



A novel series of vectors for chromosomal integration in fission yeast

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ABSTRACT

A series of fission yeast targeting vectors that can be used for wild-type strains having no selectable markers have been designed. The functions of one of three marker genes, *lys1*⁺, *arg1*⁺, and *his3*⁺, involved in amino acid synthesis, are impaired by integration of the fragments generated by restriction enzyme digestion of the plasmids. Successful integration of the fragments into the targeted loci can be readily verified by their requirement for amino acids, or by the PCR diagnostic analysis. Since these selection markers are not used commonly in fission yeast, these plasmids are likely to facilitate studies that require the co-expression of genes such as co-localization and co-immunoprecipitation experiments, by employing them in combination with most of the previously reported markers.

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Expression of cloned genes of interest under the desired conditions is important for cell biology and biochemistry in yeast. For this purpose, a variety of cloning vectors have been developed in the fission yeast *Schizosaccharomyces pombe* as described by Siam et al. and references therein [1]. However, integration of a transgene into the chromosome or direct tagging of a gene has become increasingly popular, since they can reflect the situations closer to the physiological conditions than extrachromosomal multicopy plasmids can. In particular, development of versatile methods for plasmid-based integration is currently required, in addition to the PCR-based direct tagging of an endogenous gene [2–4]. To meet these necessities, we previously constructed a new series of vectors that enable two modes of introduction of a cloned gene into cells [5]. Similar to most other vectors, these vectors can be directly used as multicopy plasmids for expression of a cloned gene, using the *ura4* gene as a selectable marker. In addition, these vectors allow chromosomal integration of a cloned gene, for expression from a single copy gene. This mode can be readily accomplished by a very simple process of the plasmid digestion with a certain restriction endonuclease before transformation of the yeast, since the resultant fragments, lacking the autonomously replicating sequence, are designed for targeting into the chromosomal *leu1* locus by homologous recombination. Introduction of a series of the vectors named pDUAL confer leucine prototrophy on the *leu1-32* mutant, since the partial *leu1* fragment contained in the plasmids fuses with the intact part of the *leu1-32*

allele when integrated, resulting in the generation of the fully functional *leu1* gene. On the contrary, the other series of vectors named pDUAL2 have another part of the *leu1* gene lacking both 5'- and 3'-ends. As a result, the function of the endogenous *leu1* gene should be impaired by insertion of the pDUAL2-derived fragment, thereby making cells leucine auxotrophy. Thus, pDUAL-series plasmids can be used for the *leu1-32* mutants, whereas pDUAL2-series plasmids are for *Leu*⁺ strains as the hosts.

For more complicated experimental design, two or more loci for expression of cloned genes should be required. However, in most cases of fission yeast studies, *ura4*⁺ [6] and *leu1*⁺ [7] have long been used as selection markers, as other markers were essentially unavailable [1]. Therefore, the lack of selectable markers sometimes limits the area of research. To overcome this limitation, we have developed a series of vectors that can be used for cells having no auxotrophy. Three genes *lys1*⁺ [8,9], *arg1*⁺ [10], and *his3*⁺ [11] were used as the target loci to integrate the expression plasmids developed in this study. When the plasmid-derived fragments are integrated into these loci, the resultant transformants are expected to exhibit histidine-, lysine-, or arginine-auxotrophy. Drug-resistant genes are also included in these vectors, allowing positive selection of transformants. Using these vectors in combination with each other, and also with pDUAL, at least four expression plasmids can be integrated simultaneously into the chromosomes in a single strain.

Materials and methods

Schizosaccharomyces pombe strains and media. *Schizosaccharomyces pombe* wild-type strains JY3 (*h*⁹⁰ prototroph) and AM1

Abbreviations: PCR, polymerase chain reaction; G418, geneticin; ORF, open reading frame; MCS, multiple cloning site.

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(*h⁹⁰ ade6-M216*) were used in this study. Complete medium YE (0.5% yeast extract, 2% glucose, and 5 µg/ml adenine) and minimal medium SD [12] were used for routine culture of *S. pombe* strains. Minimal medium MM [13] was used for expression of genes driven by the *nmt1* promoter [14]. G418 disulphate, hygromycin B, and blasticidin S [15] were used at the final concentrations of 100 µg/ml, 300 µg/ml, and 250 µg/ml, respectively. Histidine, lysine, arginine, and adenine were used at the final concentration of 50 µg/ml when needed.

Genetic methods and transformation of *S. pombe*. General methods to handle fission yeast cells were as described [16]. A high efficiency protocol for transformation of *S. pombe* cells was carried out

as described previously [17]. For chromosomal integration of a series of plasmids, each DNA was digested with *NotI* in a volume of 10–20 µl, and 3–4 µl of the resultant solution was directly used. Drug-resistant transformants were selected as described [2].

Vector construction. For construction of the pHIS3K vector, a part of the *his3⁺* ORF was amplified by PCR using the genome DNA of the wild-type strain JY3 as a template. To create the *NotI* recognition site within the *his3* fragment, this fragment was initially amplified as two fragments using two sets of primers, namely *his3*-F1 (GGGTCGACTAGTGTGGCAGCGATGAAATTAT) and *his3*-R1 (TTGCATGCGGCCGCTGTTGGATTACCAGGGGAAC), and *his3*-F2 (TTGCATGCGGCCGCTCTTAAACTCGAGGATATTAAG) and *his3*-R2

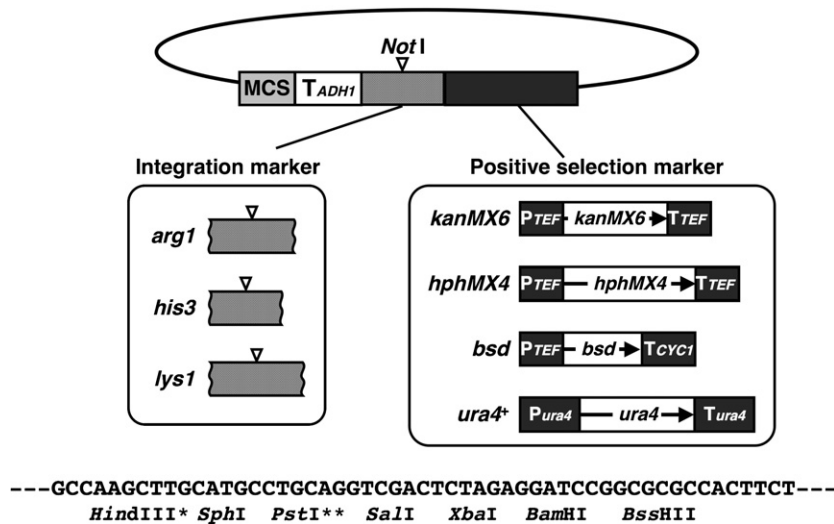


Fig. 1. Schematic representation of the construction of the vectors. Arrowheads indicate restriction sites required for linearization of the plasmids to allow targeted integration. The fragments of *arg1⁺*, *his3⁺*, and *lys1⁺* lack both 5'- and 3'-ends of the ORFs as well as their authentic promoters. The nucleotide sequence of the multiple cloning sites (MCS) is also indicated at the bottom. *, *HindIII* can be used only against pARG1B, pARG1H, and pARG1U. **, *PstI* can be used only against *bsd*- or *ura4⁺*-containing plasmids.

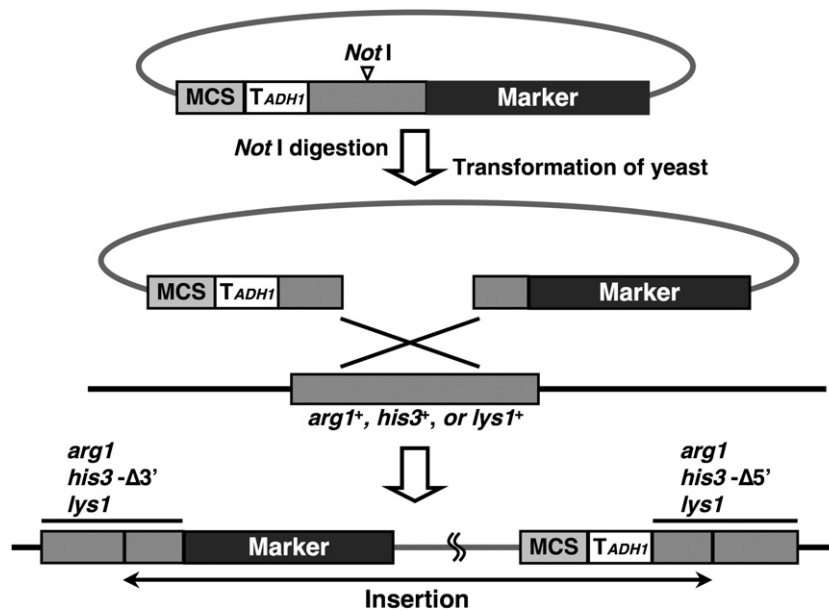


Fig. 2. Strategy for chromosomal integration of the plasmid-derived fragment. All plasmids can be linearized at the middle of the integration marker genes by *NotI* digestion, resulting in the generation of the fragment targeted for their endogenous loci. The resulted fragment introduced in a yeast cell is expected to be integrated at the chromosomal marker gene locus by homologous recombination. Transformants in which each linearized fragment was successfully integrated are expected to show drug-resistance or amino acid auxotrophy according to the markers used. The schematic diagrams are not to scale.

(GGTCTAGATCTAATGTTATAAGGAGCCTTTAATG). The *kanMX6* cassette was excised from pFA6a-*kanMX6* [2] using *Bgl*III and *Sac*I. Essentially, the 3'-fragment of *his3*⁺ and the *kanMX6* cassette were ligated to the pUC119-TADH-Pleu1 plasmid that contains the budding yeast *ADH1* terminator and a part of the fission yeast *leu1*⁺ gene [5], replacing the 3'-region of the *leu1*⁺ gene. The remaining 5'-fragment of *leu1*⁺ was then replaced by the 5'-fragment of *his3*⁺, resulting in the generation of pHIS3K. Similarly, parts of the *lys1*⁺ and the *arg1*⁺ genes were individually amplified and ligated to the same backbone plasmid, resulting in the generation of pLYS1K and pARG1K, respectively. Primers used for amplification of these genes were as follows: *lys1*-F1: GGGTCGACTAGTACT TTGTAGTACCTGCGCGG, *lys1*-R1: TTGCATGCGCGCGCTACATTCTAC ATTACCAG, *lys1*-F2: TTGCATGCGCGCGCCGACGATCAAATTTAAATTCG, *lys1*-R2: GGCTAGATCTTTCCATTAGGATTGAGAGG, *arg1*-F1:

GGGTCGACTAGTCTAGCTGCTAAAGGTG, *arg1*-R1: TTGCA TGCGGCCGCTTACGAGCGAAGCTCAAGG, *arg1*-F2: TTGCATGCGG CCGCGTTTGAGAAGTATGGAGAAG, and *arg1*-R2: GGCTAGATCTG CGACCAAGTCCGCATTG. pHIS3U, pLYS1U, and pARG1U were constructed by replacing the *kanMX6* cassette of pHIS3K, pLYS1K, and pARG1K, respectively, with the fission yeast *ura4*⁺ gene. Similarly, pHIS3H, pLYS1H, and pARG1H were constructed by using the hygromycin-resistance *hphMX4* cassette excised from the pAG32 plasmid [18] instead of *kanMX6*. Furthermore, pHIS3B, pLYS1B, and pARG1B were constructed by using the blasticidin-resistance *bsd* cassette. The pTEF1/Bsd vector containing the *bsd* cassette was purchased from Invitrogen. The *bsd* cassette was amplified by PCR from this plasmid using the following primers: *Bgl*III-*zeo*-F: GGATCCAGATCTAGCCCCACACCATAGCTTC, and *Sac*I-*zeo*-R: GAATTCGAGCTCGTTTAAACAGCTTGCAAATTTAAAGCC. Nucleotide

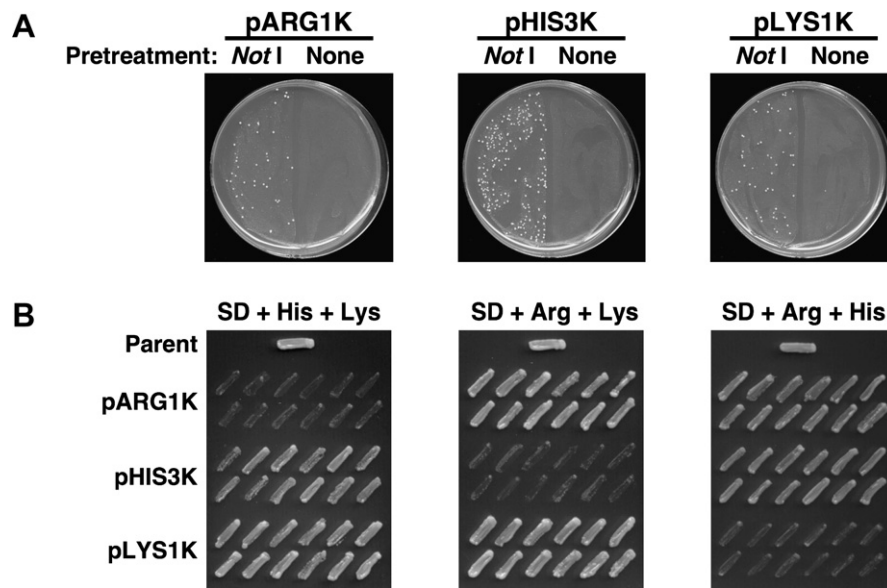


Fig. 3. Confirmation of successful integration of the vectors. (A) The wild-type strain JY3 was transformed with each of pARG1K, pHIS3K, and pLYS1K with or without pretreatment with *Not*I. Transformants initially grown on a YE solid medium were replicated on a YE plate containing G418. (B) Transformants initially selected on a YE plate containing G418 were then streaked on each of the SD plates supplemented with indicated amino acids.

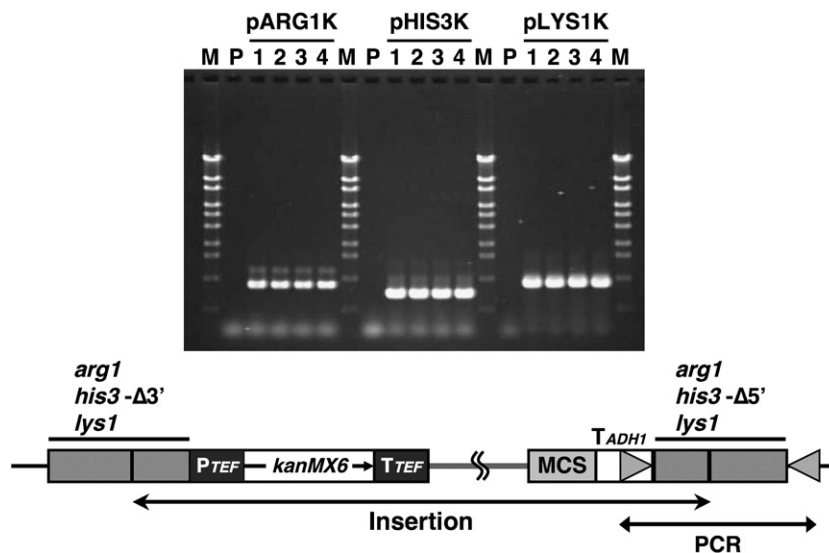


Fig. 4. Verification of successful integration by PCR. Successful integration of the fragments at the targeted chromosomal loci was verified by PCR. Cells transformed with indicated fragments were directly subjected to PCR using the primers designed at the indicated positions (inverted triangles). The parental strain AM1 was also subjected to PCR using the same primer sets, serving as a negative control M, λ /Eco114I marker.

sequence data of the plasmids constructed in this study are available in the DDBJ/EMBL/GenBank databases under the accession No. AB364151–62. These vectors including their derivatives that have several promoters and tags will be available from RIKEN BioResource Center (http://www.brc.riken.jp/lab/dna/en/yoshidayeast_en.html).

Verification of chromosomal integration by PCR. For verification of chromosomal integration, *S. pombe* transformants initially selected on appropriate media were directly subjected to PCR using primers described below in combination with ADHterm-F: CTCTTATTGACCACACTTACC. *arg1*-Rev (TCTTACTGGAGACAATGGTGGC), *his3*-Rev (AGCATCCAAAGCTAAACGAGAGG), and *lys1*-Rev (GTGATGTGTCTGGGAAAGGCAGAG) were used for checking integration of the plasmid-derived fragments at the *arg1*⁺ gene, the *his3*⁺ gene, and the *lys1*⁺ gene loci, respectively.

Results and discussion

Construction of a series of vectors for chromosomal integration

To utilize the wild-type strain as the host for chromosomal integration of plasmids, we developed a series of vectors consisting of two parts that allow targeted chromosomal integration and selection of proper transformants (Fig. 1). Three genes *lys1*⁺, *arg1*⁺, and *his3*⁺, encoding components of amino acids synthesis were selected as the targeted loci. Similar to the *leu1* fragment in pDU-AL2 [5], the marker fragments in these vectors were incomplete at both 5'- and 3'-ends, which allows selection of proper integrants by histidine-, lysine-, or arginine-auxotrophy after homologous recombination in cells. For linearization of the plasmids, the restriction enzyme recognition sites for *NotI* were created at the middle of the marker fragments. When these plasmids are linearized by *NotI* digestion followed by the introduction into the cell, they would integrate by single-crossover homologous recombination into the chromosomal locus whose sequence is identical to the corresponding fragment in the plasmid (Fig. 2). Integration of these fragments into the chromosomal marker loci was expected to result in amino acid auxotrophy, since the endogenous marker genes should be separated into two fragments, both of which are expected to lose their function. To select transformants, we incorporated one of the drug-resistant cassettes, *kanMX6* (G418) [2], *hphMX4* (hygromycin B) [18], and *bsd* (blasticidin S) [15], and the fission yeast *ura4*⁺ gene into these vectors. Therefore, transformants can be selected initially on a medium containing an appropriate drug or lacking uracil before checking for their amino acid requirement.

Verification of chromosomal integration

For typical examples of practical use, we used pHIS3K, pLYS1K, and pARG1K, which have the G418-resistant *kanMX6* marker for positive selection. As expected, G418-resistant transformants were obtained only when *S. pombe* cells were transformed with these fragments pre-digested with *NotI* (Fig. 3A). This result suggested that these vectors without modification cannot be maintained in a cell, due to a lack of an autonomously replicating sequence. To verify that the linearized fragments were successfully integrated into the proper chromosomal loci, we examined the amino acid auxotrophy of the transformants. When G418-resistant transformants were streaked on a medium containing two of three amino acids, arginine, histidine, and lysine, only one of three transformant groups showed the specific amino acid auxotrophy according to the fragment used for transformation (Fig. 3B). For example, only cells that were transformed with the pHIS3K-derived fragment showed histidine auxotrophy. These results strongly suggested that the vectors were successfully integrated into the targeted

genes on the chromosome and impaired the function of the endogenous genes.

Successful integration can finally be verified by PCR. By selecting an appropriate set of primers for each marker, a positive band can be obtained only when the introduced fragment is integrated into the proper locus. A universal primer set can be used for this confirmation regardless of sequences to be cloned, since the region required for PCR check is completely independent of MCS (Figs. 1 and 4). Indeed, PCR products with expected sizes were generated in each pair of primers (Fig. 4). These results confirmed the successful integration of the linear fragments into the targeted loci.

Conclusion

The plasmids described herein enable ectopic expression of genes from three loci of the chromosome. All these loci targeted by the plasmid-derived fragments can be used simultaneously in one strain by selecting an appropriate set of positive selection markers. In addition, each of these three genes is located on different chromosomes, which allows easy construction of a double mutant by genetic crosses. Since these markers have not been used commonly so far in fission yeast unlike *leu1*⁺ and *ura4*⁺ [1], the plasmids developed in this study can be used in combination with most of the previously reported markers. Thus, these vectors will serve as a valuable tool to perform co-localization and co-immunoprecipitation experiments especially in strains in which all nutritional markers are already used up for gene disruption.

Acknowledgments

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