Modification of Yeast Estrogen Screen (YES) by Knocking Out of Double ATP-Binding Cassette (ABC) Transporter Genes

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Abstract

ATP-binding cassette (ABC) transporter superfamily is the largest transporter. These help to translocate a wide variety of substrates across extra- and intracellular membranes. Double deletion of the ABC transporter *PDR5* and *SNQ2* genes led to increase accumulation of steroids in yeast cells. Furthermore, Yeast Estrogen Screen (YES) strain harboring human estrogen receptor alpha (hERα) or beta (hERβ) with devoid of each gene could enhance the capacity to detect estrogenic and estrogenic like compounds. This research is aimed to modify YES strain by double deletion of *PDR5* and *SNQ2* using Cre-Lox system with *KanMX* marker. Multicopy plasmid harboring *Cre* recombinase gene under the controlled of *PHO84* promoter was constructed for removal of *KanMX* marker from chromosome of YES strain with first knocking out of *pdr5* gene upon induction by low-Pi condition. The YES-*Δpdr5Δsnq2* strain was obtained by repeatedly knocking out of target gene using *KanMX* marker gene. Growth rate of YES strain with single and double deletion of ABC transporter genes in rich medium were not difference compared to YES wild type. The newly developed YES strain will be useful for screening of estrogenic like compounds.

Keywords: Yeast Estrogen Screen, ABC transporter, *KanMX* gene

Introduction

ATP binding cassette (ABC) transporter is the largest transporter gene family that conserved from bacteria to man (Dean et al. 2001; Higgin 1992). In the yeast *Saccharomyces cerevisiae*, the completed sequence of the genome predicts approximately 29 proteins belonging to the genes of ABC transporter family (Decottignies and Goffeau 1997). Among the yeast ABC transporter proteins, plasma membrane ABC transporters are considered to play a crucial function in the first line defend in *S. cerevisiae* because of their translocation of drugs, herbicides, phospholipids, steroids and peptides. The two plasma membrane ABC transporter proteins, Pdr5 (pleiotropic drug-resistance) and Snq2 (sensitivity to 4-nitroquinoline-N-oxide) are a pleiotropic drug resistance (PDR) subfamily, which confer resistance to a wide variety of compounds including cycloheximide, rhodamin 6G, tamoxifen, steroid, 4-nitroquinoline-N-oxide (4-NQO), and cercosporin (Servos et al. 1993; Kolaczkowski et al. 1996; Ververidis et al. 2001). The transcription of *PDR5* and *SNQ2* are controlled by transcriptional regulatory protein Pdr1/Pdr3 (Katzmann et al. 1996; Hlavácek et al. 2009). Over expression of *PDR5* and *SNQ2* were illustrated to increase resistance to cation such as NaCl, LiCl and $MnCl_2$ (Miyahara et al. 1996) and influence to population quorum sensing (Hlavácek et al. 2009). Regarding to the detection of estrogenic compounds, Yeast Estrogen Screen (YES) assay has been shown to be one of the most popular technique to detect phytoestrogens (Zacharewski 1997), and xenoestrogens (Denier et al. 2009). One of the YES assay is based on yeast two–hybrid system, in which human estrogen receptor and coactivator are inserted in yeast cells, leading to the interaction of ligand to estrogen receptor and

coactivator prior to their interface with the yeast transcription machinery and thereby induces the expression of reporter gene (*LacZ*) (Nishikawa et al. 1999). By single knocking out of *PDR5* and *SNQ2* genes in yeast strain with human estrogen receptor alpha (hERα) and beta (hERβ), the increasing of estrogenic activity of pure estrogenic compounds (Hasenbrink et al. 2006) and estrogenic like compounds in crude plant extracts (Sophon 2012) were observed. Previous study demonstrated that estradiol was accumulated in yeast cell by double deletion of the yeast *PDR5* and *SNQ2* genes (Mahé et al. 1996). The Cre-lox system plays a vital role as the site-specific recombination in genome engineering which enables deletion of multiple genes in single yeast strain (Tahimic et al. 2013). Cre-lox site recombination system of bacteriophage P1 was established to be widely use to rescue marker gene in eukaryote such as in the yeast *S. cerevisiae* (Güldener et al. 1996) and mammalian cells (Sauer and Henderson 1988) as well as in plants (Chong-Pérez et al. 2013). In the yeast *S. cerevisiae*, many dominant marker genes are reported to be applicable for disruption of gene, while the dominant marker *KanMX* gene was demonstrated to be an important marker for gene disruption (Güldener et al. 1996).
In this study, we described the removal of *KanMX* marker gene in YES-*Δpdr5* strain by the Cre-Lox system and the construction of double deletion *PDR5* and *SNQ2* genes by using PCR disruption method. The goal is to improve sensitivity of YES based on yeast two–hybrid system for detection of low concentration of estrogenic like compounds in various samples.

Materials and Methods

Strains, media, plasmids and cultivation conditions
Escherichia coli DH5α (BRL®) was used for standard DNA manipulation. The strain was cultivated in LB (1.0% tryptone, 1.0% NaCl, 0.5% yeast extract) medium supplement with 100 µg/mL ampicillin at 37 °C for 16 h. *Saccharomyces cerevisiae*Y190 (*MATα, ura3-52, his3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, 112, gal4Δ gal80Δ, cyhr2, LYS2::GAL1*$_{UAS}$*- HIS3*$_{TATA}$*-HIS3, MEL1, URA3::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-lacZ)* (Invitrogen®) was employed for preparation of genomic DNA. It was propagated in YPD (1% yeast extract, 2% peptone, 2% glucose) medium. Selective media for yeast transformation was Synthetic Dextrose (SD) medium (0.67% yeast nitrogen base without amino acid, 2% glucose, 20 µg/ml of required growth supplements). Synthetic complete (SC) medium (0.67% yeast nitrogen base without amino acid, 2% glucose, 0.2% dropout mix). Unless otherwise indicated, yeasts were grown at 30 °C for 3-5 days. Plasmid pSH47, pJW1 and BYP654 were employed for construction of plasmid harboring *Cre* recombinase gene.

Preparation of yeast genomic DNA and plasmid
Genomic DNA of *S. cerevisiae* was prepared using the procedure described in A Cold Spring Harbor Laboratory Course Manual (Amberg et al. 2005). Plasmid from *E. coli* was performed by rapid boiling method (Holmes and Quigley 1981).

Yeast transformation
Lithium acetate (Ito et al. 1983) was used for transformation of plasmid into yeast cells. The Trp^+ colonies were selected and confirmed growth on SD containing L-adenine and L-leucine. For knocking out the gene, the DNA fragments of the disruption cassette were introduced into the cell by electroporation method (Amberg et al. 2005). The $G418^r$ transformants were selected on YPD supplemented with 50 µg/ml of G418.

Construction Cre plasmid pSD4 with *PHO84* promoter

The *Sma*I-*Pvu*II DNA fragment (1824bp) containing Cre-*CYC1* terminator fragment from plasmid pSH47 was cloned into plasmid BYP654 containing *TRP*1 as selectable marker at *Sma*I sites to obtain the new plasmid pSD3. Then 1122 bp *Sma*I-*Pvu*II fragment comprising *PHO84* promoter from plasmid pJW1 was inserted into pSD3 at *Sma*I site to obtain plasmid pSD4.

Removal of *KanMX* marker gene from YES-*Δpdr5* strain and curing of Cre plasmid pSD4

The Cre gene under the controlled of *PHO84* promoter was expressed by transferring YES-*Δpdr5*($G418^r$, Trp^+) cells harboring pSD4 from high-Pi to low-Pi medium (Wongwikarn 2001). Cells were grown in high-Pi medium until OD_{660} reached 0.5 to 1.0, and then cells were resuspended and cultivated in low-Pi medium for 24h. $G418^s$ colonies were selected on YPD + 200 µg/ml of G418 and further confirmed by colony PCR. To cure plasmid pSD4, colonies were grown in YPD broth for 36 generations. Trp^- colonies were selected on SC medium.

Construction YES-*Δpdr5Δsnq2* strain

YES-*Δpdr5Δsnq2* (*Δpdr5Δsnq2::LoxP-KanMX-LoxP)* was constructed from YES-*Δpdr5* (*Δpdr5::LoxP-KanMX-LoxP)* by using Cre-Lox system (Güldener et al. 1996). The chromosome of wild type yeast strain Y190 and pUG6 (harboring*loxP::KanMX::loxP* marker) were used as a template for amplification of *SNQ2* and *KanMX* cassette, respectively. The two step of PCR was conducted (Figure 1) to obtain two *SNQ2* deletion cassettes which overlapped at *KanMX* marker. Two pair of primers were used: SNQ2-L1_AAATATTAAAAGTTTA CTCATACCT with KanA-R_CGACTGAATCCGGTGAGAAT for downstream fusion; and SNQ2-L4_CAACCAAGCTGTCGAATGAA with KanA-F_CCGCGATTAAATTCCAACAT for upstream fusion.

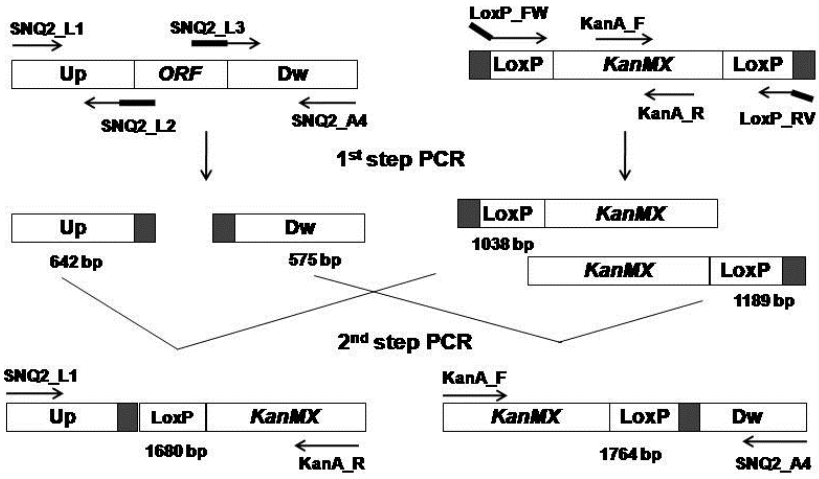

Figure 1: Schematic diagram for construction of *Δsnq2::KanMX* deletion cassette module.

Results and Discussion

Construction Cre recombinase plasmid with *PHO84* promoter

Due to background of YES strain (Boonchird et al. 2010) was Y190 which was *gal4* mutant and available Cre recombinase plasmid in pSH47 was controlled by *GAL*1 promoter. Thus the *Cre* gene with *GAL*1 promoter was not suitable for existing YES strain. In this case, *GAL*1 promoter was replaced with *PHO84* promoter in order to control the expression of the *Cre* gene. The two step of cloning was conducted in order to construct the Cre expression plasmid pSD4 with *PHO84* promoter, and *TRP1* as a selectable marker (Figure 2).

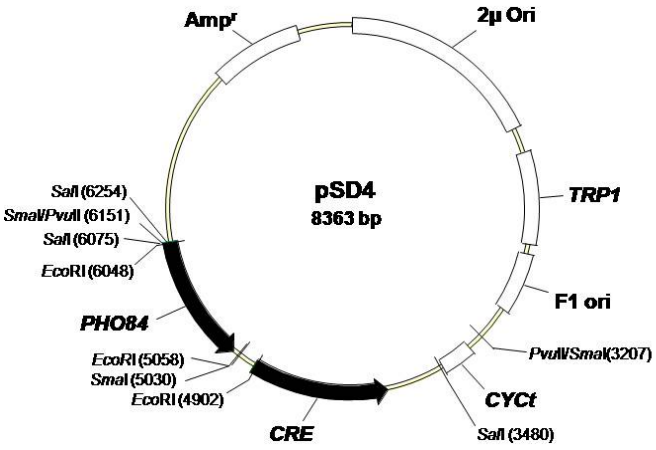

Figure 2: Multicopy plasmid pSD4 harboring *Cre* recombinase gene fused with *PHO*84 promoter.

Removal of *KanMX* marker gene

In order to make double deletion genes in single yeast strain, the *KanMX* marker gene at the first deletion gene on chromosome was removed by Cre recombinase. YES-*Δpdr5::KanMX* strain was transformed with plasmid pSD4. The expression of *Cre* gene was induced by shifting the cell from high-Pi to low-Pi medium. After 24-48 h of incubation in low-Pi medium, *KanMX* marker gene was rescued from 70-80% of colonies on YPD. The corrected excision of *KanMX* marker gene was confirmed by colony PCR (Figure 3). PCR products using *KanMX* primers could not be generated in YES-*Δpdr5* when *KanMX* was removed from chromosome.

Construction YES-*Δpdr5Δsnq2* strain

To knock out *SNQ2* gene in YES-*Δpdr5*, the *Δsnq2::KanMX* disruption cassette was introduced into the YES-*Δpdr5* cells. After selection of the recombinant yeast on YPD +50 µg/ml G418, the colonies were further confirmed by colony PCR of which 90% of colonies shown the corrected integration at *SNQ2* locus in chromosome (Figure 4). PCR products with correct sizes were generated from YES-*Δpdr5Δsnq2::KanMX* using 2 pairs of primers. Growth rate of the YES-Δ*pdr5Δsnq2* with hERα and hERβ were similar to the wild type (YES-hERα and YES-hERβ) and single deletion (YES-hERαΔ*pdr5*, YES-hERβΔ*pdr5*, YES-hERα*Δsnq2* and YES-hERβ*Δsnq2*) strains (Table 1). In addition, the yeast cell with devoid of *pdr5* and *snq2* was more sensitive to G418 as compared with single deletion (*pdr5* or *snq2*)

strain. The double deletion yeast cells could grow on YPD medium + G418 at the concentration 150 µg/ml, while the single loss of *pdr5* or *snq2* cells were able to grow at the concentration 200 µg/ml (data not shown). This result demonstrated that G418 was accumulated in double deletion strains.

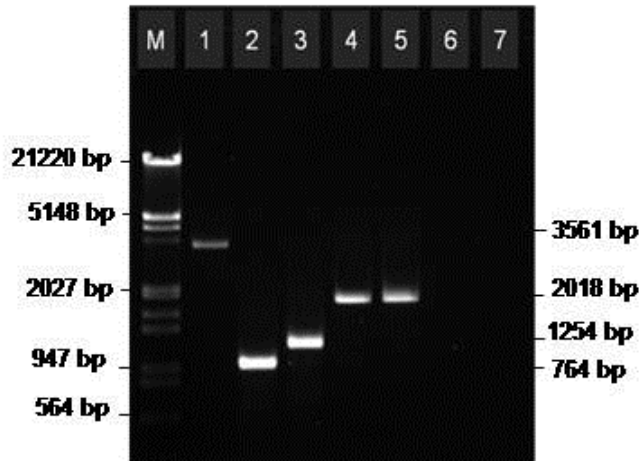

Figure 3: Analysis of *KanMX* marker gene removal in YES-*Δpdr5* strain by colony PCR. Lane M: λDNA cut with *Eco*RI and *Hind*III; Lane 1, 2 and 3: PCR product of full length (3561 bp), upstream (764 bp), and downstream (1254 bp) of *Δpdr5::LoxP-KanMX* cassette by using pair of primers: PDR5-A1 (upstream of *PDR5* locus) and PDR5-A4 (downstream of *PDR5* locus), PDR5-A1 and KanR (middle of *KANMX* marker), PDR5-A4 and KanV (middle of *KANMX* marker), respectively. Lane 4, 5, 6 and 7 represent the PCR product of *Δpdr5::LoxP* cassette (removal *KanMX* marker) in full length (2018 bp), upsteam (non), downstream (non) by using the same pair of primer as the negative control in lane 1 to 3, respectively.

Table 1 Growth rate of YES strains in YPD medium at 30 °C with vigorous shaking.

YES strains	Specific growth rate (μ, h^{-1})
YES-hERα	0.3080
YES-hERβ	0.3160
YES-hERα Δ*pdr5*	0.3017
YES-hERβ Δ*pdr5*	0.2991
YES-hERα Δ*snq2*	0.2931
YES-hERβ Δ*snq2*	0.3181
YES-hERα Δ*pdr5*Δ*snq2*	0.2872
YES-hERβ Δ*pdr5*Δ*snq2*	0.3233

Conclusion

In this study, the Cre expression plasmid pSD4 under the control of *PHO84* promoter was successfully expressed in YES-*Δpdr5* strain which caused the removal of *KanMX* marker gene from the chromosome in the low-Pi condition. The newly YES-*Δpdr5Δsnq2* strain was obtained by repeatedly use of *KanMX* marker disruption cassette. Moreover, the sensitivity of yeast strain with double knocking out *pdr5* and *snq2* to G418 provided the information to predict the related function of *pdr5* and *snq2* with the efflux of this toxic compound in yeast cell. The double deletion strain will be further used for detection of estrogenic like compounds.

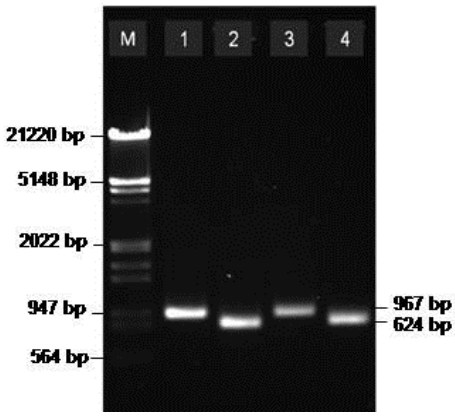

Figure 4: Analysis of YES-Δ*pdr5Δsnq2* by colony PCR.
Lane M:λDNA cut with *Eco*RI and *Hind*III; Lane 1 and 2: YES-*Δsnq2* (control) generated upstream (967 bp), and downstream (624 bp) PCR products using pair of primers: *SNQ2*-A1 (upstream of *SNQ2* locus) and KanR (middle of *KANMX* locus), *SNQ2*-A4 (downstream of *SNQ2* locus) and KanV(middle of *KANMX* locus), respectively; Lane 3, and 4: YES-*Δpdr5Δsnq2* generated PCR product as control.

Acknowledgements

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