# THE LOCALIZATION, FUNCTION AND APPLICATIONS OF THE STRESS RESPONSE PROTEIN HSP12P IN THE YEAST *SACCHAROMYCES CEREVISIAE*.

**by**

**Robert Jan Karreman**



**A thesis submitted for the degree of**

**Doctor of Philosophy**

**in the Department of Molecular and Cell Biology, Faculty of Science**
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# PREFACE

The work presented in this thesis was performed under the supervision of Associate Professors George G. Lindsey and Wolf F. Brandt at the Department of Molecular and Cell Biology, University of Cape Town, South Africa. I hereby declare that this thesis, submitted for the degree of Doctor of Philosophy in Molecular and Cell Biology at the University of Cape Town, is the result of my own investigation, except where the work of others is acknowledged.

Robert Jan Karreman
University of Cape Town
March 2006

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## ABSTRACT

Since 1990, the yeast *Saccharomyces cerevisiae* small heat shock protein Hsp12p, has continuously appeared in data associated with stress responses in this organism. Hsp12p is expressed abundantly in response to a large variety of different stresses, but for many years has eluded researchers as to its function, primarily because the viability of yeast strains lacking *HSP12* are unaffected by osmotic stress and heat shock. Subsequent studies indicated that Hsp12p played a role in the adaptation of the cell wall of *Saccharomyces cerevisiae* to conditions of stress. However, the exact *in vivo* localization, specific function and mediation of function of Hsp12p had yet to be elucidated.

The localization of Hsp12p was determined by fusion to the green fluorescent reporter protein, Gfp2p and a combination of epifluorescent microscopy and confocal imagery. Chemical extraction revealed that Hsp12p was present in the cell wall while fluorescent imagery was not conclusive. This fluorescent Hsp12p construct was later employed in a novel application to sense the stress status of yeast, which bears future promise for use in an industrial setting.

The localization of Hsp12p in the cell wall and its massive induction upon stress prompted the investigation into stress factors affecting cell wall status. Yeast cells were exposed to the cell wall damaging agent, Congo Red (CR) and the viability, morphology and dimorphic (invasive) growth of yeast cells lacking *HSP12*

investigated. Hsp12p was found to play an essential role in the viability of yeast cells exposed to CR and in addition, was required for invasive growth in the presence of CR. Since these phenotypes were very similar to those observed in yeast cells lacking another class of proteins, the PIR (proteins with inverted repeats) proteins, levels of these proteins were compared in the wildtype and *HSP12*-deficient yeast strains, but no differences were observed. The method by which Hsp12p mediated its function was investigated by *in vitro* studies using chitin and CR, which revealed that Hsp12p bound this polymer and prevented CR binding. The role of Hsp12p in invasive growth was determined by assessing the regulation of proteins involved in this process (flocculins) using Realtime PCR. These studies revealed that Hsp12p did not affect flocculin regulation and thus acted through a different mechanism.

Throughout these experiments, the *HSP12*-deficient strain showed an increase in flocculation ability. The nature of this effect was determined by hydrophobicity studies using octane partitioning, but these studies indicated that Hsp12p did not affect cell hydrophobicity and thus flocculation occurred through a different mechanism.

These findings implied that Hsp12p acted directly on the yeast cell wall and did not affect other proteins in this locus. Thus the direct effects of Hsp12p on the cell wall were investigated. These studies included the use of agarose as a model system to simulate the yeast cell wall, which showed that Hsp12p increased cell wall flexibility. Further studies using atomic force microscopy confirmed that Hsp12p increased the flexibility of the cell walls of live yeast cells. Studies of cell electrophoretic mobility revealed that cells lacking Hsp12p had stiffer cell walls. Finally, cell wall chemistry was assayed with infrared spectroscopy. Decreased phosphorylation of peptidomannan residues and a lower β-1,3-glucan content was observed in Hsp12p-deficient cells, possibly due to differential regulation of calcium ion flux. The decrease in cell wall polysaccharides was proposed to result in a compensatory increase in the amount of chitin in the cell wall. This increase correlated with the flocculation and CR-sensitive phenotype displayed by cells lacking Hsp12p, since chitin levels must be controlled for optimal cell separation and chitin is the target for CR binding.

# ABBREVIATIONS

| | |
|---|---|
| α | alpha |
| β | beta |
| $\delta$ | delta |
| Δ | delta, indicates gene mutations |
| λ | lambda |
| ρ | rho |
| ∑ | sigma |
| μ | micro, indicates $10^{-6}$ |
| | |
| °C | degrees Celcius |
| μg | microgram ($10^{-6}$ g) |
| μl | microliter ($10^{-3}$ ml) |
| | |
| 3-D | three dimensional |
| | |
| A | adenine |
| AFM | atomic force microscopy |
| *A. nidulans* | *Aspergillus nidulans* |
| ARE | AP1 response element |
| ATP | adenosine triphosphate |
| ATR | attenuated total reflectance |
| | |
| BCIP | 5-bromo-4-chloro-3-indolyl phosphate |
| BLAST | Basic Local Alignment Search Tool |
| bp | base pair(s) |
| BSA | bovine serum albumin |
| | |
| C | cytosine |
| $Ca^{2+}$ | calcium ion |
| *C. albicans* | *Candida albicans* |
| cAMP | cyclic adenosine monophosphate |
| cDNA | complementary DNA |
| $CH_3CN$ | acetonitrile |
| CHCA | α-cyano-4-hydroxy cinnamic acid |
| CIS | cell integrity stress |
| cm | centimeter |
| CR | Congo Red |
| CSM | complete synthetic growth medium |
| cw | cell wall |
| | |
| Da | dalton ($g.mol^{-1}$) |
| DNA | deoxyribonucleic acid |
| dNTP | deoxyribonucleotide triphosphate |
| DTT | dithiothreitol |
| | |
| *E. coli* | *Escherichia coli* |
| EDTA | ethylenediaminetetra-acetic acid |

| | |
|---|---|
| ELISA | enzyme-linked immunosorbance assay |
| EtBr | ethidium bromide |
| FTIR | Fourier Transform Infrared |
| G | guanine |
| g | gram |
| *g* | standard gravitational acceleration (9.8 $m.s^{-2}$) |
| GFP | green fluorescent protein |
| GSR | general stress response |
| h | hours |
| HIS | histidine |
| HOG | high osmolarity glycerol |
| Hsp | heat shock protein |
| HSE | heat shock element |
| HSR | heat shock response |
| IPTG | isopropyl-β-D-thiogalactopyranoside |
| IR | infrared |
| *k* | spring constant |
| KCl | potassium chloride |
| kb | kilobase pairs ($10^3$ bp) |
| KBr | potassium bromide |
| kDa | kilodalton ($10^3$ Da) |
| kN | kilonewton ($10^3$ N) |
| $KNO_3$ | potassium nitrate |
| KO | *hsp12ΔURA3 S. cerevisiae* strain |
| l | liter |
| LB | Luria-Bertani growth medium |
| LEA | late embryogenesis-abundant |
| m | meter |
| M | molar ($mol.l^{-1}$) |
| m/z | mass per charge |
| mA | milliampere ($10^{-3}$ A) |
| MALDI-TOF | matrix assisted laser desorption/ionisation time of flight |
| MAP | mitogen-activated protein |
| MCS | multiple cloning site |
| mg | milligram ($10^{-3}$ g) |
| $MgCl_2$ | magnesium chloride |
| min | minutes |
| ml | milliliter ($10^{-3}$ l) |
| mm | millimeter ($10^{-3}$ m) |
| mM | millimolar ($10^{-3}$ M) |
| MPa | megapascal ($10^3$ Pa) |
| mRNA | messenger RNA |
| MW | molecular weight in daltons |

| | |
|---|---|
| n | number (of) |
| NaCl | sodium chloride |
| NaOH | sodium hydroxide |
| $(NH_4)_2SO_4$ | ammonium sulphate |
| $NH_4HCO_3$ | ammonium hydrogen carbonate |
| NBT | nitro blue tetrazolium |
| NCBI | National Centre for Biotechnology Information |
| ng | nanogram ($10^{-9}$ g) |
| nm | nanometer ($10^{-9}$ m) |
| | |
| $OD_{600}$ | optical density at 600 nm |
| ODU/ml | optical density units at 600 nm / ml |
| oligo dT | oligonucleotide deoxythymine |
| ORF | open reading frame |
| | |
| Pa | pascal (pressure) |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PDS | post-diauxic shift |
| PEI | polyethyleneimine |
| PKA | protein kinase A |
| PKC | protein kinase C |
| pmol | picomole ($10^{-12}$ mol) |
| | |
| RNA | ribonucleic acid |
| RNAse | ribonuclease |
| ROS | reactive oxygen species |
| RSA | Republic of South Africa |
| RT | room temperature (~23°C) |
| RTPCR | realtime polymerase chain reaction |
| | |
| s | seconds |
| SAB | sample application buffer |
| *S. cerevisiae* | *Saccharomyces cerevisiae* |
| SDS | sodium dodecyl sulphate |
| SDS-PAGE | sodium dodecyl sulphate-polyacrylamide gel electrophoresis |
| smHsp | small heat shock protein |
| *S. pombe* | *Schizosaccharomyces pombe* |
| STRE | stress response promoter element |
| | |
| T | thymine |
| Taq | *Thermophilus aquaticus* |
| TBE | Tris-borate-EDTA |
| TE | Tris-EDTA |
| TEMED | N,N,N',N'-tetramethylethylene-diamine |
| temp. | temperature |
| $T_g$ | gelation temperature |
| $T_m$ | melting temperature |
| Tris | 2-amino-2-(hydroxylmethyl)-1,3-propanediol |

| | |
|---|---|
| Triton X-100 | 4-octylphenol polyethoxylate |
| Tween | polyoxyethylenesorbitan monolaurate |
| U | unit of enzyme activity |
| UK | United Kingdom |
| URA | uracil |
| USA | United States of America |
| V | volt |
| v/v | volume / volume (1 ml / 100 ml) |
| w/v | weight / volume (1g / 100 ml) |
| WT | wildtype *S. cerevisiae* strain |
| X-gal | 5-bromo-4-chloro-3-indoyl-β-D-galactoside |
| YEPD | yeast extract peptone dextrose (glucose) growth medium |
| YNB-AA | yeast nitrogen base lacking amino acids |

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# CHAPTER 1

# GENERAL INTRODUCTION

## 1.1. GENERAL INTRODUCTION

### 1.1.1. Yeast stress responses

Studies of stress responses in the budding yeast *Saccharomyces cerevisiae* have been of particular interest to many researchers, since this yeast represents a genetically tractable eukaryotic model organism. These studies have application in the optimisation of fermentation processes in industry and also provide a model of stress responses in higher eukaryotes.

Yeast cells possess a series of stress signalling mechanisms to relay signals from sensing components (usually at the cell surface) to transcription factors within the nucleus culminating in the repression, derepression or induction of target genes. Proteins synthesised in response fulfil various functions such as the balancing of osmotic potential, the efflux of toxic substances, the alteration of morphology and other adaptations to the stress. Most signalling mechanisms employ Mitogen-Activated-Protein (MAP) Kinases which mediate a series of phosphorylation events to relay stress signals (Posas *et al.*, 1998). These proteins function by phosphorylating the next MAP Kinase downstream, so that the cascade is usually of the form where a MAP Kinase Kinase Kinase (MAPKKK) phosphorylates a MAP Kinase Kinase (MAPKK) which in turn phosphorylates a MAP Kinase (MAPK). The MAPK then phosphorylates a variety of transcription factors. In this way, a relatively small signal from a sensor is amplified and converted to a sensitive switching response dependent on the threshold magnitude of the original stress (Ferrell, 1996; Huang and Ferrell, 1996). Other stress signalling mechanisms include the Target of Rapamycin (Tor) pathway (Thomas and Hall, 1997; Dennis *et al.*, 1999) and direct sensing mechanisms, where stressful conditions directly affect the conformation and/or localization of specific transcription factors.

The stress responsive pathways in *S. cerevisiae* can be divided into three major groups: the heat-shock response, the general stress response and the cell integrity pathway. The heat shock response (HSR) pathway is induced upon exposure to sublethal heat shock, alcohol, heavy metals and anoxia (Mager and de Kruijff, 1995). Heat shock factor 1 (Hsf1p) was identified as the effector of the HSR in yeast (Sorger *et al.*, 1988). This homotrimeric protein responds directly (Harrison *et al.*, 1994) and binds promoter sequences known as

heat shock elements (HSEs), comprising three or more contiguous inverted repeats of the 5-base pair sequence nGAAn (Pelham and Bienz, 1982; Xiao *et al.*, 1991; Morimoto *et al.*, 1993; Bonner *et al.*, 1994;). The HSR results in the expression of a variety of heat shock proteins (HSPs) which are divided into 5 groups on the basis of molecular mass and their ability to induce various downstream events. These groups are: Hsp70p, Hsp100p, Hsp90p, Hsp60p, and Small Heat Shock Proteins (smHsps). Heat shock proteins perform a wide variety of protective functions but their general function is to control the accurate folding and translocation of polypeptides in the different cellular compartments under stressful conditions. Hsp104p, a member of the Hsp100p multi-gene family, is responsible for resolubilisation and reactivation of folded aggregated proteins (Sanchez and Lindquist, 1990). Hsp60p mediates the folding of newly synthesized proteins, thus preventing subsequent formation of undesirable and potentially toxic proteins (Craig *et al.*, 1993). Hsp90p interacts with non-native proteins that have already attained a high degree of secondary structure, preventing their aggregation and maintaining them in folding-competent conformations that can complete folding upon the addition of other chaperones (Bose *et al.*, 1996; Freeman and Morimoto, 1996). Hsp70p prevents premature folding of incomplete polypeptides and is crucial for growth processes within the cell. (Craig and Jacobsen, 1984; Wiech *et al.*, 1993; James *et al.*, 1997) The last group, the small Hsps (smHsps), contain a consensus α-crystallin domain (DeJong *et al.*, 1993) and prevent the aggregation and in some cases promote the renaturation of unfolded polypeptides *in vitro* (Horwitz, 1992). *In vivo*, however, their function is largely unknown but it is believed that they may play a protective role (Yeh *et al.*, 1994) and aid in cytoskeleton assembly (Lavoie *et al.*, 1993). Small heat shock proteins are often present as large oligomeric complexes (Chang *et al.*, 1996).

The general stress response (GSR) pathway is induced by a large variety of stresses including heat shock, osmotic stress, alcohol, sorbate, nitrogen starvation, acidic external pH, oxidative stress (Ruis and Schüller, 1995) and nutrient limitation (Thevelein and de Winde, 1999). Signals within this stress response pathway are modulated mainly by the cAMP-protein kinase A (PKA) pathway (For a review, see Igual and Estruch, 2000) which negatively regulates many stress-responsive genes, including some Hsps. When cells are exposed to nutrient-limiting or certain stressful conditions, levels of cAMP decline, PKA activity decreases and repression is relieved. This pathway also affects the localization and phosphorylation state of the major transcriptional regulators of this system, Msn2p and

Msn4p, hereafter referred to as Msn2/4p (Görner *et al.*, 1998). These homologous, functionally redundant transcription factors (Marchler *et al.*, 1993; Ruis and Schüller, 1995; Mager and de Kruijff, 1995; Martinez-Pastor *et al.*, 1996; Siderius and Mager, 1997) dissociate from the 14-3-3 (footnote 1) protein, Bmh2p (Beck and Hall, 1999) and accumulate in the nucleus under stressful conditions (Görner *et al.*, 1998) where they bind sequences known as stress responsive elements (STREs), characterized by the core sequence 5'-AGGGG-3' (Marchler *et al.*, 1993; Martinez-Pastor *et al.*, 1996; Schmitt and McEntee, 1996) in the promoters of many stress-responsive genes.

GSR pathways are varied and can be divided according to the type of stress encountered, but it should be noted that there is often regulatory cross-talk between these pathways due to secondary effects. The first type of stress, oxidative stress, is encountered during normal cellular aerobic respiration or when yeast cells are subjected to hydrogen peroxide or heavy metals. Yeast cells must activate oxidative stress pathways to counter the resultant formation of reactive oxygen species (ROS) (Santoro and Thiele, 1997) in these cases. The main mediator of the oxidative stress pathway, Yap1p, possesses a cysteine-rich domain which serves as a redox state sensor when the cysteines are oxidized (Kuge *et al.*, 1997). It then translocates to the nucleus (Toone and Jones, 1998) and binds AP1-response elements (AREs) characterized by the sequence TGACTCA found in the promoters of many antioxidant enzymes (Risse *et al.*, 1989) Other types of stress, namely heat shock and osmotic stress, are alleviated by the High Osmolarity Glycerol (HOG) pathway, a MAP kinase pathway which is crucial for tolerance to these forms of stress (Brewster *et al.*, 1993). Components of the HOG Pathway include the plasma membrane-localized osmosensors, Sho1p and Sln1p (Maeda *et al.*, 1995) and the crucial effector Hog1p, which activates Msn2/4p (Brewster *et al.*, 1993; Schüller *et al.*, 1994), Mcm1p (Maeda *et al.*, 1994) and other transcription factors. Msn2/4p binds STREs and induces many genes, including *CTT1* (Wieser *et al.*, 1991), *DDR2* (Kobayashi and McEntee, 1993) and *HSP12* (Varela *et al.*, 1995) while Mcm1p stimulates cell cycle control genes via MADS box (footnote 2) type activation (Nurrish *et al.*, 1995). Thus the GSR can be mediated both through a series of derepressive and inductive events as well as by direct sensing.

Footnotes:

1. 14-3-3 proteins are a family of conserved regulatory molecules expressed in eukaryotic cells which bind a variety of functionally diverse signalling proteins, including kinases, phosphatases and transmembrane receptors. These proteins contain a number of known modification domains.
2. "An acronym for the DNA-binding domain of a gene family that is derived from the initials of the founding members MCM1, AGAMOUS, DEFICIENS and SRF, in yeast, *Arabidopsis*, *Antirrhinum* and humans, respectively".

The final pathway, the cell integrity pathway, is a MAP kinase pathway which responds to stresses which directly affect cell wall assembly and integrity (For a review, see Levin, 2005). Such stresses include mechanical forces, hypo-osmotic conditions (Davenport *et al.*, 1995), increased temperature (Kamada *et al.*, 1995), cell wall disrupting agents including Congo Red, caffeine and Calcofluor White (Martin *et al.*, 2000; Garcia *et al.*, 2004) and antifungal agents such as chitinases and glucanases (de Nobel *et al.*, 2000). The protein kinase C (PKC) cell integrity pathway is activated in response to these stresses and comprises the cell wall localized sensors, Wsc1/2/3p (Gray *et al.*, 1997; Verna *et al.*, 1997) and Mid2p (Ketela *et al.*, 1999; Rajavel *et al.*, 1999) which act, via the small GTPases Rho1p, Rom2p and Sac7p (Philip and Levin, 2001), to activate Protein Kinase C (Pkc1p). Pkc1p is the initiator of the MAP kinase cascade acting through Slt2p/Mpk1p. This eventually leads to activation of Swi4/6p, which regulates cell cycle genes (Ogas *et al.*, 1991) and cell wall biosynthesis (Igual *et al.*, 1996), and Rlm1p, involved in a variety of regulatory functions, including cell wall synthesis and assembly (Jung and Levin, 1999). It has been proposed that cell wall stress is also signalled via the common condition exhibited by all stressed cells, namely plasma membrane stretch, since cells exposed to hypo-osmotic stress, which blocks membrane stretch, cannot activate cell wall integrity pathway components (de Nobel *et al.*, 2000). This presumably has an effect on stretch-activated ion channel proteins such as Mid1p (Kanzaki *et al.*, 1999; Kanzaki *et al.*, 2000; Yoshimura *et al.*, 2004) which affect intracellular ion levels, including calcium. Calcium is involved in the activation of calcineurin, a serine/threonine specific protein phosphatase which dephosphorylates and activates transcription factors such as Crz1p/Tcn1p in response to extracellular stress. Moreover, the $Ca^{2+}$/calcineurin and Pkc1p-Slt2p pathways act synergistically to induce Fks2p, a subunit of the cell wall biosynthetic enzyme β-1,3-glucan synthase, in response to cell wall damage (Zhao *et al.*, 1998; Lagorce *et al.*, 2003).

In summary, therefore, yeast cells display many varied patterns of stress response through intricate pathways. These pathways are often closely related and share common functional aspects, ensuring harmony within the stressed yeast cell.

### 1.1.2. Hsp12p

Hsp12p is a small 12kDa stress-response protein present in the budding yeast, *S. cerevisiae*. This protein was discovered almost simultaneously in 1990 by two separate laboratories

(Praekelt and Meacock, 1990; Stone *et al.*, 1990). The protein was discovered largely because of its induction due to either removal of glucose from the medium or to an increase in temperature. The *HSP12* gene was initially named *GLP1* (glucose and lipid regulated protein) and was localized to chromosome VI in the yeast genome (Stone *et al.*, 1990).

Hsp12p is rich in alanine, glycine and charged amino acids such as aspartic acid, glutamic acid and lysine (Figure 1.1 A). It is believed to possess a random coil structure with one putative alpha helix domain (unpublished data). Due to its high hydrophilic amino acid content (Figure 1.1 A, B) and lack of structure (Figure 1.1 C), this protein is water-soluble at 80°C (Mtwisha *et al.*, 1998). These factors have prompted it to be classified as a late embryogenesis abundant (LEA)-like protein. LEA proteins are typically found in plants in conditions of water deficit (Mtwisha *et al.*, 1998). Unlike other small heat shock proteins found in yeasts, such as Hsp26p, Hsp12p does not possess an α-crystallin domain (Praekelt and Meacock, 1990; Stone *et al.*, 1990) and is loosely classified as a general stress response protein.

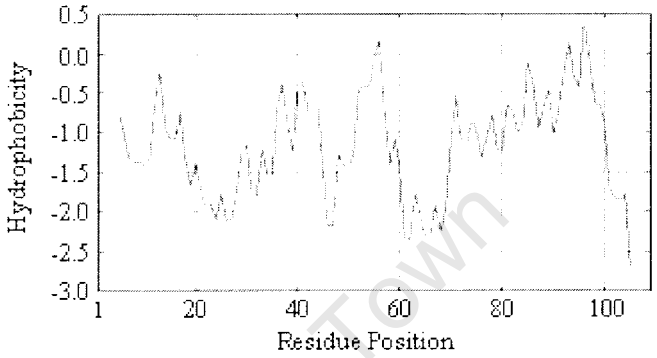
Computer-aided searches of protein databases revealed three proteins in other yeast species that are sequence-related to Hsp12p. These are the colony switching protein Whs11p in *Candida albicans* (47 % identity), the morphogenic transition protein, Awh11p in *Aspergillus nidulans* (41 % identity) and the small heat shock protein Hsp9p in *Schizosaccharomyces pombe* (40 % identity). Whs11p is a cytoplasmic protein active in the spherical, budding growth form but not the elongate hyphal form of *C. albicans* (Srikantha *et al.*, 1997). Similarly, Awh11p, regulated by StuAp, is expressed during the hyphae to budding growth transition in *A. nidulans* (Dutton *et al.*, 1997). Finally, Hsp9p is expressed in *S. pombe* under very similar conditions to Hsp12p in *S. cerevisiae*, such as heat shock, entry into stationary phase and glucose limitation (Jang *et al.*, 1996). Yeast 2-hybrid assays have suggested that Hsp12p interacts with Cpr1p (Ho *et al.*, 2002), a Cyclosporin A binding protein, but the significance of this interaction remains unclear.

A

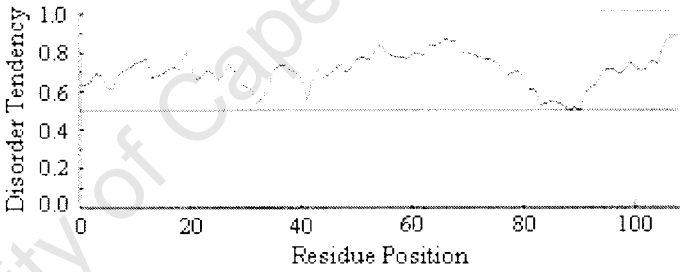
| Name (Symbol) | % mol/ mol | % w/w |
|---|---|---|
| Alanine (A) | 11.9 | 8.5 |
| Arginine (R)* | 2.8 | 3.8 |
| Asparagine (N)* | 2.8 | 2.9 |
| Aspartic acid (D)* | 10.1 | 10.7 |
| Cysteine (C) | 0.0 | 0.0 |
| Glutamic acid (E)* | 10.1 | 11.9 |
| Glutamine (Q)* | 5.5 | 6.4 |
| Glycine (G) | 11.9 | 7.2 |
| Histidine (H)* | 1.8 | 2.3 |
| Isoleucine (I) | 0.9 | 1.0 |
| Leucine (L) | 2.8 | 2.9 |
| Lysine (K)* | 13.8 | 16.1 |
| Methionine (M) | 1.8 | 2.2 |
| Phenylalanine (F) | 1.8 | 2.4 |
| Proline (P) | 2.8 | 2.5 |
| Serine (S) | 7.3 | 6.2 |
| Threonine (T) | 1.8 | 1.7 |
| Tryptophan (W) | 0.0 | 0.0 |
| Tyrosine (Y) | 3.7 | 5.3 |
| Valine (V) | 6.4 | 6.0 |

* Hydrophobicity < 0.3

B




C




D

```
1  MSDAG RKGFG EKASE ALKPD SQKSY AEQGK
31 EYITD KADKV AGKVQ PEDNK GVFQG VHDSA
61 EKGKD NAEGQ GESLA DQARD YMGAA KSKLN
91 DAVEY VSGRV HGEED PTKK
```



Figure 1.1. (A) Amino acid composition, (B) Hydrophobicity plot, (C) Structural properties and (D) Amino acid sequence of Hsp12p. Hydrophobicity was plotted according to the method of Kyte and Doolittle (Kyte and Doolittle, 1982). Structural disorder tendency was calculated using IUPRED analysis which calculates total pairwise amino acid interaction energy (Dosztányi *et al.*, 2005). Hsp12p has a predicted molecular weight of 11680 Da and a theoretical pI of 5.04, as determined using DNAMAN 4.13 software (Lynnon Biosoft).

## 1.2. *HSP12* REGULATION

### 1.2.1. *HSP12* possesses a complex, highly regulated promoter region

*HSP12* is extensively regulated in *S. cerevisiae* and responds to nearly all stresses imposed on the yeast cell. The promoter of *HSP12* consequently contains multiple consensus sequences (Table 1.1) for a number of transcription factors modulating stress responses and metabolic programming. Using a combination of *in silico* analyses, microarray and direct binding experiments, several authors have classified these promoter regions (Figure 1.2). Although the regulation of this gene has been studied extensively, this has predominantly been at the transcriptional level. Some studies have revealed that Hsp12p protein levels are lower at elevated temperatures whereas mRNA levels are correspondingly higher (Mtwisha *et al.*, 1998), implicating mRNA stability and translational control in the regulation of Hsp12p. In addition, studies of *HSP12* regulation have often involved observations after single mutations, which hinder conclusive statements since the transcription factor/s in question may be affected in localization, phosphorylation or even unknown factors by unrelated gene disruptions.



Table 1.1. Known and putative consensus sequences for various transcription factors in yeast.

| Name | Consensus sequence | Regulatory function | Reference/s |
|---|---|---|---|
| STRE (Msn2/4p) | AGGGG | General stress | Martinez-Pastor *et al.*, 1996. |
| HSE (Hsf1p) | AAG(G/A)$N_6$AAG | Heat shock | Sorger and Pelham, 1988. |
| PDS (Gis1p) | T(T/A)AGGGAT | Nutrient limitation | Boorstein and Craig, 1990. |
| RLM1 | (C/G)TA(A/T)$_4$TAG | Cell wall integrity | Dodou and Treisman, 1997; Watanabe *et al.*, 1997. |
| | CCCN$_{10}$GGC | Cell wall integrity | Boorsma *et al.*, 2004. |
| CRE-like (Sko1p) | ACGTCA | Osmotic stress | Proft and Serrano, 1999. |
| Zymolyase response element (?) | ATGACGT | Cell wall integrity | Boorsma *et al.*, 2004. |
| ABF1 | (A/G)TC(A/G)(C/T)$N_5$ACG | Global transcription | Miyake *et al.*, 2004 |
| TEC1 | CATTC(C/T)(C/T) | Filamentous growth | Madhani and Fink, 1997. |
| MIG1 | GGGG | Glucose repression | Nehlin *et al.*, 1991. |
| RAP1 | ACCCANNCA | General stress | Eriksson *et al.*, 2000. |
| DRE (Tac1p)* | CGGNNNCGG | Drug response | De Micheli *et al.*, 2002. |

* This element has only been classified in *C. albicans*.


| Colour | Position (relative to ATG) | Element name |
|---|---|---|
| Light green | -520 to -505 | Rlm1p binding domain |
| Red | -435 to -431 | STRE 5 |
| | -414 to -410 | STRE 4 |
| | -377 to -373 | STRE 3 |
| | -232 to -228 | STRE 2 |
| | -190 to -186 | STRE 1 |
| Orange | -332 to -327 | Zymolyase response element |
| Dark green | -145 to -139 | TATA box |
| | -77 to -72 | |
| Purple | 0 | *HSP12* ORF |
| Light blue | -265 to -253 | Abf1p binding domain |
| Dark blue | -542 to -536 | Tec1p binding site |
| Red italicized | -576 to -572 | Postdiauxic Shift Element (PDSE) |
| Purple italicized | -459 to -450 | Heat Shock Element (HSE) |
| | -177 to -170 | |
| Dark blue italicized | -566 to -555 | Rap1p binding site |

Figure 1.2. Analysis of the *HSP12* promoter reveals many putative regulatory elements. Only 606 bp upstream are depicted, since investigations revealed that this is the minimum region required for proper induction (Varela *et al.*, 1995). Some regulatory motifs overlap, for example the Mig1p glucose repression site closely resembles an STRE and the ZRE closely resembles the CRE. Refer to Table 1.1 for further details.

### 1.2.2. *HSP12* is induced during conditions of glucose limitation, and upon entry into stationary phase

Adenylate cyclase activity is diminished when yeast cells enter stationary phase, are grown on non-fermentable carbon sources or when glucose is limited. This causes yeast cells to produce lower levels of cyclic AMP (cAMP) which directly affects protein kinase A (PKA) activity as cAMP is required for activation of its catalytic subunits, Tpk1/2/3p (Thevelein and de Winde, 1999). This leads to derepression of many stress responsive genes including *HSP12*. Initially, the induction of *HSP12* by nutrient limitation was thought to be controlled by the general stress transcription factors, Msn2/4p, since (i) these proteins accumulate in the nucleus upon starvation (Gorner *et al.*., 1998; Smith *et al.*, 1998), (ii) PKA phosphorylates and activates these factors (Chi *et al.*, 2001) and (iii) the promoter of *HSP12* contains many stress-responsive elements (STREs), the binding sites for these proteins. Indeed, STRE mutations led to complete loss of early stationary phase induction of *HSP12* (Varela *et al.*, 1995). However, recent findings showed that when PKA activity is abolished, thus simulating starvation conditions, *HSP12* is induced independently of Msn2/4p (Ferguson *et al.*, 2005). Instead, induction appeared to be heat shock factor (Hsf1p) dependent, indicating possible regulatory cross-talk between the heat shock response and glucose-limiting response, when PKA activity is abolished. Alternatively, Hsf1p might directly induce *HSP12* via the STREs under nutrient limiting conditions. Further regulatory complexities are revealed by the fact that another level of nutrient limiting control is mediated via the post-diauxic shift (PDS) elements, which are acted upon by the effector of Rim15p, Gis1p (Pedruzzi *et al.*, 2000). Since the promoter of *HSP12* contains a putative PDS element (Figure 1.2) and a *gis1Δ* strain exhibits defective derepression of *HSP12* (Pedruzzi *et al.*, 2000), we would envisage that *HSP12* is controlled via both Hsf1p and Gis1p mediated derepression. Other authors have suggested another mechanism of glucose-mediated control, the Pho85p pathway (Timblin and Bergman, 1997). Pho85p is a cyclin dependent glycogen synthase kinase catalytic subunit which associates with Pho80p to repress many stress-responsive genes, including *HSP12* (Measday *et al.*, 1997). Pho85p is inactivated by glucose 6-phosphate (Huang *et al.*, 1997; Pederson *et al.*, 2004), which functions during early stationary phase as a trigger to stimulate cells to hyper-accumulate glycogen via the predominant yeast glycogen synthase, Gsy2p (Lillie *et al.*, 1980; Francois *et al.*, 1997). This may explain another mechanism of

Hsp12p induction upon entry into stationary phase since Pho85p inhibition by glucose-6-phosphate would lead to consequent derepression of Hsp12p.

*HSP12* is also induced when yeast cells are grown in the presence of non-fermentable carbon sources such as oleic acid (Stone *et al.*, 1990). The exact mechanism governing this process is not known, but experimental evidence points to the involvement of inositolphosphoceramide-phospolipase C (Isc1p), since removal of this protein from yeast cells results in a 4 fold increase in *HSP12* transcript levels (Cowart *et al.*, 2005). A possible mechanism is that oleic acid acts via negative feedback to inhibit Isc1p, which in turn inhibits Sst2p and increases *HSP12* induction. Another possibility is that oleic acid acts directly on protein kinase C (PKC), since PKC in human cells, which is homologous to yeast PKC (Jacoby *et al.*, 1997), displays increased activity when exposed to oleic acid (Khan *et al.*, 1992). PKC is the main mediator of the cell integrity pathway in yeast, which is a known inducer of *HSP12*. However, no studies have been conducted on the effect of oleic acid on protein kinase C in yeast cells.

*HSP12* is rapidly repressed when glucose is added to starved cells. This process is evident even at very low concentrations (0.02 % w/v) of glucose (de Groot *et al.*, 2000). A role independent of the Mig1p general glucose repression pathway for glucose-6-phosphate has been proposed in this process via an unknown mechanism affecting STRE2 at position -232 in the *HSP12* promoter (de Groot *et al.*, 2000). Intriguingly, it was later demonstrated that glycogen synthase kinase (Gsk2p) is essential for Msn2p/STRE binding complex stabilization (Hirata *et al.*, 2003). Since glucose 6-phosphate inactivates Gsk2p (Huang *et al.*, 1997), this would lead to Msn2p/STRE instability and consequently *HSP12* expression would be halted.

Thus it appears that glucose-6-phosphate is a major factor governing *HSP12* expression in response to nutrient limitation by Pho85p-mediated derepression, and to nutrient abundance by loss of Gsk2p stabilization of Msn2p/STRE complexes and down-regulation of *HSP12*. However, how the regulatory pathways respond differentially to glucose-6-phosphate under both extremes is not known, but may involve differences in cAMP synthesis and/or removal, which would affect transcription factor localization.

### 1.2.3. Temperature changes induce *HSP12* expression

Heat stress affects many physiological processes of the yeast cell, including increased plasma membrane fluidity and protein denaturation. *HSP12* acquired its name since it was originally identified as a gene which was induced in response to heat shock (Praekelt and Meacock, 1990). Although several authors have subsequently validated this finding, Mtwisha *et al.* demonstrated that Hsp12p levels are lower during growth at elevated temperatures (Mtwisha *et al.*, 1998), suggesting the presence of other regulatory mechanisms in the heat shock response.

As detailed previously, the promoter of *HSP12* contains two putative Hsf1p heat shock elements (HSEs) (Sorger *et al.*, 1988). These elements, however, appear not to be important for *HSP12* induction in response to heat stress, since disruption of all these elements had little effect on *HSP12* induction. In contrast, disruption of the second most proximal CCCCT motif (STRE2), caused a dramatic decrease in *HSP12* induction (Varela *et al.*, 1995). This finding was later confirmed using a strain lacking Hsf1p activity, although Hsf1p was required for optimal *HSP12* induction in response to heat stress (Treger *et al.*, 1998). *HSP12* induction upon heat shock is also mediated independently of the STRE-binding transcriptional activator, Yap1p (Gounalaki and Thireos, 1994; Varela *et al.*, 1995). This transcription factor possesses a cysteine-rich domain, which acts as a redox sensor which responds directly to oxidative stress, independently of cell surface localized sensors. Thus *HSP12* cannot be induced directly by stress, but its expression is instead accurately controlled by MAP kinase pathways. In addition, *HSP12* is not activated by heat stress when intracellular PKA activity is elevated (Varela *et al.*, 1995), probably due to unavailability of the Msn2/4p transcriptional activators (Görner *et al.*, 1998). Finally, since heat shock can result in activation of the cell integrity pathway, the cell integrity responsive transcription factor, Rlm1p, may play a role in *HSP12* induction upon this form of stress (Kamada *et al.*, 1995). Thus, in summary, *HSP12* is induced during heat shock by an Hsf1p and Yap1p-independent mechanism putatively involving Msn2/4p and Rlm1p, which act via binding to STREs and the Rlm1p domain respectively in the promoter of this gene.

In the inverse situation, yeast must also cope with cold stress. Upon exposure to cold, membrane fluidity decreases leading to impaired membrane transport functions (Vigh *et al.*, 1998) and upon freezing, water forms jagged crystals which can readily rupture cell walls

and membranes and interfere with protein structure. In addition, yeast cells must maintain a basal level of translational activity and metabolism to survive extended cold periods. Yeast cells have thus evolved a cold stress response, which has not been extensively studied. Recently, investigations have revealed that when yeast cells are exposed to temperatures between 10°C and near freezing, several genes encoding proteins involved in trehalose synthesis are reversibly induced (Kandror *et al.*, 2004; Schade *et al.*, 2004). This results in an increased level of intracellular trehalose, which acts as a compatible solute, thus protecting membranes (Iwahashi *et al.*, 1995; Hochachka and Somero, 2002) and proteins (Viner and Clegg, 2001) by minimizing water crystal formation. Besides trehalose synthesis, cells also respond by inducing the expression of general stress response proteins, including Hsp12p (Kandror *et al.*, 2004; Schade *et al.*, 2004). Interestingly, the heat shock protein Hsp26p, which is often co-regulated with Hsp12p in response to stress, was not induced in response to cold (Kandror *et al.*, 2004). Since the promoter of *HSP26* and *HSP12* both contain several STREs, this suggests that the response of *HSP12* to cold stress is regulated by elements other than STREs. However, the transcription factors Msn2/4p, which bind STREs, are required for viability and optimal Hsp12p expression following cold stress response (Kandror *et al.*, 2004; Schade *et al.*, 2004). This may indicate that the Msn2/4p transcription factors affect other transcription factors modulating *HSP12* expression, but not *HSP26* expression, such as the PKC cell integrity pathway effector, Rlm1p.

### 1.2.4. Barometric pressure induces *HSP12* expression

In the environment, yeast cells are rarely subjected to barometric stress. Yeast cells can survive pressures of up to 220 MPa (Palhano *et al.*, 2004) and respond to this stress by inducing the synthesis of small heat shock proteins such as Hsp12p, Hsp26p and Hsp30p (Fernandes *et al.*, 2004). The transcription factors involved in the regulation of these genes in response to barometric stress are not known, since the heat shock protein Hsp30p possesses no known stress response regulatory elements (Seymour and Piper, 1999). This has prompted researchers to believe that the pressure stress response is due to regulatory overlap rather than a specific response, as an increased hydrostatic pressure causes protein denaturation (Silva *et al.*, 2001), reduced membrane fluidity (Mentre *et al.*, 1999) and cell wall damage (Fernandes *et al.*, 2001; Motshwene *et al.*, 2004), all factors common to heat shock and cell integrity stresses. However, the effect of hydrostatic pressure is more drastic

than these stresses alone as pressure-stressed cells take longer than heat shocked cells to resume normal growth after relief of the stress (Palhano *et al.*, 2004). It has been proposed that trehalose plays a role in the acquisition of yeast barotolerance. However, cells unable to synthesize trehalose can be rescued after high pressure stress by prior heat treatment, implying a role for heat shock response proteins in the acquisition of barotolerance (Fernandes *et al.*, 2001). Indeed, a direct relationship between Hsp12p interaction with the yeast cell wall and the barotolerance of yeast cells has been recently documented (Motshwene *et al.*, 2004).

### 1.2.5. Salt stress causes induction of *HSP12*

*HSP12* is induced to high levels upon salt stress (Varela *et al.*, 1995; Siderius *et al.*, 1997). The main pathway responsible for the modulation of *HSP12* in response to salt stress is the High Osmolarity Glycerol (HOG) MAP kinase pathway (For a review, see Hohmann, 2002) since induction of *HSP12* is impaired in *hog1Δ* strains (Varela *et al.*, 1995; Siderius *et al.*, 1997). Upon salt stress, Hog1p is phosphorylated by Pbs2p and rapidly migrates to the nucleus (Ferrigno *et al.*, 1998; Reiser *et al.*, 1999) where its export is inhibited by a number of factors including Msn2/4p (Reiser *et al.*, 1999), Hot1p and Msn1p (Rep *et al.*, 1999). Hog1p activates Msn2/4p (Schüller *et al.*, 1994), Hot1p (Rep *et al.*, 1999), Sko1p (Proft *et al.*, 2001), Sgd1p (Akhtar *et al.*, 2000), Gen4p (Pascual-Ahuir *et al.*, 2001) and Skn7p (Brown *et al.*, 1994) to induce osmotic stress responsive genes, including *HSP12*. Hog1p also acts directly on the promoter of *HSP12* via various mechanisms. Firstly, Hog1p interacts physically with the histone deacetylase Rpd3p, which facilitates entry of RNA polymerase II to chromatin and induces *HSP12* expression (De Nadal *et al.*, 2004). Secondly, Hog1p appears to have a direct effect on Msn2/4p regulatory activity, but physical interactions have not been demonstrated (Rep *et al.*, 2000). In summary, the regulation of *HSP12* in response to salt stress appears to require the full complement of transcription factors, namely Hog1p, Hot1p, Msn1p and Msn2/4p (Table 1.2) with Hog1p recruiting Hot1p (Alepuz *et al.*, 2001).

Table 1.2. The effects of deletion of various transcription factors on *HSP12* gene expression in response to 0.7 M NaCl. Grey and white background alternations depict separate experiments.

| Mutation | Level after 60 min | Max level,time at max level | Change in max level | Reference/s |
|---|---|---|---|---|
| None | 100% | 100%, 60 min | 0% | Rep *et al.*, 1999 |
| hot1 | 77% | 77%, 60 min | -23% | |
| msn1 | 64% | 75%, 45 min | -36% | |
| msn2/4 | 20% | 20%, 60 min | -80% | |
| hot1 msn1 | 40% | 45%, 45 min | -60% | |
| msn1 msn2/4 | 18% | 19%, 45 min | -82% | |
| hot1 msn2/4 | 2% | 2%, 60 min | -98% | |
| hot1 msn1 msn2/4 | 1% | 1%, 60 min | -99% | |
| None | 100% | 100%, 90 min | 0% | Siderius *et al.*, 1997 |
| tpk2 tpk3 | 87% | 87%, 60 min | -13% | |
| tpk1,tpk2,tpk3 | 87% | 114%, 45 min | -13% | |
| tpk2,tpk3,bcy1 | 2% | 2%, 60 min | -98% | |
| None | 100% | 100%, 60 min | 0% | Siderius *et al.*, 1997 |
| hog1 | 50% | 88%, 120 min | -50% | |
| msn2/4 | 18% | 20%, 90 min | -82% | |
| None | | 100%, 30 min | 0% | Varela *et al.*, 1995 |
| tpk2,tpk3,bcy1 | | 25%, 30 min | -75% | |
| tpk1,tpk2,tpk3 | | 350%, 30 min | +250% | |
| tpk1,tpk2,tpk3,bcy1 | | 580%, 30 min | +480% | |



An extensive deletion analysis of the *HSP12* promoter is response to salt stress has been conducted (Varela *et al.*, 1995) and is summarized in Figure 1.3. This study showed that removal of the region between -606 and -535 had little effect, but further deletion of the region between -535 and -513 caused a substantial drop in salt stress induction. Interestingly, this region contains a putative Rlm1p cell integrity regulatory motif, $GCCN_{10}GGG$ (Boorsma *et al.*, 2004) and no STREs. This provides evidence of the regulatory relationship between salt stress and the cell integrity pathway.

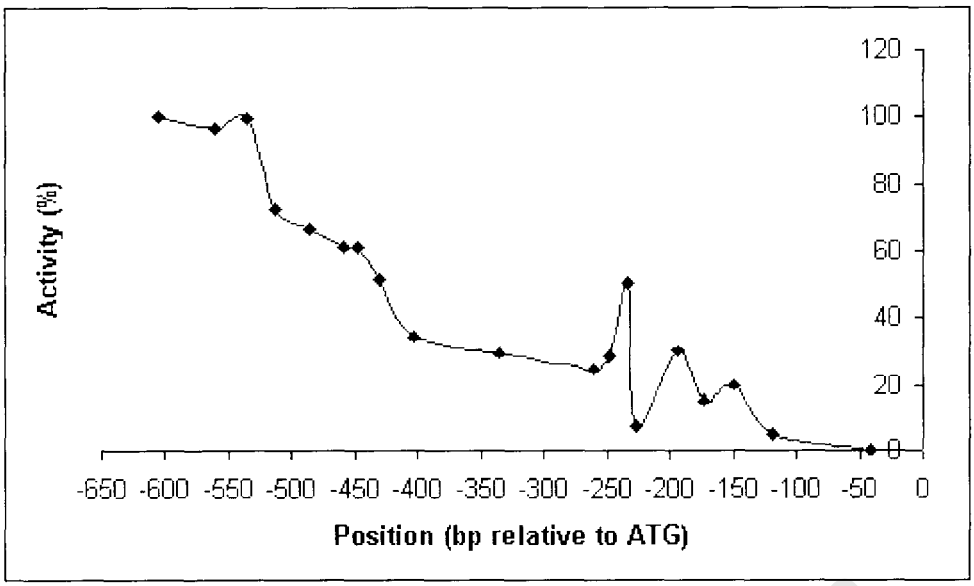



Figure 1.3. Analysis of promoter deletions assembled from the data of Varela *et al.*, 1995, in response to salt stress. Activity was determined relative to control levels, hence in certain cases, derepression is not indicated, but this indeed occurred when the region from -260 to -150 was deleted. Zero (0) on the X-axis represents the start codon for the *HSP12* ORF.

Further deletions of successively -513 to -460 resulted in an almost linear loss of salt stress induction. Deletions from -460 to -448 had no effect but deletions from -448 to -336 showed another almost linear loss of activity, coinciding with the loss of STRE5 (-435 to -431), STRE4 (-414 to -410) and STRE3 (-377 to -373). A subsequent deletion from -336 to -260 had little effect except mild stress-independent HSP12 induction, possibly by removal of glucose repression elements. This region contains a putative binding site at -333 for Sko1p, a transcription factor regulated by Hog1p in response to either osmotic stress (Proft and Serrano, 1999) or cell integrity stress (Boorsma *et al.*, 2004). Sko1p would therefore not appear to be essential for *HSP12* induction upon salt stress. A subsequent deletion of -260 to –234 resulted in partial restoration of the stress response. Interestingly, this region contains a putative binding site for the global transcription factor, Abf1p (-265 to -253). However, subsequent point deletions of this region indicated no contribution of Abf1p in the salt stress response, but since the rest of the promoter was present in this case, the contribution of upstream regulatory elements cannot be ignored. Nevertheless, this region (-336 to -150) appears to play an important role in glucose repression. Further deletions from -234 to -228 caused a sharp decline in *HSP12* induction, probably due to removal of STRE2 (-232 to -228). Interestingly, this particular STRE has also been shown to be essential for proper glucose repression, heat shock and early stationary phase induction, indicating multiple roles for STRE2 in *HSP12* modulation (Varela *et al.*, 1995; de Groot *et al.*, 2000). Subsequent deletions from -228 to -194 again resulted in a mild restoration of

salt stress inducibility but this was curtailed by subsequent deletion of -194 to -173, presumably due to loss of the last remaining STRE, STRE1 (-190 to -186). Finally, deletions from -173 to -150 resulted in a slight restoration of *HSP12* induction while deletions from -150 to -41, which resulted in TATA box (-145 to -139 and -77 to -72) removal, gradually caused a total loss of activity.

In summary, Hog1, Hot1, Msn1, Msn2/4 acting on the STREs (primarily STRE2) and Rlm1p acting through its binding site play a vital role in salt stress induction of *HSP12*. The Abf1p region appears to play a repressive role, possibly by enhancing glucose repression, but the mechanism of this is unknown. Taken together, these data can be represented as a model for the *HSP12* salt stress response (Figure 1.4). In this model, contributions of various factors are indicated by percentages. Thus when Hot1p and Msn1p are removed, induction drops by ~60% by allowing Hog1p export from the nucleus. When Hog1p is removed, induction drops to ~50%, and when Msn2/4p is removed, low levels of induction (~20%) are observed, possibly due to Rlm1p. Thus Msn2/4p and Rlm1p account for ~50% net induction, which is enhanced to ~100% by Hog1p, possibly by histone deacetylase recruitment. The discrepancy between Hot1p and Msn1p combined effects (60%) and Hog1p overall contribution (50%) may indicate enhancement of Hog1p activity by these transcription factors in addition to nuclear retention. Another possibility is that Msn2/4p itself plays a role in preventing export of Hog1p from the nucleus, thus enhancing its apparent activity (Reiser *et al.*, 1999). Future experiments to verify this model should include analysis of an *msn1Δ hot1Δ hog1Δ* mutant, which should be equivalent to an *msn2/4Δ* mutant and an *rlm1Δ* mutant to investigate the contribution of this factor in response to salt, since Rlm1p plays an essential role in *HSP12* induction in response to cell wall damage (Garcia *et al.*, 2004).

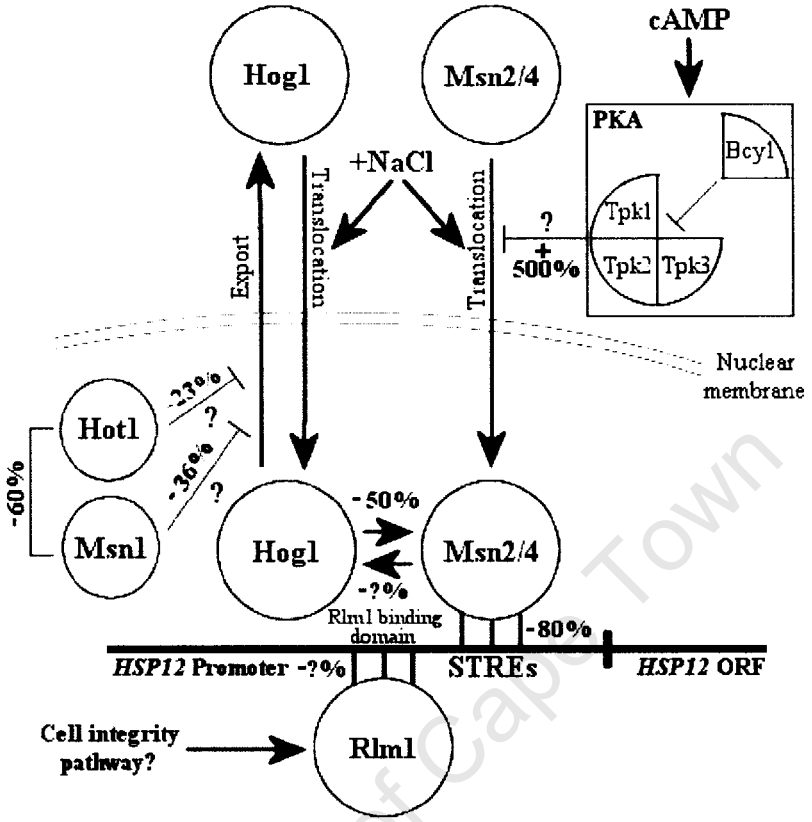



Figure 1.4. A simplified model of *HSP12* induction following salt stress. Question marks represent unknown mechanisms and/or upstream pathways. Arrows represent positive effects, blunt lines repressive effects. Effects are quantified by percentages indicative of effects of mutations on overall *HSP12* expression levels, with positive and negative values depicting enhancement and impairment of *HSP12* induction respectively.



### 1.2.6. *HSP12* is induced in response to cell wall damage

Most stressful conditions encountered by yeast cells have an effect on the cell wall of this organism. Salt (hyper-osmotic) stress causes cell shrinkage (Motshwene *et al.*, 2004), while hypo-osmotic stress causes the cell to swell (Smits *et al.*, 1999). Temperature influences the kinetics of molecules in the cell wall and also the effect of various intracellular enzymes on the wall. Certain compounds such as Congo Red (CR) or Calcofluor White respectively cause hyper-accumulation (Kopecka and Gabriel, 1992) or coating of cell wall components (Bartnicki-Garcia *et al.*, 1994). Finally, antifungal agents such as chitinases and glucanases

specifically target and destroy this vital cellular component (Garcia *et al.*, 2004). Most of the studies of *HSP12* expression in response to cell wall damage have been conducted using CR or Zymolyase (a cocktail of chitinases and glucanases) as the source of stress. The effects exerted by these compounds can however be extrapolated to the cell integrity response in general, since a large cluster of stress responsive genes, including *HSP12*, responds to a variety of different cell wall stresses (Boorsma *et al.*, 2004; Garcia *et al.*, 2004).

Recently, Hsp12p has been localized to the yeast cell wall, where it has been proposed to increase cell wall flexibility (Motshwene *et al.*, 2004). Accordingly, authors have reported strong Hsp12p induction following cell integrity stress, particularly after Zymolyase treatment (Garcia *et al.*, 2004). The transcription factors modulating these events are primarily components of the protein kinase C (Pkc1p) pathway, since over-expression of Pkc1p or its sensor Rho1p, results in a massive induction of *HSP12* (Garcia *et al.*, 2004). Pkc1p serves to activate the downstream MAP Kinases, Slt2p/Mpk1p and the final targets Rlm1p and Swi4/6p, both of which are involved in cell wall construction and growth phase-dependent events (For a review, see Levin, 2005). Interestingly, regulatory overlap is again observed when cells are treated with the cell wall damaging agent Congo Red, since *HSP12* induction in this case is Hog1p dependent (Rodriguez-Pena *et al.*, 2005). As mentioned previously, salt stress also depends on Hog1p and since the promoter of *HSP12* contains a regulatory motif for the cell integrity factor, Rlm1p, this leads to the possibility that Hog1p and Slt2p/Mpk1p both exert an effect on Rlm1p, thus linking the two pathways (Figure 1.5). Further evidence is provided by the fact that cells lacking Hog1p are hypersensitive to cell wall degrading enzymes (Alonse-Monge *et al.*, 2001) and cells lacking Rlm1p display virtually no *HSP12* induction upon cell integrity stress (Garcia *et al.*, 2004). Thus, in summary, Slt2p/Mpk1p and to a lesser extent, Hog1p, act synergistically via the transcription factor, Rlm1p, to induce *HSP12* and other cell integrity responsive proteins upon cell integrity stress.

### 1.2.7. Other factors resulting in *HSP12* induction

Aside from "common" stress conditions, a number of studies have shown *HSP12* induction in response to a diverse array of agents. These include metals such as copper (van Bakel *et al.*, 2005), cadmium (Momose and Iwahashi, 2001) and lithium (Bro *et al.*, 2003) as well as

DNA damaging agents such as cisplatin, methyl methanesulfonate (MMS), bleomycin and neocarzinostatin (Treger *et al.*, 1998; Schaus *et al.*, 2001; Caba *et al.*, 2005). *HSP12* is also induced in response to toxins such as herbicides (Simoes *et al.*, 2003; Teixeira *et al.*, 2005). The multiple stress-responsive behaviour of this protein thus makes it potentially useful as an environmental sensor as well as having several applications in other areas (Section 1.4).

Unfortunately, the role of Hsp12p in the yeast cell in these responses has not been reported and these findings are merely mentioned to further demonstrate the multitude of stresses that induce *HSP12* expression.

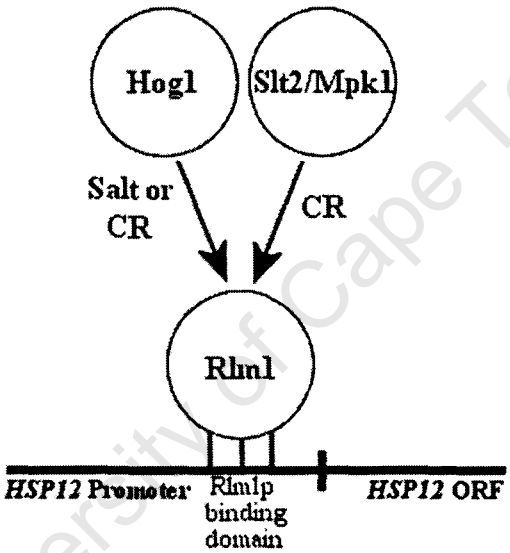



Figure 1.5. Simplified model of regulatory crosstalk to induce *HSP12* via both salt stress and cell integrity stress. CR: Congo Red.

## 1.3. HSP12P LOCALIZATION AND FUNCTION

Studies concerning the function of Hsp12p were initially hampered by the fact that the most commonly tested stress states, heat shock and osmotic stress, had the same effect on cells whether they possessed Hsp12p or not (Praekelt and Meacock, 1990). The discovery of Hsp12p in the cell wall vicinity by a combination of immunocytochemistry and chemical extraction (Sales *et al.*, 2000; Motshwene *et al.*, 2004) and the discovery that biofilm formation in a sherry yeast strain was Hsp12p-dependent (Zara *et al.*, 2002) prompted investigations into the effects of CR, a cell wall damaging agent, on cells lacking Hsp12p (*hsp12ΔURA3* cells). Hsp12p was found to be necessary for proper growth in the presence of this compound. In addition, when these cells were exposed to elevated barometric pressure, they displayed a marked loss of viability compared to wild type strains (Motswhene *et al.*, 2004). Further evidence of a cell wall location for Hsp12p is that cells exposed to the cell wall destructive agent Zymolyase displayed a 35-fold induction of *HSP12* (Garcia *et al.*, 2004). In addition, an Hsp12p homologue, Hsp9p, was able to suppress a *CDC4* mutation in *S. pombe* which resulted in improper septation, implying a further role for Hsp12p in cell wall modelling (Jang *et al.*, 1996). It is intriguing to note that this yeast does not replicate by budding, but via binary fission. This may therefore exclude the possibility that Hsp12p is involved in the actual budding process of *S. cerevisiae*.

The regulation of Hsp12p also implicates Hsp12p interaction with the cell wall, since as shown previously, most stressful conditions affect cell wall integrity (Levin, 2005). This may explain why cells pre-treated with less severe stresses display markedly enhanced survival rates following subsequent severe stress, since in this case cell integrity proteins, including Hsp12p, may "adapt" the cell wall to counter other stresses.

## 1.4. APPLICATIONS OF *HSP12*

Due to the multistress-responsive behaviour of this protein, many investigators have demonstrated the use of *HSP12* expression as a reliable method to quantify the stress status of yeast cells. One such use is in an agriculture, where herbicides such as 2,4-dichlorophenoxyacetic acid are frequently employed to combat invasive vegetation. *HSP12* was used as an indicator of the potential detrimental effects on secondary, non-target organisms in the environment (Teixeira *et al.*, 2005). Other environmental studies involved the use of *HSP12* induction as an indicator in a bioassay system screening for cadmium, a potent cell poison (Momose and Iwahashi, 2001). Another form of assay using *HSP12* induction as a marker has been developed to discriminate between genotoxic and cytotoxic compounds (Caba *et al.*, 2005). From a commercial aspect, an important area of investigation is the monitoring of fermentative processes. *HSP12* has been used as a marker to quantify stresses during the first few hours of fermentation (Perez-Torrado *et al.*, 2002), during fermentation (Ivorra *et al.*, 1999; Higgins *et al.*, 2003; Perez-Torrado *et al.*, 2005) and during aging of sherries and wines (Aranda *et al.*, 2002). In this way, new potentially stress-resistant industrial strains of yeast can be identified (Carrasco *et al.*, 2001). In addition, the promoter of *HSP12* has been fused upstream of various genes to allow growth-phase specific regulation of these genes during fermentation (Donalies and Stahl, 2001). Thus *HSP12* has proved very useful and it will be interesting to see future applications of this uniquely responsive and intriguing protein.

## 1.5. MOTIVATION FOR THE STUDY OF HSP12P IN *S. CEREVISIAE*

Large amounts of Hsp12p accumulate in yeast cells upon exposure to stress, yet the phenotype of yeast cells lacking this protein is largely unaffected by stresses such as heat shock and osmotic stress. It was thus desirable to determine the function of such a highly induced protein with no apparent function and to see why this strain of yeast had retained the gene encoding this protein. Furthermore, identification of the location of such a highly expressed protein would be important since Hsp12p is highly hydrophilic and may affect the chemistry of other molecules in the target locus. Finally, it was important to determine the expression characteristics and identify possible applications of this stress-responsive, highly expressed protein.

Investigations were conducted to:

- Determine the location of Hsp12p in yeast cells *in vivo*.
- Identify applications of Hsp12p in biosensors as a marker for compounds which may affect yeast cells and hence other eukaryotic organisms.
- Gain further understanding of regulatory systems in yeast, since Hsp12p is induced by a variety of stresses, enabling the elucidation of regulatory cross-talk between various stress pathways.
- Determine the function of Hsp12p in yeast cells.

# CHAPTER 2

# THE LOCALIZATION AND APPLICATIONS OF HSP12P

## 2.1. SUMMARY

Immunocytochemical analysis and alkali extraction revealed that Hsp12p had a cell wall location. In order to confirm this finding, the *HSP12* gene and its promoter was inserted upstream of the fluorescent marker protein, Gfp2p, in the yeast shuttle vector, pYES2. Subsequent expression, epifluorescent microscopy and confocal microscopy indicated that Hsp12p was predominantly localized near the cell periphery. This fluorescence was dependent on the growth phase of the cell, a determinant for Hsp12p expression. Studies into whether the amount of fluorescence produced was proportional to the stress conditions affecting the yeast cell were therefore undertaken. It was found that the cells emitted fluorescence commensurate with the amount of stress (salt, ethanol, mannitol and temperature stress) the cells were subjected to and this fluorescence could be readily quantified using fluorimetry. The cells were subjected to stress and the intensity of fluorescence measured. Results indicated that 400 mM NaCl represented the most severe stress while ethanol and temperature effects were not as pronounced. Thus the yeast cells expressing an Hsp12-Gfp2p fusion protein can be used as a reliable and non-invasive indicator of the stress status of yeast cells, with possible future application in industrial fermentation processes such as ethanol production.

## *2.2. LOCALIZATION STUDIES*

### 2.2.1. INTRODUCTION

Hsp12p is expressed at high levels in yeast cells upon exposure to many forms of stress (Varela *et al.*, 1995). Previous immunocytochemical studies revealed that this protein was localised near the cell membrane (Sales *et al.*, 2000). However, subsequent studies using cryo-immunocytochemistry revealed that Hsp12p was in fact mainly present in the cell wall (Motshwene *et al.*, 2004). Since Hsp12p possesses no known cell wall targeting sequence, cell wall proteins were extracted with alkali solutions and analysed for the presence of Hsp12p. Furthermore, a fusion protein comprising Hsp12p and green fluorescent protein (Gfp2p) was produced to investigate the kinetics of translocation to the cell wall after expression. Gfp2p is a commonly used protein marker and allowed viewing of expressed Hsp12p directly via epifluorescent and confocal scanning microscopy.

## 2.2.2. MATERIALS AND METHODS

### 2.2.2.1. Materials

Yeast extract, peptone, tryptone and bacteriological agar were supplied by Biolabs (RSA). All chemicals used were of high purity. Double distilled water was used for all experiments except for PCR experiments, where double distilled water passed through a Milli-Q filtration apparatus (Millipore, USA) was used.

### 2.2.2.2. Organisms and culture conditions

The *S. cerevisiae* yeast strain used in this study was from the W303 genetic background (a/α, *ade2-1/ade2-1, trp1-1/trp1-1, leu2-3/leu2-112, his3-11/his3-15, ura3/ura3, canr1-100/CAN*) and was used in the haploid form. Yeast cells were routinely cultured in 1 % (w/v) yeast extract, 2 % (w/v) peptone, 2 % (w/v) glucose (YEPD) media at 30°C. Aeration was achieved by continuous shaking on a rotary shaker. For solid media (plates), agar was added to a final concentration of 1.5 % (w/v). Positive transformants were selected on synthetic complete dropout media with uracil omitted (SC-URA). This contained 0.67 % (w/v) yeast nitrogen base without amino acids (YNB-AA, Difco, USA), 0.077 % (w/v) complete synthetic medium without uracil (CSM-URA, BIO-101, USA) and 2 % (w/v) glucose. Yeast growth, determined by measuring the optical density at 600 nm ($OD_{600}$) of the culture, was expressed as optical density units / ml (ODU/ml), where 1 ODU / ml is equivalent to *ca.* 1 X $10^7$ yeast cells.

*E. coli* DH5α ($F^-$ ϕ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17 ($r_k^-$, $m_k^+$) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 $\lambda^-$) competent cells were a kind gift from C. Hendrickse (University of Cape Town, Cape Town, RSA). These cells were cultured in Luria-Bertani (LB) broth comprising 1 % (w/v) tryptone, 0.5 % (w/v) yeast extract and 0.5 % (w/v) NaCl. For solid media (Luria plates), agar was added to a final concentration of 1.5 % (w/v). Positive transformants were selected on Luria plates supplemented with 100 μg / ml ampicillin (Boehringer Mannheim GmbH, Germany).

### 2.2.2.3. Cell wall protein extraction and classification

*2.2.2.3.1. β-elimination reaction*

Yeast cells from overnight cultures were collected by centrifugation at 3000 *x g* for 10 min at 10°C. Cells were washed twice with 50 mM phosphate, 150 mM NaCl pH 7.4 (PBS) and the wet mass of the pellet determined. The pellet was resuspended in 1 µl 0.6 M NaOH/mg cells and incubated on ice for 30 min before being centrifuged at 5000 *x g* for 10 min at 4°C. The supernatant was kept at -20°C for subsequent experiments.

*2.2.2.3.2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)*

Proteins were separated on the basis of mass and visualised by SDS-PAGE as previously described (Laemmli, 1970). A total extract of chicken histones served as a standard, with approximate molecular weights (kDa) of: H1: 22.5; H5: 20.6; H3: 15.4; H2B: 13.7; H2A: 14.0 and H4: 11.2.

*2.2.2.3.3 Hsp12p purification*

A crude cell wall protein extract which contained Hsp12p was extracted using the β-elimination method but from wet packed baker's yeast (Anchor Yeast, RSA) to maximise Hsp12p quantity. The extract was dialysed against 10 l of distilled water overnight and dried by lyophilisation. Approximately 50 mg extract in 5 ml deionised water was applied to a Sephadex G-50 (Amersham Biosciences AB, Sweden) gel filtration column (100 X 3 cm) equilibrated with 50 mM phosphate buffer pH 7.4 at 4°C. Approximately two hundred 5 ml fractions were collected over two days and assayed for protein content by spectrophotometric determination at 230 nm. Ten samples corresponding to the tubes exhibiting the greatest absorbance were pooled, dialysed overnight as before and lyophilised. The lyophilate was weighed and resuspended to 20 mg/ml in deionised water. Purity was checked by SDS-PAGE gel which confirmed that Hsp12p was 90 % pure in the collected fractions.

*2.2.2.3.4 Anti-Hsp12p antibody production, ELISA and western blotting*

Anti-Hsp12p antibody was produced by inoculating 1 mg 90 % pure Hsp12p in Freund's complete adjuvant (Difco, USA) into rabbits. Rabbits were given further weekly booster injections of protein over 4 weeks followed by a final inoculation after a two week interval. Rabbits were bled at weekly intervals and the presence of antibody determined by enzyme-linked immunosorbance assay (ELISA): *ca.* 1 μg of pure protein antigen was applied to 6 wells of a 96-well plate microarray plate (Nunclon, USA) and incubated at 4°C for 20 h. The plate was washed 3 times with PBS containing 0.05 % (v/v) Tween (PBS-T) after which the wells were blocked to eliminate non-specific binding by incubation in 1 % (w/v) bovine serum albumin (BSA) in PBS at RT for 1 h with shaking. After washing the plate 3 times with PBS-T, the primary antibody (anti-Hsp12p) was added in various dilutions between $10^{-1}$ to $10^{-6}$ in PBS, and incubated at RT for 1 h. The plate was then washed with PBS, after which goat anti-rabbit antibody conjugated to alkaline phosphatase (Pierce, USA) diluted 1:100 in PBS was added. The plate was incubated for a further 30 min. Antibodies were detected using 300 μg p-nitrophenylphosphate (N-4645, Sigma-Aldrich, USA), 10 % (v/v) diethanolamine (D-8885, Sigma-Aldrich, USA) pH 9.6 and the absorbance determined at 405 nm in a Titertek Multiscan PLUS MKII instrument (Labsystems, Helsinki, Finland).

Proteins were separated on SDS-PAGE gels and transferred to Nitrobind nitrocellulose (Osmonics Inc., USA) by electroblotting: the gel was rinsed with 192 mM glycine, 20 % (v/v) methanol, 25 mM Tris-HCl pH 8.5 (transfer buffer) and applied to a pre-soaked (in transfer buffer) nitrocellulose membrane supported on pre-soaked Whatman 3 MM paper (Whatman, UK) which was in turn supported on a pre-soaked absorbent cloth on a carbon block (anode). The gel was covered with pre-soaked Whatman 3 MM paper and another absorbent cloth followed by another carbon block (cathode). Proteins were transferred to the nitrocellulose at 80 mA for 5 h. Nitrocellulose blots were visualised by Ponceau staining by immersing blots in 0.1 % (w/v) Ponceau S (P-3504, Sigma-Aldrich, USA), 5 % (v/v) acetic acid for 5 min, followed by washing with shaking in PBS for 20 min. Blots were blocked in 10 ml PBS containing 5 % (w/v) skim milk powder for 30 min at RT

with shaking. Primary anti-Hsp12p antibody was added to a final dilution of 1:500 after which blots were incubated overnight at 4°C with shaking. The blot was washed 3 times with PBS-T and once with PBS. The blot was then incubated for 1 h at RT in PBS containing the secondary goat anti-rabbit antibody conjugated to alkaline phosphatase (1:5000 dilution) and 5 % (w/v) skim milk powder. The blot was washed a further 3 times with PBS-T and once with 10 mM Tris-Cl, 150 mM NaCl pH 7.4. Finally, the blot was incubated for 5 min at RT in 10 ml 100 mM NaCl, 100 mM $MgCl_2$, 100 mM Tris-Cl pH 9.5 with 0.25 mg/ml nitro blue tetrazolium (NBT, N-6876, Sigma Aldrich, USA) and 0.25 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP, B-8503, Sigma Aldrich, USA) to visualise proteins. Once the colour had developed to an appropriate extent, the blot was washed in deionised water and allowed to air dry.

*2.2.2.3.5. MALDI-TOF- assisted peptide fingerprinting*

Proteins were identified by in-gel tryptic digestion followed by matrix-assisted laser desorption ionisation, time-of-flight (MALDI-TOF)-assisted peptide fingerprinting essentially as previously described (Shevchenko *et al.*, 1996). Protein bands of interest were excised from SDS-PAGE gels and cut into small blocks (1 x 1 mm). The gel blocks were washed by sequential incubation in 50 % (v/v) acetonitrile ($CH_3CN$) for 5 min, in 50 % (v/v) $CH_3CN$, 50 mM $NH_4HCO_3$ for 30 min and in 50 % (v/v) $CH_3CN$, 10 mM $NH_4HCO_3$ for 30 min. Gel pieces were dried under vacuum in a microcentrifuge for 30 min and rehydrated with a minimal volume of 1 mg/ml trypsin (Promega, USA) in 10 mM $NH_4HCO_3$. The sample was incubated at 37°C for 20 h after which 1 µl was mixed with 1 µl α-cyano-4-hydroxy cinnamic acid (CHCA, C-2020, Sigma-Aldrich, USA), applied to a gold-plated MALDI-TOF sample plate and allowed to dry. Sample plates were inserted into a Perseptive Biosystems MALDI-TOF mass spectrometer (Perseptive Biosystems GmbH, Germany) and analysed using the following conditions: Method: HCD1002, Mode: linear positive ion, Accelerating voltage: 20000 V, Grid voltage: 94 %, Guide wire voltage: 0.05 %, Laser intensity: 2200, Low mass gate: 600 Da.

### 2.2.2.4. Construction of pYES2-HSP12-GFP2 (pYH12G2)

*2.2.2.4.1. Outline*

GFP (GFP Mutant 2, Cormack *et al.*, 1996) was amplified from YCplac22-GFP (a gift from F. Bauer, Stellenbosch University, Stellenbosch, RSA) with the flanking restriction sites, EcoRI (5') and XbaI (3'). The PCR product was digested with EcoRI and XbaI, gel purified and ligated into the plasmid shuttle vector pYES2 (Invitrogen, USA) to produce the vector pYES2-GFP. This plasmid was transformed into *E. coli* DH5α competent cells using standard methods. After positive transformant selection, the presence of GFP in the construct was verified by PCR.

*HSP12* and its promoter (HSP12+PROM) were amplified from pGEM-T-HSP12 (a kind gift from A. Kessler, University of Cape Town, Cape Town, RSA) with XhoI (5') and EcoRI (3') flanking restriction sites. The resulting fragment was digested with BamHI (the HSP12 promoter contains a naturally occurring BamHI site at position –606) and EcoRI, gel purified and ligated into the pYES2-GFP construct to produce pYES2-HSP12-GFP2 (Figure 2.2.1), designated pYH12G2. This plasmid was transformed into *E. coli* DH5α and the fidelity of the construct was verified by PCR and sequencing. The plasmid was then transformed into yeast cells and the presence of the construct verified by colony PCR.

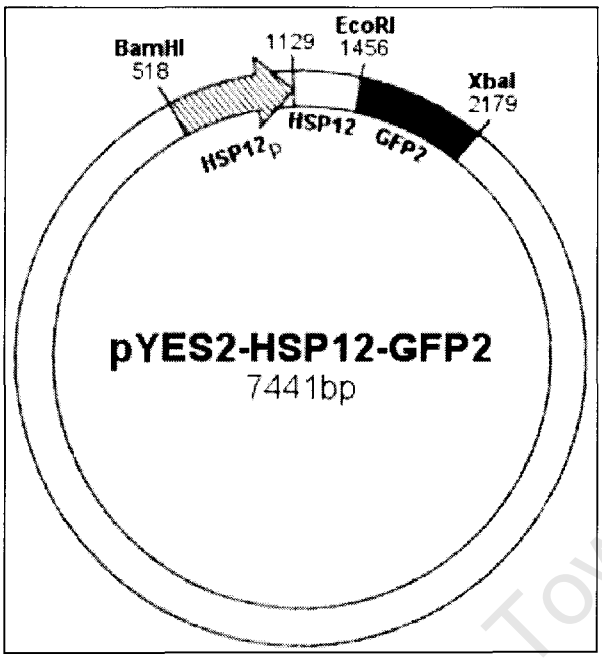



Figure 2.2.1. The pYES2-HSP12-GFP (pYH12G2) construct. *GFP2* was inserted into pYES2 using EcoRI and XbaI restriction sites. *HSP12* and its promoter were cloned upstream of *GFP2* using BamHI and EcoRI sites. The construct was verified by DNA sequencing.

*2.2.2.4.2. Agarose gel electrophoresis*

DNA was visualised on 1 % (w/v) agarose (D1-LE, Hispanagar, Spain) slab gels in a horizontal tank containing 0.09 M Tris-Cl, 0.09 M boric acid, 0.002 M ethylenediaminetetraacetic acid (EDTA, Biolabs, RSA) pH 8.0 (TBE). Prior to casting the gel, 5 µl of 10 mg/ml ethidium bromide (EtBr, Boehringer Mannheim GmbH, Germany) was added per 50 ml agarose solution. Samples were mixed with 5 µl DNA sample application buffer (DNA-SAB) which contained 0.25 % (w/v) bromophenol blue, 40 % (w/v) sucrose and 20 mM EDTA pH 8.0, applied to the gel and run at a constant voltage of 120 V for *ca.* 1 h at RT. DNA was visualized by viewing the gels on an ultraviolet light source, photographed and printed. In all cases the standard was a digestion of λ phage DNA (D-1501, Promega, USA) with PstI or BamHI and HindIII.

*2.2.2.4.3. Plasmid DNA extraction and purification*

Plasmid DNA was extracted and purified from *E. coli* cells using a QIAprep Spin Miniprep kit (Qiagen, Germany) as per manufacturer's instructions.

*2.2.2.4.4. Agarose gel extraction*

DNA fragments of interest were purified by agarose gel electrophoresis and gel extraction using a QIAquick gel extraction kit (Qiagen, Germany) as per manufacturer's instructions.

*2.2.2.4.5. Ethidium bromide DNA quantification*

The concentration of sample DNA was estimated by visual comparison with known amounts of λ phage DNA (5 ng, 10 ng and 20 ng DNA) on 1 % (w/v) agarose gels.

*2.2.2.4.6. Polymerase chain reaction (PCR)*

The *GFP2* sequence was obtained using provided sequence information and the Pubmed Basic Local Alignment Search Tool (BLAST) at the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/blast). The *HSP12* gene and its promoter sequence were obtained from the *S. cerevisiae* genome database (http://www.yeastgenome.org). All PCR reactions were performed in a Sprint Thermal Cycler (Hybaid, UK). Taq polymerase, Taq polymerase reaction buffer and $MgCl_2$ were from Promega (USA). PCR primers were from the Synthetic DNA Synthesis Facility at the University of Cape Town, Cape Town, RSA and were produced on a 1000M DNA synthesizer (Beckman-Coulter, USA) using the high purity program. Nucleotides (dNTPs) were obtained from Bioline (USA).

The oligonucleotide primers for *GFP2* had the following sequences (restriction sites underlined): forward primer: 5'-ACG-AAT-TCA-TGA-GTA-AAG-GAG-AAG-3' and

reverse primer: 5'-CGT-CTA-GAT-TAT-TTG-TAT-AGT-TCA-TC-3'. The PCR reaction mixture contained 50 pmol forward primer, 50 pmol reverse primer, 2 µl 6.25 mM dNTPs, 5 µl 25 mM $MgCl_2$, 5 µl 10X Taq reaction buffer, 2 U Taq polymerase and Milli-Q water to yield a final reaction volume of 50 µl. PCR conditions were as follows: 92°C (5 min); 5 cycles of 92°C (30 s), 44°C (30 s), 72°C (30 s); 25 cycles of 92°C (30 s), 68°C (30 s), 72°C (30 s); 72°C (5 min) and finally 4°C (10 min).

The following oligonucleotide primers were used to amplify *HSP12* and its promoter (restriction sites underlined): forward primer: 5'-CAC-TCG-AGG-CAA-ATC-CAA-GTG-AAA-ATC-TC-3' and reverse primer: 5'-CGG-AAT-TCC-TTC-TTG-GTT-GGG-TCT-TC-3'. 50 pmol forward primer, 50 pmol reverse primer, 2 µl 6.25 mM dNTPs, 5 µl 25 mM $MgCl_2$, 5 µl 10X Taq reaction buffer, 2 U Taq polymerase and Milli-Q water to yield a final reaction volume of 50 µl. PCR conditions were as follows: 92°C (5 min); 5 cycles of 92°C (30 s), 50°C (30 s), 72°C (30 s); 25 cycles of 92°C (30 s), 70°C (30 s), 72°C (30 s); 72°C (5 min) and finally 4°C (10 min).

*2.2.2.4.7. Restriction endonuclease digestion*

All restriction enzymes and digestion buffers were obtained from Roche, Germany. DNA fragments and plasmids were digested using the following general protocol: Approximately 1.5 µg DNA was incubated in the presence of 2 µl 10X Buffer B (100 mM Tris-Cl, 50 mM $MgCl_2$, 1 M NaCl, 10 mM 2-mercapto-ethanol pH 8.0), 2 U of each restriction enzyme (BamHI, EcoRI or XbaI) and Milli-Q water to a final volume of 20 µl. Reactions were incubated for 3 h at 37 °C and stopped with 5 µl DNA-SAB. Agarose gel DNA standards were prepared in the same manner except 20 µg λ phage DNA and 40 U enzymes were used.

*2.2.2.4.8. Bacterial transformation*

Eppendorf tubes (1.5 ml) containing ligation reactions were centrifuged briefly in a microcentrifuge and 2 µl of each reaction mixture transferred to a fresh 1.5 ml Eppendorf

tube. Thawed *E. coli* DH5α competent cells (50 µl) were transferred carefully to each tube, the tube gently flicked and incubated on ice for 30 min. Cells were heat shocked by incubation at 42°C for 45 s followed by incubation on ice for 2 min. An aliquot of 950 µl 1.6 % (w/v) tryptone, 1 % (w/v) yeast extract, 0.5 % (w/v) NaCl growth medium was added to each tube and incubated for 2 h at 37°C. Cells were screened for successful transformation by plating 100 µl aliquots on LB agar plates containing 0.1 mg/ml ampicillin, 0.5 mM 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal, Bioline, USA) and 80 µg/ml isopropyl-β-D-thiogalactopyranoside (IPTG, Bioline, USA).

*2.2.2.4.9. Yeast transformation*

Electro-competent yeast cells were prepared as follows: an overnight W303 wildtype yeast culture was diluted to an optical density at 600nm ($OD_{600}$) of *ca.* 25 OD units/ml (*ca.* 2.5 x $10^8$ cells/ml), harvested by centrifugation at 3000 *x g* for 10 min at 4°C, washed twice with ice-cold sterile water, washed twice with ice-cold 1 M sorbitol, and then resuspended in a minimal volume of ice-cold 1 M sorbitol. Then, 1 µg DNA was added to 50 µl yeast cells and pulsed once for 5.1 ms on the SC2 setting using a Biorad Micropulser (Biorad, CA, USA). A 500 µl aliquot of ice-cold 1 M sorbitol was added immediately and the cells were plated on SC-URA medium containing 1 M sorbitol to select for positive transformants.

*2.2.2.4.10. Ligation into pYES2 and fidelity testing*

pYES2 is a 5.9 kb episomal shuttle vector designed for expression of recombinant proteins in *S. cerevisiae*. The multiple cloning site (MCS) and other features are depicted in Figure 2.2.2.

PCR products and the pYES2 plasmid were digested with the appropriate restriction enzymes and gel purified to remove contaminating fragments. The following ligation conditions were used for production of pYES2-GFP2: To obtain a vector:insert ratio of 1:1 and a final DNA concentration of 1 pmol/µl, 39.1 ng pYES2 and 4.7 ng GFP2 was

mixed with 2 µl 2X Rapid Ligation Buffer (60 mM Tris-Cl, 20 mM $MgCl_2$, 20 mM dithiothreitol (DTT), 2 mM adenosine triphosphate (ATP), 10 % (v/v) polyethylene glycol MW8000, Promega, USA), 3 U T4 DNA Ligase (Promega, USA) and Milli-Q water to a final volume of 10 µl. The reaction was incubated at 4°C overnight and transformed into *E.coli* DH5α competent cells. pYES2-HSP12+PROM-GFP2 (pYH12G2) was produced in a similar manner except that 43.8 ng pYES2-GFP2 and 6.16 ng HSP12+PROM was used. This product was transformed into *E.coli* DH5α and extracted.

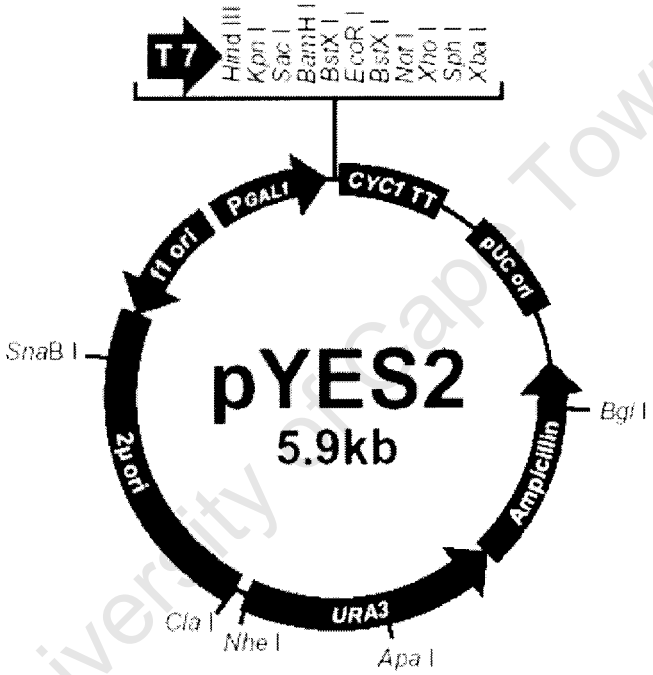



Figure 2.2.2. Vector map of the pYES2 yeast shuttle vector depicting the multiple cloning site, ampicillin resistance and uracil biosynthesis (*URA3*) genes, replication origins (2 µ ori, f1 ori and pUC ori), terminator (*CYC1*), T7 primer, the galactose promoter ($p_{GAL1}$) and various restriction digestion sites (Invitrogen).

The fidelity of the construct was verified by PCR using *GFP2* forward and reverse primers or *HSP12* forward and reverse primers to verify the presence of *GFP2* and *HSP12* respectively (Figure 2.2.3). The sequence of the construct was verified on a MegaBACE 500 DNA sequencer (Amersham Biosciences, UK) (data not shown) using the *GAL1* forward and reverse primers supplied with the pYES2 plasmid as per manufacturer's instructions. The construct was therefore deemed valid for insertion into yeast cells by transformation. Yeast cells were transformed with the construct by

electroporation and transformation verified by colony PCR (data not shown). For colony PCR, yeast cells were picked directly from freshly grown SC-URA plates and suspended in 100 µl 0.2 % (w/v) SDS. After incubation for 10 min at 95°C, 5 µl was used as a template in PCR using *HSP12* forward and *GFP2* reverse primers. The PCR reaction mixture contained 50 pmol forward primer, 50 pmol reverse primer, 2 µl 6.25 mM dNTPs, 5 µl 25 mM $MgCl_2$, 5 µl 10 X Taq reaction buffer, 2 U Taq polymerase and Milli-Q water to yield a final reaction volume of 50 µl. PCR conditions were as follows: 92°C (5 min); 5 cycles of 92°C (30 s), 44°C (30 s), 72°C (30 s); 25 cycles of 92°C (30 s), 68°C (30 s), 72°C (30 s); 72°C (5 min) and finally 4°C (10 min).

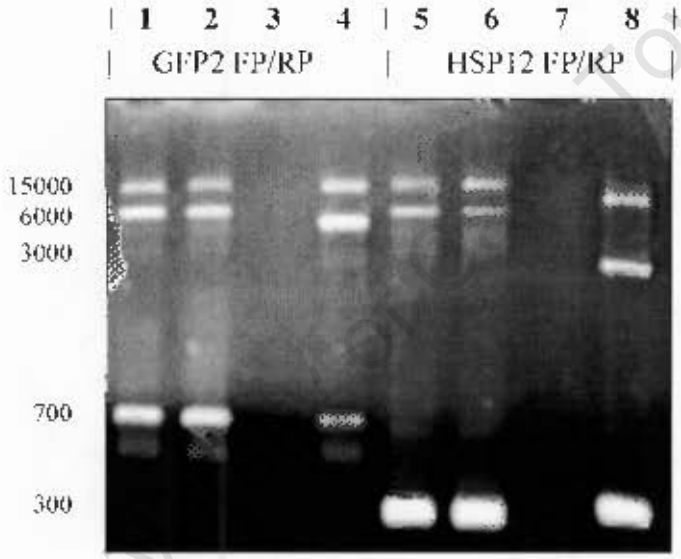



Figure 2.2.3. Confirmation of the fidelity of the pYH12G2 plasmid. Two individual clones (A and B) containing putative pYH12G2 were picked from selective agar plates, cultured, plasmid DNA extracted and subjected to PCR with either *GFP2* (Lane 1 – 4) or *HSP12* (Lane 5 – 8) primers. Lane 1: pYH12G2 template A; Lane 2: pYH12G2 template B; Lane 3: No template; Lane 4: pYES2-GFP2 template; Lane 5: pYH12G2 template A; Lane 6: pYH12G2 template B; Lane 7: No template; Lane 8: pGEM-T-HSP12 template. Approximate fragment lengths (base pairs) are indicated on the left.

### 2.2.2.5. Cell wall staining, epifluorescent and confocal microscopy

Differential cell wall staining was performed by suspension of cells (*ca.* 0.5 x $10^6$ cells/ml) in 100 µl of 1 mg/ml solution of Calcofluor White (Fluorescent Brightener 28, F-3543-1G, Sigma-Aldrich, USA) at RT for 10 min. This stain binds to chitin (found only

in the cell wall of yeast) and fluoresces with similar excitation and emission wavelengths to Gfp2p.

Fluorescence and phase contract microscopy was performed using an Axiovert 200-m microscope (Zeiss, USA) equipped with a 100-fold magnification oil immersion objective lens. Fluorescence data were obtained using 488 nm excitation and 515 – 565 nm emission filters and captured using an Axiocam camera equipped with Axiovision 3.1 software. Optical sections (1 μm) were captured using the Z-stack module of the Axiovision software, and the images were deconvoluted using the 3-D deconvolution module. Confocal microscopy was performed at the University of the Orange Free State (UOFS), Bloemfontein, RSA using a Nikon TE-2000 confocal laser scanning microscope (Nikon, Japan) with the assistance of Prof. P.W.J. van Wyk.

## 2.2.3. RESULTS

### 2.2.3.1. Cell wall protein extraction and analysis

Cell wall proteins were extracted from stationary phase yeast cells using 0.6 M NaOH and separated by SDS-PAGE (Figure 2.2.4). A large band corresponding to a molecular weight of approximately 11 kDa was noted (arrow).

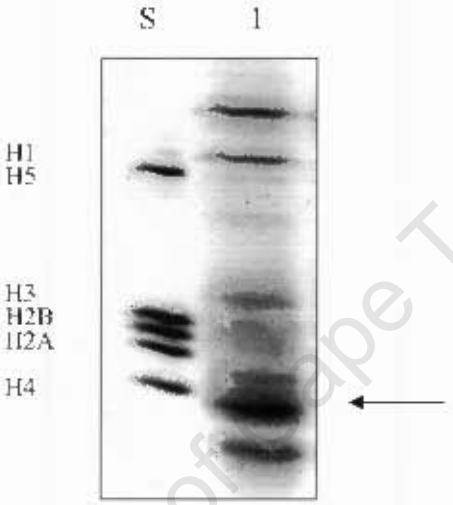



Figure 2.2.4. SDS-PAGE of proteins extracted with 0.6 M NaOH from whole yeast cells. The band corresponding to a molecular weight of *ca.* 11 kDa (Arrow, lane 1) was excised and subjected to peptide fingerprinting using MALDI-TOF mass spectrometry. Lane S contains an extract of total chicken histones, used as a standard. H1: 22.5; H5: 20.6; H3: 15.4; H2B: 13.7; H2A: 14.0 and H4: 11.2 kDa.

This band was excised and subjected to in-gel tryptic digestion and peptide fingerprinting using MALDI-TOF mass spectrometry. The tryptic fragment peaks were analysed using Peptident (http://www.expasy.ch/tools/peptident.html) which confirmed that the band corresponded to Hsp12p. The peptide masses obtained from MALDI-TOF are presented in Table 2.2.1.

Table 2.2.1. Peptide masses of the predominant 0.6 M NaOH extractable protein band digested with trypsin and subjected to MALDI-TOF mass spectrometry. Peaks not present (X) in Hsp12p were from trypsin, which digests itself during the digestion reaction.

| Mass (Da) | Presence in Hsp12 |
|---|---|
| 805.7594 | X |
| 827.6368 | ✓ |
| 849.527 | ✓ |
| 1059.664 | ✓ |
| 1140.249 | ✓ |
| 1175.255 | ✓ |
| 1223.244 | ✓ |
| 1273.976 | ✓ |
| 1297.448 | ✓ |
| 1314.939 | ✓ |
| 1438.48 | ✓ |
| 1501.445 | ✓ |
| 1531.938 | ✓ |
| 1561.46 | ✓ |
| 1601.676 | ✓ |
| 1746.323 | ✓ |
| 1784.302 | ✓ |
| 1845.984 | ✓ |
| 1887.962 | ✓ |
| 1941.41 | ✓ |
| 2009.966 | ✓ |
| 2083.552 | ✓ |
| 2162.563 | X |
| 2201.232 | ✓ |
| 2276.541 | X |
| 2353.591 | ✓ |
| 2412.908 | ✓ |
| 2464.507 | ✓ |
| 2530.556 | ✓ |
| 2569.614 | ✓ |
| 2614.217 | ✓ |
| 2647.797 | ✓ |
| 2722.394 | ✓ |
| 2800.927 | X |
| 3000.932 | ✓ |

The gel was repeated and subjected to western blotting using the anti-Hsp12p antibody. Unfortunately, the antibody was not of good quality and displayed cross-reactivity by displaying an affinity for Hsp12p as well as two other proteins (data not shown).

### 2.2.3.2. Expression and visualisation of the Hsp12-Gfp2p fusion protein

Yeast cells containing pYH12G2 were grown in YEPD for approximately 6 h till the culture had attained an optical density of approximately 0.3 ODU/ml. No green fluorescent protein (GFP) fluorescence was observed when exponentially growing yeast cells, grown in glucose-containing medium, were examined using an epifluorescent microscope. This agrees with previous studies that expression of *HSP12* is suppressed by even small quantities of glucose in the growth medium (de Groot *et al.*, 2000). Continued growth into the stationary phase (*ca.* 5 ODU/ml) resulted in the yeast appearing fluorescent (Figure 2.2.6 B). All the yeast cells appeared to fluoresce to approximately the same extent, suggesting that *HSP12* was uniformly induced under these conditions.

The *HSP12-GFP2* fusion protein product (Hsp12-Gfp2p) was localized throughout the yeast cell, except in the dark region in the centre of the cell. This was assumed to be a vacuole because an outline was visible under phase contrast microscopy (Figure 2.2.6 A). Yeast *ade2* cells grown under adenine-limiting conditions are known to accumulate a pink pigment in the vacuole (Weisman *et al.*, 1987). This pigment coincidentally fluoresces with similar excitation and emission maxima to Gfp2p, allowing the use of the same filter set on the fluorescence microscope. Examination of yeast cells containing pYH12G2 grown under adenine-limiting conditions displayed fluorescence throughout the cell (Figure 2.2.6 C). Thus the dark region in the centre of the yeast cell was indeed the central vacuole and Hsp12-Gfp2p was present throughout the remainder of the cell.

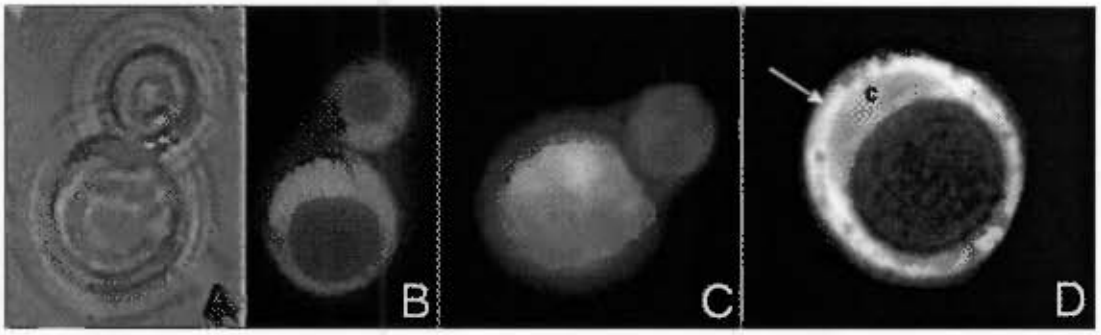



Figure 2.2.6. The Hsp12-Gfp2p fusion protein is localized to the cytoplasm, the cell wall, and the tonoplast of the yeast cell. Phase contrast microscopy (A) and corresponding epifluorescence microscopy (B-D). The large nonfluorescent region in the centre of the cell (B) is the vacuole. In (C), the vacuole also fluoresces due to accumulation of fluorescent *ade2* pathway intermediates. (D) is a deconvoluted cross section of a fluorescent yeast cell. The fusion protein is clearly visible throughout the cell but appears more concentrated at the cell periphery (arrow) and tonoplast than in the cytoplasm (c).

Previous studies have shown that Hsp12p is present both in the cytoplasm, in close proximity to the plasma membrane, and also in the cell wall (Motshwene *et al.*, 2004). A series of images, focused on different focal planes of the yeast cell approximately 1 µm apart, was captured. These images were digitally deconvoluted, allowing the observation of the fluorescence in different planes of the yeast cell. Fluorescence was observed in the cytoplasm, the cell periphery and the tonoplast. A view of the central plane of the yeast cell is shown (Figure 2.2.6 D). This suggested that the localization of Hsp12-Gfp2p was the same as for the native protein, namely in the cell wall and cytoplasm. Subsequent studies using confocal microscopy revealed that very little Hsp12-Gfp2p was present in the cell wall, presumably due to the presence of the large Gfp2p protein size (data not shown). Differential staining of the cell wall using Calcofluor White and alignment with images of cells expressing Hsp12-Gfp2p confirmed these observations (data not shown).

### 2.2.4. DISCUSSION

A host of proteins in the yeast cell have recently been discovered in the cell wall. These proteins, including metabolic enzymes such as enolase (Pardo *et al.*, 1999) and phosphoglycerate mutase (Motshwene *et al.*, 2003), have no apparent cell wall localization sequences and the mechanism of translocation and indeed the function of these proteins in this locus remains a mystery. One possible explanation is that during periods of stress, the yeast cell must rapidly remodel its cell wall (Firon *et al.*, 2004) to accommodate for changes in cell volume and membrane fluidity. This is achieved by energy-dependent vesicular transport of cell wall supporting enzymes such as β-glucan synthases and chitin synthases. Thus, metabolic enzymes may produce the energy needed for these processes.

The presence of Hsp12p and Hsp26p (van Rooyen, J., unpublished data) in the cell wall is peculiar. These proteins possess no known targeting sequences but are produced in the cell wall in abundance during stressful conditions. However, other proteins possessing no signal sequence have been reported to exit the yeast cell via non-classical protein export pathways (Cleves *et al.*, 1996).

The fact that Hsp12p is extractable by β-elimination using NaOH indicates that the protein is attached either via a strong alkali-labile linkage or is simply free in the periplasmic space. Confocal microscopy revealed that Hsp12-Gfp2p is strongly expressed throughout the cytoplasm and near the tonoplast but not in the cell wall, although it can be argued that this is due to Gfp2p interfering with Hsp12p targeting either sterically (Gfp2p is three times larger than Hsp12p) or by Gfp2p blocking unknown targeting sequences. This may also explain the findings obtained in the *S. cerevisiae* protein localization project (Huh *et al.*, 2003) where Hsp12p is described as possessing a cytoplasmic locus. Indeed these authors also found that several other known cell wall proteins were not localized to the cell wall.

## 2.3. *APPLICATIONS OF YEAST CELLS EXPRESSING FLUORESCENT HSP12P*

### 2.3.1. INTRODUCTION

*S. cerevisiae* is an essential industrial organism used in the baking industry as well as for many fermentative and biotechnological applications. This yeast is often subjected to a variety of stressful conditions during these processes, which may adversely affect the final industrial product. As an example, during the brewing process the yeast is subjected to stresses including osmotic, temperature and mechanical as well as to increasing concentrations of ethanol as fermentation progresses. Currently brewers assess the number of fermentatively active yeast cells by determining the viability of the cells using methylene blue exclusion. Since this method has been reported to both under- and over-represent the number of cells present, flow cytometric methods have been recently introduced to more accurately evaluate the number of viable cells present in a culture (Boyd *et al.*, 2003). Since viability is not an indicator of fermentative capacity and since yeast is used reiteratively in the brewing process, the brewing industry requires a method to relate the stress status of the yeast to the fermentative capacity. This will allow industrial applications to be tailored to minimally stress the yeast whilst still allowing optimal output.

The synthesis of Hsp12p is induced by a wide variety of stresses including high osmolarity, oxidative stress, heat shock, the presence of ethanol, nutrient limitation by growth on non-fermentable carbon sources and by agents affecting cell wall integrity (Praekelt and Meacock, 1990; Gupta *et al.*, 1994; Varela *et al.*, 1995; Siderius *et al.*, 1997; Mtwisha *et al.*, 1998; Sales *et al.*, 2000; Garcia *et al.*, 2004). The *HSP12* promoter region contains numerous known stress-related response elements within 700 bp of the start of transcription, thus emphasising the importance of this gene in the stress response of *S. cerevisiae* (Chapter 1, section 1.2.1). It is therefore not surprising that *HSP12* expression was one marker used to analyse the stress resistance of commercial wine yeast strains (Carrasco *et al.*, 2001). Analysis of gene expression is widely used as a determinant of changes that occur due to changes in, for example, growth conditions.

Such analyses assume that the corresponding protein synthesis occurs, although it has been documented that this is not always the case (Gygi *et al.*, 1999; Li *et al.*, 2003). Additionally it has been shown that yeast grown continually at elevated temperature have decreased Hsp12p present (Mtwisha *et al.*, 1998) despite evidence that *HSP12* expression is up-regulated after heat shock (Praekelt and Meacock, 1990).

Experiments were thus performed to ascertain whether fluorescent Hsp12p might be a good indicator of yeast stress. Such a fluorescent protein would not only reassure the investigator that protein synthesis had indeed occurred but determining the fluorescence of a yeast culture would be a far simpler, quicker and cheaper methodology than either northern blotting or flow cytometry. A fusion protein was constructed by fusing the green fluorescent protein (*GFP2*) gene downstream and in-frame to that of *HSP12* and using the *HSP12* promoter to drive synthesis of this construct. It was found that yeast containing this plasmid were not fluorescent when grown on media containing glucose but responded to a variety of stresses by exhibiting significant fluorescence, which could readily be quantified.

## 2.3.2. MATERIALS AND METHODS

### 2.3.2.1. Materials

Materials for the production, selection and growth of the yeast cells expressing Hsp12-Gfp2p fusion proteins (pYH12G2 cells) are detailed in section 2.2. All chemicals used were of high purity and water was deionised and distilled.

### 2.3.2.2. Organisms and culture conditions

The yeast strain containing the pYH12G2 plasmid expressing Hsp12-Gfp2p was constructed as described in section 2.2.2.4. Yeast cells were cultured as previously described in section 2.2.2.2.

### 2.3.2.3. Stress assays and fluorimetry

Yeast cells containing pYH12G2 were grown in YEPD at 30°C to early log phase (0.3 - 0.5 ODU/ml). For stress studies, cells were subjected to various stresses by adding the stressing agent (NaCl, ethanol or mannitol) directly to the culture after which samples were taken after various times. In the case of temperature shock, cells were shifted to 37°C for heat shock or 4°C for cold shock. In a typical assay, 500 µl of culture was transferred to a 1.5 ml Eppendorf tube at each timepoint, the cells pelleted by centrifugation in a microcentrifuge for 5 seconds, the cell pellets snap-frozen in liquid nitrogen and stored at –20 °C. Once all samples were collected, frozen cell pellets were resuspended in 1 ml PBS and the fluorescence was measured using an Aminco SPF-500 fluorimeter (American Instrument Company, USA) using an excitation wavelength of 450nm and an emission wavelength of 507 nm (Cormack *et al.*, 1996). The $A_{600}$ was determined using a Shimadzu UV-2201 spectrophotometer (Shimadzu Corporation, Japan).

## 2.3.3. RESULTS

### 2.3.3.1. Salt induces *HSP12* in a concentration-dependent manner

Yeast cells harbouring the pYH12G2 plasmid were grown to early log phase in YEPD medium, at which time various concentrations of salt were added. Cells were removed after 40, 60 and 80 minutes and the relative fluorescence determined (Figure 2.3.1). Whereas no fluorescence was observed if salt was omitted from the growth medium, the presence of 200 mM salt resulted in noticeable fluorescence after 40 min. No change in the fluorescence was observed after 60 min growth and a decreased fluorescence was observed after 80 min growth. When the NaCl concentration was increased to 400 mM, the fluorescence observed after 40 min increased to approximately 150 % of that observed with 200 mM salt. In addition, the fluorescence continued to increase, with the fluorescence observed after 60 min being approximately 30 % greater than that observed after 40 min and approximately double that observed in the presence of 200 mM NaCl after 60 minutes. The fluorescence observed at 60 min was the maximum fluorescence observed, with a slight decrease found after 80 min growth. In contrast, the presence of 800 mM salt resulted in a slight increased fluorescence only after 80 min growth. Interpretation of this data is that the presence of salt in the medium resulted in the production of Hsp12-Gfp2p despite the presence of glucose. The amount of Hsp12-Gfp2p synthesised reflected the stress that the yeast were subjected to, up to 400 mM salt.



### 2.3.3.2. Osmotic shock and ethanol stress induce *HSP12*

Yeast containing the pYH12G2 plasmid were grown to early log phase, at which time 800 mM mannitol was added to the medium. This concentration was chosen as it resulted in the same increase in medium osmolality as the addition of 400 mM NaCl. Cells were removed at various times and the relative fluorescence determined (Figure 2.3.2). The timing of the response of these yeast cells to mannitol appeared very similar to that seen when the medium osmolality was increased by the addition of 400 mM NaCl with a peak after 65 min and a slight decrease thereafter.

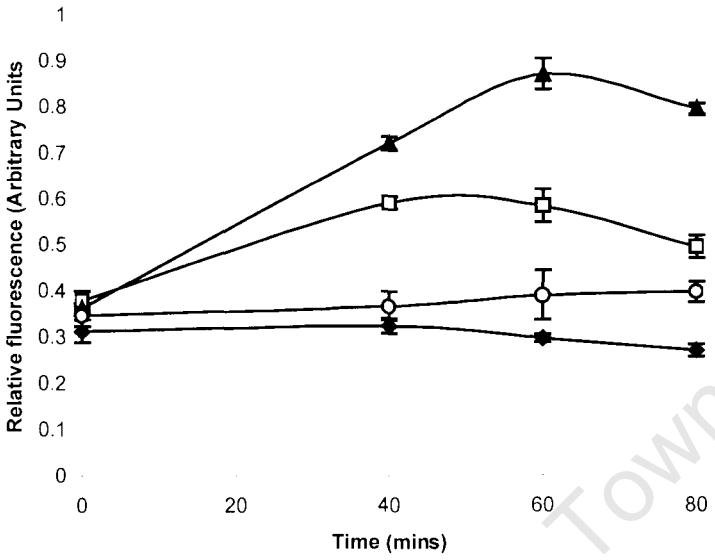



Figure 2.3.1. Various concentrations of NaCl were added to exponentially growing yeast cells and the fluorescence determined at various times thereafter. Control (YEPD only): •; 200 mM NaCl: □; 400 mM NaCl: ▲; 800 mM NaCl: ○. The data shown are the means and standard deviations from three separate experiments. Error bars not visible are within the data points.

Previous studies have shown that the presence of 5 % ethanol in the growth medium resulted in the induction of Hsp12p and that Hsp12p protected the yeast cell against the deleterious effects of ethanol (Sales *et al.*, 2000). To investigate the effect of ethanol on Hsp12p synthesis, yeast cells containing the pYH12G2 plasmid were grown to mid-log phase (*ca.* 0.5 ODU/ml), at which time 7 % v/v ethanol was added to the medium. This concentration was chosen since this concentration was used by another study which investigated the global gene response of *S. cerevisiae* to ethanol (Alexandre *et al.*, 2001). The kinetics of the response to ethanol was markedly different to those observed for NaCl and mannitol, with fluorescence only observed after 50 min incubation; thereafter the fluorescence continued to increase throughout the duration of the experiment (Figure 2.3.2).

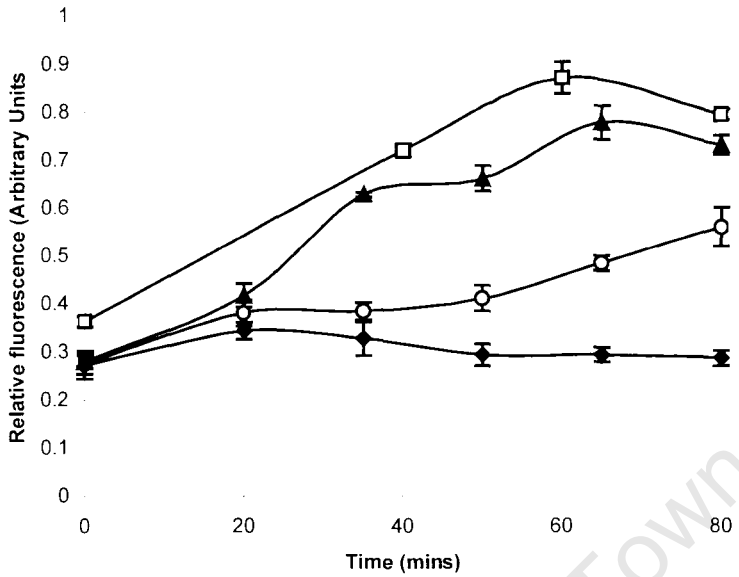



Figure 2.3.2. 7 % ethanol (○) or 800 mM mannitol (▲) was added to exponentially growing yeast cells and the fluorescence determined at various times thereafter. Control (YEPD only): ●. The response to 400 mM NaCl (□) is shown for comparison since 800 mM mannitol imparts an equivalent increased osmolality to 400 mM NaCl. The data shown are the means and standard deviations from three separate experiments.

### 2.3.3.3. Heat shock but not cold stress induces Hsp12p synthesis

Yeast containing the pYH12G2 plasmid were grown to early log phase at 30°C before the growth temperature was changed to 37°C. This heat shock resulted in a fluorescent response with a magnitude similar to that seen with 200 mM NaCl (Figure 2.3.3). In contrast, changing the temperature to 4°C resulted in little fluorescence being observed (Figure 2.3.3).

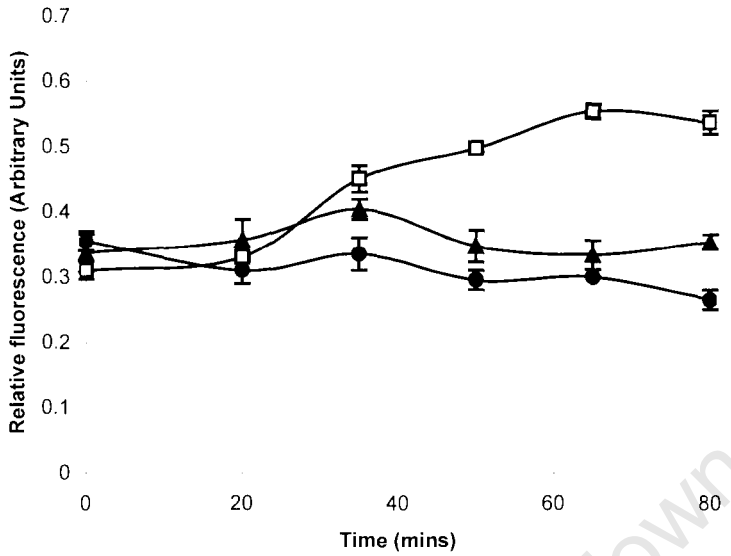



Figure 2.3.3. The growth temperature (30 °C) of exponentially growing yeast cells was changed to either 37 °C (□) or to 4 °C (▲) and the fluorescence determined at various times thereafter. Control (continued growth at 30 °C): ●. The data shown are the means and standard deviations from three separate experiments. Error bars not visible are within the data points.

### 2.3.4. DISCUSSION

Yeast containing the pYH12G2 plasmid grew with a normal appearance and at the same rate as yeast without this plasmid. No GFP fluorescence was observed when exponentially growing yeast grown in glucose-containing medium were examined using a fluorescence microscope. This agrees with previous studies that expression of the *HSP12* gene is suppressed by even small quantities of glucose in the growth medium (de Groot *et al.*, 2000). Continued growth into stationary phase resulted in the yeast appearing fluorescent, which was due to *HSP12* induction upon entry into stationary phase (Varela *et al.*, 1995). All the yeast cells appeared to fluoresce to approximately the same extent, suggesting that the *HSP12* gene was uniformly induced under stressful conditions.

Upon salt stress, yeast cells activate the High Osmolarity Glycerol (HOG) pathway, which acts to synthesize glycerol and stress-responsive proteins to alleviate the symptoms of this stress (Brewster *et al.*, 1993). When cells containing pYH12G2 were stressed with various concentrations of salt, Hsp12-Gfp2p was expressed in a dose-dependent manner analogous to native Hsp12p and could be used as a marker to quantify the extent of induction of stress-responsive proteins and hence the stress status of the cell. The anomalous result after the addition of 800 mM salt was attributed to the fact that such a severe stress either inhibited the yeast transcriptional and translational machinery or that the transcriptional and translational machinery was dedicated to systems involved in $Na^+$ efflux, such as the $Ca^{2+}$/calmodulin-dependent phosphoprotein phosphatase (calcineurin) pathway (Hirata *et al.*, 1995).

Previous studies have shown that the presence of 800 mM mannitol in the growth medium results in the additional induction of *HSP12* during early (and late) stationary phase (Mtwisha *et al.*, 1998). The magnitude of the mannitol response was slightly lower than that with NaCl suggesting that NaCl triggered expression of *HSP12* by mechanisms in addition to the HOG pathway, such as the cell integrity pathway (Garcia *et al.*, 2004).

The mechanism of induction of Hsp12p by ethanol was clearly different to that by osmolytes. It has been shown that one effect of ethanol on yeast cells is that there is a change in the lipid composition (Gupta *et al.*, 1994) and it has been postulated that the resultant change in membrane fluidity somehow triggers expression of *HSP12* (Piper, 1995) since heat shock, which also causes increased membrane fluidity, is a potent stimulant of *HSP12* expression (Praekelt and Meacock, 1990).

Finally, when yeast cells were exposed to heat stress, *HSP12* was again induced, but to a lesser extent than salt stress. Interestingly, despite several reports that *HSP12* is induced by cold stress (Rodriguez-Vargas *et al.*, 2002; Sahara *et al.*, 2002; Kandror *et al.*, 2004), very little fluorescence was detected in these experiments, but it should be noted that induction of cold-responsive genes often takes several hours (Kandror *et al.*, 2004).

In summary, the construct presented here, a fusion protein between the stress response protein Hsp12p and the fluorescent indicator protein Gfp2p, allowed quantification of the response of *S. cerevisiae* to a variety of different forms of stress typically encountered both in an industrial and a laboratory environment. In addition, this construct allowed conclusions to be drawn regarding the regulatory mechanisms of *HSP12*, since relative fluorescence indicated the extent of regulation of *HSP12* in response to various stress conditions. Thus *HSP12* was induced most strongly following salt stress, followed by general hyper-osmotic shock, ethanol, heat and cold shock. This correlates well with assumptions made in Chapter 1, section 1.2.5, where salt stress was postulated to act via other pathways in addition to the HOG pathway.

It is envisaged that this fluorescent construct will be useful to allow processes to be adapted to result in the minimal stress to the yeast whilst allowing maximum output. This will be of particular importance for processes that use the yeast preparation reiteratively.

# CHAPTER 3
# THE FUNCTION OF HSP12P

## 3.1. SUMMARY

The expression of Hsp12p under stressful conditions and the localization of this protein in the yeast cell wall prompted investigations into whether Hsp12p was responsible for cell wall adaptations and integrity in response to stress conditions. Yeast cells were treated with the cell wall damaging agent, Congo Red (CR), and assayed for changes in viability and morphology. CR affected the cells dramatically and to determine whether Hsp12p was involved in these differences, yeast cells lacking Hsp12p (the *hsp12ΔURA3* / knockout / KO strain) were exposed to similar conditions and assayed. The KO strain displayed an increased sensitivity to CR. In addition, only the WT strain displayed dimorphic growth (invasive growth) when grown for prolonged periods on CR-supplemented agar plates. The morphology and nature of invasive growth was investigated, since the W303 yeast strain does not normally exhibit this property. Cells under the agar surface were examined microscopically and found to form clumps and prolate spheroids. Restoration of invasive growth in W303 by CR was attributed to the formation of cellular chains, which allowed agar penetration, sub-surface cell division, water depletion, cracking of the agar and spheroid formation. CR also affected the aggregation of cells and resulted in flocculation solely in KO cells, implicating Hsp12p in the prevention of cell aggregation. Furthermore, KO cells displayed a marked increase in clumping, even in the absence of CR. Since clump formation resulted in flocculation, settling experiments were conducted to quantify these observations. Stationary phase KO cells settled faster than WT cells, implicating Hsp12p in the flocculation process. Hsp12p may have mediated these effects by altering cell surface hydrophobicity, a factor affecting flocculation, due to its high hydrophilic amino acid content (Chapter 1, section 1.1.2). Hydrophobicity was thus compared in the WT and KO strains, but was not markedly different in the two strains. This implied a different mechanism whereby Hsp12p affected flocculation, which possibly involved alteration of the cell wall chitin content. Furthermore, Hsp12p inhibited CR binding to chitin *in vitro*, suggesting that Hsp12p may act as a chitin protectant.

The Hsp12p and CR-dependent invasion and flocculation may also have been due to restoration of function of flocculin activity in W303, which cannot normally induce Flo11p expression due to a lack of the Flo8p transcription factor (Liu *et al.*, 1996). The regulation of the flocculins, *FLO1*, *FLO5*, *FLO8*, *FLO9* and *FLO11* was therefore investigated by real-time quantitative polymerase chain reaction (qPCR), but results indicated that flocculin expression was not restored in either strain. Thus CR acted independently of flocculins on yeast cell division to stimulate invasion and flocculation.

These phenotypes (cellular aggregation and sensitivity to CR) were similar to those observed for cells lacking cell wall-localised PIR proteins (proteins with inverted repeats). These proteins, like Hsp12p, were alkali-extractable and possessed no known function. The WT and KO strains were thus tested for differences in the expression of these PIR proteins by biotinylation and mild alkaline extraction, but no difference was observed.

Motshwene *et al.* showed that Hsp12p was required to enhance the barotolerance of yeast cells, possibly by increasing cell wall flexibility (Motshwene *et al.*, 2004). This hypothesis was tested by employing agarose as a model system for the yeast cell wall. Studies on this polymer indicated that various stresses, including salt and alcohols, hardened the agarose matrix, whereas Hsp12p softened it.

The effects of Hsp12p on cell wall properties were ascertained for live yeast cells using Atomic Force Microscopy (AFM). AFM measurements confirmed that Hsp12p played a role in promoting cell wall flexibility. Further studies of the permeability of WT and KO cells by electrophoretic mobility assays revealed that KO cells were slightly more permeable and exhibited impaired mobility, possibly due to an increase in cell wall stiffness. Furthermore, the KO strain had altered cell wall chemistry, as revealed by infrared spectroscopy, with lowered levels of phosphorylated peptidomannans and glucans. These differences were possibly due to decreased cell wall flexibility.

In summary, the results suggest that Hsp12p may function in the cell wall as:

- A protectant, by shielding chitin from cell wall damaging agents such as CR which allows invasive growth and viability.
- A plasticizer to maintain cell wall flexibility, which is required for the control of calcium flux and consequently the amount of polysaccharides in the cell wall.

## 3.2. *HSP12P ENHANCES THE ADAPTATION OF YEAST CELLS IN RESPONSE TO CELL WALL DAMAGE BY CONGO RED (CR) AND IMPAIRS THE FLOCCULATION OF YEAST CELLS*

### 3.2.1. INTRODUCTION

Under favourable growth conditions, cells of the budding yeast *Saccharomyces cerevisiae* exist predominantly in the unicellular "globular" form (Kron *et al.*, 1997). However, when cells are exposed to a variety of stressful conditions including nutrient limitation, mild heat shock, osmotic stress, the presence of aliphatic alcohols, cell wall damaging agents or an increase in the extracellular cAMP concentration, certain strains of yeast undergo a morphological transition (Gimeno *et al.*, 1992; Zaragoza and Gancedo, 2000). The type of transition is determined by the ploidy of the yeast cell. Pseudohyphal growth occurs during nitrogen-limiting conditions in diploid cells. The cells elongate, bud in a unipolar fashion, do not separate and appear as chains of multinucleated hyphal structures. In contrast, haploid cells display invasive growth when cultured for extended periods on nutrient rich agar plates. Invasive growth is similar to pseudohyphal growth, but elongation is not as pronounced and multinucleated structures are not observed (Gimeno *et al.*, 1992; Roberts and Fink, 1994). Many laboratory strains, including the commonly used W303 strain, have been selected for their inability to flocculate and invade nutrient rich agar plates (Liu *et al.*, 1996). The inability to undergo these adaptations has been ascribed to a lack of flocculin function, particularly Flo11p. Flo11p is involved in morphological adaptations and calcium-dependent cell-cell adhesion in response to nutrient deprivation (Lo and Dranginis, 1996).

Invasive growth has been reported to occur after addition of the dye, Congo Red (3,3'-[[1,1'-biphenyl]-4,4'-diylbis(azo)]-bis[4-amino-1-naphtalenesulfonic acid] disodium salt), even in strains such as W303 not normally associated with this form of growth (Zaragoza and Gancedo, 2000). This dye interferes with cell wall assembly and cell separation by complexing with chitin (poly-N-acetyl-D-glucosamine) present in the cell walls of yeast (Pancaldi *et al.*, 1985; Vannini *et al.*, 1985; Kopecka and Gabriel, 1992). Electron

diffraction studies have demonstrated an interaction between CR and newly synthesised chitin *in vitro* (Bartnicki-Garcia *et al.*, 1994). Chitin, an important constituent of the yeast cell wall, is found in small amounts dispersed over the entire cell periphery in normally growing unbudded yeast cells (Molano *et al.*, 1980). Chitin is the primary component of the primary septum, a stabilising ring which facilitates cytokinesis during the budding process (Cabib, 1981). Chitin is thus essential for cell separation (Bulawa, 1993; Cabib *et al.*, 2001). Furthermore, chitin appears to act as a compensatory element for cell wall integrity, since chitin levels are enhanced in response to cell integrity stress and in cell wall mutants (Popolo *et al.*, 1997; Dallies *et al.*, 1998; Ram *et al.*, 1998). These mutants include those lacking glucan synthase (Fks1p) activity and *gas1Δ* mutants, which cannot insert β-1,3-glucans into the cell wall but instead secrete this compound into the medium (Ram *et al.*, 1998).

A recent microarray study showed that incubation of yeast cells with CR or Zymolyase resulted in the induction of numerous genes including *HSP12*. These authors did not detect any difference in sensitivity to CR between the BY4741 strain used and an *hsp12Δ* mutant of this strain (Garcia *et al.*, 2004). However, Motshwene *et al.* found that W303 KO cells were more sensitive to CR (Motshwene *et al.*, 2004). The genetic background of the yeast strain used is thus of paramount importance for the display of phenotypes due to certain mutations, as has been noted by other authors (Rondón *et al.*, 2003).

Flocculation is the process whereby yeast cells aggregate into clumps (flocs) and sediment (lager yeasts) or rise (ale yeasts) out of solution (Verstrepen *et al.*, 2003). This process is exploited in the brewing industry to allow for the rapid separation and re-use of yeast cells in the fermentation process. Flocculation is governed by the presence of proteins known as flocculins, which are activated by calcium and bind mannose residues in other cells (Bony *et al.*, 1997). Two types of flocculation have been identified, dependent on the specificity for mannose residues. NewFlo type flocculation is inhibited by the presence of glucose, maltose, mannose and sucrose whereas Flo1 type flocculation is inhibited only by mannose (Stratford and Assinder, 1991). Most laboratory strains, including W303, tend not to flocculate due to lack of flocculin induction by the Flo8p

transcription factor (Sieiro *et al.*, 1995; Liu *et al.*, 1996). Besides flocculin-mannose interactions, flocculation is dependent on physical interactions between cells. This includes the frequency of collisions between cells, the hydrophobicity and the number of negatively charged groups in the cell wall (Stratford, 1992). Yeast cell texture also appears to play a role, since older cells often have a rougher surface texture, facilitating adherence and settling faster than younger cells (Barker and Smart, 1996). Furthermore, pH, temperature and ethanol content influence flocculation, presumably by alteration of cell surface charge, flocculin conformation or flocculin expression (Jin and Speers, 2000, Jin *et al.*, 2001). These parameters are, however, strain specific (Jin and Speers, 2000). Flocculation is typically initiated during stationary phase, when nutrients are limited (Stratford, 1992). This process appears to be dependent on oxygen content, since poorly aerated cultures display early, incomplete flocculation, while normal aeration results in delayed, strong flocculation. Addition of oleic acid or ergosterol to poorly aerated cultures, however, restores normal flocculation ability, indicating that oxygen may act indirectly by regulating unsaturated fatty acid and sterol synthesis (Straver *et al.*, 1993).

## 3.2.2. MATERIALS AND METHODS

### 3.2.2.1. Materials

Yeast extract, peptone, tryptone and bacteriological agar for growth media were supplied by Biolabs (RSA). All other chemicals used were of high purity. Double distilled water was used for all experiments except for PCR and Realtime PCR reactions, where double distilled water filtered through a Milli-Q filtration apparatus (Millipore, USA) was used.

### 3.2.2.2. Organisms and culture conditions

The *S. cerevisiae* yeast strains used were from the W303 background (a/α, *ade2-1/ade2-1, trp1-1/trp1-1, leu2-3/leu2-112, his3-11/his3-15, ura3/ura3, canr1-100/CAN*) and were used in the haploid form. Yeast strains were kind gifts from Dr P. Meacock, University of Leicester, Leicester, U.K (wildtype, WT and *hsp12ΔURA3*, knockout, KO). The KO strain was constructed and tested as described (Praekelt and Meacock, 1990). Yeast cells were routinely grown in 1 % (w/v) yeast extract, 2 % (w/v) peptone, 2 % (w/v) glucose (YEPD) media at 30°C on a rotary shaker at *ca.* 200 rpm. For solid media (plates), agar was added to a final concentration of 1.5 % (w/v). Yeast growth was monitored by measurement of the optical density of the culture at 600nm and expressed in optical density units (ODU)/ml.

### 3.2.2.3. Congo Red (CR) assays

#### *3.2.2.3.1. Morphology, viability and flocculation*

A 1 ml aliquot from an overnight culture of WT or KO yeast cells was inoculated into 50 ml fresh YEPD medium and allowed to grow to mid-exponential phase (0.5 – 0.6 ODU/ml). Cell viability was determined by spotting 10 µl volumes of 10-fold serial dilutions onto YEPD agar plates containing 0.01, 0.02 and 0.03 mg/ml CR (BDH Laboratory Supplies, England). The plates were inspected and photographed after growth

at 30°C for 2 days. For morphology studies, CR was added to mid-exponential phase cells to a final concentration of 0.01, 0.02, 0.05, 0.1, 0.2 or 0.3 mg/ml which were then allowed to grow at 30°C for a further 20 h. Cells were then examined using an Olympus CK40-F200 microscope (Olympus Optical Co., Japan). CR-dependent flocculation was quantified by transferring 1 ml culture to a 3 ml spectrophotometric cuvette, vigorously shaking and then allowing the cells to settle whilst measuring the decrease in solution turbidity over time using a Beckman DU-650 Spectrophotometer (Beckman, CA, USA) at 600 nm. CR-independent flocculation was determined by transferring 5 ml of an overnight culture of WT or KO cells to a glass test tube (13 x 100 mm) and vortexing. Cultures were then allowed to settle and photographed at various time intervals. Flocculation rate, which resulted in a gradual decline of the turbidity of the solution, was determined by measuring the rate of change of the cleared height of the cells in solution. Spectrophotometry was not used as for CR-dependent flocculation experiments since yeast cells sedimented *ca.* 12X slower and the clear region was above the incident beam of the spectrophotometer. The contribution of hydrophobicity to CR-independent flocculation behaviour was quantified as described (Reynolds and Fink, 2001). Briefly, WT or KO cells were grown in YEPD to mid-exponential phase, washed once in water, and resuspended in 1 % (w/v) yeast extract, 2 % (w/v) peptone, 0.1% (w/v) glucose to a concentration of *ca.* 0.5 ODU/ml. Cells were incubated at RT for 3 h after which the $OD_{600}$ was measured. A 1.2 ml aliquot of the culture was transferred to a borosilicate glass test tube (13 mm X 100 mm), 600 µl octane added and the tube vortexed for 3 min. Aqueous and organic phases were allowed to separate, the $OD_{600}$ of the aqueous layer measured and compared to the initial $OD_{600}$ before octane addition.

*3.2.2.3.2. Invasive growth*

A 10 µl aliquot of mid-exponential phase cultures of WT or KO cells in YEPD medium was spotted onto YEPD plates with or without a sublethal dose of CR (0.02 mg/ml). The plates were incubated for up to 6 days at 30°C; non-invasive colonies were removed under running water whilst rubbing the surface with a latex rubber gloved finger (Zaragoza and Gancedo, 2000). Plates were photographed before and after washing.

Since abnormal structures and growth were observed when invasive yeast cells were observed microscopically, yeast cells were examined using confocal microscopy. The experiment was therefore repeated using a yeast strain containing the fluorescent Hsp12p construct (pYH12G2) produced as outlined in Chapter 2. This provided fluorescence to allow for 3-D imaging using a Nikon TE-2000 confocal laser scanning microscope (Nikon, Japan), since Hsp12p-Gfp2p was induced in response to CR.

*3.2.2.3.3.* In vitro *chitin binding*

The *in vitro* interaction between Hsp12p and chitin was investigated by suspending crab shell chitin (C-9213, Sigma-Aldrich, USA) at a final concentration of 15 mg/ml in distilled water together with purified Hsp12p up to a final concentration of 2.5 mg/ml at RT. CR solution (final concentration 5 μg/ml) was added after 5 min and after a further 5 min, the suspension was centrifuged (12000 *x g*, 5 min) and the absorbance of the supernatant at 500 nm determined.

### 3.2.2.4. PIR protein analysis

The clumping and CR sensitivity phenotypes observed were similar to those observed in cells lacking the cell wall-localised PIR proteins, Ccw5p, Ccw6p, Ccw7p and Ccw8p. These PIR proteins, members of a family of proteins containing one or more internal repeats and an N-terminal signal peptide, have unknown functions (Toh-E *et al.*, 1993; Mrša and Tanner, 1997).These proteins are putatively *O*-linked to β-1,3-glucans in the cell wall and are extractable with mild alkali solutions. Potential differences in the protein expression levels of these PIR proteins in WT and KO cells were investigated by biotinylation essentially as previously described (Mrša *et al.*, 1999). Overnight cultures (50 ml) of WT and KO cells were harvested by centrifugation at 3000 *x g* for 10 min at 10°C. Cells were resuspended in 1 ml 50 mM potassium phosphate buffer pH 8.0 (Buffer K), transferred to a 1.5 ml Eppendorf tube, washed once with the same buffer by microcentrifugation for 30 seconds at 4°C and resuspended in 1 ml of 1 mg/ml EZ-Link Sulpho-NHS-LC-Biotin (#21335, Pierce Biotechnology, USA). After incubation for 1 h

at RT, cells were washed once with 50 mM Tris-Cl pH 7.5, twice with Buffer K after which 200 µl glass beads (425 – 600 µm, G-9268, Sigma-Aldrich, USA) were added. Tubes were vortexed for 3 min (30 seconds vortex, 1 min on ice for 6 cycles) to rupture cell walls and release cytoplasmic contents. Beads and cell walls were collected by microcentrifugation as before and the supernatant discarded. Cell walls were separated from glass beads by puncturing the tube and eluting into a 1.5 ml Eppendorf tube by a quick spin in a microcentrifuge. Cell walls were washed 3 times with Buffer K and treated twice with 200 µl 0.075 M Tris-Cl pH 6.8, 2 % (w/v) SDS, 20 % (v/v) glycerol, 5 % (v/v) 2-mercaptoethanol for 10 min at 95°C to remove non-alkali-extractable mannoproteins (Mrša *et al.*, 1997). Finally, cell walls were washed twice with Buffer K.

Biotinylated proteins were extracted from prepared cell walls by resuspension in 100 µl 30 mM NaOH and overnight incubation at 4°C. Cell walls were collected by microcentrifugation and the supernatant retained. The supernatant was subjected to SDS-PAGE and then western blotted (Chapter 2, section 2.3.3.4), except that a 1:5000 dilution of 1mg/ml Immunopure streptavidin conjugated to alkaline phosphatase (#21324, Pierce Biotechnology, USA) was used to detect biotinylated proteins.



### 3.2.2.5. Quantification of flocculin regulation

#### *3.2.2.5.1. Outline*

The expression of flocculins was examined using realtime quantitative PCR (qPCR), which allowed comparison of RNA levels. RNA was extracted from unstressed as well as CR-stressed WT and KO cells.

#### *3.2.2.5.2. Primer design*

Primers for qPCR (Table 3.2.1) were designed using DNAMAN (Lynnon Biosoft) according to the following parameters: (i) the melting temperature ($T_m$) of forward and reverse primers should be approximately equal, (ii) primers should be 15-30 bases in

length, (iii) the G / C content should be 30-80%, (iv) runs of four or more Gs are not allowed, (v) the total number of Gs or Cs in the last 5 nucleotides at the 3' end of the primer may not exceed two, (vi) maximum product size must not exceed 200 bases and (vii) there should be more Cs than Gs.

Table 3.2.1. Primers designed for qPCR quantification of flocculin expression.

| Gene | Forward primer sequence (FP)<br>Reverse primer sequence (RP) | Primer length (bases) | GC content (%) | Product size (bases) | $T_m$ (°C) |
|---|---|---|---|---|---|
| FLO1 | FP: TGTTCTAATAGTCAAGGAATTGCAT | 25 | 32 | 142 | 58.4 |
| | RP: CAACTGTAGCAAACTTGAATGTGT | 24 | 42 | | 58.4 |
| FLO5 | FP: TGTCTACATGTATGCAGGCTACTATTAT | 28 | 36 | 139 | 58.8 |
| | RP: AGAGTAAACGTACCCTTCAAAGTTATC | 27 | 37 | | 58.8 |
| FLO8 | FP: AATGGCAACGAATAGTGAACA | 21 | 38 | 132 | 58.2 |
| | RP: TTGAGCGTATTCTTGCAGTT | 21 | 43 | | 58.2 |
| FLO9 | FP: CGTCACATTGCTGGGATTA | 19 | 47 | 132 | 58.1 |
| | RP: TTCGAATATGTGGAGGAATCTC | 22 | 41 | | 58.1 |
| FLO11 | FP: CCGAAGGAACTAGCTGTAATTCT | 23 | 43 | 105 | 58.2 |
| | RP: AAGTCACATCCAAAGTATACTGCAT | 25 | 38 | | 58.2 |
| HSP12 | FP: CACTGACAAGGCCGACAA | 18 | 56 | 149 | 59.3 |
| | RP: CCATGTAATCTCTAGCTTGGTCTG | 24 | 46 | | 59.3 |
| ACT1 | FP: TTACTCACGTCGTTCCAATTTAC | 23 | 39 | 107 | 58.2 |
| | RP: ACTCAAGATCTTCATCAAGTAGTCAGT | 27 | 37 | | 58.2 |

*3.2.2.5.3. RNA extraction and cDNA synthesis*

Eight flasks containing 50 ml YEPD were inoculated with either 1 ml of an overnight culture of WT (4 flasks) or KO (4 flasks) and grown to an optical density of 0.6 ODU / ml. At this point, CR was added to a final concentration of 0.02 mg / ml to two of the flasks from each strain and all cultures grown for a further 4 h. Yeast cells were harvested by centrifugation at 3000 *x g* for 10 min at 10°C, washed twice with 50 mM phosphate, 150 mM NaCl pH 7.4 buffer (PBS) and resuspended in a minimal volume of PBS. RNA was extracted by the phenol/guanidinium thiocyanate/chloroform method (Chomczynski and Sacchi, 1987). Approximately 50 ODU (*ca.* 5 x $10^9$ cells / ml) of harvested cells was resuspended in 1 ml 4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5 % sarcosyl, 0.1 M 2-mercaptoethanol pH 7.0 (Solution D). Cells were ruptured by the addition of 200µl glass beads and vigorous shaking for 5 min at 4°C, and cellular debris removed by centrifugation. Supernatants were transferred to 4 ml polypropylene tubes

and 0.13 ml 1.5 M Na-acetate pH 4, 1ml Tris-saturated phenol pH 4.0 and 0.2ml chloroform-isoamyl alcohol (49:1) added sequentially. Tubes were vortexed for 10 seconds and incubated on ice for 15 min. Phenol and aqueous phases were separated by centrifugation at 10000 *x g* for 20 min at 4°C and the aqueous, top layer containing RNA transferred to a clean 2 ml Eppendorf tube. A 1 ml aliquot of isopropanol was added and the mixture incubated at -20°C for 2 h. RNA pellets were collected by centrifugation as before and dissolved in 300 µl Solution D. One volume of isopropanol was added and tubes again incubated at -20°C for 2 h. RNA pellets were collected as before, washed once in 75 % ethanol and vacuum dried. Finally, the washed RNA pellet was resuspended in 40 µl RNAse-free water, treated with 0.1% (v/v) diethylpyrocarbonate (DEPC). The quality and purity (no genomic DNA contamination) of RNA was verified by agarose gel electrophoresis.

Complementary DNA (cDNA) was prepared by reverse transcription as follows: RNA from each of the eight samples (2X WT stressed, 2X WT unstressed, 2X KO stressed and 2X KO unstressed) was adjusted to a concentration of 5 µg/µl, combined with 250 pmol oligo-dT primer and water to a final volume of 25 µl and transferred to a sterile 0.5 ml thin-walled PCR tube. Tubes were briefly spun in a microcentrifuge and incubated at 72°C for 2 min, followed by incubation for 2 min on ice. A mixture of 5 µl 10X M-MuLV RT Buffer (obtained with the enzyme), 50 nmol dNTPs, 250 U M-MuLV Reverse Transcriptase (M-0253S, New England Biolabs, USA) and water up to 25 µl was added to these tubes and incubated at 42°C for 1 h, where after 20 U RNase H (M-0297S, New England Biolabs, USA) was added to remove RNA from the RNA/cDNA complexes. Incubation was for a further 15 min at 37°C followed by storage at -20°C.

Synthesized cDNA was quantified by spectrophotometry on a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, USA) at 260nm. Prior to realtime PCR, the qPCR primers and cDNA were tested by PCR as follows: 25 pmol forward primer, 25 pmol reverse primer, 1 µl 6.25 mM dNTPs, 2.5 µl 10X Taq Buffer, 2 U Taq polymerase, 2.5 µl 25 mM $MgCl_2$, 0.5 µl cDNA and Milli-Q water up to 25 µl. The PCR program comprised: 94°C, 5 min followed by 30 cycles of 94°C, 30 seconds, 58°C, 30 seconds,

72°C, 30 seconds followed by 72°C, 10 min and finally 4°C, 10 min. Products were visualized on a 1.5 % (w/v) agarose gel using a HpaII digest of pBR322 as a DNA standard to allow size determination of small (*ca.* 150 bp) PCR products.

*3.2.2.5.4. Realtime quantitative PCR (qPCR)*

qPCR was performed using the Sensimix Kit (Quantace Ltd, UK) which contained 50X SYBR Green and Sensimix Solution (Heat-activated Taq DNA Polymerase, an internal reference, dNTPs and 25mM MgCl2). Each qPCR reaction comprised 12.5 µl Sensimix Solution, 50 pmol forward primer, 50 pmol reverse primer, 0.5 µl 50X SYBR, *ca.* 100 ng cDNA and water to a final volume of 25 µl. The reactions were performed in a Corbett Rotor Gene RG-3000A qPCR instrument (Corbett Research, Australia) using Rotorgene 6.0 software in the FAM/SYBR acquisition mode using a hold step of 95°C for 10 min, cycling at 95°C for 5 seconds, 58°C for 8 seconds and 72°C for 10 seconds, a melt step comprising a ramp from 72 to 95°C, rising in 1°C increments with a wait of 45 seconds for the first and 5 seconds for each subsequent increment.

## 3.2.3. RESULTS

### 3.2.3.1. Effects of CR

#### *3.2.3.1.1. Viability*

Serial dilutions of WT and KO yeast cells were spotted onto YEPD agar plates containing various concentrations of CR and grown for 48 h at 30°C. Whereas growth of the WT strain was largely unaffected by the presence of low concentrations of CR, the disruption mutant (KO) was found not to grow at concentrations of CR above 0.03 mg/ml (Figure 3.2.1). These results indicated that Hsp12p played a role in maintaining cell wall integrity in the presence of CR.

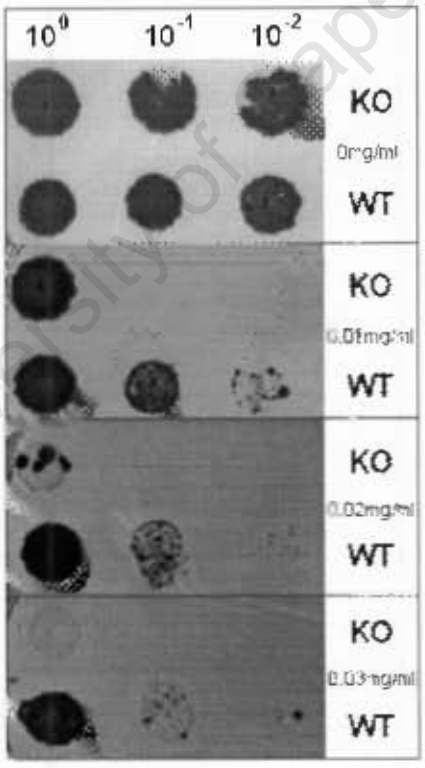



Figure 3.2.1. Effect of CR on the growth of WT and KO yeast. Cells were serially 10-fold diluted from mid-logarithmic cultures and 10 µl culture was applied to YEPD plates containing 0; 0.01, 0.02 and 0.03 mg/ml CR respectively. The plates shown represent growth after 48 hours.

*3.2.3.1.2 Morphology and flocculation*

The WT and KO strains were next examined by light microscopy after growth in YEPD liquid media alone or containing various concentrations of CR (Figure 3.2.2). In the presence of CR, both yeast strains displayed aberrant cell growth. These effects were more pronounced in the KO strain, and at a concentration of 0.1 mg/ml CR and above, this strain formed large, dense groups of cells. These clumps of cells could not be separated by detergents (5 % (w/v) SDS, 5 % (v/v) Triton X-100) or vigorous shaking (data not shown), thus the cells did not aggregate as a result of weak interaction forces. It was thus proposed that the clumps were due to improper cell separation (Figure 3.2.3), where daughter cells remained attached to the mother cell.

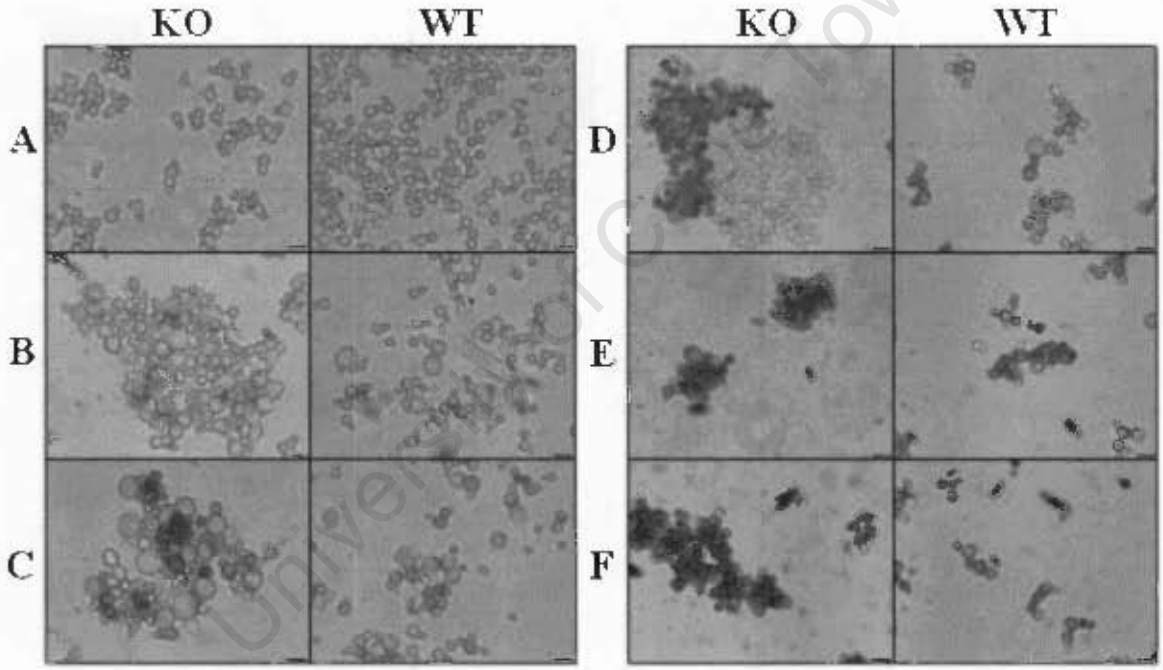



Figure 3.2.2. CR altered the morphology of the KO strain after 20 hours. A: YEPD; B: 0.02 mg/ml; C: 0.05 mg/ml; D: 0.1 mg/ml; E: 0.2 mg/ml; F: 0.3 mg/ml CR. Dead cells appear dark due to a lack of active transport of CR out of these cells. The bar represents 10 μm.

Indeed, upon closer investigation, the KO strain displayed up to 5 buds per cell and cellular distortion in the presence of CR, whereas the WT displayed a maximum of 2 buds per cell (Figure 3.2.3). These results suggested that Hsp12p partially prevented the effects of CR on chitin and hence cell separation.

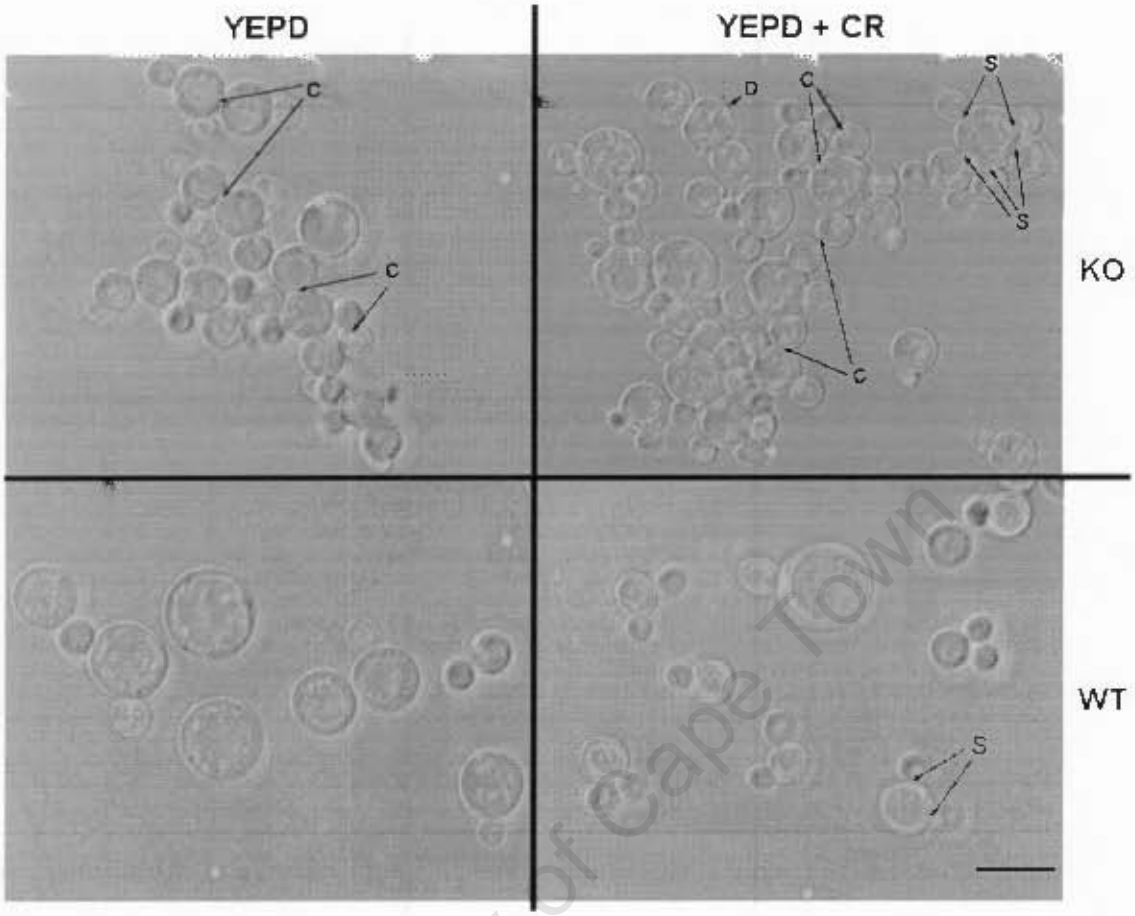



Figure 3.2.3. Closer examination of KO cells in response to 0.02 mg/ml CR revealed that cellular aggregation was due to improper septation (S) which resulted in cellular clumping (C). Bar: 5 µm.

Cellular aggregation was quantified and compared by measuring the decrease in solution turbidity in spectrophotometric cuvettes over time at 600 nm. In contrast to WT cells, the KO strain displayed a marked increase in sedimentation (Figure 3.2.4), indicating increased cell-cell interaction due to multiple daughter cells on each mother cell.

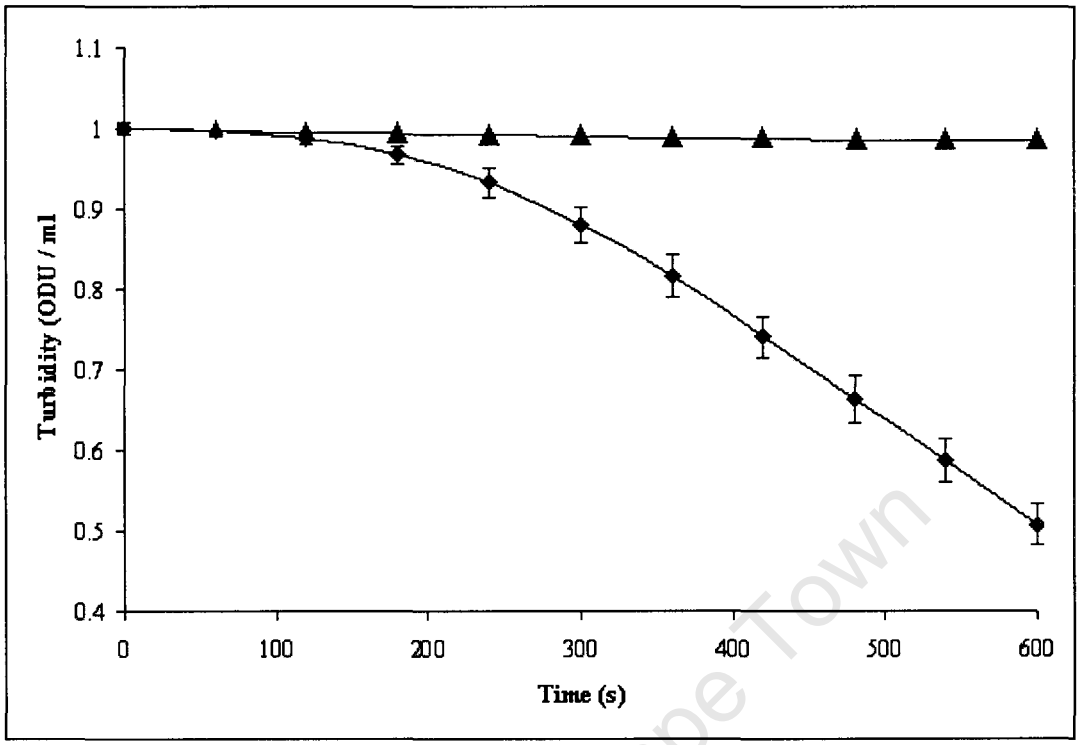



Figure 3.2.4. The flocculation of KO cells stressed with 0.05 mg/ml CR was markedly more pronounced than WT cells or unstressed cells, due to greater cell-cell interactions and cell size. ♦: KO stressed, ▲: KO unstressed, □: WT stressed and ■: WT unstressed. Values expressed are the means from three separate experiments.

This aggregation was also noticed to a lesser extent in KO cells not exposed to CR (Figure 3.2.2 A), which prompted investigation into CR-independent flocculation. In a settling experiment, where cells were transferred to a glass test tube, vortexed and allowed to settle, the KO strain displayed a markedly faster rate of settling (Figure 3.2.5 A), due to the cell aggregation previously observed. This behaviour was quantified by measuring the cleared zone over time and indeed the KO strain sedimented approximately twice as fast as the wildtype (Figure 3.2.5 B). The contribution of hydrophobic effects to this phenomenon was determined by comparing the hydrophobicity of the WT and KO strains by partitioning into an organic layer (octane). Cells were dispersed throughout the organic and aqueous layers, the layers allowed to separate and the loss of cells from the aqueous layer quantified by spectrophotometry. The results indicated that *ca.* 14±1 % of KO cells entered the organic phase compared to *ca.* 10±1 % WT cells. The KO strain was therefore only slightly more hydrophobic than

the WT strain and hydrophobicity was possibly only a contributing factor for the flocculation observed.

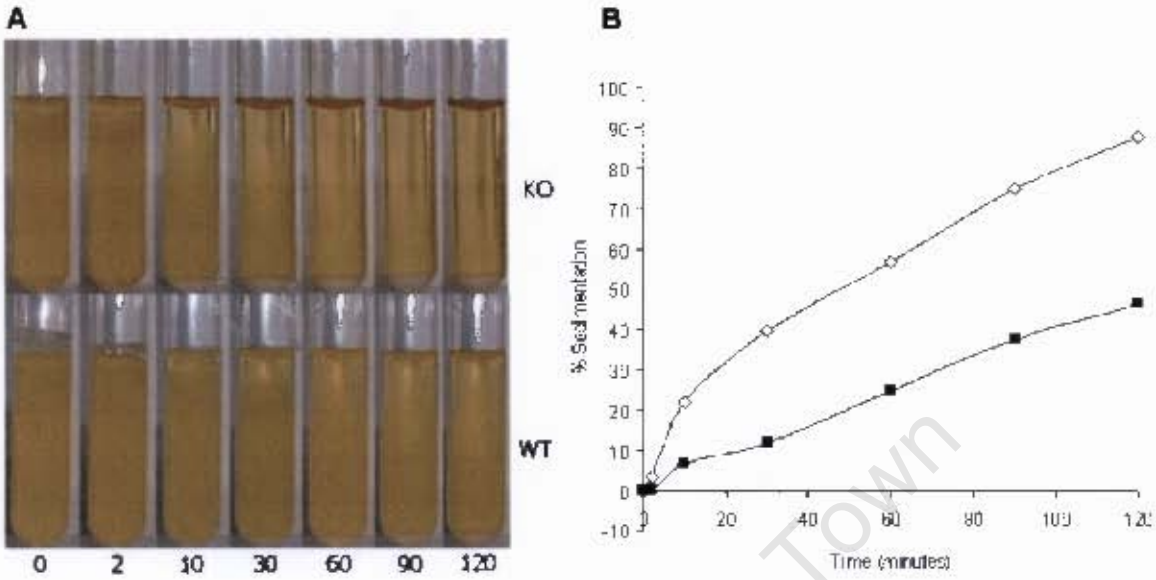



Figure 3.2.5. A: KO cells displayed aggregation and improper cell separation under non-stress conditions. This resulted in a marked increase in the rate of settling, indicated by an increase in the transparency of the YEPD media. Time is indicated in minutes along the bottom. B: WT (■) cells sedimented out of suspension slower than KO (◊) cells over time. Data was obtained by measurement of the cleared zones in A.

*3.2.3.1.3. Invasion*

Other authors have shown that CR stimulates invasive growth in strains not normally associated with this form of growth, including the W303 strain used in these studies (Zaragoza and Gancedo, 2000). The mechanism of this action is unknown, but may involve alteration of flocculin expression or of cell wall charge, hydrophobicity or surface texture. The contribution of Hsp12p to invasive growth was investigated by exposing WT and KO strains to 0.02 mg/ml CR (a sublethal dose) on agar plates for an extended period of up to six days and washing off visible colonies. The WT and KO strains did not invade YEPD agar but in the presence of CR, the WT strain displayed invasive growth whereas the KO strain did not. This process appeared time-dependent, since WT cells only markedly invaded the agar growth medium after 3 days (Figure 3.2.6).

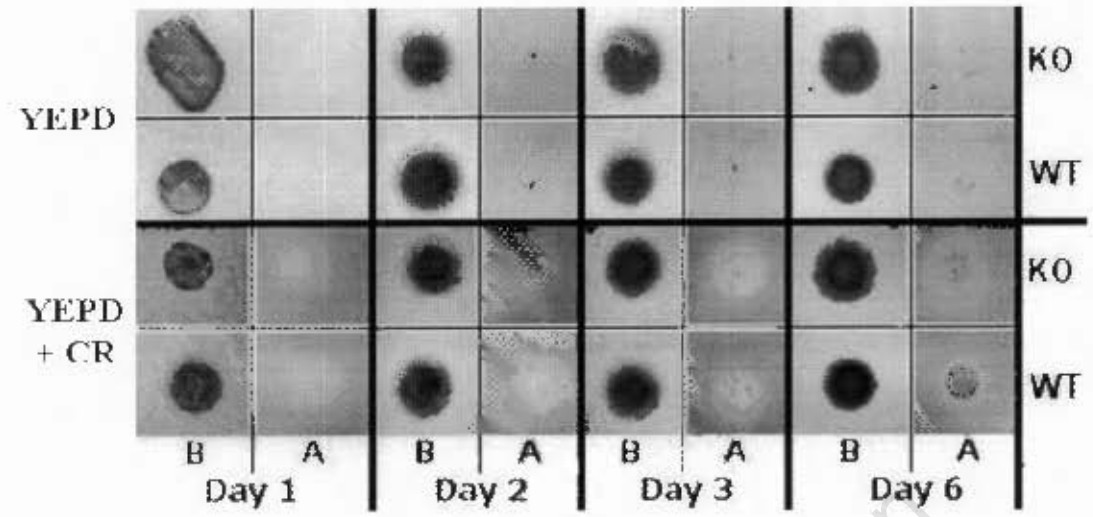



Figure 3.2.6. 0.02 mg/ml CR enhanced the invasiveness of WT yeast in an Hsp12p and time-dependent manner after extended growth at 30°C. B: Before washing, A: After washing.



The extent and nature of invasiveness of WT yeast growing in the presence of CR was ascertained by microscopy. Surprisingly, an altered morphology resembling prolate spheroids as well as large clumps was observed (Figure 3.2.7 A). Furthermore, invasion appeared to coincide with the formation of chains of cells, which may have facilitated penetration into the agar (Figure 3.2.7 B).

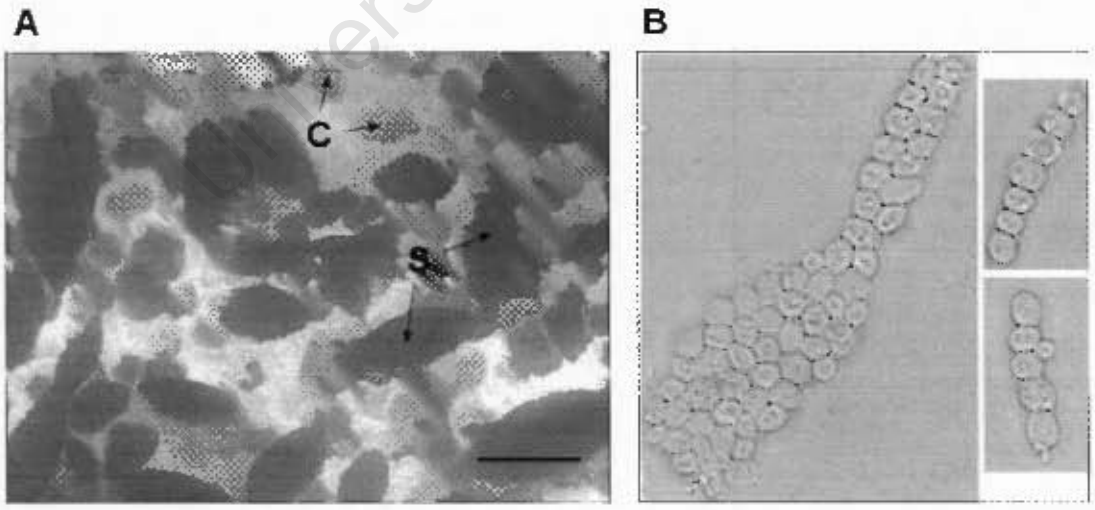



Figure 3.2.7. A: Invasive cells examined by phase contrast microscopy displayed spheroid (S) and clumped (C) structures. These structures were examined in greater detail using confocal microscopy (Figure 3.2.8); B: Invasion was possibly due to penetration of the agar by chains of cells. Bar: 500 µm.

Spheroids were examined in greater detail by repeating the experiment with a fluorescent strain of yeast, containing the pYH12G2 plasmid expressing Hsp12-Gfp2p, and viewing the invasive cells by confocal microscopy. The average spheroid was 600 µm in length and approximately 200 µm wide (Figure 3.2.8 A). Further investigation of these structures by excision, immersion in glycerol and phase contrast microscopy (Figure 3.2.8 B) or confocal microscopy of whole spheroids (Figure 3.2.8 C) revealed that the structures were composed of dense accumulations of cells. These structures were unstable and fragmented in the presence of water or when removed from the agar environment.

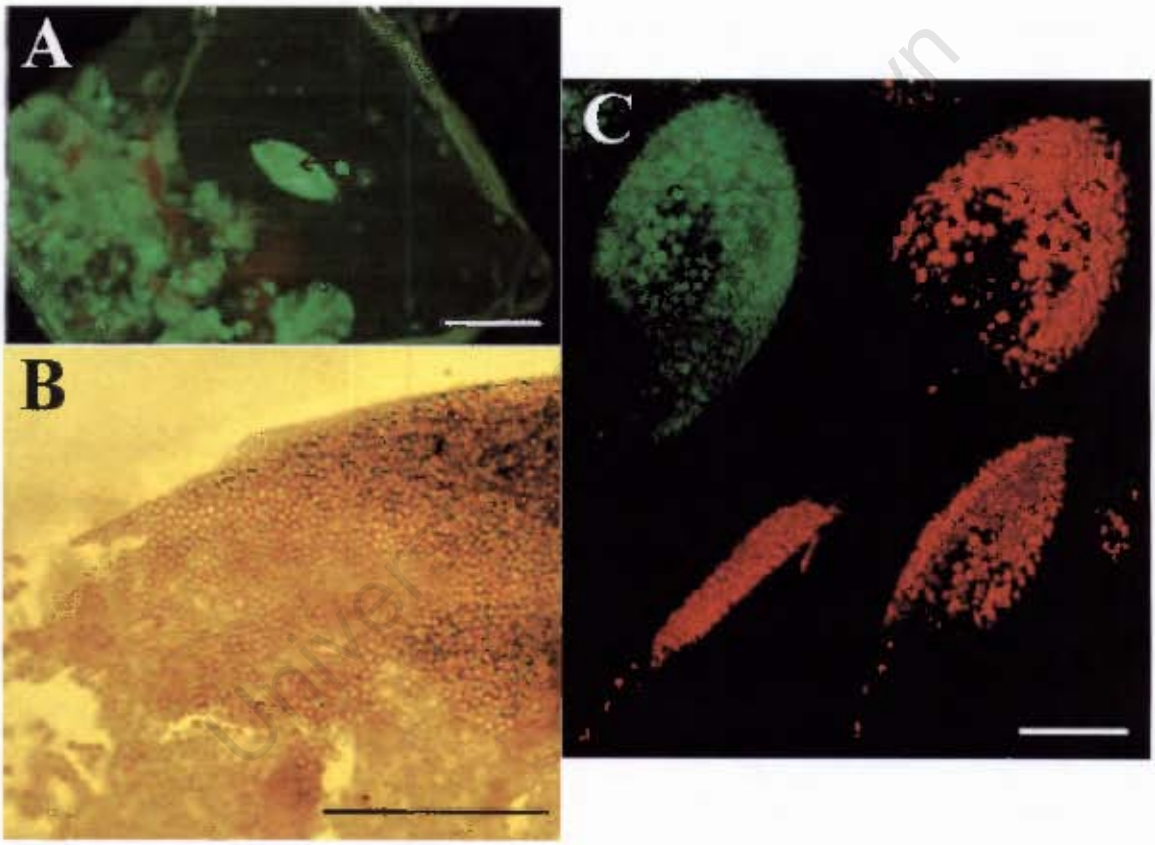



Figure 3.2.8. Investigation of the morphology of invasive yeast cells by confocal (A, C) and phase contrast (B) microscopy. A: Overview of a spheroid (arrow) to depict size, bar = 500 µm, B: phase contrast microscopy of a spheroid structure after excision from agar and immersion in glycerol. Note the fragmentation to the left of the spheroid, bar = 100 µm, C: A rotation through a fluorescent spheroid, depicting individual cells, bar = 500 µm.

*3.2.3.1.4. Chitin interaction*

Previous studies have shown that CR bound to chitin as it was synthesized. This prevented chitin fibre formation, which resulted in the disruption of the septum and consequently the budding process (Pancaldi *et al.*, 1985; Bartnicki-Garcia *et al.*, 1994; Raclavsky *et al.*, 1999). WT cells were viable in the presence of CR, whereas KO cells displayed sensitivity to low concentrations of CR. This prompted the investigation of whether Hsp12p played a protective role in the cell wall by binding to chitin to facilitate budding in the presence of CR. Chitin was incubated with increasing concentrations of Hsp12p before exposing this treated chitin to CR. Hsp12p appeared to block CR binding to chitin in a concentration-dependent manner (Figure 3.2.9).

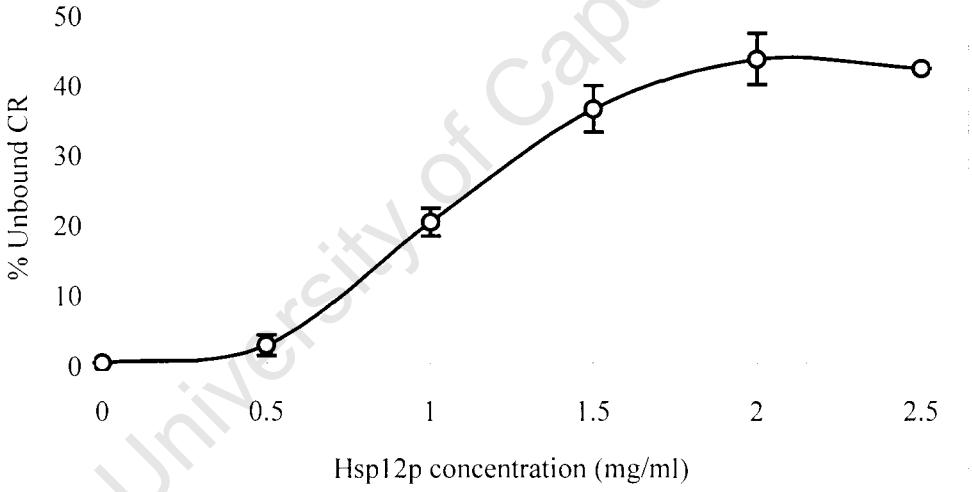





Figure 3.2.9. Hsp12p binds chitin *in vitro* and prevents CR binding. 15 mg of chitin was exposed to increasing concentrations of Hsp12p for 10 minutes at 25°C before CR was added to a final concentration of 0.005mg/ml. The total sample volume was 1ml. The % unbound CR was determined by measuring the absorbance at 500nm.

### 3.2.3.2. PIR proteins

Biotin is a small vitamin (244 Da) that efficiently binds avidin and streptavidin (Green, 1963; Tausig and Wolf, 1964). Esters of biotin, including sulfo-N-hydroxysuccinimide (Sulfo-NHS) are water soluble and in addition to avidin, bind primary amine groups in

other proteins to form amides. The plasma membrane of yeast is impermeable to this compound, thus only cell wall proteins are bound in this manner. WT and KO yeast cells were subjected to this treatment and the resultant alkali-extracted biotinylated proteins were visualised using streptavidin conjugated to alkaline phosphatase and a modified form of Western Blotting. The blot showed no difference in the expression of PIR proteins (Figure 3.2.10), implying that Hsp12p mediates its effects through another mechanism.

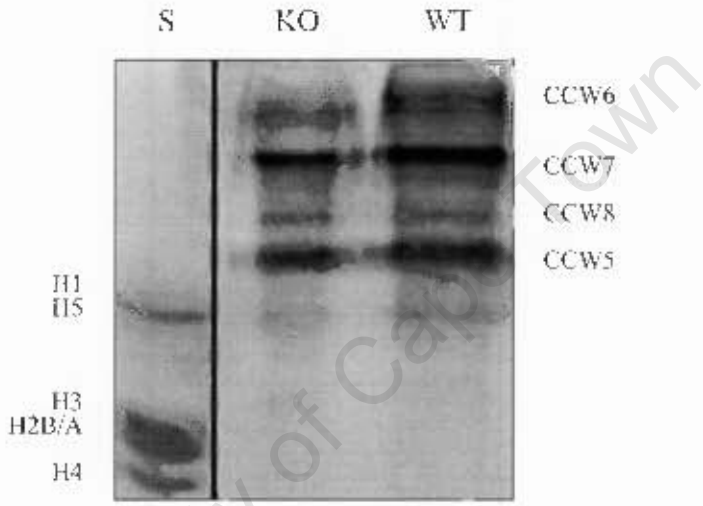



Figure 3.2.10. PIR protein expression was not affected by loss of Hsp12p. PIR proteins were extracted from biotinylated cell walls by mild alkali extraction, subjected to SDS-PAGE, transferred to nitrocellulose and visualised using streptavidin (conjugated to alkaline phosphatase), BCIP and NBT. PIR protein names are depicted on the right, as shown in Mrša and Tanner, 1999. Lane S is an extract of total chicken histones for size comparison. H1: 22.5; H5: 20.6; H3: 15.4; H2B: 13.7; H2A: 14.0 and H4: 11.2 kDa.



### 3.2.3.3. Flocculins

The main proteins involved in invasive growth and flocculation belong to the flocculin family. The most important protein, Flo11p, is essential for flocculation (Lo and Dranginis 1996), invasive growth, pseudohyphal development (Lambrechts *et al.* 1996; Lo and Dranginis 1998) and biofilm formation (Reynolds and Fink, 2001).

Possible restoration of flocculin expression by the action of CR, presence of Hsp12p or lack of Hsp12p was investigated since (i) the invasive behaviour of W303 cells was restored by CR in an Hsp12p-dependent manner, (ii) KO cells displayed enhanced flocculation and (iii) previous studies implicated Hsp12p in biofilm formation (Zara *et al.*, 2002). WT and KO cells were stressed with CR, their mRNA extracted and quantified by realtime quantitative PCR (qPCR). The results indicated that flocculins were not induced by CR and there was no difference between WT and KO flocculin regulation (data not shown). The realtime PCR graphs obtained for *HSP12* are shown (Figure 3.2.11), since *HSP12* was found to be induced in response to CR (*ca.* 2 fold), in correlation with previous findings (Garcia *et al.*, 2004). The invasive behaviour of the WT strain and flocculation of the KO strain was thus not as a result of restoration of flocculin expression.

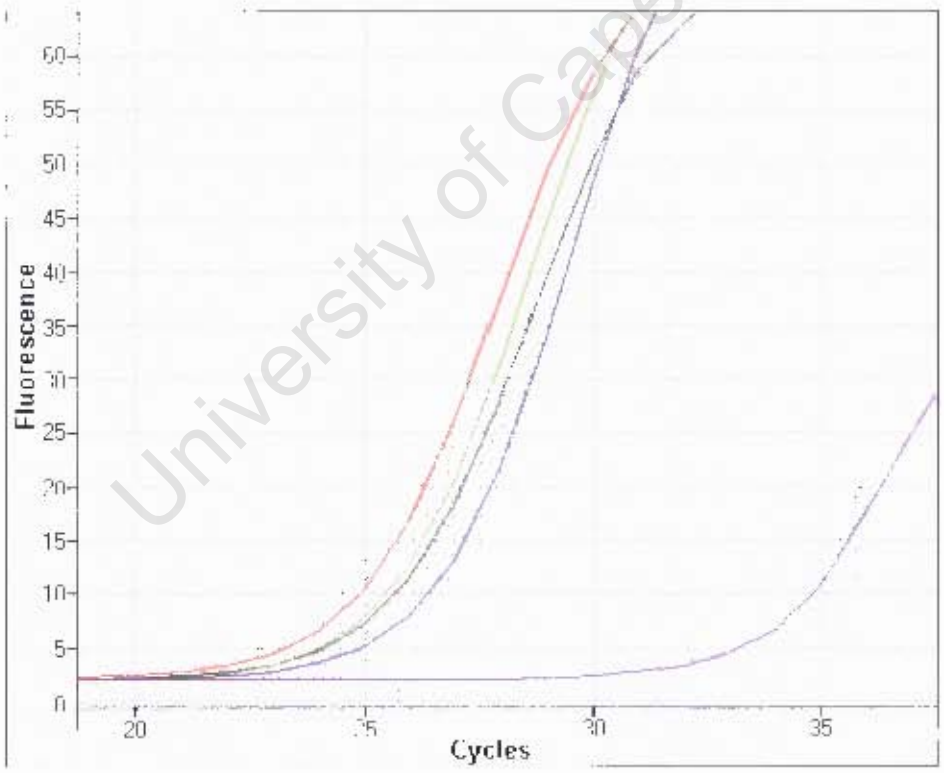



Figure 3.2.11. Realtime PCR analysis of *HSP12*. *HSP12* levels increased *ca.* 2-fold in response to CR stress, as indicated by higher fluorescence after *ca.* 24 cycles. Black: *ACT1*, unstressed cells, Blue: *HSP12*, unstressed cells, Green: *ACT1*, stressed cells, Red: *HSP12*, stressed cells, Purple: no template control.

### 3.2.4. DISCUSSION

In this section, the inter-relationship between Hsp12p, CR, flocculins, flocculation and yeast morphology was investigated.

Maintenance of cell wall integrity through the action of the stress-responsive protein, Hsp12p, appeared essential for the survival of yeast grown in the presence of CR. Furthermore, CR altered the morphology of yeast cells, with the greatest effects observed on KO cells. These cells displayed enhanced cellular aggregation and improper septation which resulted in flocculation. The maintenance of cell morphology and survival of WT cells was possibly achieved by the interaction of Hsp12p with chitin, where it prevented CR binding to chitin.

Flocculation was also observed in KO cells not exposed to CR. Investigation of the effects of hydrophobicity and flocculin expression in WT and KO strains revealed only a minor difference. Furthermore, cell aggregation was not abolished by detergents or mechanical force. This implicated other parameters, such as defective cell separation, as the main factor governing flocculation in the W303 genetic background.

A possible explanation for the effects of CR on KO cells is that these cells possessed higher chitin content than WT cells. Previous studies have shown that cells with lowered glucan content display enhanced cell wall chitin levels. Elevated chitin content would render the yeast cells more vulnerable to CR, which binds to chitin and disrupts budding. Furthermore, an increase in cell wall chitin would result in a less flexible cell wall. Finally, an excess of chitin would cause a thicker primary septum to form, which would possibly hamper cell separation, resulting in clumps of cells and flocculation. These hypotheses were investigated in section 3.4.

Yeast cells grown for prolonged periods on agar plates containing CR exhibited invasive growth. The dependence on Hsp12p for invasive growth was initially suspected to involve alteration of flocculin proteins, which are known mediators of invasive growth

(Lo and Dranginis, 1996). However, flocculin levels were not raised or different in the WT and KO strains. The dependence on CR for invasion by normally non-invasive W303 yeast cells was possibly mediated by direct effects on cell division due to CR complexing with chitin, as has been previously demonstrated in liquid cultures (Pancaldi *et al.*, 1985; Vannini *et al.*, 1985; Kopecka and Gabriel, 1992). This led to cellular chain formation, which enabled penetration of the agar surface resulting in pseudo-invasive growth. In the KO strain, cell separation was inhibited to such an extent that even chains were not formed, resulting in the abolishment of pseudo-invasive growth. Furthermore, mother cells with up to 5 daughter cells attached were observed in the KO strain in response to CR. In contrast, the WT strain only had a maximum of 2 daughter cells per mother cell, suggesting that the likelihood of chain formation due to unidirectional growth was greater in the WT strain.

An intriguing form of growth was observed when invasive WT cells growing in the presence of CR were investigated microscopically, namely the formation of prolate spheroids. These structures were postulated to be formed when yeast cells penetrated the agar surface and divided beneath it, exhausting water, causing crack formation in the agar which then led to spheroid formation as cells continually divided outwards.

In summary, Hsp12p is required for viability and proper cell separation of yeast cells in the presence of CR possibly by protecting chitin or by ensuring proper levels of polysaccharides in the cell wall.

## 3.3. *HSP12P INCREASES THE FLEXIBILITY OF THE YEAST CELL WALL IN RESPONSE TO STRESS: AN AGAROSE MODEL-BASED APPROACH*

### 3.3.1. INTRODUCTION

The cell wall of the yeast *S. cerevisiae* is composed of an outer mannoprotein layer involved in cell-cell recognition and an inner carbohydrate layer that provides much of its physical strength (Klis, 1994; Lipke and Ovalle, 1998). The glucans are the major components of this inner layer, which can be divided into inner and outer components. The outer amorphous component is enriched in β-1,6 glucans whereas the fibrillar inner component comprises mainly β-1,3 glucans. The β-1,3 glucan layer is an insoluble triple helical arrangement of β-1,3 linked polymers of D-glucose. This layer is thought to provide the yeast cell with its mechanical stability, since removal of the mannoprotein layer and the β-1,6 glucan layer with pronase and β-1,6 glucanase respectively, has no effect on cell shape (for a review, see Klis *et al.*, 2002).

Immunocytochemical analysis and alkaline extraction of Hsp12p has demonstrated that this protein is primarily located in the cell wall (Motshwene *et al.*, 2004) although a small quantity is found surrounding the plasma membrane (Sales *et al.*, 2000). Since KO yeast cells displayed markedly less cell volume change upon osmotic shock, Hsp12p was proposed to play a role in the enhancement of cell wall flexibility (Motshwene *et al.*, 2004). Indirect evidence in support of such a function is that trehalose deficient (*tpsΔ*) yeast are sensitive to hydrostatic pressure (Iwahashi *et al.*, 1997), displaying an altered morphology with cytoskeletal deformation after the application of pressure. This altered morphology, ascribed to resultant changes in the cell wall, was not observed when the yeast cells were heat shocked prior to the application of pressure (Fernandes *et al.*, 2001). Rescue of the barosensitive *tpsΔ* phenotype by heat shock thus supports the hypothesis that Hsp12p influences cell wall flexibility.

Hsp12p is postulated to act by interrupting the normal hydrogen bonding pattern of the glucan polymers in a manner analogous to plasticizers in plastic polymers. In order to test

this hypothesis, Hsp12p would have to be incorporated with β-1,3 glucan extracted from yeast cell walls and the elasticity determined. The β-1,3 glucan matrix can, however, only be solubilised after partial phosphorylation (Williams *et al.*, 1991), which would introduce anionic groups and alter the nature of the inter-chain interactions. Hsp12p was thus incorporated into the polysaccharide agarose, which has a number of similarities to β-1,3 glucan, and the resultant effects on gel strength and gelling kinetics quantified. Agarose is also a neutral copolymer but composed of alternating 1,3-linked β-D-galactopyranose and 1,4-linked 3,6-anhydro-α-L-galactopyranose residues (Arnott *et al.*, 1974). X-ray diffraction and optical rotation studies have elucidated the structure of agarose to be a parallel double helix, which adopts a left-handed three-fold symmetry (Arnott *et al.*, 1974). Unlike β-1,3 glucan, agarose readily dissolves in water at 80°C. On cooling, it remains in a sol state to the gelation temperature, whereupon gelling begins. The mechanism for the gelation process is believed to involve the assembly of random coils in solution to more ordered double helices by hydrogen bonding and, finally, aggregation of these helices to form a porous matrix (Griess *et al.*, 1989). This process is accompanied by an increase in the viscosity and light scattering properties of the solution (Arnott *et al.*, 1974). Proteins can be readily incorporated into the agarose matrix, for example the incorporation of specific antibodies for antigen quantification via "rocket" electrophoresis (Laurell, 1966).

## 3.3.2. MATERIALS AND METHODS

### 3.3.2.1. Materials

All chemicals used were of high purity. Double distilled water was used for all experiments.

### 3.3.2.2. Gelation temperature and gel strength assays

The gelation temperature was investigated using a Shimadzu UV 2201 recording spectrophotometer (Shimadzu Corporation, Japan) with a temperature controlled cuvette holder. The wavelength for analysis (500 nm) was chosen by determining the lowest wavelength exhibiting zero absorption for an agarose solution. A typical analysis involved placing 1 % (w/v) liquid Molecular Grade agarose (Whitehead Scientific, RSA) at 70°C in a plastic cuvette in the spectrophotometer with the cuvette holder maintained at 0°C, and allowing cooling to occur to 50°C. The absorption and sample temperature, monitored using a Fluke Multimeter (Fluke, USA) equipped with a thermocouple, was then determined over the next 300 seconds. Turbidity was defined as the absorption at 500 nm at 11°C. The gelation temperature, $T_g$, was defined as the temperature at which the maximum rate of change of absorption occurred.

For gel strength studies, agarose was dissolved at the stated concentration in water or the solution of choice at 80°C and the solution poured into cylindrical nylon moulds (10 mm diameter by 10 mm height). Propanol (1-propyl alcohol) was used instead of ethanol since the boiling point of 97°C is greater than the temperature used to dissolve the agarose. Each mould was covered with a glass plate and allowed to cool to RT at 100% humidity to minimise loss of liquid from the gel. Gels were removed from the moulds immediately prior to use. Compression was applied to the agarose cylinder using an Instron 5567 Universal Tester (Instron Corporation, MS, USA) using a cylindrical nylon probe with flat face geometry at a crosshead speed of 60 mm/min. This crosshead speed was used to minimise loss of water due to the porous nature of the agarose, so that the

cylinders essentially represented solid structures. The force exerted on the probe (stress) was determined as a function of probe extension (strain). Gel strength was defined as the maximum force that the sample could withstand before permanent deformation and was characterised by a sharp decline in the force exerted on the probe by the agarose at a certain critical extension.

### 3.3.3. RESULTS

A variety of salts have been characterised into lyotropic, neutral and chaotropic solutes on the basis of their effects on ribonuclease stability (Von Hippel and Wong, 1965). Incorporation of the lyotropic solute ammonium sulphate ($(NH_4)_2SO_4$) into the agarose matrix showed a large increase in the turbidity as well as the gelation temperature. The magnitude of these increases was linearly dependent on the $(NH_4)_2SO_4$ concentration (Figure 3.3.1). In contrast, incorporation of the chaotropic agent, urea, into the agarose matrix had the opposite effect, with a concentration dependent decrease in both the turbidity and the gelation temperature observed (Figure 3.3.1).

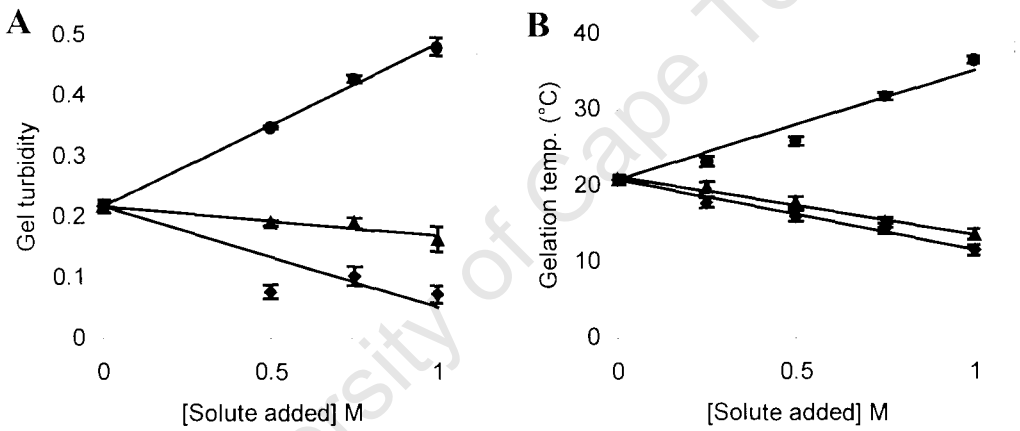



Figure 3.3.1. The effects of included solutes on (A) the gel turbidity ($A_{500}$ at 11°C) and (B) gelation temperature ($T_g$) of 1 % (w/v) agarose. The included solutes used were $(NH_4)_2SO_4$ (•—•), urea (♦—♦) and propanol (▲—▲). The data shown are the means ± the standard deviation of three separate experiments.

Agarose gelation has been postulated to be brought about by hydrogen bonding between agarose chains. To investigate whether there was any hydrophobic contribution to this process, propanol was incorporated into the agarose matrix and the change in the turbidity on cooling investigated. Propanol behaved in a similar manner to urea (Figure 3.3.1) with regard to alteration of the gelation temperature, where a concentration dependent decrease of a similar magnitude was observed. Little change in the turbidity of

the agarose was observed, however, suggesting that the interaction of individual agarose chains was largely unaltered by propanol.

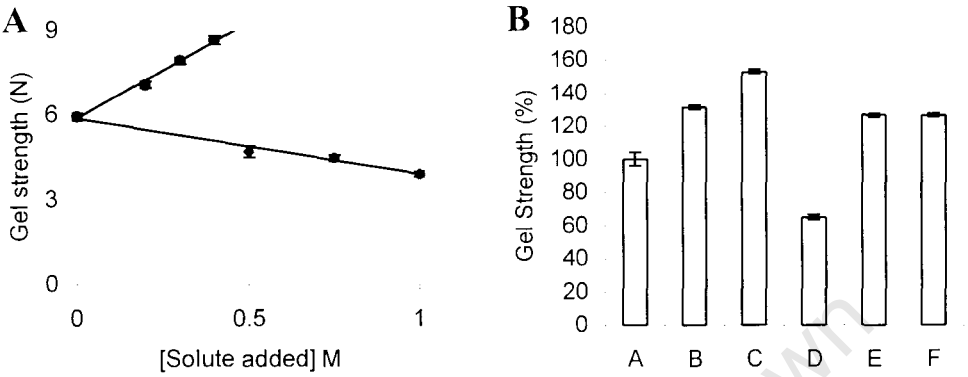



Figure 3.3.2. (A) The effects of included solutes on the gel strength of 1 % (w/v) agarose. The included solutes used were $(NH_4)_2SO_4$ (•—•) or urea (♦—♦). (B) Comparison of the effects of inclusion of 1 molar concentrations of various solutes on the relative gel strengths of 1 % (w/v) agarose. A: no addition, B: $(NH_4)_2SO_4$, C: KCl, D: urea, E: n-propanol, F: sucrose. The results shown are the means ± the standard deviation of three separate experiments.

The changes observed in the turbidity after incorporation of various solutes suggested an alteration in the strength of the agarose matrix. The gel strength, the maximum force that the sample could withstand before permanent deformation, was therefore determined for agarose incorporating a variety of solutes at different concentrations. Solutes were found to affect gel strength in a concentration-dependent manner with lyotropic solutes such as $(NH_4)_2SO_4$ increasing the gel strength and the chaotropic solute urea decreasing the gel strength (Figure 3.3.2 A). Comparison of the effects of the incorporation of the lyotropic solutes $(NH_4)_2SO_4$ and KCl showed that the presence of 1 M $(NH_4)_2SO_4$ increased the gel strength by 30 % whereas the presence of 1 M KCl increased the gel strength by 50 %. In contrast, the presence of 1 M urea was found to decrease the gel strength by 40 % (Figure 3.3.2 B). The stress response protein Hsp12p has been shown to be expressed when yeast are exposed to hyper-osmotic conditions due to the presence of a variety of different solutes including salts, ethanol and sugars in the medium. Thus the effect of incorporation of propanol or sucrose on gel strength was investigated. Both sucrose and propanol increased gel strength in a concentration dependent manner (data not shown) with

incorporation of 1 M sucrose or 1 M propanol resulting in an identical increase of 25% in the gel strength (Figure 3.3.2 B).

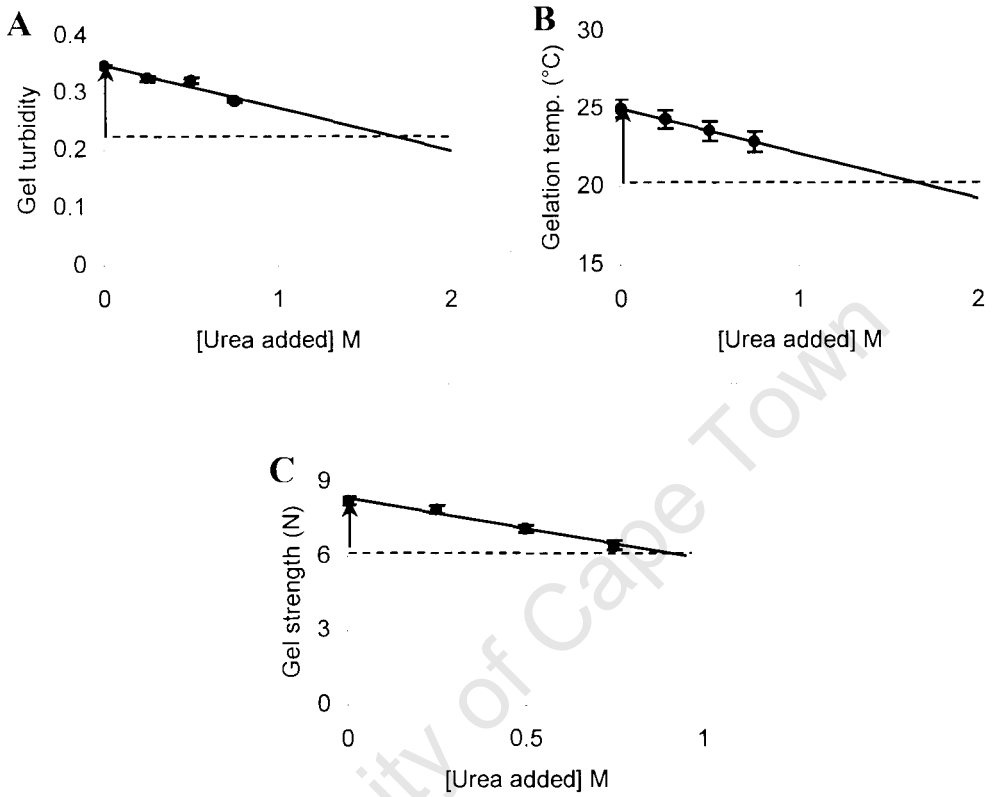



Figure 3.3.3. Effect of the incorporation of increasing concentrations of urea on the turbidity (A), gelation temperature (B) and gel strength (C) of 1 % (w/v) agarose incorporating 0.5 M $(NH_4)_2SO_4$. The arrow (↑) on the ordinate shows the effect of the addition of 0.5 M $(NH_4)_2SO_4$. The results shown are the means ± the standard deviation of three separate experiments. A least squares regression line was fitted to the data and extrapolated to cross the dashed line (- - -) representing the turbidity, gelation temperature or gel strength of 1 % (w/v) agarose prior to the addition of 0.5 M $(NH_4)_2SO_4$.

Competition between the various factors affecting agarose were investigated by simultaneous incorporation of a chaotropic and a lyotropic solute into the gel matrix, which was expected to result in intermediate values of the turbidity, gelation temperature and gel strength dependent on the relative concentration of the chaotropic and the lyotropic solutes used. Agarose incorporating 0.5 M $(NH_4)_2SO_4$ together with various concentrations of urea up to 0.75 M was prepared and the effect of the urea concentration

on the above parameters investigated. Increasing the urea concentration resulted in a concentration-dependent reduction in the increased turbidity (Figure 3.3.3 A), gelation temperature (Figure 3.3.3 B) and gel strength (Figure 3.3.3 C) brought about by the incorporation of 0.5 M $(NH_4)_2SO_4$. The effect was most marked on the gel strength, where extrapolation of the data showed that the theoretical incorporation of approximately 0.9 M urea would have restored the gel strength to that prior to incorporation of the 0.5 M $(NH_4)_2SO_4$. The effect of the urea on the gelation temperature and the turbidity was less marked. Extrapolation of the data showed that restoration of these parameters to their original value required a higher concentration of urea, approximately 1.7 M urea in both cases.

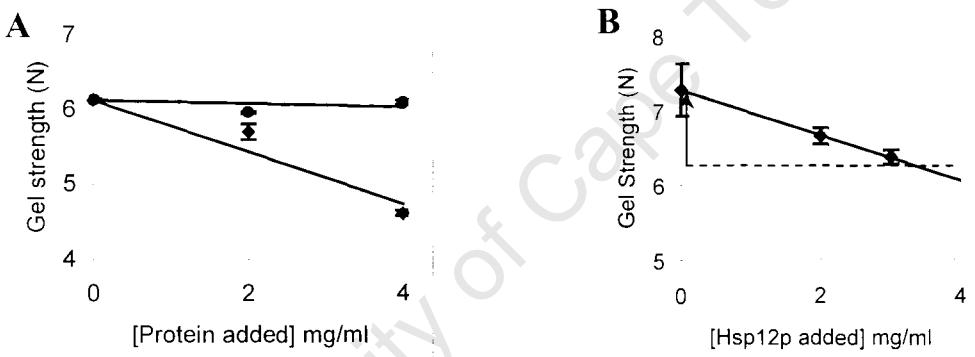



Figure 3.3.4. (A) Effect of the incorporation of increasing concentrations of the proteins Hsp12p (♦—♦) or lysozyme (●—●) on the gel strength of 1 % (w/v) agarose. (B) Effect of the incorporation of increasing concentrations of Hsp12p on the gel strength of 1 % (w/v) agarose incorporating 0.25 M $(NH_4)_2SO_4$. The arrow (↑) on the ordinate shows the effect of the addition of 0.25 M $(NH_4)_2SO_4$. A least squares regression line was fitted to the data and extrapolated to cross the dashed line (- - -) representing the turbidity, gelation temperature or gel strength of 1 % (w/v) agarose prior to the addition of 0.25 M $(NH_4)_2SO_4$. All results shown are the means ± the standard deviation of three separate experiments.

Studies on whole yeast (Motshwene *et al.*, 2004) showed that the presence of Hsp12p markedly affected the ability of the yeast to withstand sudden pressure changes. This was ascribed to an alteration in the elasticity of the cell wall brought about by Hsp12p interacting with the β-glucan matrix. Hsp12p was therefore incorporated into the agarose matrix at concentrations of up to 4 mg/ml and the effect on the turbidity, gelation temperature and gel strength investigated. Incorporation of Hsp12p had no effect on

either the gelation temperature or the turbidity of the agarose matrix (data not shown) but the protein appeared to act as a chaotrope, reducing the gel strength in a concentration-dependent manner (Figure 3.3.4 A). The gel strength was found to diminish by just over 20 % when Hsp12p was present at a concentration of 4 mg/ml (0.25 mM). In contrast, incorporation of the 14.3 kDa protein lysozyme (C-62971, Sigma-Aldrich, USA) as a control at the same concentration failed to elicit any response (Figure 3.3.4 A). Since changes in the gel strength of the agarose on incorporation of Hsp12p might have been due to homogeneous separation of the protein and agarose on cooling, resulting in the formation of agarose "islands", the distribution of Hsp12p in the agarose matrix was determined by microscopy after staining with the protein dye Coomassie Brilliant blue. A uniform distribution of stain throughout the agarose matrix was observed (data not shown), suggesting that incorporation of Hsp12p into the agarose matrix had occurred during cooling. Since Hsp12p acted in a manner analogous to urea in that the agarose gel strength decreased upon its incorporation, competition studies were conducted to ascertain if Hsp12p would restore the gel strength of agarose incorporating a lyotropic solute to that of the agarose alone. Indeed, extrapolation of the data presented in Figure 3.3.4 B showed that Hsp12p at 3.4 mg/ml would have restored the gel strength of agarose incorporating 0.25 M $(NH_4)_2SO_4$ to that found for agarose alone. A similar result was found using 0.75 M sucrose as the lyotropic solute (data not shown).

### 3.3.4. DISCUSSION

The results reported here show an intriguing correlation between the gel strength of the agarose matrix and factors known to stimulate Hsp12p synthesis, such as salt, alcohols and sugars. Incorporation of lyotropic solutes into the agarose matrix resulted in increased gel strength whereas incorporation of the chaotropic solutes resulted in decreased gel strength. Since gel strength measurements were all performed on agarose discs of identical dimensions, these results suggested an increased value of the Young's modulus on incorporation of lyotropic solutes and a decreased Young's modulus on incorporation of chaotropic solutes. Young's moduli were not determined as the methodology used would not produce reliable values for these parameters due to a number of factors including friction between the agarose disc and the compression platforms, non-elastic compression and non-uniaxial compression. The increased gel strength observed on incorporation of lyotropic solutes could be restored to its original value by the simultaneous incorporation of Hsp12p. This is analogous to the situation in yeast, where growth in hyper-osmotic media or growth in media containing ethanol results in an increased Hsp12p content (Mtwisha *et al.*, 1998; Sales *et al.*, 2000). Thus it appears that yeast senses the increased cell wall rigidity, possibly via the cell wall integrity pathway (Hohmann, 2002; Garcia *et al.*, 2004) or through plasma membrane stretch (Kamada *et al.*, 1995; de Nobel *et al.*, 2000; Harrison *et al.*, 2001; Torres *et al.*, 2002) and responds by increasing the Hsp12p content in the cell wall to restore flexibility. Recently the pressure needed to break a yeast cell has been determined (Smith *et al.*, 2000). This pressure, *ca.*4810 kN/m$^2$, is equivalent to an agarose concentration of approximately 0.25 % (w/v).

The gelation of agarose has been proposed to be a two-step process, with random coils in solution initially assuming double helical conformations. Upon further cooling, bundles of helices form (Arnott *et al.*, 1974). It has been suggested that these bundles can be described as long rods, which affect the turbidity and gel filtration characteristics of the matrix (Obrink, 1968). The results reported here suggest that the size of these rods is affected by the presence of solutes during the cooling process that results in the formation

of the agar matrix which in turn alters the physical properties of the matrix. Since both helix and rod formation are brought about by hydrogen bonding between agarose molecules, factors that affect hydrogen bonding would be expected to alter this process. Thus the presence of chaotropic solutes, which are known to disrupt hydrogen bonding, would result in a reduced helix formation and therefore smaller rods. These rods would in turn reduce the turbidity due to their smaller size and the gelation temperature and gel strength due to reduced hydrogen bonding. Lyotropic solutes, by increasing the bound water content of the solution, would have the opposite effect. The reduced free water content would favour the hydrogen bonding of agarose monomers to other monomers rather than to free water thereby promoting the formation of longer helical segments and therefore larger rods. Larger rods more strongly hydrogen bonded to one another would account for the increased gel strength observed. This explanation might well be an over-simplification of a complex phenomenon since the turbidity is dependent not only on the size of the particles but also on the relative refractive indices of particles and solvent.

The absence of effect of the incorporation of Hsp12p into the agarose matrix on the turbidity and the gelation temperature is perhaps not surprising. The concentration of Hsp12p used was extremely low, less than 1 mM, in contrast to the molar concentrations of solutes required to elicit a response. These data suggest that Hsp12p does not affect either the sizes of the rods formed on cooling the agar or the overall interactions between them that occur during the cooling process. The effect of Hsp12p on the gel strength is presumably brought about by Hsp12p interchelating between the agarose rods. It has been shown that LEA proteins have little structure in solution (Russouw *et al.*, 1995; Lisse *et al.*, 1996), a property that would enable such proteins to form hydrogen bonds to agarose rods at different positions along the primary sequence. This presumably allows the agarose rods greater movement with respect to one another without breaking hydrogen bonds, a property similar to that of plasticizers. In contrast, lysozyme failed to alter the gel strength of the agarose matrix. This is presumably because lysozyme is a globular protein (Phillips, 1966) with a defined structure. This defined structure would prevent agarose rod movement without breaking hydrogen bonds, which would account for the observed lack of effect on the agarose gel strength. Thus Hsp12p appears, as least in this

model system for the yeast cell wall, to act as a chaotropic agent and plasticizer to increase flexibility of gel matrices.

## 3.4. *INVESTIGATION INTO THE EFFECTS OF HSP12P ON CELL WALL PARAMETERS BY DIRECT MEASUREMENTS ON LIVE YEAST CELLS*

### 3.4.1. INTRODUCTION

Previous studies using agarose as a model system indicated that Hsp12p may act as a plasticizer in the yeast cell wall. It was, however, desirable to investigate whether these effects occurred in live yeast cells. Atomic Force microscopy (AFM) is a relatively new method that allows one not only to scan the surface of biological samples but also to determine the force required to cause deformation. One major problem with AFM is the attachment of the biological sample to the solid support in a manner which immobilises it yet affects neither the biological activity nor the surface properties of the sample. Previous methods used to immobilise yeast cells for AFM have included trapping the cells within polycarbonate pores (Dufrêne *et al.*, 2001) and drying yeast cells onto glass slides (Adya *et al.*, 2006). However, dried yeast cells poorly represent cells in an aqueous environment as such cells would most likely have very different surface properties as a result of the drying process. These would include an increased cell wall rigidity brought about by the increased osmolarity of the sample as well as the presence of cell wall proteins which are known to be expressed during desiccation. Although trapping yeast cells within polycarbonate pores retains the cells in an aqueous environment, the yeast cells may not be completely immobile within the pore, giving rise to the possibility of anomalous data when determining the force curves.

Physical adsorption of yeast cells onto polyethyleneimine (PEI) coated glass plates (Burks *et al.*, 2003; Camesano and Logan, 2000; Rodriguez *et al.*, 2002; Vadillo-Rodriguez *et al.* 2004) to immobilise W303 WT and KO yeast cells was thus considered the least invasive and was used in these studies. In addition, this method allowed the determination of the spring constant of the stiff PEI-coated glass surface and the yeast cell from the same plate.

Further assays to determine the flexibility of the yeast cell wall included electrophoretic mobility assays. The stiffness of the cell wall could be calculated using the data from these assays (Dague *et al.*, 2006). Finally, the yeast cell wall was investigated using infrared (IR) spectroscopy, to establish whether the chemical composition of the cell wall was altered in KO cells.

## 3.4.2. MATERIALS AND METHODS

### 3.4.2.1. Materials

All chemicals used were of high purity. Double distilled water was used for all experiments.

### 3.4.2.2. Organisms and culture conditions

Yeast strains and growth conditions were as previously described (this chapter, section 3.2).

### 3.4.2.3. Atomic Force Microscopy (AFM)

Polyethylimine (PEI, P-3143, Sigma-Aldrich, USA) covered glass slides were prepared by flooding one side of a nitric acid washed glass slide with 0.2 % (w/v) PEI after which it was allowed to stand at RT for 1 h. Slides were thoroughly washed with deionised water, allowed to dry and used immediately. An overnight culture of WT or KO yeast cells was washed twice with 1 mM $KNO_3$ pH 6.5 at 3000 *x g* for 10 min at 10°C and resuspended to a concentration of *ca.* 0.2 ODU/ml in the same buffer. This concentration of cells represented the optimum coverage of the glass slide as determined microscopically (Figure 3.4.1). The PEI-coated side of each slide was covered with washed yeast cells in 1 mM $KNO_3$ pH 6.5 and incubated at RT for 1 h. Slides were gently rinsed three times with deionised water and rapidly immersed in 1 mM $KNO_3$ pH 6.5. Immediately prior to analysis, slides were removed from the 1 mM $KNO_3$ pH 6.5 solution, fixed onto the AFM sample holder and covered by a drop of 1 mM $KNO_3$ pH 6.5. All AFM measurements were performed in this solution using a commercial microscope (Thermomicroscope Explorer Ecu+, Veeco Instruments, USA). Samples were initially imaged at low resolution to identify individual cells on the PEI-coated glass plate. Interaction forces were measured between a single silicon nitride tip ($k_c$ = 0.03

N/m, MLCT-EXMT-BF, Veeco Instruments, USA) and the cell surface. A single force measurement was performed on an individual cell to avoid potential hysteresis.

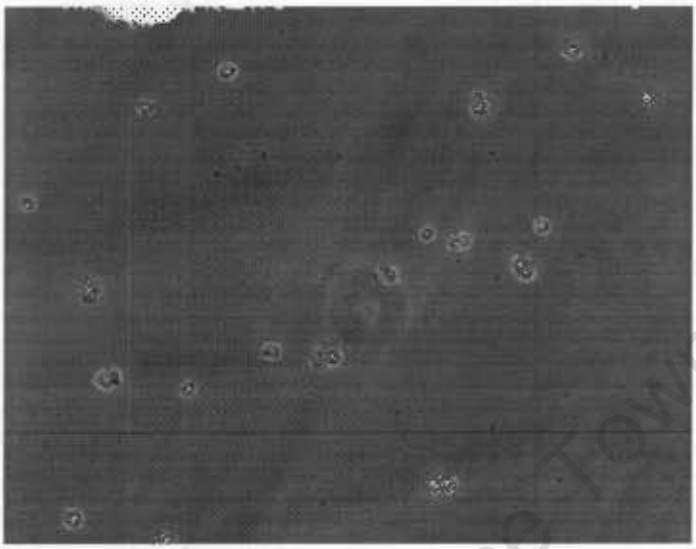


Figure 3.4.1. Glass slides were examined prior to AFM to determine the optimum optical density of cells required for even, non-overlapping cell coverage of the glass slide.

A minimum of 10 individual cells from two separate yeast cultures were used for such measurements. The X, Y and Z axis piezo-electric arms were used to position the tip. These piezo-electric arms could move minute distances in response to an applied electric current, allowing precise positioning of the tip. Raw deflection-piezo displacement curves were converted into force curves using the sensitivity of the photodetector and the spring constant of the cantilever. Force curves and standard deviations were calculated for each sample ($n = 10$). The first noticeable increase of force as the tip approached the sample was used as a reference for zero piezo displacement. After AFM measurements, slides were immersed in YEPD and incubated overnight to confirm the viability of the yeast cells used.

### 3.4.2.4. Electrophoretic Mobility Assays

Electrophoretic mobility measurements were performed on a Zetaphoremeter IV (SEPHY-CAD Instrumentation, France) equipped with laser illumination and a video interface. At least 50 measurements of cell mobility for each condition were performed in

a Suprasil quartz cell (Perkin-Elmer, USA) at RT. Yeast cells were prepared as for AFM and mobilities recorded at various ionic strengths ranging between 1 mM and 30 mM $KNO_3$ pH 6.5. The relationship between electrophoretic mobility and ionic strength was analysed using soft particle analysis (Duval and Van Leeuwen, 2004; Yezek *et al.*, 2005). Briefly, the electrokinetic (electrostatic and hydrodynamic) response of a soft particle (defined as an impermeable hard-core component covered with a permeable polyelectrolyte layer) can be described quantitatively using rigorous numerical evaluations without any restriction of size, charge and Debye thickness. The spherical yeast cells used were assumed to be soft particles with a core diameter $a$ close to 3 μm and with a cell wall thickness $\delta$ of 100 nm as identified from electronic images (Sales *et al.*, 2002). The experimental data were fitted using the method of least mean squares to determine the permeability parameter ($\lambda_0$) and the volumic charge density of the polyelectrolyte layer ($\rho_0$) of each cell.

### 3.4.2.5. Infrared Spectroscopy

Attenuated Total Reflectance Fourier Transform infrared (ATR-FTIR) spectra of yeast suspensions in Milli-Q water were measured between 4000 and 600 $cm^{-1}$ on a Perkin-Elmer 2000 spectrometer (Perkin-Elmer, USA) equipped with a KBr beam splitter and a DTGS (deuteriated triglycine sulphate) thermal detector. In a typical experiment, 100 μl of an overnight culture of WT or KO yeast cells was applied to the ATR accessory, which comprised a horizontal Diamond crystal prism (ASI Applied Systems, USA) with 9 internal reflections on the upper surface, an angle of incidence of 45° and a penetration depth at 1500 $cm^{-1}$ of 1 μm. The sample was covered with water and analysed at a spectral resolution of 4 $cm^{-1}$ for 4 min. Irradiance throughout an empty cell (without the ATR accessory) was about 20 % of full signal. ATR spectra of the sample were plotted using the absorbance of the sample together with the internal reflectances (R) of the device as log ($R_{reference}/R_{sample}$) versus wavenumber. The spectrum of water was used as an absorbance reference. The ATR spectra are not strictly proportional to the absorption coefficients at each wavenumber since no further correction has been applied (Mirabella and Harrick, 1985), but the results can still be compared relative to one another at each

wavenumber. The contribution of water vapour and carbon dioxide signals to the spectra was minimised using the $H_2O/CO_2$ procedure of the Spectrum 5.0.1 software.

## 3.4.3. RESULTS

### 3.4.3.1. Atomic Force Microscopy

PEI-coated glass slides with adhered yeast cells were incubated in YEPD after AFM analysis. Yeast cells remained viable after AFM determinations, indicating the non-invasiveness of this methodology (data not shown). A typical AFM image (Figure 3.4.2) of yeast cells physically adsorbed onto PEI-coated glass slides revealed spherical shapes with diameters ranging from 2.5 to 6 µm. These initial scans allowed the selection of individual cells for AFM force measurements of the cell wall. Regions of the cell wall with bud scars were not selected, since these regions exhibit increased rigidity due to the presence of chitin (Touhami *et al.*, 2003). All curves recorded displayed non-linear behaviour at low loading forces and a near-linear domain at high loading forces (Figure 3.4.3). Preliminary attempts to physically adsorb yeast to the slides and then perform AFM analysis in the presence of 100 mM salt were unsuccessful. This salt level prevented electrostatic interactions by shielding cell surface charge, lowering the adhesion of cells to the slide. Measurements were thus performed in 1 mM $KNO_3$, but this hampered nanomechanical interpretation of low loading force-dependent non-linear behaviour due to increased electrostatic interactions (Gaboriaud *et al.*, 2005; Dufrêne *et al.*, 2001). Fortunately, the high-loading force-dependent linear behaviour corresponded to the mechanical deformation of the cell wall and the spring constant $k_{cw}$ could be calculated using the following equation:

$$k_{cw} = -k_c \cdot \left( \frac{S}{S + k_c} \right)$$

where $k_c$ represents the spring constant of the cantilever and $S$ is the slope in the linear domain of the force curves.

In contrast with the stiff behaviour displayed by the glass surface, the force curves recorded for the two yeast strains exhibited soft behaviour with a non linear component at low loading forces and an almost linear domain at high loading forces (Figure 3.4.3). The

nanomechanical properties of 2.5 µm diameter cells of the two strains were different with the KO strain appearing stiffer ($k_{cw}$ = 72 ± 3 mN.m$^{-1}$) than the WT strain ($k_{cw}$ = 17 ± 5 mN.m$^{-1}$). Interestingly, the size of the cell affected the nanomechanical properties of the cell, as the spring constant of the cell walls of 4.5 µm diameter KO cells was lower ($k_{cw}$ = 19 ± 2 mN.m-1). This phenomenon was possibly due to larger cells presenting a flatter surface to the tip, allowing increased lateral force distribution.

The flexibility of agarose, used as a model system for the yeast cell wall, decreased upon incorporation of osmolytes. The effect of mannitol on the spring constant of the cell wall, $k_{cw}$, of WT yeast was thus investigated by incubating a PEI-coated glass slide containing this yeast strain in 0.8 M mannitol for 10 min before determining the force curves. The spring constant in the presence of mannitol increased 8-fold to 141 ± 1 mN.m$^{-1}$, in agreement with the previous data obtained using agarose.

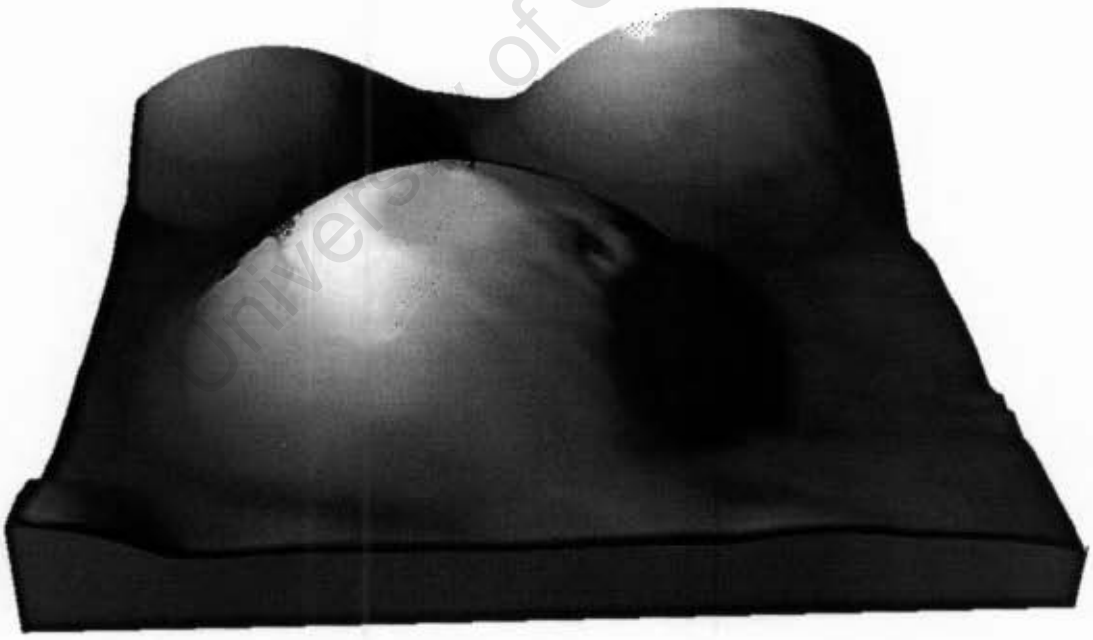


Figure 3.4.2: A typical AFM height image (12 x 12 µm; Z-Range: 3 µm) of *S. cerevisiae* WT cells recorded in aqueous solution (1 mM $KNO_3$ pH 6.5).

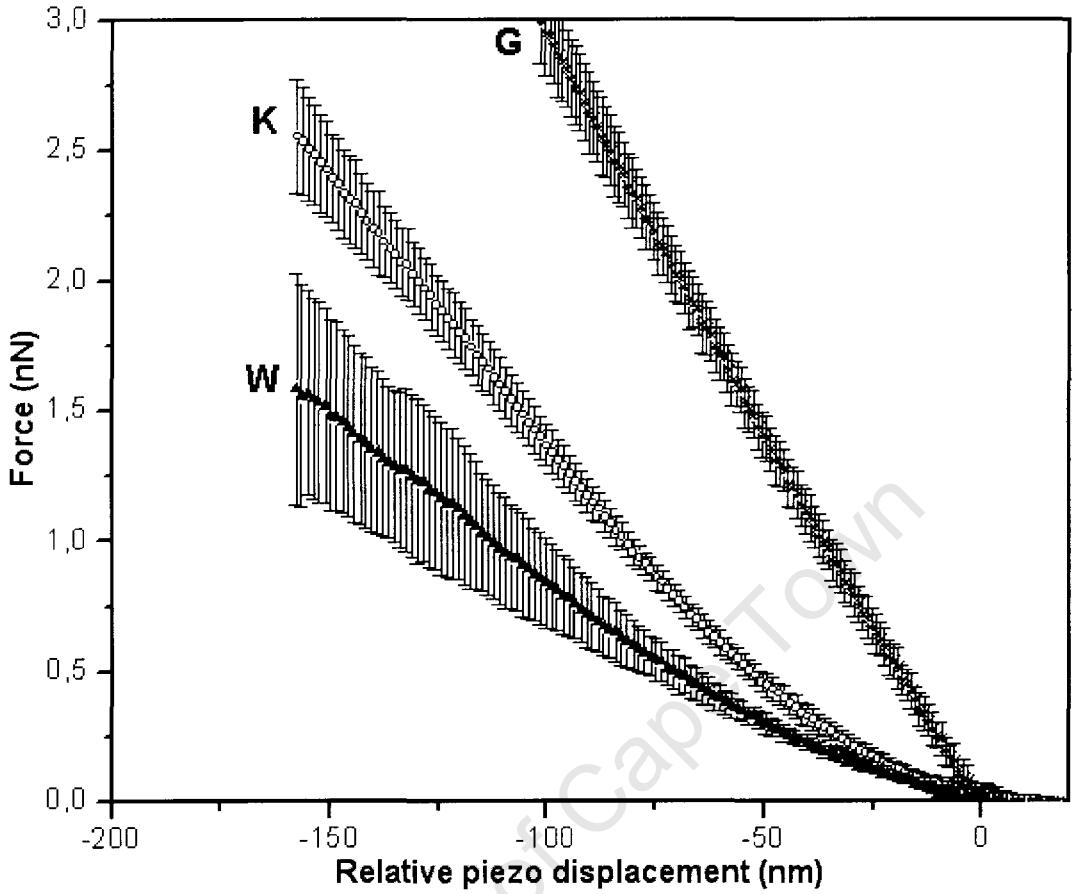



Figure 3.4.3: Mean and respective standard deviations (n = 10) of force curves measured on WT (W) and KO (K) yeast strains in aqueous electrolyte solution (1 mM $KNO_3$, pH 6). Typical experimental data obtained on a stiff glass surface (G) is plotted to illustrate the deformation of yeast. The size of all cells investigated was *ca.* 2.5 µm.



### 3.4.3.2. Electrophoretic Mobility Assays

The ionic strength dependence of the electrophoretic mobility of a particle relies on the softness of the particle. In the case of a rigid particle, the mobility tends to zero at high ionic strength, and the zeta potential at the slip plane of the particle can be calculated from the classical theoretical concepts of the Smoluchowski-Henry equations.

In contrast, soft particles such as biological cells possess non-zero asymptotic electrophoretic mobilities at high ionic strengths due to non-negligible contributions of the hydrodynamic properties. This behaviour is characteristic of particles with a soft, hydrodynamically permeable layer surrounding the particle.

The experimentally determined electrophoretic mobilities of WT and KO yeast cells at different ionic strengths and at neutral pH value are shown in Figure 3.4.4. The electrophoretic mobilities of both strains reached a non-zero constant value upon increasing the ionic strength, as expected from the theoretical considerations of soft particle behaviour.

Yeast cells were modelled as a particle with a hard core of diameter 3 μm coated with a polyelectrolyte permeable layer 100 nm thick. By fitting the experimental data, the permeability parameter ($\lambda_0$) and the volumic charge density ($\rho_0$) of the soft layer were determined for the two strains using methodology described for bacterial systems and humic substances (Duval *et al.*, 2005). The resulting best fits are shown in Figure 3.4.4 as plain lines. While the volumic charge densities were similar for the two strains, the softness parameter was higher for WT yeast (3 nm) compared with the KO strain (2.3 nm). This result indicated that the external layer of the KO cells was stiffer than the corresponding layer of WT cells.

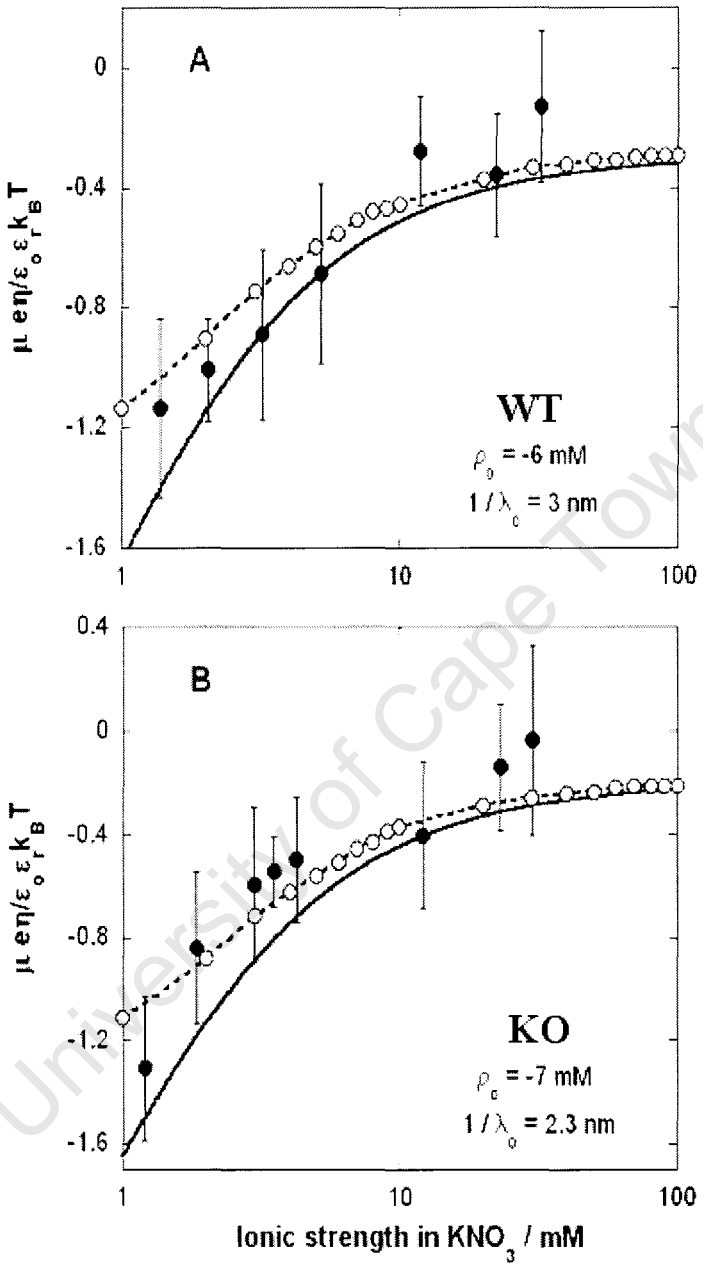



Figure 3.4.4. Electrophoretic mobilities as a function of ionic strength ($KNO_3$, pH 6.5) for the two types of *S. cerevisiae* strains. Experimental data (filled circles) were fitted by using the rigorous numerical evaluations (plain lines) with free parameters, $1/\lambda_0$ characterising the typical flow penetration length within the soft polymeric layer and $\rho_0$ the volumic charge density of that layer. The thickness of the permeable polyelectrolyte layer ($\delta$) has been considered to be 100 nm with a core radius close to 1.5 μm.

### 3.4.3.3. Infrared Spectroscopy

Yeast cells were 2.5 to 6 μm in diameter as demonstrated by AFM and optical images. The vertical thickness of ATR analysis from the crystal to the top vertical dimension in the solution was approximately 1 μm. Thus ATR spectra were related only to the spectral features of the periphery of the cells in contact with the diamond crystal. Figure 3.4.5 depicts the 1800 - 800 $cm^{-1}$ region of the ATR-FTIR spectra obtained for the WT and KO strains of yeast. The assignments of the spectra are summarised in Table 3.4.1. Bands near 1104 and 1075 $cm^{-1}$ were assigned to β-(1-3)-glucans, and bands at 977, 1050 and 1128 $cm^{-1}$ were assigned to mannans based on the known composition of the yeast cell wall (Klis *et al.*, 2002).

Table 3.4.1. Assignment of infrared vibrational bands in the region 1800-900 $cm^{-1}$ of the ATR-FTIR spectra of *S. cerevisiae* yeast cells in water suspension.

| Wavenumber/s ($cm^{-1}$) | Assignment* |
|---|---|
| 1644 | Amide I band (νC=O, δN-H, νC-N); δH2O (possible residual band arising from non-perfect compensation of water) |
| 1540 | Amide II band (νC-N, CNH) |
| 1448 | $CH_2$ bending |
| 1407 | CH bending |
| 1244 | Amide band III (νC=O, CN-H, νC-N); β–glucans |
| 1218 (sh) | P=O stretching in phosphate esters |
| 1200-950 | C-O-C, C-O, C-C stretchings and $CH_2$ (sugars); $PO_2$, C-O-P and P-O-P stretchings (weak intensity) |
| 1128, 1051, 977 | Mannans |
| 1104, 1075, 1071 | β-1-3-glucans |

*: Michell and Scurfield, 1970; Galichet *et al.*, 2001; Machova *et al.*, 1999
ν: stretching, δ: bending, sh: shoulder

The spectra of WT and KO strains were normalised with respect to the amide II (protein) band at 1540 $cm^{-1}$ and the difference spectrum between the two strains calculated (Figure 3.4.5, inset). The KO strain displayed a markedly lowered β-1-3-glucan and mannan (1150, 1240 and 950 $cm^{-1}$) content. In addition, the band at 1218 $cm^{-1}$ indicated lower phosphate group content in the KO strain, possibly due to lowered levels of phosphopeptidomannans.

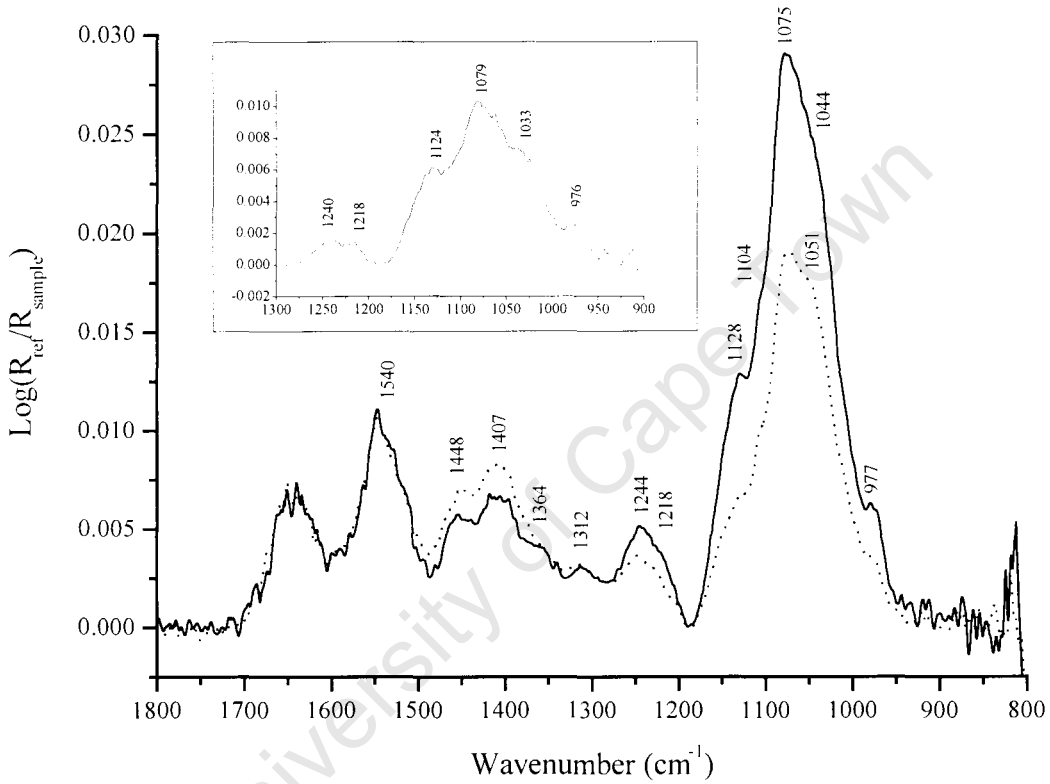



Figure 3.4.5: ATR-FTIR spectra (1800 to 800 $cm^{-1}$) of WT and KO cells in water. Solid line: WT; broken line: KO. Spectra are normalised with respect to the amide II band. Inset: The 1300 to 900 $cm^{-1}$ region of the difference spectrum (spectrum of the WT – spectrum of the KO), depicting differences in cell wall chemistry between the two strains.

### 3.4.4. DISCUSSION

These studies have shown that Hsp12p affects the flexibility, texture and chemistry of the yeast cell wall. Cells lacking Hsp12p were markedly stiffer and consequently did not deform easily. Furthermore, yeast cell walls were hardened by osmolytes (mannitol), as previously suggested by incorporation of osmolytes into agarose. Electrophoretic mobility of KO cells was markedly reduced due to a stiffer cell wall. Finally, the cell wall chemistry of cells lacking Hsp12p was altered and displayed a lower amount of glucans, mannans and phosphopeptidomannans. This may have been due to the following:
(i) Cells lacking Hsp12p would have less flexible cell walls and would thus have hampered insertion of these residues into the stiff cell surface,
(ii) Hsp12p may have acted through Mid1p to alter levels of intracellular calcium and consequently the function of Pmr1p and Fks2p, which process peptidomannans and synthesize glucans respectively (Section 3.5),
(iii) Yeast cells compensate for lack of certain cell wall components. Thus KO cells may have synthesised less glucans, which would decrease cell wall flexibility, to compensate for increased cell wall rigidity.

The lowered amount of glucan in the cell wall of KO cells might possibly result in a compensatory increase in chitin content, as has been observed in other cell wall mutants lacking glucan synthase activity (Ram *et al.*, 1998). Consequently, KO cells would have stiffer cell walls since previous studies have shown that the bud scar, mostly comprised of chitin, is stiffer than the surrounding cell wall (Touhami *et al.*, 2003). Furthermore, cells with elevated chitin levels in the cell wall would be vulnerable to agents such as CR, which bind chitin. In addition, the primary septum between mother and daughter cells, which comprises mostly chitin, would be altered, leading to impaired cell separation. This would explain our previous findings where KO cells were more vulnerable to CR and displayed cellular aggregation due to impaired cell separation.

## 3.5. GENERAL DISCUSSION

The data presented in this thesis suggest that Hsp12p plays multiple related roles in the cell wall. These roles are mediated independently of flocculin or PIR protein regulation. One such role is an interaction with chitin, since cells lacking Hsp12p were markedly more sensitive to CR, which binds chitin and disrupts cellular morphology. Interestingly, chitin is, like Hsp12p, synthesised in response to all forms of stress affecting cell integrity (Dallies *et al.*, 1998; Kapteyn *et al.*, 1999).

Studies employing agarose as a model system for the cell wall indicated that Hsp12p enhances cell wall flexibility, possibly to alleviate the effect of various stresses on the cell wall. This increased flexibility was postulated to involve interchelation of Hsp12p between agarose helices, facilitating their movement. This increase in elasticity was confirmed by atomic force microscopy, which allowed measurements of cell wall flexibility on live yeast cells. The effects of Hsp12p on cell wall chemistry included marginal enhancement of cell electrophoretic mobility, possibly by increased cell wall rigidity. Furthermore, KO cells displayed lower levels of phosphorylated peptidomannans and β-1,3-glucans. The decrease in glucan content may have led to an increased amount of chitin, due to the compensatory response exhibited by cell wall mutants. This would have led to a further increase in cell wall rigidity and impaired cell division.

These multiple roles can be presented as a model (Figure 3.5.1), where the binding target for Hsp12p is chitin. In this locus Hsp12p prevents Congo Red binding and interchelates between adjacent glucan chains, allowing them to move relative to one another, enhancing cell wall flexibility.

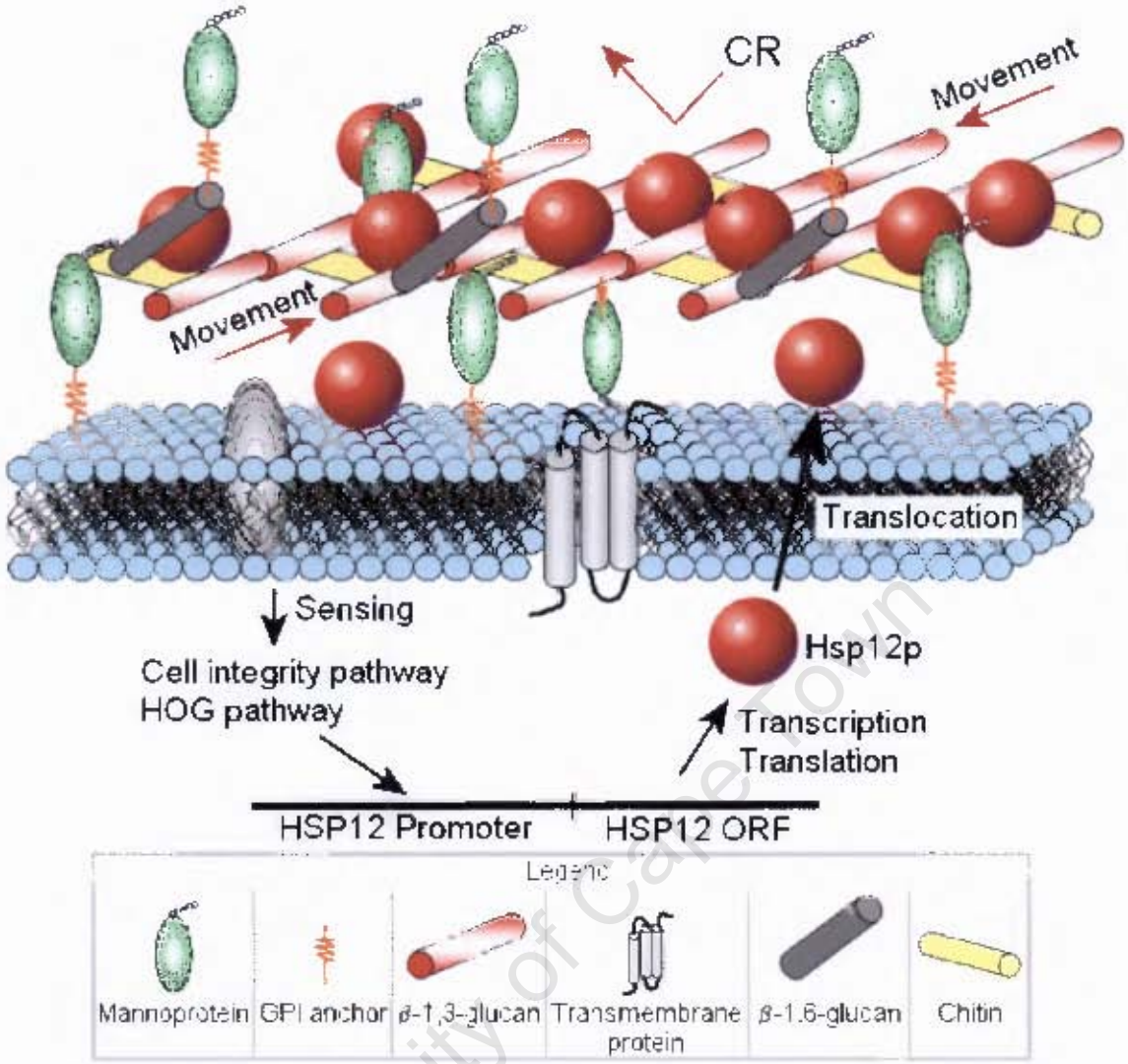



Figure 3.5.1. A simplified model depicting the multiple roles of the stress-responsive protein Hsp12p in the yeast cell wall. Hsp12p binds chitin and prevents CR binding, enhancing cell viability. Furthermore, Hsp12p interchelates between β-1,3-glucans, facilitates movement and increases cell wall flexibility.

What is the role of Hsp12p-mediated cell wall flexibility in yeast? One possibility is the modulation of calcium ($Ca^{2+}$) flux. Yeast cells require cytosolic calcium for the activity of a variety of enzymes responsible for cell wall synthesis and secretory protein processing, sorting and modification (Mazur *et al.*, 1995; Stathopoulos and Cyert, 1997; Dürr *et al.*, 1998, Zhao *et al.*, 1998). In addition, calcium is required for the unfolded protein response, which is activated upon stresses which result in an increased concentration of misfolded or unfolded proteins in the endoplasmic reticulum (ER) (Bonilla *et al.*, 2002). Rapid oscillations of calcium levels have also been shown to be

important for optimum gene regulation (Dolmetsch *et al.*, 1998). Yeast cells accumulate and store calcium in the ER through internal $Ca^{2+}$ pumps, such as Pmr1p, where it is available for rapid responses (Bonilla *et al.*, 2002). These stores are replenished by calcium uptake from the environment, through putative plasma membrane stretch-activated ion channels such as Cch1p/Mid1p (Iida *et al.*, 1994; Batiza *et al.*, 1996; Kanzaki *et al.*, 1999; Kanzaki *et al.*, 2000; Yoshimura *et al.*, 2004). Most $Ca^{2+}$-regulated pathways are initiated by the calcium and calmodulin-dependent serine/threonine-specific protein phosphatase, calcineurin (Rusnak and Mertz, 2000). This protein is responsible for the dephosphorylation of several targets such as the transcription factor, Crz1p (Matheos *et al.*, 1997; Stathopoulos and Cyert, 1997), which in turn induces cell wall construction genes, such as *FKS2* (Mazur *et al.*, 1995; Stathopoulos and Cyert, 1997; Zhao *et al.*, 1998). *FKS2* encodes a subunit of glucan synthase which produces β-1,3-glucans to increase cell wall strength to withstand turgor pressure and mechanical damage during stressful conditions.

Upon exposure to a variety of stresses, the $Ca^{2+}$ storage system is rapidly depleted, since large amounts of $Ca^{2+}$ are required for secretion and activation of cell wall remodelling enzymes and for the UPR. Moreover, these stresses harden the yeast cell wall (as shown using AFM) or prevent its outward expansion (Kamada *et al.*, 1995; de Nobel *et al.*, 2000; Harrison *et al.*, 2001; Torres *et al.*, 2002), limiting plasma membrane stretch and concomitantly $Ca^{2+}$ replenishment. We have found that the small heat shock protein, Hsp12p, increases the flexibility of the yeast cell wall and is expressed in response to a wide variety of stresses. We propose that Hsp12p may play a role in the replenishment of internal $Ca^{2+}$ stores by enhancing the activity of stretch-activated ion channels through enhancing cell wall flexibility. In this manner, Hsp12p would also enhance downstream cell wall remodelling events during continued stress, promoting cell viability.

Several threads of evidence support this model, depicted in Figure 3.5.2). Under conditions of hypo-tonic shock, which inhibits outward membrane expansion, a $Ca^{2+}$ pulse is rapidly observed within seconds, indicative of the release of internal stores of $Ca^{2+}$. Thereafter, the yeast cell gradually takes up calcium (Batiza *et al.*, 1996). This time

lapse is indicative of a pause where proteins must be expressed before ion channels can be activated. Since Hsp12p is induced through multiple pathways and expression is only pronounced after 20 minutes (Varela *et al.*, 1995), this time lapse may be explained by an increase in Hsp12p-mediated cell wall flexibility. Furthermore, in cells pre-stressed by exposure to cold for 6 days, calcium uptake was markedly enhanced in response to hypo-tonic shock, implying that an additional co-factor expressed in response to cold was necessary to ensure optimum $Ca^{2+}$ uptake (Batiza *et al.*, 1996). Kandror *et al.* showed that besides trehalose synthesizing enzymes, a variety of heat shock proteins, including Hsp12p, were slowly induced in response to cold (Kandror *et al.*, 2004). Moreover, calcium uptake in cells exposed to glucose limiting conditions, where cAMP concentrations are low and PKA activity is diminished, showed high levels of $Ca^{2+}$ influx. These levels were not restored to basal levels, implying continuous $Ca^{2+}$ uptake and loss of $Ca^{2+}$ flux control (Batiza *et al.*, 1996). This may be explained by a lack of PKA activity, which results in a strong induction of *HSP12* gene expression (Varela *et al.*, 1995). This would result in a much more flexible cell wall and subsequent continuous activation of stretch-activated channel proteins.

Kamada *et al.* observed that yeast cells activate the cell integrity protein kinase C (PKC) pathway in response to mild heat shock (Kamada *et al.*, 1995). This response was abolished in cells where plasma membrane stretch was inhibited, prompting the postulation that downstream components of this pathway were activated by weakness of the cell wall. Subsequent plasma membrane stretch would result in calcium influx, and PKC1 is activated directly by this ion (Levin *et al.*, 1990). In addition, Kamada *et al.* found that expression of Mpk1p, the MAP kinase mediating this pathway, was only observed after 20 minutes. Our model can possibly explain the nature of these findings, where expression of Hsp12p, which initiates at 20 minutes and is maximal at 30 minutes, gradually weakens the cell wall, enhancing $Ca^{2+}$ uptake and resulting in Mpk1p activation. Mpk1p and $Ca^{2+}$-activated calcineurin then act synergistically to stimulate Fks2p to reinforce the yeast cell wall (Zhao *et al.*, 1998). Furthermore, Mpk1p has been shown to be directly involved in Mid1p activation (Bonilla and Cunningham, 2003) and over-expression of Pkc1p in *S. pombe* resulted in a high level of intracellular $Ca^{2+}$

(Arellano *et al.*, 1999; Calonge *et al.*, 2000) which was dependent on the presence of Ehs1p, a Mid1p homologue (Iida *et al.*, 1994; Carnero *et al.*, 2000). Over-expression of Pkc1p in *S. cerevisiae* has been shown to result in *HSP12* induction, thus linking the two processes (Garcia *et al.*, 2004).

The importance of regulating *HSP12* in response to hyper-osmotic shock is two-fold. Firstly, in response to salts such as NaCl or LiCl, intracellular levels of $Na^+$, $Li^+$ or $Cl^-$ increase. Some authors have demonstrated that calcineurin is essential in high salt stress adaptation of yeast (Mendoza *et al.*, 1994; Nakamura *et al.*, 1993). Since cell wall flexibility and $Ca^{2+}$ uptake are necessary for sustained calcineurin function, Hsp12p may be needed for the adaptation of yeast cells to salt stress. Secondly, Alonse-Monge *et al.* showed that cells lacking the general osmotic stress responsive factor, Hog1p, were more susceptible to cell wall degrading enzymes, indicative of a common function of these two independent pathways. Hog1p was believed to act by controlling vesicle transport of cell wall enzymes, enhancing cell wall integrity (Alonse-Monge *et al.*, 2001). Furthermore, Hog1p stimulates *HSP12* induction (Siderius *et al.*, 1997). The cell integrity pathway and osmotic stress pathway thus share functional aspects and are both characterised by *HSP12* induction.

Further evidence is provided by the fact that (i) chitin synthesis is activated in response to cell wall stress and this is the binding target for Hsp12p, (ii) stress in general has been postulated to be sensed by plasma membrane stretch (Kamada *et al.*, 1995; de Nobel *et al.*, 2000; Harrison *et al.*, 2001; Torres *et al.*, 2002) and (iii) Hsp12p may directly affect the mobility and hence function of Fks2p by promotion of actin patch mobility (Delley and Hall, 1999; Utsugi *et al.*, 2002), since Jang *et al.* found that a mutant lacking proper cytokinesis could be rescued by Hsp9p over-expression, a protein in *S. pombe* extremely similar to Hsp12p. (Jang *et al.*, 1996).

All these findings and evidence can be represented as a model for Hsp12p function (Figure 3.5.2). In this model, a role for Hsp12p's interaction with Cpr1p (see chapter 1, section 1.2) is also tentatively proposed. This protein is the binding target for Cyclosporin

A (CsA), an inhibitor of calcineurin secreted by a variety of organisms as an antifungal agent. Thus by Hsp12p competing with CsA for Cpr1p, calcineurin activity is prolonged, giving the cell time to fortify its cell wall. Our model also predicts that cells lacking Hsp12p would have less post-translationally processed cell wall peptidomannans and lowered glucan content, which was indeed indicated by infrared spectroscopy.

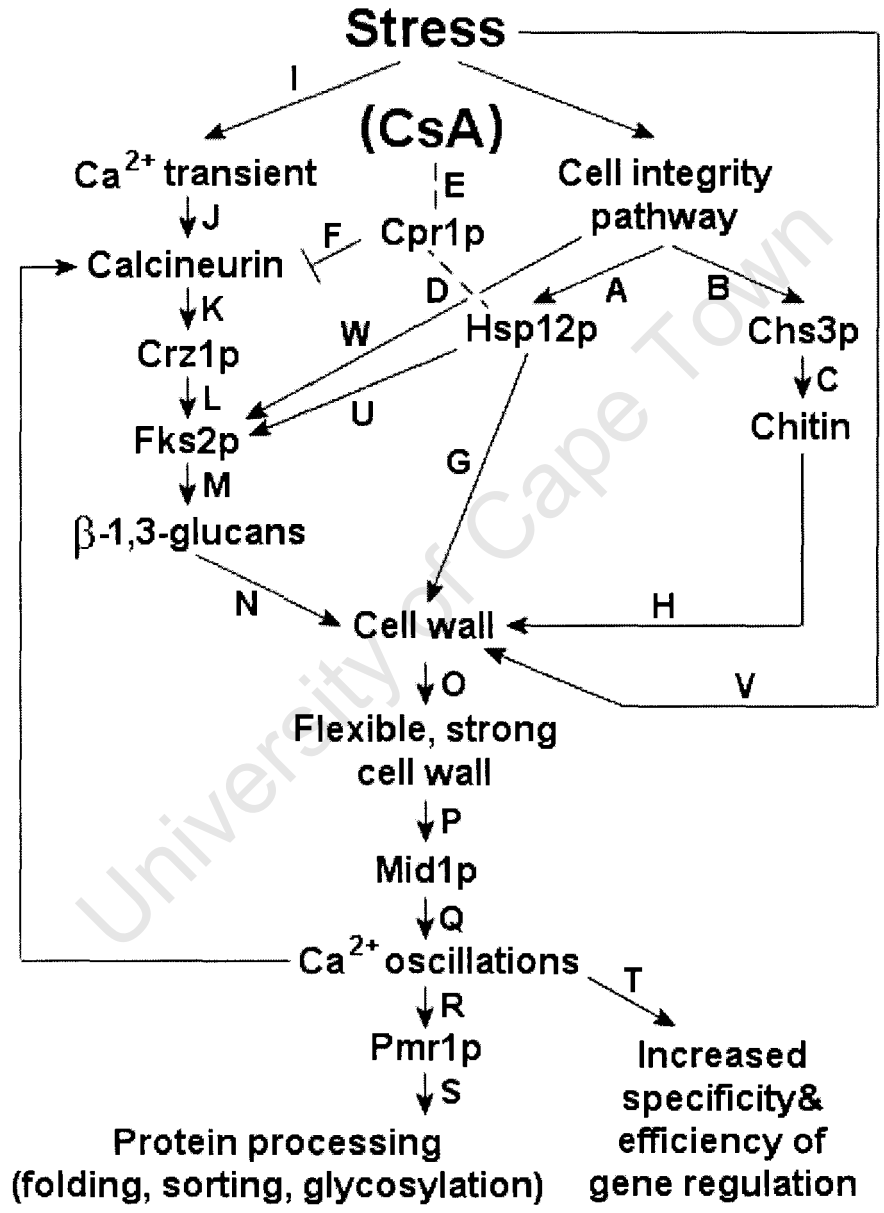



Figure 3.5.2. A model for Hsp12p function in *S. cerevisiae*. References are indicated by letters of the alphabet. Further details are provided in Table 3.5.1. Arrows: induction / stimulation, blunt-ended lines: repression / inhibition and broken lines: interaction.

Table 3.5.1. A summary of the references and findings used to compile the model presented in Figure 3.5.1.

| Name | Reference/s | Brief description |
|---|---|---|
| A | Garcia *et al.*, 2004. | *HSP12* is induced by the cell integrity pathway in response to stress. |
| B | Jung and Levin, 1999; Roberts *et al.*, 2000. | The cell integrity pathway induces chitin synthesis. |
| C | Popolo *et al.*, 1997; Ram *et al.*, 1998. | Chs3p synthesizes chitin in response to stress. |
| D | Ho *et al.*, 2002. | CsA binding protein Cpr1p associates with Hsp12p. |
| E | Breuder *et al.*, 1994. | CsA associates with Cpr1p. |
| F | Breuder *et al.*, 1994. | The Cpr1p / CsA complex inhibits calcineurin. |
| G | Motshwene *et al.*, 2004; This study. | Hsp12p is present in the yeast cell wall. |
| H | Orlean, 1997. | Chitin is a cell wall component. |
| I | Batiza *et al.*, 1996. | Stress causes a $Ca^{2+}$ transient. |
| J | Rusnak and Mertz, 2000. | $Ca^{2+}$ activates calcineurin. |
| K | Matheos *et al.*, 1997. | Calcineurin acts via Crz1p to mediate transcription. |
| L | Mazur *et al.*, 1995; Stathopoulos and Cyert, 1997; Zhao *et al.*, 1998. | Crz1p induces *FKS2*. |
| M | Douglas *et al.*, 1994. | *FKS2* encodes a subunit of glucan synthase. |
| N | Smits *et al.*, 1999. | β-1,3-glucan is the main component responsible for cell wall strength. |
| O | This study | Hsp12p enhances cell wall flexibility. |
| P | Kanzaki *et al.*, 1999; Kanzaki *et al.*, 2000; Yoshimura *et al.*, 2004. | Mid1p is stretch-activated. |
| Q | Iida *et al.*, 1994. | Mid1p is needed for calcium flux. |
| R | Dürr *et al.*, 1998. | $Ca^{2+}$ is needed for Pmr1p function. |
| S | Dürr *et al.*, 1998. | Pmr1p is required for protein glycosylation and sorting. |
| T | Dolmetsch *et al.*, 1998. | $Ca^{2+}$ oscillations optimise gene regulation. |
| U | Jang *et al.*, 1996; Delley and Hall, 1999; Utsugi *et al.*, 2002. | Hsp12p affects actin parameters since a mutant lacking proper cytokinesis could be rescued by Hsp9p, an Hsp12p homologue in *S. pombe*; Actin patch mobility is needed for Fks2p function. |
| V | This study. | Agarose (model cell wall) is hardened by stress. |
| W | Mazur and Baginsky, 1996. | Fks2p is activated directly by the cell integrity pathway factor, Rho1p. |

# CHAPTER 4
# GENERAL DISCUSSION AND FUTURE PROSPECTS

## GENERAL DISCUSSION

These studies have shown that Hsp12p resides in the yeast cell wall from where it can be extracted with alkali solutions.

What are the practical consequences of Hsp12p's presence in the cell wall on the yeast cell? One could envisage that yeast cells in the environment require a mechanism to alleviate stresses induced by, for example, the osmotic shock of sudden rainfall or the heat of the sun. These stresses require stress response proteins which require calcium signalling for optimum synthesis. Thus by increasing cell wall flexibility, optimum recovery and continued colonization of the nutrient-rich surface would be facilitated. In addition, Hsp12p would aid the yeast cell in invading the substrate and allow it to forage for nutrients. Hsp12p would also protect chitin from the cell wall degrading enzymes secreted by many plants and insects. Indeed, studies on wild yeast may provide even more functions for this multi-stress responsive multi-functional protein.



We have discovered that Hsp12p mediates the flexibility of the cell wall in response to a variety of stresses. These stresses are viewed as affecting cell wall flexibility (salts, alcohol, osmotic shock, heat) or cell wall integrity (damaging enzymes and compounds), as well as resulting in increased membrane fluidity (heat) and an increase in unfolded proteins (all stresses). Furthermore, we have shown that Hsp12p does not mediate its effects through alteration of the expression levels of flocculins or cell wall localised PIR proteins. Instead, Hsp12p appears to alter the levels of cell wall polysaccharides and protects cell wall chitin.

These studies have provided other yeast stress response researchers with a rapid method to quantify stress using a strain of yeast expressing fluorescent Hsp12p. This strain may also be employed in future in an industrial setting to monitor industrial

processes such as ethanol production, enzyme manufacture, etc. and the stresses encountered in these operations. In addition, the use of this strain of yeast as a biosensor to rapidly assess secondary effects of cleaning agents, herbicides, etc. in the environment and to identify new toxins is proposed.

## FUTURE PROSPECTS

Further studies could involve:

1. The extraction of cell wall proteins in response to various stresses at various times and at different times in the yeast cell cycle, to determine the kinetics of translocation of Hsp12p into the cell wall.
2. Investigations into the interaction of Hsp12p with Cyclosporin A binding proteins.
3. Northern blot analysis of *HSP12* mRNA levels with simultaneous determination of chitin synthase expression to establish a stoichiometry between the effects of stress on the yeast cell wall and Hsp12p expression and binding to chitin.
4. The chemistry of Hsp12p-chitin interactions.
5. Investigations into regulatory cross-talk between the cell integrity pathway and other stress response pathways in an *rlm1Δ* mutant. This mutant, in accordance with the model proposed in Chapter 1 (Figure 1.4), would possibly exhibit decreased expression in response to a variety of stresses.
6. Determination of the effects of oleic acid on *HSP12* induction by analysing the effect of oleic acid on protein kinase C activity, since a human homologue of yeast PKC is activated by this fatty acid.
7. Measurements of intracellular $Ca^{2+}$ and cell wall components in response to various stress conditions as well as at different points in the yeast cell cycle, to confirm the model of Hsp12p function.

However, these studies may be hampered by the following:

1. Several authors have reported the plasticity of gene regulation in compensating for mutations affecting cell wall integrity, such as enhanced levels of chitin and cross-linking components (Hong *et al.*, 1994; Gentzsch and Tanner, 1996; Popolo *et al.*, 1997; Ram *et al.*, 1998).

2. Some cell wall proteins are not essential for viability but do result in decreased cell wall stability (Hagen *et al.*, 2004).
3. The genetic background of the yeast strain has in many cases been shown to affect phenotypes such as flocculation and invasion. Thus the necessity of Hsp12p in response to stress and $Ca^{2+}$ signalling may only be observed in actual "wild" yeast cells, which must be optimally adapted for stress in an unforgiving environment.

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