

# **Over-expression of *FLO* genes in *Saccharomyces cerevisiae* BY4742 strains bearing a deletion in genes related to cell wall biogenesis**

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by

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School of Life Sciences  
College of Agriculture, Science and Engineering

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## SUMMARY

Mannoproteins form the outermost layer of the cell wall in *Saccharomyces cerevisiae*. These glycoproteins are first synthesized in the endoplasmic reticulum and undergo posttranslational modification before they are transported through the secretory pathway. Some of the glycosylphosphatidyl inositol (GPI) anchored proteins are incorporated into the cell wall where their GPI-anchor is first trimmed off before they are anchored into the  $\beta$ -glucan network in the cell wall. The yeast cells are constantly faced with different environmental conditions and the cell surface mannoproteins are responsible for different morphological transitions that allow the cell to survive harsh conditions. The Flo proteins or adhesins encoded for by the family of *FLO* genes are known to confer adhesion to biotic and abiotic surfaces, hydrophobicity, biofilm and pseudohyphal filamentation. These phenotypes are suggested to be passive mechanisms employed by the cells to escape from stress or to prevent being washed away. The adhesion properties conferred by the adhesins are important in biotechnological processes. Identification of genes that have the potential to release more adhesins into the culture media will facilitate studies on the fine structural details and functional domains in these glycoproteins. The knowledge will also help in the formulation of fungal drugs since the adhesion of fungal pathogens to host such as humans is known to be the first step of infection.

In this study, yeast strains with a deletion in *KNR4* or *GPI7*, which are genes related to the biogenesis of the cell wall were employed to over-express *FLO* genes. Flocculation intensity and hydrophobicity of cells in the stationary phase were used as a measure of phenotypic changes of the cell surface. The effects of these deletions on the cell surface phenotypes in transgenic strains over-expressing *FLO* genes were assessed. We found that the *KNR4* deletion resulted in a 50% decrease in cell-cell adhesion compared to the wild type. The deletion in *GPI7* was found to have no effect in flocculation or the cell initiated a response that resulted in the expression of other genes to compensate for the loss of *GPI7*. The ability of the yeast cells to invade agar surfaces was not affected by the deletion of *GPI7* or *KNR4*. The observed flocculation intensity was found to correlate with cell surface hydrophobicity. A decrease in the level of flocculation was also accompanied by a decrease in cell surface hydrophobicity. The results of this study indicate that deletion of the *KNR4* gene affects the adhesins more than the deletion of the *GPI7* gene. A screen of other genes related to cell wall biosynthesis will allow for a selection of genes with the potential to release adhesins to the cell culture medium.

This dissertation is dedicated to my family

## **BIOGRAPHICAL SKETCH**

Sizwe Innocent Mhlongo was born on the 13<sup>th</sup> of October 1989 and raised in Durban in the area of Adams Mission. He matriculated in 2006 from KwaMakhutha Comprehensive High School at KwaMakhutha and achieved a distinction with merit. He then enrolled for a Bachelor of Science degree in 2007 at the University of KwaZulu-Natal, majoring in Biochemistry and Microbiology. In 2009 he managed to finish his undergraduate degree and continued for a BSc Honours degree in 2010 in the field of Biochemistry. Sizwe is the first born out of five siblings and a cousin brother. He is passionate about research, specifically the biotechnology field. He hopes to have a career in this field and contribute to the bio-economy of the country by research that can produce products for the market, not only theoretical research. He is also passionate about understanding the sociology and making scientific research to benefit the society. He is keen to gain knowledge in the field of bioprocess engineering.

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# PREFACE

This dissertation is presented as a compilation of 5 chapters.

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Cell wall anchorage and genetic regulation of adhesion encoding genes in *Saccharomyces cerevisiae*

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# Chapter 1

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## **GENERAL INTRODUCTION AND PROJECT AIMS**

# 1 INTRODUCTION AND PROJECT AIMS

## 1.1 INTRODUCTION

In nature fungal populations respond to environmental stress or nutrient limitations by adhering to each other, or to surfaces and by the formation of biofilms. These morphological transitions are essential for the survival of cells and are suggested to be a passive transport mechanism employed by the cells to escape from stress (Verstrepen and Klis, 2006) or to prevent cells from being washed away (Linder and Gustafsson, 2008; Van Mulders, et al., 2009). The adhesion properties of fungal species are also essential for various biotechnological processes. Adhesion is exploited in bioremediation of pollutants in water (Gonzalez, et al., 2010; Ryan, et al., 2005) and in the production of fermented beverages for easy separation of product from the yeast cells (Cunha, et al., 2006; Govender, et al., 2008). Adhesion is also of medical relevance because fungal pathogens adhere to human tissues or plastic devices, which is the first step of infection before they can access internal organs (Fichtner, et al., 2007; Gonzalez, et al., 2010).

The budding yeast *Saccharomyces cerevisiae* provides a model system for understanding the mechanisms associated with adhesion (Fichtner, et al., 2007). The events of adhesion require the expression of specialised cell surface proteins which are encoded by a family of *FLO* genes. There are five dominant *FLO* genes, *FLO1*, *FLO5*, *FLO9*, *FLO10* and *FLO11* (Guo, et al., 2000). In the *S. cerevisiae* S288c background strain, these genes are silenced. The silencing of *FLO1*, *FLO5*, *FLO9* and *FLO10* is genomic location specific and in *FLO11* it is promoter specific (Bester, et al., 2006; Halme, et al., 2004). The *FLO* genes are epigenetically silenced by different histone deacetylases (HDAC) and also by a nonsense mutation in one of their transcriptional regulators, *FLO8* gene (Halme, et al., 2004; Liu, et al., 1996).

The synthesis of Flo proteins or adhesins starts in the endoplasmic reticulum (ER) bound ribosomes. These proteins undergo three posttranscriptional events where they receive a glycosylphosphatidyl inositol (GPI) anchor. They are transported to the cell wall through a secretory system and their GPI anchor is trimmed off, leaving a remnant which anchor these proteins into the cell wall (Pittet and Conzelmann, 2007). The adhesins are tightly anchored into the cell wall through covalent linkage to the  $\beta$ -glucan polymer. Defects in the cell wall result in cell lysis and release of mannoproteins which make the outermost layer of the cell wall. Therefore targeted deletion of the cell wall biosynthesis related genes would result in poor anchorage of the adhesins in the yeast cell walls (Gonzalez-Ramos and Gonzalez, 2006; Gonzalez, et al., 2010).

## 1.2 AIMS OF THIS STUDY

This work was aimed at the construction of *FLO* gene over-expressing strains bearing a deletion in cell wall synthesis related genes. The constructed strains were expected to express the *FLO* gene towards the end of exponential or at stationary phase of growth due to an *ADH2* promoter placement upstream of the *FLO* gene open reading frame (ORF). The *ADH2* promoter is a glucose repressible promoter and is therefore expressed when glucose is exhausted in the medium. Also the effect of deleting *KNR4* or *GPI7* genes on adhesion phenotypes conferred by the over-expression of *FLO* genes would be assessed. The outcomes of this work would assist in the development of strains with the potential to overproduce these adhesins into the cell culture media. Furthermore, the work carried out would assist in the identification of genes in the cell wall that are required for proper anchorage of adhesins which could be used as potential target sites in the development of novel antifungal drugs.

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# Chapter 2

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## LITERATURE REVIEW

**Cell wall anchorage and genetic regulation of  
adhesion encoding genes in *Saccharomyces  
cerevisiae***



## 2 CELL WALL ANCHORAGE AND GENETIC REGULATION OF ADHESION ENCODING GENES IN *SACCHAROMYCES CEREVISIAE*

### 2.1 INTRODUCTION

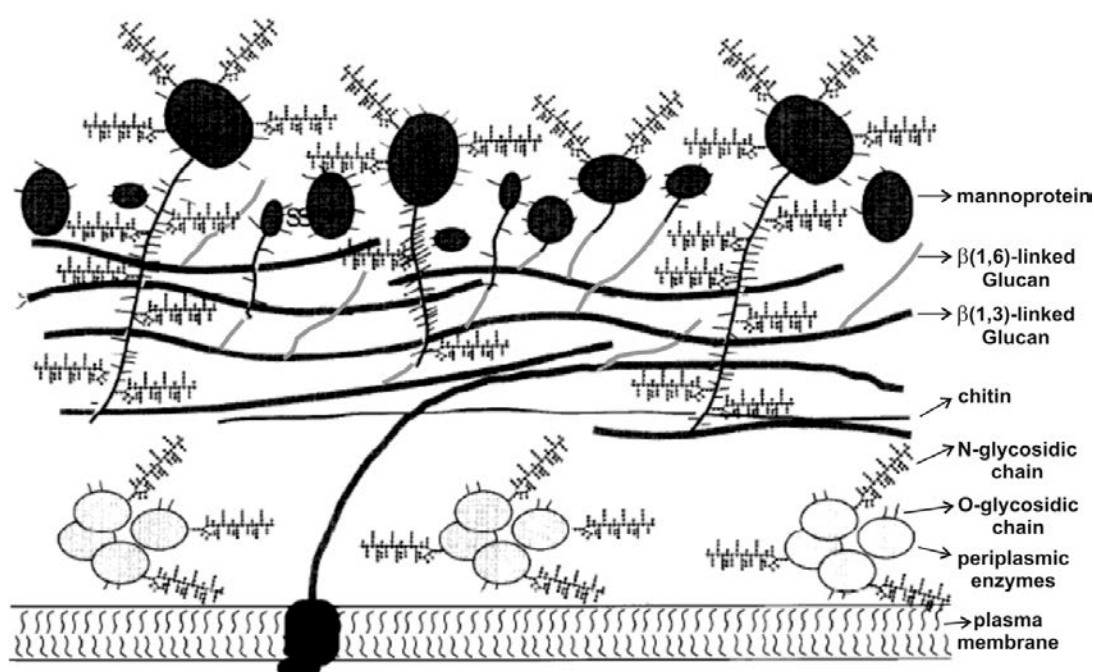
The yeast, *Saccharomyces cerevisiae* is employed in alcoholic fermentation processes to convert sugars to ethanol and other metabolites such as glycerol, acetate, succinate, pyruvate, and other esters, all which contribute to the sensorial properties of the final product (Gonzalez-Ramos and Gonzalez, 2006). Yeasts release cellular constituents such as proteins and polysaccharides during autolysis (Gonzalez-Ramos, et al., 2010). The yeast derived cell wall constituents, particularly mannoproteins, have attracted much attention in the wine making industry for the past decade due to their reported contribution in the improvement of wine quality and physicochemical stability (Cebollero, et al., 2009). The general functions of mannoproteins in the yeast cell wall is to provide the cell wall with its active properties such as mating and flocculation ability and to control wall porosity (Cebollero, et al., 2007).

The cell wall in *S. cerevisiae* is a rigid structure that surrounds the cell and therefore serves as a protective barrier, providing mechanical support and enabling selective uptake of macromolecules (Abramova, et al., 2001; de Nobel, et al., 2000; Martin, et al., 1999). This allows the cell to adapt to changing environmental conditions and also prevent it from lysing due to osmotic differences with the surroundings (Cid, et al., 1995). The cell wall in eukaryotes is shaped by a cytoskeleton which contains a highly conserved secretory system. This secretory system moves relevant construction machinery for the construction of the extracellular matrix (Lesage and Bussey, 2006).

The maintenance of cell shape is essential for the formation of a bud and hence cell division (Levin, 2005). The structure of the cell wall is dynamic and adapts to different physiological states (ie. conjugation, sporulation, stationary phase) and morphological changes (i.e., pseudohyphal and agar-invasive growth) (Martin, et al., 1999). The cell wall structure is remodelled to allow for cell expansion during events such as vegetative growth, mating, pheromone-induced morphogenesis and nutrient driven filamentation (Levin, 2005).

## 2.2. MOLECULAR ARCHITECTURE OF THE CELL WALL

Yeast cells invest considerable energy in the biogenesis of their cell walls which comprise about 10-25% of their cell dry weight (Bayly, et al., 2005). The cell wall in *S. cerevisiae* is composed mainly of polysaccharides (~85%) and proteins (~15%) (Lesage and Bussey, 2006). These components are packed in two layers which consist of an inner and an outer layer. The relative proportions of each layer vary with growth conditions and development stages such as budding, mating, sporulation, etc. (Figure 2.1) (Kapteyn, et al., 1999; Lesage, et al., 2004; Valdivieso, et al., 2000). The inner layer consists mainly of  $\beta$ -1,3-glucan chains and a small amount of chitin. This layer plays a role in providing mechanical strength and elasticity to the cell wall. The second layer consists of a lattice of highly glycosylated mannoproteins and some  $\beta$ -1,6-glucan branches. All major components in the cell wall are cross linked to  $\beta$ -1,3-glucan, which makes it a principal cell wall component. It is clear that  $\beta$ -1,3-glucan is required for construction of a strong cell wall and the glucan structure in general is responsible for cell wall rigidity (Levin, 2005). Therefore, proper synthesis and assembly of this polymer is crucial for construction of functional cell walls (de Nobel, et al., 2000).



**Figure 2.1** Composition and structure of the cell wall of *S. cerevisiae*. The cell wall, which is located outside the plasma membrane, consists of two layers. The inner layer provides cell wall strength, and is made of  $\beta$ -1,3- and  $\beta$ -1,6-glucan that is complexed with chitin. The outer layer consists of mannoproteins, and determines most of the surface properties of the cell. The majority of mannoproteins are covalently linked to the inner glucan layer. Periplasmic enzymes are trapped between the plasma membrane and the inner skeletal layer (Schreuder, et al., 1996).

### 2.2.1 Synthesis and assembly of $\beta$ -1,3-glucan

The  $\beta$ -1,3-glucan forms a fibrous network visible by scanning electron microscopy. It is a linear molecule (Lipke and Ovalle, 1998) participating in branching through C6 atoms [Table 1, (Kapteyn, et al., 1999)]. It has a coiled spring-like structure that allows for the elasticity and tensile strength of the cell wall. The non-reducing ends of the  $\beta$ -1,3-glucan chain allows this molecule to cross-link with other molecules in the cell wall. The non-reducing terminal glucose of  $\beta$ -1,3-glucan is cross-linked to the reducing end of  $\beta$ -1,6-glucan through an unidentified linkage. It is also linked to the reducing ends of chitin chains through a  $\beta$ -1,4 link and O-mannosylated cell wall proteins are linked to the  $\beta$ -1,3-glucan through an alkali sensitive bond (Kapteyn, et al., 1999; Lesage and Bussey, 2006).

**Table 1** Macromolecules of the cell wall in *S. cerevisiae* (Klis, et al., 2006)

Macromolecules	Wall dry weight (%)	Average Mw (kDa)
Mannoproteins	30-50	Highly variable
1,6- $\beta$ -Glucan	5-10	150
1,3- $\beta$ -Glucan	30-45	1500
Chitin	1.5-6	120

Synthesis of  $\beta$ -1,3-glucan starts in the plasma membrane bound protein complex and it is thereafter extruded through the plasma membrane to the cell wall (Goossens, et al., 2011; Lesage, et al., 2004). The protein complex contains both the catalytic and regulatory subunit. The enzyme implicated in the synthesis of  $\beta$ -1,3-glucan is  $\beta$ -1,3-glucan synthase (GS). This enzyme contains a catalytic subunit, encoded by two homologous genes, *FKS1* and *FKS2/GSC2* and a regulatory subunit which is a small guanosine triphosphatase (GTPase) Rho1p. The *FKS* genes form large proteins (more than 200 kDa) with 16 predicted transmembrane helices. These proteins also have a central hydrophilic domain of approximately 580 amino acids showing an approximate 80% degree in homology to other *FKS* genes that have been sequenced. Rho1p forms the regulatory subunit of the  $\beta$ -1,3-glucan synthase. This regulatory subunit switches between a guanosine diphosphate (GDP)-bound inactive state and a guanosine triphosphate (GTP)-bound active state. Rho1p is first synthesized in the endoplasmic reticulum (ER) and then it is geranylated in order to attach to the membrane to be transported (Goossens, et al., 2011).

The modified Rho1p together with Fks1p localizes to sites of cell wall remodelling in the plasma membrane (i.e., the bud tip during bud growth and the bud neck during cytokinesis) as an inactive complex. On its arrival to the plasma membrane, Rho1p is activated by Rom2p which is a GDP/GTP exchange factor of Rho1p that is only localized at the plasma membrane. The activation of Rho1p and movement of Fks1p to the plasma membrane is required for proper localization of the cell wall  $\beta$ -1,3-glucan (Goossens, et al., 2011). The GS is suggested to play a role in the transportation of glucan chains from the plasma membrane into the cell wall. Deletion of both *FKS1/FKS2* is lethal to the cell. However, deletion of *FKS1* only leads to a decrease in  $\beta$ -glucan. A third gene known as *FKS3* has been identified but its function has not yet been characterized (Goossens, et al., 2011; Lesage, et al., 2004).

### 2.2.1.1 Identification of cell wall mutants

The construction of proper cell walls in *S. cerevisiae* is a tightly controlled process. It has been shown through cell wall perturbants such as Calcofluor white and Congo red (Garcia, et al., 2004; Kim, et al., 2010), that there are about 1 200 genes that directly or indirectly affect the biogenesis of cell walls. These genes are divided into various classes based on the function of the proteins they encode. These classes include proteins involved in the synthesis of cell wall macromolecules, proteins that interconnect the cell wall polymers and proteins involved in the regulation of cell wall construction (Klis, et al., 2002).

*GAS1* gene encodes for a protein which has  $\beta$ -1,3-glucanotransferase activity and is required in the elongation of  $\beta$ -1,3-glucan chains (Cebollero, et al., 2009). The Gas1p is a glycosylphosphatidyl inositol (GPI)-anchored protein that is located in the plasma membrane (de Nobel, et al., 2000; Popolo and Vai, 1999). Synthesis of this protein starts in the endoplasmic reticulum where the carbohydrates and glycolipids are transferred to the precursor protein. The protein undergoes three types of post-translational modifications (N-glycosylation, O-mannosylation, and also receives a GPI anchor) during its maturity along the secretory pathway, resulting in a mature Gas1p. The GPI anchor is not cleaved off in the complete protein and the Gas1p remains anchored in the plasma membrane. Gas1p appears to be responsible for polymer cross-linking in the cell wall (Popolo and Vai, 1999).

Deletion of Gas1p leads to cells losing their typical ellipsoidal shape. They also become swollen and appear larger than the wild type cells. In addition they are defective in bud maturation and in cell separation. The cell wall of *gas1* null mutants is sensitive to Calcofluor white, which is a cell wall perturbing agent, its shows resistance to zymolase ( $\beta$ -1,3-glucanase), and is more sensitive to osmotic destabilizing agents such as sodium dodecyl sulfate (Popolo and Vai, 1999). Loss of *GAS1* also results in a significant reduction in the levels of  $\beta$ -1,3-glucan, hence the cells will be swollen and

the cell wall integrity altered. The *gas1* and *fsk1* mutant cells show similar phenotypes and have been reported by researchers to express high levels of chitin and the alternative  $\beta$ -1,3-glucan synthase catalytic subunit, Fks2 (de Nobel, et al., 2000). This is probably a compensatory mechanism to ensure integrity where a loss of polymer is compensated by an increase in another polymer such as chitin. The mutant cells with these deletions seem to be dependent on protein kinase C because its depletion is lethal to the mutant cells. This suggests that the viability of these cells is dependent on the signalling through the PKC 1-mediated cell wall integrity pathway (de Nobel, et al., 2000).

*GPI7* together with *GPI13* and *MCD4* encode phosphoethanolamine (EtN-P) transferases. *Gpi7p* is responsible for the addition of an ethanolphosphate side chain to the second mannose side chain of the glycan part of the GPI anchor. The other EtN-P transferases, *Gpi13p* and *Mcd4p* add the EtN-P to the first and the second mannose chains respectively. *GPI7*-deficient strains will result in cells failing to form the protein GPI-amide linkage and the GPI-anchored proteins are therefore released into the extracellular environment (Gonzalez-Ramos, et al., 2009; Gonzalez, et al., 2010). The increase in chitin was also noted in other cell wall mutants that include  $\Delta KNR4$ . These cell wall mutants have a weakened cell wall resulting from a reduction in the amount of  $\beta$ -1,3-glucan due to the reduction of  $\beta$ -1,3-glucan synthase activity. The *GPI7* gene is not required for viability of the cell. Though a loss of this gene results in GPI lacking a side chain in ethanolamine phosphate (EtN-P), the protein is still transferred. However, at high temperatures and in the presence of cell wall perturbants, the cells lacking a *GPI7* have defective growth. A deletion mutation in this gene also results in the release of cell surface anchored proteins (Fujita, et al., 2004).

The *KNR4* gene is required for correct targeting of the Stl2p MAP kinase to the downstream transcriptional factors, Rim1p and Sw1p. Deletion of this gene results in higher chitin content of the cell wall and reduction in the acidity of  $\beta$ -1,3- glucan synthase since it is involved in the biosynthesis of  $\beta$ -1,3-glucan. It is also a part of the main cell integrity pathway and plays a role in the co-ordination of cell wall synthesis with bud emergence (Gonzalez-Ramos, et al., 2008; Gonzalez-Ramos and Gonzalez, 2006).

### 2.2.1.2 Cell wall integrity pathway

The cell wall integrity (CWI) pathway is a signalling transduction pathway that is responsible for maintaining defective cell walls (Popolo and Vai, 1999). The CWI mitogen-activated protein (MAP) kinase cascade is one of the five most studied pathways. It is a linear pathway comprising of Pkc1, MEKK (Bck1), a pair of redundant MEKs (MKK1/2), and a MAP kinase (Mpk1/Slk2) (Gustin, et al., 1998; Kim, et al., 2010). In the absence of genes such as those coding for Gas1p or Fsk1p, the Rho1p is activated and then interacts with Pkc1p, Bni1p and  $\beta$ -1,3-glucan synthase through the GDP/GTP exchange factor Rom2p (Popolo and Vai, 1999).

Activation of this signalling pathway is suggested to be mediated by plasma membrane located sensors that transmit the signals to the MAP kinase cascade. These sensors include WSC genes which encode for putative receptors that maintain cell wall integrity (WSC 1/HCS77/SLG1, WSC2, WSC3 and WSC4/YFW1) (Zu, et al., 2001), and the partially redundant Mid2p and Mtl1p cell surface proteins which act as mechanosensors of cell wall stress during the events of budding or pheromone-induced morphogenesis, high temperature and other factors disturbing the cell wall (de Nobel, et al., 2000; Reinoso-Martin, et al., 2003).

Activation of the CWI pathway is sequential, where Pkc1 activates Bck1 by phosphorylating it *in vitro* at several sites (Ser939, Thr1119, and Ser1134) in a hinge region between its putative regulatory domain and its catalytic domain. The activating mutations in *BCK1* are suggested to also cluster in this hinge region. Disruption of an interaction involving Thr1119, by either phosphorylation or mutation, is believed to be a key to activation of MEKK which activates MKK1/2, which in turn activates Mpk1 by phosphorylating it on neighbouring tyrosyl (Tyr<sup>192</sup>) and threonyl (Thr<sup>190</sup>) residues (Jung and Levin, 1999; Levin, 2005; Vilella, et al., 2005). This cascade amplifies a small signal initiated at the cell surface and converts it to a highly sensitive, switch like response. The loss of any protein kinase below Pkc1 results in cell lysis at high temperatures (Levin, 2005).

The activation of Slt2 correlates to the activation of two downstream translational factors; Rlm1 which is involved in the activation of cell wall genes, and Swi6 which is involved in cell cycle regulation (Vilella, et al., 2005). In response to these changes the cell can express genes such as *FSK2*, which is an alternative catalytic subunit of  $\beta$ -1,3-glucan synthase, and also increases chitin levels (de Nobel, et al., 2000).

### 2.2.2 The $\beta$ -1,6-glucan and chitin network

The  $\beta$ -1,6-glucans are found as small molecules made up of about 130-140 glucose residues and forms about 5% of the cell wall (Jin and Speers, 1998; Kapteyn, et al., 1999). The mature  $\beta$ -1,6-glucan is a highly branched molecule with multiple non-reducing ends. These non-reducing ends function as attachment sites for other molecules in the cell wall such as chitin and other  $\beta$ -1,6-glucans. Chitin is found to strengthen the skeletal framework of the cell wall. The chitin is found close to the plasma membrane and it is also linked to the short side chains of  $\beta$ -1,6-glucan (Smits, et al., 1999).

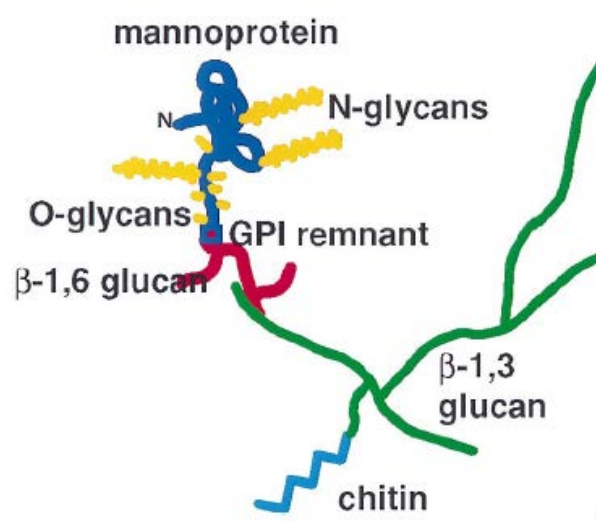
Chitin is a minor component as it constitutes approximately 1-2% of the cell wall dry weight (Klis, et al., 2002). Although this polymer is found in small quantities in yeast cell walls, its function is essential for cell viability. Chitin is deposited at the site of bud emergence during vegetative growth where it forms a ring that surrounds the neck between the mother and the daughter cell. It forms the

primary septum and after cell division is recognized as a bud scar in the mother cell and as a birth scar in the daughter cell. The deposition of chitin is a function of three chitin synthase isoenzymes (Chs1p, Chs2p, and Chs3p) which differ in their optimum pH, metal specificity and susceptibility to inhibitors (Valdivieso, et al., 2000).

The chitin synthases are a family of integral membrane proteins with a molecular weight between 100-130 kDa. Synthesis of the bulk protein of the cell wall and increase of chitin as a response to cell wall stress are a function of *CHS3* which codes for a non-zymogenic Chs3p. Chs2p is responsible for the biosynthesis of septal chitin and therefore acts in the formation of the primary septum. The linear chains of  $\beta$ -1,4-N-acetylglucosamine are synthesized by three chitin synthases from a substrate, uridine diphosphate (UDP)-N-acetylglucosamine (Goossens, et al., 2011).

### 2.2.3 Cell wall mannoproteins

The outermost layer of yeast cell walls consists of mannoproteins which are highly glycosylated proteins containing over 90% sugars (Figure. 2.2). Mannoproteins form radially extending fibrilliae at the surface of the cell wall and represent about 35-40% of cell wall dry weight. They act as structural components giving the cell wall its active properties such as permeability (Caridi, 2006).

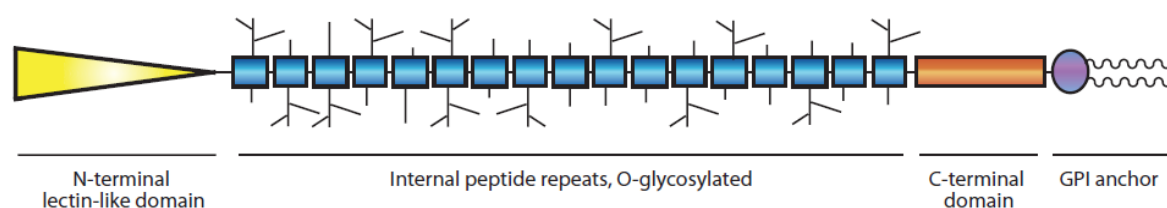


**Figure 2.2** Relationships among components of the *S. cerevisiae* cell walls: The mannoprotein polypeptide and the N or O linked oligosaccharide chains (in blue and yellow), respectively (Lipke and Ovalle, 1998).

They are secreted through a secretory pathway and most receive a GPI anchor and can also be N-glycosylated or O-glycosylated (Kapteyn, et al., 1999). Mannoproteins have gained attention over the past as interesting candidates for improvement of technological processes during wine production and sensorial properties of the final product (Quiros, et al., 2010).

Mannoproteins are either linked to the cell wall by covalent linkages to the  $\beta$ -glucans or weakly associated with cell wall components by non-covalent bonds through hydrogen bonding or disulfide bridges. The weakly associated mannoproteins can be released in large amounts by treatment with hot sodium dodecyl sulphate (SDS)- $\beta$ -mercaptoethanol. This is probably due to the unusually high molecular weight (>350 kDa) of this protein as SDS-extractable proteins are small. The strong glycosylation of Flo proteins as identified for Flo1p is important for the structure of this protein. The glycosylation is supported by several repeated serine and threonine sequences that allow these proteins to have most of the O-glycosylation and adopt a stiff and extended conformation. This conformation makes the Flo proteins readily available to drive cell-cell interactions in the outermost part of the cell wall. The highly repeated sequences function as spacers to expose a reacting domain of Flo protein at the cell surface (Bony, et al., 1997).

There are two classes of cell wall proteins that have been detected based on their extraction, i.e. glycosylphosphatidylinositol anchored cell wall proteins (GPI-CWP) and the proteins with internal repeats (PIR-CWPs). The PIR-CWPs are directly linked to the  $\beta$ -1,3-glucan without interconnection with  $\beta$ -1,6-glucan molecules and are released by mild alkali treatment (Smits, et al., 1999). The linkage in GPI-CWPs seems to be structurally complex and they can be released by  $\beta$ -1,3- or  $\beta$ -1,6-glucanase digestion of the glucan layer of the cell wall (Kondo and Ueda, 2004). There are approximately 40 predicted GPI-CWPs in yeasts. GPI-CWPs share a common structural organisation (Figure. 2.3) which consists of a carboxyl terminal region, containing a putative GPI attachment signal, a central domain rich in serine and threonine residues, and an N-terminal domain with a signalling sequence (Smits, et al., 1999; Van der Vaart, et al., 1995; Verstrepen and Fink, 2009).



**Figure 2.3** Three domain structure of fungal adhesions ( Verstrepen and Fink, 2009).



### 2.2.3.1 PIR proteins

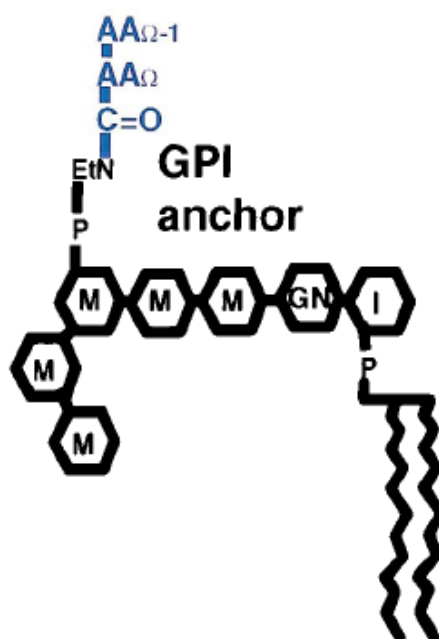
The protein with internal repeat sequences consists of an N-terminal signal peptide, a Kex2p recognition site and several internal repeats. These proteins are not linked to the cell wall by a GPI-anchor but it is possible that they might be linked by their O-linked side chains. The PIR proteins are similar to GPI-CWPs in the sense that they are highly O-glycosylated but they differ because PIR proteins lack a GPI-anchor. These proteins are found to be cleaved from cell walls under mild alkali treatment (30 mM NaOH at 4°C overnight) which suggests that they are cross-linked to the cell wall by O-linked saccharides since O-chains tend to be cleaved off in the beta-elimination process. These proteins are suggested to be linked to  $\beta$ -1,3-glucan reducing chains (Kapteyn, et al., 1999; Klis, et al., 2002).

### 2.2.3.2 GPI proteins

Cell wall proteins anchored through a GPI-remnant anchor include agglutinins (Ag $\alpha$ 1 and Ag $\alpha$ 1) (Klis, et al., 2002), flocculins (Flo1, 5, 9, 10, and 11) (Bayly, et al., 2005), Sed1, Cwp1 (Caro, et al., 1998) and a family of proteins encoded by *DAN/TIR* genes (Abe, 2007; Abramova, et al., 2001). The anchoring of these proteins to the cell wall plays a major role in surface expression and the essential viability of the yeast. In biotechnology, the cell surface proteins can be exploited in the production of microbial biocatalysts, whole cell adsorbents and live vaccines. Furthermore, cell wall proteins have been implicated in different functions that help the cell to survive in the environment (Kondo and Ueda, 2004). Two of the GPI-CWPs,  $\alpha$ -agglutinins and the core subunit  $\beta$ -agglutinin, are involved in mating. Furthermore, a group of Flo proteins have been determined to be responsible for Flo formation, adhesion and flocculation (Kapteyn, et al., 1999; Smits, et al., 1999; Van der Vaart, et al., 1995).

### 2.2.3.3 Synthesis of GPI-anchored mannoproteins

In all eukaryotes most cell surface proteins are anchored through a GPI anchor. The GPI anchor is added posttranslationally to the precursor proteins in the ER. GPI proteins form the major components of the mannoproteins at the cell wall and are also involved in cell wall construction and assembly (Fujita, et al., 2004). The GPI anchor is biosynthesized through modification of EtN-P by sequential addition of sugars to form a complete precursor lipid in the ER (Figure. 2.4). The modified EtN-P is added as a side chain to the second mannose portion of the GPI core glycan structure by a gene known as *GPI7* (Fujita, et al., 2004). The GPI anchors are added posttranslationally to the C-terminus of precursor proteins in the ER. The protein precursors contain two signal sequences for import into the ER which are located at both the N-terminus and GPI anchoring signal at their C-terminus (Caro, et al., 1997; Pittet and Conzelmann, 2007).

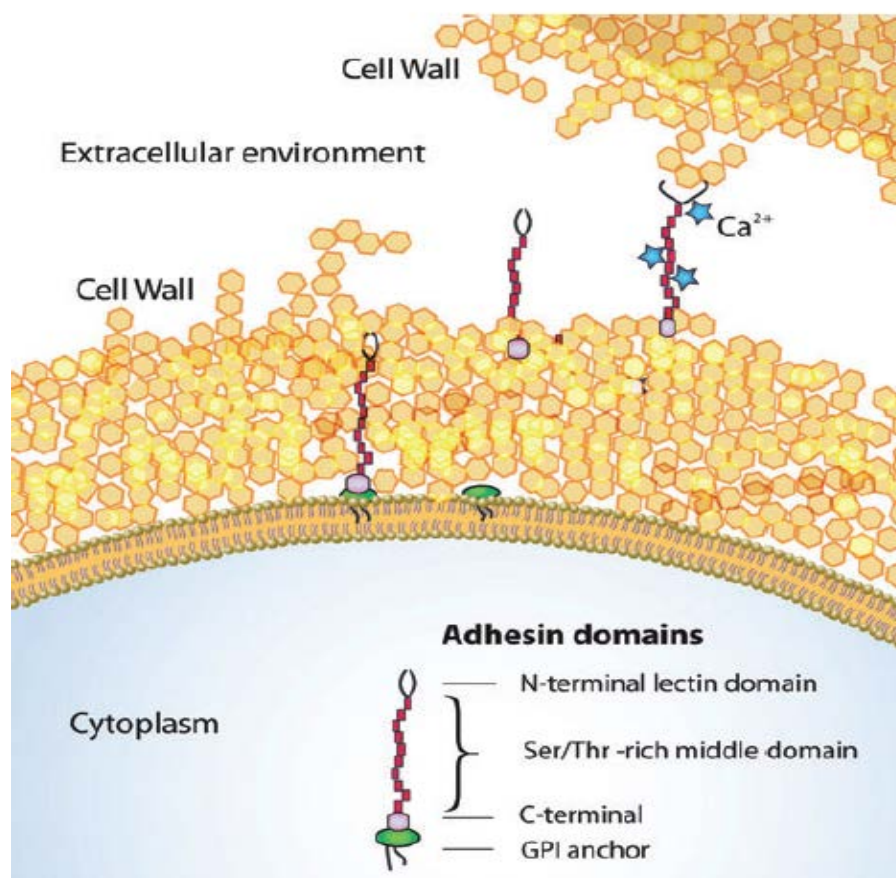


**Figure 2.4** Structure of yeast GPI anchor. AA, amino acid residues; EtN, ethanolamine; P, phosphate; M, mannose; GN, glucosamine; I, myo-inositol. At the bottom right is a phospholipid, which may be glycerol or sphingosine based (extracted from Lipke and Ovalle, 1998).

These proteins are then transported to the cell surface through a secretory pathway. Transport of these proteins to the cell surface is facilitated by the amino terminus hydrophobic signaling sequence on the protein precursor which directs the protein to the secretory pathway (Caro, et al., 1997). In the secretory pathway, the GPI transamidase complex recognizes and cleaves the GPI anchor in the carboxyl-terminus and it is replaced by a preformed GPI anchor. The C-terminal hydrophobic domain of the GPI anchor is separated by a spacer domain of about 8-12 amino acids from the cleavage/attachment site ( $\omega$  site) (Fujita, et al., 2004; Pittet and Conzelmann, 2007). Proteins are then transported to the golgi apparatus where they are subsequently transported to the plasma membrane in membrane-enclosed vesicles (Kondo and Ueda, 2004). The GPI-anchored proteins in yeast are an intrinsic part of the cell wall (Caro, et al., 1997; Kapteyn, et al., 1999) and they are largely regulated by nutrient availability and environmental conditions (Smits, et al., 1999).

### 2.3 THE Flo PROTEINS

Flocculins are yeast proteins which stick out of the cell walls of flocculent cells and therefore bind mannose residues on the neighboring cells (Figure 2.5). The calcium ions are important in this binding to activate the flocculins (Verstrepen, et al., 2003). The fungal adhesion proteins are a group of GPI-linked cell wall proteins. These adhesion proteins are characterized by a three domain structure, namely, the N-terminal signal sequence, a central domain containing a highly repeated serine/threonine-rich sequence, and a C-terminal domain containing a GPI anchoring sequence (Guo, et al., 2000; Van Mulders, et al., 2009).



**Figure 2.5** Secretion and cell anchoring of adhesins (Adapted from Verstrepen and Klis, 2006).

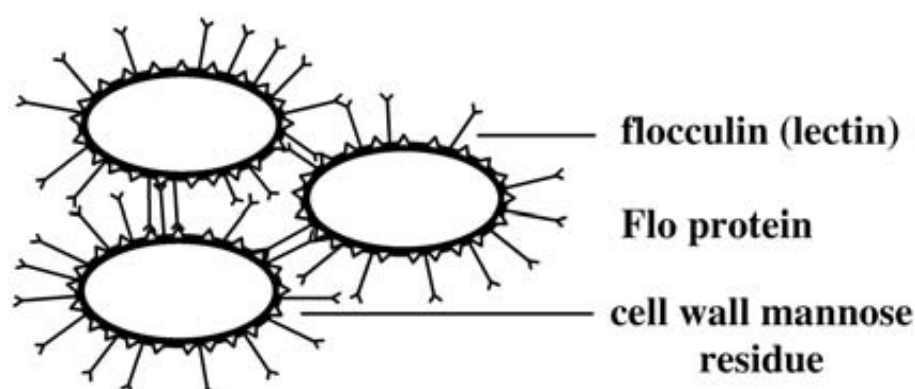
## 2.4 THE MECHANISM OF FLOCCULATION

There are two mechanisms of flocculation that have been proposed based on sugar inhibition studies. They are the lectin-like adhesion (sugar sensitive) mechanism (Miki, et al., 1982) and the mannose insensitive adhesion mechanisms (Govender, et al., 2011). The sugar sensitive phenotypes are further divided into two sub-phenotypes which include the commonly reported Flo1 sub-phenotype that is identified by mannose sensitivity, and the NewFlo sub-phenotype that is identified by mannose and glucose sensitivity (Kobayashi, et al., 1998; Van Mulders, et al., 2009).

### 2.4.1 Sugar-sensitive phenotypes

In the sugar sensitive phenotype it is proposed that a lectin-like protein with a carbohydrate domain binds to sugar chains on the surface of adjacent cells (Zhao and Bai, 2009). There is increasing evidence of the lectin-like theory as an explanation for flocculation observed when yeast clump together and sediment to the bottom of the media. This is suggested to be caused by a specific binding between a sugar chain located at the cell surface and the lectin-like proteins which are sugar binding proteins on an adjacent cell (Figure 2.6). The specificity of binding of N-terminal domain of the Flo protein to the sugar receptors is believed to depend on the specific amino acid sequence found in the

N-terminal domain rather than the altered number of tandem repeats in the central part of a *FLO* gene as suggested (Liu, et al., 2007). According to the data accumulated thus far the binding is sensitive to proteinase, inhibited by sugars such as mannose and depends on  $\text{Ca}^{2+}$  ions. This suggests that a protein is involved in the binding interaction. This protein recognizes a sugar domains and is a lectin-like protein (Kobayashi, et al., 1998).



**Figure 2.6** The lectin-like model of flocculation (Adapted from Verstrepen, et al., 2003).

The first proposed theory for yeast flocculation was Mill's theory which stated that flocculating cells are held together by salt bridges with  $\text{Ca}^{2+}$  ions joining the two carboxyl and phosphate groups at the surface of two cells. However, the calcium theory could not explain the inhibition of flocculation by sugars. The flocculation theory seemed more complex than the simple formation of a  $\text{Ca}^{2+}$  bridge. Then Miki et al. (1982) suggested a new flocculation mechanism which involves lectin-like binding of surface proteins to sugars on adjacent cells. The role of  $\text{Ca}^{2+}$  in this mechanism would be to maintain the lectins in an active conformation rather than playing the central role in flocculation (Domingues, et al., 2000).

#### 2.4.1.1 Flo1 phenotype

The strains which display Flo1 phenotype are generally laboratory strains bearing *FLO1* gene and other genes known to be involved in flocculation (Domingues, et al., 2000). In the Flo1 phenotype the binding is thought to be caused by a specific N-terminal mannose binding domain found in *Flo1p* and highly related proteins such as *Flo5p* and *Flo9p* (Van Mulders, et al., 2009). The binding in Flo1 phenotype was found to be mannose specific. The flocculation behavior of Flo1 phenotype in yeast is not affected by the physiological stage of growth in yeast (Chang, et al., 2005). Therefore strains presenting with the Flo1 flocculation phenotype were found to constitutively flocculate.

### 2.4.1.2 NewFlo phenotype

The NewFlo phenotype is of the utmost important in the modern brewing industry. This phenotype presents a desirable characteristic in that industrial yeast strains to start flocculating late during the brewing process when all sugars have been exhausted. Research shows that NewFlo phenotypes have a relaxed sugar consumption profile (Verstrepen and Klis, 2006). The NewFlo phenotype is prevalent in brewing yeast strains but its genotype remains unknown. The relaxed sugar inhibition in NewFlo phenotype-displaying strains is evidenced by the inhibition of flocculent transformants YTS-S and YTS-L by glucose, sucrose, maltose and mannose (Liu, et al., 2007). Glucose inhibits flocculation more easily than other sugars. Some lectin-like proteins in other strains show a unique character and are inhibited by galactose which is not known to inhibit both Flo1 and NewFlo flocculation phenotypes. This relaxed sugar consumption character has many advantages to brewers because it minimizes the cost of centrifugation and separation of yeast from the final product (Van Mulders, et al., 2009).

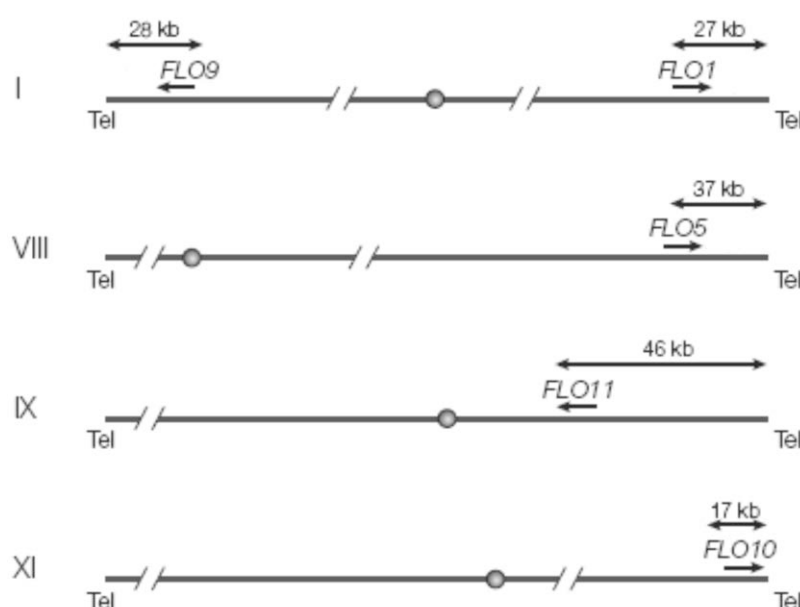
### 2.4.2 Sugar insensitive phenotype

The sugar insensitive adhesion is characterized by binding of adhesins to peptides instead of sugar chains or increases in the cell surface hydrophobicity. Flo11p binds to abiotic surface upon increased hydrophobicity. This phenotype is independent of calcium ions (Verstrepen and Klis, 2006; Zhao and Bai, 2009).

## 2.5 GENETICS OF YEAST FLOCCULATION

There are five dominant members of the *FLO* gene family that are found in a commonly used laboratory strain, S288c, which are capable of producing distinct cell surface variations. This family is composed of *FLO1*, *FLO5*, *FLO9*, *FLO10* and *FLO11* of which *FLO1*, *FLO5*, *FLO9*, *FLO10* are subtelomeric and *FLO11* is neither telomeric nor centromeric (Figure 2.7) (Kobayashi, et al., 1998). The subtelomeric *FLO* genes are about 10 to 40 kb from the telomeres, they are transcriptionally silent and their location near the telomere sequences is found to play a role in their regulation and evolution (Halme, et al., 2004). The only gene expressed in this family is the *FLO11* gene which is neither adjacent to a telomere nor a centromere. The expression of *FLO11* is important to *S. cerevisiae* for developmental diversity since it is responsible for adhesion to agar and plastic surfaces, formation of pseudohyphal filament and sliding motility (Halme, et al., 2004). The *FLO1* gene has been used as a reference *FLO* gene as most of the *FLO* genes share similarity with this gene. *FLO5*, *FLO9* and *FLO10* shares 96, 94 and 58% similarity, respectively to *FLO1* gene while there is only 37% similarity between Flo1p and Flo11p (Domingues, et al., 2000; Kobayashi, et al., 1998).

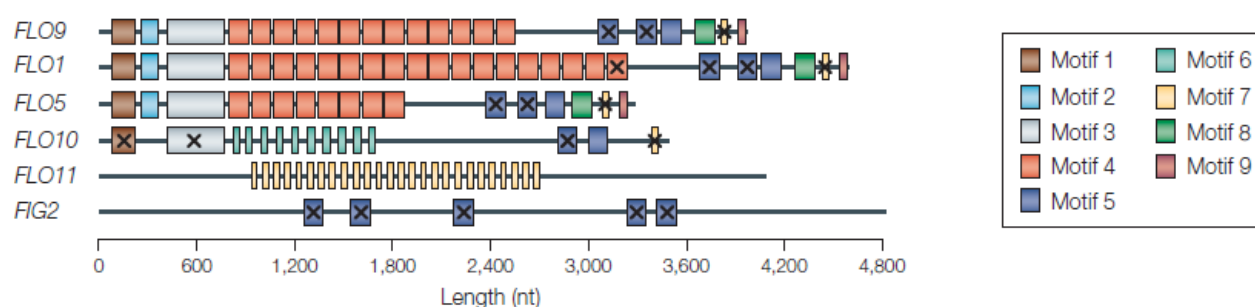
Research has shown that expression of the silent *FLO* genes by a heterologous promoter confers adhesive phenotypes that are different from those shown by *FLO11* (Govender, et al., 2008; Verstrepen, et al., 2003). Flo1p expression results in cell to cell adhesion which causes cells to flocculate. The phenotypes that are conferred upon expression of Flo10p overlap phenotypes of both Flo1p and Flo11p since it promotes adhesion and pseudohyphal filamentation and also can enhance cell to cell adhesion (Halme, et al., 2004). There is little research on the mechanism of regulation of these genes, but Halme and coworkers (2004) suggested that the regulation of these genes is under genetic and epigenetic control.



**Figure 2.7** Chromosomal localization of adhesion genes and pseudogenes in *S. cerevisiae*. Roman numerals on the left indicate the yeast chromosomes on which the *FLO* gene resides. The dots represent the centromeres. The silent *FLO* genes are all located within 40 kb of the telomeres (TEL). *FLO11* is neither telomeric nor centromeric (Adapted from Verstrepen, et al., 2004).

### 2.5.1 Recombination of intragenic repeats generates variability

Evolutionary studies suggest that the adhesion genes are the rapidly expanding groups of paralogues in the genome of *S. cerevisiae* and related species (Verstrepen and Fink, 2009). This variability was also observed in industrial *S. cerevisiae* brewer's strains which are closely related but carry *FLO* genes that are significantly different in length. The variation in the *FLO* gene length is responsible for changes in phenotypes in closely related strains (Verstrepen and Klis, 2006). There is increasing evidence that the central domain of Flo proteins is the driving force in the variability of adhesins. The central domain is highly unstable and consists of tandem repeats rich in serine and threonine residues (Figure 2.8). These repeats are thought to drive the evolution and divergence of the *FLO* genes. They are also said to drive slippage and recombination reactions between *FLO* genes (Van Mulders, et al., 2009).

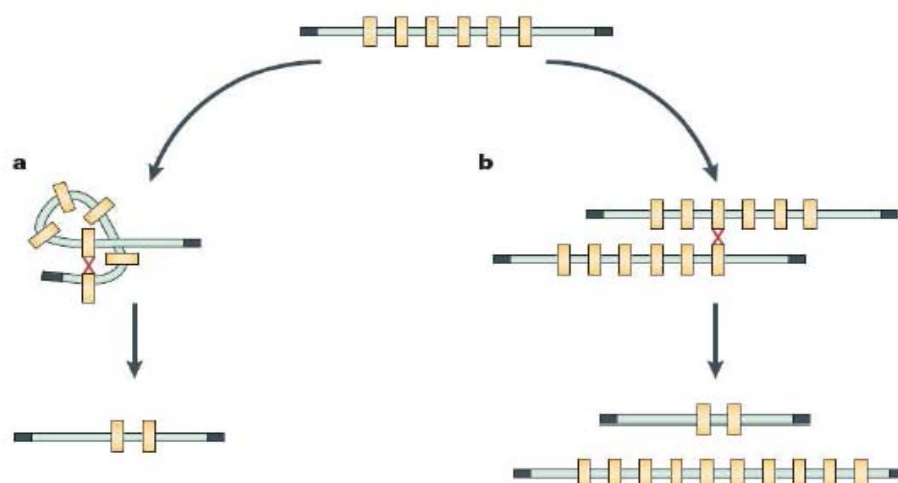


**Figure 2.8** Repeated nucleotide motifs in the *FLO* genes. The five *FLO* genes are shown. The boxes show the highly conserved DNA sequence motifs. The number below the sequences shows the distance in nucleotide from the translational start signal. Boxes marked with an 'X' represent the repeated amino acid motifs that are not conserved in the DNA sequence. These sequences are thought to have undergone genetic drift over time, resulting in many third-position nucleotide changes within the codons (Adapted from Verstrepen, et al., 2004).

The instability is suspected to be caused by the frequent addition and deletion of tandemly repeated sequences. High sequence similarity allows for recombination during DNA replication. The sequences upstream are said to recombine with sequences downstream leading to the removal or addition of repeat units (Figure 2.9). This results in adhesion genes of different length and strength in flocculation. The longer flocculins flocculate or adhere to surfaces strongly and smaller flocculins cause weak flocculation or adherence to surfaces. Smukalla, et al. (2008) and Verstrepen, et al. (2004) over-expressed a series of *FLO1* alleles with an increasing number of repeats which resulted in stress resistance to increases with the number of tandem repeat units in the *FLO1* gene.

Liu *et al.* (2007) found that deletion in internal repeats in the Flop influenced the sugar binding strength. This report also suggests that increased affinity for sugars in FLONL protein was due to the C-terminal domain. They suspected that the deletion of internal repeats activated a latent high affinity conformational state of FLON proteins for both the C-2 hydroxyl group of glucose and the C-4 hydroxyl group of galactose. This confirmation caused increased affinities for all the sugars used.

The number of repeated sequences between Flo1 and NewFlo varies to a certain extent and this is suggested to play a role in the differing flocculation characteristics and evolution of these genes (Liu, et al., 2007). Research shows that DNA motifs in the central domain are conserved among the *FLO* genes and this is also suggested to promote diversity of adhesions by frequent intragenic recombination events (Govender, et al., 2008).



**Figure 2.9** Recombination between repeated DNA motifs in adhesion genes generates new alleles. Boxes indicate nucleotide motifs; the region shown in black at the end of the chromosomes represents the telomeres. (a) Intra-chromosomal pairing coupled with the recombination event that generates a short gene with a reduced number of repeats. (b) An unequal crossover between two identical *FLO* genes on homologous chromosome that have not aligned perfectly is shown. Similarly *FLO* genes with significant homology that are located on different chromosomes could recombine to produce new chimeric genes. This event would generate both the long and the short form of a gene. Although simple reciprocal events of recombination events are shown, the amplification and loss of repeat motifs could occur by many mechanisms, including spillage during replication and double strand break (Adapted from Verstrepen, et al., 2004).

### 2.5.2 Adhesion is controlled by several signalling pathways

The *FLO* genes are not constitutively expressed under growth conditions but are under tight transcriptional control by several interacting regulatory pathways. Several phenotypic changes in yeast cells such as flocculation, biofilm formation or substrate adhesion are triggered by changes in environmental conditions which lead to stress conditions and nutrient limitation. Environmental conditions such as carbon source limitation or nitrogen starvation, changes in pH, ethanol levels and other environmental triggers that have not yet been described, activate the signalling pathways which therefore activate the adhesion-encoding genes (Verstrepen and Klis, 2006).



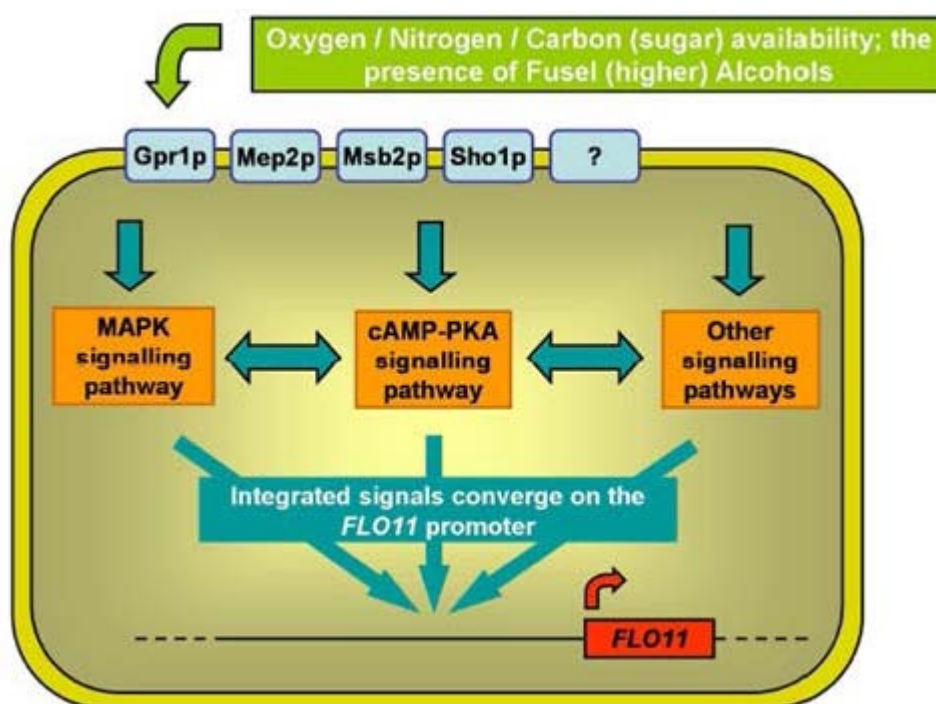
The growth form of yeast is determined by the nutrient availability in the environment. In low nutrient concentrations invasive growth is supported as opposed to nutrient rich environments where unicellular multiplication is the preferred form of cellular growth. The switch from unicellular growth to invasive growth is controlled by complex regulatory networks that respond to environmental signals (van Dyk, et al., 2005). Although some of the signaling pathways are well characterized, the upstream sensors of some of these cascades are still unknown. These sensors are probably triggered by conditions such as nutrient starvation, oxidative stress and ethanol concentrations (Verstrepen and Klis, 2006).

The activation of adhesion-encoding genes under such conditions is suggested to allow cells to adapt to stress. The adhesion protein expression confers cell-cell adhesion and cells form flocs that rapidly sediment to the bottom of the medium. This may therefore provide means of passive transport in the medium away from stress (Verstrepen and Klis, 2006). The *FLO* encoded cell-surface adhesions allow cells to adhere or penetrate to surfaces and prevent being washed away or to use it as an attempt to forage for new nutrients (Linder and Gustafsson, 2008; Van Mulders, et al., 2009).

The regulation of adhesive phenotypes in solid substrate (invasive growth or pseudohyphal formation) have received wide spread attention compared to the regulation of flocculation phenotypes. The transcriptional regulation of *FLOI* which codes for cell-cell adhesion has been shown to depend on transcription factors such as Mss11p and Flo8p. These transcriptional factors are also central in *FLOII* transcriptional regulation (Bester, et al., 2006). The putative GCN4-box at position 268 in *FLOI* is found to repress expression of this gene under high nitrogen conditions. These findings indicate that it is possible to adapt the time of flocculation onset by changing the carbon and nitrogen content in the medium since the flocculation is triggered by carbon and/ or nitrogen starvation (Verstrepen, et al., 2003).

The various signaling cascades that control adhesion translate environmental conditions into the transcriptional response of adhesion genes. The transcriptional signaling pathways which regulate *FLOII* are well characterized (Figure 2.10). The three best described pathways which include the mitogen activated protein kinase (MAPK) pathway, Ras-cAMP pathway and the glucose repression pathway. The transcriptional signaling pathways which regulate the other *FLO* genes are still not known but it is suggested that they are controlled by similar pathways with *FLOII*, but the environmental triggers and transactivator pathways are different (Verstrepen and Klis, 2006).

The *FLO8* gene encoding the transcriptional factor Flo8p, has been identified as an activator of both *FLO1* and *FLO11* and has also been found to have a nonsense point mutation in laboratory strain S288c resulting in this strain being unable to flocculate. The different phenotypes are encoded by different dominant *FLO* genes. This suggests that these genes are structurally similar and their regulatory mechanisms are the same (Bester, et al., 2006).



**Figure 2.10** The genetic regulation of *FLO11*. Nutritional status is converted to a signal in a specific pathway by regulatory proteins. These signals converge to the promoter of *FLO11* and regulates its expression. In response to specific stimuli, different phenotypes of *FLO11* are generated (Adapted from Verstrepen and Klis, 2006).

Flo11p has a number of properties that distinguish it from the other flocculins. The other flocculins confer cell-cell adhesion but Flo11p is essential for the formation of pseudohyphae and biofilm, invasive growth (also *FLO10*), and adhesion to agar and other surfaces such as plastic. The promoter of the *FLO11* is the largest promoter found in the yeast genome and it is not surprising that this gene codes for multiple phenotypes and that its regulation is complex since its expression is regulated by several major pathways, including the MAP kinase pathway and the protein kinaseA/cAMP pathway (Bayly, et al., 2005).

### 2.5.3 Epigenetic regulation of *FLO* genes

The *FLO* genes are not only under signalling cascade control, they are also under epigenetic silencing and de-silencing. The expression of *FLO* genes is reversible and cells can switch between expressed *FLO* genes and the silenced state. This epigenetic control is suggested to be caused by the location of *FLO* genes near the telomere sequence. The replacement of the *FLO11* promoter with another promoter and the relocation of the *FLO11* gene with its promoter to another locus on a chromosome abolished epigenetic control. Therefore, the epigenetic control of this *FLO11* gene was shown to be promoter-specific (Verstrepen and Klis, 2006) and location specific. The epigenetic silencing of *FLO* genes is found to play different roles including helping the cells adapt to fluctuating environments, allowing cells to adhere to specific surfaces by switching appropriate adhesins and also allowing for balance between adhering and colonizing, and non-adhering cells to re-attach to new sites (Verstrepen and Klis, 2006).

The *FLO11* gene is neither subtelomeric nor centromeric as it is more than 40 bp away from the telomeres. The metastable silencing of *FLO11* is similar to the silencing of subtelomeric *FLO* genes. Halme *et al.* (2004) determined that the silencing of the *FLO11* gene is not dependent upon genomic location, but it is promoter specific. The *URA3* gene with its promoter placed at the *FLO11* locus was not silenced. The factor believed to specifically recognize the *FLO11* promoter at this locus is Sfl1p. Furthermore, these studies showed that histone deacetylase protein (Hda1p) is also required for silencing of *FLO11*. In a genome wide analysis, this protein was identified to participate in the deacetylation of subtelomeric regions of the yeast genome. These subtelomeric regions extend much further away from telomeres. It is known that Hda1p is recruited to specific promoters by Tup1p which in turn have been shown to be recruited to the *FLO11* promoter by Sfl1p. The conclusion that can be reached in these findings is that Sfl1p recruits Hda1p to silence *FLO11* (Halme, et al., 2004). The studies show that in conditions of nitrogen starvation some diploid cells express Flo11p on their surfaces and some do not. This suggest that *FLO11* is metastable silenced in the conditions that initiate pseudohyphal filamentation (Halme, et al., 2004).

The epigenetic silencing effects observed at *FLO11* were found to extend to the *FLO10* gene. Silencing of *FLO10* was shown to be both metastable and reversible. *FLO10* promoter silencing is also likely to be directed by Sfl1p which represses transcription in *FLO11*. In contrast to the metastable silencing observed in the *FLO11* promoter, *FLO10* promoter silencing was shown to be dependent on its genomic positioning. This is not surprising since *FLO10* is a subtelomeric gene (Halme, et al., 2004). The signaling cascade has also been shown to play a role in epigenetic control. Ras/cAMP proteins such as Sfl1 and Hda1 play a role in promoter-dependent epigenetic control in *FLO11*. *FLO10* shows that epigenetic silencing is intervened by Hda1 and Hda2 (Verstrepen and Klis, 2006).

The *FLO8* gene was found to encode a transcriptional activator of *FLO1*, *FLO9* and *FLO11*. Furthermore, the *FLO11* gene was found to be subjected to regulation by complex cascade mechanisms such as cAMP/PKA and MAP kinase pathways. This is not surprising since the *FLO11* gene encodes different adhesive phenotypes which are crucial for cellular development and cell survival. The other genes in the *FLO* gene family may be regulated by different complex mechanisms and factors. The instability of *FLO* genes also lead to great differences in flocculation profiles in generations of the yeast strains due to continuous recombination. Flocculation is also dependent on factors such as temperature and pH which influence cell-cell interaction (Verstrepen, et al., 2003).

The transcriptional control of *FLO* genes is complex and not well understood. It has been shown that genetically modified laboratory yeast strains carrying specific *FLO* genes under transcriptional control of stationary phase-specific promoters, have the potential of driving the expression of these *FLO* genes and to meet the requirements of the industry (Cunha, et al., 2006; Govender, et al., 2010).

## 2.6 GENETIC INTERACTIONS AND NETWORK IN YEAST

The complete sequence of the *S. cerevisiae* genome serves as a powerful genomic tool that allows for systematic analysis (Pretorius and Bauer, 2002). This organism is used in many studies to understand how genes function as networks to carry out and regulate cellular processes (Boone, et al., 2007; Kelly, et al., 2001). In yeast, the expression of genes is tightly controlled by a number of factors including promoter strength, cis- and transacting factors, cell growth rate and other gene level regulation. These factors limit the possibilities of studying gene functions (Wang, et al., 2008).

The common strategy used to elucidate the function of each gene in the regulatory network is either deletion of the gene and replacement with a genetic marker, or strong over-expression using controllable promoters (Wang, et al., 2008). Phenotypic analysis of deletion mutants serves as a powerful tool in gene function studies. An international consortium was set up in a gene deletion project in yeast using S288C derivative strains as a genetic background (Winzeler, et al., 1999).

The direct approach of gene deletion in yeast by polymerase chain reaction (PCR) takes advantage of a high level of homologous recombination in yeast. The deletion primers employed in the deletion project were four oligonucleotides (two 74mer and two 45mer) for both the deletion and confirmation of each deletion. The 74mer primers are made of a short sequence from the upstream and downstream sequence of the antibiotic resistance marker gene KanMX4 (U2, D2), A DOWNTAG and an UPTAG which serve as a unique molecular barcode for each deletion and sequences

complementary to the upstream and downstream sequence of the target open reading frame (ORF). The entire ORF is removed but the start and the stop codons are left intact. The 74mer primer was then used to amplify the KanMX4 marker gene containing a constitutive promoter from the *Ashbya gossypii* yeast strain. A second PCR using 45mer homologous to the upstream and downstream regions of the targeted ORF was then employed to improve further targeting. The constructed cassette was then used to replace the target gene with the kanamycin marker gene through transformation. The transformants were then selected by growth on complete media containing the antibiotic Geneticin (Kelly, et al., 2001; Winzeler, et al., 1999).

Successful homologous recombination of the deletion cassette was verified by template DNA isolated from the Geneticin resistant colonies with two primers common to the KanMX4 module: KanB 5'-CTGCAGCGAGGAGCCGTAAT-3' and KanC 5'-TGATTTTGATGACGAGCGTAAT-3'. Confirmation of integration of the deletion cassette to the correct locus was verified with primers named A, B, C and D. The primer combination A and D were from regions up to 400 bases upstream and downstream of the start and stop codons respectively, and the B and C combination was from within the ORF (Kelly, et al., 2001; Winzeler, et al., 1999).

## 2.7 CONCLUSION

Understanding the genetics and physiology of yeast is the key to the development of improved industrial yeast strains with desirable properties. This is limited by the fact that industrial strains are mainly homothallic and often heterozygous which makes them difficult to manipulate under laboratory conditions. In an approach to overcome this limiting factor, tractable versions of these commercial strains have been developed. Single-gene deletion strains, which employ a commonly used laboratory strain to create both a set of deletion mutants (BY4741-BY4743) and over-expression library, have been constructed. These libraries are a very important tool for elucidating genetic pathways and gene functions (Harsch, et al., 2010). On the other hand, the *FLO* genes which encode for cell surface proteins (adhesins) are silenced. When expressed by a phase inducible promoter, these genes show desirable characters such as flocculation towards the end of fermentation when all sugars have been utilised (Govender, et al., 2008; Verstrepen, et al., 2003), and adhesion to surfaces (Govender, et al., 2011; Palecek, et al., 2000).

Mannoproteins in general also shows enologically desirable properties such as chemical and physicochemical stabilisation of the final product in fermented beverages. This study is aimed at investigating the effect of deleting cell wall biogenesis related genes in the cell surface phenotypes conferred by the expression of Flo proteins in the cell wall. The *FLO* genes encoding cell surface proteins are over-expressed using a glucose repressible promoter, *ADH2* and the yeast strains employed as genetic background are the single deletion mutants with deletions in genes related to cell wall biogenesis.

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# Chapter 3

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## RESEARCH RESULTS I

**Construction of transgenic *Saccharomyces cerevisiae* *GPI7* and *KNR4* gene deletion strains displaying controlled *FLO* gene expression**

## Construction of transgenic *Saccharomyces cerevisiae* *GPI7* and *KNR4* gene deletion strains displaying controlled *FLO* gene expression

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### 3.1 ABSTRACT

Adhesins encoded by the *FLO* genes (*FLO1*, *FLO5*, *FLO9*, *FLO10* and *FLO11*) are extracellular facing glycosylphosphatidylinositol (GPI)-anchored cell wall glycoproteins that are involved in numerous adhesion phenotypes including flocculation; biofilm and flor formation; attachment to biotic and abiotic surfaces; invasive growth; and pseudohyphal formation. To date, and to the best of our knowledge, our understanding of the fine molecular structure of these glycoproteins is fairly limited. Generally the amino acid sequences of their protein moieties have been established from structural and functional analysis of the genomic sequence of these yeasts whilst far less information is available on the glycosyl moieties of these adhesins. Moreover, the involvement of cell wall biogenesis related genes in adhesin biochemical processing for incorporation into the cell wall is not well defined. In this study, the open reading frame (ORF) of the five dominant *FLO* genes were placed under transcriptional control of the *ADH2* promoter in laboratory BY4742 strains bearing a gene deletion in either *KNR4* or *GPI7*. The *KNR4* gene encodes for a regulatory protein involved in cell wall integrity pathway whilst *GPI7* encodes for a phosphoethanolamine transferase enzyme that is responsible for the synthesis of the GPI anchor. The BY4742 wild type strain was also transformed to generate positive control transgenic strains. All transgenic strains were verified for the presence of the kanamycin (KanMX) deletion cassette. In addition, the integration of the promoter cassette into the correct *FLO* gene locus was verified using homologous primer pairs. Moreover, a heterologous primer set was used to confirm that individual *FLO* genes were in fact under the transcriptional control of the *ADH2* promoter sequence.

### 3.2 INTRODUCTION

The cell wall in *Saccharomyces cerevisiae* is composed of three polymers, namely the glucan network, chitin, and mannoproteins (Rowe, et al., 2010). The cell wall polymers are arranged into two layers, the inner layer composed of chitin and  $\beta$ -1,6-glucans, and the outer layer composed of  $\beta$ -1,3-glucans and mannoproteins (Cid, et al., 1995). Mannoproteins are highly glycosylated proteins rich in serine, threonine and asparagine. Mannoproteins contain over 30% peptides and 70% sugar residues of which 98% are mannose residues. The relative proportions vary according to growth conditions and developmental stages (Bony, et al., 1998; Giovani, et al., 2010).

The cell wall is responsible for providing the cell with mechanical strength, protecting the cell from the external environment, maintaining cell shape and acting as a sieve to filter large molecules (Dagkessamanskaia, et al., 2010; Francois, 2007). The cell wall achieves these functions by combining physical rigidity and dynamic remodelling. Dynamic remodelling allows the cell to undergo developmental processes and to adapt to changing environmental conditions (Klis, et al., 2002).

The microbial cell surface has evolved diverse mechanisms to be highly flexible and adaptable in response to environmental conditions. One of the mechanisms involves classic signalling pathways through which the cell senses the stimulants in the external environment and switches on appropriate genes, or the response can be through genetic and epigenetic changes that may result in expression, silencing or even generation of novel genes (Halme, et al., 2004; Van Mulders, et al., 2009).

The changes in the cell surface of fungal species are mostly driven by families of genes encoding cell surface proteins. High frequency of recombination in these genes allows each family member to produce distinct cell surface phenotypes. In *S. cerevisiae*, a well known cell surface encoding gene family is a group of five dominant *FLO* genes (*FLO1*, *FLO5*, *FLO9*, *FLO10* and *FLO11*) (Halme, et al., 2004). These *FLO* genes encode for cell surface flocculins which allows for cell-cell or cell-substrate adhesion and leads to flocculation phenotypes (*FLO1*, 5, 9 and 10) (Bony, et al., 1997; Verstrepen and Klis, 2006), invasive growth or pseudohyphae formation, biofilms, adherence to plastic surfaces and flor or velum formation (*FLO11*) (Van Mulders, et al., 2009; Verstrepen and Klis, 2006). The *FLO* genes contain internal tandem repeats that allow for recombination which may alter the length of these genes (Liu, et al., 2007). Straver, et al. (1994) showed that *FLO* gene expression could also impact on cell wall hydrophobicity.

Most studies have reported on *FLO11* and to a lesser extent on *FLO1*. Little information is known about the other family members such as the *FLO1* homologues, *FLO5* and *FLO9* (Bester, et al., 2006). However, it has been reported that four members of the *FLO* gene family are located near the telomeres, a location suggested to play a major role in their regulation and evolution. When one gene member is expressed, the other silent genes serve as a reservoir of variation. *FLO1*, *FLO5*, *FLO9* and *FLO10* are approximately 10-40 kb from the telomeres and *FLO11* is neither adjacent to a telomere nor a centromere (Halme, et al., 2004).

The flocculins encoded by *FLO* genes have a similar three domain structure with an N-terminal domain, which possesses a lectin-like binding property, a variable repeated amino acid motif and a carboxyl terminal glycosylphosphatidylinositol (GPI) moiety which is an anchor of the protein to the cell wall (Verstrepen and Klis, 2006). Teunissen and Steensma (1995) proposed that surface glycoproteins or flocculins specifically recognise and bind to  $\alpha$ -mannan carbohydrates of adherent cells. Previously Miki, et al. (1982) suggested that  $\text{Ca}^{2+}$  ions act as cofactors in maintaining the active conformation of surface proteins. This enhances the capacity of lectins to interact with  $\alpha$ -mannan carbohydrates. This phenomenon is known as flocculation and is defined as the asexual, reversible and calcium-dependent aggregation of yeast cells to form flocs containing thousands of cells that rapidly sediment to the bottom of the liquid medium (Bony, et al., 1997).

The Flo proteins are covalently anchored into the  $\beta$ -glucan network in the cell wall through a GPI-anchor remnant. Synthesis of proper cell wall requires approximately 1 200 genes (Klis, et al., 2002). The defects in the cell wall are detected through a cell wall integrity (CWI) pathway (Krause, et al., 2008). Mutations affecting different elements of the CWI pathway leads to cell lysis because the components in the cell wall are not correctly assembled (Arias, et al., 2011). The deletion of genes that are functionally relevant for cell wall biogenesis and hence the CWI pathway has been used as a target for the release of cell wall components, particularly mannoproteins (Cebollero, et al., 2009).

Van der Vaart, et al. (1995) studied the effect of deleting genes responsible for cell wall integrity and biogenesis in the releasing of mannoproteins. These researchers deleted genes such as *GAS1* and *GPI7* which encode for different proteins or enzymes related to the biogenesis of the cell wall, and *KNR4* which is a regulatory gene required for correct targeting of Stl2 MAP kinase to its downstream transcriptional factors, Rlm1p and Swi4p. The strains defective in these genes released higher amounts of polysaccharides, particularly mannoproteins in the medium. In a more recent study Penacho, et al. (2012) showed that deletion of *KNR4* in a recombinant strain increased flocculation to 30% as opposed to 3% displayed by the wild type yeast strain. The transcriptomic study showed expression of *FLO8* gene which is involved in the regulation of flocculation.

The current study was aimed at over-expressing five flocculation genes, *FLO1*, *FLO5*, *FLO9*, *FLO10* and *FLO11* in a BY4742 laboratory strain with deletion in either the *GPI7* or *KNR4* gene. BY4742 and its derived deletion strains over-expressing *FLO* genes showed a similar growth pattern compared to the wild type strains when it was grown in complex medium for 24 hours. The deletion strains contained a kanamycin deletion cassette, confirming the absence of a respective cell wall related gene. Transformed strains were confirmed by polymerase chain reaction (PCR) for successful integration of an *ADH2*-promoter cassette in the targeted locus, upstream of each respective *FLO* gene open reading frame (ORF).

### 3.3 MATERIALS AND METHODS

#### 3.3.1 Strains

The *FLO-ADH2-SMR-FLO* cassettes used in this study were amplified from FY23 transgenic strains previously constructed by Govender, et al. (2008) through a polymerase chain reaction (PCR) strategy. All the other strains used in this study were derived from the BY4742 strain as listed in Table 3.1.

#### 3.3.2 Media and cultivation conditions

Yeast strains were routinely cultivated at 30°C in rich YPD medium, containing 1% (wt/vol) yeast extract, 2% (wt/vol) peptone and 2% (wt/vol) glucose. Single yeast colonies from three-day old YPD plates were used to inoculate experimental cultures in 10 mL YPD broth contained in 100 mL Erlenmeyer flasks, which were incubated at 30°C in an Infors HT multitron cell shaker (United Scientific, South Africa) at 160 rpm for 48 hours. In the selection of sulfometuron methyl (SM) resistant yeast transformants, chemically defined synthetic complete (SC) medium containing 0.67% (wt/vol) Yeast Nitrogen Base (Difco™) and 2% (wt/vol) glucose was supplemented with 50 µg/mL SM (Du pont Agricultural Products, France). Yeast mutants were further selected in SC medium supplemented with 200 µg/mL G418 (Thermo Scientific, South Africa). Selection media were also supplemented with histidine, leucine, lysine and uracil (Sigma Aldrich, Germany) to complement the BY4742 strain auxotrophies and with leucine, uracil and tryptophan to complement FY23 strain auxotrophies. In this study 2% (wt/vol) agar (Difco™) was used for all solid media. Yeast strains were stored at -80°C in YPD supplemented with 15% (vol/vol) glycerol (univAR, South Africa) respectively (Govender, et al., 2008). All the chemicals used in this study were from Merck, South Africa, unless otherwise stated.



**Table 3.1** Strains employed in this study

Strain	Genotype	Reference
BY4742	<i>MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3-Δ0</i>	EUROSCARF
BY4742-F1A	<i>MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3-Δ0 FLO1p::SMR1-ADH2p</i>	This study
BY4742-F5A	<i>MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3-Δ0 FLO5p::SMR1-ADH2p</i>	This study
BY4742-F9A	<i>MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3-Δ0 FLO9p::SMR1-ADH2p</i>	This study
BY4742-F10A	<i>MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3-Δ0 FLO10p::SMR1-ADH2p</i>	This study
BY4742-F11A	<i>MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3-Δ0 FLO11p::SMR1-ADH2p</i>	This study
BY4742-ΔGPI7	<i>MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3-Δ0 YJL062w::KanMX</i>	EUROSCARF
BY4742-ΔGPI7-F1A	<i>MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3-Δ0 YJL062w::KanMX FLO1p::SMR1-ADH2p</i>	This study
BY4742-ΔGPI7-F5A	<i>MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3-Δ0 YJL062w::KanMX FLO5p::SMR1-ADH2p</i>	This study
BY4742-ΔGPI7-F9A	<i>MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3-Δ0 YJL062w::KanMX FLO9p::SMR1-ADH2p</i>	This study
BY4742-ΔGPI7-F10A	<i>MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3-Δ0 YJL062w::KanMX FLO10p::SMR1-ADH2p</i>	This study
BY4742-ΔGPI7-F11A	<i>MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3-Δ0 YJL062w::KanMX FLO11p::SMR1-ADH2p</i>	This study
BY4742-ΔKNR4	<i>MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3-Δ0 YGR229c::KanMX</i>	EUROSCARF
BY4742-ΔKNR4-F1A	<i>MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3-Δ0 YGR229c::KanMX FLO1p::SMR1-ADH2p</i>	This study
BY4742-ΔKNR4-F5A	<i>MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3-Δ0 YGR229c::KanMX FLO5p::SMR1-ADH2p</i>	This study
BY4742-ΔKNR4-F9A	<i>MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3-Δ0 YGR229c::KanMX FLO9p::SMR1-ADH2p</i>	This study
BY4742-ΔKNR4-F10A	<i>MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3-Δ0 YGR229c::KanMX FLO10p::SMR1-ADH2p</i>	This study
BY4742-ΔKNR4-F11A	<i>MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3-Δ0 YGR229c::KanMX FLO11p::SMR1-ADH2p</i>	This study
FY23	<i>MATa leu2 trp1 ura3 flo8-1</i>	(Winston, et al., 1995)
FY23-F1A	<i>MATa leu2 trp1 ura3 flo8-1 FLO1p::SMR1-ADH2p</i>	(Govender, et al., 2008)
FY23-F5A	<i>MATa leu2 trp1 ura3 flo8-1 FLO5p::SMR1-ADH2p</i>	(Govender, et al., 2008)
FY23-F9A	<i>MATa leu2 trp1 ura3 flo8-1 FLO9p::SMR1-ADH2p</i>	(Ramadhin, et al., 2009)
FY23-F10A	<i>MATa leu2 trp1 ura3 flo8-1 FLO10p::SMR1-ADH2p</i>	(Ramadhin, et al., 2009)
FY23-F11A	<i>MATa leu2 trp1 ura3 flo8-1 FLO11p::SMR1-ADH2p</i>	(Govender, et al., 2008)

### 3.3.3 Gene knockouts

Deletion mutant strains generated by the *S. cerevisiae* Genome Deletion Project (Winzeler, et al., 1999) were used in this study. The kanamycin (KanMX) deletion cassette was inserted to replace the ORF of the target gene, which was deleted, leaving the start and the stop codons intact. This cassette was flanked by two distinct molecular bar codes (20 nucleotides) which allowed for each deletion to be uniquely identified (Bauer, et al., 2010; Kelly, et al., 2001). The mutant strains bearing a deletion in either *KNR4* or *GPI7* ORF were further confirmed to carry a deletion in these ORF sites. For each deletion mutant, a set of confirmation primers (conf A, B, C and D) were used together with KanB and KanC primers for verification (Table 3.2). The primer set conf A and conf D were designed from regions 200-400 bp from the start and the stop codons of the gene respectively, and conf B and conf C were located within the coding regions of the *KNR4* or *GPI7* ORF. Absence of a PCR product in the combination of primer conf A with conf B and primer conf C with conf D indicated that the native ORF was successfully deleted (Kelly, et al., 2001; Winzeler, et al., 1999). Replacement of the *KNR4* and the *GPI7* native ORFs with a KanMX marker cassette was confirmed by a PCR reaction employing the following primer set: conf A with KanB and KanC with conf D. The PCR product of these combinations confirmed that the native ORF was replaced by a KanMX marker cassette.

**Table 3.2** KanMX4 deletion cassette confirmation primers (Winzeler, et al., 1999)

Primer name	Primer sequence (5'→3')
KanB	CTGCAGCGAGGAGCCGTAAT
KanC	TGATTTTGATGACGAGCGTAAT
ΔKNR4-CONF A	CAACTGAAAAGGTTGTGTTTTCTTT
ΔKNR4-CONF B	GGGATAAACCTTGTTGAGATCTTTT
ΔKNR4-CONF C	TTTAAAAAGAAGAACGTGGATCAAG
ΔKNR4-CONF D	GCCAACCGTTTTGTATATATTGATT
ΔGPI7- CONF A	GCTGTTCTTCTCTCCTCTTTTGTA
ΔGPI7-CONF B	CGTCACATTATTATCGACTTGAGTG
ΔGPI7-CONF C	GGTGTTCCTCAGACTACAACATCT
ΔGPI7-CONF D	GCACTTAACCTACTAGATTGGGACA

*Nucleotide numbering has been done by assigning the A in ATG start codon of the open reading frame as base 1.*

### 3.3.4 Isolation of yeast chromosomal DNA

Chromosomal DNA was isolated by a method described by Ausubel and co-workers (1995). Yeast strains were grown at 30°C with shaking (160 rpm) in an Infors HT multitron cell (United Scientific, South Africa) for 48 hours in 10 mL YPD medium. The cells were collected by centrifugation at 3000 rpm at 4°C for 2 minutes (Eppendorf, centrifuge 5417R, Germany). The medium was then discarded and the cells were resuspended in 500 µL of distilled water. The cell suspension was then transferred to a 2 mL microcentrifuge tube and centrifuged at 2 000 rpm at 4°C for 30 seconds. The supernatant was discarded and the pellet was resuspended by vortexing briefly in the residual liquid. Thereafter 200 µL of breaking buffer [2% (v/v) Triton X-100, 1% (v/v) SDS, 100 mM NaCl, 10 mM Tris-HCL pH 8, 1 mM EDTA pH 8], 0.3 g of glass beads and 200 µL of a phenol/chloroform/isoamyl alcohol [25:24:1 (v/v/v)] mixture were added to the cell suspension. This was then followed by vortexing at high speed for 5 minutes. Subsequently 200 µL of a buffer containing 10 mM Tris-HCL and 1 mM EDTA pH 8.0 (TE) was added to the mixtures and the tubes were briefly vortexed. Reaction mixtures were centrifuged at 12 000 rpm at 4°C for 5 minutes. The aqueous layer (~400 µL) was transferred into a clean 1.5 mL microcentrifuge tube and a 1 mL of 100% ethanol was added to the aqueous fraction. The contents were mixed by inverting the microcentrifuge tube to facilitate chromosomal DNA precipitation.

The mixtures were stored at -20°C for 20 minutes. The tubes were then microcentrifuged at 12 000 rpm at 4°C for 5 minutes. The supernatant was discarded and the pellet was resuspended in 400 µL Tris-EDTA (TE), pH 7. Contaminating RNA was degraded by the addition of 3 µL of RNase (10 mg/ml) and incubating at 37°C for 5 minutes. Thereafter chromosomal DNA was precipitated by the addition of 10 µL of 4M ammonium acetate and 1 mL of 100% ethanol. The contents were mixed by inversion and stored at -80°C for 20 minutes. Tubes were then centrifuged at 12 000 rpm at 4°C for 3 minutes and supernatants discarded. The pellet was dried in a vacuum concentrator (Eppendorf, concentrator 5301, Germany) and pelleted DNA was resuspended in 100 µL of TE (Govender, et al., 2008).

### 3.3.5 Amplification of promoter replacement cassettes

All PCR reactions were performed in a “G-star master cycler-GS2A” (UK). To ensure high fidelity amplification, a Kapa HiFi DNA Polymerase PCR system (Kapa Biosystems, Cape Town) was employed in all amplification reactions in which the amplicon was to be used as a

DNA template in a subsequent PCR amplification or as a vector cassette for yeast transformation. The *ADH2-SMR-410* integration cassettes previously constructed by Govender, et al. (2008) to fine tune the expression of dominant flocculation genes in FY23 background strain were amplified using genomic DNA isolated from FY23-F1A, FY23-F5A, FY23-F9A, FY23-F10A and FY23-F11A transgenic laboratory yeast strains as templates.

The *FLO1p-SMR1-ADH2-FLO1p* cassette was PCR amplified by using short primers FLO1-F and FLO1-R and genomic DNA from the FY23-F1A transgenic strain as template. A similar strategy was employed for the amplification of *FLO5p-SMR1-ADH2-FLO5p*, *FLO9p-SMR1-ADH2-FLO9p*, *FLO10p-SMR1-ADH2-FLO10p* and *FLO11p-SMR1-ADH2-FLO11p* integrating promoter replacement cassettes, using genomic DNA from FY23-F5A, FY23-F9A, FY23-F10A and FY23-F11A as templates, respectively. The primer pairs for different ORFs are listed in Table 3.3.

### 3.3.6 Purification of amplified promoter replacement cassettes

The integrating *FLO* promoter replacement cassettes were purified using a high pure PCR purification kit (Roche diagnostics, Germany), following the manufacturer's instructions for the extraction and purification of promoter replacement cassettes. Briefly, multiple amplification reactions (50 \* 20  $\mu$ L reactions) were combined into 200  $\mu$ L per 1.5 mL microcentrifuge tube. Binding buffer (1 mL) was added into each tube and mixed with the cassette. A high pure filter tube was inserted into a collection tube and the replacement cassette was transferred to the upper reservoir of the filter tube. This was centrifuged for 1 minute at 12 000 rpm using a standard table microcentrifuge (Eppendorf, centrifuge 5417R, Germany). The flow through solution was discarded and the integration cassette was washed twice with 500  $\mu$ L and once with 200  $\mu$ L of washing buffer to ensure optimal purity and complete removal of washing buffer from the glass fibers. The filter tube was reconnected with a clean 1.5 mL microcentrifuge tube. The purified promoter replacement cassette was eluted using 500  $\mu$ L sterile distilled water and 10  $\mu$ L of eluent buffer. The replacement cassette was allowed to stand on the bench top for 4 minutes and then centrifuged. Thereafter the volume of the eluent was reduced under vacuum to 10  $\mu$ L and used as transforming DNA in subsequent electroporation protocols. The same instructions were followed for the purification of all replacement cassettes used in this study.

### 3.3.7 Yeast transformation

Freshly prepared or cryopreserved electro-competent cells were electroporated with approximately 10 µg of a purified promoter replacement cassette according to the electroporation protocol described by Ausubel and coworkers (1995). The remaining freshly prepared electro-competent cells were cryopreserved according to the method described by Suga and co-authors (2000) and employed in subsequent transformations. Electroporation of yeast was performed with a Bio-Rad MicroPulser™ (Bio-Rad Laboratories, USA) using the instrument's pre-programmed setting for *S. cerevisiae* (Sc2) and HiMax electroporation cuvettes (Cell Projects Ltd., UK) with a 0.2 cm electrode gap. To limit the carry-over of untransformed cells, single colonies of putative transformants following initial selection on SC plates containing SM, were inoculated individually onto fresh SC plates containing 50 µg mL<sup>-1</sup> SM and cultivated at 30°C for 5 days.

Chromosomal integration was achieved by a double cross-over homologous recombination event, in which the *FLO1*, *FLO5*, *FLO9*, *FLO10* or *FLO11* gene was placed under transcriptional control of the *ADH2* promoter. The deletion of native promoters was confirmed by PCR using homologous primer sets. The primer pairs for transgenic strains are listed. In addition, the integration of promoter replacement cassettes in the desired locus in transformed yeast was further confirmed by PCR using heterologous primer set that contained a forward primer from outside the region of integration and genomic DNA isolated from transformants. The primer pairs for different transformants were as follows: for BY742-F1A, BY4742-ΔKNR4-F1A and BY4742-ΔGPI7-F1A, were FLO1-F2 and ADH2-R; for BY4742-F5A, BY4742-ΔKNR4-F5A and BY4742-ΔGPI7-F5A, were FLO5-F2 and ADH2-R; for BY4742-F9A, BY4742-ΔKNR4-F9A and BY4742-ΔGPI7-F9A, were FLO9-F2 and ADH2-R; for BY4742-F10A, BY4742-ΔKNR4-F10A and BY4742-ΔGPI7-F10A, were FLO10-F2 and ADH10-R; and for BY4742-F11A, BY4742-ΔKNR4-F11A and BY4742-ΔGPI7-F11A, were FLO11-F2 and ADH2-R (Table 3.3). The wild type BY4742, BY4742-ΔKNR4 and BY4742-ΔGPI7 strains served as controls in these confirmation experiments.

**Table 3.3** Primers employed in this study

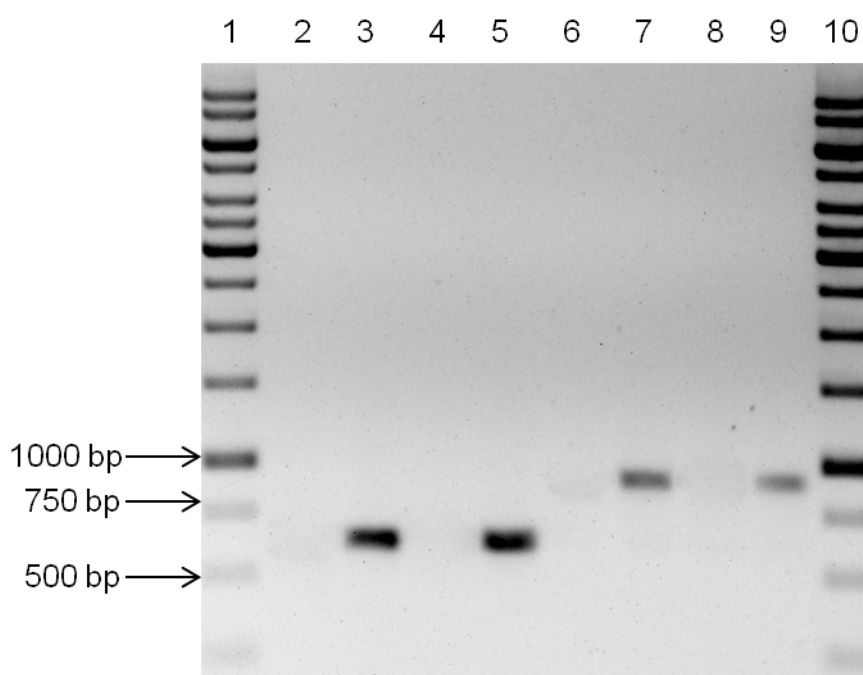
Primer name	Primer sequence (5'→3')
ADH2-R	TGATAGTTGATTGTATGCTTTTTGTAGC
FLO1-F	AGTGTTATGCTAGCCAGTTTCAGG
FLO1-F2	GCACATGCCAATTGCTGTGCATAGC
FLO1-R	CGTCAGAAATGTGAAGACCGTG
FLO5-F	CAATAACGAGATTCGCGACG
FLO5-F2	GGTTGTGTTCTA GGACTTTCTGACG
FLO5-R	AGTGGTGCTAATCAATTAAAGAA
FLO9-F	GCCGCTGCTAGCTTAAACAG
FLO9-F2	TGCGCCTTTCCTTCGCCAAG
FLO9-R	GTTATTTTCGGGGTTTTTCAGAGACCG
FLO10-F	GACCATCGGCCTGTTAATAAGG
FLO10-F2	CCATTGTTTCGCAAGGCAGATCAAG
FLO10-R	AAGCACCTAAAAGAAATCTTCAACG
FLO11-F	TTACGGCCTAATGTCGAGAC
FLO11-F2	GCTGCTTGTCTCACATCTAAACTTCG
FLO11-R	CTGGGAAATCCGTTTGAGAC

*Nucleotide numbering has been done by assigning the A in ATG start codon of the open reading frame as base 1. F and R denote the forward and reverse primers, respectively, and F 2 denotes a forward primer upstream of the region of ADH2-promoter cassette integration.*

### 3.4 RESULTS

#### 3.4.1 Confirmation of *GPI7* and *KNR4* ORF deletion in BY4742 mutant strains

The deletion of either the *GPI7* or *KNR4* ORF was confirmed in all relevant yeast strains *via* a PCR strategy (Figure 3.1) according to the verification method as described by Kelly, et al. (2001). The primers that were used in the confirmation of deletion strains are listed in Table 3.2. In this strategy, the genomic DNA of transgenic deletion strains was used as template for all PCR reactions.

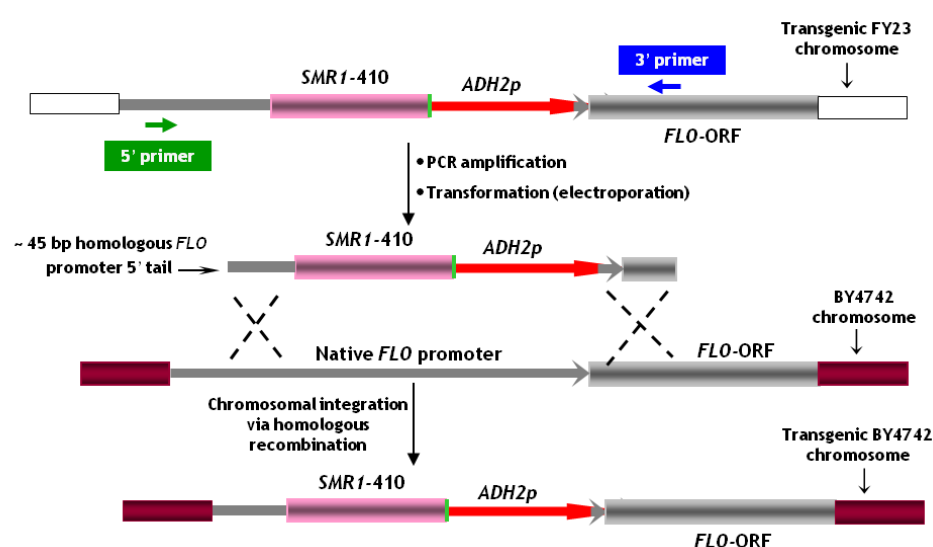


**Figure 3.1** Confirmation of the integration of kanamycin (KanMX) deletion cassette in BY4742 deletion strains. The KanMX deletion cassette was integrated to knockout either KNR4 ORF or GPI7 ORF. Lanes 2 and 4 show deletion of KNR4 ORF and GPI7 ORF respectively (primer set: Conf A and Conf B was used); Lanes 3 and 5 show replacement of KNR4 ORF and GPI7 ORF respectively with KanMX cassette (primer set: Conf A and Kan B was used); Lanes 6 and 8 show deletion of KNR4 ORF and GPI7 ORF respectively (primer set: Conf C and Conf D was used); Lanes 7 and 9 show replacement of KNR4 ORF and GPI7 ORF respectively (primer set: Kan C and Conf D was used).

The wild type BY4742 yeast strain and its deletion mutants were used as controls in these confirmation experiments. Primers A and D were located 200 and 400 bp upstream and downstream of the start and stop codons respectively. Primers B and C (Table 3.2) were located within their respective ORFs. To further verify the deletion of the native ORF by the KanMX

deletion module, two primers common to regions within the KanMX deletion module were used which included KanB and KanC. The deletion of the ORF was confirmed by the absence of a PCR product when primers A with KanB and D with KanC were used. The correct integration of the *KanMX* gene was verified in the transformants by the appearance of a PCR product. To ascertain that all yeast transformants were derivatives of their parental strains, it must be highlighted that an identical strategy was employed in the verification of all transgenic strains generated in this study (data not shown).

### 3.4.2 Construction of promoter replacement cassettes, yeast transformation and screening.



**Figure 3.2** Promoter replacement strategy. The *ADH2*-promoter was amplified by PCR reactions from the previously constructed transgenic FY23 strains using short 5' and 3' homologous primer sequences. The amplified cassette was integrated by homologous recombination.

The *SMR1-ADH2* promoter replacement vector cassette with homologous tail regions to the respective *FLO* gene integration site was PCR-generated from transgenic yeast strains constructed in previous studies. After column purification, the PCR products were transformed into BY4742-based strains and integrated through homologous recombination (Figure 3.2).



Approximately 200 colonies representing putative transformants were screened using both the PCR strategy and visual detection of the flocculation phenotype. The transformation efficiency was low in all the strains transformed with *FLO1p-SMR1-ADH2p-FLO1p*, *FLO9p-SMR1-ADH2p-FLO9p* and *FLO10p-SMR1-ADH2p-FLO10p* cassettes, with  $\pm 5$  transformants per 200 putative transformants screened.

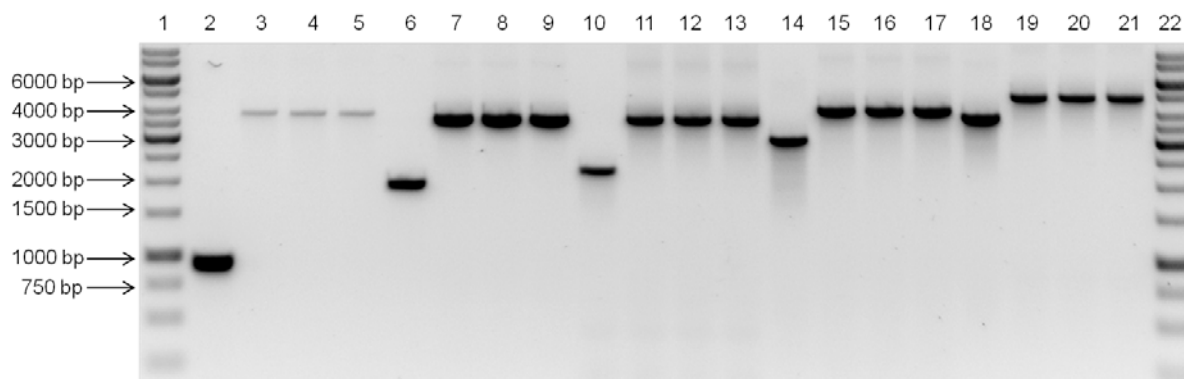
Generation of *FLO5*-based transgenic strains proved to be difficult and there were no putative transformants growing in the SC selection media. After two attempts of transforming the BY4742 based strains with *FLO5p-SMR1-ADH2p-FLO5p* replacement promoter cassette, there were BY4742-F5A and BY4742- $\Delta$ GPI7-F5A putative transformants growing in the selection media. The BY4742- $\Delta$ KNR4-F5A putative transformants were only found growing in SC media after three transformation attempts.

The *FLO11*-based transformants were selected using two strategies, the ability of putative transformants to invade agar surfaces and by a PCR screening strategy. The template DNA isolated from 100 colonies per strain was used in a PCR reaction for screening. The primer pairs employed were the same as the one used to construct these transgenic strains (Table 3.3). The *FLO11*-based transformants showed approximately 70% transformation efficiency in the wild type, the *KNR4* deletion and the *GPI7* deletion strains.

Three independent transformants in each strain were selected for further analysis. The transformants bearing a deletion in either *KNR4* or *GPI7* ORF were first selected in SC plates supplemented with 200  $\mu$ g/mL geneticin (G418) to verify that they carried a deletion (ORF replaced with a kanamycin cassette showing resistance to geneticin).

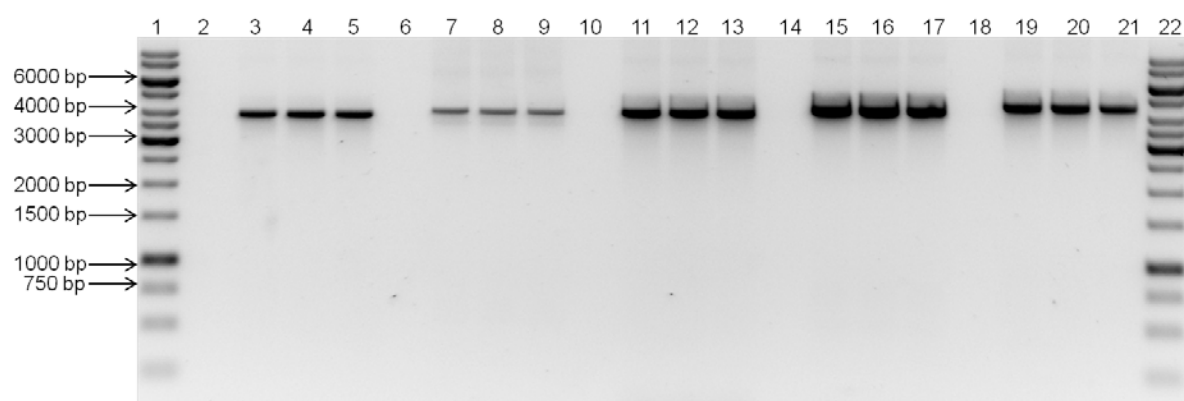
### 3.4.3 Integration of promoter replacement cassettes.

To confirm the integration of the promoter replacement cassette in the transformed strains, the three selected putative transformants in each strain were further screened by PCR using homologous primer pairs as follows: BY4742-F1A, BY4742- $\Delta$ KNR4-F1A and BY4742- $\Delta$ GPI7-F1A (*FLO1*-F and *FLO1*-R); BY4742-F5A, BY4742- $\Delta$ KNR4-F5A and BY4742- $\Delta$ GPI7-F5A (*FLO5*-F and *FLO5*-R); BY4742-F9A, BY4742- $\Delta$ KNR4-F9A and BY4742- $\Delta$ GPI7-F9A (*FLO9*-F and *FLO9*-R); BY4742-F10A, BY4742- $\Delta$ KNR4-F10A and BY4742- $\Delta$ GPI7-F10A (*FLO10*-F and *FLO10*-R) and BY4742-F11A, BY4742- $\Delta$ KNR4-F11A and BY4742- $\Delta$ GPI7-F11A (*FLO11*-F and *FLO11*-R). The correct sized PCR products (Figure 3.3) in all putative transformants showed successful integration of each promoter replacement cassette in each putative transformant and confirms that the native promoter was knocked out to put the *FLO* ORF under transcriptional control of the glucose repressible promoter, *ADH2p*.



**Figure 3.3** Chromosomal integration of *ADH2* promoter upstream of a dominant *FLO* gene in *S. cerevisiae* BY4742 and BY4742-based deletion strains. The replacement of native *FLO* promoters with the *ADH2* cassette was confirmed by PCR using homologous primers described in materials and methods. In lane 2, lane 6, lane 10, lane 14 and lane 18, native promoter sequences in wild type BY4742 strain were amplified with sizes corresponding to *FLO1* (937 bp), *FLO5* (2003 bp), *FLO9* (2270 bp), *FLO10* (3068 bp) and *FLO11* (3890 bp) respectively. The integration of a promoter replacement cassette was only amplified in *FLO1p-SMRI-ADH2-FLO1p* (BY4742, lane 3;  $\Delta$ KNR4, lane 4;  $\Delta$ GPI7, lane 5, (3707 bp); *FLO5p-SMRI-ADH2-FLO5p* (BY4742, lane 7;  $\Delta$ KNR4, lane 8;  $\Delta$ GPI7, lane 9, respectively; 3706 bp); *FLO9p-SMRI-ADH2-FLO9p* (BY4742, lane 11;  $\Delta$ KNR4, lane 12;  $\Delta$ GPI7, lane 13, respectively; 3708 bp); *FLO10p-SMRI-ADH2-FLO10p* (BY4742, lane 15;  $\Delta$ KNR4, lane 16;  $\Delta$ GPI7, lane 17, respectively; (4127 bp). and *FLO11p-SMRI-ADH2-FLO11p* (BY4742, lane 19;  $\Delta$ KNR4, lane 20;  $\Delta$ GPI7, lane 21, respectively; 4938 bp). Lane 1 and 22 contained a DNA molecular weight marker (phage lambda DNA restricted with *HindIII*).

To confirm the integration of these promoter replacement cassettes in the correct locus, heterologous primer pairs, with a forward primer from the region outside the cassette integration as explained in Section 3.3 were employed. The correct sized PCR product (Figure 3.4) shows that the promoter replacement cassette in each transformant was integrated into the desired locus. In all confirmation PCR reactions, the template DNA isolated from the transformants was used and the BY4742 wild type and its untransformed deletion derivatives were used as controls.



**Figure 3.4** The integration of promoter replacement cassettes at the correct chromosomal location were confirmed by PCR using heterologous primer sets that contained a forward primer from outside the region of integration and *ADH2* primer described in materials and methods. In lane 2, lane 6, lane 10, lane 14 and lane 18, a native promoter sequence in wild type BY4742 is not present, indicating that the wild type does not contain the promoter cassette. The integration of a promoter replacement cassette was only amplified in *FLO1p-SMR1-ADH2-FLO1p* (BY4742, lane 3;  $\Delta$ KNR4, lane 4;  $\Delta$ GPI7, lane 5, respectively; 3865 bp; *FLO5p-SMR1-ADH2-FLO5p* (BY4742, lane 7;  $\Delta$ KNR4, lane 8;  $\Delta$ GPI7, lane 9, respectively; 4058 bp; *FLO9p-SMR1-ADH2-FLO9p* (BY4742, lane 11;  $\Delta$ KNR4, lane 12;  $\Delta$ GPI7, lane 13, respectively; 3871 bp; *FLO10p-SMR1-ADH2-FLO10p* (BY4742, lane 15;  $\Delta$ KNR4, lane 16;  $\Delta$ GPI7, lane 17, respectively; 4293 bp and *FLO11p-SMR1-ADH2-FLO11p* (BY4742, lane 19;  $\Delta$ KNR4, lane 20;  $\Delta$ GPI7, lane 21, respectively; 4332 bp). Lane 1 and 22 contained a DNA molecular weight marker (phage lambda DNA restricted with *HindIII*).

### 3.5 DISCUSSION AND CONCLUSION

The goal of this study was to over-express the *FLO* genes in yeast strains with deletion in genes that play a crucial role in cell wall synthesis. A genetic system over-expressing *FLO* genes, which can sense sugar concentration in the medium and only start flocculating after all the sugars have been exhausted was developed in previous studies (Cunha, et al., 2006; Govender, et al., 2008). The strains used were naturally non flocculent, probably due to a nonsense point mutation in the *FLO8* gene encoding a transcription factor required for the transcription of *FLO1* and *FLO11* (Bester, et al., 2006). The over-expression of *FLO* genes in these non flocculent strains conferred different phenotypes such as flocculation, adhesion, hydrophobicity and mat formation (Govender, et al., 2010; Govender, et al., 2008).

The site directed method of genome modification based on homologous recombination between the transforming DNA and yeast genomic sequence was exploited in the construction of transgenic strains used in this study. The *ADH2* promoter fused with the *SMR1-410* marker

gene for selection of putative transformants was extracted from the previously constructed FY23 transgenic strains. The regions for homologous recombination with the target allele were fused with the 5' and 3' ends of the promoter cassette (Govender, et al., 2008). The marker-promoter cassette was amplified by PCR using primers with 5'- or 3'-extension homologous to the target sequence. The amplified product was integrated upstream of the target gene by double crossing over. Consequently a *FLO* gene was then placed under transcriptional control of the *ADH2* promoter.

The integration of the *ADH2*-promoter cassette was facilitated by the high level of homologous recombination which allows for insertion to the specific *loci*. However, the genetically modified organisms must fit the regulations and be acceptable by consumers (Cebollero, et al., 2007; Pretorius and Bauer, 2002). The advantage to the procedure employed in this study is that it does not introduce foreign DNA sequences in the modified yeast strains since all the genes introduced are yeast derived sequences. The other advantage of this technique is that, unlike plasmid mediated expression where there is variable copy number, this strategy is free of plasmid-related artefacts (Govender, et al., 2010). Yeast derived dominant selectable marker, sulfometuron (*SMR1-410*) was employed for selection of transgenic strains rather than antibiotic based markers. The use of stationary phase inducible promoter in this study ensured that the strains with a deletion in *KNR4* or *GPI7* are allowed to first generate biomass before over-expressing adhesins. The over-expression directs most of the cell energy in the production of the over-expressed gene and this would have not been favourable in the cells with defective cell walls.

The transgenic strains were selected using two strategies, a PCR based strategy and by the ability to form visible flocs in rich medium. The transformation efficiencies were low across all the transgenic strains, except the *FLO11* transgenic strains which showed  $\pm 70\%$  positive putative transformants after the PCR screening. The high transformation efficiency displayed by *FLO11*-based transformants has also been noted in a study by Govender, et al. (2008). The generation of *FLO5*-based transgenic strains proved to be difficult, especially BY4742- $\Delta$ *KNR4*-*F5A* as this had the lowest transformation efficiency amongst all transgenic strains. The *FLO1*, *FLO5*, *FLO9* and *FLO10* over-expressing transgenic strains were screened by both the methods employed but *FLO11* over-expressing transgenic strains were only screened using a PCR method and their ability to invade agar surfaces because they did not display any visible flocculation. The only reported flocculation expression by *FLO11* gene was in wine fermentation conditions (Govender, et al., 2011) and also by *Saccharomyces var. diastaticus* (Bayly, et al., 2005; Douglas, et al., 2007).

In conclusion, all the transgenic strains constructed were shown to contain the *FLO* replacement vector cassette but the transformation efficiencies were very low for every transgenic strain constructed. In future studies, a real time PCR method can be used to assess the expression of the *FLO* genes. The screening method was also laborious and time consuming and in future studies, a marker that can allow selection based on colour can be useful in the selection of positive transformants.

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# Chapter 4

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## RESEARCH RESULTS II

**Effect of cell wall biogenesis related gene deletions on adhesion phenotypes of *FLO* gene over-expressing BY4742 strains**



## Effect of cell wall biogenesis related gene deletions on adhesion phenotypes of *FLO* gene over-expressing BY4742 strains

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### 4.1 ABSTRACT

The Flo proteins in *Saccharomyces cerevisiae* strains are encoded by a family of five dominant *FLO* genes. These genes are anchored through a glycosylphosphatidyl inositol (GPI) remnant and are covalently linked to the  $\beta$ -1,6-glucan network in the cell wall. The expressed Flo proteins confer cell wall surface phenotypes such as flocculation, invasive growth (*FLO10* and *FLO11*), hydrophobicity, biofilm formation and pseudohyphal filamentation. The cell wall  $\beta$ -glucan network or GPI-anchor synthesis has been reported to be the primary targets for the release of GPI-anchored cell wall proteins. To date and to the best of our knowledge, the disruption of surface protein linkages in the cell wall have no known or reported effects on the adhesion phenotypes. In this study, the effect in adhesion phenotypes in *FLO* genes over-expressing deletion mutant strains is assessed. The *FLO* over-expressing BY4742 strains with deletion in *KNR4* or *GPI7* were employed in assessing the effect of the deletion in adhesion encoded phenotypes. It was found that flocculation was decreased in *KNR4* deletion mutant strains over-expressing *FLO* genes, but in *GPI7* deletion background, the flocculation was the same as that of their wild type BY4742 *FLO* over-expressing strain counterparts. The hydrophobicity is increased to varying degrees in different transgenic strains, whilst only *FLO10* and *FLO11* over-expressing strains displayed an invasive growth phenotype.

## 4.2 INTRODUCTION

The glycosphosphatidyl inositol (GPI) anchored proteins in *Saccharomyces cerevisiae* are functionally diverse, some serve as cell surface receptors, structural components, hydrolytic enzymes and adhesion proteins in mating, flocculation, biofilm formation or in cell to surface adhesion (Gonzalez, et al., 2010; Orlean and Menon, 2007). Some of the morphological transitions conferred by GPI-anchored proteins in yeast cell walls are largely regulated by nutrient limitation and environmental conditions (Smits, et al., 1999; Van Mulders, et al., 2009). One special GPI-anchored family of proteins are known as flocculins or adhesins (Halme, et al., 2004; Verstrepen and Klis, 2006). The flocculins are encoded by a family of five unlinked dominant *FLO* genes revealed by structural and functional analysis of the genomic sequence of a laboratory strain of *S. cerevisiae* (Bauer, et al., 2010; Verstrepen, et al., 2004).

These genes include *FLO1*, *FLO5*, *FLO9*, *FLO10* and *FLO11*. The *FLO1*, *FLO5*, *FLO9* and *FLO10* generate flocculation phenotypes (Verstrepen and Klis, 2006) and are located 10-40 kb from the telomeres. These genes are epigenetically silenced by different histone deacetylases (HDAC) (Halme, et al., 2004) and this silencing is also partially attributed to a nonsense mutation in the *FLO8* gene encoding one of the transcriptional activators of *FLO* genes (Liu, et al., 1996). Flocculation is defined as an asexual, calcium dependent and reversible aggregation of cells that results in flocs which sediment to the bottom of the final product such as alcoholic beverages (Bony, et al., 1997). The mechanism of flocculation in yeast strains is a very complex process that is governed by numerous factors such as the yeast strain genetics, physiological state and metabolism, the growth medium composition and environmental conditions such as temperature, agitation and aeration. The well understood and generally accepted mechanism is the lectin hypothesis (Dengis, et al., 1995). According to the proposal by Teunissen and Steensma (1995), cell surface adhesion proteins specifically recognise and bind to  $\alpha$ -mannan carbohydrates of adjacent cells. According to the first proposal by Miki, et al. (1982) the active conformation of Flo proteins is maintained by  $\text{Ca}^{2+}$  ions which act as cofactors. Lectins in their active conformation have increased capacity to interact with  $\alpha$ -mannan carbohydrates.

*FLO11* is essential for the formation of pseudohyphae, invasive growth and adhesion to agar and surfaces such as plastics. It is neither telomeric nor centromeric. The *FLO11* promoter is the largest in the yeast genome and is regulated through a complex system which may explain the multiple phenotypes associated with this gene (Bayly, et al., 2005; Gonzalez, et al., 2010; Halme, et al., 2004). The *FLO* genes contain internal tandem repeats that allow for recombination which may alter the length of these genes (Verstrepen, et al., 2004).

The synthesis of Flo proteins starts in the endoplasmic reticulum (ER)-bound ribosomes where they receive a GPI-anchor. The glycoproteins are further modified by N- and O-glycosylation in the ER and golgi apparatus. The GPI-anchor in Flo proteins is trimmed off at the plasma membrane before incorporation into the cell wall (Lipke and Ovalle, 1998; Pittet and Conzelmann, 2007). The glycoprotein in the cell wall is attached through a GPI remnant and also becomes transglycosylated to the cell wall through  $\beta$ -1,6-glucan (Orlean and Menon, 2007). The GPI-anchor is involved in the maintenance of normal cellular morphology (Gonzalez, et al., 2010).

Mannoproteins together with  $\beta$ -glucans are the major polymers of the cell wall (Cid, et al., 1995). The construction of proper cell walls in *S. cerevisiae* is a tightly controlled process. It involves approximately 1 200 genes that encode for proteins responsible for cell wall synthesis, cross-linking of cell wall polymers, and regulation of the cell wall (Klis, et al., 2002).

The Flo proteins are strongly anchored into the cell wall, which make their extraction more difficult when compared to low covalently linked glycoproteins like  $\alpha$ -agglutinins (Bony, et al., 1997). Recent studies reported that mutations in specific genes, related to cell wall biosynthesis or regulation could result in mannoproteins being released to the external environment (Gonzalez-Ramos, et al., 2008; Gonzalez-Ramos and Gonzalez, 2006; Penacho, et al., 2012; Van der Vaart, et al., 1995). The strains employed had deletions in *FKSI*, *GSI*, *GPI7* or *KNR4*, which encode for different proteins or enzymes related to the biogenesis of the cell wall. The strains defective of these genes were suggested to release higher amounts of polysaccharides, particularly mannoproteins in the medium. The effects of cell wall biogenesis related gene deletion in the phenotypes conferred by the adhesins in the cell wall surface have not been reported.

In this study, phenotypic changes were assessed in BY4742 wild type strain and its derived  $\Delta GPI7$  or  $\Delta KNR4$  mutant strains over-expressing *FLO* genes (*FLO1*, *FLO5*, *FLO9*, *FLO10*, and *FLO11*). It has been previously reported that over-expression of *FLO* genes confirms certain cell surface phenotypes such as flocculation, hydrophobicity and invasive growth (Govender, et al., 2008; Van Mulders, et al., 2009). Our data shows that deletion of *KNR4* causes a decrease in flocculation while deletion of *GPI7* seems to have no effect on flocculation. The cell surface hydrophobicity is increased to varying degrees for all the strains compared to wild type strains. The exception was in four strains, BY4742- $\Delta KNR4$ -F5A, BY4742- $\Delta GPI7$ -F5A, BY4742- $\Delta KNR4$ -F10A and BY4742- $\Delta GPI7$ -F11A which did not show cell surface hydrophobicity. Invasive growth was only observed in strains over-expressing *FLO10* and *FLO11*.

### 4.3 MATERIALS AND METHODS

#### 4.3.1 Strains

The strains employed in this study are listed in Table 4.1.

**Table 4.1** Strains employed in this study

Strain	Genotype	Reference
BY4742	<i>MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3-Δ0</i>	EUROSCARF
BY4742-F1A	<i>MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3-Δ0 FLO1p::SMR1-ADH2p</i>	This study
BY4742-F5A	<i>MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3-Δ0 FLO5p::SMR1-ADH2p</i>	This study
BY4742-F9A	<i>MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3-Δ0 FLO9p::SMR1-ADH2p</i>	This study
BY4742-F10A	<i>MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3-Δ0 FLO10p::SMR1-ADH2p</i>	This study
BY4742-F11A	<i>MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3-Δ0 FLO11p::SMR1-ADH2p</i>	This study
BY4742-ΔGPI7	<i>MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3-Δ0 YJL062w::KanMX</i>	EUROSCARF
BY4742-ΔGPI7-F1A	<i>MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3-Δ0 YJL062w::KanMX FLO1p::SMR1-ADH2p</i>	This study
BY4742-ΔGPI7-F5A	<i>MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3-Δ0 YJL062w::KanMX FLO5p::SMR1-ADH2p</i>	This study
BY4742-ΔGPI7-F9A	<i>MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3-Δ0 YJL062w::KanMX FLO9p::SMR1-ADH2p</i>	This study
BY4742-ΔGPI7-F10A	<i>MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3-Δ0 YJL062w::KanMX FLO10p::SMR1-ADH2p</i>	This study
BY4742-ΔGPI7-F11A	<i>MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3-Δ0 YJL062w::KanMX FLO11p::SMR1-ADH2p</i>	This study
BY4742-ΔKNR4	<i>MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3-Δ0 YGR229c::KanMX</i>	EUROSCARF
BY4742-ΔKNR4-F1A	<i>MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3-Δ0 YGR229c::KanMX FLO1p::SMR1-ADH2p</i>	This study
BY4742-ΔKNR4-F5A	<i>MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3-Δ0 YGR229c::KanMX FLO5p::SMR1-ADH2p</i>	This study
BY4742-ΔKNR4-F9A	<i>MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3-Δ0 YGR229c::KanMX FLO9p::SMR1-ADH2p</i>	This study
BY4742-ΔKNR4-F10A	<i>MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3-Δ0 YGR229c::KanMX FLO10p::SMR1-ADH2p</i>	This study
BY4742-ΔKNR4-F11A	<i>MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3-Δ0 YGR229c::KanMX FLO11p::SMR1-ADH2p</i>	This study

### 4.3.2 Media and cultivation conditions

Yeast strains were routinely cultivated at 30°C in rich YPD medium, containing 1% (wt/vol) yeast extract, 2% (wt/vol) peptone and 2% (wt/vol) glucose. Single yeast colonies from three day old YPD plates were used to inoculate cultures in 10 mL YPD broth in 100 mL Erlenmeyer flasks, which were incubated at 30°C on a shaker (160 rpm) for 48 hours. For the selection of sulfometuron methyl (SM) resistant yeast transformants, chemically defined synthetic complete (SC) medium containing 0.67% (wt/vol) Yeast Nitrogen Base (Difco™) and 2% (wt/vol) glucose was supplemented with 50 µg/mL SM (Du pont Agricultural Products, France). Yeast mutants were further selected in SC medium supplemented with 200 µg/mL G418 (Thermo Scientific, South Africa). Selection media were also supplemented with histidine, leucine, lysine and uracil (Sigma Aldrich, Germany) to complement the BY4742 strain autotrophies. In this study 2% (wt/vol) agar (Difco™) was used for all solid media. Yeast strains were stored at -80°C in YPD supplemented with 15% (vol/vol) glycerol (univAR, South Africa) respectively (Govender, et al., 2008). All the chemicals used in this study were from Merck, South Africa, unless otherwise stated.

### 4.3.3 Growth and enumeration of yeast populations

Yeast suspension cultures were diluted appropriately (30 mM EDTA, pH 7) and the cell density was determined using a hemocytometer. This was also complemented with measuring absorbance at 600 nm in an Analytik Jena UV-visible spectrophotometer (Germany).

### 4.3.4 Flocculation assay

The percentage of cell flocculation was measured using the modified Helm's assay as described by D'Hautcourt and Smart (1999). Single colonies were grown for 20 hours in 10 mL of 2% (wt/vol) YPD and incubated in an Infors HT multitron cell (United Scientific, South Africa) at 160 rpm at 30°C. The 20 hour culture was used to inoculate 250 mL flasks containing 50 mL YPD and incubated for 48 hours with shaking at 160 rpm. Thereafter, cells were transferred to sterile 50 mL Falcon tubes, deflocculated by adding 250 µL of 0.4 M EDTA and 1 mL of 30 mM EDTA. Cells were subsequently harvested by centrifugation at 4 000 rpm for 5 minutes at 4°C (Eppendorf, centrifuge 5417R, Germany). Cells were then washed once with 20 mL of 30 mM EDTA (pH 7) and the supernatant discarded. The cells were resuspended in 20 mL of 30 mM EDTA and diluted to a cell concentration of  $1 \times 10^8$  cells/mL. Ten 1 mL samples were then harvested by centrifugation at 12 000 rpm for 1 minute at 4°C. Five of the 1 mL samples were resuspended in 1 mL of 100 mM EDTA (pH 7) and served as the control experiment. The remaining five samples served as the test experiment and they were first washed with 1 mL buffer Wash buffer containing  $0.51 \text{ g L}^{-1}$  of calcium chloride and this was followed by

resuspending pellets in 1 mL buffer Suspension buffer containing 0.44 g L<sup>-1</sup> of calcium chloride, 6.8 g L<sup>-1</sup> sodium acetate, 4% ethanol (vol/vol) and the pH was calibrated to 4.5 using acetic acid. Tubes were vigorously vortexed for 30 seconds followed by inverting the microcentrifuge tubes five times for 15 seconds to promote flocculation. Samples were then left to stand undisturbed at room temperature for 15 minutes. Thereafter 100 µL from each control and test sample was removed from below the meniscus and diluted in 900 µL of 100 mM EDTA (pH 7). The absorbance of the cell suspension was determined at 600 nm in an Analytik Jena UV-visible spectrophotometer (Germany). Flocculation is expressed as a function of the mean absorbance of the control assay by the equation:

$$\text{Percentage flocculation} = [(\text{mean control Abs} - \text{experimental Abs}) / \text{mean control Abs}] \times 100.$$

#### 4.3.5 Determination of hydrophobicity of yeast cell surfaces

The hydrophobicity of yeast cell surfaces was determined by measuring the distribution ratio of yeast cells in a biphasic system consisting of a buffered solution and xylene (Merck, South Africa) (Powell, et al., 2003). Single colonies were grown for 18 hours with shaking at 30°C in 10 mL of 2% YPD. Thereafter, 50 µL were inoculated into 50 mL of 2% YPD and grown for 48 hours with shaking at 30°C. The cells were harvested by centrifugation at 4000 rpm for 3 minutes at 4°C, deflocculated and washed as previously described (Section 4.3.4). Cells were then diluted to a concentration of  $5 \times 10^6$  cells/mL with phosphate-urea-magnesium (PUM) buffer (22.2 g K<sub>2</sub>HPO<sub>4</sub> · 3H<sub>2</sub>O, 7.26 g KH<sub>2</sub>PO<sub>4</sub>, 0.02 g MgSO<sub>4</sub> · 7H<sub>2</sub>O, 18 g urea) (pH 7.1). The initial (I) absorbance was then measured at 660 nm. Three replicates of 2.4 mL aliquots of the suspension were then transferred to glass borosilicate tubes (15 by 75 mm) and 200 µL of xylene was then layered on the yeast suspension. The tubes were then capped with rubber and the samples were vortexed at maximum speed for 30 seconds and allowed to stand undisturbed for 15 minutes. The absorbance of the aqueous fraction was then measured at 660 nm and taken as the final (F) absorbance. The average Modified Hydrophobic Index (MHI) was expressed as:  $\text{MHI} = 1 - (F/I) \times 100$ .

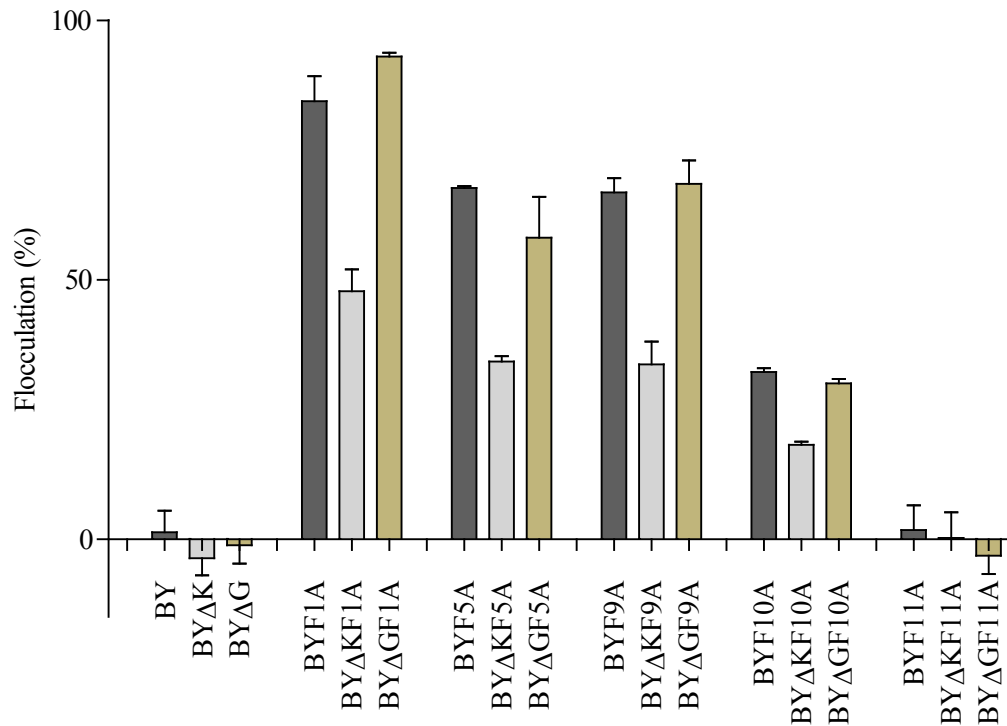
#### 4.3.6 Invasive growth plate assays

Yeast cultures, processed as described in section 4.3.4, were adjusted to an optical density (measured at a wavelength of 600 nm) of 1.0 and 10 µl aliquots were dropped onto 0.2% SC plates (histidine, leucine, lysine and uracil) without piercing the agar surface and incubated for five days at 30°C (van Dyk, et al., 2005). The plates were washed with a copious amount of running water and allowed to air dry. The plates were then photographed to assess the invasive cells in the plates.

## 4.4 RESULT

### 4.4.1 Flocculation assays

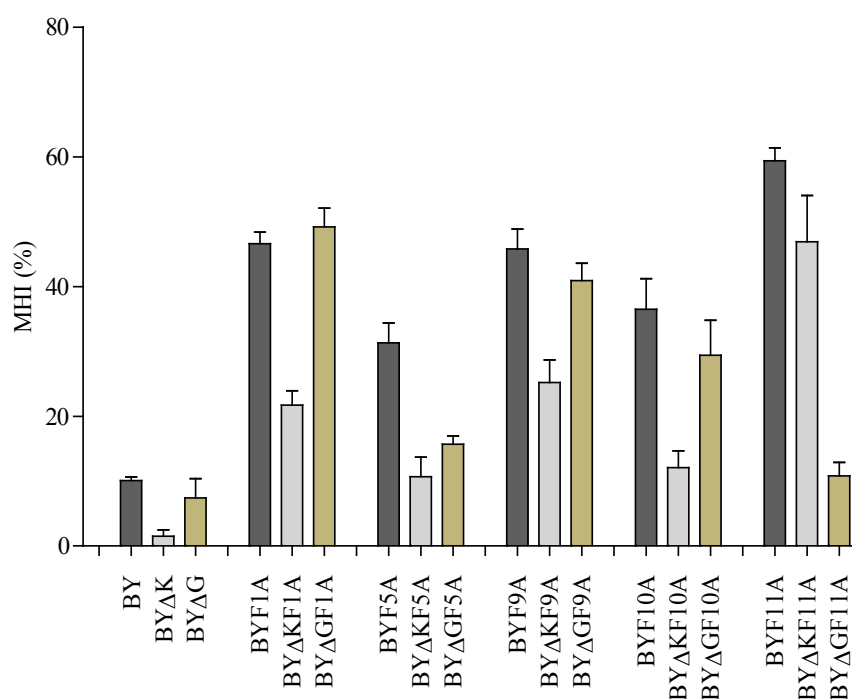
Strong flocculation was induced in the BY4742-F1A ( $84.4\% \pm 4.8$ ) and BY4742- $\Delta$ GPI7-F1A ( $93\% \pm 0.74$ ) strains. The flocculation observed in BY4742-F5A ( $67.7\% \pm 0.36$ ), BY4742-F9A ( $66.8\% \pm 2.8$ ), BY4742- $\Delta$ KNR4-F1A ( $47.8\% \pm 4.2$ ), BY4742- $\Delta$ GPI7-F5A ( $58.21\% \pm 7.88$ ) and BY4742- $\Delta$ GPI7-F9A ( $68.5\% \pm 4.47$ ) was moderate, and BY4742-F10A ( $32.2\% \pm 0.75$ ), BY4742- $\Delta$ KNR4-F5A ( $34.2\% \pm 1.09$ ), BY4742- $\Delta$ KNR4-F9A ( $33.7\% \pm 4.36$ ), and BY4742- $\Delta$ GPI7-F10A ( $30\% \pm 0.87$ ) were weakly flocculent. BY4742- $\Delta$ KNR4-F10A and all *FLO11* over-expressing strains were non flocculent. The wild type BY4742 and two mutant strains,  $\Delta$ KNR4 and  $\Delta$ GPI7 derived from the wild type were used as the untransformed controls and all the transgenic wild type BY4742 strains served as transformed controls (Figure 4.1).



**Figure 4.1:** Effect of cell wall biogenesis related gene deletion in the flocculation intensity of BY4742 strains over-expressing *FLO* genes. The results show the average of three independent determinations and error bars represent standard deviations. The results were analysed using the statistical software package GraphPad InStat version 3.05 32 bit for Windows 95/NT (GraphPad Software, San Diego, California). The abbreviations in the strain names are as follows: BY: BY4742;  $\Delta$ K:  $\Delta$ KNR4;  $\Delta$ G:  $\Delta$ GPI7.

#### 4.4.2 Hydrophobicity of yeast cell surfaces

The hydrophobicity was measured based on the affinity of cells to associate with the organic solvent after standing undisturbed for 15 minutes. The results that cells that over-expressed of *FLO* genes displayed an increase in hydrophobicity since the wild type BY4742 strain ( $10.1\% \pm 0.52$ ) did not associate with the xylene layer while the wild type strains over-expressing Flo1p, Flo5p, Flo9p, Flo10p and Flo11p associated with the hydrophobic layer,  $46.6\% \pm 1.8$ ,  $31.3\% \pm 3.07$ ,  $45.8\% \pm 3.05$ ,  $36.5\% \pm 4.7$  and  $59.4\% \pm 1.98$ , respectively. In the  $\Delta KNR4$  deletion strains hydrophobicity was only increased in the strain over-expressing Flo11p ( $46.9\% \pm 7.15$ ) compared to the BY4742- $\Delta KNR4$  ( $1.49\% \pm 0.995$ ). The other over-expressing strains showed hydrophobicity within a range of 10%-25% which is not considered to be significant. In the *GPI7* deletion strains, the order of hydrophobicity was BY4742- $\Delta GPI7$ -F1A ( $49.2\% \pm 2.9$ ), BY4742- $\Delta GPI7$ -F9A ( $40.9\% \pm 2.7$ ) and BY4742- $\Delta GPI7$ -F10A ( $29.4\% \pm 5.4$ ). The other two over-expression strains, BY4742- $\Delta GPI7$ -F5A ( $15.7\% \pm 1.28$ ) and BY4742- $\Delta GPI7$ -F11A ( $10.8\% \pm 2.08$ ), were considered not hydrophobic (Figure 4.2).

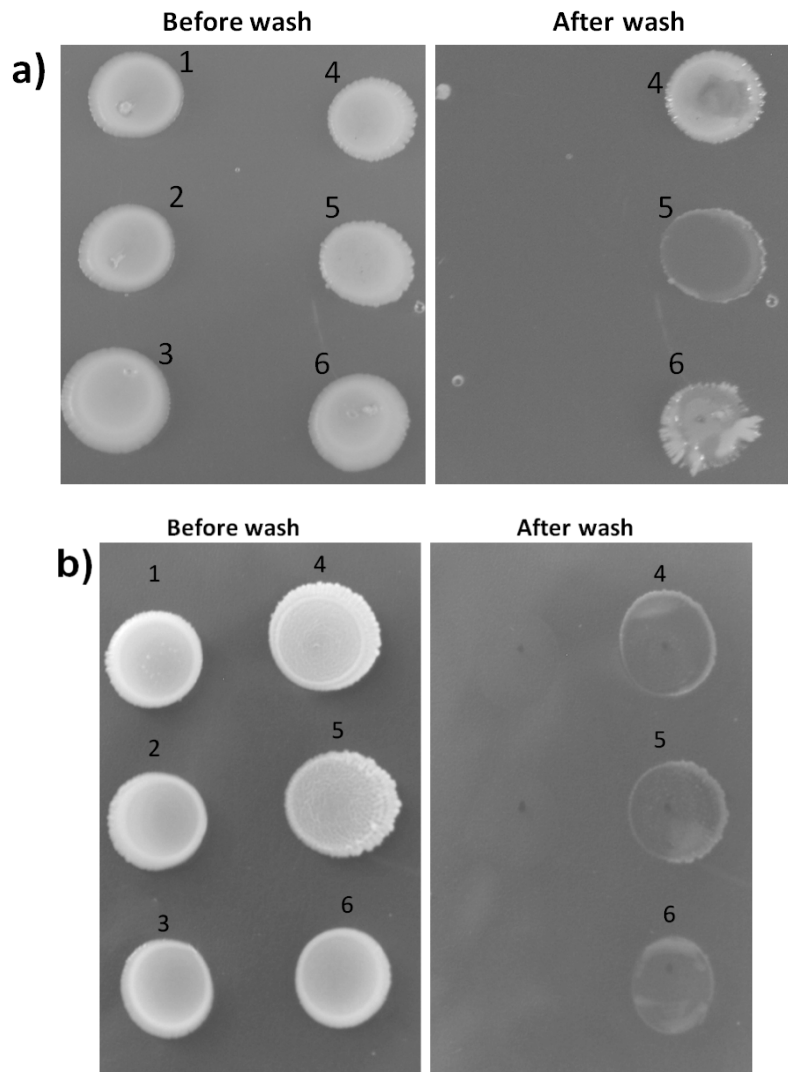


**Figure 4.2:** The impact of cell wall biogenesis related genes deletion on cell surface hydrophobicity (CSH) of BY4742 strains over-expressing *FLO* genes. The modified hydrophobic index (MHI) was determined according to the biphasic-solvent partition assay described by Hinchcliffe (1985). The results are averages of three independent determinations, and error bars represent standard deviations. The results were analysed using the statistical software package GraphPad InStat version 3.05 32 bit for Windows 95/NT (GraphPad Software, San Diego, California). ). The abbreviations in the strain names are as follows: BY: BY4742;  $\Delta K$ :  $\Delta KNR4$ ;  $\Delta G$ :  $\Delta GPI7$ .



#### 4.4.3 Invasive growth

The wild type BY4742, and deletion mutants BY4742- $\Delta$ KNR4 and the BY4742- $\Delta$ GPI7 did not show invasive growth. Haploid invasive growth was only observed in BY4742-F10A, BY4742- $\Delta$ KNR4-F10A and BY4742- $\Delta$ GPI7-F10A strains (Figure 4.3a) and in BY4742-F11A, BY4742- $\Delta$ KNR4-F11A and BY4742- $\Delta$ GPI7-F11A strains (Figure 4.3b). These strains adhered to the agar surface even after being washed with copious amount of water.



**Figure 4.3:** Haploid invasive growth in *FLO10* and *FLO11* over-expressing strains. Shown in a) are the wild type BY4742, BY4742- $\Delta$ KNR4 and the BY4742- $\Delta$ GPI7 labelled as 1, 2 and 3 respectively, and the BY4742-F10A, BY4742- $\Delta$ KNR4-F10A and BY4742- $\Delta$ GPI7-F10A strains labelled as 4, 5, and 6 respectively. Shown in b) are the BY4742, BY4742- $\Delta$ KNR4 and the BY4742- $\Delta$ GPI7 labelled as 1, 2 and 3 respectively and the BY4742-F11A, BY4742- $\Delta$ KNR4-F11A and BY4742- $\Delta$ GPI7-F11A strains labelled as 4, 5, and 6 respectively.

## 4.5 DISCUSSION AND CONCLUSION

The Flo proteins encode for different cell wall-related adhesion phenotypes. The spatial distribution of Flo proteins at the cell surface is controlled *via* a multitude of transcriptional regulators and the cell wall anchoring of the Flo proteins is dependent on the constraints of protein secretion and cell wall biogenesis (Bony, et al., 1998). There are several genes or enzymes that play a role in the synthesis, transport and cell wall anchoring of the Flo proteins. We have mentioned in the introduction that the *FLO* genes which encode for the Flo proteins are epigenetically silenced by different HDAC and by a nonsense mutation in the *FLO8* gene (Liu, et al., 1996, Halme, et al., 2004). Advances in genetic engineering have allowed for the overexpression of *FLO* genes where it was shown that placing the *FLO* genes under the control of the *ADH2* promoter allows for the selective expression of the different Flo proteins that display differing adhesive properties (Govender, et al., 2008, Van Mulders, et al., 2009). In this study, the effect of deletions of either of two cell wall biogenesis related genes; *KNR4* or *GPI7*, in the presence of *ADH2* promoter mediated *FLO* gene expression were investigated.

The flocculation intensity was dependent on a specific *FLO* gene with the BY4742-F1A-based wild type transgenic derivatives displaying the most intense flocculation ability, whilst the wild type transgenic derivatives, BY4742-F5A and BY4742-F9A displayed moderate flocculation. The flocculation phenotype of the BY4742-F10A transgenic strain was negligible. Gonzalez-Ramos and Gonzalez (2006) have reported that the deletion of *KNR4* gene in BY4741 strains resulted in the moderate release of mannoproteins into the culture medium whereas a deletion of the *GPI7* gene in the same background resulted in 9 fold increase in released mannoproteins compared to the wild type strain. Consequently, in this study it was hypothesised that strains bearing a deletion in cell wall biogenesis related genes would result in a decrease in *FLO* gene-based adhesion phenotypes due to inefficient incorporation of flocculins in the cell surface.

Flocculation in strains bearing a deletion of the *KNR4* gene was decreased by almost 50% when compared to its BY4742 wild type transgenic *FLO* gene over-expressing counterparts. This finding is in contrast with the data reported by Penacho, et al. (2012) where the industrial strain EC1118 with a *KNR4* deletion displayed 30% basal flocculation when compared to 3% flocculation displayed by the wild type strain. However, this could be due to genetic-based differences in the laboratory and industrial strains, mainly because laboratory strains are haploid and industrial strains are diploid or polyploid.

The *KNR4* gene product is essential to cell wall biogenesis and integrity. It is involved in the coordination of cell wall synthesis with bud emergence (Giovani, et al., 2010). It encodes for a protein required for correct targeting of Stl2p MAP kinase to downstream transcriptional factors, Rim1p and Swi4p, which in turn initiate a response by facilitating the expression of relevant genes (Gonzalez-Ramos, et al., 2008, Gonzalez-Ramos, et al., 2009). This is essential for proper yeast cell wall maintenance. Therefore, it is expected that a *KNR4* gene deletion in yeast cells compromises the cell in such a manner that the cell wall becomes leaky which may promote the loss of cell wall surface proteins.

The *GPI7* gene encodes for a phosphoethanolamine transferase enzyme that is required for the addition of the ethanolamine phosphate (EtN-P) to the second mannose residue of the GPI-anchor. There are other transferases, *MCD4* and *GPII3* that add the EtN-P residue to the first and third mannoses of the GPI anchor respectively. The deletion of *GPI7* results in the failure of yeast cells to form an amide linkage to what would be normally GPI-anchored proteins (Orlean & Menon, 2007). In this study it was found that deletion of *GPI7* resulted in the same or slightly enhanced flocculation intensities for strains over-expressing *FLO1*, *FLO5* and *FLO9* genes when compared to their wild type *FLO* over-expressing counterparts. The flocculation profiles displayed by these strains suggests that the cells respond to *GPI7* deletion by activating a cell wall integrity pathway that subsequently upregulates other GPI-transferases or other GPI-proteins to repair or stabilise the cells in response to the *GPI7* deletion (Gonzalez, et al., 2010).

A similar behaviour was reported for *GPII3*, a transferase which like *GPI7*, is involved in the addition of EtN-P to the mannose residue of the GPI-anchor. The strains with a deletion in *GPII3* when fused with a plasmid containing a green fluorescent protein (GFP)-Sag1p, resulted in increased fluorescence in their cell walls when compared with the non mutant strain. This suggested that the cell upregulates alternate genes in response to the deletion (Gonzalez, et al., 2010). Consequently, it can be assumed that the *FLO* over-expressing strains bearing a deletion in *GPI7* gene do not release adhesins into the fermentation medium. However, it should be noted that this is in contrast with a study by Gonzalez-Ramos and Gonzalez (2006) where a 9 fold increase in the release of cell wall-based mannoproteins was reported for BY4741 strains with a deletion in *GPI7* gene.

Previous studies have reported that flocculation or availability of Flo proteins on the cell surface is accompanied by an increase in cell surface hydrophobicity (Straver, et al., 1994, Van Mulders, et al., 2009). Our data confirms that hydrophobicity is increased when the Flo proteins are expressed. The expression of *FLO5* in the wild type BY4742 displayed the least hydrophobicity when compared to other overexpressed *FLO* genes. The most hydrophobic was BY4742-F11A. The deletion in *KNR4* caused a drastic decrease in hydrophobicity in all *FLO* over-expressing strains when compared to their wild type over-expressing counterparts which displayed increased hydrophobicity. The exception to this was observed in BY4742-Δ*KNR4*-F11A strain which showed increased hydrophobicity. The deletion in *GPI7* greatly decreased BY4742-Δ*GPI7*-F11A hydrophobicity.

Adhesion to agar surface was only displayed by *FLO10* and *FLO11* over-expressing strains. The deletion in *KNR4* or *GPI7* did not affect the invasiveness of the *FLO11* transgenic strains but *KNR4* deletion caused a slight decrease in the ability of *FLO10* transgenic strain to invade agar surfaces. The ability of the transgenic yeast strains to invade agar suggest that in natural environments the yeast strains express *FLO10* and *FLO11* genes to switch from unicellular growth form to invasive growth from under nutrient limiting conditions.

In conclusion, we have shown that *ADH2*-based promoter mediated overexpression of *FLO1*, *FLO5*, *FLO9*, and *FLO10* encoded adhesins is affected by a deletion in the *KNR4* gene but not by the *GPI7* deletion. On the other hand, *FLO11*-mediated adhesion phenotype was affected by the *GPI7* gene deletion, and not by the *KNR4* gene deletion. It must be noted that the four dominant *FLO* genes (*FLO1*, *FLO5*, *FLO9* and *FLO10*) are telomeric, whilst they are related to the *FLO11* gene which is neither telomeric nor centromeric. Consequently, the data of this study seem to suggest that the *KNR4* gene product is intricately involved in the cell surface-localization pathways of the telomeric-based *FLO* genes as the *KNR4* gene deletion noticeably affects the adhesion phenotypes encoded by these four dominant flocculation genes. Interestingly the invasiveness of the overexpressed *FLO10* and *FLO11* encoded-adhesins were not affected by a deletion of either *GPI7* or *KNR4*. Conversely, a similar scenario prevails with respect to a deletion in the *GPI7* gene and the *FLO11*-encoded phenotype.

#### 4.6 ACKNOWLEDGEMENTS.

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# Chapter 5

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## GENERAL DISCUSSION AND CONCLUSIONS

## 5 GENERAL DISCUSSION AND CONCLUSION

### 5.1 DISCUSSION

The Flo mannoproteins are components of the outermost layer of the cell wall. Mannoproteins define morphological transition in yeast cells. One family of genes encoding for specialised cell surface proteins known to display various cell surface phenotypes are known as *FLO* genes. The Flo proteins are also termed adhesins and they mediate phenotypes which include; flocculation (Bony, et al., 1997), invasive growth, pseudohyphal filamentation or biofilm formation and adhesion to surfaces (Guo, et al., 2000; Verstrepen and Klis, 2006). The *FLO* genes contain internal tandem repeats that may alter the length of by recombination events. The increase in length of the internal repeats has been associated with increased Flo-dependent phenotypes such as flocculation and adhesion (Verstrepen, et al., 2005). The availability of Flo proteins in the cell wall has also been shown to result in increased hydrophobicity (Govender, et al., 2008; Straver, et al., 1994).

The adhesive behaviour conferred by Flo proteins can be exploited in the fermentation processes such as those in wine, beer and ethanol production. The late onset of flocculation can be exploited for easy separation and recovery of the final product from the yeast cells. However, the *FLO* genes (*FLO1*, *FLO5*, *FLO9*, and *FLO10*) are epigenetically silenced by different histone deacetylases (HDAC) (Halme, et al., 2004). The silencing is also partially attributed to a nonsense mutation in the *FLO8* gene which encode for one of the key transcriptional activators of *FLO* genes (Bester, et al., 2006; Liu, et al., 1996). In this study, a self-cloning strategy, with only yeast derived sequences was employed (Govender, et al., 2008; Guo, et al., 2000). The *ADH2* promoter was used to replace the native *FLO* promoter of non-flocculent yeast strains. This resulted in transgenic strains with the ability to flocculate at the stationary phase of growth. This strategy has been reported as key to the adhesive phenotypes and has the potential to reduce the costs associated with downstream processing of fermented beverages (Cunha, et al., 2006; Govender, et al., 2008).

In the cell wall, the adhesins are covalently linked to  $\beta$ -1,6-glucan and are anchored through a GPI remnant (Klis, et al., 2002). In this study, the effect of deleting genes associated with the localisation of adhesins or involved in the synthesis of a cell wall component that is critical to the viability of *FLO* gene-based adhesion phenotypes was assessed. The *FLO* genes were over-expressed in strains bearing a deletion in cell wall biogenesis associated genes. The rationale for this was that a deletion of these genes will result in the release of adhesins into the cell culture media and therefore should affect the adhesive phenotypes conferred by the over-expressed *FLO* genes.



In this study, *FLO1*, *FLO5* and *FLO9* over-expressing laboratory strains with a *KNR4* deletion showed a range of 30-50% flocculation when compared to their wild type *KNR4* deletion strain which was not observed to be flocculent. The observed differences in the results of this study may be attributed to background differences between industrial and laboratory strains and growth conditions. This suggests that the loss of *KNR4* is integral to the anchoring of Flo proteins to the cell wall. The ability of the *KNR4* deletion strains to retain 50% flocculation when compared to their transgenic wild type over-expressing *FLO* gene strains most probably resides in that the *KNR4* deletion results in increased chitin content to compensate for loss of  $\beta$ -glucan polymer. This will alter the degree of cross-linking in the cell wall and chitin may prevent excessive loss of the glycoprotein (Gonzalez-Ramos, et al., 2008).

*GPI7* together with *MCD4* and *GPII3* encode for phosphoethanolamine (EtN-P) transferases involved in GPI-anchor biosynthesis. They are critical for the addition of the ethanolamine phosphate side chains to the mannose residues of the GPI anchor. The deletion of *GPI7* gene in yeast leads to several defects such as impaired transport of GPI-anchored protein from the endoplasmic reticulum to Golgi apparatus and also inefficient cross linking of the GPI-proteins into the cell wall (Orlean and Menon, 2007). In the determination of cell densities using a light microscope, it was noticed that *GPI7* mutant cells formed clumps and this suggest that they had not separated efficiently after cell division. This behaviour of *GPI7* deleted yeast cells has been previously reported by Fujita, et al. (2004) to be attributed to mistargeting of Egt2p, a daughter cell specific protein which is involved in degradation of the septum division. This suggests that the Man-2 attached EtN-P serves as a signal that is recognised by proteins involved in cross-linking GPI-proteins to the cell wall and lipid-remodelling enzymes (Orlean and Menon, 2007). However, the GPI precursors lacking EtN-p on Man-2 are transferred to the protein, but this transfer is suggested to be suboptimal.

Results from this work showed that deletion in *GPI7* gene had no effect on flocculation. The deletion in *GPI7* seems to initiate a response that results in more adhesins available in the cell wall surface. This was evidenced by a slight increase in flocculation percentage of *FLO1* and *FLO9* over-expressing strains with a deletion in *GPI7* gene when compared to their transgenic wild type *FLO* over-expressing counterparts. Similar behaviour by *GPII3* has been reported by Gonzalez-Ramos, et al. (2010) which showed that strains with a deletion in *GPII3*

when fused with a plasmid containing GFP-Sag1p displayed increased cell surface fluorescence compared to the strains without *GPII3* deletion. Although a GPI protein lacking an EtN-P is transferred to the cell wall, this is subject to suboptimal growth conditions such as low temperature. The flocculation behaviour shown by *GPI7* deletion strains suggest that deletion in *GPI7* responsible for EtN-P addition into the GPI anchor result in the upregulation of other transferases such as *MCD4* or *GPII3* in order to compensate for the failure to form an amide linkage in the Flo proteins.

The expression of adhesins in the cell wall surface is known to coincide with the hydrophobicity of the cell surface in yeast. In this study a similar trend was also observed. In all transgenic wild type *FLO* gene over-expressing strains there was an increase in cell surface hydrophobicity. Hydrophobicity was decreased by the deletion of the *KNR4* gene except in the *FLO11* over-expressing strain. The GPI deletion strains were hydrophobic except in the *FLO11* over-expressing strain. It is known that the *FLO* genes serve as a reservoir of phenotypes required for survival under starvation conditions (Verstrepen and Klis, 2006). The yeast strains are known to switch from unicellular growth to invasive growth under nutrient limiting conditions. This study found that only *FLO10*- and *FLO11*-encoded adhesins are employed by these transgenic yeast strains to invade agar surfaces under nutrient limiting conditions. The deletion in *KNR4* and *GPI7* had no effect on the invasiveness of the yeast strains, except in *FLO10* over-expressing strain where the *KNR4* deletion transgenic strain displayed slightly washed off cells compared to its transgenic wild type over-expressing strain.

## 5.2 CONCLUSION

The adhesion in Flo proteins is conferred through two distinct mechanisms, one through flocculation, where cells adhere to each other by a sugar-receptor interaction. The other mechanism is conferred through cell adhesion to surfaces and appears to be mediated by *FLO10* and *FLO11*. Cell-surface adhesion is believed to be facilitated by increased hydrophobic interaction between yeast cell surfaces. The deletion in *KNR4* greatly affects cell wall anchorage of the adhesion proteins, but the cell wall responds by changing the polymer cross-linking. This gene can be a potential target for release of adhesins from the cell wall. *GPI7* appears to not result in the release of adhesins from the cell wall to cell culture media.

In future studies, the scope of cell wall biosynthesis associated genes can be increased to assess genes that can result in more adhesins being released. The identification of genes that can result in the release of fungal adhesins is also of medical importance. Understanding the role of proteins and enzymes involved in the anchorage of adhesins is the key in developing antifungal drugs. It is known that fungal species form biofilms that aggressively adhere to abiotic and biotic surfaces.

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