Over-expression of *FLO11* encoded mannoprotein in *Saccharomyces cerevisiae*

by

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I, Dr Patrick Govender as supervisor of the MSc study hereby consent to the submission of this MSc dissertation.

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SUMMARY

Yeast cell wall mannoproteins (glycoproteins) released during fermentation have captivated researchers world-wide due to their medical and industrial significance. These mannoproteins have the potential to contribute positively to oenological properties of wine. In *Saccharomyces cerevisiae FLO* genes encode for mannoproteins that are associated with a multiplicity of adhesion phenotypes such as invasive growth, pseudohyphal formation, flocculation and biofilm formation. The *FLO* gene family contains five dominant *FLO* genes; *FLO1*, *FLO5*, *FLO9*, *FLO10* and *FLO11*. Furthermore, there is a paucity of knowledge encompassing the biochemical insight into the fine molecular structure of these glycoproteins. The amino acid sequences of their protein moieties have been established from structural and functional *in silico* analysis of the genomic sequence of *S*. *cerevisiae*, whereas far less structural details are available on the glycosyl moieties of these glycoproteins.

In order to understand the mechanism of action of the glycoproteins, it is imperative to elucidate the structure of these mannoproteins, however, when over-expressed the *FLO* encoded mannoproteins are attached to the cell wall thus making it difficult to isolate for analysis. Consequently, a strategy to isolate these mannoproteins must be formulated. As such, to produce significant quantities of the desired cell wall mannoprotein, a novel strategy was envisaged which utilized the genetic manipulation of *S*. *cerevisiae* strains to over-express and release the *FLO11* cell wall mannoprotein into the spent growth medium. This study utilized a polymerase chain reaction (PCR)-based cloning strategy in order to produce transgenic strains of *S. cerevisiae* in which the native *FLO11* open reading frame (ORF) was placed under the transcriptional control of the constitutive *TEF1* promoter. A flocculation and invasive growth assay was conducted on the three *FLO11*-based transgenic strains. Furthermore, the *FLO11* encoded mannoprotein was over-expressed in *S. cerevisiae* strains containing either a *KNR4* or *TDH3* gene deletion related to cell wall biosynthesis which was previously shown to promote extracellular hyper-secretion.

The research data revealed that the novel strategy employed resulted in the constitutive expression of the *FLO11* ORF in all transgenic strains generated in this study. Interestingly, the *KNR4* and the *TDH3* deletion-based transgenic yeast strains displayed a weaker invasive growth phenotype. The research data tentatively suggest that the over-expression of the *FLO11* encoded mannoprotein in the *KNR4* and *TDH3* deletion strains have the potential to release the *FLO11* encoded mannoprotein into the culture medium.

DEDICATION

This dissertation is dedicated to my incredible parents, Parma and Maeshwari and wonderful sister, Sophie.

BIOGRAPHICAL SKETCH

Kamini Govender was born on the 23rd of April 1991. She is the youngest daughter of Parma and Maeshwari Govender. Kamini Govender matriculated in 2008 with six distinctions and received an overall distinction aggregate score. In 2009, she enrolled at the University of KwaZulu-Natal to study towards a Bachelor of Science degree in Biochemistry and Microbiology, because she enjoyed the intricacies of Science. She completed her degree in 2011. She completed her honours degree in 2012. Due to her fascination with molecular work in yeast in 2013 she enrolled to study towards a Master of Science degree in Biochemistry.

She received a distinction in her Honours dissertation project. She is currently under the supervision of Dr Patrick Govender and Dr Karen Pillay. Kamini is currently researching yeast cell wall mannoproteins of *S. cerevisiae*.

In her spare time she enjoys cooking, watching her favourite soccer team and whenever possible she goes fishing with her dad.

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PREFACE

This dissertation is presented as a compilation of 5 chapters.

Chapter 1 **General Introduction and Project Aims**

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CONTENTS

ABBREVIATIONS

ALS	Alkaline sensitive
AC	Activator Core
ADH_2	Alcohol dehydrogenase 2
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
ANOVA	Analysis of variance
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
cAMP-PKA	Cyclic adenosine monophosphate- protein kinase A
Chs1p	Chitin synthase 1 protein
Chs2p	Chitin synthase 2 protein
Chs3p	Chitin synthase 3 protein
CO_2	Carbon dioxide
Conf	Confirmation
CWP	Cell wall protein
CWI	Cell Wall Integrity
DMSO	Dimethysulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediamine tetra-acetic acid
ER	Endoplasmic reticulum
EUROSCARF	European *Saccharomyces cerevisiae* archive for functional analysis
g	Grams
GPI	Glycosyl Phosphatidyl Inositol
GRAS	Generally Regarded As Safe
GTP	Guanosine triphosphate
IPTG	Isopropyl β-D thiogalactoside
kb	Kilo-base
kV	Kilo-volts
K^+	Potassium
L	Litres
LB	Luria-Bertani Broth
µL	Microliter
µg	Microgram
mg	Milligram
mL	Milliliter
mM	Millimolar
ms	Milliseconds
n	sample size
Na^+	Sodium
NaCl	Sodium Chloride
nm	Nanometres
$OD_{600\ nm}$	Optical density at 600 nm
PCR	Polymerase Chain Reaction

PGK1	Phosphoglycerate kinase 1
PGU1	Polygalacturonase 1
PIR	Protein with internal repeats
pUC118	Plasmid University of California 118
QS	Quorum sensing
rpm	revolutions per minute
S. cerevisiae	*Saccharomyces cerevisiae*
SC	Synthetic complete medium
SDS-PAGE	Sodium Dodecyl Sulphate Poly-Acrylamide Gel Electrophoresis
TEF1	Translation-Elongation Factor 1
UV	Ultraviolet
UAS	Upstream Activator Sequence
V	Volts
vol/vol	Volume/volume
wt/vol	Weight/ volume
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside
YEPD	Yeast Extract Peptone Dextrose
Zeo^R	Zeocin™ resistance gene

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Chapter 1

General Introduction and Project Aims

1. General Introduction and Project Aims

1.1 Introduction

Mannoproteins are polypeptides that are modified by covalently bound branched polymers of mannose or glucose residues and they have been reported to have numerous beneficial effects on wine production (Caridi, 2006; Lesage and Bussey, 2006). *FLO* encoded mannoproteins function in adhesion, as well as cell-substrate and cell-cell recognition (Dranginis *et al.*, 2007), and these mannoproteins are bound covalently to the *β*-1,3-glucan found in the yeast cell wall (Quiros *et al.*, 2010). To accomplish successful application in industry, there is a need for a broader understanding and characterization of these mannoproteins.

The *FLO* gene family of *S*. *cerevisiae* consists of five *FLO* genes, *FLO1*, *FLO5*, *FLO9*, *FLO10* and *FLO11* (Halme et al., 2004). The adhesin Flo11, is the most diverged Flo family member (Van Mulders *et al.*, 2009). The *FLO11* encoded mannoprotein has a number of unusual properties that distinguish it from the rest of the flocculins. It is associated with a variety of adhesive phenotypes such as invasive growth, adhesion to agar as well as plastic, pseudohyphal formation, flor and biofilm formation (Bayly *et al.*, 2005). There is limited knowledge regarding the role of *FLO11* in biofilm formation in *S*. *cerevisiae* in the liquid environment.

The switch from non-adherence to adherence is important for fungal pathogenesis and allows pathogenic yeasts to detect and respond to the unfavourable environmental conditions depending on the host and also assists the pathogen in colonizing tissue (Verstrepen and Klis, 2006). Understanding the mode of yeast biofilms would contribute towards the advancement of immobilized yeast cell technology used in the beer production industry (Bányik *et al.*, 2005). Therefore, this study aims to control the expression of *FLO11* by replacing its native promoter with the constitutive translation-elongation factor one (*TEF1*) promoter.

This study will look at the genetic manipulation of *S. cerevisiae* laboratory strains to over-express and release the *FLO11 encoded* cell wall mannoprotein into the growth medium. This innovative strategy can provide researchers with knowledge to produce significant quantities of the desired cell wall mannoprotein which would provide a reservoir for biochemical structural analysis. According to Gonzalez-Ramos and Gonzalez (2006) and Gonzalez *et al.* (2010), *S*. *cerevisiae* strains which contain a gene deletion related to cell wall biogenesis such as *KNR4 and TDH3* exhibited an increase in the release of mannoproteins. Strains that contained the above gene deletions were regarded as hyper-secretors of mannoproteins (Gonzalez-Ramos and Gonzalez, 2006; Gonzalez *et al.*, 2010).

To analyse the above concept this study attempts to over-produce *FLO11* encoded mannoproteins into the liquid growth medium by utilizing *S*. *cerevisiae* deletions strains that contain a specific deletion of genes related to cell wall biogenesis. The expression of these mannoproteins in deletion mutant strains of BY4741 may have the capacity of over-expression of these mannoproteins into the extracellular environment.

In metabolic and genomic engineering, the optimization of gene expression requires strategies to regulate a desired gene (Nevoigt *et al.*, 2006). This study will focus on the construction of a constitutive based promoter replacement cassette which will be used to drive the expression of yeast cell wall encoded mannoproteins in *S. cerevisiae*. Zeocin™ resistance (Zeo^R) is encoded for by the *Sh ble* gene which originated from *Streptoalloteichus hindustanus* (Alderton *et al.*, 2006).

1.2 The aims of this study

This study aims to construct a promoter replacement cassette via a PCR based strategy that consists of Zeocin™ as a selection marker and the constitutive promoter *TEF1* regulating *FLO11* gene expression. This innovative strategy utilizes hybrid primers that are homologous to the 5' and 3' ends of native *FLO11* ORF respectively. Haploid, non-flocculent and non-invasive *S. cerevisiae* laboratory strains BY4741-wild type, BY4741Δ*KNR4* and BY4741Δ*TDH3* deletion strains will be transformed with the PCR generated promoter cassette (*FLO11*p-*Zeocin*™-*TEF1*-*FLO11*p) via homologous recombination.

The second aim of this study is to determine the effect of the adhesion potential in BY4741 mutant strains containing either *KNR4* or *TDH3* in terms of flocculation and invasive growth intensity. The flocculation and invasive growth adhesion phenotypes of containing the *FLO11p-Zeocin*™*-TEF1-FLO11p* will be investigated in the transgenic mutant strains and will be compared to the wild type. The outcomes of this work would assist in the development of strains with the potential to over-produce adhesins into the cell culture media.

This dissertation thus aims to investigate a biotechnological strategy that would enhance the release of extracellular mannoproteins into the culture medium which would normally be attached to the yeast cell wall in the wild type strains. This dissertation is divided into five chapters, including this **Chapter 1**.

Chapter 2 provides an overview on the current understanding of mannoproteins present in *S*. *cerevisiae* and certain characteristics of their biosynthesis. It also encompasses the promoters employed in this genetic engineering strategy.

Chapter 3 reveals the novel molecular strategy used to construct the promoter replacement cassette *FLO1*p-Zeocin™-*TEF1-FLO1*p.

Chapter 4 pertains to the over-expression of the dominant flocculation gene *FLO11*, of the haploid non-flocculent and non-invasive *S*. *cerevisiae* strain which was placed under the transcriptional control of the constitutive *TEF1* promoter. The effects of cell wall based gene deletion strains *KNR4* and *TDH3* in *S*. *cerevisiae* was assessed. The adhesion potential of these deletion transgenic strains in terms of flocculation and invasive growth intensity was determined and compared to the wild type parental strain.

Lastly, **Chapter 5** describes conclusions drawn from this study, and reflects on future research endeavours.

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Chapter 2

Literature Review

2 Mannoproteins of *Saccharomyces cerevisiae*

2.1 Introduction

The aim of genetically engineering *S. cerevisiae* strains is to optimise biotechnological applications in industry. *S. cerevisiae* is an important industrial and research microorganism utilized for metabolic protein expression and pathway engineering (Fang *et al.*, 2011). The use of *S. cerevisiae* in biotechnology is lucrative because it has the ability to be genetically modified by recombinant DNA (deoxyribonucleic acid) technology, a process that was enhanced by the discovery of the complete genome sequence of *S. cerevisiae*, which was established by Goffeau *et al.* (1996) (Idiris *et al.*, 2010; Ostergaard *et al.*, 2000).

Yeast is a model system which has been in the forefront for development of analytical techniques aiding in the understanding of the transcriptome in addition to the cellular genome (Lashkari *et al.*, 1997), metabolome (Smedsgaard and Nielsen, 2005) and proteome (Pham and Wright, 2007). Proteomic analysis can be defined as the study of the protein complement that is expressed by a genome (Pennington *et al.*, 1997). Proteomic analysis has the ability to elucidate the properties of biological systems which cannot be elucidated by the DNA sequence or mRNA (messenger ribonucleic acid) expression only (Gygi *et al.*, 1999). Yeast expression systems are cost-effective in comparison to mammalian expression systems; however a yeast expression system produces a low level of recombinant proteins. Consequently, new methods which increase the levels of recombinant proteins in *S. cerevisiae* are necessary (Sugano *et al.*, 2010).

Optimization strategies of engineered pathways contain static controls for fine-tuning of promoter strength to allow for a balanced pathway reaction flux thereby resulting in a higher product yield (Blazeck and Alper, 2013). *S. cerevisiae* is a vital industrial and laboratory microorganism for biotechnological applications. *S. cerevisiae* is easily cultured and classified as a GRAS (generally recognized as safe) microorganism (Da Silva and Srikrishnan, 2012). It has been used extensively in the wine, brewing, and bio-ethanol industries. Metabolic engineers have also used it in the production of biochemical products, such as, isoprenoids and ergosterol (Nevoigt, 2008). The development and optimization of strategies and tools are essential for promoter regulation which ultimately controls the expression as well as the relative yield of the desired product (Teo and Chang, 2013).

Flo mannoproteins enhance the outer cell wall structure as well as participate in various cellular adhesion interactions. Mannoproteins are highly glycosylated in nature, thereby making the direct analysis of the cell wall mannoproteins problematic (Bester, 2010). Genome transcription analysis as well as proteome analysis of the cell wall can provide more information regarding *FLO* encoded mannoproteins. This information would be valuable in the understanding of cell wall dynamics and for numerous industrial applications (Bester, 2010). By over-expressing the yeast cell wall mannoproteins possible insight can be established to help optimize biotechnological industrial processes.

Research studies have indicated that the expression of particular *FLO* genes in *S*. *cerevisiae* are responsible for specific cell wall-dependent phenotypes, such as cell to cell adhesion, flocculation, surface hydrophobicity, substrate adhesion, invasive growth on substrates, and biofilm formation (Cunha *et al.*, 2006; Govender *et al.*, 2010; Govender *et al.*, 2008; Guo *et al.*, 2000; Van Mulders *et al.*, 2009; Verstrepen and Klis, 2006). In non-flocculent laboratory yeast strains, it has been established that it is possible to regulate adhesion and flocculation phenotypes to the desired specifications by modifying the expression of silent but dominant flocculation (*FLO*) genes (Bauer *et al.*, 2010). Refinement of yeast flocculation phenotypes could lead to the desired phenotype and result in the improvement of biotechnological fermentation products for example biofuels, food, beverages, and the processing of industrially produced peptides (Bauer *et al.*, 2010). Recombinant DNA techniques can be used to genetically modify industrial strains of yeast and control the expression of their *FLO* genes, thereby generating more stable strains with the desired flocculation phenotype (Govender *et al.*, 2008; Verstrepen *et al.*, 2004). This study will use recombinant DNA techniques as well as a PCR based strategy to construct a promoter replacement cassette to regulate the expression of *FLO11* encoded mannoprotein in *S*. *cerevisiae* strains.

2.2 The cell wall structure of *S. cerevisiae*

Yeast cell walls are semi-rigid structures composed primarily of highly glycosylated proteins and sugar polymers. The cell wall proteins perform vital roles in the cell wall structure and the regulation of numerous phenotypes. Adhesins form part of these cell wall proteins, and they confer an assortment of adhesion phenotypes such as flocculation (Lambrechts *et al.*, 1996; Lo and Dranginis, 1996; Teunissen and Steensma, 1995). In *S. cerevisiae*, the cell wall composition varies according to the strain, culture conditions, and the genetic background of yeast strains utilised. The cell wall is comprised of three macromolecules: mannoproteins, *β*-glucans and chitin (Figure 2.1). The composition of the yeast cell wall is illustrated in Table 2.1.

According to Aguilar-Uscanga and Francois (2003) mannoproteins account for approximately 40% of the yeast cell's dry mass and are located in the outer layer of the yeast cell wall. The *β*-glucans make up approximately 60% of the yeast cell's dry mass with chitin, which is a polymer of *N*-acetyl-glucosamine. The inner layer of the yeast cell wall consists of *β*-glucans that are attached to chitin. The *β*-glucans can be sub-divided into two polymer categories, *β*-1,3-glucan and *β*-1,6-glucan polymers (Aguilar-Uscanga and Francois, 2003; Govender, 2009).

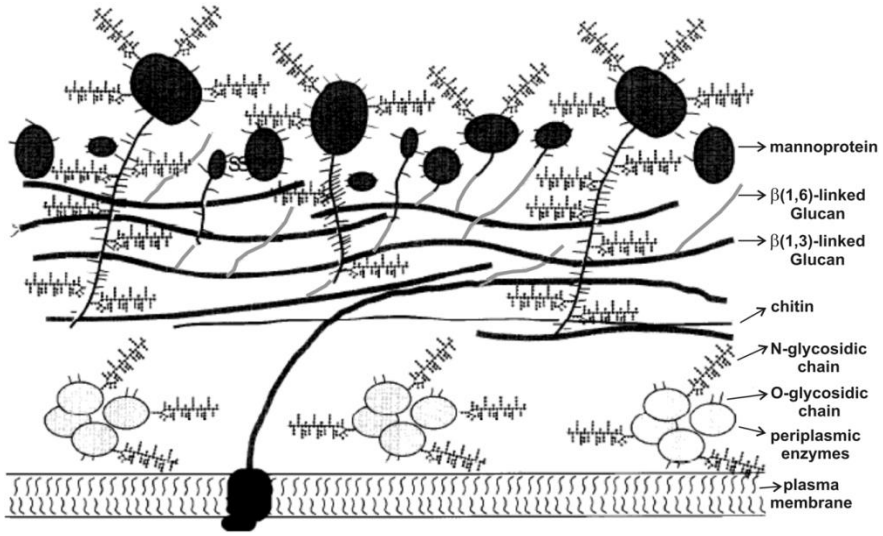


Figure 2.1: The structure and composition of the cell wall of *S. cerevisiae*. The cell wall comprises of two layers. The inner layer provides the cell wall with strength and consists of *β*-1,3-glucan and *β*-1,6-glucan which is linked to chitin. The outer layer of the cell wall consists of mannoproteins which are covalently linked to the inner glucan layer. (Adapted from Schreuder *et al.*, 1996).

In *S. cerevisiae* the cell wall determines the cell's size, provides the cell with mechanical and osmotic protection and allows selective uptake of macromolecules (Penacho *et al.*, 2011; Verstrepen and Klis, 2006). The yeast cell wall is 100-200 nm thick and is found surrounding the plasma membrane (Dallies *et al.*, 1998). The cell wall is strong and elastic in nature which is vital for the maintenance of cell integrity, shape and development through the cell cycle. In morphogenesis, growth and the cell's response to environmental stresses, the cell wall is altered in a highly regulated manner (Levin, 2011; Verstrepen and Klis, 2006), a process that is regulated mainly by the cell wall integrity (CWI) signalling pathway (Levin, 2011). The CWI signalling pathway transmits the cell wall's stress signals from the surface to the enzyme Rho1 GTPase, which mobilizes a response via an assortment of effectors (Levin, 2011). The activation of the CWI signalling controls the production of numerous carbohydrate polymers of the cell wall and their delivery to the location of the cell wall remodelling (Levin, 2011).

Table 2.1: The cell wall composition of *S. cerevisiae* (Aguilar-Uscanga and Francois, 2003; Govender, 2009; Klis *et al.*, 2002; Verstrepen and Klis, 2006)

Type of molecule	Dry cell weight (%)	Level of branching	Polymerization
Chitin	1.5-6	Linear branching	120
Mannoproteins	30-50	High branching	Variable
β-1,3-glucans	30-45	High branching	1500
β-1,6-glucans	5-10	Moderate branching	150

2.2.1 *β*-1,3-glucans

The *β*-1,3-glucan provides mechanical strength to the cell wall and its chains belong to the hollow helix family. Their shape is similar to a wire spring and they exist in many states of extension, a property that provides elasticity to the cell wall (Klis *et al.*, 2002). The degree of polymerization may vary based on environmental and growth conditions. The moderate degree of branching in mature *β*-1,3-glucan molecules can prevent large-scale crystallization on the surface of regenerating spheroplasts. There are two *β*-1,3-glucan synthase complexes that are found in yeast and they consist of either *FKS1* or *GSC2*/*FKS2* depending on the environmental conditions. *FKS1* and *GSC2*/*FKS2* are multiple-spanning trans-membrane proteins which are vital for the synthesis of *β*-1,3-glucan (Klis *et al.*, 2002).

2.2.2 *β*-1,6-glucan

β-1,6-glucan in its mature state is extremely branched, water-soluble polymer that consists of approximately 130 glucose monomers. The *β*-1,6-glucan is located in the cell wall is used to connect GPI-dependent yeast cell wall proteins to the *β*-1,3-glucan network. It can also function as an acceptor site for chitin under physiological conditions of cell wall-related stress. Various cell surface proteins, endoplasmic reticulum (ER), resident proteins and Golgi proteins strongly influence the *β*-1,6-glucan levels in the cell wall (Klis *et al.*, 2002).

2.2.3 Chitin

Chitin is composed of *β*-1,4-*N*-acetylglucosamine residues that are located in and around yeast cell's bud scars which occur as linear chains. In cells with a genetically weak cell wall, chitin synthesis is triggered as a salvage mechanism and the levels of chitin in the lateral cell walls can become as high as twenty percent.

The reducing end of the chitin chain is available for coupling to the acceptor sites of *β*-1,3-glucan and *β*-1,6-glucan, when it surfaces from the pore of the plasma membrane (Klis *et al.*, 2002). There are three chitin synthases in yeast, Chs1p, Chs2p, and Chs3p. The first synthase, Chs1p, is a repair enzyme which adds chitin to the birth scar on daughter cells towards the end of cytokinesis. The second chitin synthase, Chs2p, is responsible for the synthesis of chitin in the primary septum. The third chitin synthase, Chs3p, is associated with the deposition of chitin as a ring at the base of a rising bud and is usually retained by the mother cell after cell division. The Chs3p synthase synthesizes approximately ninety percent of the chitin in *S. cerevisiae* (Bulik *et al.*, 2003).

2.2.4 Cell wall mannoproteins

2.2.4.1 Mannoprotein cell wall attachment

Yeast cell wall proteins are mainly located in the outer cell wall and carry out essential functions in defining the cell wall's characteristics (Bester, 2010). These cell wall proteins undergo extensive modification and intra-cellular post-translational processing (Bester, 2010). The above modification and processing takes place prior to cell wall incorporation and secretion. This includes the large-scale addition of un-branched or branched sugar polymers (glycosylation) mainly comprising of mannose residues. Yeast cell wall proteins are highly mannosylated polypeptides, often comprising fifty to ninety percent carbohydrate by weight and these modified cell wall proteins are referred to as mannoproteins or yeast proteoglycans (Bester, 2010; Govender, 2009).

The majority of mannoproteins are covalently linked to the cell wall matrix and the rest are either anchored to the plasma membrane, released into the extra-cellular medium or non-covalently linked with the cell wall. Mannoproteins that are covalently linked to the cell wall can be categorized into three groups, which is determined by the precise nature of their covalent bond (De Groot *et al.*, 2005). The first group is attached to the cell wall by a glycosylphosphatidylinositol (GPI) remnant; the second group of mannoproteins are linked by an unidentified linkage, which is alkali sensitive, and the third group may contain mannoproteins that are bound to the cell wall by an ionic interaction or by weak disulphide bonds. The extraction of mannoproteins from the cell wall is facilitated by reducing agents such as dithiothreitol (DTT) (Cappellaro *et al.*, 1998) or *β*-mercaptoethanol (Mrsa *et al.*, 1997) which provides evidence of a disulphide linkage.

Yeast cell wall mannoproteins are commonly referred to as yeast mannans because of their high mannose content (Gemmill and Trimble, 1999). The mannoprotein function can be grouped into two categories. The first category of mannoproteins acts as enzymes which modify the cell wall, whilst the second category comprise of components known as adhesins or flocculins which determine the adhesion interactions and the cell wall structure. The exact cell wall mannoprotein content differs in response to growth phase, cell age and the surrounding environment of the cell (Klis *et al.*, 2006).

These mannoproteins form the outer cell wall layer and are highly glycosylated. The outer layer of the mannoproteins are less permeable to macromolecules as compared to the internal fibrillar layer because of the presence of disulphide bridges and highly branched carbohydrate side chains which are linked to asparagine. Mannoproteins are linked to the cell wall by a covalent linkage to the *β*-glucans. There are two types of cell wall glycosylation modifications in *S. cerevisiae*, *N*-linked glycosylation and the *O*-linked glycosylation (Figure 2.2). The *N*-linked glycosylation involves a glycosidic-linked oligosaccharide to an amide group of asparagine residues. An *O*-linked glycosylation involves the attachment of an oligosaccharide moiety to the hydroxyl group of serine and threonine residues (Govender, 2009; Klis *et al.*, 2002).

In *S. cerevisiae*, proteins that are targeted for incorporation into the cell wall or secretion are glycosylated by passing through the Golgi apparatus and endoplasmic reticulum (ER). The attachment of structures and mannosyl groups are initiated in the ER whilst further modification or elongation of structures occurs in the Golgi apparatus. Mannosyl groups can be modified further by the addition of a covalent mannosyl-phosphate which carries an overall net negative charge (Jigami and Odani, 1999). The negatively charged phosphodiester and mannosyl-phosphate bonds enhance the hydrophobicity of mannoproteins, whereas glycosylation structures are hydrophilic.

The overall cell surface hydrophobicity may vary due to the degree to which mannoproteins are modified by the glycosylation structures as well as the extent these amino acid side chains of proteins and structures are exposed to the environment. Protein glycosylation is imperative for cell survival and integrity because it contributes to the correct functioning and folding of mannoproteins. The precise molecular function of the yeast cells glycosylation structures remains unclear (Arnold and Tanner, 1982; Dean, 1999; Gentzsch and Tanner, 1996).

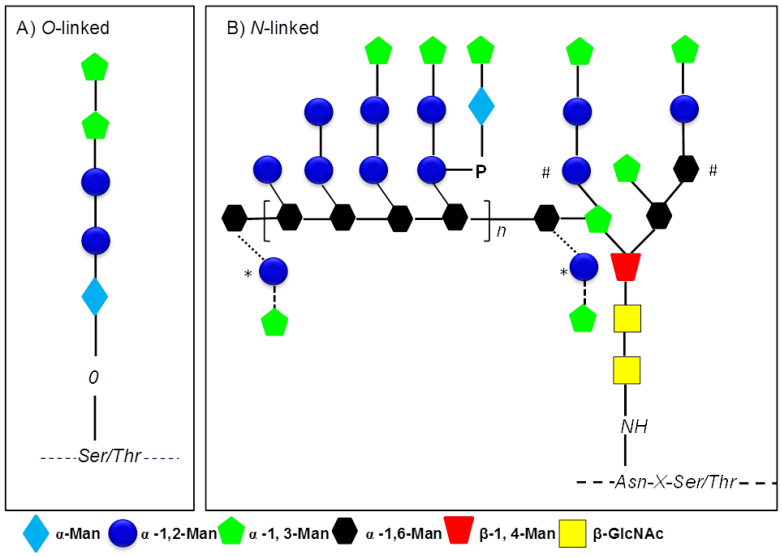


Figure 2.2: Glycosylation of cell wall proteins in *S. cerevisiae*. A) *O*-linked mannosylation is the attachment of short mannosyl chains linked to the hydroxyl group of threonine and serine residues. B) *N*-linked glycosylation occurs when carbohydrate side chains are linked to an amide group of asparagine residues. The number of repeating units is represented by (*n*), the *N*-chains vary and can reach a value as high as fifteen (Ballou, 1990). The asterisk (*) denotes the alternative positions of *α*-1,2-linked mannose. This addition is suggested to prevent elongation and is not located in the cores to which the outer chains are added. The hashtag (#) denotes additional sites of phosphorylation. Man, Mannose; GlcNAc, *N*-acetyl-glucosamine; P, phosphate. (Adapted from Govender, 2009; Herscovics and Orlean, 1993).

2.2.4.2 *O*-linked glycosylation

O-linked glycosylation structures comprise of one to five linear arranged mannose residues (Goto, 2007). Glycosylation is initiated in the ER by adding a single mannose residue to either threonine or serine of the target protein. The rest of the glycosylation structure is completed in the Golgi apparatus. These structures may be modified further by the addition of a mannosylphosphate group (Nakayama *et al.*, 1998). *O*-linked glycosylation groups, which resemble short and rigid “stalk like” structures, are considered to function in the maintenance of the tertiary structure of various mannoproteins. It was hypothesised that *O*-linked glycosylation of long threonine or serine repeats would result in mannoproteins of an elongated structure which would allow the protein to stretch throughout various sections of the cell wall matrix (Jentoft, 1990). Consequently, the extra-cellular domains for example adhesin receptors would be found on the surface of the cell wall, thereby enabling the adhesion receptors to interact freely with substrates (Dranginis *et al.*, 2007; Verstrepen and Klis, 2006). It is approximated that *O*-linked glycosylation constitutes a major proportion of the total glycosylation of mannoproteins, about double that of *N*-linked glycosylation (Strahl-Bolsinger *et al.*, 1999).

2.2.4.3 *N*-linked glycosylation

N-linked glycosyl groups are highly branched structures that display variability and contain a branched pre-formed core structure consisting of approximately nine mannose residues covalently linked to Asparagine (Asn) residues of specific target proteins in the ER (Figure 2.2). Downstream in the secretory pathway the core structure would be modified and extended by the addition of mannose polymer structures that are branched (outer chain). The hyper-mannosylated proteins could comprise of more than two-hundred mannose residues located in the outer chain and more than fifty mannose residues found in the core region of *N*-linked glycosylation groups (Dean, 1999). *N*-linked glycosylation structures are further modified by the negatively charged mannosylphosphate similar to *O*-linked glycosylation (Wang *et al.*, 1997). The degree of *N*-linked glycosylation can be controlled by nutrients and the degree of modification of *N*-linked glycosyl groups was shown to be affected by specific carbon source (Kukuruzinska and Lennon, 1994) and nitrogen source availability (Nakamura *et al.*, 1993).

2.2.4.4 Alkaline sensitive (ALS) linked mannoproteins

Mannoproteins that are alkaline sensitive (ALS) linked are emitted from the cell wall by a mild alkali (NaOH) treatment. These mannoproteins are attached to *β*-1,3-glucan polymer located in the inner cell wall layer via an uncharacterised linkage (Kapteyn *et al.*, 1999). These proteins consist mainly of the Pir protein family that comprise of high number of serine and threonine internal repeats.

2.2.4.5 Proteins with internal repeats (PIR-CWPs) and glycosylphosphatidylinositol linked mannoproteins

Glycosyl-phosphatidyl inositol-anchorage is vital in yeast for cell viability and the maintenance of normal cell morphology. Glycoproteins act as surface receptors, structural components, hydrolytic enzymes and adhesion proteins in mating. These GPI glycoproteins are biosynthesized on ribosomes that are ER-bound and have a pre-assembled GPI-anchor. This anchor is substituted for a C-terminal hydrophobic signal via a transamidation reaction in the lumen of the ER membrane before the GPI glycoproteins are exported to the cell surface in specialized vesicle structures. The proteins which have GPI-anchors are modified with *N*- and *O*-linked sugars in the Golgi apparatus and ER before they are transported to the outer portion of the plasma membrane, where they stay linked via the lipid part of the GPI anchor (Gonzalez *et al.*, 2010). The GPI-attachment signal consists of an attachment domain, a spacer domain which is about eight to twelve amino acids long and a terminal domain of about eleven amino acids. The GPI-anchored proteins in yeast forms an integral part of the yeast cell wall (Verstrepen and Klis, 2006).

There are two classes of cell wall proteins, the GPI-anchored cell wall proteins (GPI-CWPs) and the proteins with internal repeats (PIR-CWPs). The PIR-CWPs have internal repeats and are linked directly to *β*-1,3-glucan. They are similar to GPI-CWPs in the fact that they are highly *O*-glycosylated but differ because PIR proteins lack GPI-anchors (Kapteyn *et al.*, 1999). The function of cell wall proteins is not clearly defined however, it was found that all *FLO* proteins are anchored to the cell wall via a GPI-anchored remnant glycoprotein (Govender *et al.*, 2008). The GPI modified proteins such as Flo1p, Flo5p, Flo9p, Flo10p and Flo11p have been associated in an array of adhesion events such as flocculation and biofilm formation (Govender *et al.*, 2008; Verstrepen and Klis, 2006).

Cell wall proteins are mainly linked by a GPI remnant to the *β*-1,6-glucan found in the outer layer in the cell wall. This layer of proteins defines the fibrillar appearance of the outer cell wall and functions either directly or indirectly in adhesion phenotypes. Proteins that are designated for the GPI-linked cell wall attachment have a supplementary C-terminal signal sequence employed in the covalent attachment of the GPI anchor and an N-terminal signal sequence used to gain access into the ER (Pittet and Conzelmann, 2007).

2.2.4.6 The structure of adhesins

Structural and functional analysis of the genomic sequence of laboratory strains of *S. cerevisiae* revealed that mature *FLO* gene-encoded mannoproteins are components of the outermost layer of the cell wall and are referred to as flocculins or adhesins. Flo proteins are glycoproteins which possess a GPI-link. A common characteristic feature shared amongst these Flo proteins is a three-domain structure (Figure 2.3) which consists of a carboxyl-terminal domain containing a GPI-anchoring sequence, a central domain that has a high number of recurring sequences with a high number of threonine and serine residues, and an amino terminal domain, known as the *N*-terminal domain which confers adhesion (Govender *et al.*, 2008; Van Mulders *et al.*, 2009; Verstrepen and Klis, 2006). The C-terminal domain has a GPI anchor which serves as an attachment site that covalently binds to the *FLO* proteins of the *β*-1, 6-glucans of the yeast cell wall by a GPI remnant. The carboxyl terminal region is glycosidically linked via a lipid-less remnant of its GPI-anchor moiety to the inner cell wall *β*-1, 6-glucan polysaccharide skeletal network. The *N*-terminal domain contains a binding site for carbohydrate receptors, such as mannan, which confers adhesion (Goossens and Willaert, 2010).

The GPI attachment site is found inside the signal sequence used for GPI attachment. GPI attachment facilitates the removal of the remainder of the protein located on the C terminal region of the GPI attachment site.

The *N*-terminal domain has the protein site responsible for the cell wall enzyme's catalytic function or the affinity in adhesion proteins. Majority of the GPI-proteins have long internal regions that are rich in serine (Ser) and threonine (Thr), and are located in the C-terminal site of the protein (Caro *et al.*, 1997; Verstrepen *et al.*, 2005). Regions rich in serine and threonine have the tendency to form tandem repeats in some GPI protein families for instance the flocculation (*FLO*) protein family (Verstrepen *et al.*, 2004).

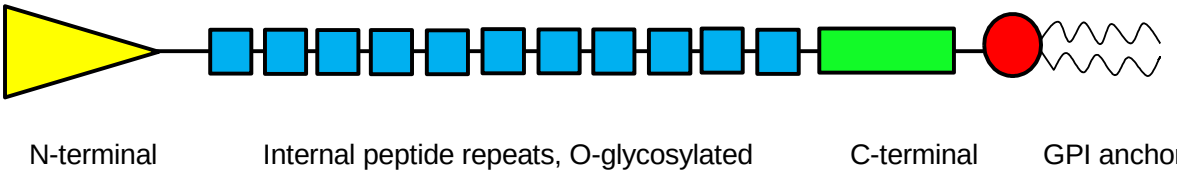


Figure 2.3: The structural domain of an adhesin. (Adapted from Verstrepen and Fink, 2009).

The lectin-like proteins or flocculins protrude out of the cell wall of yeast cells and selectively bind to cell-wall mannose residues of adjacent cells. Calcium ions are essential for the activation of flocculins (Goossens *et al.*, 2011; Verstrepen *et al.*, 2003). The cell surface anchoring and secretion of yeast adhesins is depicted in Figure 2.4.

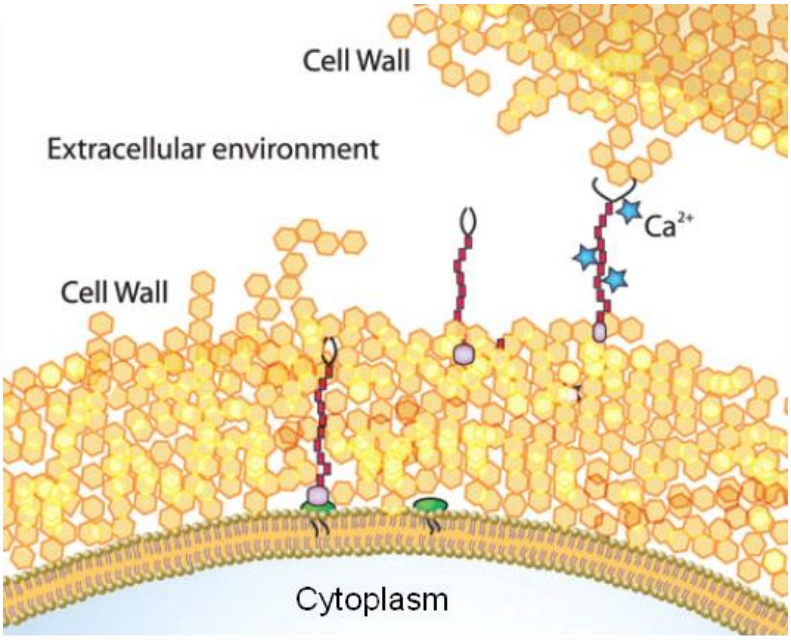


Figure 2.4.: Cell surface anchoring and secretion of yeast adhesins (Verstrepen and Klis, 2006).

2.2.5 Cell wall integrity in deletion strains *KNR4* and *TDH3*

The synthesis and expression of cell wall proteins is highly controlled and regulated (Klis *et al.*, 2002). This study will focus on the yeast strains containing either a *KNR4* or *TDH3* gene deletion. This study aims to assess the hyper-secretion ability of yeast strains bearing a deletion in *KNR4* or *TDH3* to over-express *FLO11* encoded mannoproteins.

The *KNR4*/*SMI1* gene was cloned via the complementation of a K9 killer toxin which is a glucan synthase inhibitor resistant mutant in *Hansenula mrakii* (Hong *et al.*, 1994). The *SMI1*Δmutant contains a highly permeable cell wall and displays decreased cell wall *β*-1,3-glucan content and glucan synthase activity (Hong *et al.*, 1994; Martin *et al.*, 1999). Biochemical and genetic evidence suggests that the Smi1p plays a role in the *PKC1-SLT2* signalling cascade by controlling the Slt2p kinase activity (Martin-Yken *et al.*, 2002; Martin-Yken *et al.*, 2003). *SMI1*/*KNR4* is a glucan synthesis regulator. The *KNR4* encoded enzyme is involved in the yeast cell wall integrity pathway. It activates two transcriptional factors, Rlm1p which is implicated in the expression of cell wall-related genes and Swi4p which participates in cell cycle regulation (Figure 2.5) (Gonzalez-Ramos *et al.*, 2008).

Mutations in *KNR4* result in the impairment of the cell wall integrity (Bulik *et al.*, 2003). The *KNR4* gene was initially isolated while researchers were investigating mutations that affect *β*-1,3-glucan biosynthesis (Gonzalez-Ramos *et al.*, 2008). The loss of *KNR4* in yeast results in an altered cell wall structure and composition, causing an increase in the release of mannoproteins (Gonzalez-Ramos and Gonzalez, 2006). The *KNR4* gene product is involved in the *β*-1,3-glucan biosynthesis (Hong *et al.*, 1994). The *KNR4* deletion was reported to reduce *β*-1,3-glucan synthase activity and cause an increase in the chitin content of the cell wall (Hong *et al.*, 1994; Martin *et al.*, 1999).

In research conducted by Gonzalez *et al.* (2010) it was observed that strains harbouring a deletion in *TDH3* exhibited a deficit in the anchoring ability of GPI–CWP reporter protein to the yeast cell wall. *TDH3* is a non-essential gene (Gonzalez *et al.*, 2010). *TDH3* is an isozyme that encodes for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and is a highly expressed glycolytic enzyme. The GAPDH is capable of directing intracellular proteins to the yeast cell wall (Alloush *et al.*, 1997; Edwards *et al.*, 1999; Pardo *et al.*, 1999). *TDH3* has the ability to express invertase activity at the yeast cell surface, likely from encoding regions of *TDH3* that are capable of targeting fusion protein to the yeast cell wall (Delgado *et al.*, 2003). Using immune-electron microscopy in *S. cerevisiae* and *C. albicans*, the GAPDH protein was detected and located at the outer surface of the yeast cell wall as well as the cytoplasm of yeast cells (Delgado *et al.*, 2001).

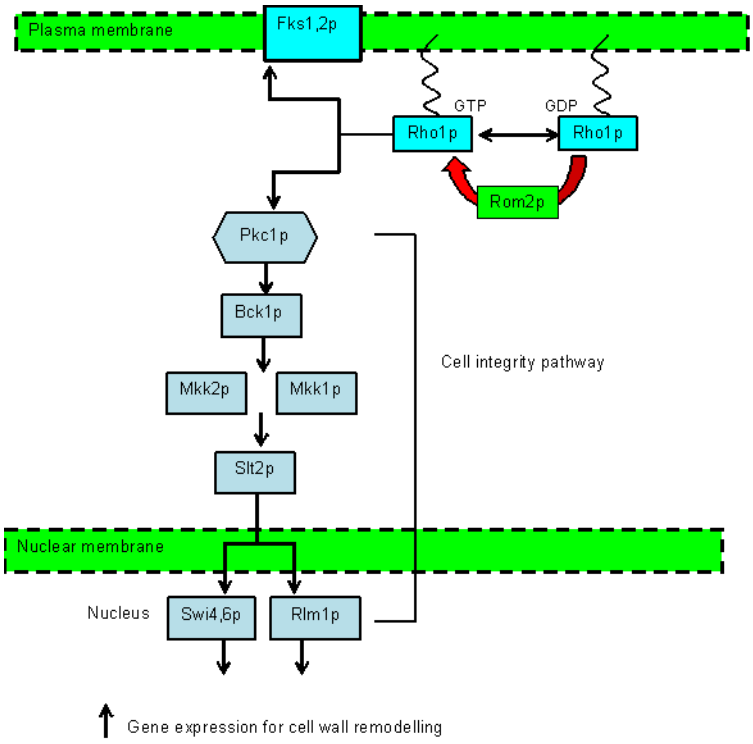


Figure 2.5: Cell wall integrity pathway (adapted from Zhong *et al.*, 2007).

2.2.6 Yeast adhesion interactions

2.2.6.1 Sexual adhesion in cells

Agglutinins are adhesins that are associated to mating in cells and function in cell to cell adherence of haploid cells of opposite mating type (α or a) following the formation of a “shmoo” (Chen and Thorner, 2007). Mating type a which is expressed by the a agglutinin which consists of two subunits of Aga1p, that is anchored to the cell wall and Aga2p, that is linked to Aga1p by disulphide bonds (Cappellaro *et al.*, 1994). Sag1p functions as the α cells agglutinin and binds with high affinity to Aga2p (Cappellaro *et al.*, 1994; Zhao *et al.*, 2001). These agglutinins are essential for the efficient mating in liquid cultures by maintaining cells in close vicinity for cell fusion to occur (Roy *et al.*, 1991). In sexual agglutination or yeast mating, haploid complementary strains α and a sexes of *S. cerevisiae* exchange a and α factors that are small peptide pheromones which promotes a succession of physiological changes. These changes aid in cell to cell aggregation to form diploids, events that take place prior to cell- and nucleus-fusion. The adhesion between cells results from protein-protein interactions between a and α agglutinins that are anchored in complementary cell walls (Calleja, 1987).

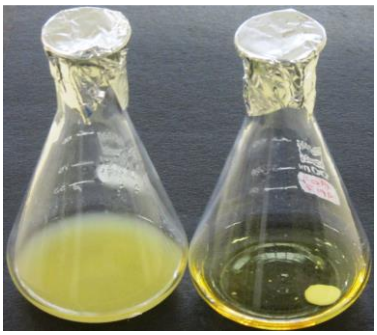
2.2.6.2 Non-sexual adhesion phenotypes

During vegetative growth, sexual agglutinins are expressed at a low level (Terrance and Lipke, 1987), hence these sexual agglutinins do not contribute substantially to cell-cell adhesion (Bester, 2010). In juxtaposition to the above, there is an assortment of non-sexual phenotypes that have been characterised. The non-sexual phenotypes comprise of “flor” formation, flocculation, agar and polystyrene adhesion, the development of pseudohyphae, agar invasion as well as “mat” formation. “Flor” formation is associated with cell aggregation and comprises of a floating air-liquid interfacial biofilm.

2.3 Flocculation

2.3.1 The mechanism of yeast flocculation

Yeast flocculation is a phenomenon that has been studied for centuries. It is referred to as reversible, asexual and a calcium dependant aggregation process of yeast cells to produce flocs (Figure 2.6). These flocs possess thousands of cells which rapidly sediment to the bottom of the liquid growth substrate (Bony *et al.*, 1997; Van Mulders *et al.*, 2009; Verstrepen and Klis, 2006). It is mediated by lectin-like proteins, which protrude out the yeast cells and bind specifically to mannose residues that are located on the surface of adjacent yeast cell walls. It provides a means to remove cell aggregates from the final product after the primary phase of fermentation (Verstrepen *et al.*, 2003; Vidgren and Londesborough, 2011).



A B

Figure 2.6: Laboratory yeast cultures of non-flocculent (A) and flocculent (B) *S. cerevisiae* strains.

Yeast flocculation in *S. cerevisiae* is initiated by specific cell surface flocculins or lectins that have the ability of binding directly to the mannose residues of mannan molecules on neighbouring cells. It results in cellular aggregation of yeast cells and floc formation resulting in the rapid settling of yeast biomass from the fermentation medium (Govender *et al.*, 2008). Yeast flocculation was suggested to be an escape mechanism from unfavourable conditions and protects the inner cells in the floc (Verstrepen and Klis, 2006).

2.4 The genetics of yeast flocculation

A family of subtelomeric genes, *FLO1, FLO5, FLO9* and *FLO10* encode for specific lectins which are responsible for yeast flocculation. *FLO1, FLO5, FLO9* and *FLO10* are found adjacent to telomeres (Figure 2.7) (Govender *et al.*, 2008; Halme *et al.*, 2004; Soares, 2010; Verstrepen *et al.*, 2004). These subtelomeric *FLO* genes are regarded as transcriptionally silent. A non-subtelomeric gene, *FLO11* encodes for a protein which has been associated with yeast flor formation, substrate adhesion and invasive growth (Govender *et al.*, 2008). *FLO11* is neither centromeric nor telomeric and it is more than forty base pairs away from the telomeres (Figure 2.7). The silencing of *FLO11* is similar to the silencing of the other four subtelomeric *FLO* genes (Govender *et al.*, 2008; Halme *et al.*, 2004; Soares, 2010; Verstrepen *et al.*, 2004).

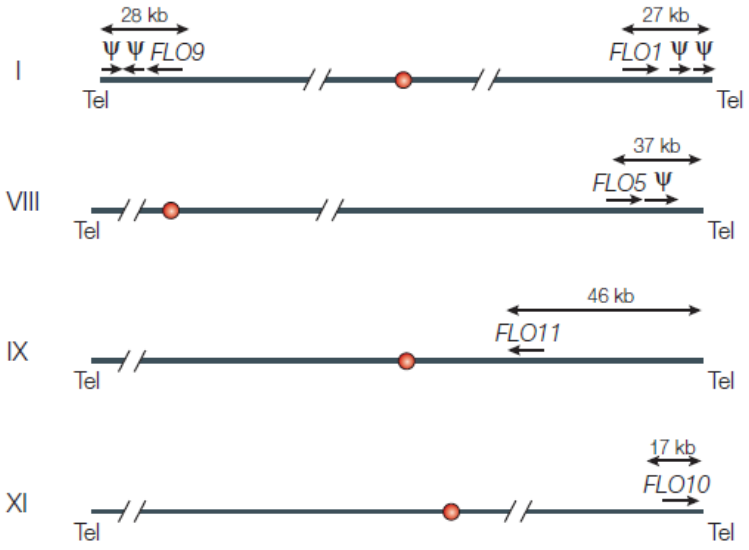


Figure 2.7: The chromosomal localization of *FLO* genes in *S. cerevisiae*. The number on the left indicates the yeast chromosomes on which the *FLO* genes are located. The dots represent the centromeres. The silent *FLO* genes are located within 40 kilo bases (kb) from the telomeres (TEL) (adapted from Verstrepen *et al.*, 2004).

FLO1 is a dominant gene and is localized twenty four kilo bases from the right end of chromosome one. *FLO5*, *FLO9* and *FLO10* and their gene products are homologous to the *FLO1* gene with 96%, 94% and 58%, respectively (Soares, 2010). *FLO11* encoded mannoprotein is approximately 37% similar to the *FLO1* encoded mannoprotein (Lambrechts *et al.*, 1996; Lo and Dranginis, 1996).

2.4.1 The generation of variability by recombination of intragenic repeats

Evolutionary studies have indicated that the recombination of intragenic repeats generates variability thereby creating adhesin genes which are rapidly expanding groups of paralogues in *S. cerevisiae*'s genome. The variability of these genes was seen in industrial *S. cerevisiae* brewer's strains that closely resemble each other but were considerably different in length. This intragenic variability was suggested to be responsible for phenotypic changes in related strains (Verstrepen and Klis, 2006). The central domain of Flo proteins is the main factor responsible for the variability of adhesin-associated phenotypes (Figure 2.8). The central domain consists of tandem repeats that are high in serine and threonine residues and are regarded as highly unstable. These tandem repeats drive evolution and divergence of *FLO* genes, by slippage and the recombination reactions between *FLO* genes (Figure 2.9) (Van Mulders *et al.*, 2009).

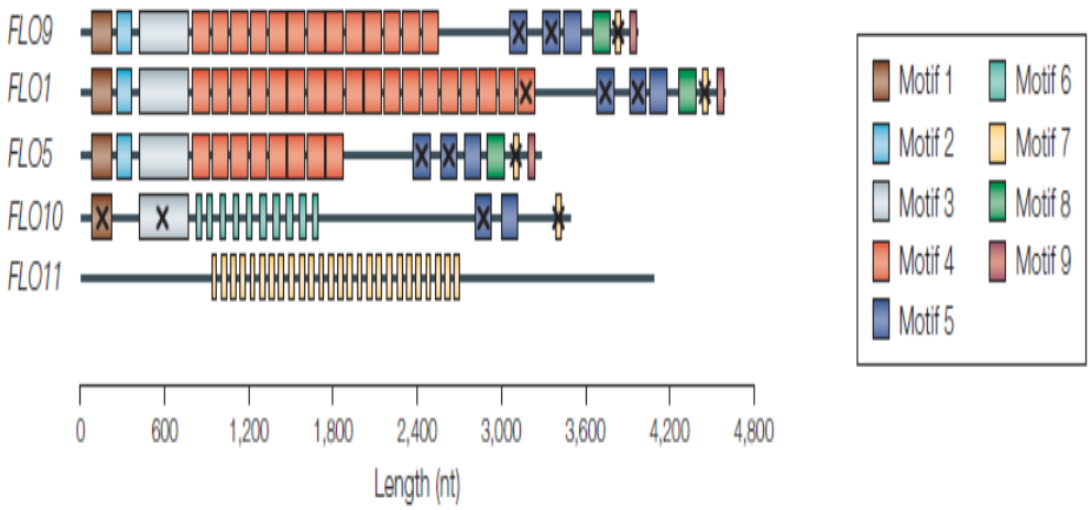


Figure 2.8: Repeated nucleotide motifs in *FLO* genes. The five *FLO* genes are depicted above. The boxes indicate the highly conserved DNA sequence motifs. The number below the sequences displays the distance in nucleotide from the translational signal start site. The boxes marked with an 'X' represent the repeated amino acid motifs which are not conserved in the DNA sequence. These sequences were assumed to have experienced genetic drift over time, resulting in numerous third-position nucleotide changes within the codons (Verstrepen *et al.*, 2004).

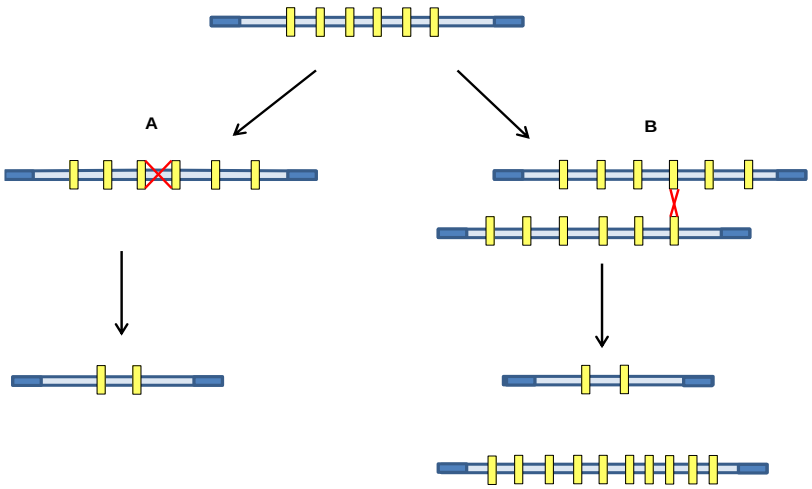


Figure 2.9: Illustrates the recombination between repeated DNA motifs in adhesin genes which synthesize new alleles. The boxes are indicative of nucleotide motifs; the regions illustrated in dark blue at the end of the chromosomes are representative of telomeres, A) depicts an intra-chromosomal pairing together with a recombination process which generates a reduced number of repeats in a short gene, B) depicts an unequal crossing over event between two identical *FLO* genes that are located on homologous chromosomes which have not aligned correctly (adapted from Verstrepen *et al.*, 2004).

FLO genes with a significant amount of homology and found on different chromosomes are able to recombine to form new chimeric genes. This process produces a long and a short form of the gene. The amplification and the loss of repeat motifs can take place by numerous mechanisms, such as slippage in replication and double-strand breaks. *FLO* genes which are adjacent to telomeres located on different chromosomes have the same orientation in relation to the centromere. Since they are transcribed towards the telomere, inter-chromosomal recombination events would result in the reconstitution of functional chromosomes with single centromeres and not chromosomes with two centromeres (dicentrics). DNA motifs located in the central domain are preserved along with the *FLO* genes and promote diversity of adhesins by recurrent intragenic recombination occurrences (Govender *et al.*, 2008).

2.4.2 The transcriptional regulation of *FLO11*

FLO11 is regulated by a large promoter where multiple signal transduction pathways converge (Nett *et al.*, 2009). *FLO11* contains one of the largest promoter regions in the yeast genome. The *FLO11* promoter is approximately 2,800 base pairs in size (Lo and Dranginis, 1998). Changes in *FLO11* or its promoter can have drastic effects on the cell surface variation (Baillie

and Douglas, 1999; Blankenship and Mitchell, 2006) and can induce unique cellular properties, such as the formation of buoyant aggregates of yeast cells on liquid surfaces (Kumamoto and Vinces, 2005). Apart from being targeted by these different signalling cascades, *FLO11* is regulated by epigenetic control and chromatin remodelling complexes (Barrales *et al.*, 2008; Frieman and Cormack, 2004; Halme *et al.*, 2004).

The regulation of *FLO11* has received widespread attention because of the gene products specific role in cellular adhesion. The *FLO11* encoded mannoprotein is associated with numerous adhesion phenotypes. It is required for the formation of pseudohyphae, agar invasion, (Lambrechts *et al.*, 1996; Lo and Dranginis, 1998), "flor" formation (Fidalgo *et al.*, 2006; Ishigami *et al.*, 2004; Ishigami *et al.*, 2006), "mat" formation and the adhesion to surfaces for instance polystyrene (Reynolds and Fink, 2001) or glass (Purevdorj-Gage *et al.*, 2007). As well as determines yeast colonies morphological appearance on agar plates (Kuthan *et al.*, 2003). *FLO11* transcriptional regulation is primarily dependent on the composition and the nutritional status of the growth environment (Figure: 2.10) (Bester, 2010). Flo11p is a vital adhesion molecule in *S. cerevisiae* because it controls filamentous growth (Beauvais *et al.*, 2009; Costerton *et al.*, 1995; Parsek and Greenberg, 2005) and the development of interconnected cells in biofilms (Smukalla *et al.*, 2008). Flo11p also known as Muc1p which is an adhesion molecule that is homologous to pathogenic adhesins (Chandra *et al.*, 2005).

There are various co-factor complexes and transcriptional regulators which contribute to the control of the transcriptional regulation of *FLO11*. Barrales *et al.* (2012) reported that two chromatin co-factor complexes, the Swi/Snf chromatin-remodelling complex and the Rpd3L deacetylase complex, contribute to the regulation of *FLO11* (Barrales et al., 2012). Mss11p is an essential factor in the regulation of *FLO11* (Van Dyk *et al.*, 2005) (Bester *et al.*, 2006). The expression of truncated forms of Mss11p revealed that they were essential for regulating the transcription of *FLO11* (Bester *et al.*, 2006). Flo8p is a transcription activator of *FLO11* (Gagiano *et al.*, 1999a; Kobayashi *et al.*, 1999; Pan and Heitman, 1999; Pan and Heitman, 2002; Rupp *et al.*, 1999). Mss11p is a transcriptional regulator which has an essential function in the regulation of pseudohyphae formation by means of regulating the expression of *FLO11* expression (Gagiano *et al.*, 1999a; Gagiano *et al.*, 1999b; Van Dyk *et al.*, 2005). Mss11p and Flo8p serve as core regulators of the transcription of *FLO11* (Gagiano *et al.*, 1999a; Kobayashi *et al.*, 1999; Pan and Heitman, 2002; Rupp *et al.*, 1999). Flo8p in addition to Sfl1p act on the *FLO11* promoter and function in both the activation as well the repression of transcription. Genetic

analysis of the factors affecting *FLO11* transcription displays that transcriptional activation is impeded by the deletion in *MSS11* (Van Dyk *et al.*, 2005). Evidence suggested that Mss11p interacts with Flo8p and binds with DNA and interacts with central transcriptional machinery to control gene expression (Kim *et al.*, 2004a).

The Srb8p/Ssn8p protein complex has a repressive effect on *FLO11* (Fichtner *et al.*, 2007; Kim *et al.*, 2004b). The Srb8p/Ssn8p protein complex interacts with the yeast mediator complex that in turn regulates the gene transcription via the interaction with RNA polymerase II (Myers and Kornberg, 2000).

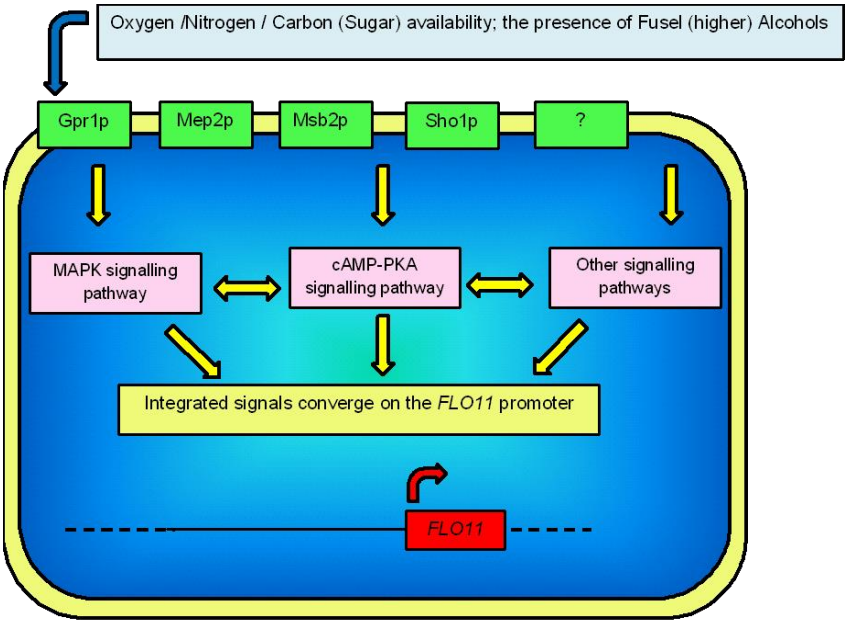


Figure 2.10: *FLO11* expression is highly regulated in response to the growth environment composition and the nutritional status. The signalling pathways involved in the transmission of the nutritional status to the *FLO11* promoter are the MAP-kinase pathway, cyclic-AMP-protein kinase A pathway and the main glucose repression pathway (adapted from Verstrepen and Klis, 2006).

Adhesion is controlled by several signalling pathways. The expression of *FLO11* is highly regulated by environmental factors as well as various signalling cascades and signalling pathways. There are three transcriptional signalling pathways (Figure 2.11) which regulate *FLO11* expression which include the Ras-cAMP pathway, mutagen activated protein kinase (MAPK) pathway and the main glucose repression pathway (Verstrepen and Klis, 2006). These signalling pathways converge at the *FLO11* promoter to control the primary *FLO11* phenotypes such as pseudohyphal differentiation and invasive growth (Figure 2.10) (Govender, 2009).

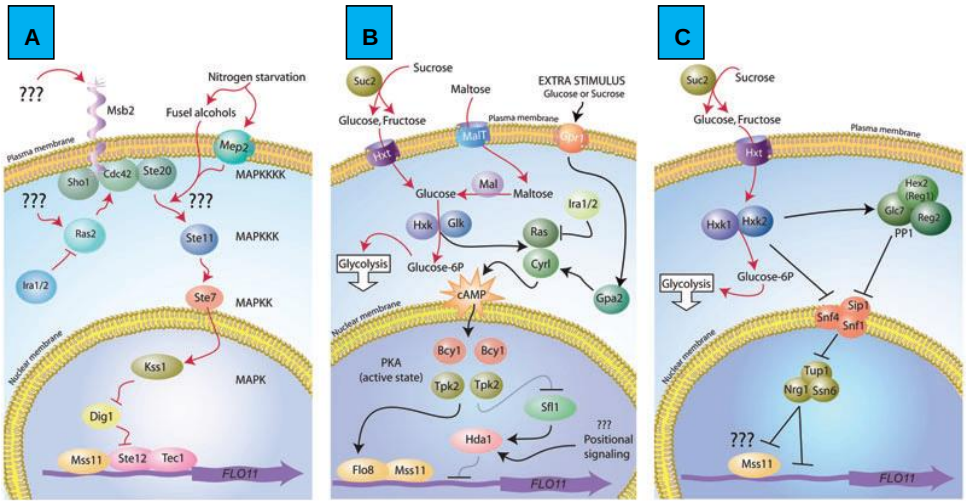


Figure 2.11: The regulation of *FLO11* by three signalling cascades, A) The MAPK-dependent filamentous growth pathway, B) The Ras/cAMP/PKA pathway, C) The main glucose repression pathway (adapted from Verstrepen and Klis, 2006).

There are two well-characterised signalling pathways, the cyclic AMP responsive protein kinase A (cAMP-PKA) and the filamentous growth mitogen activated protein kinase (FG MAPK). These two pathways constitute an important signalling component that control *FLO11* transcription (Gagiano *et al.*, 2002; Verstrepen and Klis, 2006). These pathways do not function in isolation however they interact and coordinate with each other in order to regulate the complex *FLO11* promoter (Gagiano *et al.*, 1999a; Rupp *et al.*, 1999). Mitogen-activated protein kinases (MAPK) are rich in serine-threonine residues which are activated by various stimuli ranging from growth factors, cytokines, hormones cell adherence, and cellular stress (Widmann *et al.*, 1999). An essential property of viable yeast cells is their ability to detect and respond to changes in the environment. A three-tiered cascade of protein kinases referred to as the mitogen-activated protein kinase (MAPK) pathway is commonly used to elicit a response to changes in the environment (Widmann *et al.*, 1999).

The MAPK pathway consists of a variety of kinases which phosphorylate each other sequentially in order to convey a signal. Upstream of the MAPK pathway the Cdc42p protein that is a GTPase that belongs to the Rho subfamily (Johnson, 1999) activates the MAPK component via interaction with Ste20p which is the first protein kinase. Ste20p phosphorylates Ste11p, which phosphorylates Ste7p, and finally transmits the signal to Kss1p (Chen and Thorner, 2007). The Kss1p performs as a downstream effector of the MAPK pathway as well as activates the Ste12p transcription factor (Bardwell *et al.*, 1998). Ste12p has various gene targets however it binds specifically to the *FLO11* promoter via a cooperative method with the Tec1p transcription factor (Madhani and Fink, 1997).

The cAMP-PKA signalling is activated via the GTP (guanosine triphosphate) binding protein activity of Gpa2p and Ras2p (Tamaki, 2007). These proteins activate an adenylate cyclase Cyr1p which converts ATP (adenosine triphosphate) into cAMP (cyclic adenosine monophosphate) the signalling molecule. The protein complex PKA comprises of the catalytic subunits Tpk1/2/3p and two regulatory subunits of Bcy1p. The cAMP attaches to Bcy1p, activating its inhibitory effect on the Tpk proteins this process has positive and negative effects on the *FLO11* transcription (Robertson and Fink, 1998). The Tpk2p has a vital role in the transcriptional signalling and determines the activity of cAMP-PKA downstream transcription factors such as Flo8p in addition to Sfl1p.

Gpr1p transmits information regarding nitrogen and carbon source availability (Lorenz *et al.*, 2000; Tamaki *et al.*, 2000). The ammonium membrane transporter, Mep2p transmits signals under nitrogen starvation conditions (Lorenz and Heitman, 1998). Ras2p affects both the cAMP-PKA and MAPK signalling, thus indicating that these signalling pathways cross communicate (Mösch *et al.*, 1999).

The transcriptional signalling pathways which regulate the other *FLO* genes are not known as yet however, it is thought that they are regulated by similar pathways to *FLO11* (Verstrepen and Klis, 2006). Environmental parameters such as nitrogen starvation, carbon limitation, fluctuations in pH and ethanol levels trigger the signalling pathways consequently activating the adhesin encoding genes (Verstrepen and Klis, 2006).

2.4.3 The epigenetic regulation of *FLO* genes

According to Verstrepen and Klis (2006) *FLO* genes are controlled by signalling cascades as well as epigenetic silencing and de-silencing. *FLO* genes have the ability to revert from a silent state to an expressed state, thus *FLO* gene expression is considered to be reversible. Epigenetic control is promoter specific and dependent on the subtelomeric location of the *FLO* gene. This was apparent when the *FLO11* promoter was replaced with another promoter and relocated to another locus on a chromosome. These events prevented epigenetic control from occurring (Verstrepen and Klis, 2006). The epigenetic silencing of *FLO* genes has various functions such as aiding cells to adapt to changing environmental conditions and enabling these cells to adhere to surfaces by triggering appropriate adhesins (Bayly *et al.*, 2005; Verstrepen and Klis, 2006).

2.5 The use of promoters in gene expression

Metabolic engineering applications have relied on the discovery of effective promoter characterization. Promoter engineering provides a means to modulate the transcriptional capacity of a promoter by enhancing, mutating or altering the promoter DNA sequence. Promoter engineering can be used in metabolic engineering applications to generate a dynamic range of promoters to optimize a gene of interest. Promoters have a pivotal role in synthetic biology because it enables a gene to perform at its optimum capacity (Young and Alper, 2010).

Chromosomal integration and plasmid vectors are often used to introduce a gene of interest into *S*. *cerevisiae*. Plasmid vectors are useful for gene expression but, there are limitations with the use of plasmids for example, the limited control of copy number (Da Silva and Srikrishnan, 2012). The PCR-based gene integration technique utilizes PCR-generated fragments which are utilized for gene insertions and deletions in yeast. PCR integration is a vital mode for insertion of genes into the yeast genome (Schaerer-Brodbeck and Barberis, 2004; Shao *et al*., 2009). The use of high-fidelity polymerases reduces the amount of errors during PCR and thereby aid in the recovery of genomic inserts which confirm the correct sequence (Da Silva and Srikrishnan, 2012).

Efficient targeting correlates to an increasing length of homology. An overlap of approximately fifty base pairs is adequate to screen and recover the integrated gene. Primers can be utilized to amplify a gene cassette with flanking regions, thereafter the PCR product is transformed into yeast via homologous recombination (Bai Flagfeldt *et al.*, 2009; Erdeniz *et al.*, 1997; Hawkins and Smolke, 2008; Shao *et al.*, 2009).

There is an assortment of promoters available for the control of gene expression at the transcriptional level in *S*. *cerevisiae*, comprising of both inducible and constitutive promoters which drive the expression of a desired gene of interest at different intensities (Da Silva and Srikrishnan, 2012). In *S*. *cerevisiae,* constitutive promoters have been extensively used in the control of gene expression. These promoters are simplistic because they do not require inducers or repressors and allow for relatively constant levels of expression (Da Silva and Srikrishnan, 2012).

An example of a constitutive promoter is *PGK1*. In *S*. *cerevisiae* the *PGK1* gene encodes for the highly abundant mRNA and protein in the cell (Ogden *et al.*, 1986). *PGK1* is considered to be one of the most efficiently expressed genes in *S. cerevisiae* (Holland and Holland, 1978). The upstream activator sequence (UAS) of the *PGK* gene consists of three sequence elements.

The first element is known as the Ypp, this region has a strong protein binding capacity, the second element is a triple repeat of a CTTCC motif and the third region is regarded as an essential activator core (AC) sequence which binds to the Rap1 protein (Stanway *et al.*, 1989). The *PGK1* promoter yields a high constitutive expression in the presence of glucose however, the promoter's expression is repressed by other non-fermentable carbon sources such as glycerol (Kingsman *et al.*, 1990).

TEF1 is another constitutive promoter, and the *TEF1-α* gene (*TEF1*) encodes for the translation elongation factor one α (Schirmaier and Philippsen, 1984). The *TEF1* gene has strong and constitutive promoter activity in *Aspergillus oryzae* (Kitamoto et al., 1998). The *TEF1* has a strong promoter activity in other microorganisms such as *S*. *cerevisiae* (Schirmaier and Philippsen, 1984). The *TEF1* promoter drives the expression of the *S*. *cerevisiae α* subunit of the translation elongation factor one (Cottrelle *et al.*, 1985).This study used the constitutive *TEF1* promoter to drive the expression of the *FLO11* encoded mannoprotein.

Inducible promoters have been extensively used in industrial-scale biotechnological applications and fundamental research for induction or repression of a gene. However, there are limitations in their use such as toxicity, they are expensive to produce, and may contain inherent regulation modalities (Nevoigt *et al.*, 2007). Inducible promoters have been important tools in applied and elementary biological research. A model inducible promoter used in industrial fermentation of yeast requires the following attributes; it must be highly regulated, be cost efficient to induce, have high levels of expression after induction, and be easy to work with. Currently there are no systems available which can induce gene expression in *S*. *cerevisiae* that satisfy all of the above requirements. The vast array of tools that are available for manipulation at a molecular level and the knowledge of *S*. *cerevisiae* metabolic pathways aids in its successful engineering which can be used for a range of applications (Da Silva and Srikrishnan, 2012).

The use of inducible promoters has disadvantages such as they are limited by their sensitivity of the promoter to the inducer, the cost of metabolites for induction is high, and background levels of expression can be present due to 'leaky' promoters. A strains response to an inducer can add to the complexity of the gene's expression (Da Silva and Srikrishnan, 2012). An example of an inducer promoter is *ADH2*. It is highly regulated by glucose repression, with approximately a hundred-fold repression when glucose is present (Price *et al.*, 1990). For high-level expression to occur with the *ADH2* promoter, the cells must be grown in a complex medium (Michael Lee and DaSilva, 2005; Price *et al.*, 1990). The intracellular metabolic flux is highly regulated via a series of distinct levels of control which occurs at the transcriptional, translational, and protein regulatory levels (Blazeck and Alper, 2010).

Regulated promoters allow for control over the level of gene expression at a specific time or growth phase (Da Silva and Srikrishnan, 2012). They are therefore more appropriate when expression of a gene of interest is required at a specific stage of yeast growth or to avoid the development of toxic pathway intermediates (Da Silva and Srikrishnan, 2012).

2.6 Conclusion

There is a plethora of information available regarding the structure of the cell wall of *S. cerevisiae*. The yeast cell wall is considered a dynamic entity because there is constant change in its structural organization and composition. Yeast cells are continuously adapting because they are exposed to harsh environmental conditions. This enables the cell to respond and adapt to environmental stresses. The regulation of these events is yet to be fully elucidated. Researchers are constantly searching for a better understanding of the yeast cell wall.

2.7 References

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Chapter 3

Research Results 1

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

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

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3.1 Abstract

The *FLO* gene family contains five dominant *FLO* genes (*FLO1*, *FLO5*, *FLO9*, *FLO10* and *FLO11*) which encode for adhesins. These adhesins are extracellular glycosylphosphatidylinositol-anchored yeast cell wall glycoproteins, which are associated with many adhesion phenotypes such as invasive growth, pseudohyphal formation, flocculation, biofilm formation, attachment to abiotic and biotic surfaces. To date, and to the best of our knowledge, understanding of the fine molecular structure of these glycoproteins is fairly limited. Amino acid sequences of *FLO* encoded mannoproteins have been discovered based on the analysis of the genomic sequence of *S. cerevisiae in silico*. It is envisaged that the over-expression of the silent *FLO11* gene will generate the desired adhesin in sufficient quantities allowing for the isolation and purification for their biochemical structural analysis. In this study a PCR-based promoter replacement strategy was utilized to bring the native ORF of *FLO11* under the transcriptional control of the constitutive *TEF1* promoter. The *FLO1*p-*Zeocin*™-*TEF1*-*FLO1*p constitutive promoter cassette was used as a tool to construct the *FLO11*p-*Zeocin*™-*TEF1*-*FLO11*p cassette. Zeocin™ was used as a selection marker in yeast and was placed under the control of the *PGK1* constitutive promoter. Integration of the *FLO1*p-*Zeocin*™-*TEF1*-*FLO1*p constitutive promoter cassette was verified by PCR and Sanger DNA sequencing. In this chapter, the functionality and the integrity of the *TEF1* promoter replacement cassette was verified by the strong flocculation phenotype displayed in the transgenic strain.

3.2 Introduction

One of the most widely researched microorganisms is *S*. *cerevisiae*, commonly referred to as baker's yeast. It is non-pathogenic and has received GRAS status (Da Silva and Srikrishnan, 2012; Klis *et al.*, 2002; Verstrepen *et al.*, 2004). It has been extensively used throughout history in the production of food and alcoholic beverages (Klis *et al.*, 2002; Verstrepen *et al.*, 2004).

Yeast strains are involved in a variety of industrial processes, ranging from the production of recombinant proteins, traditional fermented foods and beverages. Current biotechnological yeast applications, such as biofuel production, present yeast with different challenges and environments which differ from those experienced in the traditional food fermentations. This is indicative that there is a need to generate yeast strains that are capable of performing better than the currently used yeast strains (Steensels *et al.*, 2014). *S*. *cerevisiae* has been used in the bioethanol industry and in the production of heterologous compounds, such as hepatitis vaccines, human insulin, and human papillomavirus vaccines (Hou *et al.*, 2013).

Yeast physiology can be altered to facilitate the downstream processing of a fermentation product. For instance, yeast flocculation allows for the removal of biomass and clarification from fermented beverages. 'Flotation' is another ability displayed by yeast cells to entrap carbon dioxide in the fermenting liquid substrate and forms a film or vellum at the surface of the fermentation bioreactor, for example in traditional ales or flor sherry (Pretorius, 2000; (Verstrepen *et al.*, 2003). Both these characteristics are associated with the expression of *FLO* genes (Ishida-Fujii *et al.*, 1998). The coupling of the expression of *FLO* genes with an appropriate promoter can enable the required intensity of yeast aggregation (Verstrepen *et al.*, 2001).

The aroma of fermented food such as beer, sake, wine and bread are affected by numerous yeast associated compounds, comprising of, higher alcohols, ketones, esters, sulphuric compounds, terpenes, and phenolic compounds. As such, an increase or decrease in these aroma-active compounds will affect the aroma of the respective fermented food (Steensels *et al.*, 2014). *S*. *cerevisiae* is used on a large scale in both the pharmaceutical and biotechnological industries because it is easily cultured (Da Silva and Srikrishnan, 2012; Van Mulders *et al.*, 2009). It is capable of growing on different types of carbon sources such as glucose, maltose and fructose (Govender *et al.*, 2008).

An optimized expression system is identified, based on their genetic and fermentative levels, several properties have to be taken into account such as the product, expression cassette and the expression host organism (Celik and Calik, 2012). This study focuses on the construction of a constitutive promoter that will be used to drive the expression of *FLO11* encoded mannoproteins in *S*. *cerevisiae*. Furthermore, this study aims to genetically engineer yeast strains bearing a constitutive promoter which was used to over-express and release mannoproteins during fermentation. This will aid in our fundamental understanding of yeast cell wall mannoproteins.

The expression of dominant *FLO* genes in *S*. *cerevisiae* laboratory strains determines specific cell-wall dependent phenotypes such as flocculation, cell-cell adhesion, surface hydrophobicity, substrate adhesion, invasive growth of substrates, and biofilm formation (Cunha *et al.*, 2006; Govender *et al.*, 2008; Guo *et al.*, 2000; Van Mulders *et al.*, 2009; Verstrepen and Klis, 2006; Wang *et al.*, 2008). The native promoter of *FLO1* the haploid non-flocculent and non-invasive *S*. *cerevisiae* BY4741 strain was replaced with the *TEF1* constitutive promoter.

The cell wall mannoproteins releasing capacity exhibited by *S. cerevisiae* during fermentation has fascinated researchers in the wine industry because the yeast mannoproteins exhibit many advantageous properties which aid in superior wine production. The aesthetic properties which mannoproteins confer to wine include colour, aroma stability, astringency, tartaric protein stability, mouth feel and foaming in sparkling wines (Gonzalez-Ramos and Gonzalez, 2006).

Mannoproteins are highly glycosylated proteins that are covalently bound to the *β*-1,3-glucan present in the yeast cell wall which has a significant role in industry (Quiros *et al.*, 2010). Flo mannoproteins are components of the outermost layer of the cell wall, collectively called flocculins (Govender *et al.*, 2010). Expression of these mannoproteins in deletion mutant strains of BY4741 has the ability to over-express these mannoproteins into the extracellular environment. This would provide a reservoir of free mannoproteins and will aid in the isolation as well as biochemical structural analysis of these mannoproteins.

In this study, transformation cassettes will be constructed via a PCR based strategy that consists of Zeocin™ as the selection marker and the constitutive promoters *PGK1* and *TEF1*. The *TEF1* and the Zeocin™ encoding fragments inclusive of a terminator (*CYC1*) was amplified from the pTEF/Zeo plasmid. The *TEF1* promoter will drive the expression of the respective *FLO* gene.

The haploid *S. cerevisiae* laboratory BY4741-wild type strain will be transformed via electroporation with PCR generated vector cassettes. The cell density and flocculation phenotype of the wild type transgenic strain will be quantified under defined laboratory cultivation conditions.

3.3 Materials and methods

3.3.1 Strains and plasmids

The strains and plasmids employed in this study are listed in Table 3.1. The *Escherichia coli* ElectroMAX™ DH5α-E™ (Invitrogen, Life Technologies, Carlsbad, California, United States) electro-competent cells were used as a host for all plasmid amplifications. The yeast strains used in this study were derived from *S. cerevisiae* BY4741 or BY4742 genetic background.

Table 3.1: Strains and plasmids employed in this study

Strain/Plasmid	Genotype	Reference
BY4741	MATa *his*3Δ1 *leu*2Δ0 *met15*Δ0 *ura*3Δ0	EUROSCARF
BY4742 PGU1	MATa; *his3*Δ1; *leu2*Δ0; *lys2*Δ0; *ura3*Δ0; *PGK1p- PGU1*	Fernández-González *et al.* (2005)
BY4741-F1P	*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 FLO1p:: Sh ble-PGK1p*	(Ngobese, 2014)
BY4741-F1T	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 FLO1p::-Sh ble-CYC1-TEF1p*	This study
ElectroMAX™ DH5α-E™	F- φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rk-, mk+) *gal- pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1	Invitrogen™
FY23	*MATa leu2 trp1 ura3 flo8-1*	(Winston *et al.*, 1995)
FY23-F1A	*MATa leu2 trp1 ura3 flo8-1 FLO1p::SMR1 ADH2p*	(Govender *et al.*, 2008)
FY23-F1H	*MATa leu2 trp1 ura3 flo8-1 FLO1p::SMR1 Hsp30p*	(Govender *et al.*, 2008)
pTEF/Zeo	pTEF/Zeo backbone (3556 bp)	Invitrogen™ Life technologies
pUC118	pUC118-*Hinc II*/BAP (3162 bp)	Takara
pUC118-PGK1	pUC118 containing *PGK1* fragment (3804 bp)	This study
pUC118-Zeo	pUC118 containing Zeocin™ fragment (3937 bp)	This study
pUC118-TEF1	pUC118 containing *TEF1* fragment (3735 bp)	This study
pUC118-FZT	pUC118 containing Zeocin™-*TEF1* fragment (4515 bp)	This study

3.3.2 Media and *E.coli* cell growth conditions

E. coli was routinely grown in Luria-Bertani (LB) broth (Merck, Germany). The bacterial transformants were screened using blue-white selection and were plated on LB agar containing 100 mg/L Ampicillin (Sigma-Aldrich Inc, USA), X-gal (30 mg/L) (Melford laboratories, United Kingdom) and IPTG (30 mg/L). (Thermo-Scientific, USA) In this study, 2% agar (Merck, Germany) was used for all solid media. The white transformant colonies from the blue-white selection plates were inoculated in LB broth containing 100 mg/L Ampicillin. The cultures were then incubated for 24 hours at 37°C in an orbital shaker at 180 revs per minute (rpm). These bacterial strains were stored in LB broth and 40% (vol/vol) glycerol (Merck, Germany) and stored in the bio-freezer (Snijders Scientific, Tilburg, Netherlands) at -80°C (Govender *et al.*, 2008).

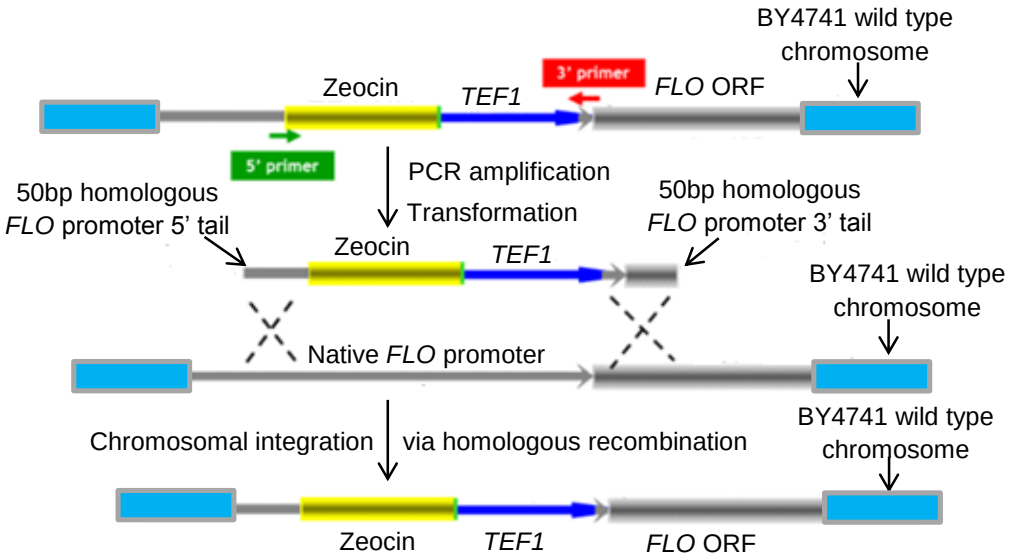

Figure 3.1: Promoter replacement strategy indicating chromosomal integration of the *TEF1* promoter upstream of the *FLO* open reading frame (ORF) (adapted from Govender *et al.*, 2008).

3.3.3 Construction of the promoter replacement cassettes

The construction of the promoter replacement cassettes was conducted using a modified PCR-based strategy from Govender *et al.* (2008) (Figure 3.1). All PCR amplifications were performed in a G-Storm Master Gradient Cycler (Somerton Biotechnology Centre, United Kingdom). PCR reactions were carried out with Takara Ex Taq™ according to the manufacturer's specifications (Takara Bio Inc., Japan).

The promoter cassette consists of three fragments; *PGK1*, Zeocin™ with a *CYC1* transcription terminator and *TEF1* (Figure 3.1 and Figure 3.2). A mass PCR amplification of the three fragments was conducted using Takara Ex Taq™ (Takara Bio Inc., Otsu, Japan). The respective amplification reactions were pooled and run on an (1%) agarose (Lonza, Rockland, ME, USA) gel. These amplification products were then extracted from agarose gels using the Zymoclean™ gel DNA recovery kit (ZYMO Research, USA) according to the manufacturer's instructions. Subsequently, the amplicons were cloned into the pUC118 vector obtained from Takara (Takara Bio Inc., Otsu, Japan) according to the manufacturer's instructions as specified in the product manual. The respective fragments were then transformed in ElectroMAX™ DH5α-E™ cells and plated on (LB) pates containing 100 mg/L Ampicillin, IPTG and X-gal. High efficiency electro-transformation of *E*. *coli* was conducted according to Ausubel *et al.* (1987) and Miller and Nickoloff (1995).

The electroporation of *E.coli* was conducted using a Bio-Rad Micro-Pulser™ (Bio-Rad Laboratories, CA, USA). The electroporation cuvettes (Cell Projects Ltd., Kent, UK) containing a one millimeter (mm) electrode gap was exposed to 1.5 kilo volts (kV) for 5.0 milliseconds (ms) The plasmid was isolated using a Zyppy™ Plasmid Miniprep Kit (ZYMO Research, USA). Thereafter, the white colonies were inoculated into 10 mL of LB broth containing 100 µg/mL Ampicillin. The cultures were incubated overnight at 37 °C with shaking at 180 rpm. These LB broths were then analysed based on their turbidity, which was indicative of growth as such freeze cultures were made up of the respective cultures. Mini-preps were then conducted on these cultures using the Zyppy™ kit (ZYMO Research, USA) as per manufacturer's instructions.

The DNA isolated from the mini-prep reactions were restricted using restriction enzymes and this was followed by ligation of the respective fragments. All restriction enzymes were purchased from Thermo-Scientific (Lithuania, Europe). The *PGK1* promoter was PCR amplified from the BY4742Δ*PGU1* chromosomal DNA, using the FLO1-PGK1-F and PGK1-R primers. The *FLO1*p-*PGK1* fragment 647 base pairs (bp) located in pUC118 was recovered by a double digestion with *Xho*I and *EcoR*I.

The Zeocin™ resistance gene is encoded by *Sh ble* gene and *CYC1* transcription termination sequence was PCR amplified from the plasmid pTEF-Zeo with the Zeocin™-F and Zeocin™-R primer pair. The Zeocin™ fragment 761 bp situated in the pUC118 was recovered by double restriction digestion with *Sal*I and *Bgl*II. While the *TEF1-FLO1*p insert 595 bp was released by double digestion with *Bam*HI and *Pst*I. The Zeocin™ and the *TEF1* fragments were ligated using the rapid DNA ligation kit which was purchased from (Lithuania, Europe). The *Zeocin™-TEF1-FLO1*p fragment was PCR amplified by using primers Zeocin™-F and TEF1-R and the ligation reaction mixture as the template for the PCR amplification.

The integrated *Zeocin™-TEF1-FLO1p promoter* replacement cassette (1,353 bp) was subsequently extracted from an agarose gels and purified using Zymoclean™ (ZYMO Research, USA). This fragment was placed in pUC118 and recovered by a double digestion using *Sal*I and *Pst*I .The *FLO1*p-*PGK1* was ligated to *Zeocin™-TEF1-FLO1p* using the rapid DNA ligation kit (Lithuania, Europe) (Figure 3.2).

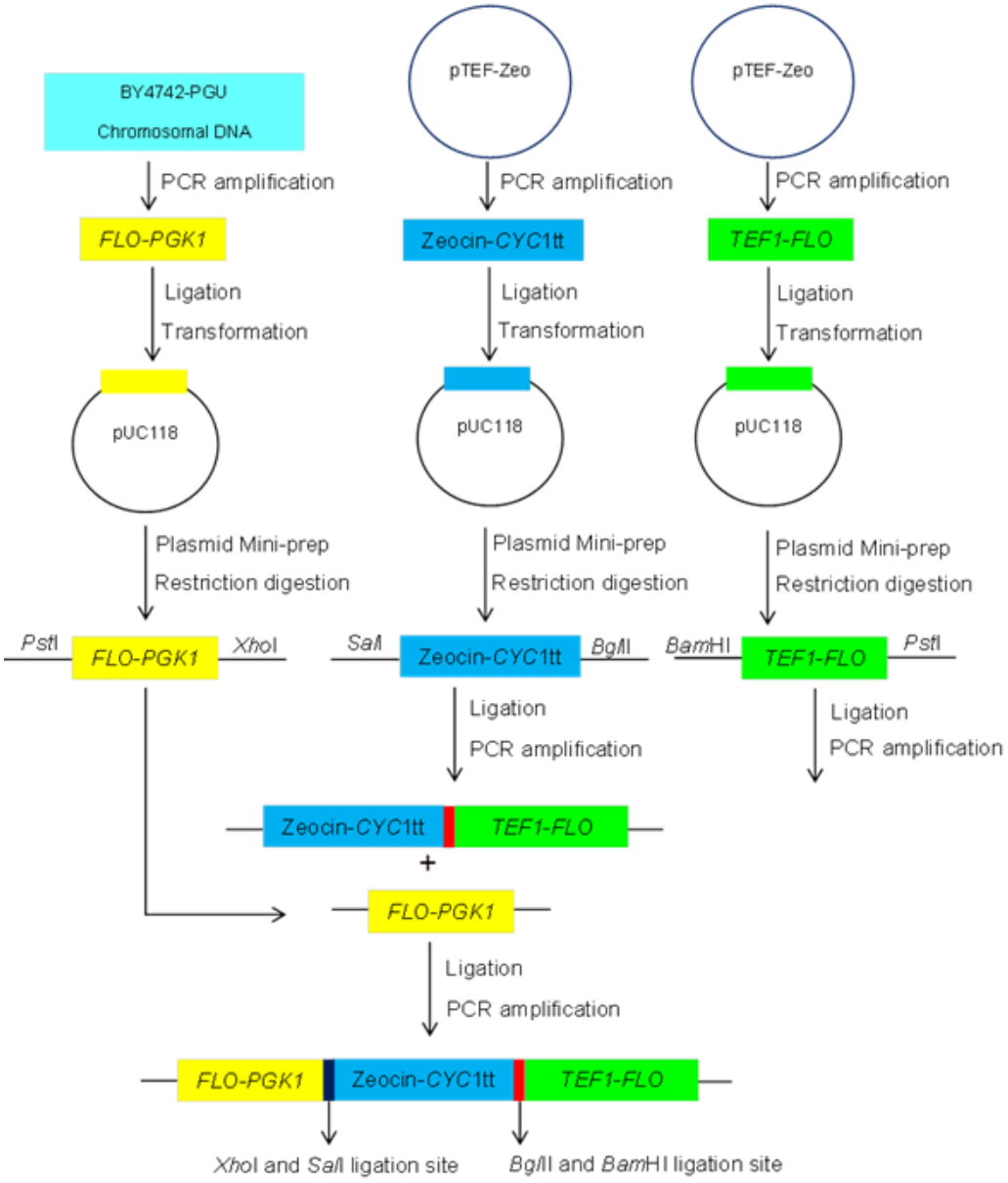

Figure 3.2: The cloning strategy used in the construction of the promoter cassette.

The *FLO1*p-*Zeocin™-TEF1-FLO1*p fragment was PCR amplified by using primers FLO1-F and FLO1-R and the ligation reaction mixture as the template for the PCR amplification. Thereafter, FLO1 primers, PGK1-FLO1-F and FLO1-R were used in the mass amplification of the cassette this was used for yeast transformation. The cassette *FLO1*p-*Zeocin™-TEF1-FLO1*p was approximately 1981 bp in length.

3.3.4 Media and yeast cell growth conditions

The selection of Zeocin™ resistant yeast transformants were plated on defined synthetic complete (SC) media. The SC (DuPont Agricultural Products, France) media contained 0.67% (wt/vol) yeast nitrogen base (YNB) (Difco™) without amino acids, 2% (wt/vol) glucose (Merck, Germany), 2% (wt/vol) agar (Merck, Germany) and 18% (wt/vol) sorbitol (Melford laboratories Ltd, Bildestond Road, Ipswich). The medium was supplemented with the auxotrophic amino acid requirements as per laboratory yeast strain (Ausubel *et al*. 1995) and 150mg/L of Zeocin™. The selection of kanamycin (*KanMX*) resistant strains was selected using YEPD medium that contained 200 µg/mL of Geneticin (G418) (Thermo-Scientific, USA).

The YEPD medium contained 1% (wt/vol) yeast extract (Merck, Germany), 2% (wt/vol) glucose (Merck, Germany) and 2% (wt/vol) peptone (Merck, Germany). The yeast cultures were then incubated for 24 hours at 30°C in an orbital shaker at 160 rpm. After 24 hours, YEPD medium, which contained yeast cell culture and 30% glycerol was placed in cryovials and placed in the -80ºC bio-freezer (Snijders Scientific, Netherlands) for long-term storage.

3.3.5 Isolation of yeast chromosomal DNA

Yeast chromosomal DNA was isolated according to the method of Ausubel *et al.* (1995). The yeast strains were grown at 30ºC in an Infors HT multitron cell orbital shaker (United Scientific, South Africa) at 160 rpm for 24 hours in 10 mL yeast extract peptone dextrose (YEPD) medium. The yeast cells were thereafter collected via centrifugation at 3000 rpm at 4ºC for two minutes (Eppendorf, centrifuge 5417R, Germany). The YEPD medium was discarded and the cells were then resuspended in 500 µL distilled water. The cells were transferred to a 1.5 mL microcentrifuge tube and centrifuged at 12 000 rpm for 30 seconds.

The supernatant was discarded and the pellet was disrupted by vortexing briefly in the residual liquid. The cells were resuspended in 200 µL of breaking buffer [2% (v/v) Triton X-100, 1% (v/v) SDS, 100 mM NaCl, 10 mM Tris-HCL pH 8, 1 mM EDTA pH 8], approximately 0.3 g of glass beads (Sigma-Alrich Inc, USA) and 200 µL of a phenol/chloroform/isoamyl (PCI) alcohol [25:24:1 (v/v/v)]. Thereafter, the yeast cells were vortexed at high speed for three minutes. This was followed by the addition of 200 µL tris-ethylene diamine tetra-acetic acid (TE) buffer (10 mM Tris-HCL and 1 mM EDTA pH 8.0) and the tubes were briefly vortexed. The microcentrifuge tubes were centrifuged at 12 000 rpm for five minutes.

The supernatant was transferred to a new maximum recovery 1.5 mL microcentrifuge tube (Lasec, Axygen Inc, 3321 Central Avenue union city, USA). Aliquots (1 mL) of 100% ethanol (Merck, Germany) was thereafter added to the supernatant and mixed via inversion of the microcentrifuge.

The samples were stored at -20ºC for 20 minutes to facilitate DNA precipitation. The microcentrifuge tubes were then centrifuged at 12 000 rpm for five minutes and the supernatant was discarded. The pellet was re-suspended in 400 µL TE buffer. RNA contaminants were removed by addition of 3 µL of Ribonuclease A (10 mg/mL) (Sigma-Aldrich Inc, USA) and incubating the solution at 37ºC for five minutes.Thereafter 10 µL of 4 M ammonium was added to the microcentrifuge tube and 1 mL of 100% ethanol, the samples were mixed by inversion. The samples were stored at -80ºC for 20 minutes. The micro-centrifuge tubes were centrifuged at 12 000 rpm for three minutes and the supernatants were discarded. The pellets were air dried and the pellets of DNA were re-suspended in 100 µL of TE buffer.

3.3.6 Transgenic yeast strain verification

All transgenic yeast strains were verified using a PCR strategy and the Takara Ex Taq™ DNA polymerase (Takara Bio Inc., Otsu, Japan) system according to the manufacturer's guidelines. All primers used in this aspect of the study are listed in Table 3.2. Genomic DNA was isolated from the positive yeast transformants using a method recommended by Ausubel *et al*. (1995). Chromosomal integration was achieved by the double-crossover homologous recombination event in which the *FLO1* gene was placed under transcriptional control of the *TEF1* promoter. The deletion of native promoters was confirmed by PCR using homologous primer sets. The primer pairs used for the transgenic wild type BY4741 strain containing the *FLO1*p-Zeocin™-*TEF1-FLO1*p was as follows: primers pair FLO1-F2 and FLO1-R.

In addition, the integration of the promoter replacement cassettes into the correct locus was confirmed by PCR using heterologous primer sets which contained a forward primer that is complementary to sequences from outside the region of integration and the TEF1-R reverse primer. The heterologous primer pair used for confirmation of the correct locus for the transformant feral strain was FLO1-F2 and TEF1-R. The wild-type BY4741 strain served as a control in these confirmation experiments.

3.3.7 Primers

The primers used in this study are listed in Table 3.2. All primers were designed using Primer Stats software and the respective primers were purchased from Integrated DNA technologies (USA).

Table 3.2: Primers used in this study

Primer Name	Primer Sequence (5'→3')
FLO1::PGK1-F	TGCGTCACTTTTCCTACGGTGCCTCGCACATGAATGTTATCCGGCGCACGTCC CTCCTTCTTGAATTGAT
FLO11::PGK1-F	TCACTGCACTTCAACTATGCCTTATAGCAACCAAGAAGCTAGAAAATGCCTCC CTCCTTCTTGAATTGAT
PGK1-R	AGGACTCGAGCATTGTTTTATATTTGTTG
Zeocin™-F	AGGCGTCGACCAATTAATCATCGGCATAG
Zeocin™-CYC1-R	AGGGAGATCTCAGCTTGCAAATTAAAGCCT
TEF1-F	ATGCTGATCATTACGGTTCCTGGCCTTTTG
FLO1-TEF1-R	CATATAGCGATGAGGCATTGTCATTTTTGGATGTTCTGTTTACTGGTGAC AACACCGCCCCTTAGATTAG
FLO11-TEF1-R	GGACCAAATAAGCGAGTAGAAATGGTCTTTGCATAGTGTGCGTATATGG AACACCGCCCCTTAGATTAG
PGK1-F	TCCCTCCTTCTTGAATTGAT
TEF1-R	AACACCGCCCCTTAGATTAG
FLO1-F	AAGTGTGCGTCACTTTTCCTACGGT
FLO1-F2	AGTGTTATGCTAGCCAGTTTCAGG
FLO1-R	AGCGATGAGGCATTGTCATTT

*F denotes forward primer and R the reverse primer.

3.3.8 Enumeration of cell density

The cell density of the wild type and transgenic strains were measured after 6, 12 and 24 hours of incubation using an automated cell counter the Countess™ (Invitrogen, Life Technologies, Carlsbad, California, United States) in conjunction with a 0.4% Trypan blue stain (Invitrogen, Life Technologies, Carlsbad, California, United States). The strains were all cultivated in YEPD which contained 1 % (w/v) yeast extract, 2% glucose (w/v) and 2% (w/v) peptone, and incubated at 30°C in an Infors HT multitron cell shaker (United Scientific, South Africa) at 160 rpm for 24 hours.

3.3.9 Flocculation assay

The intensity of yeast cell flocculation was measured using a modified Helm's assay as described by D'Hautcourt and Smart (1999). Single yeast colonies were grown for 18 hours in 50 mL of YEPD which contained 1 % (w/v) yeast extract, 2% glucose (w/v) and 2% (w/v) peptone and incubated at 30°C in an Infors HT multitron cell shaker (United Scientific, South Africa) at 160 rpm. This starter culture was used to inoculate 50 mL of YEPD broth that was placed in a 250 mL Erlenmeyer flask and grown at 30°C for 48 hours with shaking at 160 rpm. The yeast cells were then transferred to 50 mL tubes (Cell Star^{R} tubes, Greiner Bio-One) and thereafter the cells were harvested by centrifugation at 4000 rpm (Eppendorf, centrifuge 5417R, Germany) for 5 minutes at 4°C. The cells were then washed once with 20 mL of 100 mM EDTA (pH 7-8), once with 20 mL of 30 mM EDTA (pH 7-8), and at each wash step the supernatant was discarded. The cell counting was conducted as in 3.3.8. Ten 1mL samples were harvested by centrifugation at 16 400 rpm for 1 minute at 4°C. Of these ten samples, five of the 1mL samples were re-suspended in 1 mL of 100 mM EDTA (pH 8) and served as the control experiment.

The remaining five samples served as the test experiment and were washed with 1 mL wash buffer that contained 0.51 g/L calcium chloride and this was followed by re-suspending pellets in 1 mL suspension buffer comprising of 0.44 g/L of calcium chloride, 6.8 g/L sodium acetate, 4.06 g/L of glacial acetic acid and 4% ethanol (v/v). These tubes were then vigorously vortexed for 30 seconds and the micro-centrifuge tubes were inverted five times in 15 seconds to promote flocculation. The samples were thereafter left to stand undisturbed at room temperature for 15 minutes. This was followed by removing 100 µL from each control and test sample from just below the meniscus and diluted in 900 µL of 100 mM EDTA (pH 7-8). The absorbance (Abs) of the cell suspension was determined at 600 nm using a Specord 220 spectrophotometer (Analytik Jena™, Germany). Flocculation is expressed as a function of the mean absorbance of the control assay by the equation:

Flocculation (%) = [(mean control Abs – experimental Abs)/ mean control Abs] × 100

3.3.10 Statistical analysis

In this study, one-way analysis of variance (ANOVA) was employed to statistically compare flocculation data obtained for wild type and transgenic yeast strains. Analyses were performed using the statistical software package Graph pad in stat version 3.05 32 bit for Windows 95/NT (Graph pad software, San Diego California).

3.3.11 Verification of DNA fragments via Sanger DNA sequencing

The DNA fragments were verified by Sanger DNA sequencing utilising the ABI BigDye® V3.1 kit (Applied Biosystems) and the ABI 3500XL genetic analyser (Applied Biosystems) at Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa).

3.4 Results

3.4.1 Blue-white selection

This study utilized Blue-white selection to screen for positive clones in *E.coli* (Figure 3.3). When *E*. *coli* was transformed with an unmodified plasmid, this resulted in the formation of blue colonies (Voet and Voet, 2011), however, when *E*. *coli* was transformed with a plasmid that contained a foreign DNA insert located in its poly-linker region this resulted in the formation of white colonies (Voet and Voet, 2011).

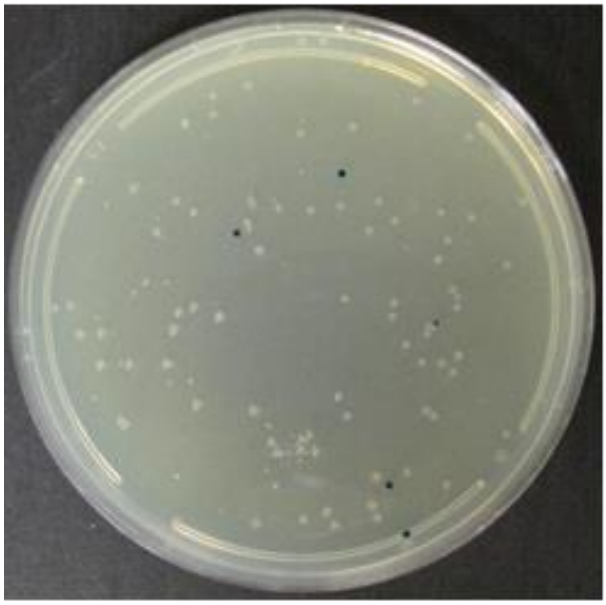

Figure 3.3: Blue-white selection was used for screening of positive clones.

3.4.2 Confirmation of fragment integration in pUC118

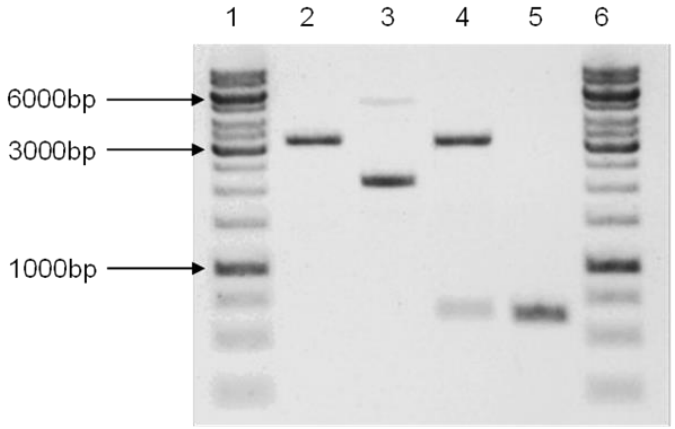

Figure 3.4: The restriction endonuclease digestion analysis of the pUC118-*PGK1* plasmid and the PCR product of the *FLO1-PGK1* fragment. Lane 1 and 6 contain the one kb molecular weight marker (Thermo-Scientific, USA). Lane 2, contains the linearized pUC118 vector. Lane 3, Mini-prep product of the recombinant pUC118-*PGK1* plasmid. Lane 4, Double digestion of pUC118-*PGK1* using *Eco*RI and *Xho*I restriction enzymes, the lower band is indicative of the released 647 bp *FLO1-PGK1* fragment and the higher band represents the linearized pUC118 vector. Lane 5, PCR amplification product of the *FLO1-PGK1* fragment.

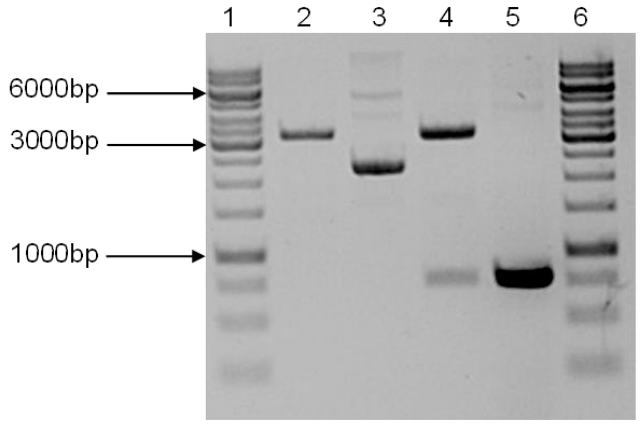

Figure 3.5: The restriction endonuclease digestion analysis of the pUC118-Zeo plasmid and the PCR product of the Zeocin™ fragment. Lane 1 and 6 contain the one kb molecular weight marker (Thermo-Scientific, USA). Lane 2, contains the linearized pUC118 vector. Lane 3, Mini-prep product of the recombinant pUC118-Zeo plasmid. Lane 4, Double digestion of pUC118-Zeo using *Sal*I and *Bgl*II restriction enzymes, the lower band is indicative of the released 761 bp *FLO1-PGK1* fragment and the higher band represents the linearized pUC118 vector. Lane 5, PCR amplification product of the Zeocin™ fragment.

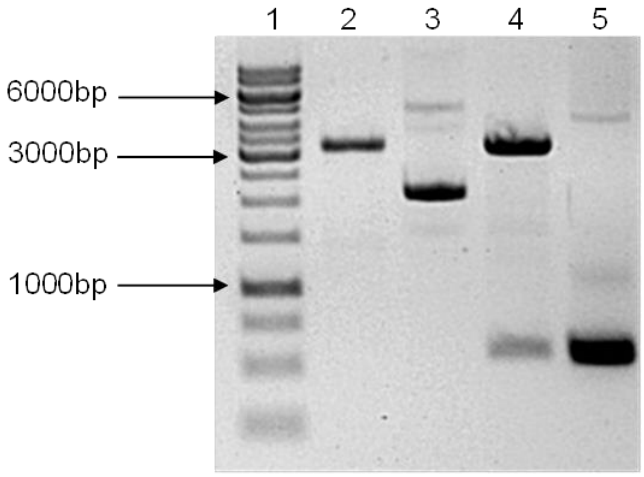

Figure 3.6: The restriction endonuclease digestion analysis of the pUC118-*TEF1* plasmid and the PCR product of the *TEF1-FLO1* fragment. Lane 1 contains the one kb molecular weight marker (Thermo-Scientific, USA). Lane 2, contains the linearized pUC118 vector. Lane 3, Mini-prep product of the recombinant pUC118-*TEF1* plasmid. Lane 4, Double digestion of pUC118-*TEF1* using *BamH*I and *Pst*I restriction enzymes, the lower band is indicative of the released 595 bp *TEF1-FLO1* fragment and the higher band represents the linearized pUC118 vector. Lane 5, PCR amplification product of the *TEF1-FLO1* fragment.

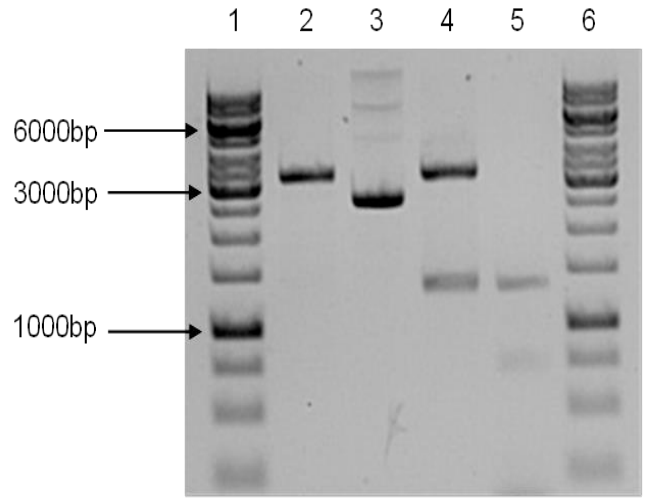

Figure 3.7: The restriction endonuclease digestion analysis of the pUC118-FZT plasmid and the PCR product of the *Zeocin*™*-TEF1* fragment. Lane 1 and 6 contains the one kb molecular weight marker (Thermo-Scientific, USA). Lane 2, contains the linearized pUC118 vector. Lane 3, Mini-prep product of the recombinant pUC118-FZT plasmid. Lane 4, Double digestion of pUC118-FZT using *Sal*I and *Pst*I restriction enzymes, the lower band is indicative of the released 1375 bp *Zeocin*™*-TEF1* fragment and the higher band represents the linearized pUC118 vector. Lane 5, PCR amplification product of the *Zeocin*™*-TEF1* fragment.

3.4.3 Yeast transformation and phenotypic characterization

After the initial selection on YEPD plates containing Zeocin™, 40 putative transformants were inoculated into YEPD broth and cultivated for 24 hours at 30°C at 160 rpm in an orbital shaker. The parental strain BY4741 displayed a non-flocculent phenotype (Figure 3.8A). Approximately 15% of the putative transgenic BY4741 strain transformed with the *FLO1p-PGK1-Zeo^{R}-TEF1-FLO1p* promoter replacement cassette displayed a strong flocculation phenotype (Figure 3.8B).

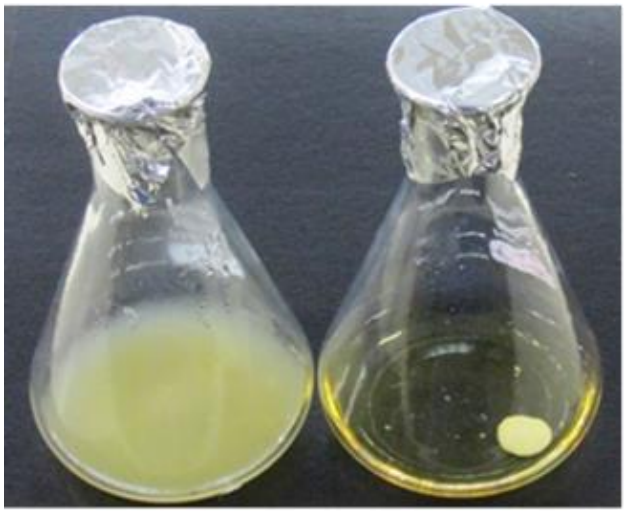

Figure 3.8: Cultures of non-flocculent wild type laboratory strain BY4741 (A) and a flocculent transgenic BY4741 strain containing the *FLO1p-Zeocin™-TEF1-FLO1p* promoter replacement cassette (B). The wild type BY4741 cells are suspended in the liquid YEPD growth medium (A), whereas the transgenic wild type strain visibly displays a strong flocculation phenotype exhibiting distinct flocs in YEPD medium.

3.4.4 PCR verification of *FLO1-based* transgenic yeast strain

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 3.9, lanes 2 and 3). 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 3.9 lanes 4 and 5).

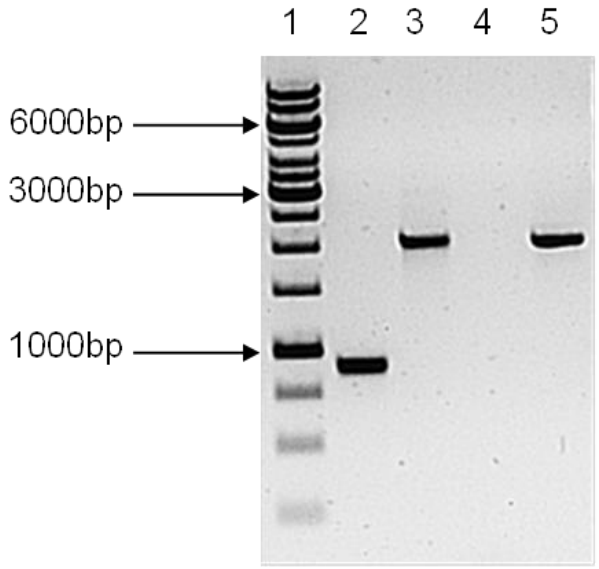

Figure 3.9: Chromosomal integration of the *TEF1* promoter upstream of the dominant *FLO1* ORF in *S. cerevisiae* strain BY4741. Lane 1 contains the 1 kb DNA molecular weight marker (Thermo-Scientific, USA). The deletion of the native *FLO1* promoter was confirmed via PCR using homologous primer pairs described in materials and methods. Lane 2 contains the PCR amplification product of the native *FLO1* promoter sequence observed in the non-transgenic wild type BY4741 strain; *FLO1p* (904 bp). Lane 3 contains the integration cassette, which was amplified from the transgenic BY4741-F1P (*FLO1*p-*Zeocin*™-*TEF1-FLO1*p, 2048 bp). The integration of promoter replacement cassette in the *FLO1* locus was confirmed via PCR using heterologous primer sets which was mentioned in the materials and methods. Lane 4 contained no PCR amplification product, the non-transgenic wild type BY4741 strain's DNA was used as the template DNA. Lane 5 contains the PCR amplification product of the *FLO1p-Zeocin*™*-TEF1p* fragment (1998 bp) promoter replacement cassette.

3.4.5 Growth curve analysis

The growth rates of the wild type strain BY4741 and *FLO1* transgenic strains regulated by *ADH2*, *HSP30*, *PGK1* and *TEF1* promoters were assessed in YEPD medium containing 2% glucose after 6, 12 and 24 hours of growth. As shown in Figure 3.10. The cell density observed in the transgenic strains BY4741-F1A and BY4741-F1H were higher than that of BY4741-F1P and BY4741-F1T.

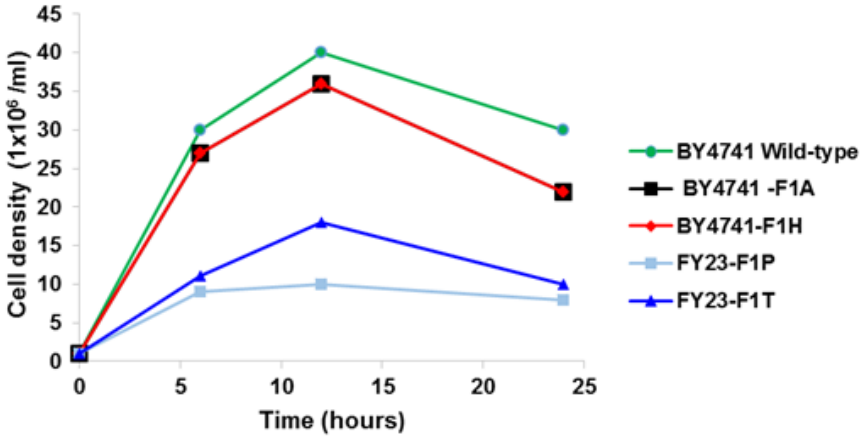

Figure 3.10: A growth curve depicting cell density of the wild type strain BY4741 and transgenic strains, BY4741-F1A, BY4741-F1H, BY4741-F1P and BY4741-F1T over time.

3.4.6 Flocculation assay

A modified Helm's flocculation assay was used to assess flocculation intensities of *FLO1* transgenic strains regulated by various promoters such as *ADH2*, *HSP30*, *PGK1* and *TEF1* (Figure 3.11). The data is presented as a mean ± standard deviation (n = 3). For the statistical analysis of the flocculation assay ANOVA was conducted to determine the p value. The p value was less than 0.0001 indicating that the results were significant.

A flocculation assay of the different promoters regulating the expression of *FLO1* was conducted as positive controls and was used to determine the different promoter strengths. The wild type FY23 was non-flocculent (1.22% ± 0.31%). The transgenic *FLO1* strains were all strongly flocculent, FY23-F1A (98.14% ± 0.12%), FY23-F1H (97.82% ± 0.46%), BY4741-F1P (98.56% ± 0.26%) and BY4741-F1T (99.53% ± 0.27%).

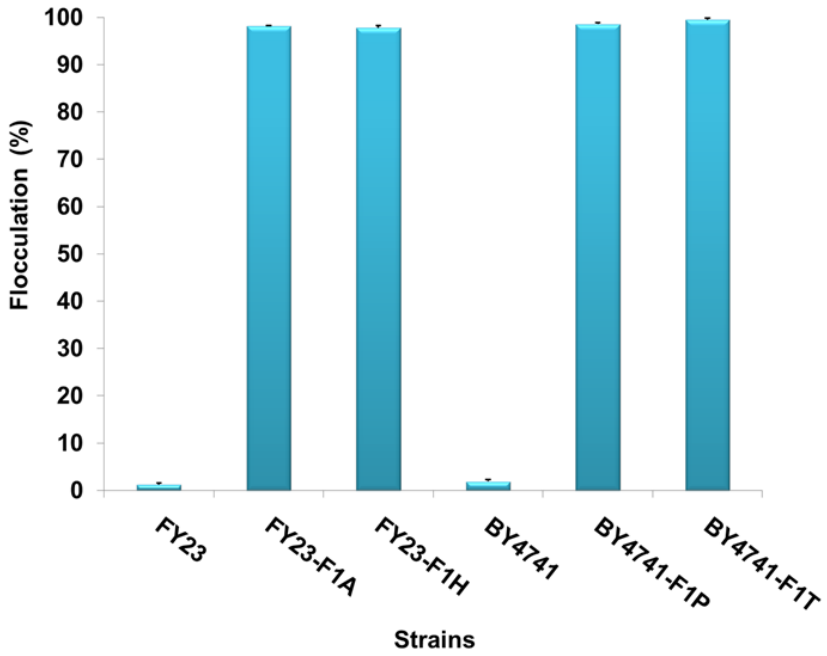

Figure 3.11: Flocculation intensities of laboratory *S. cerevisiae* strains. Each bar is indicative of the percentage of flocculation and the error bars represent the standard deviation of triplicates.

3.5 Discussion

The cloning strategy that was employed in this study has an advantage over strategies used by other research groups (Cunha *et al.*, 2006) since this novel strategy did not require sub-cloning of the *FLO1* gene which is known as a difficult ORF to clone due to the number of intragenic tandem repeats in its ORF (Govender *et al.*, 2008). This study employed recombinant DNA strategies and constructed a promoter replacement cassette that was similar to that of Govender *et al.* (2008) and Verstrepen and Thevelein (2004). PCR amplicons *FLO1-PGK1*, Zeocin™ and *TEF1-FLO1* fragments containing the restriction endonuclease digestion enzymes *Xho*I, *Sal*I, *Bgl*II and *Bam*HI respectively were not directly digested due to the low efficiency associated with the restriction digestion of PCR amplicons which contained restriction sites at the 5' or 3' termini. Consequently, these fragments were sub-cloned into a linearized pUC118 plasmid vector and thereafter recovered by double digestions which are associated with higher recovery efficiency when performed on plasmid DNA. Blue-white selection was used as a selection tool to screen the positive transformants. These plasmid derived fragments were subsequently ligated to generate the promoter replacement cassette.

The *PGK1* promoter sequence was amplified from a polygalactorunic acid producing strain BY4742-PGU1. The *PGU1* gene located in most *S*. *cerevisiae* strains encodes for an endo-polygalacturonase (Louw *et al.*, 2010; Van Wyk and Divol, 2010). The activity of polygalacturonase is strain specific in *S*. *cerevisiae* (Louw *et al.*, 2010). The *S*. *cerevisiae PGU1* is located on chromosome X and there is only a single copy which is present per haploid genome (Van Wyk and Divol, 2010). The endo-polygalacturonase is a pectinolytic enzyme which cleaves bonds found between non-methylated galacturonic acid units that are found in pectin (Van Wyk and Divol, 2010). The *PGK1* genomic DNA was isolated from a BY4742 strain which contained PGU1.

The *PGK1* will drive the expression of the Zeocin™ selection marker and a *CYC1* transcription terminating promoter will end the termination of the Zeocin™ fragment. Zeocin™ is composed of phleomycin D1. Zeocin™ is basic in alkalinity and water-soluble copper chelated glycopeptide which is isolated from *Streptomyces verticillus*. This copper-chelated glycopeptide form is inactive. However, when Zeocin™ enters the cell, this results in the reduction of the copper cation which is reduced from Cu^{2+} to Cu^{1+}. The copper is removed by sulfhydryl compounds, which results in the activation of Zeocin™. Zeocin™ will thereafter bind and cleave DNA, leading to cell death (Berdy, 1980).The Zeocin™ resistance protein is a 13,665 Da protein (Drocourt *et al*., 1990).

The use of Zeocin™ as a selection tool for screening was based on the research conducted by Alderton *et al.* (2006) and Higgins *et al.* (1998) which indicated that there was a high yield of transformation efficiency because the insert size was smaller than other selectable markers obtained from yeast genomic sequences such as *SMR1* (Alderton *et al.*, 2006; Higgins *et al.*, 1998; Johansson and Hahn-Hägerdal, 2002). Zeocin™ selection was conducted because it had a broad selection range as it inhibits the growth of prokaryotes and eukaryotes, thus the efficiency of the transformation process as well as the sensitivity of the screening process was increased (Higgins *et al.*, 1998).

The constitutive *PGK1* promoter was fused to the Zeocin™ marker gene which was used for the selection of transformants. The *PGK1* promoter regulates Zeocin™ expression in the transformants and the positive transformants would be Zeocin™ resistant. The *TEF1* promoter was ligated to the *PGK1*-Zeocin™ fragment to form the promoter replacement cassette. All the fragments used in the promoter replacement cassette were verified by Sanger DNA sequencing.

It was established that feral laboratory strains of *S. cerevisiae* do not have the innate ability to express the *FLO* encoded phenotype due to the non-sense mutation in *FLO8*, which is a transcription activator of the dominant *FLO* gene family (Liu *et al.*, 1996; Verstrepen *et al.*, 2005). As such the expression of *FLO1* transcriptional control was driven by the *TEF1* promoter. The promoter replacement cassette was designed to integrate into the genome and constitutively drive the expression of *FLO1* throughout all growth phases. Consequently, the *TEF1* promoter was selected on the basis of its attributes such as the high constitutive expression of a desired gene (Van Mulders *et al.*, 2009).

A PCR-based strategy was employed in this study whereby the regions for homologous recombination with the target allele were incorporated into the 5' and 3' termini of the *FLO1*p-*Zeocin*™-*TEF1-FLO1*p promoter replacement cassette. The integration of the *TEF1* driven promoter replacement cassette was facilitated by homologous recombination which allowed for the site specific integration into the yeast genome. A study conducted by Van Mulders *et al.* (2009) used the constitutive *TEF1* promoter to drive *FLO* gene expression in BY4742, however their cloning strategy utilized plasmid derived gene expression. This method is flawed and problematic due to curing and the size of the integrating plasmid. In contrast to plasmid derived gene expression, site-directed chromosomal integration strategy prevents variation in gene expression due to plasmid copy number and curing. In addition, site-directed chromosomal integration does not depend on selection pressure to maintain the expression vector because the cassette integrates into the yeast genome into a specific locus (Govender *et al.*, 2010).

FLO11 transgenic strains display a non-flocculent phenotype. Contrary, *FLO1* transgenic strains display a visibly strong flocculation phenotype this allowed for easy detection of the functionality of the *TEF1* promoter. This rationale was employed as a basis for using *FLO1* to construct the initial promoter replacement cassette which facilitated the construction of the *FLO11* promoter replacement cassette.

The integration of the promoter replacement cassette *FLO1p*-Zeocin™-*TEF1-FLO1p* into the feral BY4741 non-flocculent laboratory strain genome resulted in the display of a distinctively strong flocculation phenotype by the transgenic strain. The transgenic BY4741 strain was verified utilizing PCR which employed homologous and heterologous primer sets respectively. This verification validated that the *FLO1* native ORF was placed under the transcriptional control of the constitutive *TEF1* promoter. The promoter replacement cassettes sequence was also verified in the BY4741-F1T transgenic strain using Sanger DNA sequencing. The DNA of this transgenic strain served as a template for the synthesis of the *FLO11p-Zeocin*™*-TEF1-FLO11p* promoter replacement cassette. The transgenic strains containing the *FLO1*p-*Zeocin*™*-TEF1-FLO1*p promoter replacement cassette exhibited low transformation efficiency.

All the transgenic strains constructed contained the *FLO1* replacement vector cassette. The *FLO1p-Zeocin™-TEF1-FLO1p* promoter replacement cassette was used as a tool to facilitate the construction of the *FLO11p-Zeocin™-TEF1-FLO11p* promoter replacement cassette.

The growth trends observed in the wild type and transgenic strains BY4741-F1A, BY4741-F1H, BY4741-F1P and BY4741-F1T were comparable. In terms of cell density the inducible promoters *ADH2* and *HSP30* driven strains cell density did not significantly differ from the wild type strain. However, the constitutive promoters *PGK1* and *TEF1* driven transgenic strains displayed a decreased cell density in comparison to the wild type strain. Low cell density is indicative of a low biomass and substrate utilization, not to mention a high probability of a stuck fermentation. As such the decreased biomass in BY4741-F1P and BY4741-F1T strains containing the constitutive promoters indicated that the substrate is partially utilized due to the onset of early flocculation. Early flocculation increases the possibility of sluggish or stuck fermentation as a result the final fermentation products would have high residual sugars, unsatisfactory aromatic characteristics and susceptible to spoilage (Verstrepen *et al.*, 2001). Given the above backdrop the constitutive promoters would not be well suited for industrial fermentation. Consequently the *PGK1* and *TEF1* promoters can be used for metabolic studies for the expression of proteins. The constitutive promoters express the *FLO1* gene throughout all growth phases. This was validated by the visibly strong flocculation phenotype observed throughout all phases of growth. The *ADH2* and *HSP30* promoters appear to be suitable promoter candidates in the brewing industry, since these promoters have a higher biomass yield which will allow for complete utilization of the substrate and ultimately the completion of fermentation.

Yeast strains are classified as strongly flocculent if they flocculate more than 85% (ASBC, 1996). The results were as follows the *FLO1* driven by the constitutive *TEF1* promoter contained the highest flocculation intensity of (99.53% ± 0.27%) followed by the constitutive *PGK1* promoter (98.56% ± 0.26%), the inducible *ADH2* (98.14% ± 0.12%) and *HSP30* (97.82% ± 0.46%) promoters. All the transgenic *FLO1* strains displayed a visibly strong flocculation phenotype.

The functionality and the integrity of the *TEF1* promoter replacement cassette was verified by the strong flocculation phenotype displayed by the transgenic parental strain. This indicated the integration of the promoter replacement cassette upstream of the *FLO1* ORF. A similar strong flocculation phenotype was stated by Van Mulders *et al.* (2009) when a promoter replacement cassette bearing the constitutive *TEF1* promoter was integrated upstream of the native *FLO1* ORF of BY4742. The strains containing the *TEF1* promoter, from this study, yielded comparable

flocculation (99.53% ± 0.27%) intensity as compared to the use of the inducible *ADH2* promoter (98% ± 1%), *HSP30* promoter (97% ± 1%) (Govender, et al., 2008) and the constitutive *TEF1* promoter (99.6%) (Van Mulders, et al., 2009).

The research data presented evidence that the promoter replacement cassette can constitutively over-express *FLO1* encoded mannoproteins in adequate amounts. This study provides a feasible strategy to generate sufficient amounts of mannoproteins which would assist in the future with regards to the characterization of in-depth biochemical structures of cell wall-related glycoproteins as there is limited knowledge encompassing the glycoproteins structure.

3.6 Conclusion

This Chapter illustrated the construction of a promoter replacement cassette *FLO1p-Zeocin™-TEF1-FLO1p* which showed the constitutive expression of *FLO1* in *S. cerevisiae*. The correct integration of the promoter replacement cassette was confirmed by the strong flocculation phenotype and Sanger DNA sequencing. The *FLO1p-Zeocin™-TEF1-FLO1p* promoter replacement cassette conferred stable flocculation throughout all the phases of growth. This work validated that the constitutive *TEF1* promoter was capable of over-expressing the dominant *FLO1* gene. The modified Helms assay was semi-quantitative in nature. As such, it was limited in differentiating between negligible differences in strong flocculation phenotypes

3.7 Acknowledgements

This work was made possible through financial support from the NRF and UKZN.

3.8 References

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Chapter 4

Research Results II

Over-expression of *FLO11* encoded mannoprotein in BY4741 strains bearing a gene deletion related to cell wall biogenesis

Over-expression of *FLO11* encoded mannoprotein in BY4741 strains bearing a gene deletion related to cell wall biogenesis

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4.1 Abstract

There are five dominant *FLO* genes that were identified in *S. cerevisiae* which encode for glycoproteins also known as mannoproteins, adhesins or flocculins. These mannoproteins are anchored through a GPI remnant and are covalently linked to the *β*-1,6-glucan network in the yeast cell wall. Numerous adhesion phenotypes are associated with these *FLO* encoded mannoproteins. There is limited information regarding the fine molecular structure of mannoproteins which mainly depends on the information produced from *in silico* based predictive research. In this study a novel genetic engineering strategy was employed to over-express the *FLO11* encoded mannoprotein in BY4741 laboratory strain and its derivative mutant strains bearing a specific gene deletion related to cell wall biogenesis. The *FLO11* ORF was placed under the control of a constitutive *TEF1* promoter. Zeocin™ was used as a selection marker and was regulated by a constitutive *PGK1* promoter. A flocculation and invasive growth assay was conducted in this study to assess the adhesion phenotypes of the transgenic stains. In this study, all transgenic strains over-expressing the *FLO11* encoded mannoprotein displayed a non-flocculent and invasive growth phenotype. Interestingly, the invasive growth intensity was visibly weaker in both the deletion strains in comparison to the transgenic parental BY4741-F11T strain utilized in this study. As such, the current study indicated that both the deletion BY4741 transgenic strains are potential hyper-secretion candidates. To determine if these transgenic deletion strains are potential hyper-secretors of mannoproteins further characterization of the liquid medium is required to validate the release of the *FLO11*-encoded mannoproteins into the extracellular medium. It is envisioned that the transgenic BY4741 deletion strains over-expressing *FLO11* mannoproteins can provide a suitable strategy for the isolation and purification of mannoproteins released into the extracellular growth medium. Furthermore, this mannoprotein reservoir can be used for the structural analysis of *FLO11* encoded mannoproteins.

4.2 Introduction

S. *cerevisiae* can exist in numerous multicellular aggregates in order for yeast cells to increase their survival rate and utilize the available resources. The ability of yeast to form cooperative communities is imperative for their survival. Yeast communities are relevant to human health as well as industry. Cooperative strategies utilized by the cells include filament formation, flocculation, adhesion and biofilm formation (Honigberg, 2011).

Fungal adhesion signifies the prelude to the development of an infection, thus it has medical significance (Verstrepen and Klis, 2006). The ability to adhere to abiotic surfaces, for instance plastic prostheses and catheters, serve as carriers of pathogenic yeast to patients' internal organs and bloodstream. This ability to adhere to surfaces is enhanced by biofilm formation and is difficult to eradicate (Kojic and Darouiche, 2004). The switch from non-adherence to adherence is important for fungal pathogenesis and allows pathogenic yeasts to detect and respond to the unfavourable environmental conditions depending on the host and also assists the pathogen in colonizing tissue (Verstrepen and Klis, 2006).

Adhesin facilitated phenotypes comprise of agar invasion, (Guo *et al.*, 2000; Verstrepen and Klis, 2006), biofilm formation (Purevdorj-Gage *et al.*, 2007; Reynolds and Fink, 2001), pseudohyphae formation (Lambrechts *et al.*, 1996; Lo and Dranginis, 1996; Lo and Dranginis, 1998), colony morphology (Kuthan *et al.*, 2003), the adherence to plastic surfaces (Mortensen *et al.*, 2007) as well as "flor" formation (Fidalgo *et al.*, 2006; Ishigami *et al.*, 2006). Flo11p is essential and contributes to "mat" formation ("biofilm formation") (Reynolds and Fink, 2001), flocculation in *S*. *cerevisiae* var. *diastaticus* (Bayly *et al.*, 2005), pseudohyphal formation (Lambrechts *et al.*, 1996; Lo and Dranginis, 1996; Lo and Dranginis, 1998) and "flor" formation (Ishigami *et al.*, 2006).

The primary objective pertaining to genetic-based research of *FLO* genes is driven by their phenotype that they display. These phenotypes have imperative roles in the brewing and pharmaceutical industries. In an attempt to genetically control the onset of flocculation in both industrial and laboratory strains, the native promoters of *FLO* genes were replaced with inducible promoters such as *ADH2* and *HSP30*. (Cunha *et al.*, 2006; Govender *et al.*, 2010; Govender *et al.*, 2008). These transgenic strains flocculated on entry into the stationary phase of growth.

Gonzalez-Ramos and Gonzalez (2006) identified gene deletion strains that contained a cell wall deletion exhibited hyper-secretion of cell wall mannoproteins. Furthermore, research studies showed that gene deletion strains such as *KNR4* (Penacho *et al.*, 2012) and *TDH3* (Gonzalez *et al.*, 2010) were able to over-express mannoproteins that will improve the organoleptic properties of wine (Gonzalez-Ramos and Gonzalez, 2006). This novel strategy was employed in this work and has the potential to benefit the local as well as international wine industries because the presence of mannoproteins in wine has the ability to enhance the quality of wine (Gonzalez-Ramos and Gonzalez, 2006; Penacho *et al.*, 2012).

To date, research studies have reported that deletions of specific genes, related to cell wall biosynthesis can result in mannoproteins being released to the extracellular environment (Gonzalez-Ramos *et al.*, 2008; Gonzalez-Ramos and Gonzalez, 2006; Gonzalez *et al.*, 2010; Penacho *et al.*, 2012). The defective strains containing a gene deletion related to cell wall biosynthesis were suggested to release higher amounts of polysaccharides, particularly mannoproteins into the medium. The effects of these cell wall biogenesis related gene deletions on the phenotypes conferred via the adhesins in the cell wall surface have not yet been reported. As such, this study hypothesised that *KNR4* and *TDH3* gene deletions strains would result in a decrease in *FLO11* gene based adhesion phenotypes due to ineffective incorporation of flocculins into the yeast cell surface which aids in the extracellular release of mannoproteins.

This study utilized a PCR based strategy to construct a *FLO11* promoter replacement cassette. The haploid *S. cerevisiae* laboratory strains BY4741 wild type, BY4741Δ*KNR4* and BY4741Δ*TDH3* was transformed via electroporation with the PCR generated vector cassette. The adhesion phenotypes of BY4741-F11 strain and its derivative transgenic strains harbouring a deletion in *KNR4* and *TDH3* were assessed. The *KNR4* gene encodes for a regulatory protein involved in cell wall integrity pathway whilst *TDH3* encodes for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

It was previously reported that the over-expression of *FLO* genes confers certain cell surface phenotypes such as hydrophobicity, flocculation, flor-formation, biofilm formation and invasive growth (Govender *et al.*, 2008; Van Mulders *et al.*, 2009). The research data showed that strains which contained a deletion of *KNR4* and *TDH3* respectively displayed weaker invasive growth intensity in comparison to the transgenic parental strain. All transgenic strains over-expressing the *FLO11* encoded mannoprotein displayed a non-flocculent and invasive growth phenotype.

4.3 Materials and methods

4.3.1 Yeast strains

The strains employed in this study are listed in Table 4.1. All yeast strains were derived from *S. cerevisiae*.

Table 4.1: Strains employed in this study

Strain/Plasmid	Genotype	Reference
BY4741	MATa *his*3Δ1 *leu*2Δ0 *met15*Δ0 *ura*3Δ0	EUROSCARF
BY4741-F1T	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 FLO1p::-Sh ble-CYC1-TEF1p*	This study
BY4741-F11T	*MATa his3Δ1 leu2Δ0 met15*Δ0 *ura3Δ0 FLO11p:: Sh ble-TEF1p*	This study
BY4741-ΔKNR4	*MATa his3Δ1 leu2Δ0 met15*Δ0 *ura3Δ0 ;YGR229c*::*KanMX4*	EUROSCARF
BY4741-ΔKNR4-F11T	*MATa his3Δ1 leu2Δ0 met15*Δ0 *ura3Δ0; YGR229c::KanMX4, FLO11p:: Sh ble-TEF1p*	This study
BY4741-ΔTDH3	*MATa his3Δ1 leu2Δ0 met15*Δ0 *ura3Δ0; YGR192c*::*KanMX4*	EUROSCARF
BY4741-ΔTDH3-F11T	*MATa his3Δ1 leu2Δ0 met15*Δ0 *ura3Δ0; YGR192c*::*KanMX4, FLO11p:: Sh ble-TEF1p*	This study

4.3.2 Media and yeast cell growth conditions

S. cerevisiae strains were cultivated in YEPD medium which contained 1% (wt/vol) yeast extract (Merck, Germany), 2% (wt/vol) glucose (Merck, Germany) and 2% (wt/vol) peptone (Merck, Germany). The yeast cultures were then incubated for 24 hours at 30°C in an orbital shaker at 160 rpm.

The selection of the deletion strains that are kanamycin (*KanMX*) resistant strains were selected in YEPD medium which was supplemented with 200 µg/mL of Geneticin (G418) (Thermo-Scientific, USA). The selection of Zeocin™ resistant yeast transformants were plated on defined synthetic complete (SC) media. The SC (DuPont Agricultural Products, France) media contained 0.67% (wt/vol) yeast nitrogen base (YNB) (Difco™) without amino acids, 2% (wt/vol) glucose (Merck, Germany), 2% (wt/vol) agar (Merck, Germany) and 18% (wt/vol) sorbitol (Melford laboratories Ltd, Bildestond Road, Ipswich). The medium was supplemented with the auxotrophic amino acid requirements as per laboratory yeast strain (Ausubel *et al*. 1995) and 150 µg/mL of Zeocin™. Thereafter, the positive transformant yeast strains were stored in YEPD medium supplemented with 30% glycerol (Merck, Germany) and placed in the biofreezer (Snijders Scientific, Tilburg, Netherlands) at -80ºC for long-term storage.

4.3.3 Primers

The primers used in this study are listed in Table 4.2. All primers were designed using Primer Stats software and the primers were purchased from Integrated DNA Technologies (USA).

4.3.4 Confirmation of deletion strains

The deletion in the *KNR4* and *TDH3* ORF's were confirmed in the transgenic mutant strains. The confirmation of the deletion strains were conducted via PCR following the verification method described by Kelly *et al.* (2001). The primers that were utilized in the confirmation of deletion strains are listed in Table 4.2. The genomic DNA of transgenic deletion strains were used as templates for the PCR reactions. The wild type BY4741 yeast strain was used as a control in this confirmation experiment. For each deletion mutant, a set of confirmation primers were used (confirmation primers A, B, C and D) in conjunction with KanB and KanC primers these were used in the verification of the kanamycin (*KanMX*) deletion cassette. To verify the deletion of the native ORF by the kanamycin deletion module, two primers common to regions within the kanamycin deletion module were used which was KanB and KanC. The correct integration of the *KanMX* gene was verified in the transformants by the appearance of a PCR product.

Table 4.2: Primers used in this study

Primer Name	Primer Sequence (5'→3')
FLO11::PGK1-F	TCACTGCACTTCAACTATGCCTTATAGCAACCAAGAAGCTAGAAAATGCCTCCCT CCTTCTTGAATTGAT
FLO11-TEF1-R	GGACCAAATAAGCGAGTAGAAATGGTCTTTGCATAGTGTGCGTATATGG AACACCGCCCCTTAGATTAG
FLO11-F	CCTCTCACTGCACTTCAACTATGC
FLO11-F2	GCTGCTTGTCTCACATCTAAACTTCG
FLO11-R	GGACCAAATAAGCGAGTAGA
KNR4-Conf A	CAACTGAAAAGGTTGTGTTTTCTTT
KNR4-Conf B	GGGATAAACCTTGTTGAGATCTTTT
KNR4-Conf C	TTTAAAAAGAAGAACGTGGATCAAG
KNR4-Conf D	GCCAACCGTTTTGTATATATTGATT
TDH3-Conf A	CATCAGTTCATAGGTCCATTCTCTT
TDH3-Conf B	ATCTTCTTACCATCGACAATGATGT
TDH3-Conf C	GTTTTCAAGGAATTAGACACTGCTC
TDH3-Conf D	AATATCCCCAAAATTATTAAGAGCG
KanB	CTGCAGCGAGGAGCCGTAAT
KanC	TGATTTTGATGACGAGCGTAAT

*F denotes forward primer and R denotes the reverse primer.

4.3.5 Purification of amplified promoter replacement cassettes and yeast transformation.

A mass PCR amplification of the *FLO11* promoter replacement vector cassette was conducted. In order to ensure high fidelity amplification, Prime STAR (Takara Bio Inc., Otsu, Japan) DNA polymerase was used in the mass amplification in which the PCR product was used as a vector cassette for the yeast transformation. The pooled amplification reactions was run on an (1%) agarose gel and purified using Zymoclean™ gel DNA recovery kit (ZYMO Research, USA) according to the instructions of the manufacturer. The vector cassette was eluted and the volume of the sample was reduced in a vacuum concentrator (Eppendorf, concentrator 5301, Germany). A 5 µL aliquot of the *FLO11* promoter replacement vector cassette was used to transform yeast by electroporation. A similar strategy was used for the transformation of the *FLO1* cassette. The FLO11-PGK1 forward primer and FLO11-TEF1 reverse primers were used on the *FLO1* cassette which served as the template DNA. Thereafter, the *FLO11* short primers FLO11-F2 and FLO11-R were used to extract the *FLO11* cassette this was used for the yeast transformation. The cassette *FLO11*p-*Zeocin*™-*TEF1*-*FLO11*p was approximately 1980 bp in length.

Preparation of electro-competent BY4741 cells and transformation was performed by a modified method of Ausubel *et al.* (1995). Yeast transformation of BY4741, BY4741-Δ*KNR4* and BY4741-Δ*TDH3* strains was achieved using cryopreserved electro-competent cells and 5 µL of purified *FLO11* promoter replacement vector cassette. Electroporation cuvettes (Cell Projects Ltd., Kent, UK) with a 2 mm electrode gap were used and a Bio-Rad MicroPulser™ (Bio-Rad Laboratories, USA) was employed in the electroporation with the following manufacturer approved settings: 1.5 kV for approximately 5-6 milliseconds. An aliquot (5 µl) of TE buffer was used instead of the DNA vector cassette as a negative control for the electroporation.

4.3.6 Transgenic yeast strain verification

All transgenic yeast strains were verified using a PCR strategy and the Takara Ex Taq™ DNA polymerase (Takara Bio Inc., Otsu, Japan) system according to the manufacturer's guidelines. All primers used in this aspect of the study are listed in Table 4.2. Genomic DNA was isolated from the positive yeast transformants using a method recommended by Ausubel *et al*. (1995). Chromosomal integration was achieved by the double-crossover homologous recombination event in which the *FLO11* ORF was placed under transcriptional control of the *TEF1* promoter. The deletion of native promoters was confirmed by PCR using homologous primer sets. The primer pairs used for the transgenic wild type and deletion strains containing the *FLO11*p-*Zeocin*™-*TEF1-FLO11*p were as follows: primers FLO11-F2 and FLO11-R.

In addition, the integration of the promoter replacement cassettes into the correct locus were confirmed by PCR using heterologous primer sets which contained a forward primer that is complementary to sequences from outside the region of integration and the TEF1-R reverse primer. The primer pairs for different transformants were as follows: FLO11-F2 and TEF1-R and FLO11-F2 and FLO11-R. The wild-type BY4741 strain's chromosomal DNA served as a control in these confirmation experiments.

4.3.7 Flocculation assay

The yeast strains flocculation ability was determined using the modified Helm's assay as described by D'Hautcourt and Smart (1999). Five replicates of the control and test reactions were conducted for each sample. The data is presented as a mean ± standard deviation (n = 3).

4.3.8 Statistical analysis

In this study, ANOVA was employed to statistically compare flocculation data obtained for BY4741 wild type and transgenic yeast strains. Analyses were performed using the statistical software package Graph pad in stat version 3.05 32 bit for Windows 95/NT (Graph pad software, San Diego California).

4.3.9 Invasive growth

Yeast cultures were grown for 48 hours with shaking at 160 rpm at 30ºC in YEPD medium. The YEPD medium contained 1% (w/v) yeast extract, 2% (w/v) glucose and 2% (w/v) peptone. These cultures were plated on SC plates which contained 0.67% (w/v) yeast nitrogen base, 0.2% (w/v) glucose, 2% (w/v) agar and the auxotrophic amino acid requirements. Thereafter, the yeast cultures were incubated at 30ºC for a period of five days. The plates were then washed under a constant supply of running water to physically remove superficial yeast growth. The plates were then air dried and photographed.

4.3.10 Verification of DNA fragments via Sanger DNA sequencing

The *FLO11* promoter replacement cassette sequence was verified by Sanger DNA sequencing utilising the ABI BigDye® V3.1 kit (Applied Biosystems) and the ABI 3500XL genetic analyser (Applied Biosystems) at Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa).

4.4 Results

4.4.1 Confirmation of *KNR4* and *TDH3* gene deletions in BY4741 strain

The deletion of the native *KNR4 and TDH3* ORFs by homologous recombination of the *KanMX4* cassette into the genome of BY4741-Δ*KNR4* and BY4741-Δ*TDH3* mutants and their transgenic strain derivatives were confirmed via a PCR-based strategy (Figure 4.1.).

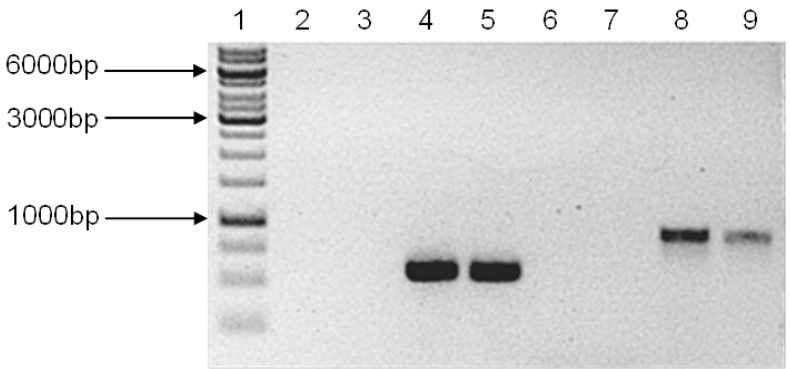

Figure 4.1: Confirmation of the integration of kanamycin (*KanMX*) deletion cassette in BY4741Δ*KNR4* and BY4741Δ*TDH3* deletion strains. Lane 1 contains a 1 kb molecular weight marker (Thermo-Scientific, USA). The *KanMX* deletion cassette was integrated to knockout either the *KNR4* or *TDH3* ORF's respectively. Lanes 2 and 3 confirms the deletion of *KNR4* and *TDH3* ORF's (primer set: Confirmation primer A and Confirmation B was used); Lanes 4 and 5 confirms the replacement of *KNR4* and *TDH3* ORF's with the *KanMX* cassette, yielding a band size of approximately 685 bp (primer set: Confirmation A and Kan B was used); Lanes 6 and 7 confirms the deletion of *KNR4* and *TDH3* ORF's (primer set: Confirmation C and Confirmation D was used); Lanes 8 and 9 confirms the replacement of *KNR4* and *TDH3* ORF's respectively with the *KanMX* cassette, yielding a band size of approximately 963 bp (primer set: Kan C and Confirmation D was used).

4.4.2 Transformation of BY4741 and its derivative deletion strains

The promoter replacement strategy presented in Figure 4.2 was utilized in the electro-transformation of the laboratory yeast strains BY4741, BY4741Δ*KNR4* and BY4741Δ*TDH3*. Fifty putative transformant colonies from YEPD selection plates containing Zeocin™ were grown in YEPD broth at 30°C for 24 hours at 160 rpm. Under these conditions, it was observed that majority of the *FLO11* transgenic strains displayed an invasive growth phenotype. The transformation efficiencies for the *FLO11* transgenic strains displayed a high degree of positive transformants. Three independent transgenic strains were selected and cryopreserved for further analysis.

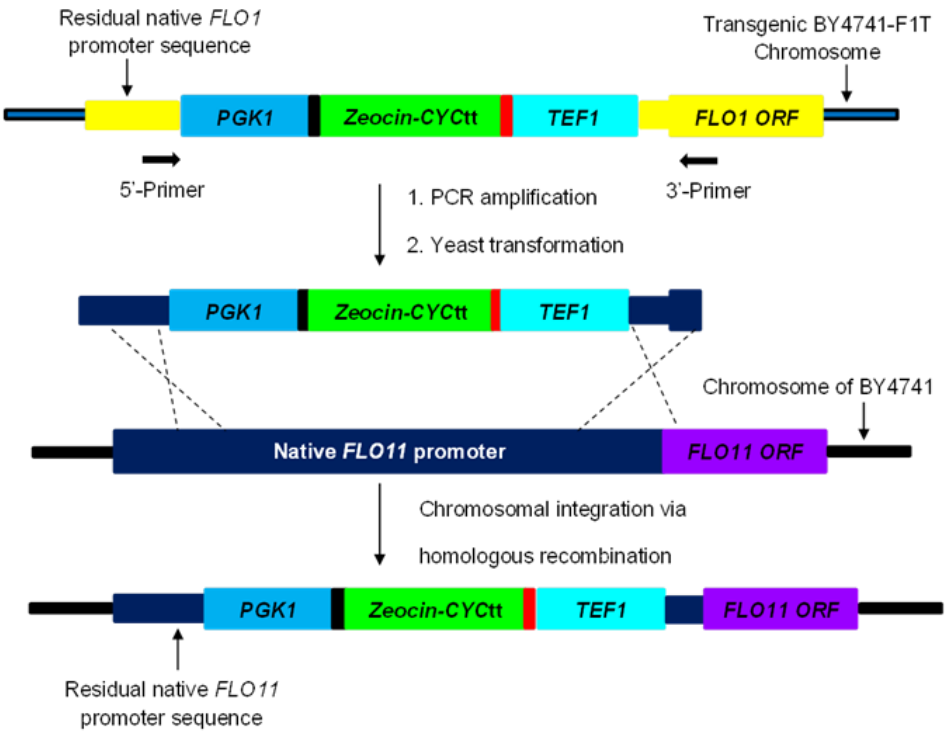

Figure 4.2 Promoter replacement strategy depicting the chromosomal integration of the *TEF1* promoter upstream of the *FLO11* ORF in BY4741 and its deletion mutant strains BY4741Δ*KNR4* and BY4741Δ*TDH3*. The promoter replacement cassette containing 5' and 3' regions which are homologous to the native promoter regions of *FLO11* ORF was amplified using the chromosomal DNA from BY4741-F1T transgenic yeast strain as a template.

4.4.3 Verification of transgenic strains

Verification of the deletion of the native *FLO11* promoter in the transgenic strains were confirmed via PCR using a homologous primer pair which consisted of a *FLO11* forward primer from outside the region of integration and a *FLO11* ORF-specific reverse primer.

The PCR amplicons obtained from the template DNA isolated from the transgenic strains BY4741-F11T; BY4741Δ*KNR4*-F11T and BY4741Δ*TDH3*-F11T correlated to the expected amplicon size of approximately 2843 bp (Figure 4.3). A larger amplicon was produced from the genomic DNA of the BY4741 wild type strain which correlated to the expected size of the native *FLO11* target gene sequence of approximately 3607 bp.

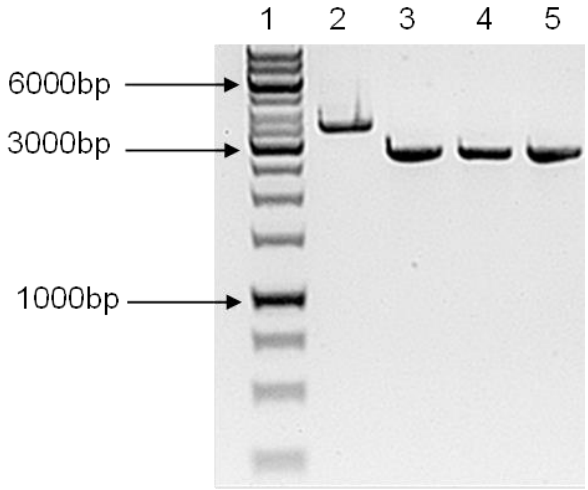

Figure 4.3: Confirmation of the deletion of the native *FLO11* promoter was verified by PCR utilizing homologous primer pairs as indicated in the materials and methods. Lane 1 contains a 1kb DNA molecular weight marker (Thermo-Scientific, USA). Lane 2 contains the PCR amplicon of the *FLO11* native promoter fragment amplified from the genomic wild type BY4741 DNA (3607 bp). In comparison to the feral BY4741 strain, a larger PCR amplicon was attained corresponding to the expected promoter replacement cassette of 2843 bp (*FLO11*p-*Zeocin*™-*TEF1*-*FLO11*p,) which was amplified from the transgenic strains i.e. Lane 3, BY4741-F11T; Lane 4, BY4741Δ*KNR4*-F11T and Lane 5, BY4741Δ*TDH3*-F11T.

The integration of the promoter replacement cassette upstream of the *FLO11* ORF of the transgenic strains were confirmed by PCR utilizing a heterologous primer set which consisted of a *FLO11* forward primer from outside the region of integration and a *TEF1* reverse primer. The correct locus of the *TEF1* promoter upstream of the *FLO11* ORF was confirmed using this primer specific combination. The correct integration of the promoter replacement cassette was verified by the presence of the expected amplicon size of approximately 2794 bp generated by all the transgenic strains. This was indicative of the correct integration of the *TEF1* promoter replacement cassette upstream of the *FLO11* ORF (Figure 4.4).

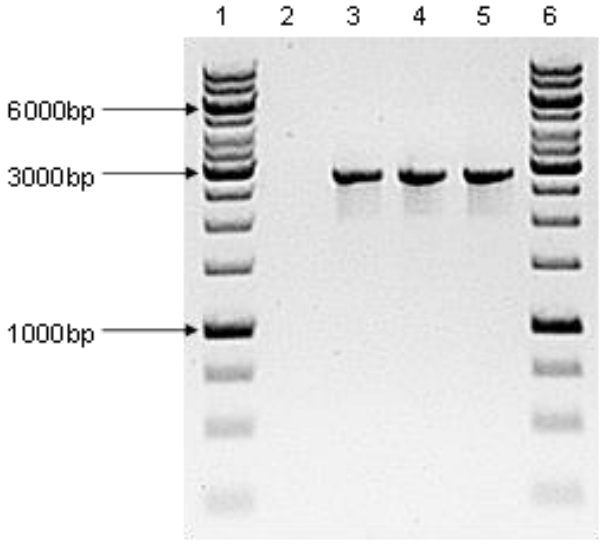

Figure 4.4: Confirmation of correct integration locus of the promoter replacement cassettes integration upstream of the *FLO11* ORF in transgenic strains was verified by PCR using heterologous primer sets as described in materials and methods. Lane 1 and 6 contains a 1kb DNA molecular weight marker (Thermo-Scientific, USA). Lane 2, no amplicon was generated from the wild type BY4741 strain. In comparison to the transgenic strains, PCR amplicons correlating to the expected promoter replacement cassette (*FLO11p-Zeocin*™*-TEF1p*, 2794 bp) was observed in all the transgenic strains i.e. Lane 3, BY4741-F11T; Lane 4, BY4741Δ*KNR4*-F11T and Lane 5, BY4741Δ*TDH3*-F11T.

4.4.4 Flocculation

The flocculation assay was conducted on the wild type BY4741 and the transgenic strains with the *FLO11* cassette under the control of the *TEF1* promoter which is upstream of *FLO11* ORF. The flocculation intensity of wild type strains and their transgenic strains were determined after 48 hours of growth in YEPD broth (Figure. 4.5). The data is presented as a mean ± standard deviation (n = 3). For the statistical analysis of the flocculation assay ANOVA was conducted to determine the p value. The p value was less than 0.0001 indicating that the results were significant. The wild type BY4741 (1.87% ± 0.46%), BY4741Δ*KNR4* (-2.03% ± 4.10%) and BY4741Δ*TDH3* (-2.07% ± 4.66%) were non-flocculent. The transgenic yeast strains, BY4741-F11T (5.41% ± 2.47%), BY4741Δ*KNR4*-F11T (4.31% ± 1.90%) and BY4741Δ*TDH3*-F11T (2.70% ± 0.49%) were non-flocculent.

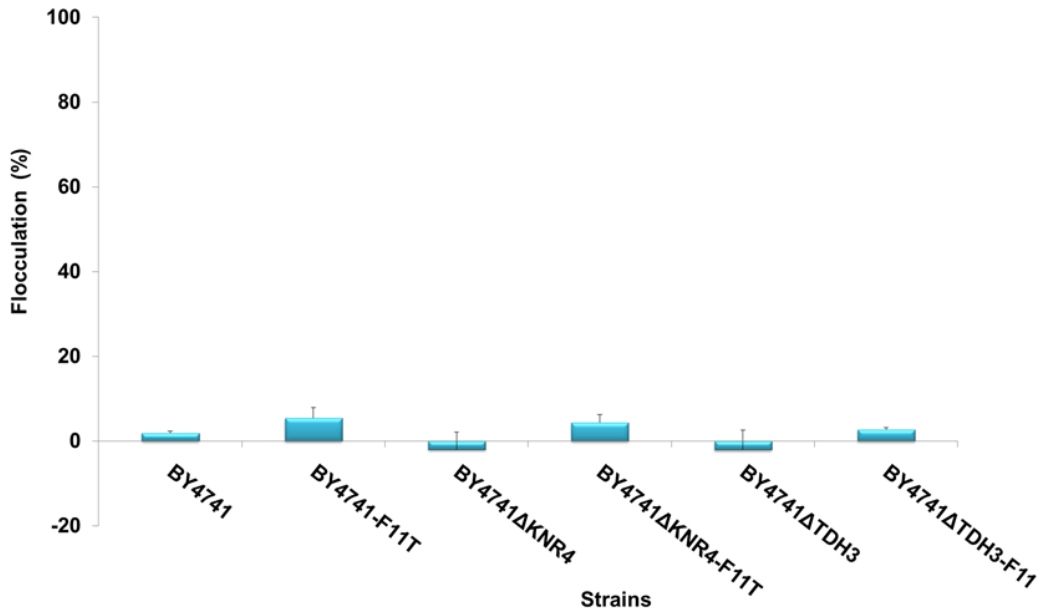

Figure 4.5: Flocculation intensities of laboratory *S. cerevisiae* strains containing a specific gene deletion related to cell wall integrity. Each bar is indicative of the percentage of flocculation and the error bars represent the standard deviation *n* =3.

4.4.5 Invasive growth

The BY4741 wild type as well as the deletion mutant strains BY4741Δ*KNR4* and BY4741Δ*TDH3* strains displayed a non-invasive phenotype. The BY4741-F1T served as a negative control, since *FLO1* encoded mannoproteins exhibit a non-invasive phenotype. The invasive growth phenotype was displayed in BY4741-F11T, BY4741Δ*KNR4*-F11T and BY4741Δ*TDH3*-F11T transgenic strains (Figure 4.6).

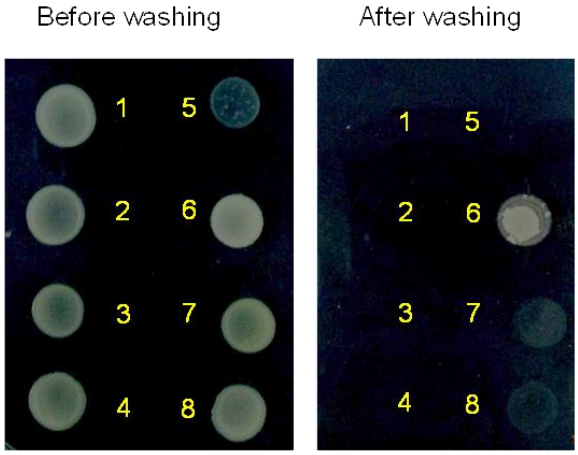

Figure 4.6: Haploid invasive growth of *FLO1* and *FLO11* over-expressing strains. Shown above is the BY4741-wild type, BY4741-wild type, BY4741Δ*KNR4* untransformed, BY4741Δ*TDH3* untransformed labelled as 1, 2, 3 and 4 respectively, and the BY4741-F1T, BY4741-F11T, BY4741-Δ*KNR4*-F11T, BY4741-Δ*TDH3*-F11T strains labelled as 5, 6, 7 and 8 respectively.

4.5 Discussion

The *FLO11* native promoter is approximately three kilo-bases in length, it is the largest described in *S*. *cerevisiae*. Its regulation is complex, with numerous inputs and pathways affecting *FLO11* expression (Barrales *et al.*, 2012; Halme *et al.*, 2004; Madhani and Fink, 1997; Octavio *et al.*, 2009; Pan and Heitman, 1999; Rupp *et al.*, 1999; Van Dyk *et al.*, 2005). *FLO11* is the most diverged gene within the *FLO* group and rarely influences flocculation significantly (Bayly *et al.*, 2005; Guo *et al.*, 2000). In comparison to yeast flocculation, the key environmental factor initiating yeast cell adhesion seems to be amino acid starvation (Braus *et al.*, 2003; Kleinschmit *et al.*, 2005; Valerius *et al.*, 2007), this affects yeast cells in the presence of adequate amount of glucose and ammonium, such compounds normally prevent adhesion phenotypes (Braus *et al.*, 2003). *FLO11* is regulated by a quorum-sensing (QS) mechanism. QS molecules are produced by the yeast cells which trigger a phenotypic response (Sprague and Winans, 2006).

There is minimal knowledge encompassing the factors which determine strain to strain *FLO11* phenotype variation (Bayly *et al.*, 2005; Douglas *et al.*, 2007). Furthermore, there is limited knowledge surrounding the *FLO11* cellular metabolic pathways which lead to their incorporation into the cell wall and biochemical synthesis. *FLO11* encodes for a protein associated with phenotypic characteristics that is considered important for virulence. The switch from a state of non-adherence to adherence in *S*. *cerevisiae* is a probable way the cells can adapt to different environmental stresses (Verstrepen and Klis, 2006). This provides an evolutionary advantage, because this plasticity is imperative from an economic perspective, as it can be exploited in numerous biotechnological applications (Brüeckner and Mösch, 2012; Verstrepen *et al.*, 2003) .
The *FLO* gene family confers phenotypic diversity to the yeast cell wall. *FLO11* encodes for a GPI-anchored glycoprotein which is rich in serine as well as threonine, and mediates homotypic adhesion between yeast cells (Douglas *et al.*, 2007). *FLO11* is involved in substrate-cell interactions such as biofilm formation on liquid and plastic surfaces (Reynolds and Fink, 2001; Zara *et al.*, 2005). The only reported flocculation expression by *FLO11* was in wine fermentation conditions (Govender *et al.*, 2011) and by *Saccharomyces diastaticus* (Bayly *et al.*, 2005; Douglas *et al.*, 2007).

This study evaluated the effect of gene deletion strains *KNR4* and *TDH3* which are devoid of a gene related to cell wall biogenesis; whereby the *FLO11* gene expression was driven by the constitutive *TEF1* promoter. The transgenic *FLO11* strains were synthesized by chromosomal integration of the constitutive *TEF1* promoter upstream of the *FLO11* ORF. The invasive growth phenotype displayed by all the transgenic BY4741 and derivative deletions strains was indicative of the integration of the *FLO11* promoter replacement cassette.

It is envisaged that the over-expression of a silent *FLO11* gene will generate the desired adhesin in ample quantities. To this end the constitutive *TEF1* promoter was engineered into a promoter replacement vector cassette containing Zeocin™ resistant marker gene to drive the *FLO11* ORF expression in BY4741 laboratory strain and gene deletion strains BY4741-Δ*KNR4* and BY4741-Δ*TDH3.* The effect of the adhesion characteristics attributed to *FLO11* gene-based transformants was evaluated. The flocculation potential of *FLO11* gene-based transformants and wild type strains were established in nutrient rich YEPD medium. It was established that *FLO11* is associated with non-flocculation (Douglas *et al.*, 2007). As such, if a strong flocculation phenotype was displayed after transformation the promoter replacement cassette could have integrated upstream of a different *FLO* ORF.

The integration of the promoter replacement cassette *FLO11p-Zeocin™-TEF1-FLO11p* into the feral BY4741, BY4741Δ*KNR4* and BY4741Δ*TDH3* strains resulted in the display of a distinctive invasive growth phenotype. The transgenic BY4741 strains were verified utilizing PCR which employed homologous and heterologous primer sets respectively. This verification validated that the *FLO11* native ORF was placed under the transcriptional control of the constitutive *TEF1* promoter. The promoter replacement cassette's sequence was also verified in all the transgenic strains using Sanger DNA sequencing.

KNR4 encodes for a protein which is involved in the regulation of the cell wall integrity pathway. The loss of *KNR4* in yeast results in an altered cell wall structure and composition, causing an increase in the release of mannoproteins (Gonzalez-Ramos and Gonzalez, 2006). *KNR4* was identified as a hyper-secretor (Gonzalez-Ramos and Gonzalez, 2006). Gonzalez *et al.* (2010) stated that Tdh3 protein could have a possible non-redundant role in yeast cell wall biogenesis. Strains bearing a deletion in *TDH3* were also identified as a hyper-secretor of proteins into the extracellular medium (Gonzalez *et al.*, 2010). Considering the background above it was expected that the transgenic deletion strains (BY4741Δ*KNR4*-F11T and BY4741Δ*TDH3*-F11T) derived from this study would most likely display decreased adhesion phenotypes when compared to the transgenic wild type strain (BY4741-F11T).

The above rationale was tentatively validated by the research data obtained from this study which indicated that the decrease in invasive growth intensity seen in BY4741Δ*KNR4*-F11T and BY4741Δ*TDH3*-F11T strains may indicate the release of flocculins into the growth substrate. The transgenic wild type strain displayed a very strong invasive growth phenotype in contrast to the transgenic deletion strains (BY4741Δ*KNR4*-F11T and BY4741Δ*TDH3*-F11T) which exhibited

a decreased intensity of the invasive growth phenotype. A strong invasive growth phenotype was observed in studies conducted by Van Mulders *et al.* (2009) where a constitutive *TEF1* promoter was used to drive *FLO11* gene expression in BY4742 and Govender *et al.* (2008) used the inducible *ADH2* promoter to control *FLO11* gene expression in FY23. The strong invasive growth phenotype observed in BY4741-F11T strain is comparable to the results obtained by Govender *et al.* (2008) and Van Mulders *et al.* (2009).

Yeast strains are classified as non-flocculent if they flocculate less than 20% (ASBC, 1996). The transgenic yeast strains containing the *FLO11* promoter replacement cassette, BY4741-F11T (5.41% ± 2.47%), BY4741Δ*KNR4*-F11T (4.31% ± 1.90%) and BY4741Δ*TDH3*-F11T (2.70% ± 0.49%) were all classified as non-flocculent. Both the transgenic deletion strains displayed a decrease in flocculation in comparison to the transgenic parental strain. The wild type BY4741 strain and its derivative deletion mutants BY4741Δ*KNR4* and BY4741Δ*TDH3* were all non-flocculent because of a mutation in *FLO8* which encodes for a transcriptional activator of *FLO* gene expression (Verstrepen and Klis, 2006). The modified Helms assay is semi-quantitative in nature. As a result it was limited in differentiating between negligible differences in weakly flocculent phenotypes (Jin and Speers, 1998).

There was a decrease in invasive growth and flocculation intensity observed in BY4741Δ*KNR4*-F11T and BY4741Δ*TDH3*-F11T strains as compared to the BY4741-F11T which may indicate the release of flocculins into the spent growth substrate. The BY4741Δ*TDH3*-F11T strain displayed a decrease in invasive growth and flocculation intensity as compared to the BY4741Δ*KNR4*-F11T strain. Furthermore, the transgenic strain containing the *TDH3* gene deletion is possibly a better hyper-secretor than the *KNR4* mutant strain.

The data from this study tentatively suggests that both the transgenic deletion strains are potential hyper-secretion candidates, however future studies must be conducted on the spent medium to validate these findings. It must be noted that the transformation and screening process was difficult due to the high levels of expression caused by the strong constitutive *TEF1* promoter. Secondary adaptations such as mutations can arise to restore the normal growth rate of the planktonic yeast cultures (Van Mulders *et al.*, 2009). The transformation and screening method was time consuming and tedious. Another disadvantage to the transformation and screening method was the presence of co-transformation and co-isolation of strains. In future studies, selection and screening of putative transformants must be detected by incorporating either bioluminescence or colour in the selection process.

4.6 Conclusion

The current study produced BY4741 transgenic strains that are capable of constitutively over-expressing the *FLO11*-ORF under the *TEF1* promoter. An invasive growth phenotype was observed in all transgenic strains. The invasive growth intensity was visibly weaker in both the deletion strains in comparison to the transgenic parental BY4741-F11T strain utilized in this study. As such this study tentatively suggests that both deletion BY4741 transgenic strains are potential hyper-secretion candidates. To determine if these transgenic deletion strains are potential hyper-secretors of mannoproteins further characterization of the liquid medium is required to validate the release of the *FLO11*-encoded mannoproteins into the extracellular medium.

4.7 Acknowledgements

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4.8 References

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Chapter 5

General Discussion and Conclusion

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5.1 General Discussion and Conclusion

FLO11 is regulated by complex mechanisms, which involve both epigenetic and genetic regulation that control the expression of the gene (Barrales *et al.*, 2008). *FLO11* encodes for an adhesin involved in, invasive growth, filamentation and the adherence to solid surfaces (Douglas *et al.*, 2007; Zara *et al.*, 2009). Biofilm formation and cell adhesion are significant processes in the pathogenicity of fungi and are mediated by a protein adhesins conserved throughout fungi and yeast (Barrales *et al.*, 2012). These phenotypic traits are putative virulence factors which have significance in the medical field.

The five dominant *FLO* genes are epigenetically and genetically silenced due to a non-sense mutation of a fundamental transcription activator, *FLO8*, consequently the adhesion phenotypes are not expressed in laboratory strains of *S. cerevisiae* (Bester *et al.*, 2006). As such, it was envisaged that the over-expression of these silent *FLO* genes in mutant strains would result in an appropriate amount of the mannoprotein. Furthermore this would then be isolated and purified for the biochemical structural analysis of the desired mannoprotein.

There is a need for a broader understanding of these mannoproteins and their characterization, in order for their successful applications in the biotechnology industry. In order to understand and utilize the functions of *FLO11* in research and industrial applications, novel molecular cloning strategies were implemented to transform laboratory BY4741 strains to display the desired adhesion phenotypes. The *FLO1*p-*Zeocin*™-*TEF1*-*FLO1*p constitutive promoter cassette was used as a tool to construct the *FLO11*p-*Zeocin*™-*TEF1*-*FLO11*p promoter replacement cassette. The promoter replacement cassette was employed to transform a non-invasive BY4741 laboratory strain in order to determine the efficacy and functionality of the *FLO11p*-*Zeocin*™-*TEF1*-*FLO11p* cassette. The genomic *FLO11* ORF was brought under transcriptional control of the *TEF1* promoter via the replacement of the native promoter sequence. This was validated by PCR and the invasive growth phenotype displayed by the transgenic strains containing *FLO11* encoded mannoprotein.

The adhesion phenotypes are determined by the availability of the cell surface *FLO11*-encoded mannoproteins. Some yeast strains that contain a gene deletion pertaining to cell wall biogenesis release an increased amount of mannoproteins into the extracellular growth medium. These strains are referred to as hyper-secretors (Gonzalez 2010).

To date, there has been minimal data reported with regards to the over-expression of a particular gene encoding for a specific mannoprotein. This study utilized two mutant strains in order to facilitate the release of *FLO11* encoded mannoproteins into the extracellular medium. Yeast strains containing a deletion of *KNR4* and *TDH3* genes were identified as hyper-secreting strains because they were capable of releasing mannoproteins into the spent growth medium (Gonzalez-Ramos and Gonzalez, 2006; Gonzalez *et al.*, 2010).

KNR4 encodes for a transcriptional activator Knr4p which was isolated by (Hong *et al.*, 1994). The deletion of *KNR4* results in changes in the cell wall structure and composition such as a decrease in *β*-glucan synthase activity as well as an increase in the concentration of chitin (Klis *et al.*, 2006). *TDH3* is an isozyme that encodes for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and is a highly expressed glycolytic enzyme. The GAPDH is capable of directing intracellular proteins to the yeast cell wall (Alloush *et al.*, 1997; Edwards *et al.*, 1999; Pardo *et al.*, 1999).

It was hypothesized that the *TEF1* driven constitutive over-expression of *FLO11* in the *KNR4* and *TDH3* deletion strains would result in a decrease of the adhesion phenotype and conversely an increase in the amount of *FLO11* encoded mannoproteins that are released into the spent growth medium. The results attained from this study tentatively suggest that the deletion of *KNR4* and *TDH3* in the BY4741 strain altered the adhesion phenotypes. There was a decrease in invasive growth and flocculation intensity in comparison to the transgenic wild type BY4741-F11T strain. This is probably attributed to the alteration in the cell wall composition of these transgenic deletion strains which consequently influenced the cellular processing of *FLO11* encoded mannoproteins. A modified Helm's flocculation assay was utilized to assess the flocculation of the transgenic strains, the parental BY4741 was used as a negative control. This assay was fast and semi-quantitative; however this method was inadequate because it could not differentiate between strongly or weakly flocculent yeast strains with the similar flocculation intensity.

In conclusion, this study constructed the promoter replacement cassette, *FLO11p-Zeocin*™*-TEF1-FLO11p* for the constitutive expression of *FLO11*. The results attained from this study tentatively suggested that the BY4741 transgenic strains were capable of constitutively over-expressing the *FLO11* encoded mannoprotein driven by the *TEF1* promoter. This was validated by the distinctive invasive growth phenotype observed. There was a decrease of invasive growth and flocculation intensity observed in BY4741Δ*TDH3*-F11T strain as compared to the BY4741Δ*KNR4*-F11T strain. As such the transgenic strain containing the *TDH3* gene deletion is possibly a better hyper-secretor than the *KNR4* mutant strain.

Furthermore, the current study also indicated that both the deletion BY4741 transgenic strains are potential hyper-secretion candidates. To determine if these transgenic deletion strains are potential hyper-secretors of mannoproteins further characterization of the liquid medium is required to validate the release of the *FLO11*-encoded mannoproteins into the extracellular medium. It is envisaged that the transgenic deletion yeast strains constitutively over-expressing the *FLO11* encoded mannoproteins will enable the isolation and purification process of the desired mannoprotein. As such, future study endeavours should utilize this glycoprotein reservoir to facilitate the biochemical structural characterization and analysis of the *FLO11* encoded mannoprotein.

5.2 Acknowledgements

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5.3 References

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Appendix I

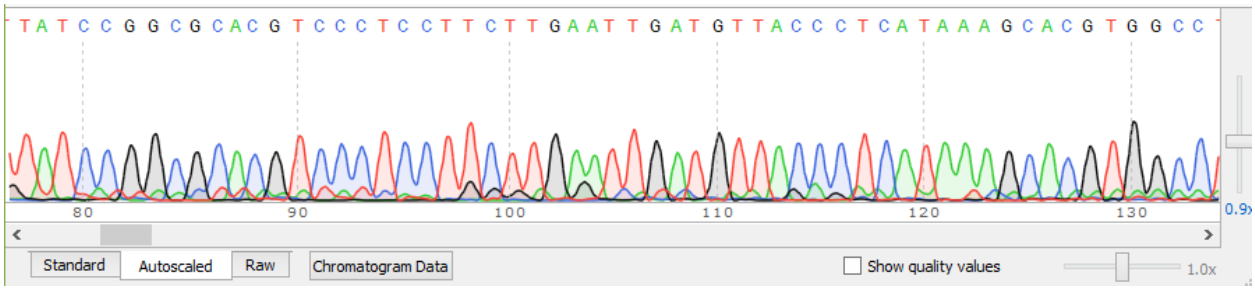

Figure AI.1: An extract of the Sanger DNA sequencing chromatogram of *FLO1*.

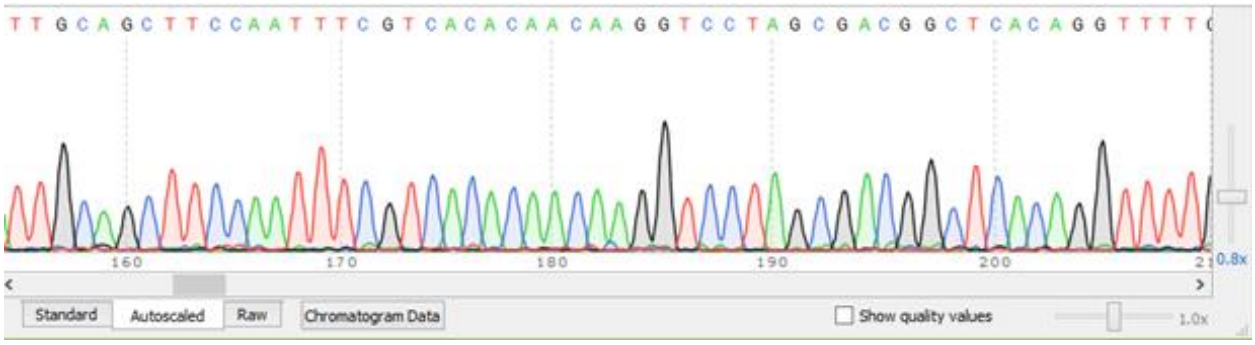

Figure AI.2: An extract of the Sanger DNA sequencing chromatogram of *FLO11*.