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Generation of Genome-wide Systematic Deletion Mutants of Fission Yeast Using PCR-based Targeted Mutagenesis

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The heterozygous genome-wide deletion mutants of *Schizosaccharomyces pombe* have been generated using PCR-based targeted mutagenesis. The deletion cassettes containing kanMX module as a selection marker, tag sequences as mutant identifiers and the flanking regions of target gene at 5'- and 3'-end were amplified by four round PCR. The deletion cassettes were used to be replaced at the target site of chromosome of diploid strain SP286 by homologous recombination. The deletions of target genes were confirmed by colony PCR of transformants. About 1000 heterozygous deletion mutants were generated with the deletion efficiency of 2-100%. Some heterozygous deletion mutants were tested for the induced haploinsufficiency phenotypes sensitive to thiabendazole (TBZ) responsible for microtubule polymerization. The deletion mutants can be exploited for the identification of target genes to specific drugs that affect the gene product in a large scale. They can be used for new drug discovery by identifying drug targets and establishing HTS drug validation system.

Schizosaccharomyces pombe has served as an excellent model organism for the study of cell cycle control, mitosis and meiosis, DNA repair and recombination, checkpoint controls, and genome stability. The completion of full genome sequence opened the era of fuctional genomics of *S. pombe* and will accelerate the use of S. pombe for functional and comparative studies of eukarytic cell processes (1).

The 13.8-Mb genome of *S. pombe* contains the smallest number of protein-coding genes of 4824 recorded for a eukaryotes. *S. pombe* has characteristic life cycle of haploid and diploid and sporulates under the unfavorable condition. The purpose of this study is to provide deletion strains as experimental model for functional genomics thousands of in a rapid and cost effective study. These results, along with the collection of individual deletion strains, will be a valuable resource for investigating the function of genes in yeast and human.

The stable non-sporulating fission yeast diploids SP286 (h^+/h^+, ade6-M210/ade6-M216 ura4-D18/ura4-

D18 leu 1-32/ leul-32) was used as the parental strain for mutant construction. The deletion cassettes that can be replaced at the target gene of chromosome contain a kanMX module as a selection marker, two tag sequences to label deletion mutants, and two universal primers to amplify tag sequences, and 80 bp of target site for homologous recombination.The tag sequences of forty thousand 20 base-oligonucleotides were designed by computer algorithm based on the several parameters and were allotted to each gene as a uptag and a downtag. Tags can be identifiers of mutant strains survived in a given competitive condition when amplified by PCR with universal primers and used in high density oligonucleotide DNA chip analysis. Universal primers in a deletion cassette can be used to amplify tag sequences for the analysis of mutants in a larger scale parallel analysis. To determine the sequence of universal primers, we designed 21 primers of 20-mer oligonucleotides that do not have sequence similarity with *S. pombe* genome. PCR analysis was performed using chromosomal DNA as template to find the best oligonucleotide pair that gave less non-specific PCR products.

The deletion cassettes consists of kanMX module containing kan^r gene, two tag sequences, two universal primers C10 and C11 and 80 base pair of flanking region of target gene were amplified by four-round PCR (figure 1). The amplified deletion cassettes were used to transform parental strain SP286 by Lithium acetate method. The transformants grown in YE plates containing G418 were examined for the deletion of a target gene by colony PCR. The primers of common sequences in kanMX module and gene-specific sequences located at 300 bp upstream or downstream from the target site for deletion were designed and used to confirm the deletion of target gene. When colony PCR were performed using 4-5 different commom primers such as CPN1, CPN3, CPN10, CPC3, and CPC9 at the same time, specific sizes of PCR products were amplified (figure 2). Southern blot analysis of 20 heterozygous mutants was performed to examine whether or not one copy of deletion cassette was replaced at target site by homologous recombination (data not shown). We exclude the possibility of second site mutation by random integration of deletion cassette since we detected only one band with DNA fragment containing kanMX module as a probe. Total more than 1000 heterozygous mutants were constructed with deletion frequency of 2-100% and average deleion efficiency is 16%.

Whether or not the tag sequences of deletion mutants could be amplified depending on the amount of cells existing in cell suspension was investigated by amplification of tag sequences of deletion mutants mixture with wild type cells using universal primers.

Haploinsufficient loci define a set of genes whose dosage and function are critical to the organism. In human, some genes have been identified as haploinsufficient. These were identified because they resulted in developmental abnormalities or other severe disease. *S. pombe* could be a quite useful organism to screen target genes for a certain drug using haploinsufficient phenotypes since the genetics and physiology of *S. pombe* have been studied well. Thiabendazole is a inhibitor microtubule formation that blocks progression of mitosis in fission yeast. The fission yeast has two alpha-tubulin genes and one beta-tubulin gene. Gene disruption experiments showed that the alpha 1-tubulin gene (nda2) is essential whereas the alpha 2 gene is dispensable. The alpha 2-disrupted cells had an increased sensitivity to an antimicrotubule drug thiabendazole, and the alpha 1(cold-sensitive [cs]) alpha 2 (disrupted) cells became not only cs but also temperature sensitive.

To test sensitivity to thiabendazole (TBZ) of heterozygous deletion mutant, parental type cells SP286, Δnda2, and Δpsp1 heterozygous deletion mutants of logarithmic phase were diluted and spotted on the plates

containing different concentrations (0, 0.5, 1.0, and 5.0 μg/ml) of TBZ and grown for 3 days at 30 °C (figure 3). To test the feasibility of using the molecular tags to monitor a population of cells using deletion mutants, equal numbers of 18 heterozygote deletion strain were pooled and grown in the absence and presence of thiabendazole (0, 5.0, 10.0, 20.0 μg/ml) for 50 h. Aliquote of cells were sampled during growth and chromosomal DNA was extracted from the cells by glassbeads and phenol extraction method. As a control, the existence of Δnda2, and Δpsp1 mutants after 50 h growth in the presence of TBZ was examined by PCR using gene-specific primers (figure 4). To characterize the deletion mutants, we are going to use high density microarray hybridization system. The deletion mutants labeled with gene-specific tag sequence will be pooled and grown in the various selective growth conditions. Some mutants could not grow and gradually disappear. To identify the survived mutants or disappeared mutants, tag sequences will be amplified by PCR using universal primer. The tag sequences labeled with fluorescence will be used for hybridization of high-density oligonucleotide DNA chip composed of oligonucleotides complementary to tag sequences.

In addition to TBZ, other chemicals or drugs that affect cell growth and phenotypes could be used for haploinsufficiency study. Determining the biological function for many of the newly identified yeast ORFs will require the thousands of different deletion strains to be tested under a large variety of selection conditions. Tagging is ideal for this task because it allows larger numbers of tagged deletion strains to be analysed simultaneously in a highly quantitatively fashion.

The entire deletion mutant strains of *S. pombe* are quite precious in many respects. The genome-wide parallel analysis of thousands of deletion mutants will be quite useful tool for functional genomics and drug target identification just by detecting lethal mutants in selective growth condition. As a part of functional genomics, the biological functions of unknown genes can be analyzed in large scale easily and rapidly and human orthologs and paralogs can be identified. And we can develop new drugs by identifying drug targets and establishing HTS drug validation system.

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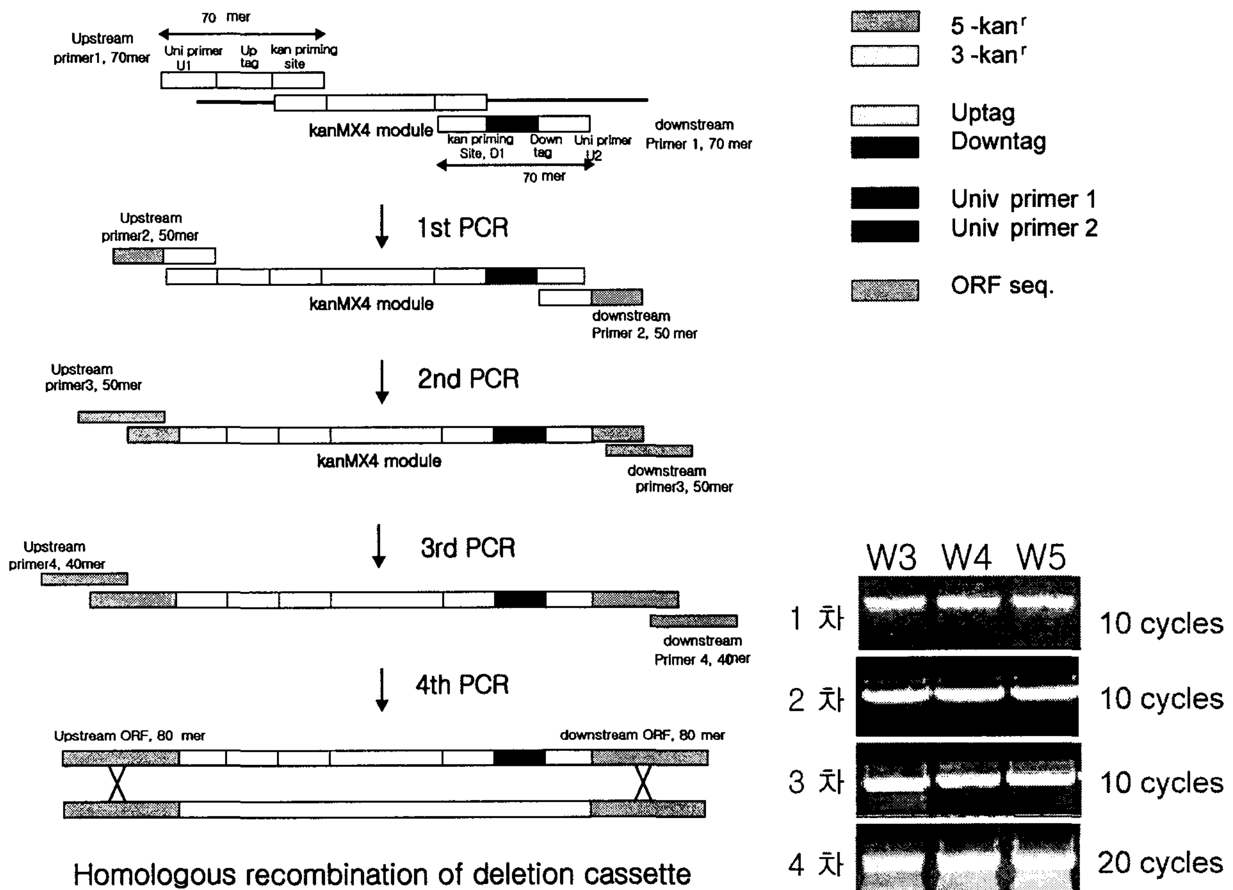


Figure 1. Amplification of deletion cassette by 4-round PCR

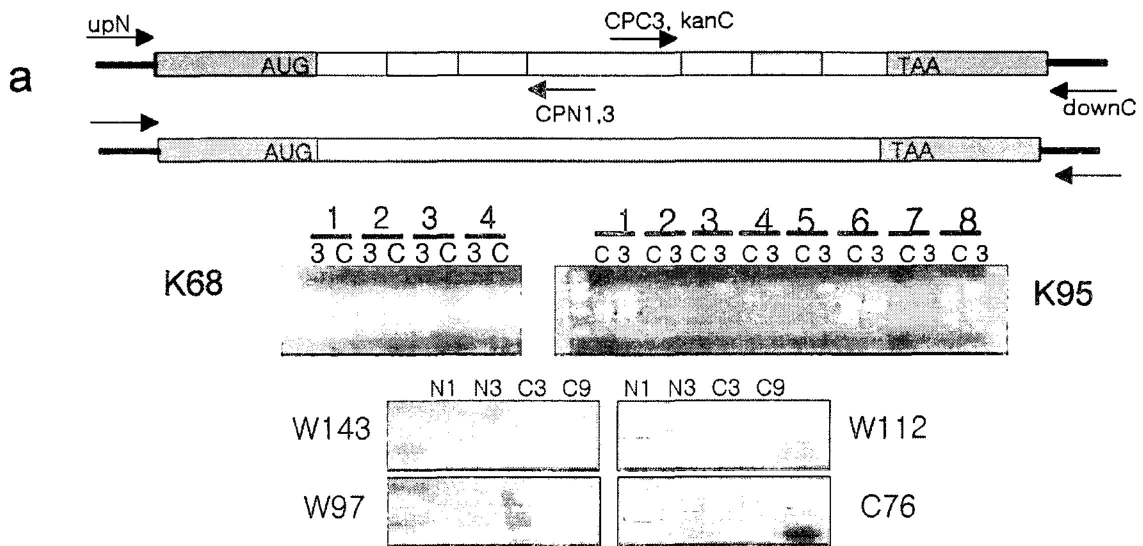


Figure 2. Confirmation of deletion of target gene by colony PCR (a) and Southern Blot analysis

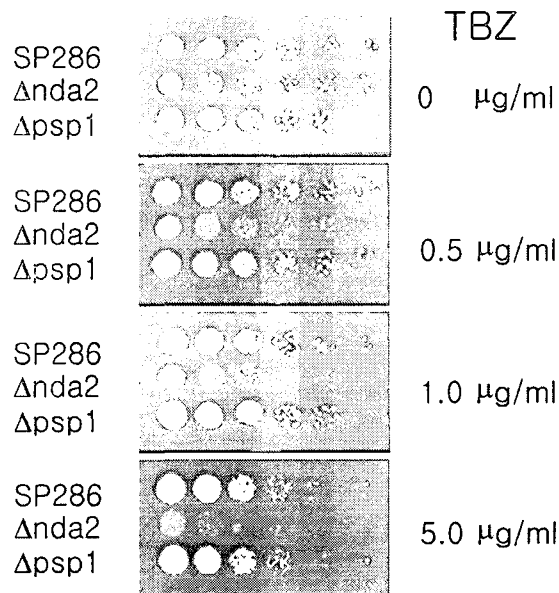
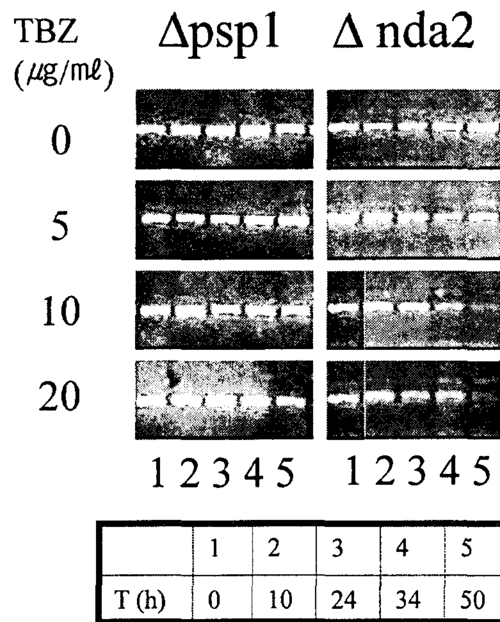


Figure 3. TBZ plate assay. To test sensitivity to thiabendazole (TBZ) of heterozygous deletion mutant, parental type cells SP286, Δnda2, and Δpsp1 heterozygous deletion mutants of logarithmic phase were diluted and spotted on the plates containing different concentrations (0, 0.2, 0.5, 1.0, 5.0, 10, and 20.0 mg/ml) of TBZ and grown for 3 days at 30°C



	1	2	3	4	5
T (h)	0	10	24	34	50

Figure 4. Detection of nda2 mutant. Equal numbers of 18 heterozygote deletion strain were pooled and grown in the absence and presence of thiabendazole (0, 5.0, 10.0, 20.0 mg/ml) for 50 h. Aliquote of cells were sampled during growth and chromosomal DNA was extracted from the cells. The existence of Δnda2, and Δpsp1 mutants after 50 h growth in the presence of TBZ was examined by PCR using gene-specific primers.