Constitutive over-expression of *FLO1* encoded mannoproteins

by

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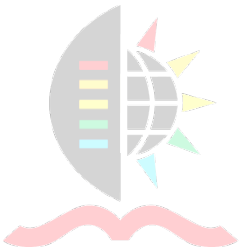

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I, Dr Patrick Govender and Prof AS Gupthar as supervisors of the MSc study hereby consent to the submission of this MSc Thesis.

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SUMMARY

The ability of yeast cell wall-derived mannoproteins (glycoproteins) to positively contribute to oenological properties has been a key factor that stimulates research initiatives into these industrially important glycoproteins. In addition, and from a fundamental research perspective, yeast cell wall glycoproteins are involved in a wide range of biological interactions. To date, and to the best of our knowledge, biochemical insight into the fine molecular structure of these mannoproteins is fairly limited. The amino acid sequences of their protein moieties have been established from structural and functional *in silico* analysis of the genomic sequence of *Saccharomyces cerevisiae* whilst far less structural details are available on the glycosyl moieties of these mannoproteins.

In an attempt to generate significant quantities of cell wall-attached glycoproteins that may be employed for the purpose of biochemical structural analysis, a novel strategy was envisaged that encompasses the genetic manipulation of *S. cerevisiae* strains to over-express and release cell wall-associated glycoproteins into the liquid growth medium. To this end, the *FLO1*-encoded cell wall mannoprotein was overexpressed in haploid *S. cerevisiae* laboratory strains bearing a specific cell wall biosynthesis-related gene deletion that has been previously shown to promote extracellular hyper-secretion of cell wall-based glycoproteins that would otherwise be attached to the cell wall in the feral strain. A polymerase chain reaction (PCR)-based cloning stratagem was employed to yield transgenic strains in which the native *FLO1* open reading frame (ORF) is placed under transcriptional control of the constitutive *PGK1* promoter.

The data reveals that the strategy employed resulted in the expected constitutive expression pattern of the *FLO1* ORF in all transgenic strains created in this study. A modified Helm's flocculation assay was employed to assess flocculation intensities of the three *FLO1*-based transgenic strains. This assay confirmed that the flocculation phenotypes observed were indeed of the Flo1-type in that they were Ca^{2+}-dependent, sensitive to ethylenediaminetetraacetic acid (EDTA) and inhibited in the presence of mannose. Interestingly, the *KNR4* deletion-based transgenic yeast strain displayed a visibly weaker different adhesion behaviour in terms of weaker flocculation intensity as evidenced by noticeably smaller floc aggregate size. In addition a greater quantity of proteins was detected at statistically significant levels in the spent culture medium of the *KNR4* deletion-based transgenic yeast strain. Although yet to be purposefully optimised, the data seemingly suggests that *PGK1* promoter-based mediated overexpression of the FLO1 ORF in a *KNR4* deletion-based genetic background has the potential to release the desired mannoprotein into the culture medium.

This thesis is dedicated to my Family and especially to my mother **Thembelihle Yvonne Ndebele**

BIOGRAPHICAL SKETCH

My name is Lethukuthula Melusi Ngobese. I was born in Durban, St Aidans Hospital on the 13th November 1990. I am a second born among four siblings. I am a resident of C132 Uitvaal area, Wasbank, Ladysmith. I attended Sigweje High School in Ladysmith, where I matriculated in 2007 with Merit. As an undergraduate, I enrolled at the University of KwaZulu-Natal for a Bachelor of Science Degree with Biochemistry and Microbiology as majors. After graduating, my passion for Biochemistry led me to read for an Honours degree and now an MSc degree in this forever interesting discipline.

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PREFACE

This dissertation is presented as a compilation of five chapters.

Chapter 1 **General Introduction and Project Aims**

Chapter 2 **Literature Review**

Chapter 3 **Research Results I**
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ABBREVIATIONS

cm	Centimetres
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FDA	US Food and Drug Administration
G	Grams
H	Hours
IPTG	Isopropyl β-D-thiogalactoside
kDa	Kilodaltons
L	Litres
LB	Luria Broth
M	Molar
mg	Milligrams
mL	Millilitres
mM	Millimolar
ms	Milliseconds
NaCl	Sodium chloride
nm	Nanometers
OD_{600}	Optical density at 600 nm
Abs_{562}	Absorbance at 562nm
Rpm	Revolutions per minute
µL	Microlitres
µg	Micrograms
V	Volts
v/v	Volume/volume
w/v	Weight/volume
Zeo^R/*Sh ble*/$Bleo^R$	Zeocin™ resistance gene

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Chapter 1

INTRODUCTION AND PROJECT AIMS

1. INTRODUCTION AND PROJECT AIMS

1.1 Introduction

Over the past decade, mannoproteins have gained ground as one of the most interesting molecules involved in the improvement of wine making processes and the sensorial properties of wine (Horie & Isono, 2001). Recent research has shown that mannoproteins play a key role in the chemical stabilization of the wine by protecting it against protein haze and tartaric acid instability (Brown*, et al.*, 2007, Caridi, 2006, Dupin*, et al.*, 2000). Other desirable oenological properties of mannoproteins are retention of aroma, reduction of astringency, increase in sweetness and increase in body and mouth feel which is appreciated in red wine (Caridi, 2006).

Another striking property of these mannoproteins is their ability to confer adhesion to abiotic (attach to plastic) and biotic (flocculation) surfaces, the latter being of interest in the fermentation industry. Flocculation is defined as the asexual, reversible and calcium-dependent aggregation of thousands of cell to form flocs that sediment to the bottom of the growth substrate (Bony*, et al.*, 1997). Flo mannoproteins, which confer cell to cell adhesion are referred to as flocculins/adhesins (Guo*, et al.*, 2000). Properly controlled flocculation has the potential to be an environmentally friendly way to remove cells from the final product of the fermentation process. Although the natural functions of these mannoproteins have not been elucidated, it has only been suggested that they play a protective role against harsh environmental conditions, and there is limited information on the structure of these proteins.

Information about the mannoprotein structure comes from partial characterization where, for example, only the glycan moiety is elucidated or the general amino acid sequences of their protein moieties have been established from structural and functional analysis of the genomic sequence. There have been no reports where the intact mannoprotein have been characterized. From the information available, mannoproteins are highly glycosylated proteins containing over 90% sugars, mainly in the form of mannose. They are located in the outermost layer of the yeast cell wall, presenting between 35 and 40% of the cell weight (Klis*, et al.*, 2006). Attempts have been made to extract and characterize the cell wall mannoproteins (Brown*, et al.*, 2007, Van der vaart*, et al.*, 1995), but to the best of our knowledge the structure of an intact mannoprotein is yet to be elucidated.

Gonzalez-Ramos and Gonzalez (2006) identified genetic determinants for mannoprotein release into the fermentation medium by employing laboratory strains of *Saccharomyces cerevisiae* that had deletions in genes implicated directly or indirectly in cell wall biogenesis. These strains showed an increase in mannoprotein release, which correlated with different genetic backgrounds and the increase in mannoprotein release was strain-specific.

In proof of concept exercise herein we attempt to overproduce *FLO1*-encoded mannoprotein into the growth medium by employing laboratory strains of *S. cerevisiae* with deletions in genes involved in the cell wall biogenesis. The strains employed in this work harbour an ORF deletion in either their *KNR4* or *GPI7* genes and have been reported to be hyper-secretors of mannoproteins (Gonzalez-Ramos & Gonzalez, 2006). *KNR4* encodes for a protein required for correct targeting of the Stl2 MAP kinase (cell wall integrity pathway transcriptional activator) to its two known downstream transcriptional targets, Rlm1p and Swi4p (Levin, 2005). As a consequence, the loss of the *KNR4* ORF leads to altered cell wall structure and composition (Gonzalez-Ramos & Gonzalez, 2006). Gpi7p encoded for by *GPI7,* acts indirectly during maintenance of cell wall integrity since it is an enzyme involved in the synthesis of the GPI anchor and deletion of its encoding gene results in inefficient linkage of GPI-anchored proteins to the cell wall (Benachour*, et al.*, 1999). The *FLO1* gene is not expressed in the laboratory strains of *S. cerevisiae* (Liu*, et al.*, 1996), it was deemed an ideal candidate for overexpression in *KNR4* or *GPI7* deletion strains with defective cell wall biogenesis as a simple and effective tool to monitor mannoprotein release. In this regard, a shift in flocculation intensity would be easily gauged via visual and photographic documentation. To this end, a promoter replacement cassette was created to overexpress the *FLO1* ORF. A vector cassette containing the zeocin™ resistance gene as the selection marker and the *PGK1* promoter was employed to constitutively express the *FLO1* gene during all phases of yeast growth.

1.2 Aims of this study

The main aim of the MSc study described within this dissertation is to investigate a biotechnological strategy that would facilitate the direct extracellular release of mannoproteins from transgenic *S. cerevisiae* strains into the culture medium that would ordinarily be attached to the cell wall of their parental wild type strains. The dissertation is divided into five chapters, including this introductory **Chapter 1**.

In **Chapter 2**, a comprehensive literature review encompassing the various cell wall-attached glycoproteins of *S. cerevisiae* and selected aspects of their biosynthesis is presented. Furthermore it also incorporates a current understanding of the promoter and selection marker entities to be employed in the genetic engineering strategy.

In **Chapter 3**, the haploid non-flocculent *S. cerevisiae* BY4741 laboratory yeast strain was employed as a model organism. This aspect involves exploitation of currently available molecular methods to construct a promoter replacement cassette *FLO1p-Zeo^R-PGK1-FLO1p* containing a zeocin™ resistant gene as the selectable marker and a constitutive promoter *PGK1* to drive expression of the transcriptionally silent, yet dominant *FLO1* gene.

In **Chapter 4**, employing the wild type transgenic strain from above the construction of *FLO1* over-expressing BY4741-derived strains bearing a specific deletion in a gene central to cell wall biogenesis is described. To this end, gene deletions corresponding to either *KNR4* or *GPI7* were purposefully identified due to their potential to release of mannoproteins into the growth medium. The adhesion potential of these deletion-based transgenic strains in terms of flocculation intensity was measured against the transgenic wild type parental control strain. In addition the protein content of spent chemically defined culture medium was also monitored as an indirect measure of the ability of transgenic strains to release mannoproteins.

Finally, **Chapter 5** reflects a general conclusion and ideas for future research.

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Chapter 2

LITERATURE REVIEW

Cell wall-attached mannoproteins of *S. cerevisiae*

2 Cell wall-attached mannoproteins of *S. cerevisiae*

2.1 Introduction

S. cerevisiae is employed in a wide range of critical processes for the conversion of biomass into desirable commercial products. This includes the production of bread, alcoholic beverages, biofuel and pharmaceutical products, with the use of yeast in the related fermentation-based industries dating back to more than 3000 years. Its significance in this arena has prompted scientific research of the species and catapulted the development of new strains to fulfil specific requirements of these efficiency-driven bioprocesses. *S. cerevisiae* was the first organism whose entire genome was sequenced and made readily accessible to the greater scientific community (Goffeau*, et al.*, 1996). The genome sequence is readily available and today represents probably the best annotated of all published genomes [*Saccharomyces* Genome Database, www.yeastgenome.org] (Cherry*, et al.*, 1998). In the wine industry alone, it was estimated that more than 300 strains are sold commercially and each strain displays a unique phenotypic trait that differentiates it from the other strains (Bauer*, et al.*, 2010).

However, over the past two decades, significant research efforts have focused on different structural aspects of the yeast cell wall. This was primarily facilitated by some of its constituents positively contributing to both the quality and chemical stability of wines (Gonzalez-Ramos*, et al.*, 2008). In wine-making cell wall derived mannoproteins have particularly been reported to chemically stabilize wines by binding to the heat-sensitive proteins and thereby rendering them soluble. They have also been reported to improve the tartaric salt stability and increase the retention of aroma (Caridi, 2006). Another striking feature of the yeast cell wall that is relevant to the brewing industry, is the ability of the yeast cell wall to confer an asexual aggregation phenomenon termed flocculation (Bauer*, et al.*, 2010).

Flocculation is defined as the asexual, reversible and calcium-dependent aggregation of yeast cells to form flocs which contain thousands of aggregated cells which rapidly settle to the bottom of the fermentation tank. The ability of the yeast cells to flocculate is of considerable importance as it provides a simple cost-effective and environmentally friendly separation of the yeast cells from the final product (Bauer*, et al.*, 2010). The self-clearing of beer at the end of primary fermentation as elicited by flocculation is a highly desirable characteristic of brewing yeast strains. In addition, the other attractive adhesion phenotypes of yeast namely flor (velum) and bioflim-formation could be theoretically beneficial for many other industrial processes (Govender*, et al.*, 2008).

In this review, a current perspective that focuses on the biochemical dynamics of cell wall turnover and genetic regulation of mannoprotein encoding genes involved is presented.

2.2 Cell wall architecture

The yeast cell wall determines the cell shape as well as integrity of the organism during growth and cell division (Aguilar-Uscanga & Francois, 2003), and initially the cell wall was believed to be an inert exoskeleton. However and more recently, the cell wall is now seen as a dynamic structure that is responsive to environmental conditions. Due to its unique biochemistry, structural organization and the absence of most constitutive components from mammalian cells; the yeast cell wall is deemed an attractive target for the development of new antifungal agents.

Electron microscopy employing the negative staining technique reveals that the cell wall is made up of two layers namely: the electron-transparent β-1,3 glucan-chitin complex which forms the major constituent of the inner wall and the electron dense outer layer consisting of mannoproteins (Figure 2.1). The inner and the outer layers of the cell wall are linked via β-1,6 glucans (Lipke & Ovalle, 1998). The inner layer of load-bearing polysaccharides act as a scaffold for the protective outer layer of mannoproteins, that extend outwards to the extracellular medium (Klis*, et al.*, 2006, Klis*, et al.*, 2002). These two layers of the cell wall are densely packed and they limit wall permeability to a range of solutes (Zlotnik*, et al.*, 1984). The percentage and stoichiometry of the components of the cell wall are not fixed. They differ from yeast strain to strain and undergo compositional flux in response to epigenetic stress and shifting culture or environmental conditions (Klis*, et al.*, 2002, Lesage & Bussey, 2006, Lipke & Ovalle, 1998).

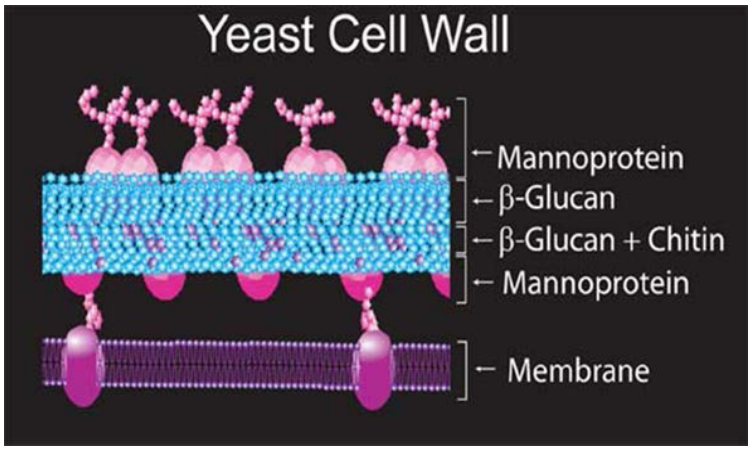

Figure 2.1: Composition and structure of the cell wall of *S. cerevisiae*. The cell wall, which is located outside the plasma membrane, consists of two layers. The inner layer provides cell wall strength and is made of β-1,3 and β-1,6 glucan that is complexed to chitin. The outer layer consists of mannoproteins 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 (adapted from Sigma-Aldrich, Biofiles, 2009).

The cell wall makes up approximately 25% of the total cell dry weight and contains three main groups of polysaccharides as follows (Table 2.1):

(i) β-glucan which is a polymer of glucose that is subdivided into two types based on the glycosidic linkages: β-1,3 glucan and β-1,6 glucan.

(ii) Chitin a polymer of N-acetyl glucosamine monomers.

(iii) Mannoprotein, a polymer of mannose.

Table 2.1 The composition of *S. cerevisiae* cell wall and site of synthesis of each polysaccharide components (adapted from Klis*, et al.* (2002)

Polysaccharide	% dry weight	Site of synthesis
Mannoprotein	35-40	Endoplasmic reticulum (Secretory pathway)
β-1,3 glucan	50-55	Plasma membrane
β-1,6 glucan	5-10	Plasma membrane
Chitin	1-2	Plasma membrane

2.2.1 Glucans and chitin

Based on the type of glycosidic linkage β-glucans can be divided into two subtypes, namely, β-1, 3 and β-1, 6 glucans. The biosynthesis of β-1, 3 glucans is a cell cycle controlled process that is associated with the participation of two β-1,3 synthases namely Fks1p and Fks2p. Both synthases are associated with a Rho1p regulatory subunit. Fks1p is activated during normal cell growth whilst Fks2p is activated in response to physiological and nutritional (carbon source) stress factors (Klis*, et al.*, 2002, Levin, 2005, Lipke & Ovalle, 1998). β-1, 3 glucan consists of long chains of glucose that are linked together via the β-1, 3 linkages and they represent approximately 85% of the total β-glucans (Aguilar-Uscanga & Francois, 2003). The β-1, 3-glucans are initially generated as long linear chains of glucans and they are modified post-synthesis to provide mechanical strength which resides in its hollow, helical structural conformation (Klis*, et al.*, 2006, Klis*, et al.*, 2002). The deletion of either one of the synthases results in cell wall damage and hence the activation of a combination of cell responses including transient depolarization of the actin cytoskeleton as well as the cell wall integrity pathway thus resulting in the increased amount of chitin. Another enzyme that plays a role in the synthesis and structural organisation of β-1,3 glucans is Gas1p, an endotransglycolase (Klis*, et al.*, 2002).

The β-1, 6 glucans are composed of shorter chains of glucose residues that are β-1,6 linked. They constitute approximately 15% of the total cell wall β-glucan component (Aguilar-Uscanga & Francois, 2003, Klis*, et al.*, 2006). They function in connecting the inner layer to glycosylphosphatidylinositol (GPI)-dependent mannoproteins of the outer layer of the cell wall. They also function as an acceptor site for chitin integration (Klis*, et al.*, 2002). The current understanding of the biosynthesis of β-1, 6 glucan is unclear and fairly limited.

Chitin synthesis and deposition are strictly cell cycle-dependent as its occurrence increases during cell division and is clearly evident in bud scars. It usually occurs after cytokinesis at low concentrations in the lateral walls. Increased chitin in *S. cerevisiae* is activated as a salvage mechanism to compensate for the loss of the other polymers such as the glucans (Aguilar-Uscanga & Francois, 2003). It plays a major role in preventing the neck from widening during septum formation in cell division (Klis*, et al.*, 2002). The synthesis of chitin makes use of three synthases namely Chs 1 p, Chs 2 p and Chs 3 p which are active in the plasma membrane.

2.2.2 Cell wall proteins

Cell wall proteins are highly glycosylated containing over 90% sugars, mainly in the form of mannose. They are located in the outermost layer of the yeast cell wall, contributing between 35 and 40% of the dry cell weight (Klis*, et al.*, 2002, Quiros*, et al.*, 2010). The mannoproteins in the cell wall act as structural components, giving the cell wall its interactive properties and being partially responsible for its permeability (Klis*, et al.*, 2002). Due to their high sugar content, they behave more like polysaccharides rather than proteins (Quiros*, et al.*, 2010). The glycan portion of the mannoproteins is composed of mannose, with some neutral oligosaccharides that contain N-acetyl glucosamine and acidic sugars which contain mannosylphosphate (Aguilar-Uscanga & Francois, 2003, Klis*, et al.*, 2006). Cell wall mannoproteins are covalently linked to the β-1,3 glucan either directly or indirectly, through a β1,6 glucan linker and most of them are attached to the cell wall through a glycosylphosphatidylinositol (GPI) anchor remnant (Klis*, et al.*, 2002). Most adhesins including *FLO* gene encoded adhesins are located in this region of the cell wall.

There are two main classes of cell wall proteins that can be distinguished on the basis of their covalent interaction with cell wall polysaccharides viz., (i) GPI-anchored proteins (GPI-CWP) and (ii) proteins with internal repeats (Pir-CWPs). This review will focus on the GPI-anchored proteins.

2.2.2.1 GPI-anchored proteins

The modular structural organisation of the greater majority of GPI-anchored proteins consists of three domains. The structure includes an N-terminal domain which contains a sugar binding site that interacts with mannose residues of mannan located on neighbouring cells thereby conferring the flocculation phenotype (Kobayashi, *et al.*, 1998). The central domain bears *O*-linked oligosaccharides (Figure 2.2) that are attached to the hydroxyl group of serine or threonine residues and consist of short linear chains consisting of only one to five mannose residues, with the first two residues being α(1,2)-linked and subsequent ones α(1,3)-linked (Herscovics & Orlean, 1993). These oligosaccharide moieties resemble short rigid rod-like stalks that elevate protein moiety from cell wall surfaces (Jentoft, 1990), presumably thereby lending them greater access to the extracellular environment. Lastly, the C-terminal domain harbours the GPI anchor that is required for attachment. These proteins receive a GPI anchor that initially anchors them into the outer face of the plasma membrane and if they are destined for cell wall construction, the anchor is trimmed and the remainder of the mature GPI remnant is attached to β-1,6 glucan component of the cell wall (Figure 2.2 and 2.4), which in turn is attached to the β-1,3 glucan network. The GPI-linked mannoproteins that play an enzymatic role are usually trapped in the plasma membrane. *In silico* studies performed by Caro, *et al.* (1997) revealed that there are between 60-70 potential GPI-anchored proteins in *S. cerevisiae* and their biosynthesis follows a secretory pathway whereby the proteins are N-glycosylated, O-mannosylated and modified by addition of a GPI-anchor.

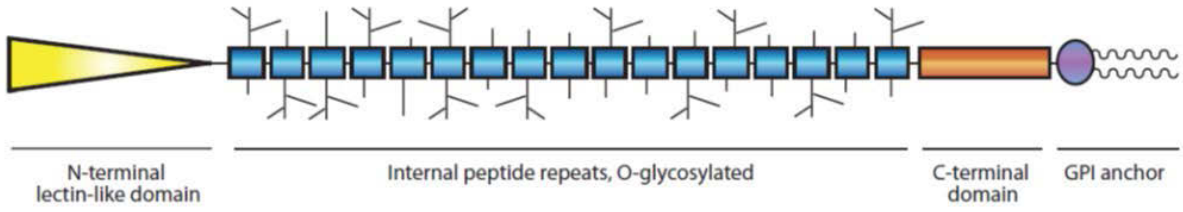

Figure 2.2 Schematic organization of GPI-linked protein domains. These proteins are comprised of three domains, the N-terminal domain which is the carbohydrate binding site, the serine/ threonine rich central domain and the carboxyl-terminal domain containing the site for covalent attachment of the GPI anchor.

2.3 Post-translational modification and transport of mannoproteins

Glycosylation of proteins is essential for cell wall integrity and survival of the cell. The process contributes to the correct folding and functioning of the mannoproteins. Glycosylation is a post-translational modification in the event proteins are targeted for secretion or incorporation into the cell wall (Aguilar-Uscanga & Francois, 2003). The glycosylation

process starts in the endoplasmic reticulum (ER) where the first mannosyl group is attached to the protein. Further modification and elongation of the structure takes place in the Golgi apparatus (Klis, *et al.*, 2006). Mannosyl groups can be modified further by covalent addition of mannosylphosphate which imparts an overall nett negative charge to mannoproteins (Klis, *et al.*, 2006).

2.3.1 N-linked glycosylation

During *N*-linked glycosylation, the mannosyl group is attached to the amide group of an asparagine (Asn) residue. There is a specific amino acid sequence at which N-linked glycosylation occurs, namely the sequence/motif Asn-Xaa-Ser/Thr (Xaa denotes any amino acid). The *N*-linked glycosyl moieties are extensively branched and variable in length (figure 2.3). In the ER, the core of the branches is formed with 9-13 mannose residues. These core entities can be further modified or extended by addition of monomers of mannose or branches of mannose chains in the Golgi apparatus (Dean, 1999, Herscovics & Orlean, 1993). Extensive glycosylation in the Golgi apparatus yields an α-1,6-linked mannose backbone chain of up to 50 mannose residues extending from the core and to which are attached shorter chains of α-1,2-linked mannose that ends in α-1,3-linked mannose residues. This forms a highly branched structure that contains approximately 200 mannose residues. The regulatory mechanism of the glycosylation is poorly understood.

2.3.2 *O*-linked mannosylation

In contrast to the N-linked glycosylation, the *O*-linked mannosylation consists of one to five mannose residues attached to the hydroxyl group of either serine or threonine. The first two residues being attached via an α-1,2-linkage and subsequent mannose residues are α-1,3-linked (Dean, 1999, Herscovics & Orlean, 1993). These mannose residues are linear in arrangement and they resemble short, rigid, rod-like stalks that elevate the protein domain from the membrane or cell wall surface (Kapteyn, *et al.*, 1999). The process of *O*-mannosylation takes place in the endoplasmic reticulum (ER) and further modification proceeds in the Golgi apparatus, where the attachment of mannosylphosphate at specific places also take place to give the mannoproteins with a negative charge (Kapteyn, *et al.*, 1999). The small oligosaccharides which are *O*-linked to mannoproteins also seem to contribute to the stability of the cell wall, since mutants defective in protein *O*-mannosylation are osmolabile (Mrsa, *et al.*, 1999).

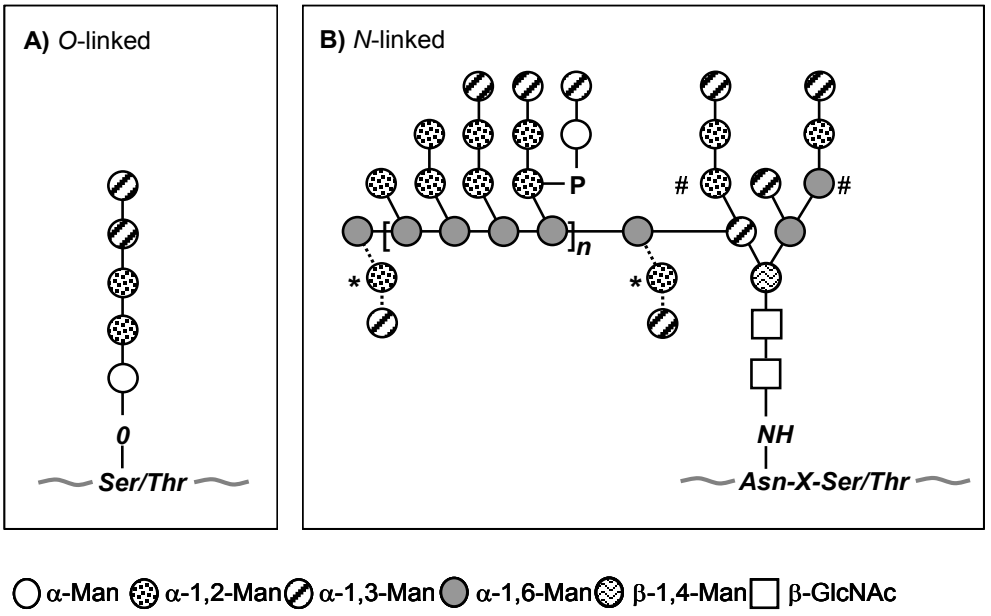

Figure 2.3 Glycosylation of cell wall proteins in *S. cerevisiae.* A) *0*-linked mannosylation, short mannosyl side-chains linked to hydroxyl group of serine or threonine residues. The *0*-chains can vary in length from one to five mannose residues. B) *N*-linked glycosylation, carbohydrate side chains linked to amide group of asparagine residues. The number of repeating units (*n*) varies and can be as high as 15. ∗ Denotes alternative positions of the α-1,2-linked mannose. This addition is proposed to prevent elongation and is not found in the cores to which outer chains are added. # Denotes additional sites for phosphorylation. Man, Mannose; GlcNAc, *N*-acetylglucosamine; P, phosphate (adapted from Herscovics & Orlean, 1993).

2.3.3 Attachment of mannoprotein to the cell wall

Glycosylphosphatidylinositol is a structurally complex glycophospholipid that facilitates the anchorage of GPI-linked proteins into the cell wall. It is added post-translationally to the C-terminal end of secretory proteins (Pittet & Conzelmann, 2007). The synthesis and addition of the GPI-anchor to synthesized proteins takes place on the ER. At least 21 proteins play a role in the synthesis and attachment of the GPI precursor (Orlean, 2012). The synthesis begins at the cytoplasmic face of the ER where a *N*-acetylglucosamine (GlcNAc) residue is transferred from uridine diphosphate (UDP)-GlcNAc to phosphatidylinositol (PI) and a complex of six proteins is involved in the transfer process (Orlean, 2012). The GlcNAc-PI undergoes deacetylation catalysed by Gpi12p. The deacetylated precursor is subsequently translocated to

the luminal side of the ER where it undergoes an acylation reaction on its 2'-OH. This acylation plays a protective role as it renders the glycolipid resistant to PI- specific phospholipase C and also prevents this GPI precursor from being translocated back to the cytoplasmic side. The GlcN-(acyl)PI is extended with four mannose residues and the first three mannose residues are concurrently modified with ethanolamine phosphate (Etn-P). There are three different Etn-P transferases that sequentially add Etn-P starting at the first mannose residue and proceeding to the third residue and they are Mcd4p, Gpi7p and Gpi13p respectively. The nascent GPI anchor can now be transferred to a protein.

A GPI anchor will be attached to a target protein on condition it contains two hydrophobic signal sequences of which one is located in the amino terminus and other at the carboxyl terminus (Caro*, et al.*, 1997). The amino terminus signal sequence directs the protein through a secretory pathway to the ER and is a precursor for the entry of the protein (Caro*, et al.*, 1998). The carboxyl terminus signal sequence is composed of a cleavage/attachment domain (ω site), a spacer domain (8 to 12 amino acids) and a terminal hydrophobic domain (at least 11 amino acids). This signal sequence is cleaved off at the ω site and replaced with a pre-formed GPI-anchor via a putative GPI-anchor transamidase complex whereby the amino group of Etn-P of the third mannose residue acts as a nucleophile (Caro*, et al.*, 1997).

Following addition of the GPI anchor, the modified proteins proceed through the secretory pathway from the ER through the Golgi apparatus undergoing further modification by means of the aforementioned *O*- and *N*-glycosylation events. The GPI proteins are transported from the late Golgi apparatus to the surface in several different types of vesicles. Some GPI-mannoproteins may be incorporated into the plasma membrane via their hydrophobic fatty acid tails. However, most GPI proteins in yeast, do not remain anchored in the plasma membrane (Caro*, et al.*, 1997). The GPI anchor at the plasma membrane is further processed to generate a lipid-less remnant of the original GPI-mannoprotein (GPIr). The GPIr-protein is then covalently linked to the outer β-1, 6 glucan polymers of the cell wall via its glycosyl moiety (Figure 2.4). The molecular mechanism for the attachment of the mannoproteins is yet to be fully elucidated, but Hamada*, et al.* (1998) speculated that the cell wall mannoproteins possess a sequence that enables their incorporation into the cell wall.

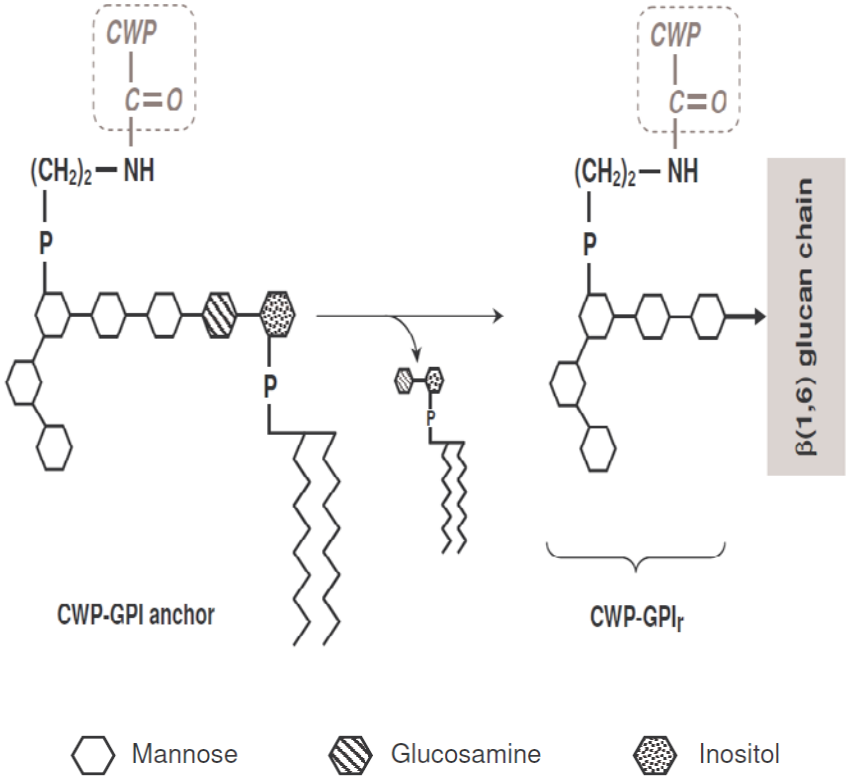

Figure 2.4. Structure of a yeast glycosylphosphatidylinositol (GPI) anchor and a proposed model for the attachment of cell wall proteins to the β-glucan inner skeletal network. Certain classes of outer-layer mannoproteins are post-translationally modified by addition of a GPI anchor. After secretion of the GPI-anchored mannoprotein to the outer layer of the plasma membrane, the anchor is internally cleaved and the lipid-less remnant forms a glycosidic linkage via the reducing end of its mannosyl moiety (indicated by arrow in bold) with the non-reducing terminus of a β-1,6 glucan chain that is intrinsic to the outer fibrillar layer of the cell wall. This complex typifies the interaction of *FLO1*-encoded proteins with the cell wall. CWP, cell wall protein; GPIr, lipidless remnant of a GPI-anchor; P, phosphate; adapted from Lipke and Ovalle (1998).

2.4 Cell wall integrity pathway

The main pathway employed in yeast for cell wall biogenesis and for the prevention of cell lysis is the PKC-SLT2 MAP kinase/cell wall integrity pathway. Activation of the cell wall integrity pathway is in response to cell stress and it results amongst other things in a massive deposition of chitin in the cell.

This pathway consists of a family of cell surface sensor proteins, Rho1p, protein kinase C (PKC) and a downstream mitogen-activated protein kinase (MAPK) cascade (Levin, 2005). Activation of the cell integrity pathway is initiated by signals transmitted from the cell wall sensor proteins to Rom 2 p, which stimulates formation of the GTP-bound activated form of

Rho1p and the subsequent activation of Pkc1p. The enzyme Pkc1p activates a cascade of phosphorylation reactions involving the MAP kinase Bck1p complex, the redundant MAP kinase Mkk1p, Mkk2p and the MAPK Slt2p (Levin, 2005). Signalling through the MAPK cascade results in dual phosphorylation and activation of Slt2p. This event exerts its effect on cell wall biogenesis via two transcription factors, Rlm1p and the Swi4-Swi6 cell cycle box-binding factor complex. These transcriptional factors activate many genes involved in cell wall biogenesis among which is *FKS2*. A constitutively activated PKC-Slt2- cell integrity pathway in cell wall mutants *fks1Δ* and *gas1Δ* leads to increased *FKS2* expression and a Slt2p-dependent increase in thermal resistance (Zhong & Ye, 2007). Four protein kinases are involved in a MAPK cascade and the last is Mpk1/Stl2. The Mpk1/Stl2 MAPK is phosphorylated and it enters the nucleus and activates the transcription of genes involved in cell wall remodelling (Gonzalez-Ramos*, et al.*, 2008). The induction occurs through activation of the transcription factor Rlm1p via the activation of Swi4p. The Knr4p is necessary for correct targeting of the Stl2 MAP kinase to its two known transcriptional targets, Rlm1p and Swi4p (Gonzalez-Ramos*, et al.*, 2008, Levin, 2005). It participates in the coordination of cell wall synthesis with bud emergence. Rlm1p and Swi4p up regulate genes involved in cell wall biogenesis.

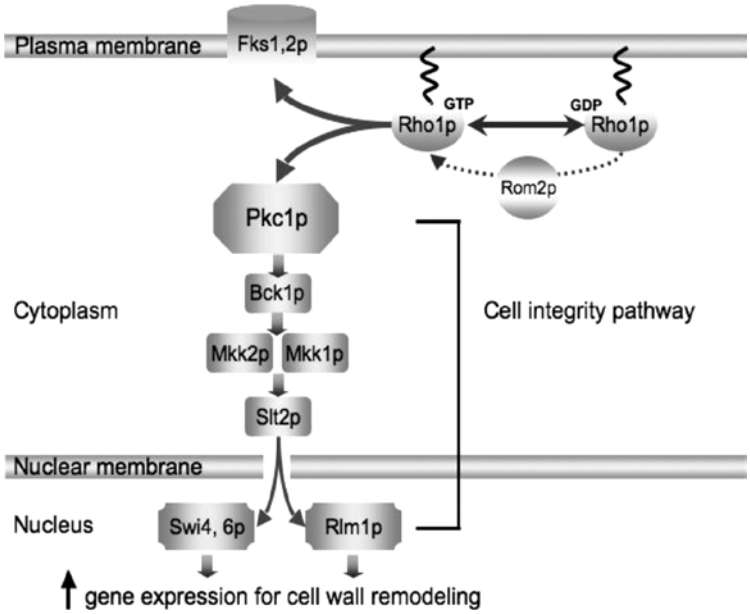

Figure 2.5 Glucan synthesis and cell wall integrity pathway (adapted from Zhong and Ye (2007).

2.5 Identification of over-secreting strains

There are about 1200 genes that directly or indirectly affect the biogenesis of cell walls (Gonzalez-Ramos, *et al.*, 2008). 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. The importance of these proteins to maintain cell wall integrity was investigated by deleting their ORFs. The cell wall integrity of these mutant strains is evaluated by employing cell wall pertubants such as Calcofluor white and Congo red.

The deletion of *KNR4* ORF leads to changes in the cell wall structure and composition that results in increased concentration of chitin, reduction of β-glucans by 50% and the reduction of the β-glucan synthase activity. In contrast to their parental strains, Δ*knr4* mutants exhibit hypersensitivity to sodium dodecyl sulphate, Congo red, Calcofluor white and their growth is arrested with small buds at elevated temperature (Gonzalez-Ramos, *et al.*, 2008). It was observed by (Gonzalez-Ramos & Gonzalez, 2006) that Δ*knr4* mutants that were derived from BY4741 and BY4743 laboratory strains released increased amount of mannoproteins.

The protein Gpi7p, encoded for by *GPI7* is also involved in the maintenance of the cell wall integrity. This protein acts indirectly during maintenance of cell wall integrity since it is an enzyme involved in the synthesis of the GPI-anchor (Gonzalez-Ramos & Gonzalez, 2006). The transferase Gpi7p is responsible for the addition of Etn-P to the second mannose of the pre-formed GPI-anchor (Benachour, *et al.*, 1999). Deletion of the *GPI7* ORF is not lethal and results in thermo-sensitive cells and also results in hypersensitivity to Calcofluor white (Benachour, *et al.*, 1999). The mutation in this gene also results in release of cell surface anchored proteins (Fujita, *et al*., 2004). Gonzalez-Ramos and Gonzalez (2006) reported increased mannoprotein release of up to nine times in BY4741-derived Δ*knr4* mutants when compared to their parental strain. However and seemingly contradictory research studies by an independent research group showed in a different but related genetic background that BY4743-derived Δ*knr4* mutants were incapable of increased mannoprotein release (Gonzalez, *et al.*, 2010).

Bearing in mind the hypothesis of this research study (Chapter 1) and in view of the above, yeast strains bearing a specific deletion in either *GPI7* or *KNR4* were selected as suitable candidates for the overexpression *FLO1*-encoded mannoprotein so as to evaluate their potential to hyper-secrete this mannoprotein.

2.6 Flocculation

Flocculation is a reversible, asexual, calcium-dependent aggregation of thousands of yeast cells to form clumps (flocs) that rapidly sediment to the bottom of the growth substrate (Figure 2.6) (Bony, *et al.*, 1997). The flocculation mechanism is not fully elucidated but the widely accepted mechanism based on evidence is the lectin hypothesis as revised by Teunissen and Steensma (1995). The hypothesis postulates that in the presence of calcium, flocculins on the cell wall are able to bind highly branched mannose from the adjacent cells therefore initiating flocculation. The role of Ca^{2+} in this mechanism would be to maintain the lectin in an active conformation rather than playing a central role in flocculation (Domingues, *et al.*, 2000).

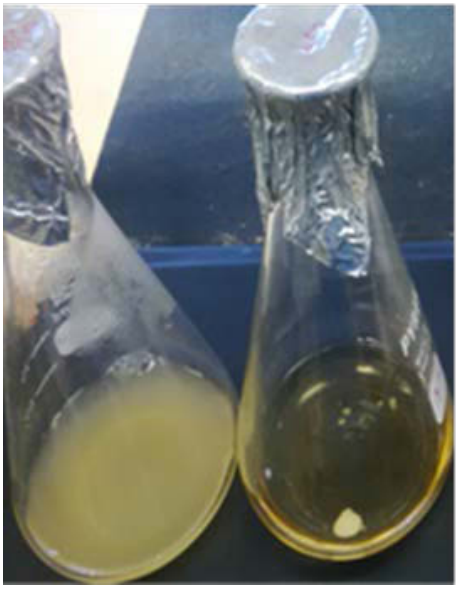

Figure 2.6 Shows the laboratory cultures of both non-flocculent (left) and flocculent cells (right, this study).

It is proposed that flocculation plays an important protective role in that cells which are contained within flocs can be shielded from harsh environmental conditions so as enhance survival when more appropriate conditions prevail. The outer most cells of the flocs offers protection to the cells enclosed within flocs. The cells located just beneath the surface of aggregates lyse and provide nutrients for the other cells in the flocs (Goossens, *et al.*, 2011, Verstrepen, *et al.*, 2003). Flocculation is inhibited by mannose in the growth medium, presumably because the free mannose occupies the N-terminal sugar binding site of flocculins so that they can no longer bind mannan-based mannose residues located on wall of adjacent cells. Based on their affinity for sugar, flocculent yeast strains can be divided into three phenotypes (Masy, *et al.*, 1992, Stratford & Assinder, 1991) as described in following section.

2.6.1 Flocculation phenotypes

2.6.1.1 Flo1 phenotype

This phenotype is most prevalent in the laboratory yeast strains and is associated with the expression of the *FLO1*, *FLO5*- and *FLO9*-encoded mannoproteins (Gonzalez-Ramos, *et al.*, 2008, Van Mulders, *et al.*, 2009). The Flo1 phenotype of wild type laboratory strans is associated with constitutive expression of dominant *FLO* gene concerned. This flocculent phenotype exclusively inhibited by the presence of mannose in the growth medium and (Masy, *et al.*, 1992) categorised this phenotype as Mannose Sensitive (MS). It has been suggested that the amino acid tryptophan in the position 228 plays a major role in the sugar recognition. It was proposed that the tryptophan 228 in Flo1p recognises the C2-hydroxy group of mannose but does not recognise the C2-hydroxyl group of glucose (Goossens, *et al.*, 2011).

2.6.1.2 NewFlo phenotype

The NewFlo flocculation phenotype, also known as GMS, is generally characteristic of brewing yeast strains and is inhibited by the presence mannose, glucose, maltose and sucrose in the growth medium. As a consequence it is deemed ideal for the process of brewing since flocculation is only initiated at the end of primary fermentation when sugars are depleted. Although this phenotype is of unknown genotype it has been associated with the *FLO1* homologues Lg-*FLO1* (Kobayashi, *et al.*, 1998) and *FLONS* encoded flocculins (Liu, *et al.*, 2007). In contrast to Flo1p, Lg-Flo1p possesses a leucine residue instead of tryptophan at position 228. It has also been proposed that either glycine or arginine at position 226 may also contribute to the NewFlo phenotype sugar sensitivity profile (Goossens, *et al.*, 2011).

2.6.1.3 Mannose insensitive (MI) phenotype

This sugar insensitive flocculent phenotype is characterized by binding of adhesins to peptides instead of sugar chains. Unlike Flo1 and NewFlo, this phenotype is not inhibited by the presence of mannose. Interestingly and unlike the sugar sensitive phenotypes (MS and GMS), this phenotype occurs independently of calcium ions (Verstrepen & Klis, 2006, Zhao & Bai, 2009). Transgenic *FLO11* overexpressing strains have been associated with this phenotype, which also promotes binding to abiotic surfaces (Govender, *et al.*, 2011).

2.7 Genetics of flocculation

The laboratory strain S288C has five dominant flocculation genes, namely *FLO1, FLO5, FLO9, FLO10* and *FLO11* (Guo, et al., 2000, Teunissen & Steensma, 1995). The *FLO1, 5, 9* and *10* genes are located 10-40 kb adjacent to the telomeres and are transcriptionally silent in laboratory yeast strains (Figure 2.7). On the contrary *FLO11* is neither sub-telomeric nor centromeric and is expressed in laboratory strains (Halme*, et al.*, 2004, Teunissen*, et al.*, 1993, Verstrepen*, et al.*, 2003). Each of the *FLO* genes when expressed induces slightly different flocculation phenotypes in terms of their intensity. For instance, *FLO1* expression results in strong flocculation whilst *FLO5, FLO9* and *FLO10* expression result in moderate to weak flocculation (Verstrepen & Fink, 2009). *FLO11* differs from the other *FLO* genes due to *FLO11*-encoded protein's stronger binding affinity to abiotic material (Guo*, et al.*, 2000, Verstrepen*, et al.*, 2003). The *FLO10*-encoded flocculin seems to overlap the phenotypes of both Flo1p and Flo11p since it promotes both cell to cell adhesion and pseudohyphal filamentation.

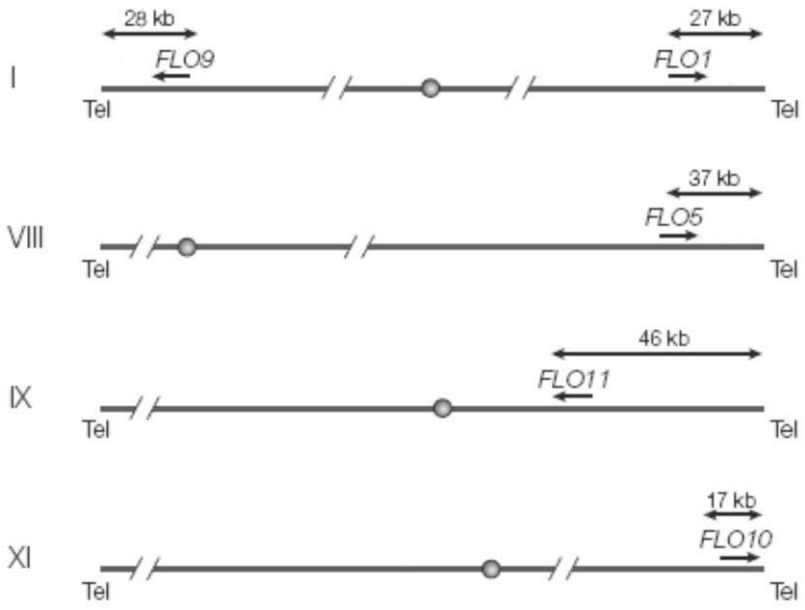

Figure 2.7 Chromosomal location of the *FLO* genes in *S. cerevisiae* strain S288C. The Roman numerals on the left represents the chromosome number, dots represent the centromere whilst Tel denotes the telomeres. (adapted fromVerstrepen & Klis, 2006).

The *FLO* ORFs contain conserved DNA motifs of approximately 100 nucleotides that are repeated 10-20 times. These regions containing tandem repeats correspond to the central domain of *FLO1*, *FLO5* and *FLO9* encoded flocculins (Figure 2.8). Comparative studies of six different *S. cerevisiae* strains showed that the size of these tandem repeats varied from strain to strain and were inherently unstable. The sequence similarities between these tandem repeats lead to frequent homologous recombination events which yield highly variable adhesion phenotypes that is proposed to enable yeast to adapt their adhesion properties to suit new environments (Gonzalez-Ramos*, et al.*, 2008, Van Mulders*, et al.*, 2009, Verstrepen*, et al.*, 2005).

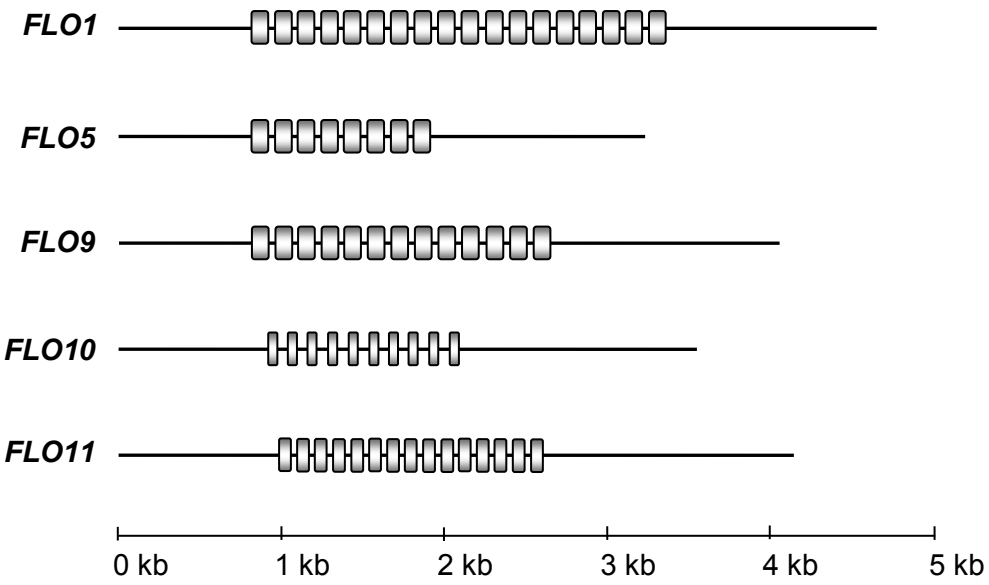

Figure 2.8 The *S. cerevisiae FLO* genes contain conserved intragenic tandem repeats. These repeats were shown to vary in size in six different strains of *S. cerevisiae*. It should be noted that the *FLO1*, *FLO5* and *FLO9* share the same repeat sequences. (adapted from Verstrepen*, et al.*, 2005).

As the cells divide or undergo DNA replication recombination events can occur either within the same gene or between adjacent genes to generate new alleles in a short space of time (Figure 2.9). The recombination process can yield *FLO* genes with central domains that are either increased or reduced in length. Longer central domains with an increased number of tandem repeats generates a stronger flocculation phenotype and *vice versa* (Goossens*, et al.*, 2011).

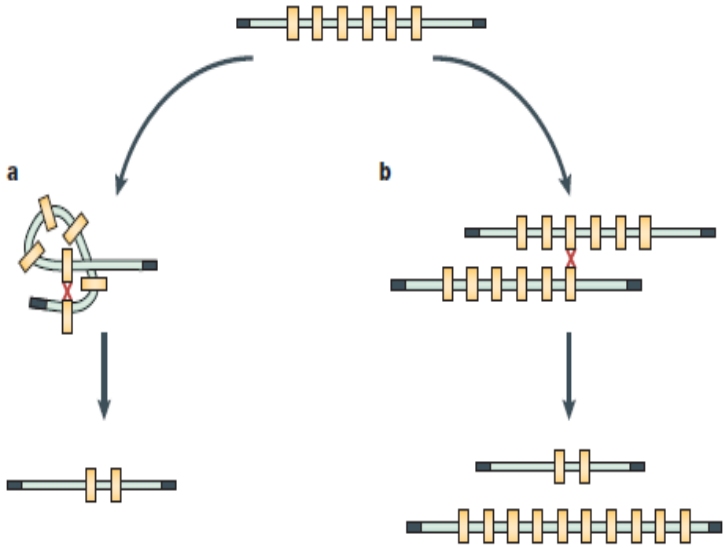

Figure 2.9 Recombination between repeated DNA motifs in flocculin genes generates new alleles. Boxes indicate nucleotide motifs; the regions shown in black at the ends of the chromosomes represent telomeres. **(a)** Intrachromosomal pairing coupled with a recombination event can generate a short gene with a reduced number of repeats. **(b)** An unequal crossover between two identical *FLO* genes on homologous chromosomes 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 a long and a short form of a *FLO* gene (Verstrepen*, et al.*, 2005).

2.7.1 Regulation of *FLO* genes

The expression of *FLO* gene-encoded adhesins is strongly regulated in response to nutrient availability and other stress factors in the environment of the cells. Studies of *FLO* gene regulation have been done intensively on *FLO11*. These studies showed that *S. cerevisiae* have evolved mechanisms to sense and respond to environmental signals by activating development switches that result in a coordinated change in cell physiology and morphology (Bauer*, et al.*, 2010, Verstrepen & Klis, 2006).

Three well-characterised signalling pathways are responsible for transmitting the stimuli of nutritional status in the environment to the promoter of *FLO11* which include the Ras-cAMP-PKA, MAP kinase invasive growth and the main glucose repression pathways (Figure 2.10), (recently reviewed in Verstrepen & Klis, 2006). The aforementioned pathways regulate *FLO11* transcription via a set of transcriptional activators and repressors, which include Flo8p (Kobayashi*, et al.*, 1999), Ste12p and Tec1p (Bester*, et al.*, 2006), and Mss11p (van Dyk*, et al.*, 2005). Data suggest that the above mentioned signalling pathways and regulatory proteins converge on the *FLO11* promoter to regulate primarily the *FLO11*-generated phenotypes of invasive growth and pseudohyphal differentiation (Verstrepen & Klis, 2006).

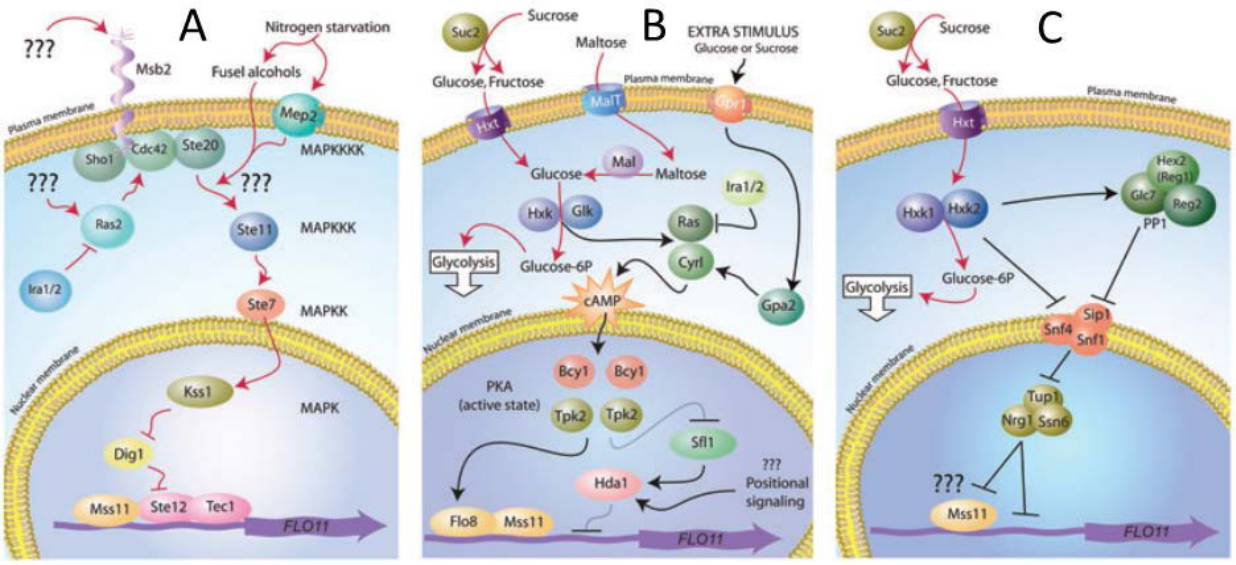

Figure 2.10 Three signalling cascades regulate *FLO11* expression in response to nutrient starvation; they all converge in the promoter region. (A) The MAP kinase invasive growth pathway. (B) Ras-cAMP-PKA pathway. (C) The main glucose repression pathway (Verstrepen & Klis, 2006).

Limited information has been presented on the genetic regulation of the other *FLO* genes. However, Verstrepen and Klis (2006) suggested that their regulation may follow similar strategies. This suggestion was substantiated by previously reported data that *FLO1* and *FLO11* are regulated by the same transcription activators Flo8p (Kobayashi, *et al.*, 1999) and Mss11p (Bester, *et al.*, 2006) and the fact that both *FLO* genes are regulated in response to nutrient availability.

Data also suggest that *FLO* genes are not only under transcriptional regulation but they are also under promoter specific epigenetic control that allows gene expression to be switched from silent to active mode or *vice versa*. This epigenetic influence on *FLO11* is a heritable trait that is regulated by an histone deacetylase (Halme, *et al.*, 2004).

2.8 Genetic manipulation of *S. cerevisiae* for mannoprotein overexpression

Genetic-based research studies pertaining to flocculation has been primarily driven by the importance of this phenotype to the brewing industry and the attractive potential to create transgenic strains with industrially desirable flocculation traits. In attempts to genetically control the onset of flocculation in both laboratory and industrial yeast strains, the native promoter of specific *FLO* genes (*FLO1*, *FLO5* or *FLO9)* was replaced with an inducible promoter which included the *ADH2* and *HSP30* promoters (Cunha, *et al.*, 2006, Govender, *et al.*, 2010, Govender, *et al.*, 2008, Verstrepen, *et al.*, 2003). These transgenic strains were observed to exclusively flocculate on entry to the stationary phase of growth and once primary fermentation was completed. The advantages of this genetic manipulation strategy

over the more traditional plasmid-based cloning approach include; stable expression, avoidance of sub-cloning of the *FLO* genes and lack of plasmid-borne expression artefacts (Govender, *et al.*, 2008). In our endeavour to design a transgenic yeast strain that would be capable of extracellularly releasing an increased quantity of a desired mannoprotein, the constitutive *PGK1* promoter was employed to drive *FLO1* expression. It was deemed that constitutive expression would yield more *FLO1*-encoded mannoproteins in comparison to the employment of a growth phase-specific promoter sequence.

2.9 Conclusion

Up until now, a multitude (thousands) of scientific research and review papers covering all aspects of the structural architecture and biosynthesis of the cells wall of *S. cerevisiae* have been reported (Figure 2.11).

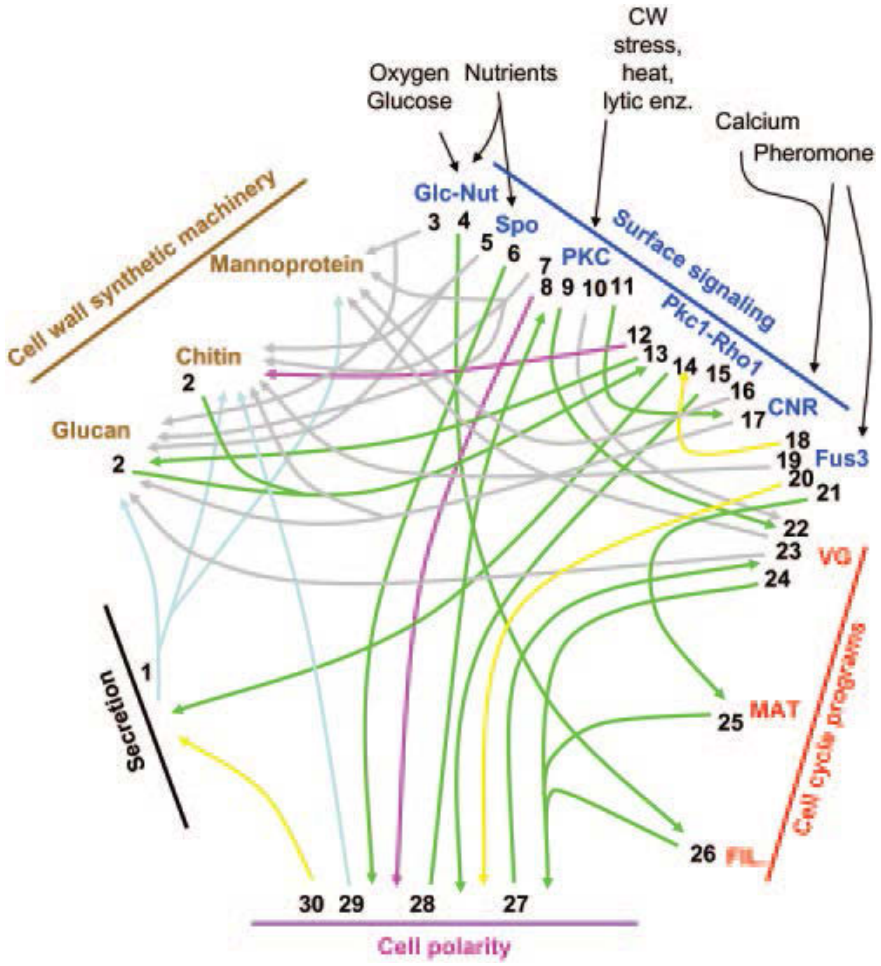

Figure 2.11 Integrated view of regulatory pathways involved in cell wall biology. Five levels of regulation are represented (cell wall synthetic machinery, brown; surface signaling, blue; cell cycle, red; cell polarity, purple; secretion/endocytosis, black. (extracted from Lesage & Bussey, 2006).

Unlike the initial proposal of earlier researchers in this field; that the cell wall is a static entity that undergoes limited or no change during the life cycle of a cell; it has increasingly become clearly evident that cell wall assembly is indeed a dynamic process that accommodates for constant change in the structural organization and composition. This scenario enables the cell to respond and adapt to ever emerging environmental stresses and triggers. However considering the large volume of studies, the regulation of these events is yet to be fully understood. As such research continuously strives towards a more comprehensive understanding of the yeast cell wall.

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Chapter 3

RESEARCH RESULTS I

Construction of a promoter replacement cassette for constitutive gene expression in *S. cerevisiae*

Construction of a promoter replacement cassette for constitutive gene expression in *S. cerevisiae*

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3.1 Abstract

Adhesins encoded by the dominant *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, understanding of the fine molecular structure of these glycoproteins is fairly limited. Generally the amino acid sequences of their protein moieties have been predicted from structural and functional analysis of the genomic sequence of SC288C laboratory yeast strain. In addition, fairly limited information is available on the glycosyl moieties of these adhesins. The majority of *FLO* genes are known not to be expressed in laboratory yeast strains. Given this backdrop it is envisaged that overexpression of a silent *FLO* gene will generate the desired adhesin in sufficient quantities which may then be isolated and purified for the purpose of their biochemical structural analysis. To this end the *PGK1* constitutive promoter is engineered into a promoter replacement vector cassette containing zeocin™ resistant marker gene to drive *FLO1* ORF expression in BY4741 laboratory strain that is isogenic to the SC288C strain. It was observed that *FLO1* overexpressing transgenic yeast strains visibly displayed a flocculent phenotype when cultivated in nutrient rich YPD liquid growth medium. Integration of the promoter replacement cassette was verified by a PCR strategy that employed either heterologous or homologous primer sets. In addition, the flocculation displayed by the transgenic strain was characterized as a Flo1 phenotype. This study demonstrates that the *PGK1* promoter is suitable for constitutive overexpression of a dominant *FLO* gene.

3.2 Introduction

Flocculation of cells is the asexual, calcium-dependent and reversible aggregation of cells to form flocs containing thousands of cells and it renders easy separation of the cell aggregates from the liquid medium by sedimentation (Bony*, et al.*, 1997). A well-known glycoprotein involved in flocculation is a product of the *FLO1* gene. The *FLO1* gene encodes a cell wall glycosylphosphatidylinositol (GPI) - linked glycoprotein that is related to Flo5p, Flo9p, Flo10p and Flo11p/Muc1p. These flocculins possess an N-terminal signal sequence, a central domain of highly repeated sequences rich in serine and threonine residues and a carboxyl terminal domain containing a GPI-anchoring sequence (Kapteyn*, et al.*, 1999). The ability of the yeast cells to flocculate is of considerable importance as it provides an environmentally friendly, simple and cost-effective separation of the yeast cells from the final product (Bauer*, et al.*, 2010).

However, in laboratory strains of *S. cerevisiae* flocculation does not occur naturally. Due to the genetic and the epigenetic silencing of the *FLO* genes, studies on these genes have been possible through genetic manipulations. The *FLO* gene encoding flocculation and other adhesion-related phenotypes are transcriptionally silent due to the nonsense mutation on the *FLO8* transcriptional activator (Guo*, et al.*, 2000). In attempts to genetically control the onset of flocculation in both laboratory and industrial yeast strains, the native promoter of specific *FLO* genes (*FLO1*, *FLO5* or *FLO9)* was replaced with an inducible promoter which included the *ADH2* and *HSP30* promoter (Cunha*, et al.*, 2006, Govender*, et al.*, 2008)

The promoter replacement cassette strategy relies heavily on PCR to construct a cassette for integration into the genome by homologous recombination and drive the expression of the target gene. The advantages of this genetic manipulation strategy over the more traditional plasmid-based cloning approach include; stable expression, avoidance of sub-cloning of the *FLO* genes and lack of plasmid-borne expression artefacts (Govender*, et al.*, 2008).

To drive the expression of a gene in yeast, generation of significantly small cassette is desirable as this allows for easy routine cloning manipulations. Larger vectors have a tendency to make the process of cloning very difficult. The use of a *Sh ble* gene from *S. verticillus* has potential to be an alternative for the previously employed large selectable markers like *HIS-4* and Ampicillin resistance. The *Sh ble* gene product confers resistance to zeocin™, a drug that introduces double-stranded breaks in chromosomal DNA. The zeocin™ resistance protein confers resistance stochiometrically rather than enzymatically by binding to the drug. The open reading frame that encodes for this resistance protein is 375 base pairs long and the protein is 13666 Da. in weight (Alderton*, et al.*, 2006).

The *PGK1* gene promoter presents high constitutive expression in the presence of glucose and is repressed by other non-fermentable carbon sources like glycerol (Fernandez-Gonzalez*, et al.*, 2005). This promoter is relatively small in size and can efficiently drive high levels of expression of genes in *S. cerevisiae* (Nevoigt, 2008, Partow, et al., 2010). The *PGK1* promoter yields sustained expression during all phases of yeast growth.

In this work a PCR based strategy was employed to engineer a promoter replacement cassette capable of integrating via double cross over homologous recombination into the genome of *S. cerevisiae* and therefore drive the expression of the *FLO1* gene. A non –flocculent, haploid laboratory strain BY4741 was transformed with a promoter replacement cassette and its *FLO1* ORF was under the transcriptional control of the *PGK1* promoter. The *PGK1* gene promoter presents high constitutive expression in the presence of glucose and is repressed by other non-fermentable carbon sources like glycerol. The data in the literature indicate that this promoter can increase the level of expression of different genes in *S. cerevisiae*. Integration of the cassette into the target *FLO1* promoter region was evident by the distinct flocculation phenotype shown by the transgenic *S. cerevisiae* BY4741 strain, while the wild type remained non-flocculent. The data also clearly demonstrate that the flocculation behaviour of yeast strains can be tightly controlled and fine-tuned to satisfy specific industrial requirements.

3.3 Materials and Methods

3.3.1 Microbial strains and plasmids

The *S. cerevisiae* strains used in this work were both derivatives of strains BY4741 and BY4742 of genotypes summarized in Table 3.1. The host used for maintenance and propagation of the plasmids was *Escherichia coli* ElectroMAX™ DHα5 cells which were grown in Luria–Bertani (LB) medium (bactotryptone 1%, yeast extract 0.5%, and NaCl 0.5%), supplemented with ampicillin (100 μg/ml).

Table 3.1 Strains employed in this study

Strain	Genotype	Reference
BY4741	*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCAF
BY4742-PGU	*MATα;his3Δ1;leu2Δ0;lys2Δ0;ura3Δ0*	(Fernandez-Gonzalez, *et al.*, 2005)
BY4741-F1P	*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0, FLO1p::Sh ble-PGK1p*	In this study
ElectroMAX™ DH5α	*F- φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rk, mk+) phoA supE44 λ- thi-1 gyrA96 relA1*	Invitrogen

3.3.2 Media and cultivation conditions

Yeast strains were routinely cultivated in yeast-peptone-dextrose (YPD) medium containing 2% (w/v) glucose, 2% (w/v) peptone and 1% (w/v) yeast extract. For selection of kanamycin (*KanMX*) resistant strains, YPD medium was supplemented with 200 μg/ml G418 (Thermo Scientific, South Africa). Single yeast colonies from three-day old YPD medium were used to inoculate 10 mL YPD broth contained in 100 mL Erlenmeyer flasks, which were incubated at 30°C in a 160 rpm mechanical shaker. Zeocin™ resistant yeast transformants were selected in a chemically defined synthetic complete (SC) medium containing 0.67% Yeast Nitrogen Base (YNB) without amino acids (Difco™), 2% (w/v) glucose and 150 μg/ml zeocin™. The selection medium was further supplemented with histidine, leucine, methionine and uracil (Sigma Aldrich, Germany) to complement the BY4741 auxotrophic nutrient requirements. In this study 2% (w/v) agar (Difco™) was used for all solid media.

3.3.3 Polygalacturonic acid plate assay

The *S. cerevisiae* BY4742-PGU strain was grown on agar plates, containing 1.25% (w/v) polygalacturonic acid, 0.68% (v/v) 10% KH_2PO_4 (pH 4.5), 0.67% (w/v) YNB without amino acids and 1% (w/v) glucose; and supplemented with histidine, lysine, leucine and uracil to complement the auxotrophies of the strain. Colonies were replica plated and incubated at 30 °C for 3 days. Thereafter colonies from one plate were washed off with distilled water and the surface covered with an aliquot of 6 M HCl. Positive clones displaying endo-polygalacturonase activity were identified as those that were surrounded by clear zones in HCL treated plates. Genomic DNA was isolated from positive clones that were propagated in YPD broth. The isolated DNA was used as template for PCR amplification of the *PGK1* promoter fragment.

3.3.4 Isolation of yeast chromosomal DNA

Chromosomal DNA was isolated by a method described by Asubel and co-workers (1995). Yeast strains were grown at 30^{o}C with shaking at 160 rpm for 18 hours in 10 mL YPD medium. Cells were collected by centrifugation at 3000 rpm at 4^{o}C for 2 minutes. The supernatant was then discarded and the cells were resuspended in 500 µL of distilled water. The cell suspension was transferred to a 2 mL micro centrifuge tube and centrifuged at 2000 rpm at 4^{o}C for 30 seconds. The supernatant was discarded and the pellet was resuspended in the residual liquid by vortexing. Into the cell suspension, 200 µL of breaking buffer containing, 0.3 grams of glass beads (Sigma-Aldrich, USA) and 200µL of a phenol/chloroform/isoamyl alcohol [25:24:1(v/v/v)] mixture were added. The mixture was vortexed at high speed for 5 minutes. Subsequently, 200µL of TE buffer containing 10mM Tris-HCL and 1mM EDTA (pH 8) was added into the tube which was briefly vortexed and centrifuged at 14000 rpm at 4^{o}C for 10 minutes. After centrifugation, the upper aqueous layer of approximately 500 µL was transferred into a clean 1.5 mL microcentrifuge tube and treated with 1 mL of 100% ethanol. The contents were mixed by inversion to precipitate the chromosomal DNA. The tube was then stored at -80^{o}C for 20 minutes, which was followed by centrifugation at 14000 rpm at 4^{o}C for 5 minutes. The supernatant was discarded and the pellet was resuspended in 400µL TE buffer and 3µL of RNase (10 mg/mL) to degrade contaminating RNA. The degradation was carried out at 37^{o}C for 5 minutes. DNA remaining in the tube was precipitated by addition of 10µL 4 M ammonium acetate and 1 mL of 100% ethanol. The contents were mixed by inversion and stored at -80^{o}C for 20 minutes followed by centrifugation at 14000 rpm at 4^{o}C for 3 minutes. The supernatant was discarded and the DNA pellet retained. The pellet was dried in a vacuum concentrator (Eppendorf, concentrator 5301. Germany) and then resuspended in 100 µL of TE buffer (Govender*, et al.*, 2010).

3.3.5 Construction of the *FLO1* promoter replacement cassette

The strategy for construction of the promoter replacement cassette is presented in Figure 3.1.

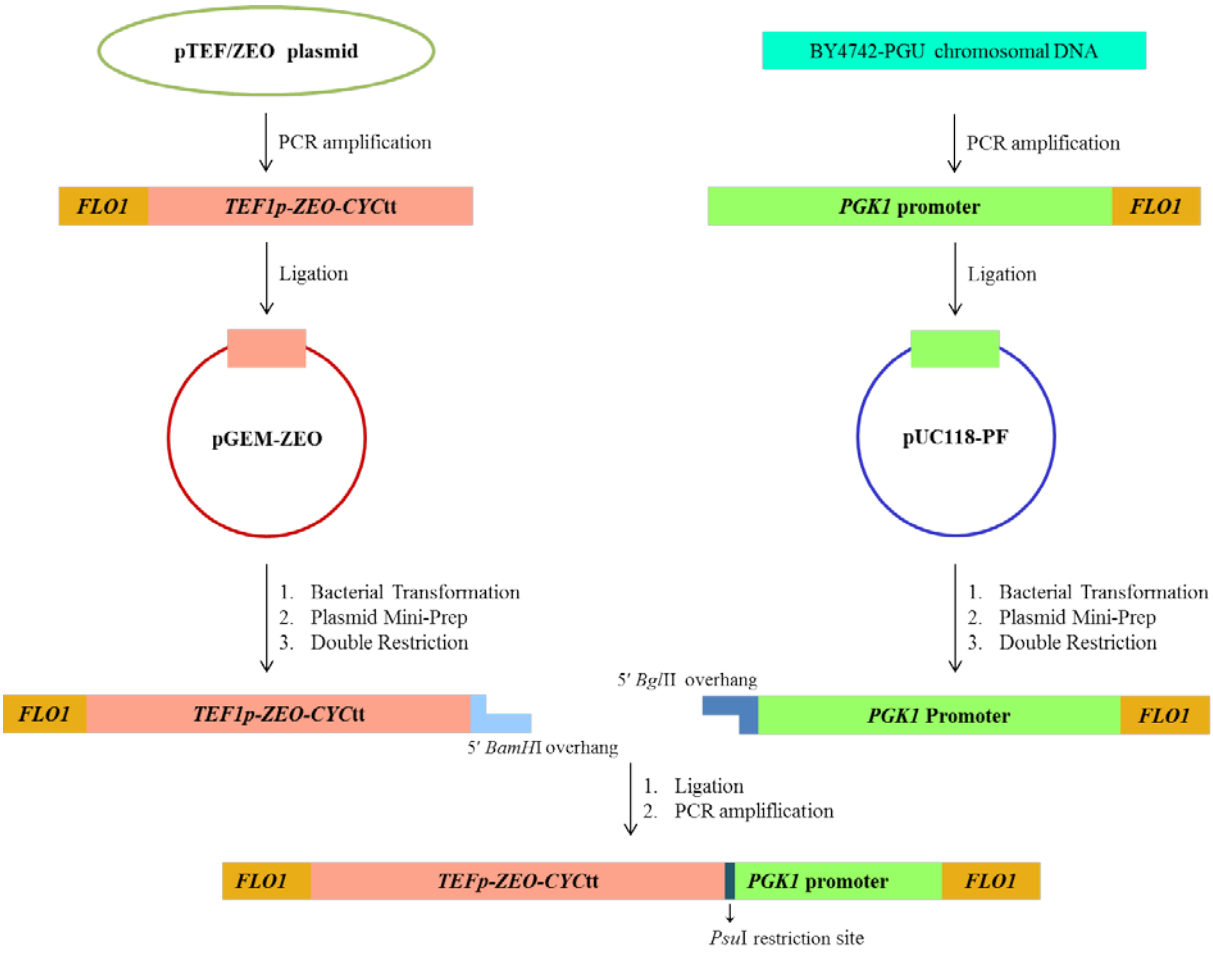

Figure 3.1 Construction of the *FLO1* promoter replacement cassette containing the promoter element of the *S. cerevisiae* 3-phosphoglycerate kinase gene (*PGK1p*) and the zeocin™ selection marker gene.

All restriction enzymes were purchased from Thermo Scientific (RSA). The Takara Ex-Taq and Primestar HS polymerase (Takara Bio inc, Japan) systems were employed for routine and high fidelity DNA amplification, respectively as per manufacturer's guidelines. The PCR amplification products were purified from 1% (w/v) agarose gels and cloned into pUC118 blunt-ended vector using the Mighty Cloning Kit blunt ends (Takara Bio inc, Japan) or the pGEM-T Easy vector systems (Promega Corporation, Madison, WI) according to the manufacturer's specifications.

The *PGK1* promoter region was amplified from the genomic DNA of the BY4742-PGU strain (Fernandez-Gonzalez*, et al.*, 2005) using the primer set PGK1-F and PGU-R (Table 3.2). The amplified fragment (595 bp) was gel-purified using a Zymo-prep DNA-gel purification kit (Zymo Scientific, USA). The purified fragment served as a template for the amplification of the *PGK1*-promoter region bearing a 60 bp 3' *FLO1* promoter homologous sequence using the primer pair PGK1-F and FLO1::PGK1-R. The amplicon corresponding to the *PGK1-FLO1* promoter fragment (652 bp) was gel purified and ligated to the blunt-ended pUC118 vector. A 2 µL aliquot of the ligation reaction mixture was used to transform ElectroMAX™ DH5α cells. Electroporation of *E.coli* was performed with a Bio-Rad MicroPulser™ (Bio-Rad Laboratories, CA, USA) using the instrument's pre-programmed setting for *E.coli* (Ec2, 1.8 kV for 5.0 ms) and HiMax electroporation cuvettes (Cell Projects Ltd., Kent, UK) with a 1 mm electrode gap.

Table 3.2 The primers employed in this study

Prime Name	**Sequence 5'-3'**
FLO1::PGK1-R	CTGCCAAAAACATATAGCGATGAGGCATTGTCATTTTTGGATGTTCTGTTTACTGGTGACTGTTTTATATTTGTTG
FLO1::TEF1-F	TGCGTCACTTTTCCTACGGTGCCTCGCACATGAATGTTATCCGGCGCACGACGGTTCCTGGCCTTTTG
CYCtt-R	ACCG**TGATCA**ATGCCTGCAGGTCGACTCT
PGU-R	GTAATGAATTAGCAGAAATCAT
PGK1-F	AGCC**AGATCT**TCCCTCCTTCTTGAATTGATG
FLO1 F	TGCGTCACTTTTCCTACGGT
FLO1 R	ACATATAGCGATGAGGCATT
FLO1-F2	GCACATGCCAATTGCTGTGCATAGC
PGK1-R	GTGACTGTTTTATATTTGTTG

F denotes the forward primer and R the reverse primer. As denoted by the primer name non-underlined regions correspond to the *PGK1* promoter and *TEF1* gene sequences. Underlined regions correspond to the *FLO1* sequences. Bold letters indicates restriction enzyme sites.

The cells were incubated overnight at 37°C in Luria Bertani (LB) agar plates supplemented with ampicillin (100 µg/ml), X-gal (20 mg/ml) and 100 µl/ml isopropyl β-D-1-thiogalactopyranoside (IPTG) for blue/white screening of transformants. White colonies representing putative transformants were inoculated into LB broth supplemented with ampicillin. The cultures were incubated overnight at 37 °C with shaking (180 rpm). The recombinant plasmid pUC118-*PGK1-FLO1* (pUC118-PF) was harvested and purified using a Zyppy Mini Prep Kit (Zymo Scientific, USA) as per manufacturer's guidelines. The plasmid was double-digested with *Pst*I and *Bgl*II restriction endonucleases to release the *PGK1-FLO1* promoter fragment with the required 5'-overhangs for further ligation (Figure 3.1). The *PGK1-FLO1* fragment was gel purified using the Zymo-prep DNA-gel purification kit.

The zeocin™ selectable marker gene which comprises of the *TEF1* promoter, *Sh ble* ORF coding for zeocin™ resistance and the CYC1 termination sequence was amplified from pTEF1/Zeo plasmid DNA using the FLO1::TEF1-F and CYCtt-R primer pair (Table 3.2). The corresponding amplicon was gel-purified and ligated into the pGEM-T Easy plasmid vector. A 2µl aliquot of the ligation mixture was used to transform electrocompetent ElectroMax™ DHα5 cells. Screening of putative transformants bearing the plasmid of interest was carried out on LB agar plates containing ampicillin and other reagents for blue-white screening. White colonies representing positive transformants were inoculated into LB broth supplemented with ampicillin. The cultures were incubated overnight at 37 °C with shaking (180 rpm). The recombinant plasmid pGEM-ZEO was harvested and purified using a Zyppy Mini Prep Kit. The *FLO1*-zeocin™ selectable marker gene fragment was recovered from the recombinant plasmid via double digestion using *Bam*HI and *Sph*I restriction enzymes. The *FLO1*-zeocin™ fragment was gel purified using the Zymo-prep DNA-gel purification kit.

The ligation of *PGK1-FLO1* and *FLO1*-zeocin™ fragments containing compatible sticky ends generated by the isoschizomers *Bgl*II and *Bam*HI respectively was carried out using the Roche Dephosphorylation and Ligation Kit (Roche Diagnostics, Germany) according to the manufacturer's instructions. A 10 µL aliquot of the ligation reaction mixture was employed as a template for PCR amplification of *FLO1p-Zeo^R-PGK1-FLO1p* promoter replacement cassette using the short primer set FLO1-F and FLO1-R. The integrating *FLO1* promoter replacement cassette (3762 bp) was extracted from agarose gels and purified using the Zymo-prep DNA-gel purification kit.

Table 3.3 List of plasmids employed in this study.

Plasmid name	Base vector	Reference
pTEF1/ZEO	pTEF1/Zeo	Life technologies
pGEM-T Easy	pGEM-T Easy™	Promega
pUC118	pUC118	Takara
pGEM-ZEO	pGEM-T Easy™	This study
pUC118-FP	pUC118	This study

3.3.6 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*, et al.* (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 zeocin, were inoculated individually onto fresh SC plates containing 150 μg/ mL zeocin and cultivated at 30°C for 5 days. Chromosomal integration was achieved by a double cross-over homologous recombination event, in which the *FLO1* ORF was placed under transcriptional control of the *PGK1* promoter.

3.3.7 PCR verification of over-expression strains

The deletion of native promoters was confirmed by PCR using homologous primer sets FLO1-F2 and FLO1-R are listed in Table 3.2. In addition, the integration of promoter replacement cassettes in the desired locus in transformed yeast was further confirmed using heterologous primer set FLO1-F2 and PGK1-R. The wild type BY4741 strain served as a control in these confirmation experiments.

3.3.8 Flocculation Assay

A modified Helms assay described by D'Hautcourt and Smart (1999) was used to estimate the percentage flocculation for each yeast strain. Single colonies were grown for 20 hours in 10 mL of 2% (w/v) 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 broth 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 4000 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 x 10^8 cells/mL. Ten 1 mL samples were then harvested by centrifugation at 12000 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 wash buffer containing 0.51 g L^{-1} of calcium chloride and this was followed by resuspending pellets in 1 mL suspension buffer containing 0.44 g L^{-1} of calcium chloride, 6.8 g L^{-1} sodium acetate, 4% ethanol (v/v) 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

Percentage flocculation = [(mean control Abs – experimental Abs)/ mean control Abs] × 100.

To investigate sugar inhibition of *FLO1* flocculation phenotype, either 1 M mannose or 1 M glucose was added to both the washing and suspension buffers that are employed in modified Helms assay.

3.4 RESULTS

3.4.1. Construction of *PGK1-FLO1* promoter fragment

The promoter replacement cassette was constructed following the strategy illustrated in Figure 3.1. The *PGK1* promoter sequence was amplified to bear a 60 bp 3' *FLO1* promoter homologous tail sequence from genomic DNA of the previously constructed polygalacturonic acid-degrading transgenic strain of *S. cerevisiae* BY4742-PGU. After DNA column purification (Figure 3.2, lane 5), the fragment was cloned into the linearized pUC118 host vector and used to transform electrocompetent *ElectroMax DHα5* cells. Following blue/white screening, positive white colonies were cultivated and the harboured recombinant plasmid pUC118-PF was isolated and purified. The three conformations of the recombinant plasmid representing linear, nicked-circular and supercoiled isoforms are reflective of the top to bottom bands respectively in Figure 3.3, lane 3. The recombinant plasmid was subjected to double digestion using *Bgl*II and *Hind*III restriction endonucleases to release the desired 652 bp *PGK1-FLO1* promoter fragment and the host vector (Figure 3.2, lane 4).

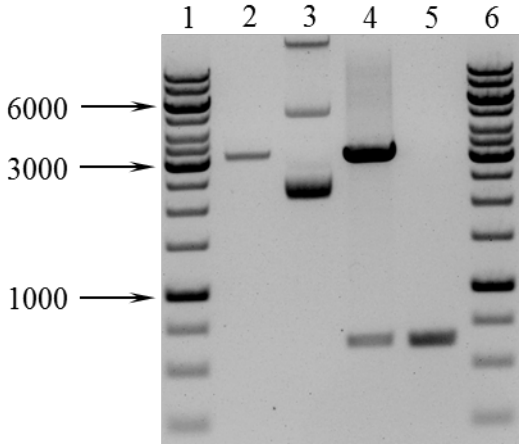

Figure 3.2 PCR amplification of *PGK1-FLO1* promoter fragment and restriction endonuclease analysis of the recombinant plasmid pUC118-FP. Lane 1 and 6, 1 kb DNA molecular weight marker (ThermoScientific, USA). Lane 2, ligation reaction negative control linearized pUC118 vector. Lane 3, Miniprep of recombinant plasmid pUC118-PF. Lane 4, Double digestion of pUC118-PF using *Hind*III and *Bgl*II restriction enzymes with the lower band to representing the released 652 bp *PGK1-FLO1* promoter fragment and top band corresponding to the linearized pUC118 host vector. Lane 5, PCR amplified *PGK1-FLO1* promoter fragment.

The band corresponding to the promoter fragment was excised from the gel, purified, vacuum concentrated and stored at -20 °C.

3.4.2 Construction of the the *FLO1*-zeocin™ selectable marker fragment

The *FLO1-zeocin*™ selectable marker sequence was amplified bearing a 50 bp 5' *FLO1* promoter homologous tail sequence from pTEF1/Zeo plasmid DNA. After DNA column purification (Figure 3.3, lane 3), the fragment was cloned into the linearized pGEM-T Easy vector and used to transform electrocompetent *ElectroMax DHα5* cells. Following blue/white screening, positive white colonies were cultivated and the recombinant plasmid pGEM-ZEO was isolated and purified. The three conformations of the recombinant plasmid representing linear, nicked-circular and supercoiled isoforms correspond to the top, middle and bottom bands respectively (Figure 3.4, lane 4). The recombinant plasmid was subjected to double digestion using *BamH*I and *Sph*I restriction endonucleases to release the desired 1362 bp *FLO1-zeocin*™ selectable marker fragment and the linearized pGEM-T Easy host vector (Figure 3.3, lane 5).

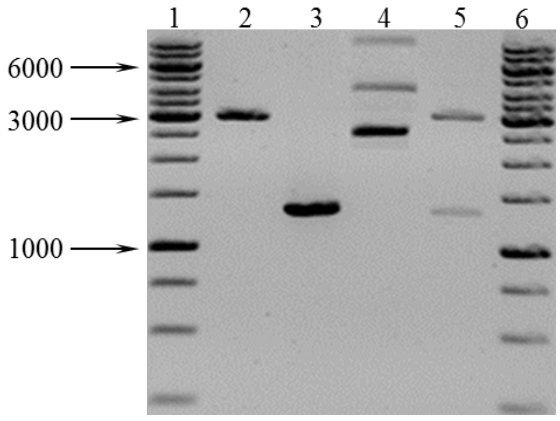

Figure 3.3 PCR amplification of *FLO1-zeocin*™ selectable marker fragment and restriction endonuclease analysis of the recombinant plasmid pGEM-ZEO. Lane 1 and 6, 1kb molecular weight marker (ThermoScientific, USA). Lane 2, Linearized pGEM-T Easy plasmid vector. Lane 3, PCR amplified *FLO1-zeocin*™ selectable marker fragment. Lane 4, Miniprep of recombinant plasmid pGEM-ZEO. Lane 5, Double digestion of pGEM-ZEO using *BamH*I *and Sph*I restriction enzymes with the lower band to representing the released 1362 bp *FLO1-zeocin*™ selectable marker fragment and top band corresponding to the linearized pGEM-T Easy host vector.

The band corresponding to the promoter fragment was excised from the gel, purified, vacuum concentrated and stored at -20 °C

3.4.3 Ligation reaction

T4 ligase was used to ligate the complimentary sticky ends of the *PGK1-FLO1* promoter and *FLO1-zeocin*^{TM} selectable marker fragment. The ligation reaction served as template for PCR amplification of the promoter replacement cassette. Using shorter primers homologous to the *FLO1* sequences located at the 3' and 5' of the ligated product (*FLO1p-Zeo*^{R}*-PGK1-FLO1p*) the desired amplicon (2014 bp) was generated (Figure 3.4, lane 4).

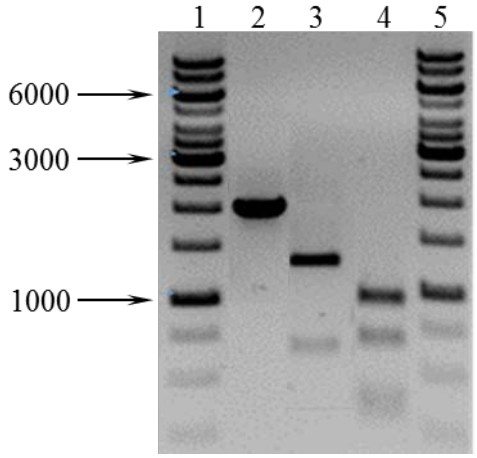

Figure 3.4 PCR amplification of the promoter replacement cassette *FLO1p-Zeo*^{R}*-PGK1-FLO1p* and restriction endonuclease verification of the amplicon. Lane 1 and 5 contain 1 kb molecular weight marker (ThermoScientific, USA). Lane 2, PCR amplification of *FLO1p-Zeo*^{R}*-PGK1-FLO1p* promoter replacement cassette using ligation reaction as template DNA. Lane 3, Digestion of promoter replacement cassette amplicon with *Psu*I restriction enzyme. Lane 4, Digestion of promoter replacement cassette amplicon with *Eco*RI restriction endonuclease.

The authenticity of the PCR generated of *FLO1p-Zeo*^{R}*-PGK1-FLO1p* promoter replacement cassette amplicon was verified using restriction endonucleases. The single *Psu*I restriction site located in the sequence of promoter replacement cassette is unique in that it was formed due the ligation of compatible sticky ends produced by restriction digestion with the isoschizomers *BamH*I and *Bgl*II. Digestion with *Psu*I validated to some extent the promoter replacement cassette amplicon in that two fragments were yielded that correspond in size to the ligated fragments i.e. the *PGK1-FLO1* promoter (652 bp) and the *FLO1- zeocin*^{TM} selectable marker (1362 bp) fragments (Figure 3.4 lane 3). DNA restriction site sequence analysis of the promoter replacement cassette identified two *Eco*RI cleavage sites. Restriction with *Eco*RI produced three fragments (991, 640 and 383 bp) which correspond to the theoretically predicted fragment sizes. The latter observation additionally confirms the integrity of the PCR amplified *FLO1p-Zeo*^{R}*-PGK1-FLO1p* promoter replacement cassette.

3.4.4 Yeast transformation and phenotypic characterization

Following initial selection on SC plates containing zeocin™, 25 putative transformants were inoculated individually into YPD broth and cultivated for 48 h at 30°C with shaking (160 rpm). No flocculent phenotype was detectable for wild type strain BY4741 (Figure 3.5A). In contrast, approximately 55% of putative transgenic strains transformed with the *FLO1p-Zeo*^{R}-*PGK1-FLO1p* promoter replacement cassette visually displayed strong flocculent phenotypes suggesting that integration had occurred at the desired locus (Figure 3.6).

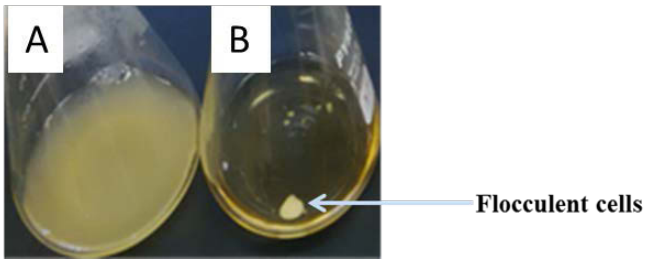

Figure 3.5 Laboratory cultures of non-flocculent wild type BY4741 (A) and flocculent transgenic BY4741 strains. Visible, distinct flocculent cells in a clear nutrient rich YPD media can be seen on the right hand side (B) while the cells are still suspended in the growth medium as shown on the left-hand side.

Three independent transgenic yeast strains (BY4741-F1P) were selected and cryopreserved for further analysis. The deletion of the *FLO1* native promoter was confirmed by PCR using homologous primer sets (Figure 3.6 A). In addition, integration at the correct gene locus was also confirmed by PCR using primers in which the upstream primer was located outside the region of integration of promoter replacement cassette (Figure 3.6 B).

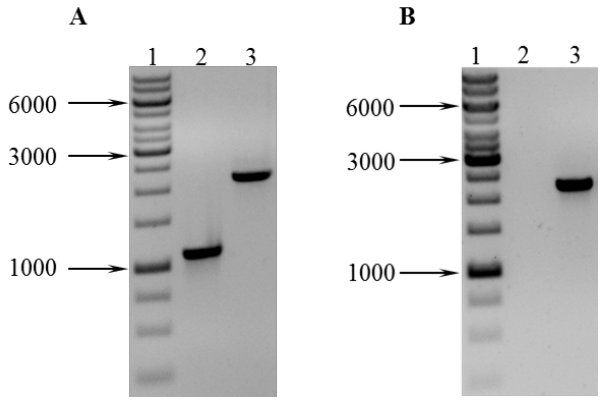

Figure 3.6 Chromosomal integration of the *PGK1* promoter upstream of the dominant *FLO1* ORF in *S. cerevisiae* strain BY4741. (A) The deletion of the native *FLO1* promoter was confirmed by PCR using homologous primer pairs described in materials and methods. Lane 1, 1kb DNA molecular weight marker (ThermoScientific, USA). Lane 2, the amplification of the native promoter sequence was only observed in the wild type BY4741 strain; *FLO1p* (1050 bp,). Lane 3, only the integration cassette was amplified in BY4741-F1P (*FLO1*p-*Zeo*^{R}-*PGK1-FLO1*p, 2253 bp). (B) The integration of promoter replacement cassettes in the FLO1 locus was confirmed by PCR using heterologous primer sets as described in materials and methods. Lane 1, 1kb DNA molecular weight marker (ThermoScientific, USA). Lane 2, no amplicon was generated from the wild type BY4741 strain. Lane 3, the amplification of *FLO1p-Zeo*^{R}-*PGK1p* fragment (2193 bp).

After 48 h growth in YPD containing 2% glucose, BY4741-F1P transgenic strains (99 ± 0.124%) were strongly flocculent whilst the wild type parental strain BY4741 (8.73 ± 2.26%) displayed no flocculation (Table 3.4). The ability of either 1 M glucose or 1 M mannose to inhibit the flocculent phenotypes of BY4741-F1P transgenic yeast strains after 48 h growth in YPD was evaluated (Table 3.4). The flocculent phenotype of the BY4741-F1P transgenic yeast strain was completely abolished on exposure to 1 M mannose. On the contrary, no inhibitory effect was evident in the presence of 1 M glucose. Since NewFlo-type flocculation is inhibited by both mannose and glucose, while Flo1-type flocculation is exclusively inhibited by mannose (Stratford & Assinder, 1991), this result clearly demonstrates that the BY4741-F1P transgenic yeast strain exhibits Flo1-type flocculation.

Table 3.4 The sugar inhibition analysis of the wild type and the transgenic strain

Strain	Flocculation (%)			
	No Mannose	1M Mannose	No Glucose	1M Glucose
BY4741	8.73 ± 2.26	6.47 ± 1.33	5.53 ± 0.96	7.81 ± 2.65
BY4741-F1P	99 ± 0.124	5.91 ± 1.89	98.43 ± 0.21	99.27 ± 0.09

The effect of either mannose or glucose on flocculation was determined using a modified Helm's assay as described in the materials and methods. Values reflect the mean of experiments performed in triplicate and error bars represent standard deviations.

3.5 Discussion

In this study, the genomic *FLO1* open reading frame in the haploid laboratory yeast strain BY4741was brought under the transcriptional control of the *PGK1* constitutive promoter by replacement of the native promoter sequence. The distinct advantage of the cloning strategy employed here over those used by other research groups (Cunha*, et al.*, 2006) is that no sub-cloning of the *FLO* gene is required which is renown as a difficult ORF to clone in terms of its intragenic tandem repeats (Govender*, et al.*, 2008). In this work recombinant DNA

strategies similar to those reported by Govender*, et al.* (2008) and Verstrepen and Thevelein (2004) were employed to construct the promoter replacement cassette. Restriction endonuclease digestion with *Bgl*II and *Bam*HI of the PCR generated amplicons corresponding to the *PGK1-FLO1* promoter and *FLO1*-zeocin™ selectable marker fragments respectively was circumvented due to the low efficiency associated with the digestion of PCR products that bear restriction sites at either their 3' or 5' termini. Instead the fragments were subcloned into linearized pUC118 and pGEM-T Easy plasmid vectors and subsequently recovered by double digestions which are most effective when performed on plasmid DNA. These plasmid born fragments were ligated to generate the complete promoter replacement cassette.

The cassette was created bearing in mind its fate, which is to integrate into the genome and constitutively drive the expression of the *FLO1* gene through all the growth phases. Therefore, the *PGK1* promoter was selected based on it attributes such as high constitutive expression of a target gene in the presence of glucose and it is only repressed by the presence of other non-fermentable carbon sources such as glycerol (Liu*, et al.*, 1996). The relative smaller size of this promoter (583 bp) also contributed in its selection as the promoter of choice.

The *PGK1* promoter sequence was fused to the chimeric zeocin™ resistance gene. The use of zeocin™ selection for screening was based on data presented by (Alderton*, et al.*, 2006, Higgins*, et al.*, 1998) that showed high throughput transformation efficiency due to the fact that the insert is smaller in size when compared with other selectable markers derived from yeast genomic sequences for example, *SMR1* (Alderton*, et al.*, 2006, Higgins*, et al.*, 1998, Johansson & Hahn-Hagerdal, 2002). It was also selected due to its broad selection range as it inhibits growth of both eukaryotes and prokaryotes, therefore increasing the sensitivity of the screening process (Higgins*, et al.*, 1998).

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 regions for homologous recombination with the target allele were incorporated via PCR-based strategy into the 5' and 3' termini of the promoter replacement cassette. The integration of the *PGK1*-based promoter replacement cassette was facilitated by the high level of homologous recombination which facilitates

insertion into the specific locus. Unlike plasmid-borne gene expression, when using the site-directed chromosomal integration method, both curing and variation in expression due to plasmid copy number are averted. Furthermore, no selection pressure is required to maintain the expression vector since the cassette integrates into the genome (Govender*, et al.*, 2010).

It has been previously established that laboratory strains of *S. cerevisiae* do not express *FLO*-gene encoded phenotype due to a nonsense mutation in *FLO8*, a transcription activator of the *FLO* genes (Liu*, et al.*, 1996, Verstrepen*, et al.*, 2005). Introduction of the promoter replacement cassette *FLO1p-Zeo^{R}-PGK1-FLO1p* into the non-flocculent BY4741 laboratory strain resulted in a visibly distinct and strong flocculation displayed by the transgenic strain BY4741-F1P. Transgenic yeast strain verification protocols including a PCR-based strategy that employs homologous and heterologous primer sets clearly demonstrated that *FLO1* ORF of the transgenic strain was placed under transcription control of the *PGK1* promoter. In addition a sugar inhibition assay confirmed the flocculation displayed is a Flo1 flocculation phenotype. The transgenic strains had a flocculation potential of 99% which indicates that the constitutive *PGK1* promoter is an ideal promoter for the overexpression of other dominant *FLO* genes. The data presented seemingly suggests that constitutive overexpression of *FLO* gene encoded mannoproteins may provide a feasible option for the generation of sufficient amounts that will enable an in depth insight into the biochemical structures of these otherwise poorly understood cell wall-associated glycoproteins.

3.6 Conclusion

In conclusion, this work reports the construction of a promoter replacement cassette *FLO1p-Zeo^{R}-PGK1-FLO1p* for constitutive expression of genes in *S. cerevisiae* laboratory yeast strain. The data show that integration of the created promoter replacement cassette confers stable flocculation during all growth phases. This study demonstrates that the *PGK1* promoter is suitable for constitutive overexpression of a dominant *FLO1* gene.

3.7 Acknowledgements

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Chapter 4

RESEARCH RESULTS II

Constitutive *FLO1* overexpression in *S. cerevisiae* strains bearing deletions in cell wall biogenesis related genes

Constitutive *FLO1* overexpression in *S. cerevisiae* strains bearing deletions in cell wall biogenesis related genes

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4.1 Abstract

To date, it has been reported that the five dominant *FLO* genes in *S. cerevisiae* encode for a family of glycosylphosphatidylinositol (GPI) linked glycoproteins that are commonly referred to as adhesins, flocculins or cell wall mannoproteins. The adhesion phenotypes that are associated with individual *FLO* genes have been extensively researched and well characterized. However, far less is understood about the cellular metabolic routes that lead to their biochemical synthesis and incorporation into the yeast cell wall. Additionally our fairly limited current understanding of the fine molecular architecture of these mannoproteins predominantly relies on data generated from *in silico* predictive research studies. In this research study a genetic engineering approach will be employed to constitutively overexpress the *FLO1*-encoded mannoprotein in the wild type BY4741 haploid laboratory *S. cerevisiae* strain and in mutant BY4741 strains bearing a disruptive deletion in either their *KNR4* or *GPI7* cell wall biogenesis related genes. As such the effect of the gene deletions on the intensity of the flocculation phenotype will shed light on the contribution of the deleted gene products in biochemical processing of *FLO1* mannoproteins. Additionally it is envisioned that the transgenic yeast strains overexpressing *FLO1* mannoproteins will provide a viable alternative for the large scale isolation and purification of the intact mannoprotein especially if it were to be released into the growth medium by *FLO1* overexpressing deletion transgenic strains. This glycoprotein reservoir can be utilised in the structural analysis of *FLO1* mannoproteins. In relation to the transgenic wild type BY4741-F1P strain decreased flocculation intensity was observed in the BY4741ΔKNR4-F1P strain. BY4741ΔGPI7-F1P displayed a flocculation intensity that was similar to the BY4741-F1P strain. Interestingly, 10% higher protein concentrations were observed in the spent growth medium of the transgenic BY4741ΔKNR4-F1P strain thereby seemingly suggesting the possibility of the FLO1 mannoprotein being released extracellularly.

4.2 Introduction

Mannoproteins are highly glycosylated cell wall proteins comprising over 90% sugar. The sugar constituent is 98% mannose and 2% glucose (Klis*, et al.*, 2006, Lipke & Ovalle, 1998) and these glycoproteins resemble carbohydrates in their properties rather than those associated with proteins. They play a major role in giving the cell wall its active properties and are partially responsible for the cell wall permeability (Caridi, 2006, Cid*, et al.*, 1995, Klis*, et al.*, 2006). Mannoproteins form the outer most layer of the cell wall and they are covalently linked directly to the ß-1, 3-glucan by the glycosylphosphatidylinositol (GPI) anchor, and indirectly via the ß-1, 6-glucan. More studies have been done on the GPI-anchored proteins and have been identified, with all having two characteristics in common. Their central domains are rich in serine and threonine and they all contain a putative (GPI) attachment signal.

It has been suggested that ageing wine on lees improves the organoleptic properties of wine, possibly due to the increased amount of mannoproteins in lees (Quiros*, et al.*, 2010) . Mannoproteins play an important role in wine stabilization which includes prevention of haze due to proteins and retention of aroma (Goncalves, et al., 2002). The mechanism by which they prevent aggregation of wine proteins is still unclear. This is due to the limited information on chemical reactive groups in them that allow them to bind to other wine proteins. To understand the mechanism of action of the mannoproteins, it is necessary for their structure to be fully elucidated.

Adhesins encoded by the *FLO* genes (*FLO1, FLO5, FLO9, FLO10 and FLO11)* are extracellular facing (GPI)-anchored cell wall glycoprotein that are involved in numerous adhesion phenotypes including flocculation, biofilm and flor formation, attachment to biotic and abiotic surfaces, invasive growth and pseudohyphal formation. They possess a common three-domain assembly with an amino-terminal domain that is proposed to contain the binding site to carbohydrate receptors (mannan) which confers adhesion (Kobayashi*, et al.*, 1999), a central domain rich in serine and threonine residues and a carboxyl-terminal region. Members of the *FLO* gene family are located near the telomeres with *FLO11* being an exception since it is neither sub-telomeric nor centromeric (Halme*, et al.*, 2004). Their location in the genome is suggested to play a major role in their regulation and evolution (Verstrepen*, et al.*, 2005). These genes are transcriptionally silent due to a point mutation on a transcriptional activator

of *FLO 8*. In order to exploit their function one needs to place them under the control of a heterologous promoter.

Genetic-based research studies pertaining to flocculation has been primarily driven by the importance of this phenotype to the brewing industry and the attractive potential to create transgenic strains with industrially desirable flocculation traits. In attempts to genetically control the onset of flocculation in both laboratory and industrial yeast strains, the native promoter of specific *FLO* genes (*FLO1*, *FLO5* or *FLO9)* was replaced with an inducible promoter which included the *ADH2* and *HSP30* promoters (Cunha*, et al.*, 2006, Govender*, et al.*, 2010, Govender*, et al.*, 2008, Verstrepen*, et al.*, 2003). These transgenic strains were observed to exclusively flocculate on entry to the stationary phase of growth and once primary fermentation was completed. To date, there exists limited information on the fine molecular structure of these mannoproteins. Generally the amino acid sequences of their protein moieties have been established from structural and functional analysis of the genomic sequence of various yeasts whilst far less information is available on the glycosyl moieties of these mannoproteins.

Gonzalez-Ramos and Gonzalez (2006) identified genetic determinants for hyper-secretion of cell wall mannoproteins. These strains had a specific deletions in genes related to cell wall biogenesis and regulation and were found to release an increased mannoprotein concentration into liquid growth medium. They showed that using strains disrupted in *KNR4*, *GPI7* and *GAS1* one might be able to over-express and release a specific protein or even better a combination of proteins that will improve the organoleptic properties of wine. This technology therefore has the potential to directly benefit the local and international wine industries since the presence of mannoproteins in wine have been shown to improve wine quality.

The current study aims at over-expressing *FLO1* ORF that has been suggested to improve flocculation in *S. cerevisiae*. This gene will be over-expressed in the laboratory strains bearing deletions in genes *GPI7* and *KNR4* which are related to cell wall biogenesis. It is envisioned that the use of these deletion strains will allow the increased production of intact *FLO1*-encoded mannoproteins into the spent medium that will allow for their isolation and further structural characterization of the mannoprotein.

4.3 Materials and methods

4.3.1 Microbial strains

The *S. cerevisiae* strains employed on this work are listed in Table 4.1.

Table 4.1 *S. cerevisiae* strains employed in this study.

Strain	Genotype	Reference
BY4741	*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF
BY4741ΔGPI7	*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 YJL062w::KanMX*	EUROSCARF
BY4741ΔKNR4	*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 YGR229c::KanMX*	EUROSCARF
BY4741-F1P	*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 FLO1p::Sh ble-PGK1p*	This study
BY4741ΔGPI7-F1P	*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0YJL062w::KanMX FLO1p::Sh ble-PGK1p*	This study
BY4741ΔKNR4-F1P	*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0YGR229c::KanMX FLO1p::Sh ble-PGK1p*	This study

4.3.2 Media and cultivation conditions

Yeast strains were routinely cultivated at 30^{o}C in rich YPD medium, containing 1% (w/v) yeast extract, 2% (w/v) D-glucose and 2% (w/v) peptone. Single yeast colonies from 3-day old YPD plates were used to inoculate experimental cultures in 20 mL YPD broth contained in 250 mL Erlenmeyer flasks, which were incubated with shaking at 160 rpm for 48 hours. The screening for positive yeast transformants was achieved by cultivating yeast in synthetic complete (SC) medium containing 0.67% (w/v) yeast nitrogen base (without amino acids) and 2% glucose (w/v), supplemented with 150 μg/mL zeocin™ (Invitrogen). Two percent (w/v) agar (Difco) was used to solidify media. Transformed yeast strains were stored at -80^{o}C in YPD broth supplemented with 15% (v/v) glycerol(Suga*, et al.*, 2000).

4.3.3 Confirmation of Gene Knockout (YKO) strains

The deletion strains, generated by the *S. cerevisiae* Genome Deletion Project were used in this study (Winzeler*, et al.*, 1999). The Kanamycin (*KanMX*) deletion cassette is inserted at the ORF of the target genes, deleting the target ORF, while leaving the start and the stop codon intact (Kelly*, et al.*, 2001). The mutant strains, bearing deletions in the *GPI7* and *KNR4* ORFs were further confirmed to carry the deletion. In each deletion mutant, a set of confirmation

primers (Conf A, B, C and D) were used together with KanB and KanC for verification (Table 4.2). The primer set Conf A and D were designed to be 200-400bp upstream and downstream from the start and the stop codon of the gene of interest, respectively and Conf B and Conf C were located within the coding regions of the gene of interest.

Table 4.2 Primers employed in this work.

Primer Name	Primer sequence (5'→3')
KanB	CTGCAGCGGAGAGCCGTAAT
KanC	TGATTTTGATGACGAGCGTAAT
ΔKNR4-CONFA	CAACTGAAAAGGTTGTGTTTTCTTT
ΔKNR4-CONFB	GGGATAAACCTTGTTGAGATCTTTT
ΔKNR4-CONF C	TTTAAAAAGAAGAACGTGGATCAAG
ΔKNR4-CONF D	GCCAACCGTTTTGTATATATTGATT
ΔGPI7-CONF A	GCTGTTCTTCTCTCCTCTTTTGTAA
ΔGPI7-CONF B	CGTCACATTATTATCGACTTGAGTG
ΔGPI7- CONF C	GGTGTTTCCTCAGACTACAACATCT
ΔGPI7-CONF D	GCACTTAACCTACTAGATTGGGACA
FLO1-F2	TGCGTCACTTTTCCTACGGT
FLO1-R	ACATATAGCGATGAGGCATT
FLO1-F3	CAGCAGTCGAATGTTCAAGATGC
PGK1-R	GTGACTGTTTTATATTTGTTG

4.3.4 PCR amplification of the promoter replacement cassette

All the PCR reactions were performed using a G-star master gradient (Vacutec, South Africa). Primestar HS DNA polymerase system™ (Takara Bio inc. Japan) was employed to ensure high fidelity amplification of vector cassettes. Chromosomal DNA of the previously engineered transgenic strain BY4741-F1P was used as a template to amplify the PGK1-based promoter replacement cassette using the primer set consisting of FLO1-F2 and FLO1-R (Table 4.2).

4.3.5 Purification of amplified PCR product

The PCR mass amplified (50 x 20 µL reactions) self-integrating *FLO1* promoter replacement cassette was purified using a high pure PCR purification kit (Roche diagnostics, Germany), following the manufacturer's instructions. Briefly, 10 x 20 µL reactions PCR mixtures were pooled (200 µL) into a 1.5 mL microcentrifuge tube to which was added 1 mL of binding buffer. A high pure filter tube was inserted into a collection tube and a 600 µL aliquot was added to the upper reservoir of the filter tube. The filter assemblies were centrifuged for 1 minute at 12000 rpm using a microcentrifuge (Eppendorf, centrifuge 5417R, Germany). The latter process was repeated with the remaining 600 µL aliquot. The flow through solution in each instance was discarded and the silica-based filter matrix washed twice with 500 µL and once with 200 µL of washing buffer to ensure optimal purity and complete removal of washing buffer. The filter tube was then inserted into a clean 1.5 mL microcentrifuge tube and 500 µL sterile distilled water as used to elute the purified promoter replacement cassette. The filter assembly was allowed to stand on the bench top for 4 minutes and then centrifuged at 12000 rpm. Thereafter the volume of the eluent was reduced under vacuum to concentrate the eluted promoter replacement cassette DNA down to 10 µL and stored at -20 °C until further use.

4.3.6 Yeast transformation

Transformation of the yeast strains BY4741ΔKNR4 and BY4741ΔGPI7 using freshly prepared electro-competent cells was performed with 10 µg of DNA 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, CA, USA) using the instrument's pre-programmed setting for *S. cerevisiae* (Sc2) and HiMax electroporation cuvettes (Cell Projects Ltd., Kent, 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 zeocin™, were inoculated individually onto fresh SC plates containing zeocin™ and cultivated at 30°C for 3 days.

4.3.7 PCR-based verification of transgenic yeast strains

Chromosomal integration was achieved by a double cross-over homologous recombination event, in which the *FLO1* ORF was placed under transcriptional control of the *PGK1* promoter. The deletion of native promoters in transgenic yeast strains (BY4741ΔKNR4-F1P and BY4741ΔGPI7-F1P) was confirmed by PCR using homologous primer sets FLO1-F3 and FLO1-R listed in Table 4.2. In addition, the integration of promoter replacement cassettes in the desired locus in transformed yeast was confirmed by a PCR strategy using the heterologous primer set FLO1-F3 and PGK1-R with the binding site of the forward primer being outside the region of integration of the promoter replacement cassette. The wild type BY4741 and parental deletion mutant BY4741ΔKNR4 and BY4741ΔGPI7 strains served as controls in these confirmation experiments.

4.3.8 Flocculation Assay

The ability of yeast strains to flocculate was established using the modified Helm's assay as described by D'Hautcourt and Smart (1999). Five replicates of the control and test reactions were performed for each sample. The percentage flocculation reported in this chapter reflects the mean of three independent determinations.

4.3.9 Bicinchoninic acid assay (BCA)

The BCA assay is a colour reaction whereby bicinchoninic acid builds a complex with peptide bonds and reduces Cu^{2+} to Cu^{+} that can be detected at (λ max $_{562nm}$). The amount of reduced copper is proportional to the number of peptide bonds and thus to the number of protein molecules in a sample. The cell-free spent medium recovered by centrifugation from cultures of wild type, deletion mutant and transgenic strains that were grown in GCY broth (2% glucose, 2% casein hydrolysate, 0.67% YNB) at 30 °C for 48 hours with shaking (160 rpm). The protein concentration of the spent medium was determined using the Pierce BCA Protein Assay Kit according to the manufacturer's instruction (ThermoScientific, USA). For each strain tested three independent samples were assessed and the concentration was determined as an average of the three samples.

4.3.10 Statistical Analysis

In this study, one-way analysis of variance (ANOVA) was employed to statistically compare flocculation data obtained for parental strains to that of transgenic yeast strains. Analysis was performed using the statistical software package GraphPad InStat version 3.05 32 bit for Windows 95/NT (GraphPad Software, San Diego California).

4.4 Results

4.4.1 Confirmation of yeast knockout strains

The deletion by integration of the *KanMX* gene in either the *GPI7* or *KNR4* ORF in the respective mutant BY4741ΔKNR4 and BY4741ΔGPI7 strains was confirmed via a PCR strategy according to the method as described by Kelly*, et al.* (2001). The correct integration of the *KanMX* gene into the *GPI7* or *KNR4* loci was verified by the appearance of relevant amplicons (Figure 4.1) using gene-specific primer sets Conf A with KanB and KanC with Conf D (Table 4.2). In addition the absence of a PCR product using gene-specific primers sets with one from inside the region of integration of the deletion cassette viz; Conf A with Conf B and Conf C with Conf D indicate the successful deletion of native ORF (Figure 4.1).

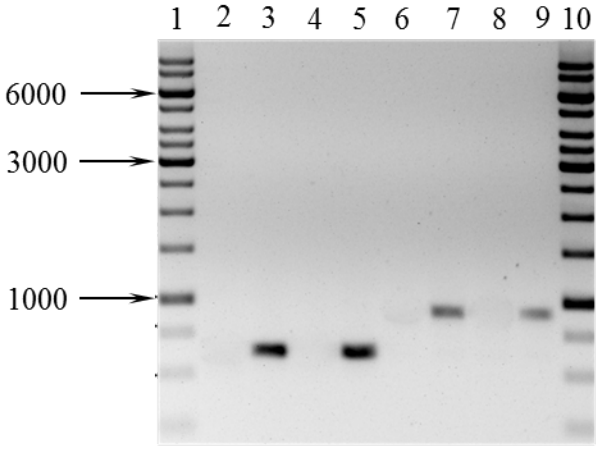

Figure 4.1 Confirmation of the integration of kanamycin (*KanMX*) deletion cassette in BY4741 deletion strains. The *KanMX* deletion cassette was integrated to knockout either *KNR4* ORF or *GPI7* ORF. Lanes 2 and 4 show deletion of *KNR4* ORF and *GPI7* ORF respectively, primer set: Conf A and Conf B; Lanes 3 and 5 show replacement of *KNR4* ORF and *GPI7* ORF respectively with the *KanMX* cassette, primer set: Conf A and Kan B; Lanes 6 and 8 show deletion of *KNR4* ORF and *GPI7* ORF respectively, using primer set: Conf C and Conf D; Lanes 7 and 9 show replacement of *KNR4* ORF and *GPI7* ORF respectively, using primer set: Kan C and Conf D. Lane 1 and 10, 1 kb molecular weight marker (Thermo Scientific, USA).

4.4.2 Transformation of BY4741 deletion mutant strains

The promoter replacement strategy presented in Figure 4.2 was employed in the electro-transformation of the haploid laboratory yeast strains BY4741ΔGPI7 and BY4741ΔKNR4. Fifty putative transformant colonies from YPD selection plates containing zeocin™ were cultivated aerobically in YPD broth at 30°C for 24 hours with shaking (160 rpm). Under these conditions, it was observed that 30 % of BY4741ΔGPI7 and 10 % of BY4741ΔKNR4 based putative transformants displayed visually intense flocculation phenotypes (Figure 4.5). Three independent strains were selected and cryopreserved for further analysis.

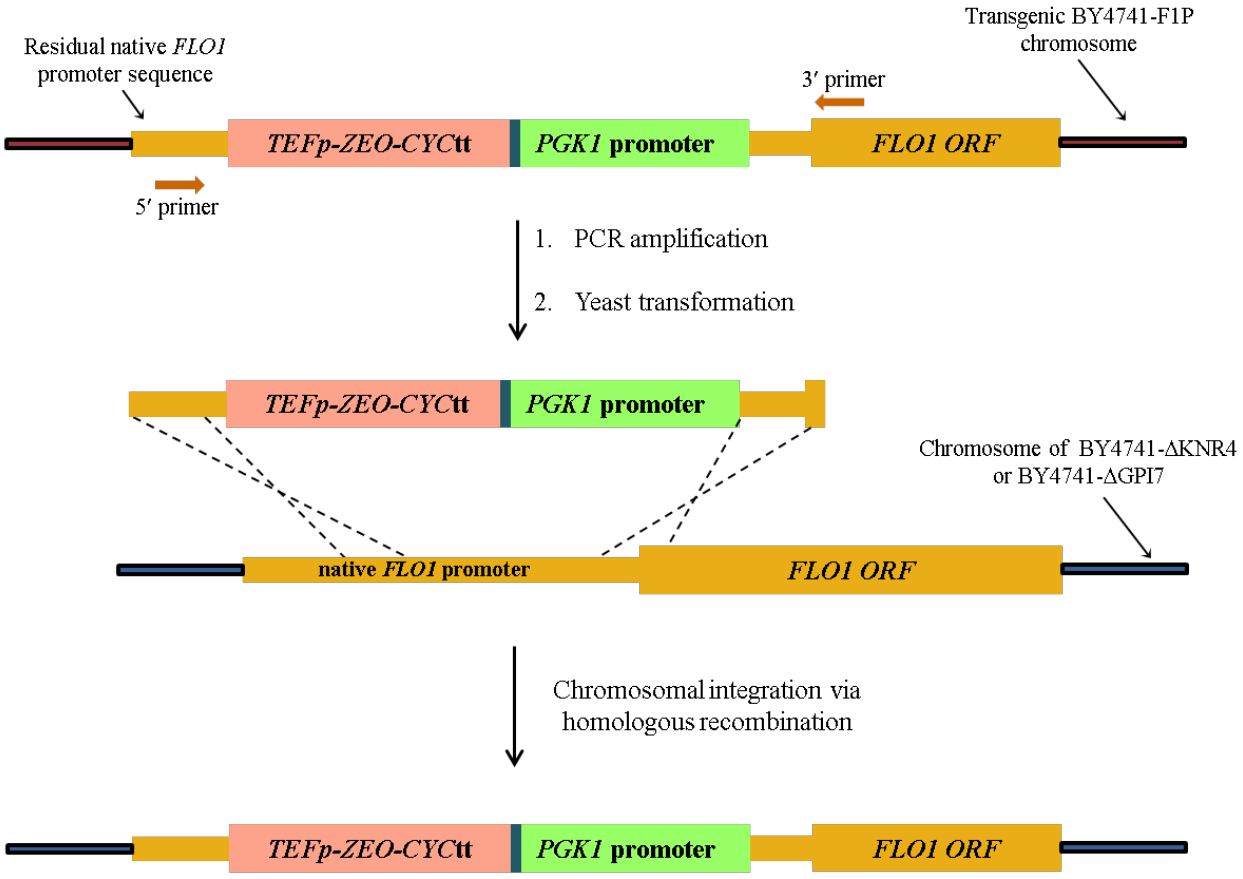

Figure 4.2 Promoter replacement strategy demonstrating the chromosomal integration of the *PGK1* promoter upstream of the *FLO1* ORF in BY4741 and its deletion mutant strains BY4741ΔGPI7 and BY4741ΔKNR4. A promoter replacement cassette bearing 5' and 3' tail regions that are homologous to the native promoter regions of *FLO1* gene were amplified using genomic DNA as a template from BY4741-F1P transgenic yeast strain.

4.4.3 PCR-based verification of transgenic strains

The deletion of the native *FLO1* promoter in transgenic strains was confirmed by PCR using a homologous primer set which contained a *FLO1* promoter-specific forward primer from outside the region of integration and a *FLO1* ORF-specific reverse primer. The generated PCR fragment (*FLO1p-Zeo*^{R}*-PGK1-FLO1p)* amplified from template genomic DNA isolated from the transgenic BY4741-F1P; BY4741ΔGPI7-F1P and BY4741ΔKNR4-F1P corresponded to the predicted amplicon size of 2349 bp (Figure 4.3). In contrast, a smaller amplicon (1050 bp) was generated from genomic DNA of BY4741 wild type strain which corresponds to the expected size of the targeted native *FLO1* gene sequence (Figure 4.3).

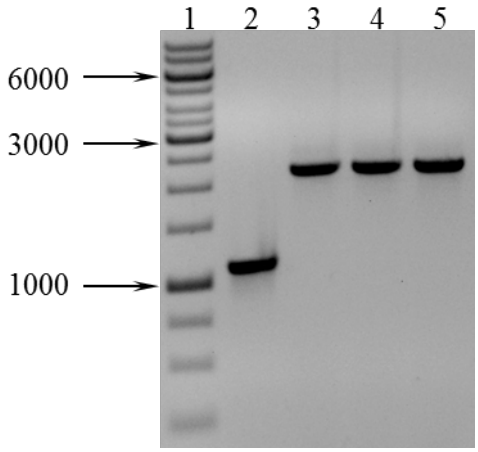

Figure 4.3 The deletion of the native *FLO1* promoter was confirmed by PCR using homologous primer pairs as described in materials and methods. Lane 1, 1kb DNA molecular weight marker (ThermoScientific, USA). Lane 2, the amplification of the targeted native *FLO1* gene sequence was exclusively observed in the wild type BY4741 strain (1050 bp). In contract to the wild type BY4741 strain, a much larger amplicon corresponding promoter replacement cassette (*FLO1*p-*Zeo*^{R}*-PGK1-FLO1*p, 2349 bp) was amplified from all transgenic yeast strains i.e. Lane 3, BY4741-F1P; Lane 4, BY4741ΔGPI7-F1P and Lane 5, BY4741ΔKNR4-F1P.

Furthermore, the integration of the promoter replacement cassette into the *FLO1* locus of transgenic deletion mutant strains was also confirmed by PCR using a heterologous primer pair containing a native *FLO1* promoter-based forward primer from outside the region of integration and a *PGK1*-specific reverse primer. This primer combination is specific for amplification of a sequence which contains the *PGK1* promoter upstream of the *FLO1* ORF. The presence of an amplicon (2289 bp) in all transgenic strains confirmed the correct placement of the *PGK1* promoter upstream of the *FLO1* ORF (Figure 4.4).

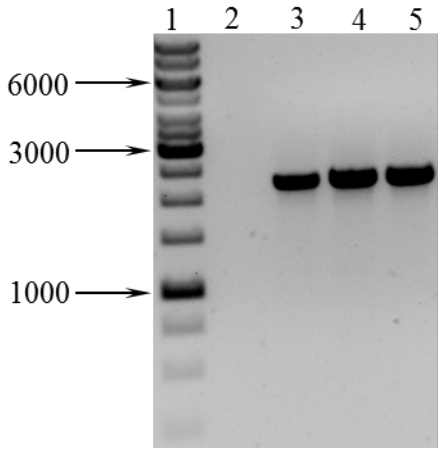

Figure 4.4 The integration of promoter replacement cassettes in the *FLO1* locus in transgenic strains was confirmed by PCR using heterologous primer sets as described in materials and methods. Lane 1, 1kb DNA molecular weight marker (ThermoScientific, USA). Lane 2, no amplicon was generated from the wild type BY4741 strain. In contract to the wild type BY4741 strain, an amplicon corresponding promoter replacement cassette (*FLO1*p-*Zeo*^{R}-*PGK1p*, 2289 bp) was amplified in all transgenic yeast strains i.e. Lane 3, BY4741-F1P; Lane 4, BY4741ΔGPI7-F1P and Lane 5, BY4741ΔKNR4-F1P.

4.4.4 Flocculation

The flocculation intensity of parental and their transgenic strains were determined after 48 hours of growth in YPD broth (Figure. 4.5). The wild type BY4741 (8.73 ± 2.26 %), BY4741ΔGPI7 (7.52 ± 2.45 %) and BY4741ΔKNR4 (7.43 ± 3.05) strains were nonflocculent. The transgenic yeast strains, BY4741-F1P (99 ± 0.124 %), BY4741ΔGPI7-F1P (99 ± 0.059 %) and BY4741ΔKNR4-F1P (98 ± 0.560 %) displayed comparable and very strong flocculation intensities.

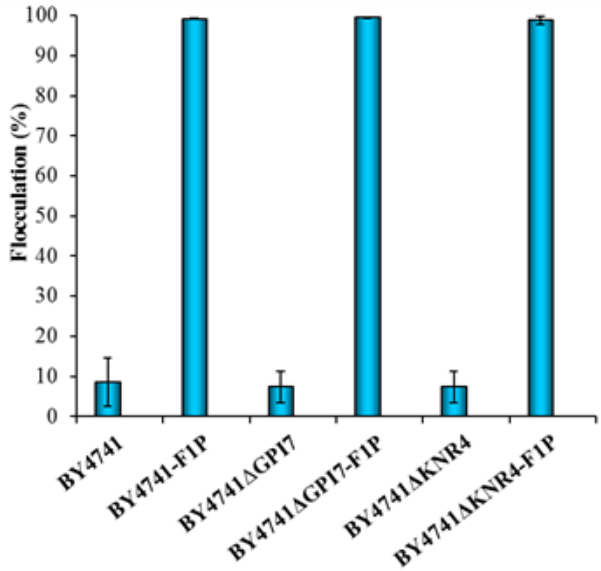

Figure 4.5 Flocculation intensities of laboratory *S. cerevisiae* strains deleted in cell wall biogenesis related genes. Each bar indicates percentage flocculation for three independent determinations and the error bars represent the respective standard deviations.

The modified Helm's flocculation assay (1999) revealed no significant differences ($p > 0.05$) in the flocculation intensities displayed by transgenic yeast strains. However, macroscopic evaluation reveals that *FLO1*-based flocculation in a *KNR4* deletion background produced smaller flocculation aggregates when compared to flocculation mediated by either the BY4741-F1P or BY4741ΔGPI7-F1P transgenic strains (Figure 4.5).

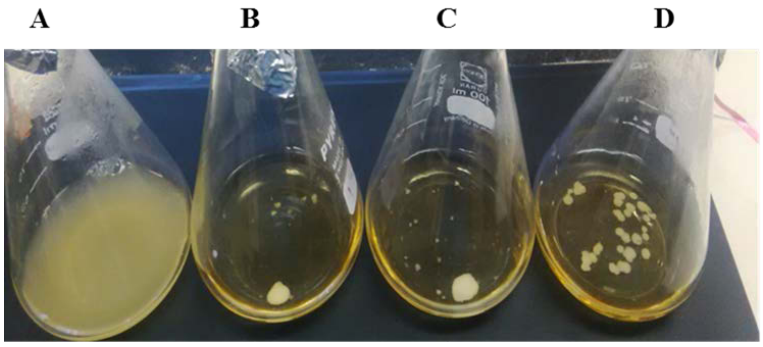

Figure 4.6 Laboratory cultures of (A) BY4741 (B) BY4741-F1P (C) BY4741ΔGPI7-F1P (D) BY4741ΔKNR4-F1P *S. cerevisiae* strains. Only transgenic strains displayed flocculation after 48 hours growth in YPD medium at 30°C with shaking (160 rpm).

4.4.5 Protein concentration determination

Protein concentration analysis of cell-free spent medium from cultures of parental and transgenic strains grown in GCY broth at 30 °C for 48 hours with shaking (160 rpm) reveals a statistically significant ($p < 0.05$) increased protein content (~ 10%) in the spent medium of BY4741ΔKNR4-F1P cultures. The protein concentrations of spent media arising from other strains were comparably lower.

Table 4.3 Protein concentration of spent culture medium

Strain	Protein concentration (µg/ml)
BY4741	4802 ± 250
BY4741-F1P	4826 ± 185
BY4741ΔGPI7	4794 ± 200
BY4741ΔGPI7-F1P	4780 ± 251
BY4741ΔKNR4	4798 ± 301
BY4741ΔKNR4-F1P	5441 ± 297

4.5 Discussion

In this study, the effect of deletions of either of two cell wall biogenesis related genes; *KNR4* or *GPI7*, in the presence of *PGK1* promoter mediated *FLO1* gene expression was investigated. Transgenic *FLO1* overexpression strains were created by chromosomal integration of the constitutive *PGK1* promoter upstream of the *FLO1* ORF. Strong flocculation phenotypes displayed by the transgenic BY4741 and deletion mutant strains was indicative of the integration of the promoter replacement cassette bearing a constitutive *PGK1* promoter upstream of the *FLO1* ORF. A similar strong flocculation phenotype was reported by (Van Mulders*, et al.*, 2009), when the promoter replacement cassette bearing the constitutive *TEF1* promoter was integrated upstream of the *FLO1* gene of BY4742. The replacement *PGK1* promoter of this study mediated a comparable flocculation (99.1%) intensity when compared to the use of the inducible *ADH2* promoter (98%) (Govender*, et al.*, 2008) or the constitutive *TEF1* promoter (99.6%) (Van Mulders*, et al.*, 2009). The engineered yeast strains displayed growth patterns that are similar to that of the host strain, indicating that the relevant characteristics were not compromised by modified *FLO*-gene expression. The wild type BY4741 yeast strain and its deletion mutants BY4741ΔGPI7 and BY4741ΔKNR4 are non-flocculent due to a mutation in *FLO8* which serves as a transcriptional activator of *FLO1* expression (Liu*, et al.*, 2007, Verstrepen & Klis, 2006).

According to the modified Helms assay (D'Hautcourt & Smart, 1999), the data presented in this work showed no significant difference in the strong flocculation intensities of the transgenic strains BY4741-F1P, BY4741ΔGPI7-F1P and BY4741ΔKNR4-F1P. This is of interest as it was previously reported that deletion mutants corresponding to BY4741ΔGPI7 and BY4741ΔKNR4 are mannoprotein hypersecreting (Gonzalez-Ramos & Gonzalez, 2006, Gonzalez-Ramos*, et al.*, 2008). It was reported that 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 a 9 fold increase in released mannoproteins compared to the wild type strain.

Given the above background and that *FLO*-encoded flocculins are mannoproteins it was expected that the transgenic mutant deletion strains (BY4741ΔGPI7-F1P and BY4741ΔKNR4-F1P) created in this study would most probably display decreased flocculation intensities when compared to the wild type transgenic strain (BY4741-F1P). This

concept arises from the virtue of the finding that *FLO1*-encoded mannoproteins would be released into the growth medium and thereby not mediate cellular aggregation. Interestingly and in support of the aforementioned concept, visual observation and documentation did not correlate with the Helms assay; instead it was observed than the transgenic BY4741ΔKNR4-F1P strain produced much smaller flocculation aggregates that BY4741-F1P and BY4741ΔGPI7-F1P transgenic strains. The discrepancy between flocculation intensities observed visually and the modified Helms assay could possibly stem from the semi-quantitative nature of the flocculation assay. As such it is limited in differentiating small differences in very strong flocculent phenotypes (Jin & Speers, 1998).

As mentioned above the transgenic strain BY4741ΔKNR4-F1P displayed a lesser aggregation of cells (smaller floc size) when compared to BY4741-F1P and BY4741ΔGPI7-F1P transgenic strains. It can be tentatively suggested that the decreased *FLO1*-encoded mediated aggregation observed in the ΔKNR4-based transgenic strain may imply release of the flocculins in to the growth medium. This suggestion is seemingly supported by the increased protein concentrations detected in spent medium of cultures of the BY4741ΔKNR4-F1P strain. This may also indicate that the transcriptional activator knr4 is important in the metabolism of *FLO1*-encoded flocculins (Levin, 2005). On the contrary it seems that the BY4741ΔGPI-F1P strain was incapable of releasing the flocculin thereby implying that the *GPI*-encoded enzyme is not critical in the biochemical processing of *FLO1*-encoded flocculins and that its involvement may be salvaged by another enzyme namely *GPI11* (Orlean, 2012).

4.6 Conclusion

The current study produced transgenic BY4741 strains that are capable of constitutively overexpressing the *FLO1*-ORF under the *PGK1* promoter. A strong flocculation phenotype was observed in all transgenic strains. The *KNR4* deletion-based transgenic strain visually displayed smaller flocculation aggregates compared with the transgenic wild type and deletion mutant *GPI7* strains. The study suggests that the BY4741ΔKNR4-F1P transgenic strain is an interesting candidate in that it may release flocculins into the culture medium. In this regard further characterization of the spent medium is required to verify that the intact *FLO1*-encoded mannoproteins are indeed released.

4.7 Acknowledgements

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Chapter 5

GENERAL DISCUSSION AND CONCLUSION

5.1 General discussion and conclusions

The *FLO1*-encoded mannoprotein, found in the outer layer of the yeast cell wall confers a cell to cell adhesion phenotype called flocculation (Guo*, et al.*, 2000). Flocculation is defined as an asexual, reversible and calcium-dependent aggregation of thousands of cells to form clumps that sediment to the bottom of the fermentation medium (Bony*, et al.*, 1997). However, due to the genetic and epigenetic silencing of the *FLO1* gene owing to the nonsense mutation on the key transcriptional activator of *FLO8* in a laboratory strain of *S. cerevisiae,* this phenotype is not expressed in laboratory *S. cerevisiae* strains (Bester*, et al.*, 2006). To study and exploit the functions of *FLO* gene in fundamental research or for industrial purposes, several molecular biological strategies have been employed to transform non-flocculent yeast strains to display the desired flocculation phenotype. The *FLO*1 ORF has been cloned in a plasmid with a heterologous promoter to drive its expression (Cunha*, et al.*, 2006) while other researchers have reported on the promoter replacement strategy using constitutive and inducible promoters to drive the expression of these genes (Govender*, et al.*, 2008, Verstrepen & Thevelein, 2004).

This study is the first to report a PCR-based construction of a promoter replacement cassette utilizing the *PGK1* promoter to drive *FLO1* gene expression. A non-flocculent, laboratory strain of BY4741 genetic background was transformed with the *FLO1p-Zeo^{R}-PGK1-FLO1p* cassette to assess the functionality. The genomic *FLO1* ORF was brought under transcriptional control of the *PGK1* promoter by the replacement of the native promoter sequence. This was evident by the visibly distinct *FLO1* flocculation trait acquired by the transgenic strain. Sugar inhibition studies revealed that the flocculation phenotype expressed was of Flo1 phenotype, since it was exclusively inhibited by the addition of 1M mannose but no inhibition was observed in the presence of the same concentration of glucose. The use of the zeocin™ resistant marker gene in the cassette denies the transgenic strains their "generally regarded as safe" (GRAS) status. However, the strains are well-suited in terms of the aims of the present study which is to evaluate the effect of the *GPI1* and *KNR4* gene deletions on *FLO1*-encoded adhesin mediated flocculation.

It is generally agreed that the yeast cell wall determines the rate and the extent of flocculation and the availability of the cell surface *FLO1*-encoded mannoproteins influence flocculation. Strains lacking genes involved in the cell wall biogenesis have been reported to release an

increased amount of mannoproteins into the growth medium but no data has been put forward that involves over-expression of a specific mannoprotein encoding gene. In an attempt to release *FLO1* encoded mannoproteins into spent medium, this study employed two gene deletion backgrounds. Deletions in *GPI7* and *KNR4* genes were previously shown to alter the composition of the cell wall and lead to hyper-secretion of intact cell wall mannoproteins into the growth medium (Gonzalez-Ramos & Gonzalez, 2006). Consequently, in this study it was hypothesised that strains bearing these gene deletions would result in a decrease in *FLO1* gene-based adhesion phenotypes due to inefficient incorporation of flocculins in the cell surface and promotes their extracellular release.

The Gpi7 protein is involved in the addition of the ethanolamine phosphate side chains to the mannose residues of the GPI anchor. Therefore, the deletion of the *GPI7* gene results in inefficient linkage of GPI-anchored proteins to the cell wall and will be probably released extracellularly (Gaynor*, et al.*, 1999). The transcriptional activator Knr4p is a product of *KNR4* gene was initially isolated by Hong*, et al.* (1994). The loss of *KNR4* leads to altered cell wall structure and composition, including increased concentration of chitin and the reduction of the β- glucan synthase activity (Klis*, et al.*, 2006)

As mentioned previously it is hypothesized that the constitutive over-expression of the *FLO1* gene in the strains lacking *GPI7* and *KNR4* will result in decreased flocculation intensity and increased amount of *FLO1*-encoded mannoproteins being released in the growth medium. However, results from this work, showed that the deletion of *GPI7* ORF in BY4741 strains had no effect on the flocculation intensity. The transgenic deletion strain BY4741-ΔGPI7-F1P showed similar flocculation intensities (>99%) as shown by the transgenic BY4741 strain. Similar behaviour by *GPI7* has been in a different study reported by Gonzalez*, et al.* (2010) which showed that strains with a deletion in *GPI7* when fused with a plasmid containing GFP-Sag1p displayed the same cell surface fluorescence compared to the wild type transgenic strains and the protein release profile was similar to the wild-type.

The deletion of *KNR4 ORF* resulted in smaller floc sizes being formed when compared to the transgenic BY4741 strain. This most probably suggests that the changes in the cell wall composition of the transgenic deletion strain had an impact on the cellular processing of *FLO1*-encoded mannoproteins. Klis and coworkers (2006) suggested that in response to cell wall stress the cell increase the concentration of chitin in the cell wall and the proteins

attached to the increase chitin via the B1,6 glucan. Transgenic wine yeast strains with a deletion of the *KNR4* ORF were reported show an increased basal flocculation (Penacho*, et al.*, 2012).

The modified Helm's assay offered a fast and semi-qualitative method to assess flocculation but the method is limited in the differentiation of strongly flocculent strains. Although there was a similar strong flocculation intensity amongst the transgenic strains, differences in the cell aggregate size were observed which probably suggests that transgenic *KNR4* deletion strains have a decreased amount of flo1 proteins on the cell wall of that strain which may imply release of non-incorporated flocculins in to the growth medium. This assumption appears to be supported by increase protein concentrations being detected in the spent medium of this transgenic strain. It is envisioned that the transgenic yeast strains overexpressing *FLO1* mannoproteins facilitaes the large scale isolation and purification of the intact mannoprotein especially if it were to be released into the growth medium. This glycoprotein reservoir can be utilised in the structural analysis of *FLO1* mannoproteins.

5.2 References

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