Efficient PCR-based epitope gene tagging in *Schizosaccharomyces pombe*

Gene deletion and tagging are important for understanding gene function in all organisms, particularly in single-cell model microorganisms like yeast. While the classical approaches involved homologous recombination to achieve gene deletion by inserting a selectable marker within the gene of insert and transforming the strain with a DNA fragment containing at least 1 kb of homology on each side of the selectable marker[1–3], the last decade has witnessed development of PCR-mediated approaches. For example, in the budding yeast *Sachharomyces cerevisiae*, short stretches of homology of ~20 bp flanking of either a prototrophic or a drug marker are sufficient to achieve homologous gene targeting with extremely high efficiency[1]. However, longer regions of at least 1 kb are required for other yeasts like *Schizosaccharomyces pombe*, *Aspergillus*, etc.[4–6]; DNA fragments generated with shorter regions of homology tend to be inserted at non-homologous sites. Attempts to circumvent this problem have involved use of mutants in the gene encoding *ku80*, which is required for nonhomologous end-joining pathway. Using *ku80Δ* strains efficient gene replacement has been reported in *Aspergillus nidulans*[4], *Neurospora crassa*[5] and *Aspergillus niger*[6].

The problems of non-homologous recombination also bedevil targeted recombination in *S. pombe*. Variable efficiency of homologous gene deletion and tagging has been reported using PCR products generated with oligonucleotides having 80 bp of homology to the targeted gene region using synthetic modules containing auxotrophic or drug markers[1]. Although this approach has become the method of choice, the efficiency is rather low and highly variable. Commercially available libraries developed using this approach[7] are not cost-effective, highlighting the need for developing more efficient and cost-effective alternative approaches.

We have exploited the information about efficient gene targeting in *ku80Δ* mutants in *Aspergillus* and *Neurospora* to study whether we can achieve high-efficiency gene deletion and tagging in these mutants in fission yeast. Here we show that while similar gene deletion efficiency was observed in *wt* and *ku80Δ* strains, a consistent and higher efficiency of gene tagging was achieved with *ku80Δ* mutants, even with shorter stretches of flanking homology of up to 40 bp.

In the present study we have used standard genetic methods of growth of strains in solid and liquid media[2] and the modules developed by Bähler *et al.*[1]. Conditions for PCR and transformation are as described by Bähler *et al.*[1]. Sequences of oligos can be provided on request. Plasmid BS-ura4 was used for deletion of *swi6*, while pFA6a-kanMX6 (ref. 1) was used for deletion of *clr4*. Plasmid pFA6a-3Flag-kanMX6 (ref. 8) was used for FLAG tagging of *swi6*, *clr4* and *polδ* genes, while plasmid pFA6a-hphMX6 (ref. 9) was used for inserting *hph*^{r} marker distal to the *swi6* 3′ end. In most cases, accurate disruption was confirmed by diagnostic PCR. In some cases where switching efficiency is known to be affected by gene disruption, an iodine staining test was considered adequate for confirmation[3]. Alternatively, Western blotting with epitope-specific antibody was used for confirmation.

We carried out two sets of experiments. In the first set, we compared the efficiency of deletion of *clr4* and *swi6* genes in *wt* versus *ku80Δ* strains, using PCR-generated fragments having gene flanking homology of 80, 60, 40 and 20 bp. No homologous recombinant was found among the transformants with PCR product of homology of 40 and 20 bp, while PCR product having 60 bp homology gave ~11% targeting to the homologous site (Table 1, negative data not shown). PCR products with 80 bp flanking homology were 87% and 60% efficient in deletion of *clr4* gene in *wt* and *ku80Δ* strains respectively (Table 1 and Figure 1 *a*). In contrast, deletion efficiency of *swi6* gene was 100% in both *wt* and *ku80*Δ strains with 80 bp flanking homology (Table 1). Shortening flanking

Table 1. Similar frequencies of gene deletions in *wt* versus *ku80Δ* strains

	wt				*ku80Δ*			
Gene	80	60	40	20	80	60	40	20
swi6	9/9(100)$^+$	1/9(11)	0/8(0)	0/8(0)	9/9(100)	1/9(11)	0/8(0)	0/8(0)
clr4	7/8 (87)	ND	ND	ND	4/7 (60)	ND	ND	ND

ND, Not detected.

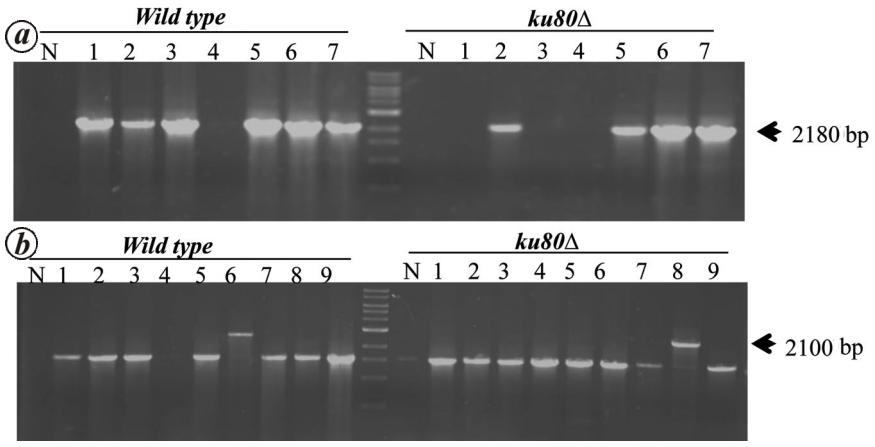

Figure 1. Deletion of *clr4* and *swi6* genes. Agarose gel photograph showing the results of diagnostic PCR to confirm homology-driven deletion of the chromosomal copy of *clr4* (***a***) and *swi6* (***b***) genes in wild type (*wt*) and *ku80Δ* strains. Correct deletion is indicated by arrow heads at (***a***) 2180 bp or (***b***) 2100 bp.

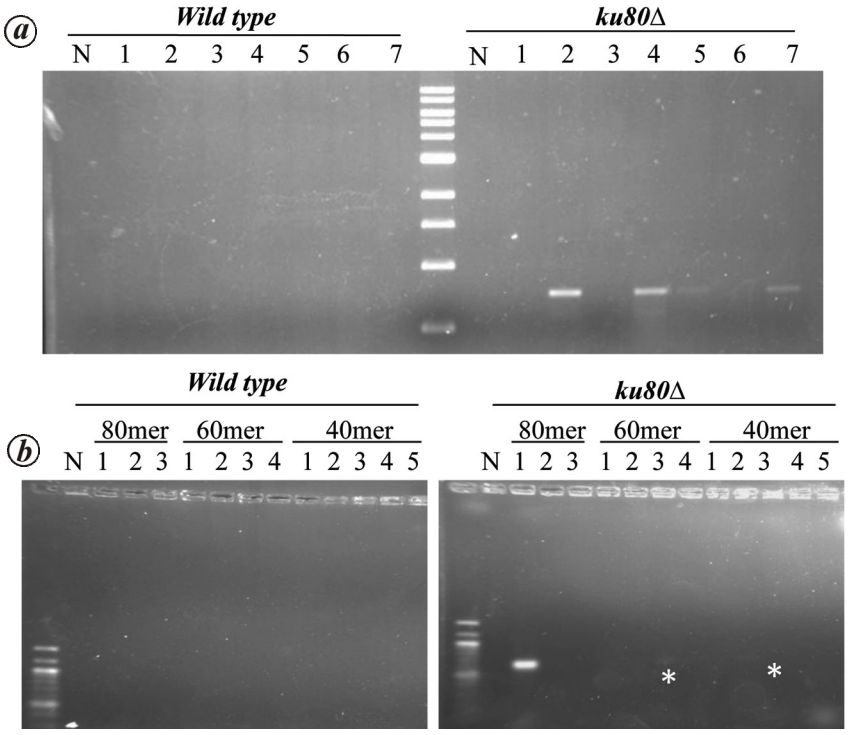

Figure 2. Epitope tagging of *clr4* and *swi6* genes. Diagnostic PCR to check the efficiency of homology-driven epitope tagging in (***a***) *clr4* and (***b***) *swi6* genes in *wt* and *ku80Δ* strains. White star sign (*) indicates the fainter bands in case of tagging with 60 and 40 bp flanking homology.

Table 2. Enhanced tagging frequency in *ku80Δ* versus *wt* strains

	wt			*ku80Δ*		
Gene	80	60	40	80	60	40
swi6[#]	0/3 (0)	0/4 (0)	0/5 (0)	1/3 (33)	1/4 (25)	1/5 (20)
*swi6-3′**	0/10 (0)			2/2 (100)		
clr4[#]	0/9 (0)			4/9 (44)		
polδ[#,@]	0/4 (0)			4/4 (100)		

[+]Assayed by iodine staining. [#]Refers to C-terminal FLAG tag. [@]Assayed by Western blotting for the epitope. *Refers to insertion of hyg[r] tag 3′ downstream of the *swi6* gene.

homology to 60 bp reduced the efficiency of *swi6* gene deletion to ~11% in both *wt* and *ku80Δ* strains (Table 1 and Figure 1 *b*). Thus, *ku80Δ* mutation does not offer any advantage in gene deletion by homologous recombination.

In the second set of experiments, we tested the efficiency of epitope tagging of *clr4*, *swi6* and *polδ* genes and insertion of just the *hph*[r] drug marker distal to the 3′-end of *swi6* gene for subsequent generation of *swi6* mutants. Interestingly, while we did not obtain tagging of any gene using 80 bp flanking homology in the *wt* strain, tagging was significantly more efficient in *ku80Δ* strain: 44% for *clr4* (Figure 2 *a*), 100% for *polδ* (data not shown), 33% for *swi6* (Figure 2 *b*) and 100% for the *swi6* 3′-distal tagging. Even with a reduced homology of 60 and 40 bp respectively, 25% and 20% tagging efficiency was obtained for the *swi6* gene (Table 2).

A major requirement for efficient gene targeting (deletion, tagging, etc.) in *S. pombe* is the presence of at least 0.5–1.0 kb of homology on both sides of the targeted region. In the absence of long flanking homology, there is an elevated level of non-homologous recombination. Since Ku proteins, Ku70 and Ku80, are required for non-homologous end-joining (NHEJ) pathway during DNA repair[10,11], we reasoned that it may be possible to achieve more efficient gene targeting with shorter flanking homology in *ku80Δ* strains. Noticeably, more efficient gene targeting has been reported in *ku* mutants in several fungi[4–6]. On the other hand, homologous tagging efficiency was improved in *ku80Δ* mutant with a significant frequency ranging from 33% to 100%, compared to 0% in *wt S. pombe* using PCR fragment with 80 bp flanking homology. Interestingly, shorter stretches of 60 and 40 bp flanking homology also facilitated moderately efficient tagging in *ku80Δ* mutants (Table 2). However, we failed to observe any increase in the efficiency of gene deletion in *ku80Δ* compared to *wt* strain (Table 1). This is in contrast to a recent report which showed enhanced gene deletion in *ku80Δ* mutants relative to *wt* strain[12]. Given the significant improvement in gene tagging, it is surprising that *ku80Δ* mutation did not affect gene deletion efficiency in the present study. The reason for this is not known; it is possible that the ends of the genes, separated by the coding region of ~1 kb, may not be efficiently aligned for homologus recombination event in *ku80Δ* mutants, at least in case of *swi6* and *clr4* genes. In contrast, tagging involves contiguous regions that are obviously in greater mutual proximity at the site of insertion, rendering them readily accessible for real-time homology search during recombination. It is also possible

that the 3′-regions of genes may have specialized chromatin structure more amenable to tagging.

In conclusion, our finding holds promise for efficient gene tagging even with shorter oligos, thus reducing the cost. This factor could be especially useful when planning large-scale gene tagging in *S. pombe*. After successful tagging, the *ku80Δ* mutation can be easily outcrossed. Further improvements can be attempted by combining *ku80Δ* with mutations in *ku70* and other genes involved in the NHEJ pathway.

Conflict of interest: The authors declare no conflict of interest.

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A new population of *Santalum album* L. (sandalwood) from Agali Forest Range, Kerala, India

Santalum album (sandalwood) is indigenous to peninsular India and is naturally distributed over 9600 sq. km (ref. 1) from Kerala in the south to Uttar Pradesh in the north, in regions with varying eco-climatic conditions and edaphic factors[2]. In Kerala, natural sandal populations have been reported from Marayur (Idukki district), Meppadi (Wayanad district), Wadakkanchery (Thrissur district), Ottapalam (Palakkad district), Kannavam (Kannur district) and some fragments of Kollam and Kasargod districts[3,4]. In India, most of the existing sandal populations are not dense. They are devoid of large girth class trees due to illicit felling, hacking, forest fire and encroachments[5]. The sandal area is declining drastically due to over-exploitation, poor seed germination, poor regeneration and failure of artificial regeneration[6]. Extensive extraction of heartwood has severely decimated the natural stands of the trees in forests and has rendered many populations fragmented[7]. Since much of sandal wealth and natural sandal-bearing areas have been lost, the remaining sandal trees are to be effectively protected and their natural habitats to be preserved[8]. In addition to the conservation of the

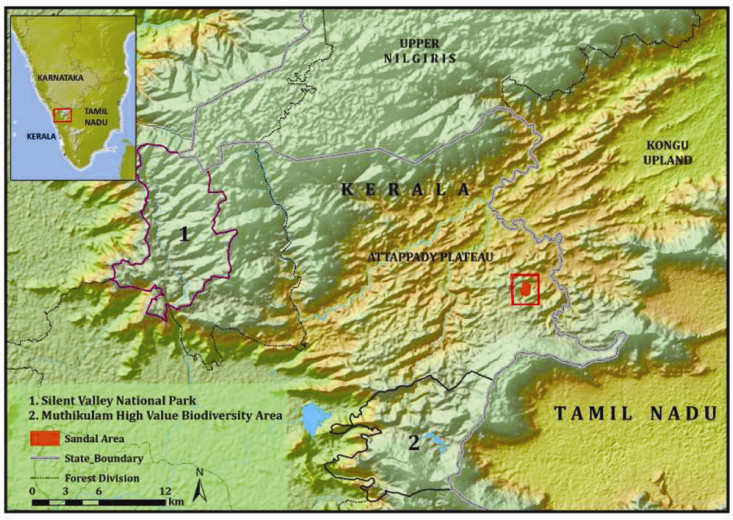

Figure 1. Map showing sandal population in Marappalam, Agali Forest Range, Kerala, India.