Chapter 2

MATERIAL AND METHODS
2.1: MATERIALS AND METHODS

2.1.1: Chemicals and Reagents
All the chemicals used in this study were obtained commercial sources and were of analytical grade. Media components were purchased either from Hi Media, India or Difco, USA. Oligonucleotides were bought from Ransom Hill Biosciences, USA, Gemini Biotech, USA or Integrated DNA Technologies, Inc., USA. Restriction/modifying enzymes were purchased either from New England Biolabs, USA or Boeheringer Mannheim, Germany. DNA sequencing kit (ABI PRISM 310 with dye termination cycle sequencing ready reaction kit) was bought from Perkin Elmer, USA.

2.1.2: Oligonucleotides
The list of Oligonucleotides used in this study is given in table 2.

2.1.3: Strains and Plasmids
The list of the yeast strains and plasmids used in this study is given in table 2.1 and table 2.2 respectively.

2.1.4: Medium and buffer composition
All the media and the buffers were prepared in double distilled water and were sterilized by autoclaving for 15 min at 15 psi except for buffers and material used for RNA work, which was autoclaved for 1 hr. at 15 psi.

2.1.4.1: Medium composition
YPD medium (Yeast extract-peptone-dextrose medium) (per litre)
Yeast extract 10 g
Peptone 20 g
Dextrose 20 g

YPG medium (Yeast extract-peptone-galactose medium) (per litre)
Yeast extract 10 g
Peptone 20 g
Galactose 30 g

SD medium (synthetic defined medium) (per litre)

Yeast nitrogen base 1.7 g
(Without amino acids)
Ammonium sulfate 5 g
Dextrose 20 g
Supplements 50-70 mg
(As per requirement)

SD-galactose medium (synthetic defined -galactose medium) (per litre)

Yeast nitrogen base 1.7 g
(Without amino acids)
Ammonium sulfate 5 g
Galactose 30 g

LB (Luria-Bertani) medium (per litre)

Yeast extract 5 g
Tryptone 10 g
Sodium chloride 10 g

The pH of the LB medium was adjusted to 7.0 with sodium hydroxide. Ampicilin was added to LB medium at a concentration of 100µg/ml for selection of the plasmids.

YES medium (Yeast extract supplement medium) (per litre)
<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract</td>
<td>5 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>30 g</td>
</tr>
<tr>
<td>Supplements</td>
<td>50 mg</td>
</tr>
</tbody>
</table>

**EMM medium (Edinburgh minimal medium) (per litre)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium hydrogen phthalate</td>
<td>3 g</td>
</tr>
<tr>
<td>Disodium hydrogen phosphate</td>
<td>2.2 g</td>
</tr>
<tr>
<td>Sodium glutamate</td>
<td>5 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>20 g</td>
</tr>
<tr>
<td>Salt stock</td>
<td>20 ml of 50X salt stock solution</td>
</tr>
<tr>
<td>Vitamins</td>
<td>1 ml of 1000X vitamin stock solution</td>
</tr>
<tr>
<td>Minerals</td>
<td>0.1 ml of 1000X mineral stock solution</td>
</tr>
<tr>
<td>Supplements</td>
<td>75 mg</td>
</tr>
</tbody>
</table>

**Salt stock (50X) (per litre)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgCl₂6H₂O</td>
<td>53.3 g</td>
</tr>
<tr>
<td>CaCl₂2H₂O</td>
<td>0.735 g</td>
</tr>
<tr>
<td>KCl</td>
<td>50 g</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>2 g</td>
</tr>
</tbody>
</table>

**Vitamin stock (1000X) (per litre)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotinic acid</td>
<td>10 g</td>
</tr>
<tr>
<td>myo-Inositol</td>
<td>10 g</td>
</tr>
<tr>
<td>Pantothenic acid</td>
<td>1 g</td>
</tr>
<tr>
<td>Biotin</td>
<td>10 mg</td>
</tr>
</tbody>
</table>

**Mineral stock (1000X) (per litre)**
The salt, vitamin and mineral stocks were autoclaved separately, were stored at 4°C and were added to the autoclaved media just before pouring the plates.

Pre-sporulation medium (per litre)

Yeast extract 8 g
Peptone 3 g
Dextrose 10 g

Sporulation medium (per litre)

Yeast extract 2.5 g
Potassium acetate 15 g

X-gal plates:

The basic ingredients of SD medium were dissolved in 900 ml double distilled water. The medium after adding 2.5% agar was autoclaved and cooled to 50°C. 100 ml of sterile 10X BU salts and 2 ml of 40 mg/ml solution of X-gal (dissolved in dimethyl formamide) were added to the medium just before pouring the plates. The plates were stored at 4°C and were protected from light.
YPD plates containing drugs:

YPD plates containing different compounds were made by adding the required volume of the stock solution of the compound to the autoclaved YPD medium (temperature of YPD media maintained at 50 - 55°C) just before pouring the plates. The stock solutions of cadmium, geneticin, t-butylhydroperoxide were prepared in water while β-estradiol and chlorodinitrobenzene were made in ethanol.

2.1.4.2: Buffer Composition

TE buffer:
10 mM tris-Cl (pH 8.0)
1mM EDTA (pH 8.0)

TAE buffer:
40 mM Tris-acetate
1mM EDTA (pH 8.0)

Orange-G dye (Gel loading dye, 6X)

0.25% orange-G
30% glycerol

DNA binding matrix solution:

Silica (1g) was suspended in 100 ml. of sterile water. The slurry was mixed well and allowed to settle down overnight. The supernatant containing fine silica particles was discarded and the pellet suspended in sterile water to a final concentration of 0.166mg silica/ml. The working solution was prepared [6.38 mg silica/ml of 6M potassium iodide (KI)] by adding 1ml of silica stock solution to the 25 ml of 6M KI (made in 50 mM Tris and 10 mM EDTA, pH 8.0) and the resulting solution was used to purify DNA from the agarose gels.
Incubation buffer (for colony PCR):
1.2 M Sorbitol
100 mM Sodium phosphate (pH 7.4)
2.5 mg/ml Zymolyase

Lysis mixture (for yeast genomic DNA isolation):
10 mM Tris-Cl (pH 8.0)
1 mM EDTA (pH 8.0)
100 mM NaCl
1% SDS
2% Triton X-100

STES lysis solution (for miniprep of yeast)
10 mM Tris-Cl (pH 8.0)
1 mM EDTA (pH 8.0)
0.1% SDS
2% Triton X-100

Lac Z buffer (for β-galactosidase activity measurement)

60 mM Na₂HPO₄·7H₂O
40 mM NaH₂PO₄·H₂O
10.06 mM KCl
1 mM MgSO₄·7H₂O
0.27% β-mercaptoethanol

10X BU salts:

10X BU stock solution was prepared by dissolving 70 g of disodium hydrogen phosphate and 30 g of sodium dihydrogen phosphate in one litre of distilled water. The pH of the stock solution was adjusted to 7.0 and autoclaved.
10X MAE buffer:

200 mM MOPS
50 mM Na Acetate
10 mM EDTA

Zymolase solution: 0.5 mg zymolase/ml of 1M sorbitol

2.2: METHODS

2.2.1: Growth and maintenance of yeast strains.

*S. cerevisiae* strains were maintained routinely on YPD medium while *S. pombe* strains were maintained on YES medium. Both the cultures were grown at 30°C with or without shaking.

2.2.2: Recombinant DNA methodology

Plasmid DNA isolation, restriction digestion, modification, ligation and all other DNA manipulations were carried out as described in Sambrook *et al.* (1989).

2.2.3: DNA purification from the agarose gels

The desired DNA fragment was excised from the gel and transferred into a preweighed microfuge tube. DNA binding matrix solution of about three times the weight of the gel piece was added to the tube and incubated at 55°C for 10 min. After incubation, the silica suspension was spun down at 12,000 rpm for 5 min. The pellet was washed with 50% ethanol followed by acetone. The silica pellet was air-dried and the DNA was eluted twice with 0.1X TE or sterile water at 45°C for 5 min. Following the elution, silica particles were removed by centrifuging at 12,000 rpm for 10 min. The DNA was also extracted from gel slices using the QIAGEN gel extraction kit.
2.2.4: Isolation of genomic DNA from yeast

Genomic DNA from *S. cerevisiae* strains was isolated as described by Kaiser *et al.* (1994) using the glass bead lysis method.

2.2.5: PCR amplification

PCR amplification was carried out using Vent DNA polymerase, *Taq* DNA polymerase, mixture of both vent and *Taq* and by *Pfu* DNA polymerase in MJ Research Minicycler or Techne Genei Minicycler with genomic DNA or plasmid DNA as a template.

2.2.6: Yeast colony PCR

Colony PCR was performed on fresh transformants as described (Ling *et al.*, 1995) with slight modifications. Briefly, a single fresh, average sized colony was transferred to an eppendorf tube containing 7 µl of incubation buffer and was mixed thoroughly. The cell suspension was incubated at 37°C for 4-6 hrs and 3 µl of this cell suspension was used as a template in 100 µl of PCR reaction mix.

2.2.7: DNA sequencing

DNA sequencing was done by using automated DNA sequencer. ABI PRISM 310 kit was used to prepare the DNA samples for sequencing.

2.2.8: YEAST TRANSFORMATION

Transformation of *S. cerevisiae*

The transformation of *S. cerevisiae* strains was carried out by lithium acetate method (Ito *et al.*, 1983). Yeast cultures were grown in YPD at 30°C with shaking for 16-18 hrs and then reinoculated in fresh YPD media to an initial OD_{600} of 0.1, cells were allowed to grow at 30°C for 4-5 hrs with shaking. Cells were harvested at 6k for 5 min, then were washed with sterile water followed by subsequent wash with 0.1M lithium acetate (LioAc) solution (prepared in TE, pH 7.5) and were finally resuspended in the same solution. Cells were
incubated at 30°C for 30 min with shaking. The cells were spun down, suspended in 0.1 M LioAc solution to a cell density of $1 \times 10^9$ cells/ml and divided into 100μl aliquots. Approximately 0.3 μg-0.7μg of plasmid/DNA fragment and 50μg (5 μl of 10 mg/ml stock solution) of heat denatured, salmon sperm carrier DNA was added to each aliquot and whole cell suspension was incubated at 30°C for 30 min. After the incubation, 0.3 ml of 50% PEG 3350 (prepared in 0.1 M LioAc, pH 7.5) was added to each tube, mixed well and was kept at 30°C for 45 min. The cell suspensions were subjected to heat shock at 42°C for 5 min. and the cells were allowed to cool to room temperature. The cells were pelleted down at 7K for 3 min. The cell pellet was resuspended in sterile water and appropriate volume of cell suspension was plated on selection plates.

Transformation of *S. pombe*

Transformation of *S. pombe* was carried out by lithium acetate method as described previously (Chaudhuri et al., 1997). A single colony from a freshly grown culture was inoculated in YES containing all required supplements and allowed to grow overnight at 30°C shaker. Cells were reinoculated at OD_{600} of 0.1 in fresh YES medium. Cultures were allowed to grow at 30°C shaker for 5-6 hrs. Cells were harvested at 7K for 3 min. Cells were washed with sterile water and 0.1 M LioAc (prepared in water and pH was adjusted with acetic acid, pH 4.9). Cells were suspended in 0.1M LioAc so as to obtain $1 \times 10^9$ cells/ml of LioAc. Aliquots of 100μl were made and 0.5-1μg plasmid/DNA fragment was added along with 50 μg of carrier DNA. Incubated for 10 min at room temperature. After incubation 500 μl of 50% PEG (prepared in 0.1 M LioAc, pH 4.9) was added to each tube and incubated at 30°C for 45 min. Heat shock was given at 46°C for 25 min. Cells were allowed to cool at room temperature for 5 min and then harvested at 7K for 4 min. The cell pellet was resuspended in sterile water and required amount of cell suspension was plated on selection plates.

2.2.9: Amplification of *S. cerevisiae* genomic DNA library

*S. cerevisiae* genomic library was used to identify novel gene/s, which play role in glutathione mediated detoxification pathway in yeast. Genomic library was amplified by transforming freshly prepared electrocompetent *E.coli* DH5α cells with 2μl (~1μg) of library
DNA. The cells were electroporated at 200 ohms of resistance, 25 μF of capacitance and 25 kV of voltage. After the electroporation, the cells were suspended in one ml of LB broth and were allowed to grow at 37°C for 1 hr. After the incubation, cells were spread onto LB plates containing ampicillin (~25 plate/transformation) followed by incubation at 37°C for overnight. The transformants were pooled into LB broth containing ampicillin. The amplification was carried out by inoculating the culture into fresh LB broth containing ampicillin to an absorbance of 0.1 followed by the incubation at 37°C with shaking for 12-15 h. Library DNA was prepared by plasmid isolation kit (QIAGEN), and the concentration of library DNA was measured by using UV spectrophotometer.

2.2.10: Isolation of Plasmids from yeast
Selected yeast transformants were inoculated in 3 ml of selection medium and the cultures were incubated at 30°C with shaking for 18-20 hr. After the incubation, the cells were harvested at 8,500 rpm for 5 min at room temperature and the pellets were suspended in 200 μl of STES lysis solution. Equal amounts of sterile glass beads (0.45-mm diameter) were added and the cell suspensions were vortexed vigorously for 1 min at room temperature. The lysed suspensions were the treated with phenol-chloroform adding 200 μl of phenol-chloroform solution and the vortexing for 1-2 min at room temperature. The lysates were spun down at 12,000 rpm for 5 min at RT and the aqueous phase was collected in a fresh microfuge tube. This lysate was further extracted with phenol-chloroform mixture and the precipitated with double amount of absolute ethanol. After centrifugation at 15,000 rpm for 15 min at 4°C, the pellets were washed with ice-cold 70% ethanol and air-dried. Each dried pellet was suspended in 10 μl of sterile water. This DNA was then electro-transformed in E.coli. Transformants were then used for making plasmid DNA.

2.2.11: Tetrad dissection and Analysis
Few of the strains described in this study were constructed through tetrad dissection as described (Kaiser et al., 1994). Briefly, two yeast strains of opposite mating type were freshly grown on YPD medium and were mixed with each other in a drop of sterile water on YPD plate. The YPD plate was incubated at 30°C for 1-2 days. To select the diploid, a pickful of
the mating mix was streaked on minimal medium plate containing appropriate selection markers. The plates were incubated at 30°C for 2-3 days till distinct colonies were seen. These were then grown on YPD for 2-3 days and were then patched on pre-sporulation medium. After 24 h of incubation the cells were patched on sporulation media. After 3-4 days of growth on sporulation plates, the ascus formation was examined under microscope. Once sufficient numbers of tetrads were detected, the sporulating culture was treated with 50µl of zymolase solution (0.5 mg/ml in 1M sorbitol) at 30°C for 10 min. After treatment, 0.8 ml of sterile water was added slowly to the tube taking care not to disturb the four-spore clusters. 10 µl of the zymolyase treated cell suspension was spread onto YPD plate in single streak and tetrads were dissected under tetrad dissection microscope. The growth of the dissected spore was checked after 2-3 days of incubation at 30°C.

In absence of any selection marker, the diploid strain was constructed by monitoring the zygote formation between the cells of the opposite mating type under the tetrad microscope as described (Kaiser et al., 1994).

2.2.12: Pigment estimation

The red pigment formed in ade2 mutants was quantitated spectrophotometrically. Cells were grown on 0.5% YPD to an OD$_{600}$ upto 3.0. An equal number of cells were harvested and their extracts were made in 5% sulphosalicylic acid as described earlier. The extent of pigment formed was estimated by measuring the absorbance at 530 nm. As a control, extracts were prepared using the ABC591 (gsh1Δ) cells grown in presence of excess adenine (Chaudhuri et al., 1997).

2.2.13: Growth curve experiments

In growth curve experiments, cultures were grown on YPD overnight and then were reinoculated at starting OD$_{600}$ of 0.1 into fresh YPD containing different concentrations of CdCl$_2$ and their growth were monitored by measuring the OD$_{600}$ at periodic interval. Total growth was determined by measuring OD$_{600}$ after 30 hrs. In growth curve experiments to examine the growth of gsh1Δ mutants of S. cerevisiae and gcs1Δ mutants of S. pombe, cells were grown in minimal medium containing glutathione. Overnight cultures were washed
thoroughly with minimal media lacking glutathione and were then reinoculated in suitable medium.

2.2.14: β-galactosidase reporter assays

β-galactosidase activity was measured in permeabilized cells as described earlier (Guarente, 1983). Yeast cultures were grown overnight either in SD-URA or SD-URA-LEU and reinoculated in fresh medium at initial OD₆₀₀ of 0.1. Cells were allowed to grow at 30°C shaker for 6-7 h. Equal number of cells (10⁷ to 10⁸) were harvested, washed with water and LacZ buffer. To permeabilize the yeast cells, 50μl of chloroform and 20μl of 0.1% SDS was added to the cell suspension and vortexed for 10s. The cell suspension was then placed at 30°C for equilibration for 5 min. β-gal substrate, ONPG (0.7 ml of 2mg/ml stock prepared in LacZ buffer) was added and reaction was allowed to proceed for 15-30 min. The reaction was terminated by adding 0.5 ml. of 1M sodium carbonate solution. The cell debris was separated by a quick spin and the absorbance of yellow coloured supernatant was measured at 420 nm.

β-galactosidase activity was measured by the following equation;

\[
\text{β-gal activity} = \frac{\text{OD}_{420} \times 1000}{\text{OD}_{600} \text{ of assayed culture} \times \text{Volume assayed} \times \text{Time}}
\]

Where \(\text{OD}_{420}\) is the optical density of the product, o-nitrophenol; \(\text{OD}_{600}\), is the optical density of the culture at the time of assay; Volume, is the volume of the culture used in the assay in ml. Time is in minutes and represents the time for which enzymatic reaction was carried out.

2.2.15: Glutathione estimation

Glutathione was estimated by the 5, 5' dithiobis- (2-nitrobenzoic acid) (DTNB) - glutathione reductase coupled assay essential as described earlier (Anderson, 1997). Briefly, Cells were harvested at desired time intervals, washed twice with water, resuspended in 0.4 ml of 5% sulhosalicylic acid, mixed with equal volume of glass beads, and broken by vigorous
bead beating in a bead beater (Biospec) at 4°C for a total of 6 min with intermittent pauses. The extracts were spun down in a microfuge to remove the glass beads and also the cell debris and protein precipitate. The supernatant was assayed to determine the amount of glutathione, by comparing it with standard curve prepared with known glutathione concentrations.

2.2.16: Yeast RNA isolation and RT-PCR

Yeast RNA was isolated by a modified hot-phenol method (Schmitt et al., 1990). Cells were washed in DEPC-treated water and then suspended in 400 µl of AE buffer (50 mM NaOAc and 10 mM EDTA). 40 µl of 10% SDS was added, after mixing equal volume an equal volume of phenol (450 µl) equilibrated in AE buffer was added and after vortexing, further incubated at 65°C for 4 min. The samples were chilled on ice and incubated until the phenol crystals appeared (2-4 hrs). Samples were centrifuged, supernatant was transferred to a fresh tube and an equal volume of phenol/chloroform was added. After mixing and incubation at room temperature for 5 minutes, samples were centrifuged and the aqueous layer was transferred to a fresh tube. To this 40 µl of 0.3M NaOAc and 2.5 volume of ethanol were added and further incubated at -20°C for 30 min to 1 hr. The RNA pellet was collected by centrifugation and washed with 70% ethanol. RNA was air dried and dissolved in 100 µl of TE buffer. Concentration of RNA was measured by monitoring absorbance at 260 nm.

2.2.17: Agarose Formaldehyde Gel

Agarose formaldehyde gel was prepared by adding 1.8 gm of agarose in 73.3 ml of distilled water and boiling it in microwave for 2 minutes. Allowed it to cool added 16.7 ml formaldehyde and 10 ml of 10X MAE buffer to it in a hood. Poured the solution into the gel mold and allowed it to solidify.

2.2.18: Preparation of cDNA

cDNA was prepared by adding mRNA as a template in a mixture containing dNTPs, oligo dT, RNAase inhibitor and 10X reverse transcriptase buffer. After addition of Reverse Transcriptase enzyme, the mixture was incubated at 42°C for 1 hr. RT PCR was performed using cDNA as a template.
2.2.19: Transport assays

For the in vitro transport measurements, vacuoles were isolated by the Ficoll floatation technique from cells grown in YPD and vesiculated as described (Li, 1996). The uptake of [3H] 2,4-dinitrophenylglutathione (DNP-GS), [3H] 17 β-estradiol 17-(β-D-glucuronide) (E2 17 βG) or [3H] glutathione (GSH) by yeast vacuolar membrane vesicles was measured at 25°C in 200 μl volumes containing, 3 mM ATP, 3 mM MgSO₄, 5 μM gramicidin-D, 10 mM creatine phosphate, 16 U/ml creatine phosphate kinase, 50 mM KCl, 1mg/ml BSA, 400 mM sorbitol, 25 mM Tris-Mes buffer (pH 8.0) and the indicated concentrations of radiolabeled transport substrate. In the case of [3H] GSH, oxidation and the formation of [3H] GSSG was minimized by degassing all solutions before the dissolution of GSH and by purging the freshly prepared GSH stock solutions and uptake media with nitrogen gas immediately before use. In all cases, uptake was initiated by the addition of membrane vesicles and terminated by the addition of 1-ml ice-cold wash medium (400-mM sorbitol in 3 mM Tris-Mes buffer, pH 8.0) and vacuum filtration of the suspension through prewetted Millipore Durapore (GVWP) filters (pore size 0.22 μm). The filters were rinsed twice with wash medium and the radioactivity retained was determined by liquid scintillation counting. Non-energized (MgATP-independent) uptake was estimated by the same procedure except that ATP was omitted from the uptake medium.

2.2.20: Computational analysis

Different computer programs were used to analyze the sequence homology and phylogenetic relation between different genes and proteins. To identify which of the yeast, human and A.thaliana MRP proteins were best-hits to each other, each of these proteins was first subjected to a BLAST analysis using the NCBI BLAST program (Altschul et al., 1997) to pull out the best hits in the three organisms. The phylogenetic analysis (Nearest neighbour joining method) was carried out after multiple sequence alignment of the sequences using the 'CLUSTAL X' program (Thompson et al., 1999) and 'CLUSTAL W' program (Thompson et al., 1994) using the default parameters. The phylogenetic trees were drawn using the 'TreeView' (Page, 1996). Hydropathy plot analysis was carried out using the method of Kyte and Doolittle (Kyte and Doolittle, 1982) with default parameters.
2.3: Construction of strains and plasmids

2.3.1: Construction of plasmids

(pABE 853): This plasmid was constructed by cloning a purified PCR product containing 448 bp upstream of the BPTI ORF into the Smal site of pBluescript (Stratagene, La Jolla, Calif.). The PCR product was amplified by using primers YLL15-BAM and YLL15-FUSE.

P\textsubscript{BPTI}-lacZ (pABE 851): This plasmid was constructed by subcloning the BPTI promoter region of 448 bp (From ABE 853) into Xhol-BamHI site of pLG699-Z (2\mu m URA3 lacZ) vector.

P\textsubscript{BPTI}-lacZ -300bp (pABE 1052): This plasmid was constructed by subcloning the BPTI promoter region of 300 bp (From ABE 851) into Xhol-BamHI site of pLG699-Z (2\mu m URA3 lacZ) vector. The PCR product was amplified by using the primers YLL15-300 and YLL15-FUSE.

P\textsubscript{BPTI}-lacZ -200bp (pABE 1053): This plasmid was constructed by subcloning the BPTI promoter region of 200 bp (From ABE 851) into Xhol-BamHI site of pLG699-Z (2\mu m URA3 lacZ) vector. The PCR product was amplified by using the primers YLL15-200 and YLL15-FUSE.

P\textsubscript{BPTI}-lacZ -150bp (pABE 1054): This plasmid was constructed by subcloning the BPTI promoter region of 150 bp (from ABE 851) into Xhol-BamHI site of pLG699-Z (2\mu m URA3 lacZ) vector. The PCR product was amplified by using the primers YLL15-150 and YLL15-FUSE.

P\textsubscript{YCFI}-lacZ (pABE 852): This plasmid was constructed by cloning a purified PCR product containing 418 bp upstream of the YCFI ORF into Xhol-BamHI site of pLG699-Z (2\mu m
URA3 lacZ) vector. The PCR product was amplified by using the primers YCF1-5-XHO1 and YCF1-FUSE.

\( P_{BAT1}-lacZ \) (pABE 1055): To construct YLL048c promoter-reporter fusion plasmid, 448 bases upstream of YLL048c ORF were amplified using primers YLL48-Bam and YLL48-Fuse and Amplified PCR products were digested with *BamH1* and *Xho1*. Digested product was ligated at BamH1-Xho1 site of *pLG 699Z* vector. The primers were designed to enable in-frame fusions with *LacZ* gene in vector.

\( P_{YHL035C}-lacZ \) (pABE 1056): To construct YHL035c promoter-reporter fusion plasmid, 456 bases upstream of YHL035c ORF were amplified using primers YHL35-XH and YHL35-Fuse and Amplified PCR products were digested with *BamH1* and *Xho1*. Digested product was ligated at BamH1-Xho1 site of *pLG 699Z* vector. The primers were designed to enable in-frame fusions with *LacZ* gene in vector.

\( pTEF-TRX1 \) (pABE 855): TRX1 gene (300 bp) of *S. cerevisiae* was cloned into *BamHI-Xho1* site of *TEF-416* plasmid. The PCR product was amplified by using the primers TRX1FP and TRX1RP.

\( pGPD-TRX1 \) (pABE 934): TRX1 was excised out from ABE 855 by *BamHI-Xho1* digestion and cloned in *BamHI-Xho1* site of *GPD-426* plasmid.

pABE 953: TRX1 gene was excised out from ABE 855 by *BamHI-Xho1* digestion and end-filled by using klenow DNA polymerase. The end-filled fragment containing TRX1 gene was cloned in *Sma1* site of *S. pombe* expression vector *pART1-114* under *ADH1* promoter.

pYEp-SKN7 (pABE 1062): YEpl3 genomic library plasmid vector containing *SKN7* gene. This plasmid was pulled from out from a genomic library screen for isolating the genes which could complement for pigmentation defect of *ycf1Δbpt1Δ* strain.
pYEp-YCF1 (ABE 1165): YEp13 genomic library plasmid vector containing YCF1 gene. This plasmid was pulled from out from a genomic library screen for isolating the genes which could complement for pigmentation defect of ycf1Δbpt1Δ strain.

pABE 1166: YEp13 genomic library plasmid vector containing several genes like, YCK1, carboxypeptidase, YHR133c, and YHR134c. This plasmid was pulled from out from a genomic library screen for isolating the genes which could complement for pigmentation defect of ycf1Δbpt1Δ strain.

pYEp-YCK1 (ABE 1208): ABE 1166 was partially digested twice with Scal and religated, selecting for plasmids which had not lost Scal sites within the genomic library fragment. Scal fragment was then removed from selected plasmids by digesting with Scal. The plasmid backbone containing only YCK1 gene was then religated.

pABE 935: A DNA fragment of 2.8 kb obtained by PstI digestion of ABE 521 (P47c 7-2) was cloned into PstI site of pSP1 vector.

pABE 946: An EcoRI fragment of size 1.8 kb was excised out from ABE 773 (BamHI-BglII drop out) and cloned into EcoRI site of pSP2 vector.

pABE 947: A DNA fragment of 3.8 kb obtained by EcoRI digestion of ABE 521 (P47c 7-2) was cloned into EcoRI site of pSP2 vector.

pABE 951: A DNA fragment of 3 kb excised from SphI digestion of ABE 521 (P47c 7-2) was end-filled with T4 DNA polymerase and subsequently cut with BglII enzyme. This fragment was then cloned in pSP2 vector into BamHI and SmaI site. pSP2 vector was cut with BamHI and SmaI and after CIP treatment, it was used for ligation.

pABE 955: A DNA fragment of 3 kb was excised out from SphI-BamHI digestion of ABE 521(P47c 7-2) and cloned into S. pombe vector pIRT2 (ABE115) cut with SphI-BglIII.
pABE 967: A DNA fragment of 2.5 kb was excised from ABE 951 by Scal-SpeI double digestion and was cloned into Smal-SpeI site of pSP2 vector.

pABE 970: A 2.11kb EcoRI DNA fragment was excised out from pABE 967. The fragment was isolated by partial EcoRI digestion of pABE 967, followed by SpeI digestion. This fragment was then cloned into the EcoRI-SpeI site of pSP2 vector.

pABE 971: A 1.7kb EcoRI DNA fragment was excised out from ABE 967. The fragment was isolated by partial EcoRI digestion of ABE 967, followed by SpeI digestion. This fragment was then cloned into EcoRI-SpeI site of pSP2 vector.

pABE 1203: *S. pombe* ura4+ cassette (1.8 kb) was inserted at HindIII site (position 1626) of apd6+ gene (2.1 kb) cloned in pSP2 vector (see ABE 970). HindIII site at position 2240 was killed by partial digestion and end filling. The resulting plasmid was digested with HindIII and *S. pombe* ura4+ cassette (1.8 kb) was ligated into it. *S. pombe* ura4+ cassette was excised by HindIII digestion of plasmid pABE 137 (*S. pombe* ura4+ gene cloned in pBSK).

pABE 1204: *S. pombe* ura4+ cassette (1.8 kb) was inserted at HindIII site (position 2240) of apd6+ gene (2.1 kb) cloned in pSP2 vector (see pABE 970). HindIII site at position 1626 was killed by partial digestion and end filling. The resulting plasmid was digested with HindIII and *S. pombe* ura4+ cassette (1.8 kb) was ligated into it. *S. pombe* ura4+ cassette was excised by HindIII digestion of plasmid pABE 137 (*S. pombe* ura4+ gene cloned in pBSK).

### 2.3.2: Construction of strains

*bpt1Δ* (ABCDE 791): *BPT1* gene was disrupted in ABC 154 (WT) strain from codon 428 to 736 by PCR-mediated gene disruption using the KanMX2 module [Wach, 1994] using the primers Y015-DELL1 and Y015-DELL2. Disruptions were made in both the MATa and MATa parental strains ABC154 and ABC579 yielding ABC791 and ABC790 respectively. The double mutant ABC794 (*ycf1Δ: KanMX2 bpt1Δ: KanMX2*) was constructed by dissecting
tetrads derived from a diploid generated by a cross between ABC470 and ABC790. Due to absence of any selective marker for diploid, diploids were selected by observing formation of zygotic canal and zygotic bud under tetrad dissecting microscope.

\[bat1\Delta (ABC 1063):\] BATI gene was disrupted in ABC 579 (WT) strain from codon 1499 to 2741 by PCR-mediated gene disruption using the \textit{KanMX2} module [Wach, 1994] using the primers Yll48 Del1 and Yll48 Del 2.

\[bat1\Delta (ABC 1145):\] BATI was disrupted in ABC 154 (WT) strain using the \textit{URA3} marker. \textit{URA3} cassette was amplified from plasmid ABE 150 (\textit{psP2}) by using the primers Yl48-URA D1 and Yl48-URA D2. Disruptions were confirmed by PCR. \textit{URA3} cassette was also integrated in ABC 154 (WT) to make a URA+ strain (ABC 1144). This strain was used as an isogenic control for \textit{bat1\Delta} (ABC 1145) strain.

\[bat1\Delta ycf1\Delta (ABC 1147):\] BATI was disrupted in ABC 470 (ycf1\Delta) strain using the \textit{URA3} marker. \textit{URA3} cassette was amplified from plasmid ABE 150 (\textit{psP2}) by using the primers Yl48-URA D1 and Yl48-URA D2. Disruptions were confirmed by PCR. \textit{URA3} cassette was also integrated in ABC 470 (ycf1\Delta) to make a URA+ strain (ABC 1146). This strain was used as an isogenic control for \textit{bat1\Delta ycf1\Delta bpt1}\Delta (ABC 1149) strain.

\[bat1\Delta ycf1\Delta bpt1\Delta (ABC 1149):\] BATI was disrupted in ABC 794 (ycf1\Delta bpt1\Delta) strain using the \textit{URA3} marker. \textit{URA3} cassette was amplified from plasmid ABE 150 (\textit{psP2}) by using the primers Yl48-URA D1 and Yl48-URA D2. Disruptions were confirmed by PCR. \textit{URA3} cassette was also integrated in ABC 794 (ycf1\Delta bpt1\Delta) to make a URA+ strain (ABC 1148). This strain was used as an isogenic control for \textit{bat1\Delta ycf1\Delta bpt1}\Delta (ABC 1149) strain.
**yhlo35cΔ (ABC 1064):** YHL035c gene was also disrupted in ABC 579 (WT) strain from codons 1704 to 3040 by PCR-mediated gene disruption using primers Yhl35 Del1 and Yhl35 Del2.

**yhlo35cΔycflΔ (ABC 1065):** The double mutant ycflΔyhol035cΔ was constructed by dissecting tetrads derived from a diploid generated by a cross between ABC 470 (ycflΔ) and ABC 1064 (yhlo35cΔ). All four spores were found to be viable. Disruption was confirmed by PCR.

**skn7Δ (ABC 1037):** The Skn7 gene was disrupted in ABC 154 (WT) strain by transforming a linearized fragment obtained by restriction digestion of ABE 1031 (Skn7 :: TRP) with SacI (Brown et al., 1993). Disruptions were confirmed by PCR.

**skn7Δ bpt1Δ (ABC 1038):** The Skn7 gene was disrupted in ABC 791 (bpt1Δ) strain by transforming a linearized fragment obtained by restriction digestion of ABE 1031 (Skn7 :: TRP) with SacI. Disruptions were confirmed by PCR.

**skn7Δ ycflΔ (ABC 1039):** The Skn7 gene was disrupted in ABC 470 (ycflΔ) strain by transforming a linearized fragment obtained by restriction digestion of ABE 1031 (Skn7 :: TRP) with SacI. Disruptions were confirmed by PCR.

**skn7Δ ycflΔbpt1Δ (ABC 1040):** The Skn7 gene was disrupted in ABC 794 (ycflΔbpt1Δ) strain by transforming a linearized fragment obtained by restriction digestion of ABE 1031 (Skn7 :: TRP) with SacI. Disruptions were confirmed by PCR.

**vph1ΔycflΔbpt1Δ (ABC 1153):** The VPH1 gene was disrupted in ABC 794 (ycflΔbpt1Δ) background by transforming a linear fragment containing LEU2 cassette obtained by restriction digestion of ABE 482 (pMM219) plasmid by BamHI-Apal and disruptions were checked by PCR.
vph1Δycf1Δbpt1Δbat1Δ (ABC 1155): The VPH1 gene was disrupted in ABC 1149(ycf1Δbpt1Δbat1Δ) background by transforming a linear fragment containing LEU2 cassette obtained by restriction digestion of ABE 482 (pMM219) plasmid by BamHI-ApaI and disruptions checked by PCR.

YPH499-GV8GHS1 (ABC 936): This strain contain an integrated copy of the GSH1 gene expressed downstream of the TEF promoter and it was constructed by transforming linearized DNA fragment obtained by digesting pGV8GSH1 plasmid with StuI. pGV8GSH1 contained the yeast GSH1 gene, which was amplified by PCR and cloned into the Hind III -BspDI sites downstream of the strong GPD promoter in a pRS306 derived integrating vector. Transformants were selected on SD-URA plates. Overproduction of glutathione was checked by glutathione-reductase assay.

apd6Δ::ura4+ (ABP 1220): S. pombe ura4+ cassette was used to disrupt the apd6+ gene by homologous recombination. S. pombe ura4+ cassette flanked by homologous regions of apd6+ gene was excised out from ABE1203 plasmid by EcoRI-PstI double digestion. The resulting fragment of 2.8 kb was transformed in WT S. pombe strain to give rise to ABP1219 strain. Similarly 2.8 kb DNA fragment containing S. pombe ura4+ cassette was excised from pABE1204 plasmid EcoRI-PstI double digestion. The resulting fragment of 2.8 kb was transformed in WT S. pombe strain to give rise to ABP1220 strain.

Table 2: List Of Oligonucleotides used in this study:

1) YLL48 DEL1: 5'- CAAgCACAggCACTATTCTTTggAAggAgAgTTTgTATCAgCTgAAgCTTCgTACgC-3'

2) YLL48 DEL 2: 5'- AACAATAAgCgATAgTgTgTTCCATT CgCATCTACATAgCCACTAgTggATCTg-3'
3) YLL48 FOR: 5'-gACCATAAaggTTAAAAaggAAgCg-3' 

4) YLL48 REV: 5'-ggTCACCggCTTTCAAgATCTC-3' 

5) YLL48 FUSE: 5'-TTgAggATCCATgCATgTTCTCCgTAgtTTTTCC-3' 

6) YLL48 XH: 5'- gTCACTCgAgAAAATATCACAACCAATAgTTAgCCA-g-3' 

7) YL48-URAD1: 5'- gTTAAAggATATTTgaggATATgATgCAAgA TTATTTCTCCACAgCTTTTCAATTCAA-3' 

8) YL48-URAD2: 5'-AAgCgTCAATAgCCATgAgATTAATAATCgCTC CAAggTAATAAACTgATATAATTAATTTg-3' 

9) YL48U-F: 5'-TggCCTTgTTTATCTCTATgATTTT-3' 

10) YL48U-R: 5'-CCAAAaggAAAgAACTgATAgACC-3' 

11) YHL35 DEL1: 5'-TgTTgTAagACTACTTTTTCAACTTTTT AggTgTTTCAgCTgAAgCTTCgTACgC-3' 

12) YHL35 DEL2: 5'-AgAAgTTATCAAAAgATAATATTgTTTTTTCAgCcTgTCATTTCAgCCACTAgTggATC-3' 

13) YHL35 FUSE: 5'- gATCggATCCCATTTCTCCAggTAATgAAACCATTg-3' 

14) YHL35 XH: 5'- CgAACTCgAgCAAACACTgAgTTTCTgAAgAAAAG-3' 

15) YHL35 FOR: 5'-gACgCTAgCACTgTTTTgAATTTTTTT-3'
| 16) | YHL35 REV: 5’- ggAAATTCTCTgTTTCTgCCCTCC-3' |
| 17) | Skn7 FOR: 5’- AggAAATgACTTgTTgCAgAgCg-3' |
| 18) | Skn7 REV: 5’- gTTTTCTTgAAgTgTAgATTg-3' |
| 19) | p47-1021F : 5’- CTACTgATTAACCTTAATgT-3' |
| 20) | p47-1892F: 5’- CTggAAgTCCTTCATAAgCCC-3' |
| 21) | p47-1920R : 5’- gTTCTATTgTgtgTTgAAG-3' |
| 22) | p47-2327F : 5’- gCATgAAACAACCACATCgTACA-3' |
| 23) | p47-3164R : 5’- CgTTATTAgATCATTATAgAT-3' |
| 24) | YCF-FP: 5’- TgggCCTgCAgACTCTgTAAC-3' |
| 25) | YCF-RP2: 5’- CACCggTAgAgAAATgTCCAg-3' |
| 26) | YCF1-5-XHO1: 5’- TATTCCTgCAgAACgTTCCACCTCgAggAgAAATg-3' |
| 27) | YCF1-FUSE: 5’- AggATTTgATCCCATTTTTCTTgTTTTACgTAATCCCATTTTCTTgTTTTACgTAAT-3' |
| 28) | YCFDEL1: 5’- TgggCCTgCAAGCTCTgTAATCCTCTCgA  
| | AgggTTTgACAATAgCTgAAgCCCTCgTACg-3' |
| 29) | YCF1DEL2: 5’- TCCTgTCTCTCACTgTTATAgTCTgTAACgA  
| | ACTTTgATTgCAgCCACTAgTggATC-3' |
| 30) | YO15DELL1: 5’- ATTggCgCTCCTATTCAAgATTATTgTTgTATT |
AACTTCCAgCTgAAgCTTCgTACgC-3'

31) YO15DELL2: 5’-ATTgACATgCTTTAATAgTgAggTCATAAT
            AATCTTggTCATAggCCACTAgTggAT-3’

32) YO15FOR: 5’-CgTAATCAgCgTggTTTCTgTg-3’

33) YO15REV: 5’-CTAACTTTCTgCATCAACAgCAG-3’

34) YLL15-BAM: 5’-gAAgggATCCTCTTATATTTAATCTTTTTTTAgAC-3’

35) YLL15-FUSE: 5’-ACTTCggATCCAgACATTgTCTTTATATTTAATCTTT-3’

36) YL15-300: 5’-CTATCTCgAgATgTTgATCgTATTgTTTgCCTAg-3’

37) YL15-200: 5’-TTggCTCgAgCgACATCgCgATAATTCCAATTCCC-3’

38) YL15-150: 5’-AgTTACTCgAgCCAggTTCTCCAAAATTAAAATCgCA-3’

39) VPH1FP: 5’-gTgACTTgAACTCTAaggTgCg-3’

40) VPH1RP: 5’-CAgTCgATggCAgTggTTCg-3’

41) TRX1FP: 5’-TCgATAggATCCATggTTACTCAATTCAAAAAACTgCC-3’

42) TRX1RP: 5’-TCATTgTCgAgTTTTAACgATTTAgCAgCAATggC-3’
Table 2.1: Strains used in this study

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TEF-GSH1
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**Table 2.2: Plasmids used in this study**

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