1}$$^+$, *LEU2-d8*$^+$, 2 μ$^+$
pXP518	pBF3055	$P_{HX7–391}$$^+$, *URA3*$^+$, *CEN/ARS*$^-$
pXP618	pBF3055	$P_{HX7–391}$$^+$, *URA3*$^+$, 2 μ$^+$
pXP522	pBF3055	$P_{HX7–391}$$^+$, *LEU2-d8*$^+$, *CEN/ARS*$^-$
pXP622	pBF3055	$P_{HX7–391}$$^+$, *LEU2-d8*$^+$, 2 μ$^-$
pXP114-*Rluc*	pXP114	*6A-Rluc* was inserted into *Spe*I and *Xho*I sites
pXP214-*Rluc*	pXP214	*6A-Rluc* was inserted into *Spe*I and *Xho*I sites
pXP116-*Rluc*	pXP116	*6A-Rluc* was inserted into *Spe*I and *Xho*I sites
pXP216-*Rluc*	pXP216	*6A-Rluc* was inserted into *Spe*I and *Xho*I sites
pXP118-*Rluc*	pXP118	*6A-Rluc* was inserted into *Spe*I and *Xho*I sites
pXP218-*Rluc*	pXP218	*6A-Rluc* was inserted into *Spe*I and *Xho*I sites
pXP318-*Rluc*	pXP318	*6A-Rluc* was inserted into *Spe*I and *Xho*I sites
pXP418-*Rluc*	pXP418	*6A-Rluc* was inserted into *Spe*I and *Xho*I sites
pXP518-*Rluc*	pXP518	*6A-Rluc* was inserted into *Spe*I and *Xho*I sites
pXP618-*Rluc*	pXP618	*6A-Rluc* was inserted into *Spe*I and *Xho*I sites
pXP120-*Rluc*	pXP120	*6A-Rluc* was inserted into *Spe*I and *Xho*I sites
pXP220-*Rluc*	pXP220	*6A-Rluc* was inserted into *Spe*I and *Xho*I sites
pXP122-*Rluc*	pXP122	*6A-Rluc* was inserted into *Spe*I and *Xho*I sites
pXP222-*Rluc*	pXP222	*6A-Rluc* was inserted into *Spe*I and *Xho*I sites
pBF3037	pCRBluntII	*URA3* was cloned into *Nhe*I and *Mlu*I flanking by two codon-optimized 200 bp GAG fragments: one was cloned into *Asc*I and *Nhe*I sites and the other was cloned into *Mlu*I and *Sal*I sites
pKN2735	pYES2	*LEU2* replaced *URA3*
pKN2736	pKN2735	The MCS was modified by introducing *Nde*I and *Xma*I
pNB3045	pXP100	*LEU2-d8* was flanked by *Sma*I-*loxP* on both sides
pBF3038	pKN2736	*creA* was cloned between *Nco*I and *Xma*I
pBF3052	pXP100	P_{PGK1}-T_{CYC1} was deleted by *Ssp*I digestion
pBF3055	pBF3052	$P_{HXT7–391}$-$T_{CYC1}$was inserted into *Nde*I and *Hin*dIII sites
pBF3060	pBF3038	*URA3* replaced *LEU2*
pBF3187	pXP522	P_{TK}-*Fluc* was inserted into *Nde*I and *Xho*I sites

*Sequence orientation in the pUC18 vector, proceeding 5′ to 3′ clockwise around the map shown in Figure 1.

+Sequence described in Supporting information S3.

−Sequence described in Supporting information S3, orientated with 5′ end distal to the *Ssp*I site.

Table 3

Low- and high-copy *MET15*- and *URA3*-marked plasmid copy number. The ratio of plasmid-borne P_{PGK1} hybridization was normalized to chromosomal P_{PGK1} hybridization and the average of three measurements is shown

	Relative copy number					
	Plasmids			Plasmids with *Rluc* expression		
Marker	*CEN/ARS*/genome	2 μ/genome	2 μ/*CEN/ARS*	*CEN/ARS*/genome	2 μ/genome	2 μ/*CEN/ARS*
MET15	1.4 ± 0.2	7.5 ± 1.0	5.2 ± 1.0	1.9 ± 0.4	4.2 ± 0.4	2.2 ± 0.6
URA3	1.5 ± 0.03	11.6 ± 0.8	7.8 ± 0.6	1.6 ± 0.1	5.6 ± 0.9	3.4 ± 0.6