AsPac J. Mol. Biol. Biotechnol. 2024
Vol. 32 (3) : 191-205

Development of selectable markers for mitochondrial transformation in yeast

Mei San Ho[a], Wai Keat Toh[a], Shu Ting Chang[a], Kiao Huio Yap[a], Pek Chin Loh[a], Parameswari Namasivayam[b], Hann Ling Wong[a*]

[a]*Department of Biological Science, Faculty of Science, Universiti Tunku Abdul Rahman, Jalan Universiti, Bandar Barat, 31900 Kampar, Perak Darul Ridzuan, Malaysia*
[b]*Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, Serdang 43400, Selangor Darul Ehsan, Malaysia*

Received 26th March 2024 / Accepted 2nd July 2024 / Published 27th August 2024

Abstract. Mitochondria, present in most eukaryotic organisms, are crucial for energy production and essential for cellular functions. Sequencing of the complete mitochondrial genome of *Saccharomyces cerevisiae* in 1998 has paved the way for mtDNA gene editing, enabling the study of mitochondrial function and potential gene therapies for mitochondrial diseases. Effective selectable markers are crucial for addressing heteroplasmic mtDNA issues after mitochondrial transformation. Antibiotic resistance (Ab^R) marker genes *aadA*1, *cat*, and *hph* confer resistance to streptomycin, chloramphenicol, and hygromycin B, respectively. This study aimed to explore the feasibility of employing these Ab^R markers for selecting transformed yeast cells. Additionally, the usefulness of these Ab^R genes as selectable markers for yeast mitochondrial transformation was assessed by fusing a mitochondrial targeting signal (MTS) to the N-terminus of these genes using overlapping PCR. The minimal inhibitory concentration (MIC) of yeast transformants expressing various Ab^R genes, with or without MTS fusion, was determined using the agar dilution method. Yeast transformants expressing *aadA*1, *cat*, and *hph*, with or without MTS fusion, displayed resistance to streptomycin (>10 mg/mL), chloramphenicol (up to 6 mg/mL), and hygromycin B (up to 4 mg/mL), respectively. MICs were similar between Ab^R and MTS-tagged Ab^R yeast transformants. To assess mitochondrial targeting, GFP was fused to the C-terminus of *cat* and MTS-*cat* gene constructs. Fluorescence microscopy confirmed MTS-tagged CAT-GFP localization to yeast mitochondria, while CAT-GFP showed cytoplasmic localization. The fluorescence microscopy results were confirmed by Western blotting. This study demonstrated that yeast transformants expressing *aadA*1 exhibit a significant level of streptomycin resistance (>10 mg/mL), suggesting that *aadA*1-mediated streptomycin resistance has the potential to serve as a selectable marker for mitochondrial transformation in yeast.

Keywords: antibiotic selectable marker, *gfp* reporter gene, minimum inhibitory concentration, mitochondrial targeting sequence, yeast transformation

INTRODUCTION

Mitochondria are double membrane-bound semi-autonomous organelles found in eukaryotic cells that play a prominent role in providing cells with energy in the form of adenosine triphosphate (ATP) through oxidative phosphorylation (Chen *et al.*, 2017; Mahapatra *et al.*, 2021). Mitochondria also participate in various crucial cellular processes, such as β-oxidation, lipid and cholesterol synthesis, apoptosis, ion homeostasis, and the generation of oxygen free radicals (Brand *et al.*, 2013). Genetic modification of the mitochondrial genome has long been deemed

*Author for correspondence: Hann Ling Wong, Department of Biological Science, Faculty of Science, Universiti Tunku Abdul Rahman, Jalan Universiti, Bandar Barat, 31900 Kampar, Perak Darul Ridzuan, Malaysia.
Email – hlwong@utar.edu.my

crucial for investigating mitochondrial gene function and the mechanisms underlying mitochondrial processes (Yang *et al.*, 2021). Currently, by harnessing the genetic information contained in mitochondria, DNA specifically located in the mitochondria (mtDNA) can be modified selectively to gain a more comprehensive understanding of diverse processes in genome evolution (Foury *et al.*, 1998; Niazi *et al.*, 2013).

Saccharomyces cerevisiae has become the primary organism central to mitochondrial studies (Solieri, 2010), with its mitochondrial DNA fully sequenced in 1998 (Foury *et al.*, 1998). This species, which is widely acknowledged, has a short life cycle and generation time and can be easily controlled in a laboratory setting or cultured economically compared to higher eukaryotes, making it an excellent model for various genetic studies (Altmann *et al.*, 2007). Nucleoids, which are clusters of multiple mitochondrial genomes, typically consist of 1.5 to 20 copies of the genome (Nosek *et al.*, 2006). Yeasts are ideal model systems for mitogenomics research because they are easily manipulated in the laboratory, and a broad diversity of different lineages is available for study. Ultimately, it is essential to recognize the importance of yeast mitogenomics in basic, applied, and medical research. As diminished mitochondrial function can lead to decreased yeast cellular proliferation or cell death, altering mitochondrial function enables the study of physiological processes in yeast cells (Rak *et al.*, 2007; Zhou *et al.*, 2010).

Mitochondrial transformation is essential for the analysis, manipulation, and expression of foreign DNA within mitochondria. Executing mitochondrial transformation is more difficult than performing nuclear transformation. This phenomenon arises because of the presence of multiple copies of mtDNA in the mitochondria, whereas nuclei contain either a single copy (haploid) or two copies (diploid) of nuclear DNA (Jensen *et al.*, 2000). Therefore, determining the desired mutation occurring in the mitochondrial genome is a challenging task, as there are numerous copies of mtDNA within a cell, and the majority of mutated mtDNA exhibits a recessive characteristic. Hence, these result in a phenomenon known as heteroplasmy, where the mutated mtDNA is masked by the wild-type mtDNA present in abundance. As a result, heteroplasmic cell cannot be differentiated from homoplasmic cell that contains only the wild-type mtDNA in terms of their physical characteristics, as the wild-type mtDNA is sufficient to contribute to its functional phenotype of the cell (Lax *et al.*, 2011; Yang *et al.*, 2021). In yeast, mitochondrial transformation has been successfully achieved by complementing host auxotrophy (Bonnefoy & Fox, 2007; Zhou *et al.*, 2010). One drawback of using auxotrophy-complementing marker genes is the need for a compatible auxotrophic strain (Pronk, 2002).

In addition to a standard set of selectable auxotrophic markers, *Saccharomyces cerevisiae* also permits the use of dominant markers for antibiotic resistance or other toxic agents to select for transformants. The chosen selectable marker must effectively and efficiently distinguish the transformed organism from a non-transformed one, for example, by hindering growth or causing cell death. Antibiotic resistance genes are commonly used as selectable markers for this purpose (Reece, 2004). The protein synthesis machinery of the mitochondria is similar to that of prokaryotes. Therefore, antibiotics that specifically target the protein synthesis machinery of prokaryotes can potentially be employed as selectable markers for mitochondrial transformation. In addition, the selectable markers used in mitochondrial transformation have a dual function. These genes not only confer antibiotic resistance to the modified mitochondria, but are also crucial for ensuring the successful preservation and duplication of the modified mitochondria that contain both the selectable marker and transgene. Moreover, the sequences of the marker genes used in mitochondrial transformation were modified according to the yeast mitochondrial genetic code. This is due to the codon usage in the nuclear genome differs from that in the mitochondria (Yoon and Koob, 2011; Mookerjee and Sia, 2013). In short, an efficient and simplified selectable marker is required to deal with the heteroplasmic mtDNA phenomenon that may occur after mitochondrial transformation (Zhou *et al.*, 2010).

Previously, antibiotic resistance genes, such as *kan*^{R} and its derivatives (resistance to G418), *nat* (to nourseothricin), *hph* (to hygromycin B), and *cat* (to chloramphenicol), have been used as

selectable markers in yeast transformation (Siewers, 2022). In this study, we used both *cat* and *hph* genes in addition to the primary target streptomycin 3'-adenylyltransferase (*aadA*1) gene to evaluate the suitability of *aadA*1 as a potential selectable marker for yeast mitochondrial transformation. The *aadA*1 marker was selected due to its specific development for tobacco plastids and chloroplast transformation, as documented by Shen *et al.* (2010), Svab & Maliga (2007), and Yang *et al.* (2013).

In addition, heterologous gene expression levels are frequently affected by gene copy number. If the transgene is located within the mitochondria, the level of gene expression may be enhanced in accordance with the number of mitochondria within each cell, rather than the mere existence of a singular recombinant DNA in the cytoplasm. Foreign genes can be effectively introduced and expressed in plant chloroplasts. The abundance of chloroplasts in plant cells has led to a significant increase in the production of foreign proteins in tobacco chloroplasts, reaching levels 300 times higher than those observed in plants undergoing nuclear transformation (Staub *et al.*, 2001; Ruf *et al.*, 2001). Thus, it is possible to utilize mitochondria as a cellular compartment to achieve a high level of foreign gene expression, especially in eukaryotes lacking plastids. Hence, this study also aimed to examine the differences in the minimum inhibitory concentrations (MIC) to determine the in vitro levels of susceptibility or resistance of yeast cells to various targeted Ab^R genes, both with and without the fusion of a mitochondrial targeting sequence (MTS).

MTS is a positively charged N-terminal peptide sequence that forms an amphiphilic α-helix with high hydrophobicity (von Heijne, 1986). MTS is nuclear-encoded and is found at the N-terminus of pre-proteins. It is responsible for targeting nuclear-expressed pre-proteins into the matrix of the mitochondria. This presequence is about 20-50 residues, and is a detachable amino acid sequence, which is removed upon entering the matrix of the mitochondria (Omura, 1998; Rapaport, 2003).

In order to import preproteins across mitochondrial membranes, the proteins are partially unfolded (Eilers & Schatz, 1986). The receptors on the surface of the outer membrane, TOM20 recognise the MTS and translocates the preproteins into the intermembrane space through the translocase of the outer membrane. The translocase of the outer membrane is connected to the translocase of the inner membrane (TIM). TOM and TIM can function independently. Two TIM complexes are responsible for mitochondrial protein import. Preprotein attached to the presequence and is translocated by the TIM23 complex, while preprotein attached to the internal targeting signal, such as for inner membrane proteins, is translocated into the matrix through the TIM22 complex. Therefore, preproteins led by MTS enter the matrix through the TIM23 complex. The mitochondrial heat shock protein 70 (mhsp70), which is found in the matrix, is an ATP-dependent import motor, that binds to TIM23 and drives the preprotein into the mitochondrial matrix across the inner membrane (Omura, 1998; Pfanner & Geissler, 2001).

Upon entering the matrix of the mitochondria, matrix processing peptidase (MPP), together with a processing enhancing protein (PEP), will perform the proteolytic cleavage of MTS. PEP enhances the function of MPP, which contains a catalytic site. The chaperonin Hsp60 in the matrix is a folding helper that folds the protein back into its active form (Pfanner and Geissler, 2001). Two metallopeptidases, Mop112 and Prd1, are present in the mitochondria to degrade free MTS in both the matrix and intermembrane space. These two enzymes have overlapping substrate specifications (Kambacheld *et al.*, 2005, Koppen & Langer, 2007).

MATERIALS AND METHODS

Microbial strains, vectors and growth conditions

The microbial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH5α and DB3.1 were regularly cultivated in 2X YT medium (10 g l^{-1} yeast extract, 16 g l^{-1} tryptone, and 5 g l^{-1} NaCl) at 37°C with 220 rpm agitation, while *S. cerevisiae* JD53 was regularly cultivated in YPD medium (10 g l^{-1} yeast extract, 20 g l^{-1} tryptone, and 20 g l^{-1} dextrose) at 30°C with 220 rpm agitation.

Table 1. List of microbe strains, plasmids used, and plasmids constructed in this study

Strain or plasmid	Description	Reference
Strain		
E. coli DH5α	F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1*gyr*A96 *rel*A1	Invitrogen, USA
E. coli DB3.1	F- *gyr*A462 *end*A1 Δ(sr1-recA) *mcr*B *mrr hsd*S20(rB-, mB-) *sup*E44 *ara*-14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20(SmR) *xyl*-5 λ- *leu mtl*1	Invitrogen, USA
S. cerevisiae JD53	MATα *his*3-D200 *leu*2-3,112 *lys*2-801 *trp*1-D63 *ura*3-52	Dohmen *et al.*, 1995
Plasmid		
pRK2073	A derivative of pRK2013 which carries the transposon Tn7 in the kanamycin gene of pRK2013	Leong *et al.*, 1982
pGWB2	A Gateway Binary Vector with constitutive expression of transgene using the cauliflower mosaic virus (*CaMV*) *35S* promoter	Nagakawa *et al.*, 2007
pGWB4	A Gateway Binary Vector with synthetic green fluorescent protein with S65T mutation (sGFP) located at the C-terminal of transgene	Nagakawa *et al.*, 2007
pYES-Dest52::*aadA*1	Yeast *GAL*1 inducible expression vector harboring *aadA*1	This study
pYES-Dest52::MTS-*aadA*1	Yeast *GAL*1 inducible expression vector harboring MTS-*aadA*1	This study
pYES-Dest52::*cat*	Yeast *GAL*1 inducible expression vector harboring *cat*	This study
pYES-Dest52::MTS-*cat*	Yeast *GAL*1 inducible expression vector harboring MTS- *cat*	This study
pYES-Dest52::*cat-gfp*	Yeast *GAL*1 inducible expression vector harboring *cat-gfp*	This study
pYES-Dest52::MTS-*cat-gfp*	Yeast *GAL*1 inducible expression vector harboring MTS-*cat-gfp*	This study
pYES-Dest52::*hph*	Yeast *GAL*1 inducible expression vector harboring *hph*	This study
pYES-Dest52:MTS-*hph*	Yeast *GAL*1 inducible expression vector harboring MTS-*hph*	This study

Molecular cloning of targeted antibiotic resistance (Ab^R) genes, both with and without mitochondrial targeting sequence (MTS) fusion

The oligonucleotides used for PCR in this study are listed in Table 2. Polymerase chain reaction (PCR) conditions were optimized for each primer pair and DNA fragments were amplified using PrimeSTAR HS DNA polymerase (TaKaRa, Kusatsu, Japan). First, *aadA1*, *cat*, and *hph* antibiotic resistance gene amplicons were amplified from pRK2073 using the primer pair F/R-aadA1, and pGWB2 using the primer pair F/R-cat and F/R-hph, respectively. Overlapping PCR (OE-PCR) was used to genetically fuse the aforementioned antibiotic resistance genes with an MTS derived from mitochondrial tryptophanyl-tRNA synthetase, as depicted in Figure 1(a). Purified *aadA1*, MTS-*aadA*1, *cat*, MTS-*cat*, *hph*, and MTS-*hph* amplicons were individually inserted into the pENTR/D-TOPO® vector through pENTR™ Directional TOPO® cloning according to the manufacturer's instructions (Invitrogen). The respective pENTR reaction mixtures were transformed into competent *Escherichia coli* DH5α cells through electroporation. The transformants were plated on 50 μg/mL of kanamycin supplemented 2X YT plate and incubated at 37ºC overnight. Putative clones of recombinant pENTR vectors harboring the desired constructs were screened by colony PCR using the primer pair F-M13 and R-Ab^R, and recombinant pENTR vectors from the positive clones were extracted for further verification through DNA sequencing. Following this, pENTR/D-TOPO® harboring *aadA1*, MTS-*aadA1*, *cat*, MTS-*cat*, *hph*, and MTS-*hph* were individually subcloned downstream of P_{GAL1} in pYES-Dest52 through Gateway® LR Recombination Cloning in accordance with the manufacturer's guidelines (Invitrogen, Waltham, Massachusetts, USA). LR reaction mixtures were transformed into competent *E. coli* DH5α cells by electroporation. The transformants were plated on 100 μg/mL ampicillin-supplemented 2X YT plates and incubated at 37ºC overnight. Putative clones of recombinant pYES-Dest52 vectors harboring the desired constructs were screened by colony PCR using the primer pair F-T7 promoter and R-Ab^R, and recombinant pYES-Dest52 vectors from the positive clones were extracted for further verification through DNA sequencing.

Table 2. List of primers used in this study

Primer	Sequence (5'-3')	Description
F-FL-MTS	CAC CAT GTC GAA TAA GCA GGC GGT TCT GAA GTT AAT CAG TAA AAG GTG GAT AAG CAC AGT GCA ACG TGC CGA TTT TAA GCT GAA TTC CGA AGC GCT TCA TAG TAA TGC T	Full length MTS serve as forward primer in relevant PCR amplification
F-aadA1	CAC CAT GAG GGA AGC GGT GAT C	To amplify *addA*1 from pRK2073
R-aadA1	CAG AAT TCA CTA GTT TAT TTG CCG ACT ACC TTG GTG	
F-cat	CAC CAT GGA GAA AAA AAT CAC TGG ATA TAC	To amplify *cat* from pGWB2
R-cat	TTA CGC CCC GCC CTG CCA	
R-cat-gfp	GCT CCT CGC CCT TGC TCA CGG ATC CCG CCC CGC CCT GCC ACT C	To serve as reverse primer (coupled with F-cat), in amplifying *cat* with its 3' end anchored with part of the N-terminal of *gfp*
F-cat-gfp	GAG TGG CAG GGC GGG GCG GGA TCC GTG AGC AAG GGC GAG GAG C	To amplify *gfp* with its 5' end anchored with part of the C-terminal of *cat* from pGWB4
R-gfp	TTA CTT GTA CAG CTC GTC C	
F-hph	CAC CAT GAA AAA GCC TGA ACT CAC C	To amplify *hph* from pGWB2
R-hph	CTA TTC CTT TGC CCT CGG AC	
F-MTS-aadA1	GAA TTC CGA AGC GCT TCA TAG TAA TGC TAG GGA AGC GGT GAT CGC CGA	To serve as forward primer (coupled with R-aadA1), in amplifying *aadA*1 with its 5' end anchored with additional 28 nt of C-terminal MTS
F-MTS-cat	GAA TTC CGA AGC GCT TCA TAG TAA TGC TGA GAA AAA AAT CAC TGG ATA TAC CAC CG	To serve as forward primer (coupled with R-cat), in amplifying *cat* with its 5' end anchored with additional 28 nt of C-terminal MTS
F-MTS-hph	GAA TTC CGA AGC GCT TCA TAG TAA TGC TAA AAA GCC TGA ACT CAC C	To serve as forward primer (coupled with R-hph), in amplifying *hph* with its 5' end anchored with additional 28 nt of C-terminal MTS

Molecular cloning of cat-gfp and MTS-cat-gfp

To further investigate and verify the mitochondrial targeting ability of MTS, green fluorescent protein (GFP), which served as a reporter gene in this assay, was fused to the C-terminus of the *cat* and MTS-*cat* genes using OE-PCR, as depicted in Figure 1(b). The purified *cat-gfp* and MTS-*cat-gfp* amplicons were first subjected to pENTR™ Directional TOPO® cloning into the pENTR/D-TOPO® vector, followed by Gateway® LR Recombination Cloning into the destination vector pYES-DEST52.

Yeast transformation

The lithium acetate transformation method was used to transform yeast JD53 cells (Clontech, 2009). Briefly, 0.1 µg of the desired plasmid DNA and 0.1 mg of salmon sperm ssDNA (Sigma-Aldrich, Burlington, Massachusetts, USA) as carrier DNA were mixed with 100 µL of freshly prepared yeast JD53 competent cells in a 1.5 mL centrifuge tube. Next, 600 µL of freshly prepared 1x lithium acetate (LiAc) [100 mM LiAc, pH 7.4]/40% *polyethylene glycol 4000 (PEG-4000)*/1x TE [10 mM Tris (pH 7.4), 1 mM ethylenediaminetetraacetic acid (EDTA)] was added to the cell mixture and vortexed vigorously for 1 min. The mixture was incubated at 30ºC with agitation at 200 rpm for 30 min. A volume of 70 µL dimethylsulfoxide (DMSO) was added to the cell mixture and mixed gently by inversion. The cells were then subjected to heat-shocked at 42ºC for 15 min and immediately incubated on ice for 2 min prior to centrifugation at 13,000 × *g* for 5 s. The supernatant was discarded, and the cell pellet was resuspended in 100 µL of 1x TE buffer, plated on a Synthetic Define, SD-(Ura-)-Glu plate, and incubated at 30ºC for 3 d.

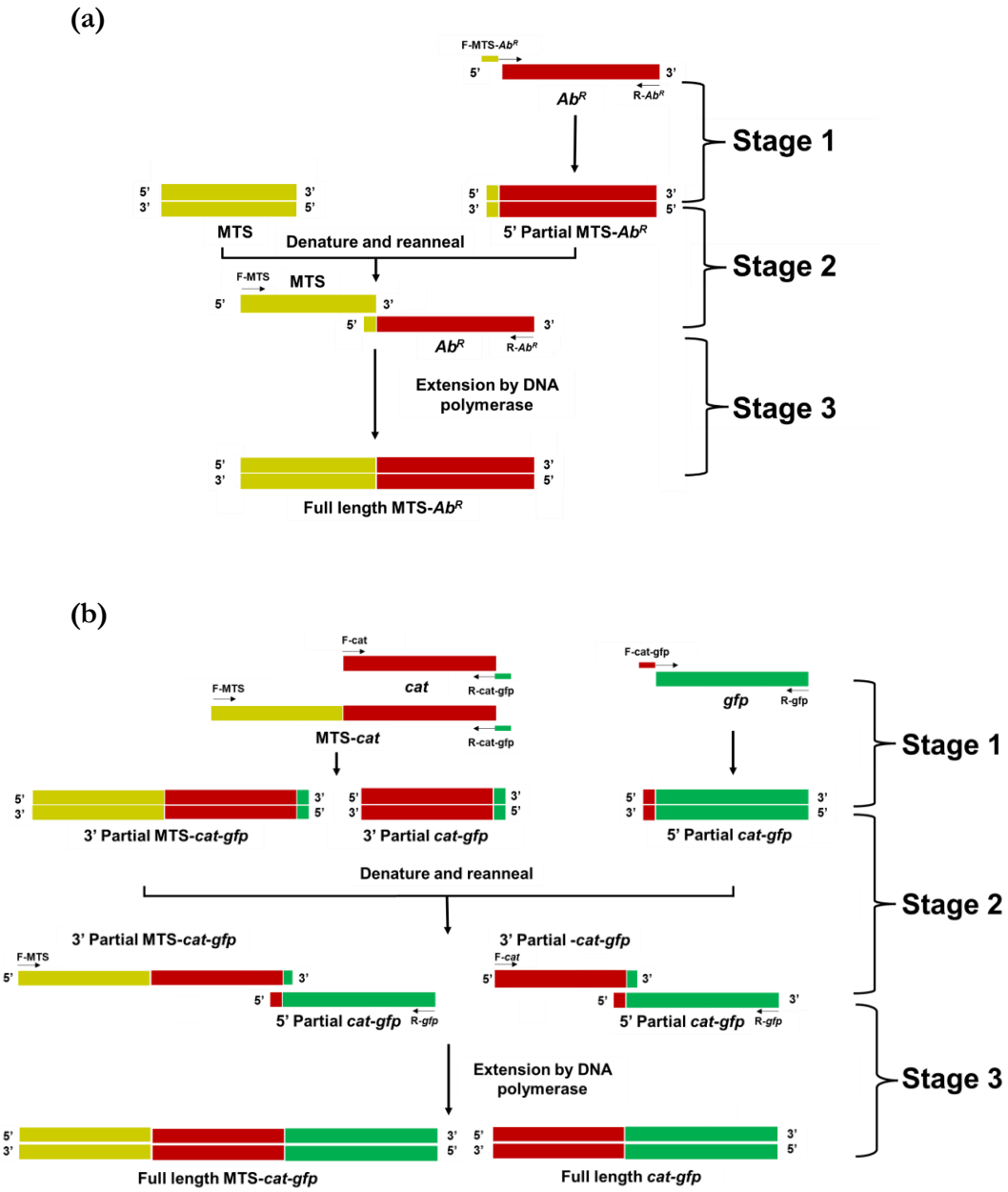

Figure 1. Schematic diagram representation of the OE-PCR for generating (a) MTS-*Ab*^{R} construct; (b) MTS-*cat-gfp* and *cat-gfp* constructs.

Screening of yeast transformants via colony PCR

Screening of yeast transformants for positive clones was adapted from Harju *et al.* (2004). In brief, a single yeast colony was picked and resuspended in 10 µL of solution I (50 mM glucose, 25 mM Tris, and 10 mM EDTA; pH 8.0). The mixture was heated at 80ºC for 5 min and another 10 µL of Solution I containing 0.6 µL of zymolyase was added to lyse the yeast cell wall. The reaction mixture was incubated at 37ºC for 30 min and then placed on ice. A total volume of 40 µL solution II [0.2 N sodium hydroxide (NaOH), 1% sodium dodecyl-sulfate (SDS)] was then added to the mixture, vortexed, and left on ice for 5 min. Subsequently, 30 µL of solution III (3M potassium acetate, 5M glacial acetate acid) was added, vortexed, and left on ice for 5 min prior to centrifugation at 4ºC, and 3,500 × *g* for 10 min. The supernatant was transferred to a new microcentrifuge tube, and an equal volume of absolute ethanol was added and mixed. The reaction was left at room temperature for 10 min before centrifugation at 4ºC, and 3,500 × *g* for 10 min. The pellet was washed once with 70% ethanol and centrifuged at 4ºC, at 3,500 × *g* for 10 min. The supernatant was discarded, and the cell pellet was vacuum-dried prior to dissolving in 10 µL TE buffer. Five microliters of the extracted yeast plasmid served as a template for subsequent colony PCR using the F-T7 promoter and respective R-Ab^R gene primers.

Minimal inhibitory concentration (MIC) test

Yeast transformants containing pYES-Dest52 harboring *aadA1*, *cat*, and *hph* genes, both with and without MTS fusion and/or *gfp*, were streaked on SD-(Ura-)-Gal-Raf agar supplemented with different concentrations of antibiotics, namely streptomycin (2, 4, 6, 8, and 10 mg/mL) (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), chloramphenicol (2, 4, 6, 7, and 8 mg/mL) (FUJIFILM Wako Pure Chemical Corporation), and hygromycin B (2, 4, 6, 8, and 10 mg/mL) (FUJIFILM Wako Pure Chemical Corporation), in triplicate manner. Wild-type (untransformed) *S. cerevisiae* strain JD53 was included in every assay plate as a negative control. The plates were incubated at 30ºC °C for three days. The MIC of the antibiotic was determined by identifying the lowest concentration at which the respective antibiotic effectively killed or inhibited the growth of yeast transformants containing either pYES-Dest52::*Ab*^{R} or pYES-Dest52::MTS-*Ab*^{R}.

Induction of cat-gfp and MTS-cat-gfp expressions

Ab^R gene expression is regulated by galactose-inducible P_{GAL1} in the yeast expression vector pYEST-DEST52. Since P_{GAL1} is tightly controlled and repressed by glucose and induced by galactose, the expression of *Ab*^{R} genes in this study was induced by 1% galactose and glucose was replaced by raffinose as the carbon source to support yeast growth in the SD medium.

Visualizing of Ab^R protein and GFP-tagged Ab^R protein using fluorescence microscopy

The location of the *Ab*^{R} protein was assessed by determining the position of the expressed *Ab*^{R}-*gfp* chimeric gene in yeast cells. Yeast transformants harboring *cat-gfp* and MTS-*cat-gfp* plasmids were separately induced for 16 h in SD induction medium supplemented with 1% galactose. The overnight culture was harvested by centrifugation at 1,000 × *g* for 5 min at room temperature. The cell pellet was then resuspended in 500 µL of SD induction medium containing the mitochondria-specific dye MitoTracker® Red CMXRos (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and incubated at 30°C for 30 min, followed by centrifugation at 1,000 ×*g* for 5 min at room temperature. The cell pellet was washed once with an equal volume of phosphate buffered saline (PBS) and pelleted at 1,000 × *g* for 5 min. Finally, the cell pellet was resuspended in 100 µL PBS and visualized under an Olympus BX41TF-FL-CCD fluorescence microscope (Olympus Tokyo, Japan).

Extraction of yeast cytoplasmic proteins and crude mitochondria fraction

The extraction of yeast cytoplasmic proteins and crude mitochondrial fraction was adapted from Gregg *et al.* (2009), with slight modifications. First, 50 ml of the induced yeast culture harboring pYES-Dest52::*cat-gfp* and pYES-Dest52::MTS-*cat-gfp* was harvested at 3,000 × *g* for 5 min at room temperature. The cell pellet was washed twice with 25 mL of dH2O and pelleted at 3,000 × *g* for 5 min at room temperature. The cell pellet was

resuspended in dithiothreitol (DTT) buffer (100 mM Tris, 10 mM DTT; pH 9.4) according to the weight of the cell pellet (2 mL buffer/g cells). The cell suspension was incubated at 30°C with agitation at 70 rpm for 20 min and centrifuged for 5 min at 3,000 × *g* at room temperature. The cell pellet was then washed twice with zymolyase buffer (20 mM potassium phosphate, 1.2 M sorbitol; pH 7.4) without the addition of zymolyase. Subsequently, the cell pellet was resuspended in zymolyase buffer (7 mL/g cells) with the addition of zymolyase (1 mg/g cells), followed by incubation at 30°C with 70 rpm agitation for 30 min.

The spheroplasts were pelleted by centrifugation at 3,000 × *g* at 4°C for 8 min and washed twice with ice-cold homogenization buffer [10 mM Tris, 0.6 M sorbitol, 1 mM EDTA, 0.2% (w/v) bovine serum albumin (BSA)] according to the weight of the cell pellet (6.5 mL buffer/g cells). The resuspension mixtures were then homogenized using a LabGEN homogenizer (Cole Parmer, Vernon Hill, Illinois, USA) for 30 s. Homogenization buffer was added to the mixture and centrifuged at 1,500 × *g* at 4°C for 5 min. The mixture was then centrifuged at 3,000 x *g* at 4°C for 5 min. The supernatant containing the cytoplasmic protein was harvested, and the remaining pellet was resuspended in ice-cold homogenization buffer (6.5 mL buffer/g cells) and centrifuged at 12,000 × *g* at 4°C for 15 min. The pellet, consisting of crude mitochondrial proteins, was dissolved in Protein Extraction Reagent Type 4 (Sigma-Aldrich).

Verification of the localization of cat-gfp and MTS-cat-gfp proteins in yeast cells

A total of 40 ng of each yeast cytoplasmic fraction and crude mitochondrial fraction, based on the Bradford assay, were separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% polyacrylamide gel with 90 min migration at 140 V. After migration, the gel was transferred onto a PVDF membrane using a semi-dry blotter (Cosmo Bio, Tokyo, Japan) for 25 min at 75 V. Blocking, probing, and washing were performed according to the manufacturer's protocol, using a Chemi-Lumi One Super detection system (Nacalai Tesque, Kyoto, Japan). Anti-GFP rabbit IgG (Abcam, Cambridge, UK) and anti-rabbit HRP-conjugated IgG from donkey (GE Healthcare, Chicago, Illinois, USA) were used as the primary and secondary antibodies, respectively. For signal detection, Chemi-Lumi One Super substrate (Nacalai Tesque) was used, and signals were detected using a ChemiDoc™ imager (Bio-Rad, Hercules, California, USA).

RESULTS

Molecular cloning of Ab^R, MTS-Ab^R, cat-gfp and MTS-cat-gfp constructs

In this study, all purified PCR amplicons (*aadA*1, MTS-*aadA*1, *cat*, MTS-CAT, *cat-gfp*, MTS-*cat-gfp*, *hph*, and MTS-*hph*) were first cloned into the pENTR-D-TOPO entry vector, followed by subcloning into the yeast destination vector, pYES-Dest52, via Gateway® LR cloning. In each step of molecular cloning, the intermediate products and the final vector were verified by DNA sequencing. Next, pYES-Dest52 harboring the different desired antibiotic resistance genes, both with and without MTS fusion or *gfp* fusion, was transformed into *S. cerevisiae* strain JD53 for subsequent analysis.

MIC determination of yeast transformants harbouring different Ab^R, both with and without MTS- and/or gfp-fusions

Yeast transformants harbouring different recombinant vectors, namely pYES-Dest52::*Ab*^{R}, pYES-Dest52::MTS-*Ab*^{R}, pYES-Dest52::*cat-gfp*, and pYES-Dest52::MTS-*cat-gfp*, were streaked on different concentrations of antibiotics (0.1 – 10 mg/mL) supplemented SD (Ura-)-Gal-Raf plates, in order to determine their respective MIC.

For hygromycin testing, a concentration ranged from 0.1 mg/mL to 10 mg/mL was tested on yeast transformants harbouring pYES-Dest52::*hph* and pYES-Dest52::MTS-*hph*. Both yeast transformants expressing *hph* and MTS-*hph* showed resistance to hygromycin at concentrations of up to 4 mg/mL. However, no significant difference in MIC was observed between the yeast transformants harboring the recombinant plasmids pYES-Dest52::*hph* and pYES-Dest52::MTS-*hph*. Figure 2(a) shows the results of the hygromycin resistance MIC plate

assay, where images of plates supplemented with < 2 mg/mL hygromycin are not shown.

On the other hand, a range of 0.1 mg/mL to 10 mg/mL chloramphenicol was tested on yeast transformants harbouring pYES-Dest52::*cat*, pYES-Dest52::MTS-*cat*, pYES-Dest52::*cat-gfp*, and pYES-Dest52::MTS-*cat-gfp*. In this study, yeast transformants expressing *cat*, *cat-gfp*, MTS-*cat,* and MTS-*cat-gfp* proteins showed resistance to up to 6 mg/mL chloramphenicol. The current results also indicated that there was no difference in terms of chloramphenicol MIC between native *cat* genes with and without MTS and/or *gfp* fusion. Figure 2(b) shows the results of the chloramphenicol resistance MIC plate assay, where images of plates supplemented with < 2 mg/mL of chloramphenicol are not shown.

Lastly, the yeast transformants expressing the *aadA*1 gene constructs, both with and without MTS fusion were subjected to streptomycin testing, with concentrations ranging from 0.1 mg/mL to 10 mg/mL. In this study, both yeast transformants harbouring the recombinant plasmids pYES-Dest52::*aadA*1 and pYES-Dest52::MTS-*aadA*1 were resistant to all tested streptomycin concentrations. This suggests that yeast transformants containing the *aadA*1 gene as a selectable marker might be able to exhibit a resistance level exceeding 10 mg/mL streptomycin. A similar trend was observed, where there was no significant difference in the MIC between yeast transformants expressing *aadA*1 with and without MTS fusion. Figure 2(c) shows the results of the streptomycin resistance MIC plate assay, where images of plates supplemented with < 2 mg/mL streptomycin are not shown.

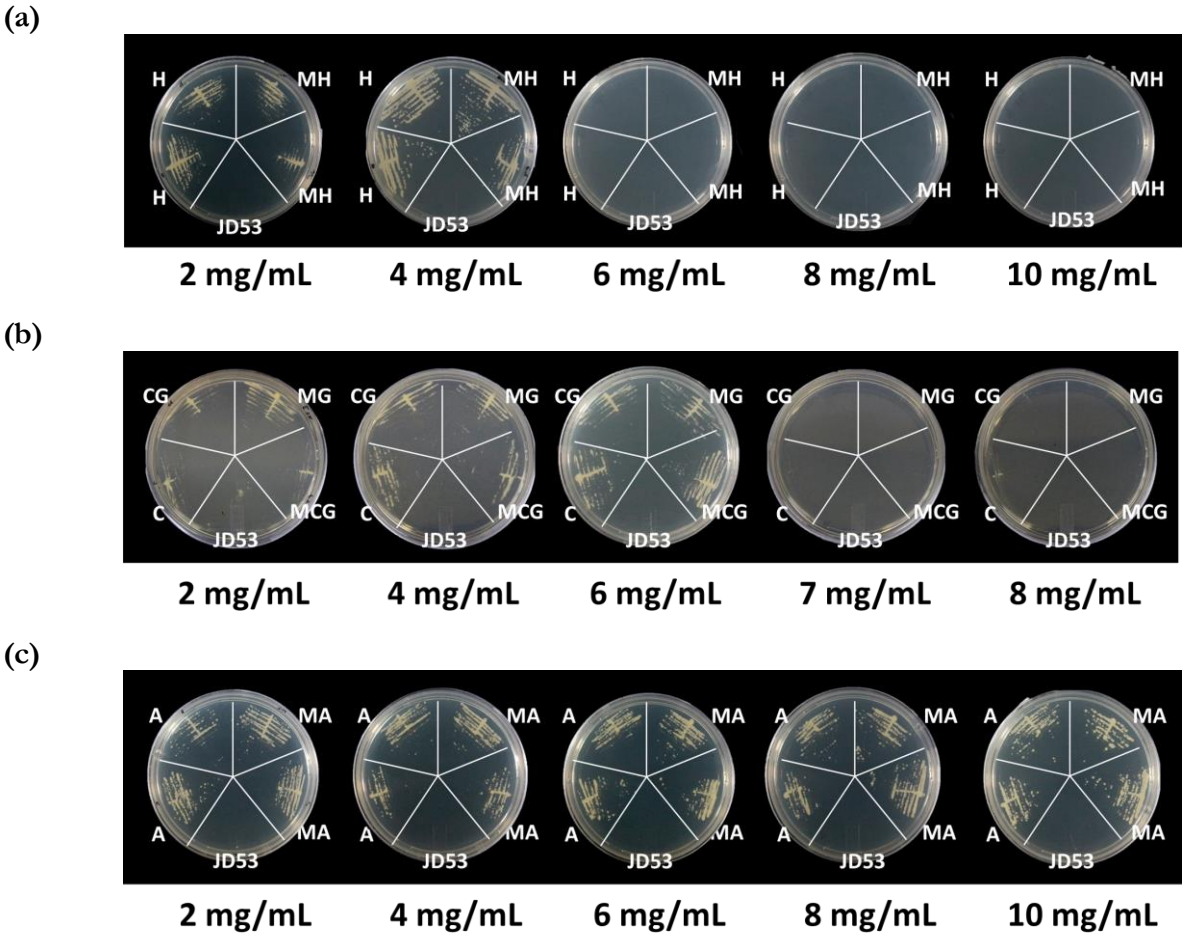

Figure 2. Representative *S. cerevisiae* MIC plates supplemented with different concentrations of (a) hygromycin B; (b) chloramphenicol; (c) streptomycin. H: *hph*; MH: MTS-*hph*; C: *cat*; CG: *cat-gfp*; MC: MTS-*cat*; MCG: MTS-*cat-gfp*; S: *aadA*1; MS: MTS-*aadA*1; JD53: wild type *S. cerevisiae* strain JD53.

Verification of the cat-gfp and MTS-cat-gfp protein localization in yeast

In this study, *gfp* was fused to the C-terminus of *cat* and MTS-*cat* to function as a reporter gene to evaluate whether MTS is capable of translocating its fusion protein into the mitochondria. Upon galactose induction, yeast transformants expressing *cat-gfp* and MTS-*cat-gfp* proteins emitted green fluorescence signals under blue light excitation. Additionally, the yeast transformants were stained using a dye specifically designed for mitochondria, known as MitoTracker® Red CMXRos. The mitochondria were stained red when exposed to green light. As shown in Figures 3(a) and 3(b), green fluorescence signals were detected in the entire yeast cell for both yeast transformants expressing *cat-gfp* and MTS-*cat-gfp*, respectively, except for the central region of the cell. In addition, red spots representing stained mitochondria were observed inside yeast cells. In merged images of the green channel of GFP and the red channel of MitoTracker CMXRos, yellow signals were observed in the transformants expressing MTS-CAT-GFP, as shown in Figure 3(b); yeast transformants expressing *cat-gfp* without MTS fusion are shown in Figure 3(a). These results indicate that only MTS-tagged CAT-GFP proteins were able to translocate into the mitochondria.

To provide additional evidence for this, cytoplasmic and crude mitochondrial proteins were extracted from yeast transformants expressing *cat-gfp* and MTS-*cat-gfp*, respectively. The extracted protein fractions were first subjected to SDS-PAGE, followed by Western Blotting, and probed with anti-GFP. Figure 4 shows the Western Blot analysis of proteins extracted from yeast transformants expressing *cat-gfp* and MTS-*cat-gfp*. Yeast transformants expressing only *cat-gfp* without MTS fusion exhibited a clear band exclusively present in the cytoplasmic fraction, but absent in the crude mitochondrial fraction. Conversely, yeast transformants expressing MTS-*cat-gfp* showed the presence of a distinct band in both cytoplasmic and mitochondrial fractions.

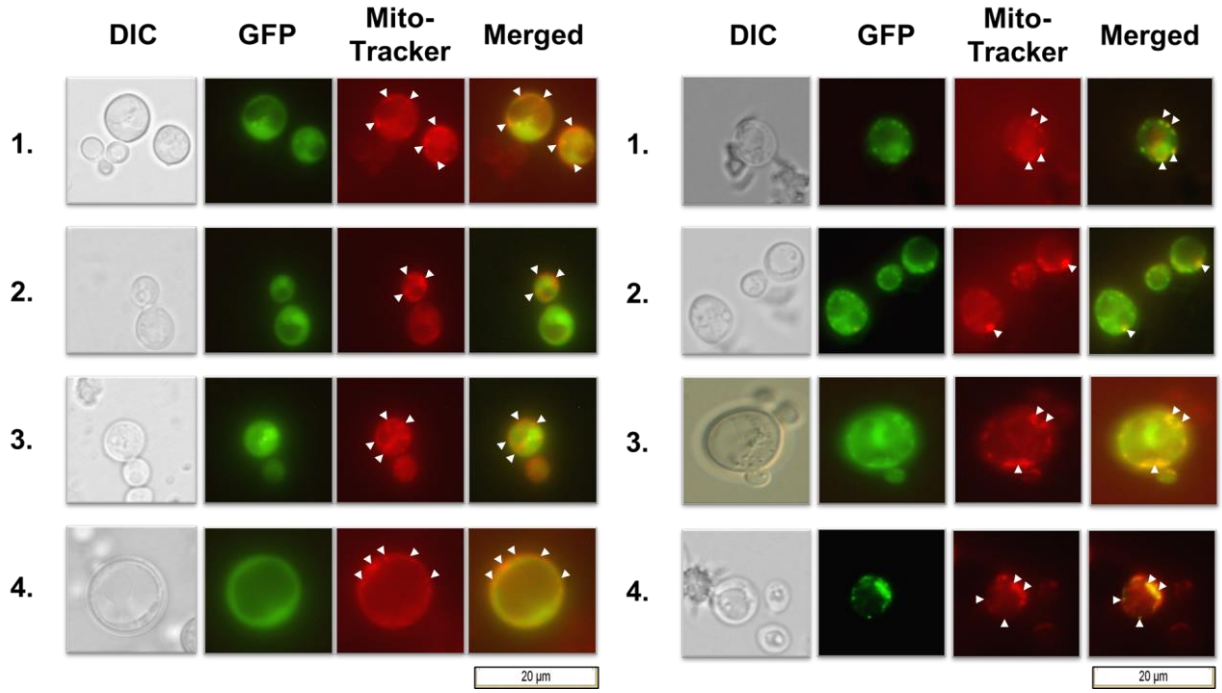

Figure 3. Fluorescence microscopy analysis of yeast transformants expressing (a) *cat-gfp* and (b) MTS-*cat-gfp*. White arrows indicating the position of mitochondria within the yeast cells. DIC: differential interference contrast; GFP: ex filter: BP460-495, em filter: BA510IF; MitoTracker: ex filter: BP530-550, em filter: BA575IF ; Merged: merged images from the GFP and MitoTracker channels.

DISCUSSION

Shockman and Lampen (1962) reported that the MIC of *streptomycin and chloramphenicol against S. cerevisiae* were 0.1 mg/mL. The present study demonstrated that yeast transformants expressing *aadA*1 or MTS-*aadA*1 proteins exhibited resistance to all tested streptomycin concentrations, ranging from 0.1 mg/mL to 10 mg/mL. This implies that yeast transformants harbouring the *aadA*1 gene as a selectable marker could potentially display a resistance level exceeding 10 mg/mL of streptomycin. A streptomycin concentration of 10 mg/mL was considered to be significantly high. Therefore, concentrations greater than 10 mg/mL were not tested in this study. The expression of all four *cat*-related genes, *cat*, *cat-gfp*, MTS-*cat*, and MTS-*cat-gfp*, increased the resistance of their respective yeast transformants to chloramphenicol, even at concentrations as high as 6 mg/ml. Finally, it was observed that yeast transformants expressing both *hph* and MTS-*hph* exhibited resistance to hygromycin B at concentrations of up to 4 mg/mL. Prior study has shown that yeast cells are generally vulnerable to hygromycin B at concentrations of 0.2 mg/mL and above (Kaster *et al.*, 1984).

Although all three tested Ab^R genes, *aadA*1, *cat*, and *hph*, were found to possess an increased MIC level for their respective antibiotics compared to that of the wild-type yeast strain JD53, the yeast transformants expressing *aadA*1 proteins demonstrated a greater level of resistance compared to those expressing *cat* and *hph*. Increased resistance to antibiotics will enhance the effectiveness and efficiency of screening for yeast mitochondrial transformants in the future. Therefore, *aad*A1 exhibits superior potential as a selectable marker compared with *cat* and *hph*. Several studies have demonstrated the use of both hph^R and neomycin phosphotransferase (neo^R) for mitochondrial transformation in mammalian cells (Yoon and Koob, 2008; Yoon and Koob, 2011). However, hygromycin B and G418 have a broad spectrum of activity, as they target the ribosomes of both prokaryotes and eukaryotes (Gonzáles *et al.*, 1978; Borovinskaya *et al.*, 2008; Brodersen *et al.*, 2000). Conversely, streptomycin selectively targets the protein synthesis machinery of prokaryotes, which resembles that of the mitochondria.

It is worth mentioning that the antibiotic resistance proteins analyzed in this study were encoded within the nucleus. For yeast mitochondrial transformation, it is necessary to recode the *aadA*1 gene using the yeast mitochondrial genetic code in order to be functional in yeast mitochondria. The mitochondrial-recorded Ab^R genes, such as *aadA1*-mt, can be inserted into yeast mitochondria using biolistic transformation and integrated into mtDNA through homologous recombination (Bonnefoy and Fox, 2007). Theoretically, the mitochondrial recoded *aadA*1-mt cannot undergo precise translation in the nucleus to generate functional Ab^R proteins. Therefore, they can be considered potential selectable markers specifically engineered for future mitochondrial transformation studies.

No disparity in the resistance level was detected among yeast transformants expressing Ab^R proteins, both with and without MTS. This could be attributed to a fraction of MTS-Ab^R proteins located in the cytoplasm rather than being completely targeted to the mitochondria. This was shown by the fact that the presence of a portion of the MTS-CAT-GFP protein in the cytoplasmic region was confirmed through fluorescence microscopy visualization and chemical verification (Figure 3 and Figure 4). If the Ab^R proteins with MTS were entirely targeted into mitochondria, the resistance level to the relevant antibiotics was expected to at higher concentration.

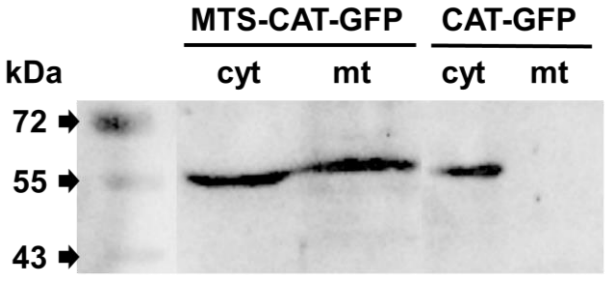

Figure 4. Western blot analysis of proteins extracted from yeast transformants expressing *cat-gfp* and MTS-*cat-gfp*. Cyt: cytoplasmic fraction; mt: mitochondrial fraction.

In this study, we ligated the *gfp* gene to the C-terminus of *cat* and MTS-*cat* constructs, allowing us to observe the spatial distribution of the proteins using a fluorescence microscope. Simultaneously, the mitochondria were labelled with MitoTracker® Red CMXRos. When the stained yeast cells were examined under a fluorescence microscope, the localization of GFP to the yeast mitochondria was shown by the presence of yellow signals in the merged images of the green and red channels. No yellow signal was detected in the merged images of yeast transformants expressing *cat-gfp* alone, indicating that the CAT-GFP fusion proteins did not localize to the yeast mitochondria, but rather to the cytoplasmic region. In contrast, yeast transformants expressing MTS-*cat-gfp* exhibited conspicuous bright yellow signals in the merged channel, demonstrating the localization of GFP-tagged MTS-CAT proteins in yeast mitochondria. Furthermore, MTS facilitated the specific targeting of CAT-GFP proteins to yeast mitochondria. Nevertheless, fluorescence microscopy analysis revealed that green fluorescence signals were detected in the cytoplasmic region, suggesting that MTS-CAT-GFP proteins were not only found in yeast mitochondria but also in the cytoplasm. The MTS-CAT-GFP proteins were localized in the cytoplasm, possibly due to inefficient targeting to the yeast mitochondria.

Western blot analysis was performed to verify the localization of MTS-CAT-GFP proteins to the mitochondria. A clear band was observed in the cytoplasmic protein extract, but not in the mitochondria for the yeast transformants expressing *cat-gfp* (Figure 4). This observation demonstrates that in the absence of MTS, CAT-GFP proteins are incapable of being localized to the mitochondria. Conversely, a clear band was observed in both the cytoplasmic and mitochondrial fractions of the yeast transformants expressing MTS-*cat-gfp* (Figure 4). This outcome provides additional evidence that the MTS successfully directed the GFP fusion proteins to yeast mitochondria. Furthermore, the Western blot analysis aligned with the fluorescence microscopy observation, where MTS-CAT-GFP proteins were also detected in the cytoplasmic region. This may be attributed to the impact of protein folding on the MTS conformation. Alterations in conformation could impede the effective translocation of mitochondria.

The length of the linker may affect MTS conformation (Schmitz and Lonsdale, 1989). The efficiency of mitochondrial protein import into the mitochondria depends on the length of the linker. Linkers consisting of 17 nucleotides exhibit a high level of efficiency, whereas linkers consisting of 16 nucleotides result in decreased efficiency (Schmitz and Lonsdale, 1989). No linker molecule was used to connect MTS and Ab^R in this study. The pre-folding of CAT-GFP protein before it enters the mitochondria may potentially affect the structure of the MTS. This could explain the ineffective localization of the protein within the mitochondria. However, further investigation is required to ascertain the ideal length of the linker to enhance the effectiveness of mitochondrial protein translocation into the mitochondria.

Moreover, a recent investigation revealed that more than 33% of the mitochondrial proteome in yeast demonstrates dual localization, as reported by Dinur-Mills *et al.* (2008) and Burak *et al.* (2013). The subcellular distribution of mitochondrial proteins is influenced by both MTS parameters and general characteristics of the proteins. Studies have shown that approximately 35% of the 126 expected mitochondrial proteins, which are found in two distinct regions, have a MitoProtII score between 0.9 and 1.0 (Dinur-Mills *et al.*, 2008). Furthermore, statistical analysis has shown that dual-localized mitochondrial proteins generally possess a low net charge and exhibit a negative charge (Dinur-Mills *et al.*, 2008). The MitoProtII score of MTS-CAT-GFP in this study was determined to be 0.9868 and carried a negative charge of -11, via MitoProtII analysis (Claros and Vincens, 1996). Together, these findings clarify the existence of MTS-CAT-GFP proteins in both the cytoplasm and the mitochondria.

An independent study illustrated that the process of folding mitochondrial proteins directly influences the positioning of these proteins. When the process of protein folding in mitochondria is disrupted, proteins may relocate from the mitochondria to the cytosol (Regev-Rudzki *et al.*, 2008). Retrograde translocation of mitochondrial proteins from the mitochondrial compartment to the cytosolic compartment has

been demonstrated by the activity of matrix processing peptidase (MPP) on these proteins. Studies have shown that missense mutations in MTS can result in the presence of a particular mitochondrial protein in both the cytoplasmic and mitochondrial areas. This phenomenon has been documented in the studies conducted by Burak *et al.* (2013) and Regev-Rudzki *et al.* (2008). Thus, protein folding plays a role in the dual localization. Following the removal of the N-terminal targeting sequence by matrix MPP, the altered protein is transported back to the cytosol through reverse translocation (Regev-Rudzki *et al.*, 2008).

CONCLUSION

Taken together, the high level of streptomycin resistance in yeast transformants expressing *aadA*1 suggests that it could be a useful selectable marker for future yeast mitochondrial transformations. Although the MTS-tagged CAT-GFP proteins were successfully localized to the yeast mitochondria, as confirmed by both fluorescence microscopy analysis and Western Blotting, the presence of these proteins in the cytoplasmic region also implies that the MTS targeting ability is not completely efficient. This may be due to the adverse effect of the fusion protein, resulting in a change in the conformation of MTS, which affects the translocation efficiency or the overexpression of the fusion protein. To improve protein targeting via MTS, it is advisable to consider modifications such as mitochondrial recoding, utilization of different MTS types, and the insertion of a linker sequence between MTS and *aadA*1 to further enhance the antibiotic resistance level conferred by the protein. This will help in distinguishing between genuine transformants. Developing an effective selectable marker for mitochondrial transformation could boost its transformation rate, making it easier to study of mitochondrial function at the molecular level, and pave the way for gene therapy approaches to treat mitochondrial diseases.

ACKNOWLEDGEMENTS

This research was supported by the Ministry of Higher Education Malaysia (MOHE) through the Fundamental Research Grant Scheme (FRGS) of Project No. FRGS/1/2021/STG01/UTAR/02/1.

CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

REFERENCES

Altmann, K., Dürr, M., & Westermann, B. 2007. *Saccharomyces cerevisiae* as a model organism to study mitochondrial biology. In: *Mitochondria: practical protocols*. Ed. Leister, D., & Hermann, J. M. pp. 81-90. Totowa: Humana Press Inc.

Bonnefoy, N., & Fox, T. D. 2007. Directed alteration of *Saccharomyces cerevisiae* mitochondrial DNA by biolistic transformation and homologous recombination. In: *Methods in molecular biology: mitochondria practical protocols*. Ed. Leister, D., & Herrmann, J. M. pp. 153-166. New Jersey: Humana Press.

Borovinskaya, M. A., Shoji, S., Fredrick, K., & Cate, H. H. D. 2008. Structural basis for hygromycin B inhibition of protein biosynthesis. *RNA* 14(8): 1590-1599. https://doi.org/10.1261/rna.1076908

Brand, M. D., Orr, A. L., Perevoshchikova, I. V., & Quinlan, C. L. 2013. The role of mitochondrial function and cellular bioenergetics in ageing and disease. *British Journal of Dermatology* 169(s2): 1-8. https://doi.org/10.1111/bjd.12208

Brodersen, D. E., Clemons, W. M., Jr, Carter, A. P., Morgan-Warren, R. J., Wimberly, B. T., & Ramakrishnan, V. 2000. The structural basis for the action of the antibiotics tetracycline, pactamycin, and hygromycin B on the 30S ribosomal subunit. *Cell* 103(7): 1143-1154. https://doi.org/10.1016/s0092-8674(00)00216-6

Burak, E., Yogev, O., Sheffer, S., Schueler-Furman, O., & Pines, P. 2013. Evolving dual targeting of a prokaryotic protein in yeast. *Molecular Biology and Evolution* 30(7): 1563-1573. https://doi.org/10.1093/molbev/mst039

Chen, Z., Zhao, N., Li, S., Grover, C. E., Nie, H., Wendel, J. F., & Hua, J. 2017. Plant mitochondrial genome evolution and cytoplasmic male sterility. *Critical Reviews in Plant Sciences* 36(1): 55-69. https://doi.org/10.1080/07352689.2017.1327762

Claros, M. G., & Vinvens, P. 1996. Computational method to predict mitochondrially imported proteins and their targeting sequences. *European Journal of Biochemistry*, 241(3): 770-786. https://doi.org/10.1111/j.1432-1033.1996.00779.x

Clontech Laboratories, Inc, 2009. Yeast Protocols Handbook. United States. https://www.takarabio.com/documents/User%20Manual/PT3024/PT3024-1.pdf

Dinur-Mills, M., Tal, M., & Pines, O. 2008. dual targeted mitochondrial proteins are characterized by lower MTS parameters and total net charge. *PLoS ONE* 3(5): e2161. https://doi.org/10.1371/journal.pone.0002161

Dohmen, R. J., Stappen, R., McGrath, J. P., Forrova, H., Kp;arov, J., Goffeau, A., & Varshavsky, A. 1995. An essential yeast gene encoding a homolog of ubiquitin-activating enzyme. *Journal of Biology Chemistry*, 270(30), pp. 18099-18109. https://doi.org/10.1074/jbc.270.30.18099

Eilers, M., & Schatz, G., 1986. Binding of a specific ligand inhibits import of a purified precursor protein into mitochondria. *Nature*, 322(6076): 228-232. https://doi.org/10.1038/322228a0

Foury, F., Roganti, T., Lecrenier, N., & Purnelle, B. 1998. The complete sequence of the mitochondrial genome of *Saccharomyces cerevisiae*. *FEBS Letters* 440(3): 325-331. https://doi.org/10.1016/S0014-5793(98)01467-7

González, A., Jiménez, A., Vázquez, D., Davies, J. E., & Schindler, D. 1978. Studies on the mode of action of hygromycin B, an inhibitor of translocation in eukaryotes. *Biochimica et Biophysica Acta* 521(2): 459-469. https://doi.org/10.1016/0005-2787(78)90287-3

Gregg, C., Kyryakov, P., & Titorenko, V. I. 2009. Purification of mitochondria from yeast cells. *Journal of Visualized Experiments* 30: 1417. https://doi.org/10.3791/1417

Harju, S., Fedosyuk, H., & Peterson, K. R. 2004. Rapid isolation of yeast genomic DNA: Bust n' Grab. *BMC Biotechnology* 4(8): 1-6. https://doi.org/10.1186/1472-6750-4-8

Jensen, R.E., Hobbs, A.E.A., Cerveny, K.L., & Sesaki, H., 2000. Yeast mitochondrial dynamics: fusion, division, segregation, and shape. *Miscroscopy Research and Technique* 51(6): 573-583. https://doi.org/10.1002/1097-0029(20001215)51:6<573::AID-JEMT7>3.0.CO;2-2

Kambacheld, M., Augustin, S., Tatsuta, T., Müllerm S., & Langer, T., 2005. Role of the novel metallopeptidase MOP112 and saccharolysin for the complete degradation of proteins residing in different subcompartments of mitochondria. *The Journal of Biological Chemistry*, 280(20):20132-20139. https://doi.org/10.1074/jbc.M500398200

Kaster, K. R., Burgett, S. G., & Ingolia, T. D. 1984. Hygromycin B resistance as dominant selectable marker in yeast. *Current Genetics* 8(5): 353-358. https://doi.org/10.1007/BF00419824

Koppen, M., & Langer, T., 2007. Protein degradation within mitochondria: versatile activities of AAA proteases and other peptidases. *Critical Reviews in Biochemistry and Molecular Biology*, 42(3):221-242. https://doi.org/10.1080/10409230701380452

Lax, N. Z., Turnbull, D. M., & Reeve, A. K. 2011. Mitochondrial Mutations: Newly Discovered Players in Neuronal Degeneration. *The Neuroscientist* 17(6), 645–658. https://doi.org/10.1177/1073858411385469

Leong, S. A., Ditta, G. S., & Helinski, D. R. 1982. Heme biosynthesis in *Rhizobium*. Identification of a cloned gene coding for delta-aminolevulinic acid synthetase from *Rhizobium meliloti*. *The Journal of biological chemistry*. 257(15):8724-8730. https://doi.org/10.1016/S0021-9258(18)34188-7

Mahapatra, K., Banerjee, S., De, S., Mitra, M., Roy, P., & Roy, S. 2021. An insight into the mechanism of plant organelle genome maintenance and implications of organelle genome in crop improvement: an update. *Frontiers in Cell and Developmental Biology* 9: 2021. https://doi.org/10.3389/fcell.2021.671698

Mookerjee, S. A., & Sia, E. A., 2013. Using genetic reporters to assess stability and mutation of the yeast mitochondrial genome. In *Genetic manipulation of DNA and protein- examples from current research*. Ed. Figurski, D. Croatia: InTech.

Nakagawa, T., Suzuki, T., Murata, S., Nakamura, S., Hino, T., Maeo, K., Tabata, R., Kawai, T., Tanaka, K., Niwa, Y. *et al.* (2007). Improved Gateway binary vectors: high-performance vectors for creation of fusion constructs in transgenic analysis of plants. *Bioscience, Biotechnology, and Biochemistry*. 71(8): 2095–2100. https://doi.org/10.1271/bbb.70216

Niazi, A. K., Mileshina, D., Cosset, A., Val, R., Weber-Lotfi, F., & Dietrich, A. 2013. Targeting nucleic acids into mitochondria: progress and prospect. *Mitochondrion* 13(5): 534-558. https://doi.org/10.1016/j.mito.2012.05.004

Nosek, J., Tomaska, L., Bolotin-Fukuhara, M., & Miyakawa, I. 2006. Mitochondrial chromosome structure: an insight from analysis of complete yeast genomes, *FEMS Yeast Research* 6(3): 356-370. https://doi.org/10.1111/j.1567-1364.2005.00016.x

Omura, T., 1998. Mitochondria-targeting sequence, a multi-role sorting sequence recognized at all steps of protein import into mitochondria. *Journal of Biochemistry*, 123(6):1010-1016. https://doi.org/10.1093/oxfordjournals.jbchem.a022036

Pfanner, N., & Geissler, A., 2001. Versatility of the mitochondrial protein import machinery. *Nature Review: Molecular Cell Biology*, 2(5), pp. 339-349. https://doi.org/10.1038/35073006

Pronk, J. T. 2002. Auxotrophic yeast strains in fundamental and applied research. *Applied and Environmental Microbiology* 68(5): 2095-2100. https://doi.org/10.1128/AEM.68.5.2095-2100.2002

Rak, M., Tetaud, E., Duvezin-Caubet, S., Ezkurdia, N., Bietenhader, M., Rytka, J., & di Rago, J.P. 2007. A yeast model of the neurogenic ataxia retinitis pigmentosa (NARP) T8993G mutation in the mitochondrial ATP synthase-6 gene. *The Journal of Biological Chemistry* 282(47): 34039-34047. https://doi.org/10.1074/jbc.M703053200

Rapaport, D., 2003. Finding the right organelle: targeting signals in mitochondrial outer-membrane proteins. *EMBO Reports*, 4(10):949-952. https://doi.org/10.1038/sj.embor.embor937

Reece, R. J. 2004. Analysis of genes & genomes. West Sussex : John Wiley & Sons.

Regev-Rudzki, N., Yogev, O., & Pines, P. 2008. The mitochondrial targeting sequence tilts the balance between mitochondrial and cytosolic dual localization. *Journal of Cell Science* 121(4): 2423-2431. https://doi.org/10.1242/jcs.029207

Ruf, S., Hermann, M., Berger, I. J., Carrer, H., & Bock, R. 2001. Stable genetic transformation of tomato plastids and expression of a foreign protein in fruit. *Nature Biotechnology* 19(9): 870-875. https://doi.org/10.1038/nbt0901-870

Schmitz, U. K., & Lonsdale, D. M. 1989. A yeast mitochondrial presequence functions as a signal for targeting to plant mitochondria *in vivo*. *The Plant Cell* 1(8): 783-791. https://doi.org/10.1105/tpc.1.8.783

Shen, H., Qian, B., Chen, W., Liu, Z., Yang, L., Zhang, D., & Liang, W. 2010. Immunogenicity of recombinant F4 (K88) fimbrial adhesin FaeG expressed in tobacco chloroplast. *Acta Biochimica et Biophysica Sinica* 42(8): 558-567. https://doi.org/10.1093/abbs/gmq060

Shockman, G., & Lampen, J. O. 1962. Inhibition by antibiotics of the growth of bacterial and yeast protoplasts. *Journal of Bacteriology*, 84(3): 508-512. https://doi.org/10.1128/jb.84.3.508-512.1962

Siewers, V. 2022. An overview on selection marker genes for transformation of *Saccharomyces cerevisiae*. *Methods in Molecular Biology* 2513: 1-13. https://doi.org/10.1007/978-1-0716-

2399-2_1

Solieri L. 2010. Mitochondrial inheritance in budding yeasts: towards an integrated understanding. *Trends in Microbiology* 18(11): 521-530 https://doi.org/10.1016/j.tim.2010.08.001

Staub, J. M., Garcia, B., Graves, J., Hajdukiewicz, P. T. J., Hunter, P., Nehra, N., Paradkar, V., Schlittler, M., Carroll, J. A., Spatola, L. *et al.* 2000. High-yield production of a human therapeutic protein in tobacco chloroplasts. *Nature Biotechnology* 18: 333-338. https://doi.org/10.1038/73796

Svab, Z., & Maliga, P. 2007. Exceptional transmission of plastids and mitochondria from the transplastomic pollen parent and its impact on transgene containment. *Proceeding of the National Academic of Science of the United States of America* 104(17): 7003-7008. https://doi.org/10.1073/pnas.0700063104

von Heijne, G, 1986. Mitochondrial targeting sequences may form amphiphilic helices. *The EMBO Journal*, 5(6):1335-1342. https://doi.org/10.1002/j.1460-2075.1986.tb04364.x

Yang, H., Gray, B. N., Ahner, B. A., & Hanson, M. R. 2013. Bacteriophage 5' untranslated regions for control of plastid transgene expression. *Planta* 237(2): 517-527. https://doi.org/10.1007/s00425-012-1770-3

Yang, X., Jiang, J., Li, Z., Liang, J., & Xiang, Y. 2021. Strategies for mitochondrial gene editing. *Computational and Structural Biotechnology Journal* 19: 3319-3329. https://doi.org/10.1016/j.csbj.2021.06.003

Yoon, Y. G., & Koob, M. D. 2008. Selection of drug resistance proteins located in the mitochondria of mammalian cells. *Mitochondrion* 8(5-6): 345-351. https://doi.org/10.1016/j.mito.2008.07.004

Yoon, Y. G., & Koob, M. D. 2011. Toward genetic transformation of mitochondria in mammalian cells using a recoded drug-resistant selection marker. *Journal of Genetics and Genomics* 38(2011): 173-179. https://doi.org/10.1016/j.jgg.2011.03.005

Zhou, J., Liu, L., & Chen, J. 2010. Mitochondrial DNA heteroplasmy in *Candida glabrata* after mitochondrial transformation. *Eukaryotic Cell*, 9(5): 806-814. https://doi.org/10.1128/ec.00349-09