The control of cellular adhesion of *Saccharomyces cerevisiae* by the *FLO* gene regulator Mss11p

by

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Declaration

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the owner of the copyright thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: 01/12/2009

Summary

The yeast *Saccharomyces cerevisiae* senses change within its environment and responds through specific adaptive cellular programmes, in particular by modifying gene expression. Many adaptive changes affect the physico-chemical properties of the cell wall, and several mechanisms that specifically affect the expression levels of genes that encode for cell wall components have been described previously. Cell wall modification directly impacts on general cell wall properties and cell-cell and cell-surface interactions. Many of these properties have been directly linked to families of cell wall proteins referred to as adhesins. In particular members of the Flocculation (*FLO*) gene family have been shown to play a crucial role in adhesion phenotypes. Flo11p functions in a variety of phenotypes including agar invasion, plastic adhesion and the formation of pseudohyphae, “flor” and “mats”, whereas Flo1p appears to control flocculation. The regulation of *FLO11* expression is well documented and is mainly controlled by the mitogen activated protein kinase (MAPK) and cyclic AMP protein kinase A (cAMP-PKA) signalling cascades. Genetic analysis shows that Mss11p acts downstream and is central to these pathways, and furthermore interacts with the cAMP-PKA component Flo8p to activate transcription. In this study we further explore additional gene targets of Flo8p and Mss11p, as well as their regulation and their impact on cell wall characteristics and associated adhesion phenotypes.

Our analysis shows that Mss11p is also required for *FLO1* expression, and functions together with Flo8p to control many Flo-dependent adhesion phenotypes. Genome-wide gene expression analysis further reveals that altered Mss11p levels leads to the change in the expression of various cell membrane and cell wall genes, notably *AQY2* and members of the *DAN* and *TIR* gene families. Further genetic analysis indicates that adhesion phenotypes display an almost exclusive dependence on *FLO* gene expression. We also demonstrate that these phenotypes require Flo10p and are thus dependent on the specific balance of Flo proteins in the cell wall. The analysis of signalling deletion mutants show that regulation of *FLO10* shares signalling components with *FLO11*, but that the two genes are differentially regulated. Unlike *FLO11*, *FLO10* transcription also does not display an absolute requirement for Mss11p but rather for the MAPK component Ste12p.

Whole genome expression analysis were also performed on strains with altered levels of Flo8p which were compared with the above mentioned transcriptome data set. This analysis shows that Flo8p and Mss11p co-regulate the *FLO* genes, as well as *AQY2* and *TIR3*, but also have significant unique gene targets. The combination of transcriptome data with current information concerning transcription factor (TF) interaction networks reveals the importance of network interaction between Cin5p, Flo8p, Mga1p and Mss11p. From these data we constructed a TF interaction model in which Flo8p acts as the predominantly activating TF component, whereas Mss11p function as a target hub TF, possibly as a mediator- or polymerase II holo-enzyme component.

Finally we provide a first report on “mat” formation by an industrial wine yeast strain, and show that by adjusting *FLO11* expression in this strain we are able to significantly change this phenotypic behaviour.

Opsomming

Die gis *Saccharomyces cerevisiae* neem veranderinge in sy omgewing waar en reageer daarop deur middel van spesifieke sellulêre programme, in die besonder deur geenuitdrukking aan te pas. Verskeie aanpasbare veranderinge beïnvloed die fisieke, asook chemiese eienskappe van die selwand, en talle meganismes is al beskryf wat die uitdrukkingsvlakke beïnvloed van gene wat vir selwandkomponente kodeer. Die modifikasie van die selwand het ’n direkte impak op selwand-eienskappe, asook die sel-sel- en sel-oppervlak-interaksies. Verskeie van hierdie eienskappe word direk gekoppel aan die selwandproteïenfamilies, wat ook as adhesie-faktore bekend staan. Veral lede van die Flokkulasie (*FLO*) -geenfamilie het ’n noodsaaklike funksie in adhesie-fenotipes. Flo11p speel ’n rol in verskeie fenotipes, wat insluit die indringende groei van agar, plastiekaanhegting en die vorming van pseudohifes, “flor“ en “matte“, terwyl Flo1p flokkulasie beheer. Die regulering van *FLO11*-uitdrukking is deeglik gedokumenteerd en dit word hoofsaaklik gereguleer deur die mitogeen-geaktiveerde proteïenkinase (MAPK) en sikliese AMP-proteïenkinase A (cAMP-PKA) seintransduksiekaskades. Genetiese analises toon dat Mss11p stroom-af en sentraal tot hierdie kaskades funksioneer, en dit aktiveer transkripsie deur interaksie met die cAMP-PKA-komponent, Flop8. In hierdie studie word ’n ondersoek gedoen na addisionele teikengene van Flo8p en Mss11p, en hoe hierdie gene gereguleer word, asook hul impak op selwandeienskappe en geassosieerde adhesie-fenotipes.

Ons analises toon dat Mss11p ook benodig word vir die ekspressie van *FLO1* en dat dit, tesame met Flo8p, beheer uit oefen oor verskeie Flo-afhanklike fenotipes. Genoomwye geenekspressie-analises wys verder daarop dat veranderde Mss11p-vlakke lei tot die aanpassing van die ekspressie van verskeie selmembraan- en selwandgene, naamlik *AQY2* asook lede van die *DAN*- en *TIR*-geenfamilies. Verdere genetiese analise dui daarop dat adhesie-fenotipes byna eksklusief afhanklik is van *FLO*-geenekspressie. Daar is verder getoon dat hierdie fenotipes ook Flo10p benodig en dus afhanklik is van die spesifieke balans van Flo-proteïene in die selwand. Die analise van seintransduksiemutante demonstreer dat *FLO10* en *FLO11* seintransduksie-komponente deel, maar dat hierdie gene verskillend gereguleer word. Anders as *FLO11*, toon *FLO10* nie ’n absolute noodsaaklikheid vir Mss11p nie, maar eerder vir die MAPK-komponent, Ste12p.

Totale genoomekspressie-analises is ook gedoen op gisrasse met aangepaste vlakke van Flo8p en dis vergelyk met bogenoemde transkripsiedatastel. Hierdie analise wys dat Flo8p and Mss11p die *FLO*-gene, asook *AQY2* en *TIR3*, koreguleer, maar ook beduidende unieke teikengene het. Die kombinasie van transkripsiedata met huidig beskikbare informasie betreffende transkripsiefaktor (TF) -interaksienetwerke dui op die relevansie van netwerkinteraksie tussen Cin5p, Flo8p, Mga1p en Mss11p. Hiervan is daar ’n model opgestel waarin Flo8p in die meeste gevalle as die aktiverende TF-komponent optree, terwyl Mss11p as TF-teiken dien, moontlik as ’n mediator- of polimerase II holoënsiemkomponent.

Laatens word daar vir die eerste keer verslag gedoen van ”mat”-vorming deur ’n industriële wyngisras en toon ons verder dat hierdie fenotipe beduidend verander word deur middel van die aanpassing van *FLO11*-uitdrukking.

Dedication

This dissertation is dedicated to

Sven Kroppenstedt (1975-2006)

Biographical sketch

Michael Christiaan Bester was born in Port Elizabeth, South Africa on 9 October 1976. He matriculated at the DF Malan High School, Bellville in 1994. In 1995, he enrolled at Stellenbosch University and obtained a BSc degree in Biochemistry and Microbiology in 1998. The following year, he completed a BSc Hons degree in Wine Biotechnology at the Institute for Wine Biotechnology, Stellenbosch University. In 2003 he completed a MSc study at the same university entitled “Functional characterisation of Mss11p, a transcriptional regulator of pseudohyphal development, starch degradation and flocculation in Saccharomyces cerevisiae”.

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Preface

This dissertation is presented as a compilation of seven chapters. Each chapter is introduced separately and is written according to the style of the journal *Yeast*.

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Chapter 1

Introduction and project aims

Chapter 1: Introduction and project aims

1.1 Introduction

The yeast *Saccharomyces cerevisiae* has long been associated with human activity. The use of *S. cerevisiae* in the leavening of bread and the preparation of fermented beverages is likely the first historical example of the application of biotechnology, and arguably has made a positive contribution to human development. As a natural consequence of this long historical association, microbiologists have gathered a substantial amount of information on the physiology of yeast. This, and the ease with which yeast is cultivated, led to the development of *S. cerevisiae* as one of the first model systems for the scientific study of the eukaryotic cellular system. It is the first organism for which the whole genome sequence became available (Goffeau *et al.*, 1996) and is genetically modified with relative ease (Sherman *et al.*, 1991). Well established systems for the deletion and over-expression of genes have proven especially valuable in the understanding of specific gene function in the broader cellular context. Furthermore yeast as model system has been at the forefront in the development of analysis techniques for the understanding of the cellular genome and transcriptome (Lashkari *et al.*, 1997) as well as the proteome (Pham and Wright, 2007) and metabolome (Smedsgaard and Nielsen, 2005).

Like all organisms, yeast has the ability to sense, and to adapt to, changes in its immediate environment to ensure survival. Changes in extra-cellular conditions are experienced in the natural habitat (one can imagine rain causing sudden shifts in osmolarity, and limitation of nutrients; or the location on ripe fruit with sudden high osmolarity and abundance of fermentable sugars) as well as under industrial conditions (inoculation into high sugar grape must with high osmolarity; high alcohol levels and low nutrient levels towards the end of wine fermentation). Information regarding the environmental status is sensed by either plasma membrane-based or intracellular sensing systems. This information is further processed and relayed to the nucleus activating specific transcriptional programmes. This ultimately results in the modification of cell physiology and for example may involve the mobilisation of storage compounds, cell cycle arrest, adjustment of intracellular osmolite levels and the modification of cell wall composition (Clotet and Posas, 2007; Lesage and Bussey, 2006; Levin, 2005; Shima and Takagi, 2009). Cell wall adjustments directly affect yeast behaviour and are the primary focus area of the research presented in this thesis.

The yeast cell wall is a semi-rigid structure composed mainly of sugar polymers and highly glycosylated proteins. These cell wall proteins perform essential roles in cell wall structure and regulate many relevant phenotypes such as cellular adhesion patterns to other cells or to organic and inorganic surfaces. Adhesins form a subgroup of such cell wall proteins that in particular confer a variety of adhesion phenotypes ranging from flocculation, a form of cellular clumping that eventually leads to the formation of multi-cellular “flocs”, to the formation of greatly elongated cells that remain attached to each other upon the completion of budding, leading to the formation of pseudohyphae. These adhesins primarily belong to the flocculation (*FLO*) gene family, of which *FLO1* and *FLO11* perform dominant roles in the respective cellular adhesion processes of flocculation and hyphal growth (Lambrechts *et al.*, 1996; Lo and Dranginis, 1996; Teunissen and Steensma, 1995).

FLO expression is responsive to various extra-cellular conditions such as nitrogen and sugar availability. These extra-cellular conditions are sensed by the cell, mainly through plasma membrane based sensors, and the information is further transmitted through intracellular signalling networks, (Gagiano *et al.*, 2002). By far the most information regarding such systems and their impact on gene regulation is available for the adhesin encoded by *FLO11*, which has been shown to respond to two core signalling modules, a mitogen activated protein kinase (MAPK) pathway as well as the cyclic AMP responsive protein kinase A (cAMP-PKA) pathway (Verstrepen and Klis, 2006). Two factors acting downstream of these signalling pathways, Flo8p and Mss11p, act as core regulators of *FLO11* transcription (Gagiano *et al.*, 1999; Kobayashi *et al.*, 1999; Pan and Heitman, 2002; Rupp *et al.*, 1999). Furthermore Mss11p plays a central role in this system as no other factors can bypass this protein (van Dyk *et al.*, 2005).

1.2 Aims of study and dissertation layout

The work presented in this dissertation aims to broaden the current knowledge available on adhesion phenotypes, the regulation of adhesins as well as the signalling mechanisms that control adhesin expression. Special attention is given to the analysis of the adhesin transcriptional regulators Flo8p and Mss11p. The thesis is divided into seven chapters, of which this introduction forms **Chapter 1**. **Chapter 2** contains a literature review concerning cell wall architecture and the role of glycosylated cell wall proteins (mannoproteins), with special focus on the Flo mannoprotein family. In **Chapter 3** we provide evidence that Mss11p, together with Flo8p, regulates not only *FLO11*, but also another *FLO* gene, *FLO1*. As is the case with the regulation of *FLO11*, Mss11p also acts as a central regulator of *FLO1*. In **Chapter 4** we aim to broaden our understanding of the genes that are targets of regulation by Mss11p by performing whole genome transcript analysis of strains either deleted in-, or over-expressing *MSS11*. We report that Mss11p affects the expression of multiple cell wall genes of the *DAN*, *FLO* and *TIR* gene families. By means of an extensive gene deletion analysis we however conclude that only Flo genes directly impact on known non-sexual adhesion phenotypes. Furthermore we highlight the minor role of Flo10p in either Flo1p- or Flo11p-dependent phenotypes. Thus it would seem that adhesion phenotypes are complex behaviours that do not only rely on the expression of a single gene, but require a certain balance of cell wall located Flo proteins. Work in **Chapter 5** further explores the transcriptional targets of Flo8p and Mss11p. Using the same strategy for analysis of Mss11p as in Chapter 4, we analyse the transcriptome of *FLO8* deletion and -over-expression strains. The combination of this data set with the Mss11p data set obtained in Chapter 4 enables us to identify common as well as unique gene targets for these two factors. By extrapolating current available data concerning transcription factor (TF) interaction networks unto these transcriptome data sets we are able to put Flo8p and Mss11p in context of this regulatory network. In **Chapter 6** we further investigate the Flo11p-dependent phenotype of “mat” formation in an industrial wine yeast strain. By altering *FLO11* expression levels, thus changing the Flop balance in the cell wall, we demonstrate that this phenotype is either partially or completely inhibited. Finally **Chapter 7** contains a general discussion and reference to ideas for future projects based on the data generated in this work.

Two articles are included at the end of this dissertation in the form of addendums and are partially based on work performed by M. C. Bester, who is listed as co-author (Govender *et al.*, 2009; Govender *et al.*, 2008). The contributions by M. C. Bester includes the development of a reliable qPCR system that can efficiently differentiate between transcripts of the different homologous *FLO* genes, the determination of *FLO* gene expression levels as

shown in both these papers, and finally, assistance provided in the discussion and preparation of these manuscripts preceding publication. These two articles have previously appeared in the dissertation of P. Govender and provide excellent examples on the industrial applications of controlled *FLO* expression made possible by the fundamental research previously generated in our research group and furthered in this dissertation.

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Chapter 2

Literature review

The role and regulation of *Saccharomyces cerevisiae* Flo mannoproteins

Chapter 2: Literature review

The role and regulation of *Saccharomyces cerevisiae* Flo mannoproteins

2.1 Introduction

Saccharomyces cerevisiae yeast cells are continuously challenged by changes in their extra-cellular environment and respond to such changes by activating specific adaptive cellular programs. These responses in part involve extensive cell wall remodelling resulting in the modification of cell wall characteristics. Tightly controlled cellular mechanisms control these responses. They consist of mainly cell membrane associated sensors that communicate information on the status of the extra-cellular environment, in particular its composition and physical parameters, to an intra-cellular network of interacting signal transduction pathways. The downstream components of these pathways in turn interact with the transcription machinery in the nucleus controlling the induction or repression of genes that encode cell wall proteins. This ultimately adjusts the protein content of the cell wall and its associated characteristics, providing the cell with the necessary adaptation.

The cell wall is a semi-rigid structure surrounding the cell to form a physical barrier to the extra-cellular environment. It functions as a protective layer of the cell and in addition provides mechanical support for the maintenance of cell shape. It is however far from a fully rigid structure and has the ability to facilitate wide-ranging changes in cell shape through its inherent dynamic properties (Slaughter and Li, 2006). Its partly fluidic nature is controlled by constant cell wall remodelling that allows for cellular processes such as vegetative cell growth, reproductive budding, “shmoo” formation preceding yeast mating, and the transition from single cell “yeast form” to multi-cellular “filamentous form” growth behaviour.

Of specific importance for the control of yeast behaviour in terms of growth phenotypes and environmental interactions is the composition of the outer layer of the cell wall. This exposed part of the cell wall serves as the physical contact point between the cell and its surroundings and yeast behaviour involving cell-cell or cell-surface interactions is directly affected by regulating the specific characteristics of this layer.

Cell wall proteins dominate in the outer cell wall and perform a critical role in defining cell wall characteristics. These proteins are subjected to extensive intra-cellular post-translational processing and modification that precedes secretion and cell wall incorporation. This involves in particular the extensive addition of branched or un-branched sugar polymers (glycosylation) mainly consisting of mannose residues. Thus the alternative referral to these modified proteins as mannoproteins.

This literature review will focus on the general role of mannoproteins in yeast physiology and -phenotypic behaviour. Specific attention will be given to Flo proteins, their involvement in cell adhesion phenotypes and the regulation of the genes that encode them. Finally, attention will be given to the relevance of these Flo proteins in industry.

2.2 The cell wall

The cell wall accounts for between 10% and 30% of the dry weight of *S. cerevisiae* yeast cells (Klis *et al.*, 2006), a figure that displays even more variation when comparing different *Saccharomyces* species (Nguyen *et al.*, 1998). In *S. cerevisiae* this cell wall variability has been shown to involve the adjustment of the specific ratio of cell wall components (see following section), which in turn is responsive to extra-cellular parameters such as carbon, nitrogen and oxygen availability as well as specific pH and temperature (Aguilar-Uscanga and Francois, 2003). Yeast cells that undergo the switch from exponential growth to stationary phase experience many of these changes in environmental cues and adjust their cell wall composition accordingly. Biophysical measurements of the mechanical properties of cells show that the switch to stationary growth causes an increase in cell wall strength due to cell wall thickening. The same analysis, however, shows that this change does not affect cell elasticity (Smith *et al.*, 2000).

Construction and compositional adjustment of the cell wall is mainly regulated by the cell wall integrity (CWI) signalling pathway although many other signalling pathways may contribute to cell wall status or interact with CWI signalling (Levin, 2005). CWI signalling is responsive to various cellular processes such as progression through the cell cycle, pheromone induced morphogenesis during mating as well as the adaptive response to osmotic shock and heat/oxidative stress.

2.3 Cell wall architecture

The cell wall is a bi-layered structure that consists of an inner layer of an interlinked network of polysaccharides and an outer layer of mannoproteins (Klis *et al.*, 2006) (Figure 2.1). The polysaccharide layer consists mainly of β(1-3) and β(1-6) linked glucose polymers complexed with a lesser amount of chitin to form an interlinked polymer network that serves as the main load bearing component of the cell wall (Lesage and Bussey, 2006). β(1-3) glucan forms the main physical structure of the polymer layer with β(1-6) glucan branching of the outside part of the β(1-3) glucan network. As a whole, the glucan layer serves as the scaffold onto which the outer layer of mannoproteins is attached. Mannoproteins are predominantly covalently linked to the glucan network, but a minority is associated via disulphide and ionic bonds (De Groot *et al.*, 2005; Klis *et al.*, 2006). Mannoproteins dominate the outermost layer of the cell wall, defining the interface that is exposed to the extra-cellular environment. Between the glucan network and the plasma membrane the periplasmic space contains various glycosylated enzymes involved with metabolism or cell wall maintenance.

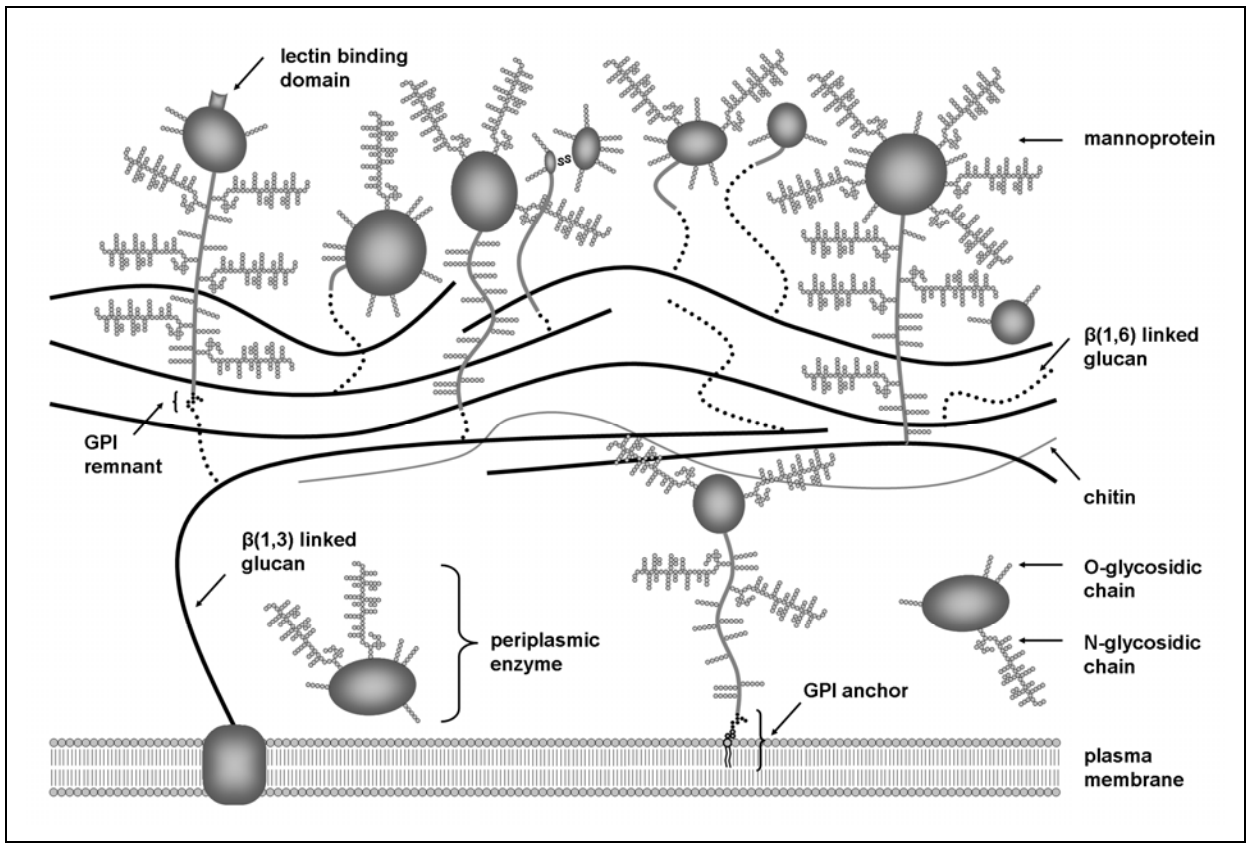

Figure 2.1 Simplified representation of the layered structure and composition of the yeast cell wall (adapted and modified from Schreuder *et al*., 1996).

2.4 Mannoproteins

Cell wall mannoproteins are often collectively referred to as yeast mannans due to their high mannose content (Gemmill and Trimble, 1999). Protein glycosylation is not limited to cell wall mannoproteins as some intra-cellular proteins may carry glycosyl chains. Nevertheless, proteins destined for either cell wall incorporation or extra-cellular secretion, carry much more mannosyl residues compared to intra-cellular proteins (Ballou, 1990). In fact the carbohydrate fraction has been reported to constitute a considerable proportion (up to 95% of the molecular mass) of cell wall mannoproteins (Dean, 1999). With the aid of transmission electron microscopy (TEM) cell wall mannoproteins are visualised as an electron dense fibrillar layer in the outer part of the cell wall (Figure 2.2) (Osumi, 1998). It has been suggested that at any given time greater than 20 different mannoproteins may be present in the cell wall (Klis *et al.*, 2006; Yin *et al.*, 2008) and that protein copies per cell are in the range of 10^3 to 10^4 as determined for Cwp1p, Crh1p, Scw4p, Gas1p, and Ecm33p (Yin *et al.*, 2007). Cell wall mannoprotein function can be roughly grouped in two categories. Firstly, mannoproteins act as enzymes that modify the cell wall itself and secondly, components referred to as adhesins or flocculins determine cell wall structure and adhesion interactions. Specific cell wall mannoprotein content varies in response to cell age, growth phase and the status of the surrounding environment. The adjustment of mannoprotein composition, concentration and location, and possibly glycosylation status all form part of this finely tuned cellular response (Klis et al., 2006). Although cell wall components often have specialised functions, such as sexual adhesins that are required for the recognition

of mating partners, integrated cellular responses most probably rely on the orchestrated balance of diverse mannoproteins in the cell wall.

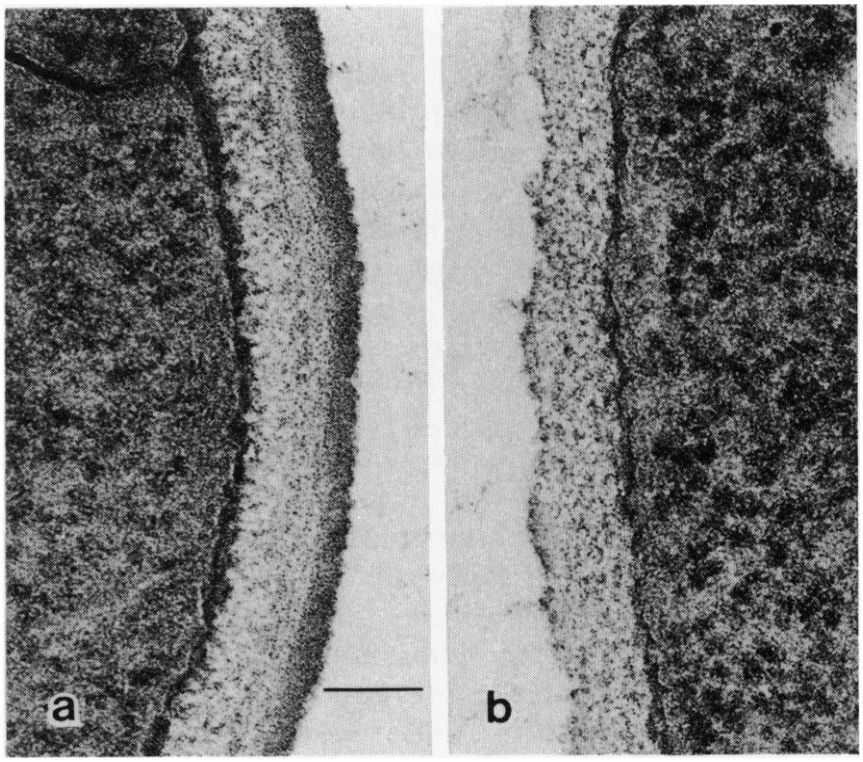

Figure 2.2 Cross section view across the cell wall of yeast grown to exponential growth phase as imaged by transmission electron microscopy (TEM). The dark stained outside fibrillar mannoprotein layer is clearly visible in the control yeast sample (a) visibly absent upon protease treatment (b) Bar, 0,1 μm Taken from Zlotnik *et al*. (1984).

2.4.1 Protein glycosylation

Once thought to be exclusive to eukaryotes, protein glycosylation is also a common feature of archaea and eubacteria (Abu-Qarn *et al.*, 2008; Yurist-Doutsch *et al.*, 2008). Furthermore glycosylation pathways show a high level of conservation from yeast to mammalian systems (Lehle *et al.*, 2006). In *S. cerevisiae* proteins targeted for secretion or incorporation into the cell wall undergo glycosylation in passing through the endoplasmic reticulum (ER) and the Golgi apparatus. Attachment of mannosyl groups and -structures is initiated in the ER and the further modification and/or elongation of structures takes place in the Golgi apparatus. Mannosyl groups may be further modified by the covalent addition of mannosylphosphate that carries a net negative charge (Jigami and Odani, 1999). Glycosylation structures are hydrophilic in nature whereas the negatively charged mannosylphosphate and phosphodiester bonds contribute to the hydrophobic nature of mannoproteins. Depending on the extent to which mannoproteins are modified by these structures, and to what degree these structures and amino acid side chains of proteins are exposed to the surrounding environment, the overall cell surface may display varying degrees of hydrophobicity. Protein glycosylation is essential for cell integrity and -survival in that it probably contributes to the correct folding and functioning of mannoproteins. However, the specific molecular function of glycosylation structures remains unclear (Arnold and Tanner, 1982; Dean, 1999; Gentzsch and Tanner, 1996).

Target proteins can be modified by the covalent addition of mannosyl groups in two ways. *O*-linked glycosylation structures are attached to the hydroxyl group of serine (Ser) or threonine (Thr) residues, whereas *N*-linked glycosylation groups are linked to the amide group of asparagine (Asn) residues (Figure 2.3). For *N*-linked modification the Asn is required to be in the context of an Asn-Xaa-Ser/Thr sequence motif where Xaa represents any amino acid.

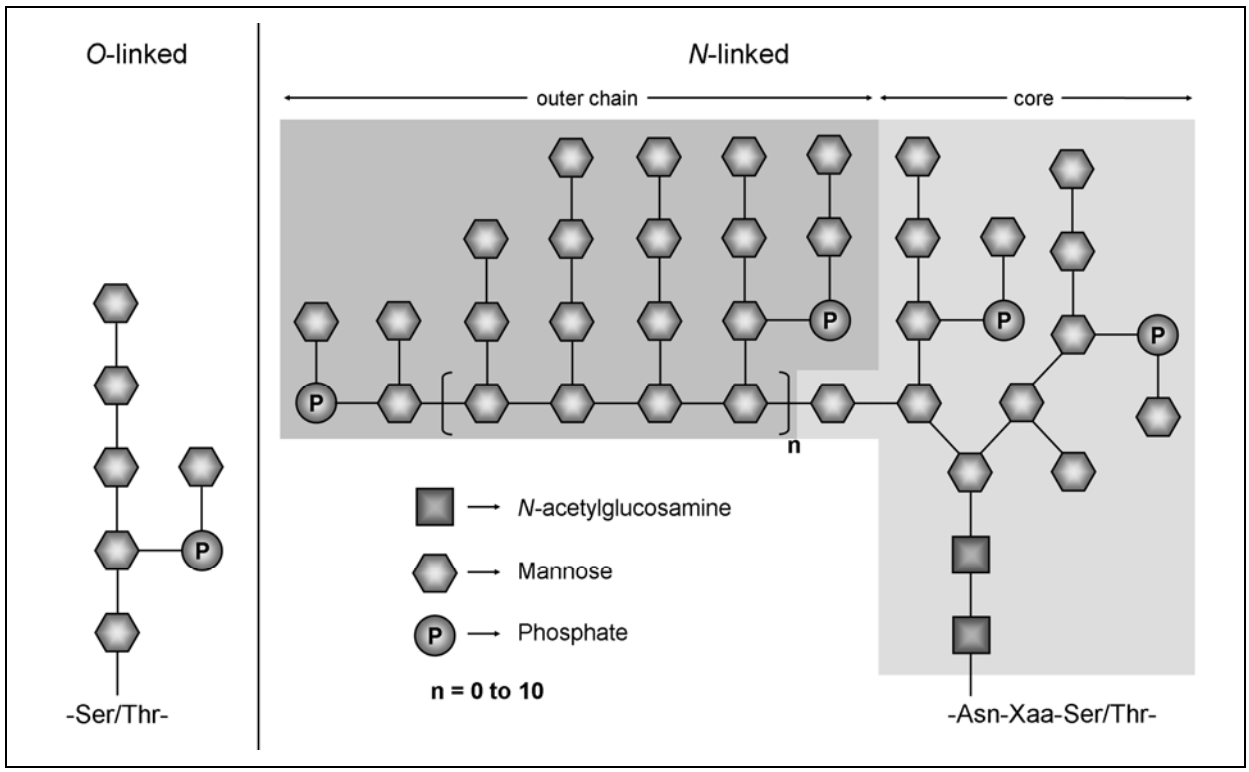

Figure 2.3 Composition of *O*-linked and *N*-linked glycosylation groups. Glycosylation events display extensive variability and it should be noted that not all mannose or phosphomannosyl residues shown are always incorporated in structures. Diagram based on information from Jigami and Odani (1999).

2.4.1.1 *O*-linked glycosylation

O-linked glycosylation structures consist of anything from one to five mostly linearly arranged mannose residues (Goto, 2007). Glycosylation is initiated in the ER by the addition of a single mannose residue to either serine or threonine of the target protein while the rest of the glycosylation structure is further completed in the Golgi apparatus. These structures may be further modified by the addition of a mannosylphosphate moiety at the position indicated in Figure 2.3 (Nakayama *et al.*, 1998). *O*-linked glycosylation groups, resembling short and rigid "stalk like" structures, are speculated to function in the proper maintenance of the tertiary structure of a number of mannoproteins. Indeed many genes have been shown to contain extensive serine/threonine repeat encoding regions (Caro *et al.*, 1997; De Groot *et al.*, 2003). It has been hypothesised that the *O*-linked glycosylation of these long Ser/Thr repeats would result in a mannoprotein of elongated structure that would enable the protein to stretch throughout sections of the cell wall matrix (Jentoft, 1990). Thus, extra-cellular domains such as adhesin receptors would be presented on the outside of the cell wall matrix, free to interact with substrates (section 2.5.4) (Dranginis *et al.*, 2007; Verstrepen and Klis, 2006). It is estimated that *O*-linked glycosylation constitute a major proportion of the total glycosylation of mannoproteins, roughly double that of *N*-linked glycosylation (Strahl-Bolsinger *et al.*, 1999).

2.4.1.2 *N*-linked glycosylation

Unlike the short linear *O*-linked glycosyl groups, *N*-linked groups are extensively branched and display considerable variability in structure. In the ER a pre-formed branched core structure (~9 mannose residues) is covalently added to Asn residues of target proteins (Figure 2.3). Further downstream in the secretory pathway this core structure may be significantly modified and extended by the further addition of branched mannose polymer structures (outer chain). The outer chain region typically displays a high degree of length variation. Hyper-mannosylated proteins may contain more than 200 mannose residues in the outer chain and more than 50 residues in the core region of individual *N*-linked glycosylation groups (Dean, 1999). As with *O*-linked glycosylation, *N*-linked structures are additionally modified by negatively charged mannosylphosphate groups (Wang *et al.*, 1997). The regulatory mechanisms that control the degree of glycosylation are poorly understood but arguably play an important role in controlling the characteristics of newly synthesised mannoproteins. Some evidence point to the role of nutrients in controlling the degree of *N*-linked glycosylation. Specific nitrogen source (Nakamura *et al.*, 1993) or carbon source availability (Kukuruzinska and Lennon, 1994) were shown to drastically affect the degree of modification of *N*-linked glycosyl groups.

2.4.2 Mannoprotein cell wall attachment

The majority of mannoproteins are covalently attached to the cell wall matrix and the remainder are either found anchored to the plasma membrane, non-covalently associated with cell wall components or secreted into the extra-cellular medium. Covalently linked cell wall mannoproteins can be classified into three groups, determined by the specific nature of the covalent linkage (De Groot *et al.*, 2005). The first group are linked to the cell wall via a glycosylphosphatidylinositol (GPI) remnant. Secondly, mannoproteins are attached through an as yet unidentified linkage that is sensitive to mild alkali treatment. These two groups are referred to as GPI- and ALS (alkali-sensitive) -linked mannoproteins, respectively and are discussed in more detail below. Thirdly, proteins may be bound to the cell wall through weaker disulfide bonds or by means of ionic interaction. Evidence for disulphide linkage is provided by the extraction of mannoproteins from cell walls by reducing agents such as β-mercaptoethanol (Mrsa *et al.*, 1997) or dithiothreitol (DTT) (Cappellaro *et al.*, 1998). Other mechanisms for the retention of mannoproteins in the cell wall are speculative but may very well involve ionic forces. These interactions could be facilitated by negatively charged mannosylphosphate groups of *O*- and *N*-linked mannosyl groups and charged protein groups interacting via salt bridges.

2.4.2.1 ALS-linked mannoproteins

ALS-linked mannoproteins are released from the cell wall by means of mild alkali (NaOH) treatment. They are directly linked to the inner β(1-3) glucan polymer layer by an as yet uncharacterised linkage (Kapteyn *et al.*, 1999). This small group of proteins mainly consists of members of the Pir protein family which contain Ser/Thr rich internal repeats and thus are very likely to be extensively *O*-linked glycosylated.

2.4.2.2 GPI-linked mannoproteins

The majority of cell wall proteins are linked via a GPI remnant to β(1-6) glucan in the outer cell wall layer. It is this layer of proteins that defines the outer cell wall's fibrillar appearance and functions directly or indirectly in cellular adhesion phenotypes. Proteins destined for GPI-linked cell wall attachment contain an N-terminal signal sequence for entry into the ER and an

additional C-terminal signal sequence for the covalent attachment of the GPI anchor (Pittet and Conzelmann, 2007). Following the addition of the pre-formed GPI anchor the proteins proceed through the secretory pathway from the ER through the Golgi apparatus, while undergoing modification by means of the aforementioned glycosylation events. Following glycosylation, some GPI-mannoproteins may be incorporated in the plasma membrane via the hydrophilic fatty acids of the GPI anchor. Most GPI proteins in yeast, however, do not remain anchored in the plasma membrane. Following additional processing of the GPI anchor at the plasma membrane, GPI-mannoproteins are covalently linked to the outer β (1-6) glucan polymers of the cell wall via the remaining part of the GPI anchor.

2.4.3 Mannoprotein cell wall distribution

For the purpose of this review examples of mannoprotein location will be discussed in order to illustrate three different organisational "levels" of distribution patterns. (1) Firstly distribution in terms of the layered cross section of the cell wall, followed by (2) the three dimensional distribution across the cell surface, and lastly, (3) differential mannoprotein expression by cells in the context of a given yeast population will be addressed. Specific cell wall location is often associated with particular protein function and brief examples will be included to illustrate this point.

2.4.3.1 Cell wall cross section distribution

While predominant in the outer cell wall layer, mannoproteins are also present in all the other architectural layers of the cell wall, as well as being integrated into the plasma membrane. At the plasma membrane, mannoproteins are incorporated either as integral- or as GPI-anchored proteins. An example of the former is the integral membrane Msb2p that functions as a cell wall sensor communicating extra-cellular cues to intra-cellular signalling pathways, (Cullen *et al.*, 2004). This protein is composed of a large extra-cellular domain containing various mannosylated Ser/Thr repeats, an integral-membrane region for insertion into to the plasma membrane and a cytoplasmic tail that interacts with intracellular signalling machinery. An example of the latter is Ecm33p, which is a GPI-anchored mannoprotein preferentially anchored in the plasma membrane. The regulatory mechanisms that control the distribution between the plasma membrane and the cell wall will be discussed in more detail in section 2.5.3. Yeast cells carrying a deletion in *ECM33* display a variety of defects such as hypersensitivity to the cell wall perturbing agent Calcofluor White as well as a temperature sensitive (TS) growth defect (Lussier *et al.*, 1997). The TS growth defect was shown to be fully repressed by the expression of the native Ecm33p in the mutant strain. When, however, a modified Ecm33p was expressed carrying a signal for attachment to the cell wall the suppressive effect was lost (Terashima *et al.*, 2003). These findings thus show that the specific attachment of Ecm33p to the plasma membrane is essential for its cellular function.

Outside of the plasma membrane the next layer is referred to as the periplasmic space. Mannoproteins present in this space generally possess enzymatic functions. Enzymes such as invertase (Carlson and Botstein, 1982) and acid phosphatase (Oshima, 1997) are involved in metabolism and functions in sugar- and phosphate utilisation, respectively. Alternatively, a variety of enzymes are involved in the maintenance and modification of the cell wall (Lesage and Bussey, 2006) such as Bgl2p (Klebl and Tanner, 1989), an endo-β-1,3-glucanase that contributes to the rigidity of the cell wall through modification of glucan polymer cross

linkages (Mrsa *et al.*, 1993). Furthermore protein glycosylation has been shown to be essential for invertase (Gallili and Lampen, 1977), acid phosphatase (Mizunaga and Noguchi, 1982) and endo-β-1,3-glucanase (Mrsa *et al.*, 1993) activity, respectively.

Surrounding the periplasmic space, the inner cell wall contains mainly β(1-3) glucan and associated covalently linked ALS-mannoproteins. The Pir protein family (Pir1/2/3/4p) is distributed throughout this cell wall layer and gene deletion studies show that these proteins function in protecting the cells from cell wall perturbing agents such as Calcafluor white, Congo red and Sodium dodecyl sulphate (SDS) (Mazan *et al.*, 2008; Mrsa and Tanner, 1999). By deleting any of the *PIR* genes the same increase in sensitivity is observed, with a cumulative effect upon the deletion of additional *PIR* genes. It appears as if Pir proteins function in the general maintenance of cell wall integrity and it has been speculated that Pir proteins strengthen the cell wall by acting as cross linkages between glycan polymers (Mazan *et al.*, 2008; Mrsa and Tanner, 1999).

Finally, the outer layer of the cell wall is predominantly made up of mannoproteins, many of which will be the subject of this review. A well characterised example is Sap1p, which functions in the recognition of, and cellular adhesion to, haploid cells of opposing mating type during yeast mating (Doi *et al.*, 1989). It is covalently linked to β(1-6) glucan via a GPI remnant (Lu *et al.*, 1994), located in the outer fibrillar part of the cell wall (Cappellaro *et al.*, 1994) and contains immunoglobulin-like domains presented to the cell surroundings that interact with strong affinity to the Aga2p agglutinin of cells of the opposing mating type (Zhao *et al.*, 2001). This interaction has been shown to be essential for mating to occur in liquid culture where Brownian motion inhibits cell fusion (Roy *et al.*, 1991). Various other outer layer mannoproteins also function in specific cellular adhesion phenotypes and are discussed in section 2.6.2.

2.4.3.2 Distribution across the cell surface

Mannoprotein distribution patterns across the cell surface can be either of a localised or diffuse nature, and combinations thereof. The cell wall sensor Msb2p (Figure 2.4) specifically concentrates at the distal pole of daughter cells (Figure 2.4). *MSB2* over-expression however results in the redistribution of Msb2p to the whole of the cell periphery. These cells undergo morphological change as a direct result of *MSB2* over-expression showing a direct correlation between altered location and cellular behaviour (Cullen *et al.*, 2004). Pir proteins, ALS-linked to the inner cell wall, display both localised and diffuse distribution patterns. Pir1p exclusively localises to the inside of cell wall bud scars following the completion of budding (Sumita *et al.*, 2005). Pir3p and Pir4p are spread uniformly across the cell wall whereas Pir2p localises to both bud scars as well as the rest of the cell wall. As mentioned previously deletion studies suggest a redundant role for the Pir proteins and thus do not explain their differential localisation patterns (Mazan *et al.*, 2008). The GPI-mannoprotein Flo11p is a well characterised adhesion protein involved in a variety of yeast phenotypes (discussed in more detail later). The Flo11p is evenly distributed across the outside cell surface of “yeast form” cells (Lo and Dranginis, 1996) but at times appears to be slightly polarised in pseudohyphal cells (Guo *et al.*, 2000).

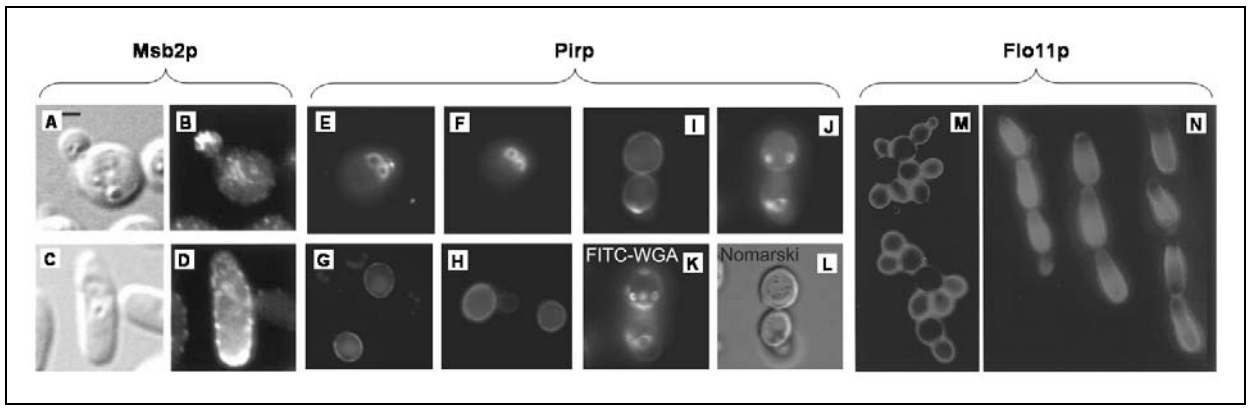

Figure 2.4 Different cell wall distribution patterns for Msb2p, the Pir proteins and Flo11p. Immunolocalisation of Msb2p in dividing (B) and elongated (D) cells also visualised under normal light microscopy (A and C, respectively) taken from Cullen *et al.* (2004). Pirp localise to the inside of bud scars as shown with respective chitin staining (E) of cell expressing GFP-tagged Pir1p (F). GFP-tagged Pir3p (G) and -Pir4p (H) distribute evenly across the cell surface. GFP-Pir2p localise to both bud scars and across the cell surface. Cells (L) were either stained for chitin (K) or fluorescence was monitored on different focal levels (I and J) taken from Sumita *et al*. (2005). Immunolocalisation of tagged Flo11p shows even distribution in normal growing (M) and slightly polarised fluorescence in pseudohyphal cells (N) respectively. Adapted from Guo *et al* (2000).

2.4.3.3 Distribution among cells in a population

Flo11p location is also used to illustrate the third organisational level of cellular mannoprotein distribution. In the context of a population of yeast cells growing in liquid culture, it has been shown that only a minority of these cells express Flo11p on their cell surface at any given moment (Guo *et al.*, 2000). An increase in the proportion of cells expressing Flo11p is observed in populations that have made the morphological switch to Flo11p-dependent phenotypes such as flor- (Reynolds *et al.*, 2008) and pseudohyphae formation (Halme *et al.*, 2004). This expression pattern has been shown to be not only the result of signalling based transcriptional regulation but also due to the epigenetic control of the *FLO11* locus (Halme *et al.*, 2004).

2.5 Outer cell wall GPI-linked mannoproteins

2.5.1 Prediction from genome sequence analysis

The minimum requirement for classification as a GPI protein is an N-terminal signal sequence for targeting entry into the ER as well as a C-terminal sequence for the attachment of the GPI anchor. All the open reading frames of yeast were screened using an algorithm that predicts the presence of a potential N-terminal ER entry signal (Caro *et al.*, 1997). Genes identified in this manner were further screened for regions that encode for a GPI attachment signal. By the addition of genes not identified in this screen but shown experimentally to contain both signals, the total number of genes identified came to 58. Further analysis showed that 20 of these genes were predicted to encode proteins destined for the plasma membrane and the other 38 for localisation in the cell wall. A further study screening open reading frames using a GPI attachment signal consensus sequence brought the *in silico* predicted GPI anchored proteins to 66 (De Groot *et al.*, 2003). Finally, a third *in silico* approach in combination with experimental evidence predicted the total number of GPI mannoproteins to be 61 (Pittet and Conzelmann, 2007).

2.5.2 Structure

GPI proteins display structural similarities to each other in that they are modularly organised (Figure 2.5). For the purpose of protein processing they contain signal sequences for entry into the ER, GPI anchor attachment and cell wall incorporation. The N-terminal signal sequence directs proteins to the ER and is cleaved off upon ER entry. The physical GPI attachment site (ω) is located within the signal sequence for GPI attachment. GPI attachment results in the removal of the rest of the protein on the C terminal side of the ω site. The N-terminal domain contains the protein region responsible for affinity in adhesion proteins or the catalytic function of cell wall enzymes. Many, but not all GPI-proteins contain long internal regions rich in serine (Ser) and threonine (Thr) at the C-terminal end of the protein (Caro *et al.*, 1997; Verstrepen *et al.*, 2005). These repeat regions are typically extensively *O*-linked mannosylated and may provide GPI proteins with a mechanism to present N-terminal domains to the extra-cellular environment as mentioned previously (Jentoft, 1990). Specifically Ser/Thr rich regions have been shown to take the form of tandem repeats in certain GPI protein families such as the flocculation (*FLO*) gene family (Verstrepen *et al.*, 2004).

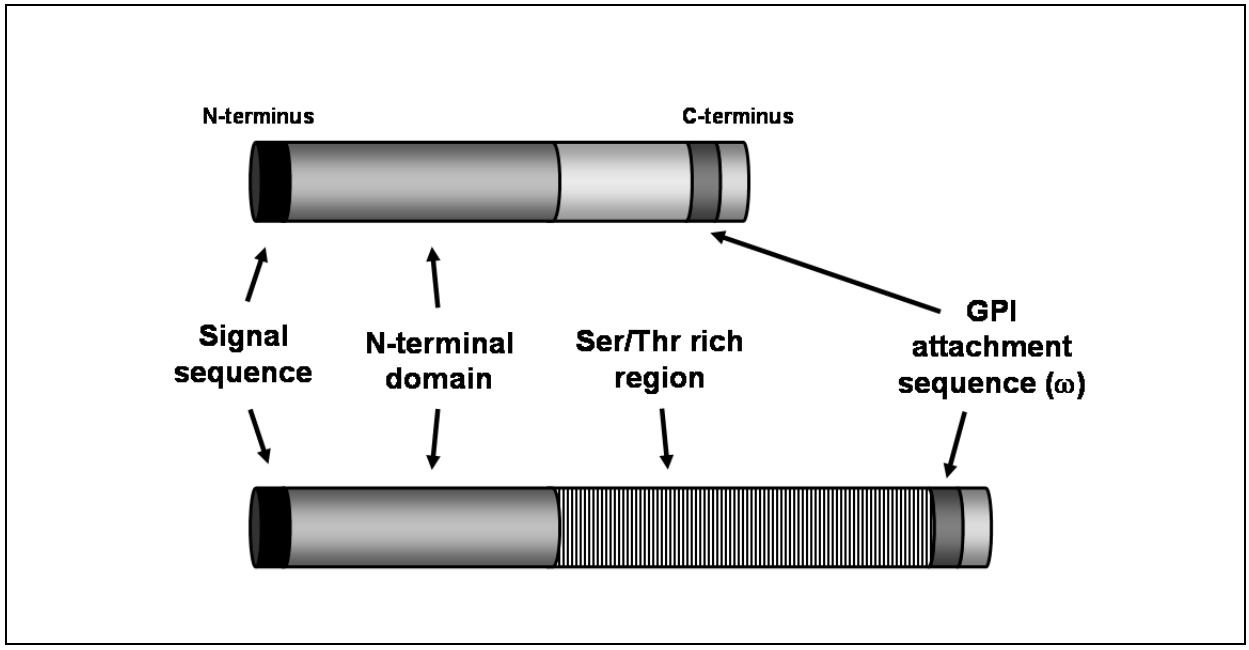

Figure 2.5 Diagram illustrating the modular domain structure of GPI-mannoproteins preceding modification in the ER. Shown is the N-terminal signal sequence for entry into the ER, followed by an N-terminal domain coding for either enzymatic activity or adhesion interaction. These genes often contain long Ser/Thr rich regions (lower example). The sequence that signals GPI attachment is located at the C-terminal with the site for GPI attachment within this sequence (ω).

2.5.3 Regulation of cell wall distribution

The regulatory mechanisms that control the distribution of GPI-mannoproteins, either being anchored in the plasma membrane or attached to β(1-6) glucan, are not well understood. Evidence suggests that specific protein sequences perform a role in directing proteins to either destination. Firstly, it was shown that specific amino acid composition upstream of the GPI anchor attachment site directs relocation to the cell wall (Hamada *et al.*, 1998; Hamada *et al.*, 1999). In fact, analysis of amino acid sequence and location of various GPI-mannoproteins suggests there might be two classes of these proteins either predominantly plasma membrane (PM) or cell wall (CW) situated. A mutational screen identified amino acid

changes that directed GPI-mannoproteins that would normally be found in the PM to the cell wall and vice versa (Frieman and Cormack, 2003). As previously mentioned, the PM located Ecm33p was relocated to the cell wall by the modification of the signal sequence near the GPI attachment site (Terashima *et al.*, 2003) supporting the above mentioned findings by Friedman and Cormack. A second line of evidence suggests that the presence of Ser/Thr repeat regions promotes protein relocation to the cell wall. Repeat regions were even shown to be able to override the amino acid signal near the GPI attachment site and the introduction of such sequences redirected a model PM GPI-mannoprotein to the cell wall. *In silico* analysis of GPI-mannoprotein sequences shows that mannoproteins with PM targeting sequences are less likely to contain Ser/Thr repeats in comparison to CW-mannoproteins (Frieman and Cormack, 2004).

2.5.4 Cell surface mannoprotein concentration and extra-cellular accessibility

Mass spectrometric analysis of the cell wall has shown that it is not uncommon for unique cell wall proteins to be in the range of 10^3 to 10^4 copies per cell (Yin *et al*., 2007). Dranginis *et al* (2007) argue that based on the assumption that a cell has $2{,}5 \times 10^4$ molecules of a certain mannoprotein attached to the outer cell wall, the local cell surface concentration of this protein should be in the region of $\sim 4 \times 10^{-4}$ M. This localised concentration effect greatly increases the probability of these cell wall proteins to interact with their respective substrates.

A second factor of great importance for extra-cellular interactions is the substrate accessibility of cell wall proteins within the context of the cell wall matrix. The specific location of cell wall attachment (outer or inner glucan layer) as well as the manner in which cell wall proteins are folded (resulting in elongated or globular secondary structure) determine the efficiency of cell wall protein substrate interaction. Cell wall attachment to the outside of the glucan layer as well as an elongated protein structure, reaching out into the extra-cellular environment, would clearly favour substrate interactions. It has been hypothesised that *O*-linked glycosylated Ser/Thr repeats in proteins aid in forming elongated proteins that stretch through the cell wall presenting interaction domain(s) to the environment without steric interference of other cell wall components (Jentoft *et al*., 1990). These repeat regions have been shown to genetically contract and expand resulting in protein variants with different abilities to participate in extra-cellular adhesion interactions (Figure 2.6). Furthermore, by expressing different variants of an adhesin, each variant in a different strain, it was shown that there exists a positive correlation between the increase in repeat length and the intensity of the resulting adhesion phenotype (Verstrepen *et al.*, 2005).

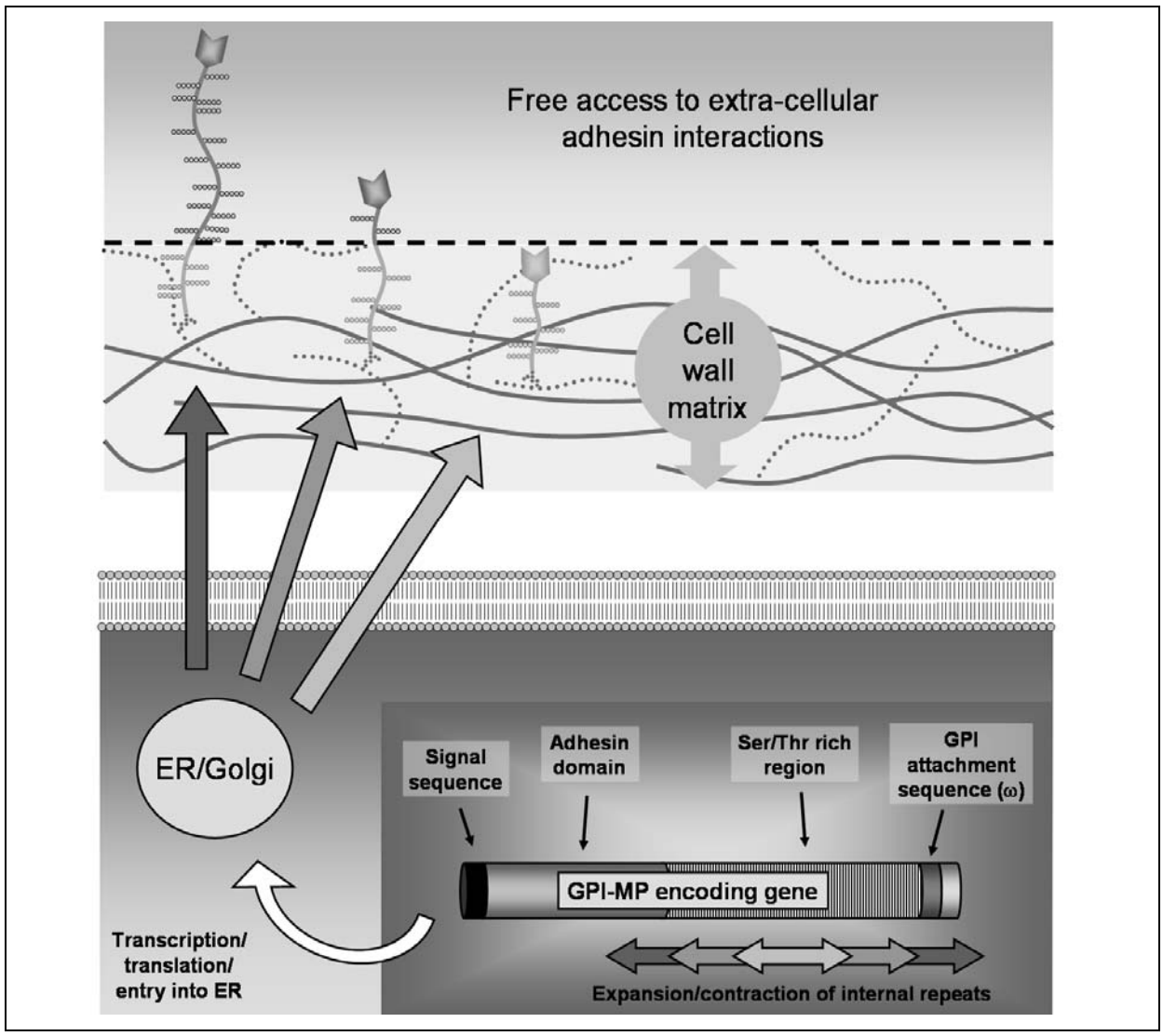

Figure 2.6 Variation in internal repeats of GPI-mannoproteins (GPI-MP) results in the corresponding length variation of the cell wall adhesins they encode. Repeat length variation is represented by different shaded arrows. Following modification in the ER and Golgi apparatus different protein variants (indicated by the same shaded arrows) are incorporated into the cell wall. Depending on peptide length these variants are either prohibited from extra-cellular substrate interaction by steric interference of the cell wall matrix (lightest grey arrow) or freely accessible to the exterior (darkest grey arrow).

2.6 Yeast adhesion interactions

2.6.1 Sexual cell-cell adhesion

Mating related adhesins are referred to as agglutinins and function in the cell-cell adherence of haploid cells of opposing mating type (a or α) following “shmoo” formation (Chen *et al.*, 2007). Cells of the a mating type express the a agglutinin composed of two subunits of Aga1p, anchored to the cell wall, and Aga2p, which is linked to Aga1p via disulfide bonds (Cappellaro *et al.*, 1994). As mentioned previously Sag1p functions as the agglutinin of α cells and interacts with high affinity with Aga2p (Cappellaro *et al.*, 1994; Zhao *et al.*, 2001). These agglutinins are expressed at very low levels during vegetative growth but are greatly up-regulated when the cell is exposed to mating pheromone. Being exclusively involved with

mating these agglutinins are required for efficient mating in liquid cultures by keeping cells in close proximity for cell fusion to occur (Roy *et al.*, 1991).

2.6.2 Non-sexual adhesion

2.6.2.1 Phenotypes

Sexual agglutinins are expressed at very low levels during vegetative growth (Terrance and Lipke, 1987) and do not contribute significantly to cell-cell adhesion under these conditions. On the other hand, a variety of very conspicuous non-sexual cell-cell and cell-surface phenotypes have been characterised (Figure 2.7). These phenotypes include the cell-cell interaction phenotypes of flocculation and “flor” formation, the cell-substrate interaction phenotypes of polystyrene and agar adhesion as well as cellular behaviour such as agar invasion, “mat” formation and the development of pseudohyphae, the latter phenotypes probably involving both cell-cell and cell-substrate related interactions. Flocculation is defined as the non-sexual, reversible and calcium (Ca^{2+})-dependent aggregation of yeast cells to form flocs that rapidly sediment in a liquid environment (Bony *et al.*, 1997). “Flor” formation involves cell aggregation and consists of a buoyant air-liquid interfacial biofilm that forms in the final stages of the production of sherry-like wine. It is speculated that “flor” formation occurs by means of hydrophobic cells that clump together and trap carbon dioxide thus rising to the liquid surface (Martinez *et al.*, 1997). Interaction between yeast and polystyrene surfaces most likely depend on the degree of cell wall hydrophobicity as such surfaces do not provide binding sites for adhesin receptors. Pseudohyphal growth results from the switching from “yeast from” ovoid shape cells that bud in an axial or bipolar pattern to a “hyphal like” growth form of elongated cells that remain attached to each other after unipolar budding (Gimeno *et al.*, 1992). This behaviour is closely associated with the directional growth of yeast into an agar substrate. “Mat” formation occurs when yeast is seeded on a low percent agar substrate in which yeast grow rapidly over the agar surface in a very specific pattern that resembles a central “hub” with several “spokes” protruding from it (Reynolds and Fink, 2001). The role of cellular adhesion with regard to pseudohyphae- and “mat” formation or agar invasion is not clear. Most likely the adhesion to agar enables yeast to proceed with directional growth. These phenotypes have been linked to nutrient availability and appear to provide yeast with the ability to search for or grow towards more favourable environments (Gimeno *et al.*, 1992; Reynolds *et al.*, 2008).

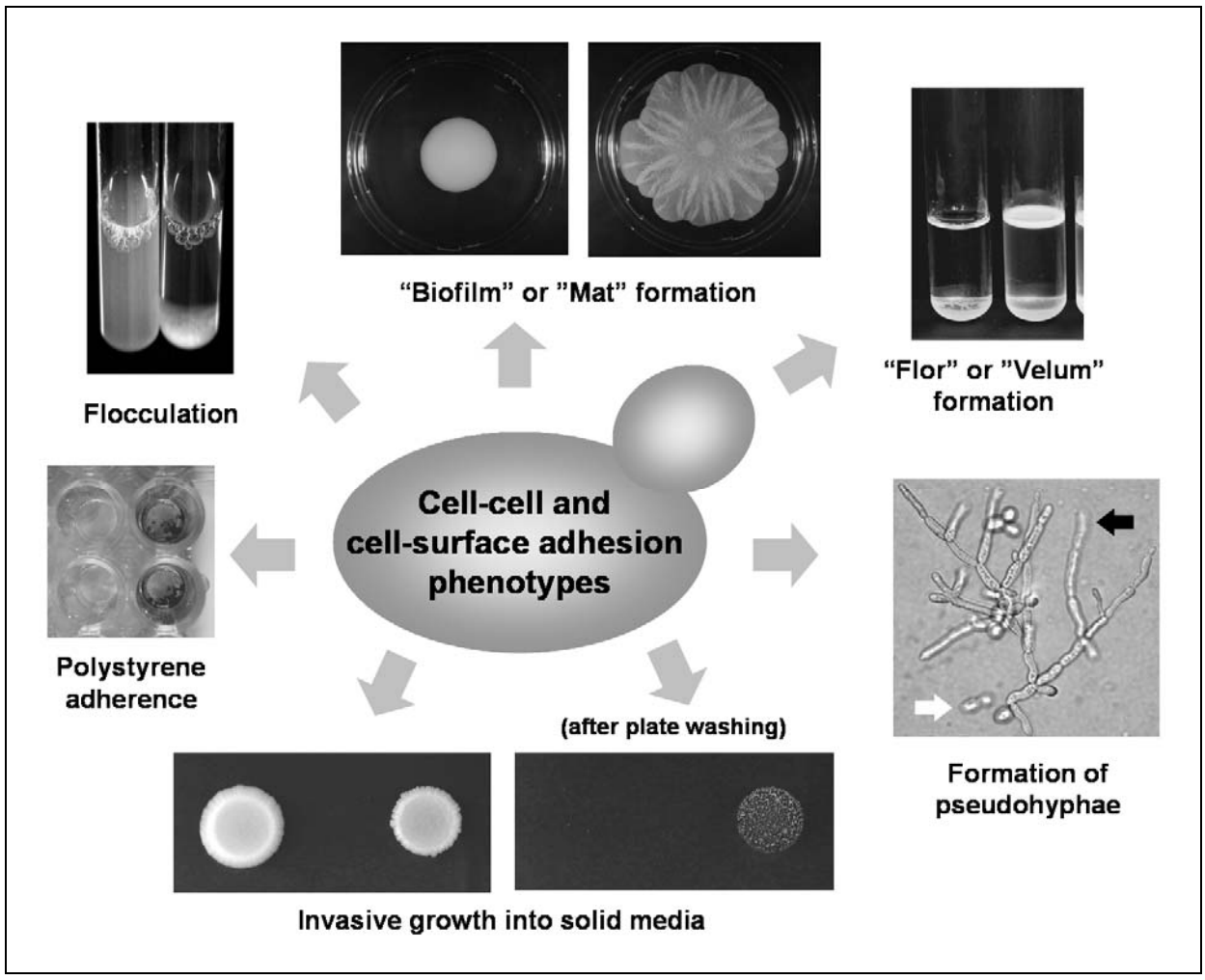

Figure 2.7 Different yeast cell-cell and cell-surface non-sexual adhesion phenotypes. The absence and presence of various phenotypes are indicated on the left and right hand sides (of test tubes, panels, microtiter wells or dropped yeast) respectively. Normal growing "yeast form" is indicated by a white arrow and pseudohyphae with a black arrow.

2.6.2.2 Flo adhesins

All of the above mentioned phenotypes require a class of adhesins encoded by the flocculation (*FLO*) gene family. The *FLO* genes that have been shown to encode for active adhesins are *FLO1*, *FLO5*, *FLO9*, *FLO10* and *FLO11* (Lambrechts *et al.*, 1996; Lo and Dranginis, 1996; Teunissen and Steensma, 1995). These genes encode proteins with the same domain organisation as other GPI-proteins and contain significant stretches of Ser/Thr motif repeats. No biochemical evidence for cell wall attachment has been reported yet, but the cell wall location has been confirmed for Flo1p (Bony *et al.*, 1997; Sato *et al.*, 2002) and Flo11p (Figure 2.4 M and N) (Guo *et al.*, 2000; Lo and Dranginis, 1996; Reynolds *et al.*, 2008) by means of immunofluorescent detection of tagged versions of these proteins. *FLO1*, *FLO5* and *FLO9* display extensive sequence similarity, with the exception of length variation in the internal homologous tandem repeat region in all these genes and can be considered as close homologues of each other. An *in silico* analysis on the phylogenetic relationships between the Flo proteins show that Flo1p, Flo5p and Flo9p group closely together while Flo10p and Flo11p group more distantly due to lower sequence similarity (Figure 2.8) (Caro *et al.*, 1997). This fits well with the observation that Flo1/5/9p, Flo10p and Flo11p are involved in different adhesion

phenotypes but it should be noted that functional overlaps between these proteins are observed. Flo1p has been identified as the dominant adhesin required for flocculation (Teunissen and Steensma, 1995) but over-expression of *FLO5* and *FLO9* also induce this phenotype (Govender *et al.*, 2008; Van Mulders *et al.*, 2009). In fact even *FLO10* and *FLO11* over-expression can lead to flocculation (Guo *et al.*, 2000) in certain conditions, as well as increased cell clumping (Van Mulders *et al.*, 2009). These flocculation phenotypes were not assessed for whether they could be reversibly inhibited by the addition of mannose or Ca^{2+}-dependent, observations typical of true Flo1p flocculation (Stratford and Assinder, 1991). Thus it cannot be ruled out that these Flo10p and Flo11p mediated phenotypes could involve cells aggregating by some other related mechanism. However in a *S. cerevisiae* var. *diastaticus* strain true flocculation was shown to be Flo11p dependent (Bayly *et al.*, 2005). The role of Flo10p is less well defined but over-expression studies suggest involvement in flocculation, agar invasion and the formation of pseudohyphae (Guo *et al*., 2000). Flo11p has been identified to be the dominant adhesin in all the above mentioned phenotypes with the exception of flocculation. It has been shown to be required for agar invasion and the formation of pseudohyphae (Lambrechts *et al.*, 1996; Lo and Dranginis, 1998), is needed for "flor" (Fidalgo *et al.*, 2006; Ishigami *et al.*, 2006) as well as "mat" formation (Reynolds and Fink, 2001) and determines even the morphological appearance of yeast colonies grown on agar plates (Kuthan *et al.*, 2003).

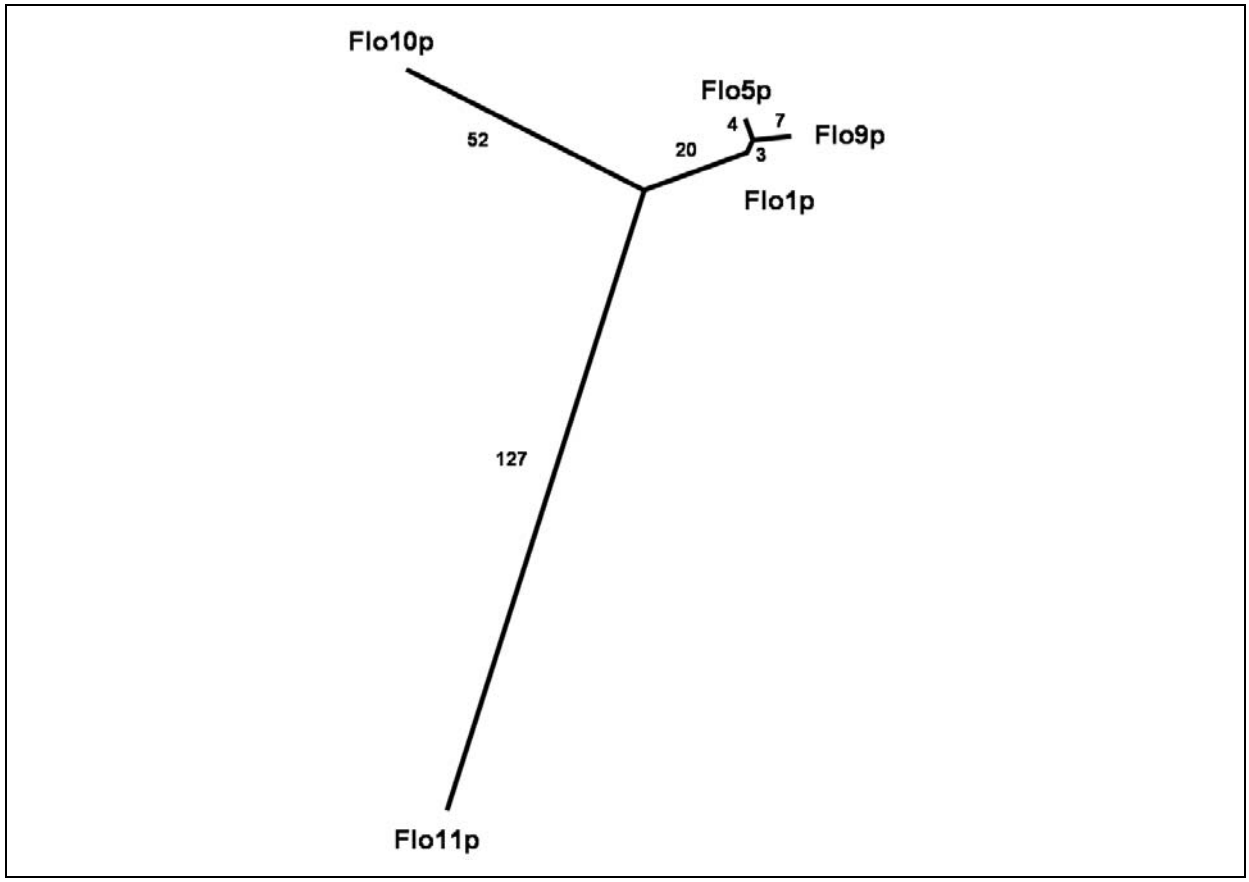

Figure 2.8 Phylogenetic relationships between the Flo protein family members. Numbers indicate the phylogenetic distance multiplied by 100 as described by Caro *et al*. (1997).

2.6.3 Functional overlap of sexual and non-sexual cell wall factors

Sexual agglutinin components function exclusively in mating but other mating factors have been shown to function in non-sexual adhesion phenotypes. Two factors first identified to function in yeast mating, Fig1p and Fig2p (Erdman *et al.*, 1998), also appear to be involved in adhesion phenotypes. Fig1p has been shown to be an integral membrane protein required for efficient cell fusion during mating (Aguilar *et al.*, 2007; Muller *et al.*, 2003), but a *FIG1* transposon insertion mutant displays decreased filamentation in response to 1-butanol, suggesting a role for Fig1p in polarised growth (Lorenz *et al.*, 2000a). Fig2p is a Ser/Thr rich mannoprotein that is GPI-anchored to the cell wall (Van der Vaart *et al.*, 1997) and is required for maintaining cellular integrity during mating (Zhang *et al.*, 2002). However, it was also shown that Fig2p can function as a substitute for other adhesion proteins such as Flo11p as Fig2p over-expression in a *flo11*Δ strain can re-establish Flo11p-dependent invasion (Figure 2.9A) (Guo *et al.*, 2000). Strikingly the over-expression of non-sexual adhesins was shown to be able to compensate for mating deficiencies caused by mating factor encoding gene deletions, as was shown by the over-expression of *FLO11* and *FLO10* suppressing the inability to mate in an *aga1*Δ *fig2*Δ strain (Figure 2.9B). In the case of Flo11p, this has been shown to be due to the proper localisation of Flo11p to the shmoo tip in pheromone treated cells (Figure 2.9C) (Guo *et al.*, 2000).

2.7 Regulation of *FLO* expression

FLO gene regulation is highly complex and has been shown to involve a number of interacting mechanisms. In laboratory conditions, Flo-dependent phenotypes appear mostly responsive to the nutrient status of the extra-cellular environment. The change from rich to nutrient limited conditions induces flocculation (Sampermans *et al.*, 2005) and agar invasion (Cullen and Sprague, 2000; Gimeno *et al.*, 1992). Furthermore the presence of nutrient gradients in the growth media appears to control “mat” formation (Reynolds *et al.*, 2008). It is well established that nutrient sensing is dependent on intracellular- and membrane-based sensors that transmit nutritional information via signalling pathways that in turn affect the expression of the *FLO* genes. Other mechanisms that control *FLO* gene expression and protein production include epigenetic gene control as well as mechanisms that specifically affect the transcription and translation of some *FLO* family members.

With the exception of *FLO11*, all of the *FLO* genes are situated adjacent to telomeric regions (Verstrepen *et al.*, 2004). *FLO1*, *FLO5* and *FLO9* have been shown to be under the control of telomeric silencing (Dietvorst and Brandt, 2008) whereas *FLO10* and *FLO11* transcription is also controlled by epigenetic mechanisms (Halme *et al.*, 2004). Efficient transcription of *FLO1* and *FLO11* has also been show to be dependent on the THO protein complex. More in-depth analysis showed that this protein complex is required for the transcriptional elongation through the repeat containing regions of the *FLO11* open reading frame (Voynov *et al.*, 2006). The signalling factor Ste12p is a known activator of *FLO11* and possibly *FLO10* (discussed in section 2.7.2). During the switch to the Flo11p-dependent phenotype of filamentous growth, an increase in Ste12p protein levels is observed with *STE12* transcript levels remaining constant (Park *et al.*, 2006). Furthermore Park *et al* (2006) showed that *STE12* mRNA was mainly associated with poly-ribosomes, greatly increasing the translational turnover of these messenger molecules. Thus by controlling gene mRNA

translation, the cell can modify Flo expression independent of epigenetic and transcriptional regulation.

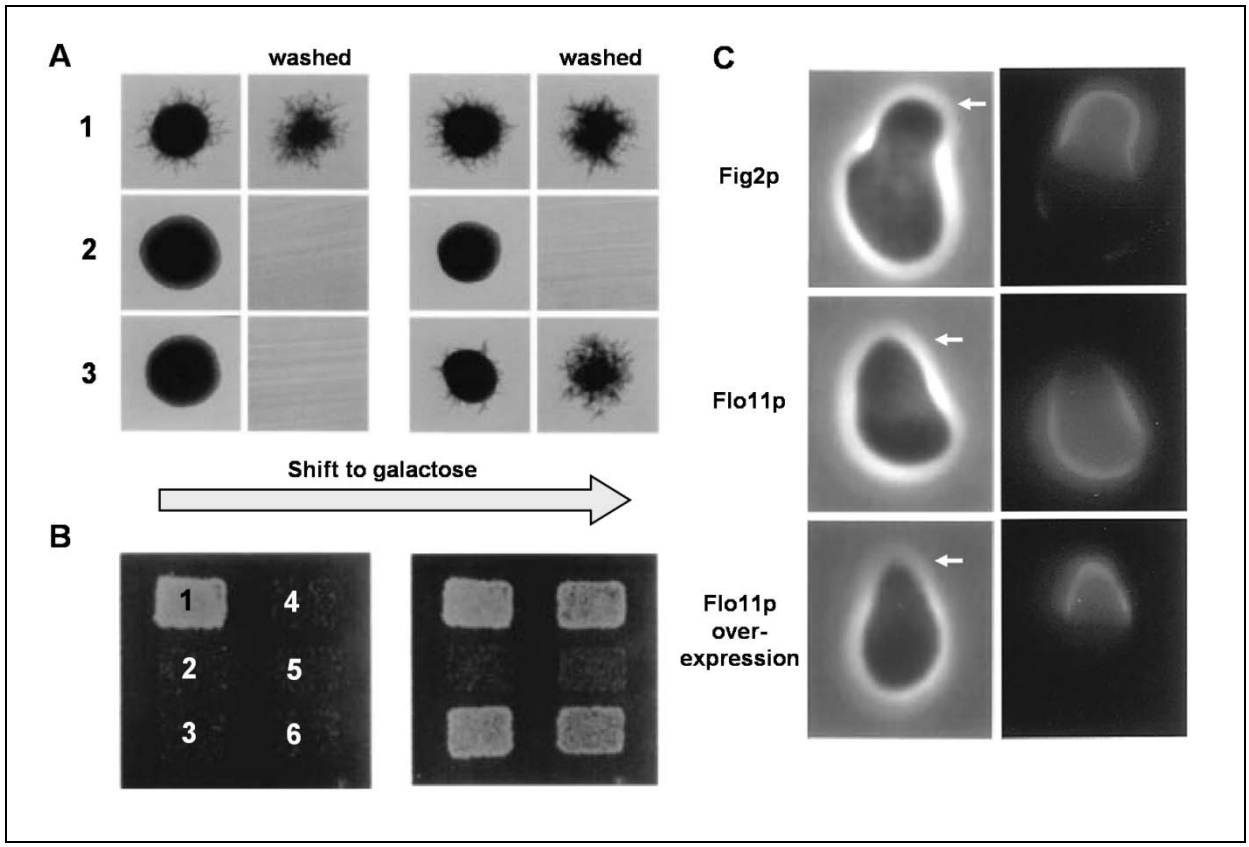

Figure 2.9 The adhesins Fig2p, Flo10p and Flo11p are able to functionally compliment each other. (A) Fig2p over-expression suppresses a *flo11*Δ deletion by inducing invasion. Total growth is shown on the left and invaded cells revealed after plate washing on the right. Shown is (1) wild type yeast, (2) yeast with *FLO11* deleted and (3) *flo11*Δ cells carrying a *FIG2* over-expressing construct that is induced upon the shift from glucose to galactose (left and right panel, respectively) in the growth media. (B) Flo10p and Flo11p over-expression can overcome the mating defects caused by *FIG2* and *AGA1* deletion. A Matα tester strain was mated with the following Mata strains (1) wild type yeast, (2) *fig2*Δ *aga1*Δ *flo11*Δ, (3) *fig2*Δ *aga1*Δ + *FLO11* over-expressing construct (↑), (4) *aga1*Δ *flo11*Δ, + *FIG2* (↑), (5) *fig2*Δ *aga1*Δ *flo11*Δ + *FLO1* (↑) and (6) *fig2*Δ *aga1*Δ *flo11*Δ + *FLO10* (↑). As with (A) the left panel shows the strains growing on glucose media and on the right galactose media. In both cases media was supplemented with the amino acids that would permit the growth of only diploid strains originating from successful mating events. Strain (2) shows an obvious mating defect which is clearly suppressed upon the over-expression of *FLO11* (3), *FIG2* (4) and *FLO10* (6) (right panel). (C) Flo11p over-expression suppresses mating defects by localising to shmoo tips (white arrow) during mating. Tagged versions of Fig2p localise to shmoo tips of yeast treated with mating pheromone (top panel). Tagged Flo11p however localise to the rest of the outer cell except the shmoo tip (middle panel). When however Flo11p over-expression was induced in combination with pheromone treatment Flo11p localised exclusively to the shmoo tip (bottom panel). Note that it is by the over-expression of Flo11p that it is able to induce mating in the mating deficient strain (B). All results shown were taken from Guo *et al*. (2000).

The specific sensing and signalling systems that control *FLO* gene expression are not well understood with the exception of *FLO11* transcriptional regulation. In fact a substantial amount of information has been collected on the subject of *FLO11* regulation. In the following sections the core signalling pathways controlling *FLO11* regulation will be briefly discussed followed by a discussion on the available information on the regulation of the other *FLO* family members.

2.7.1 Regulation of *FLO11* expression

Two well characterised signalling pathways, the filamentous growth mitogen activated protein kinase (FG MAPK) and the cyclic AMP responsive protein kinase A (cAMP-PKA) pathways have been shown to constitute important signalling modules controlling *FLO11* transcription (Figure 2.10) (Gagiano *et al.*, 2002; Verstrepen and Klis, 2006). These pathways do not signal in isolation but interact with each other in a coordinated manner to regulate the complex and unusually large (~3kb) *FLO11* promoter (Gagiano *et al.*, 1999; Rupp *et al.*, 1999). The FG MAPK pathway is composed of various kinases that sequentially phosphorylate each other in order to transmit a given signal. Upstream of this pathway the rho like GTP-binding protein Cdc42p (Johnson, 1999) activates the MAPK module by physical interaction with the first protein kinase, Ste20p. Ste20p phosphorylates Ste11p, which in turn phosphorylates Ste7p, which finally by the same mechanism transmits the signal to Kss1p (Chen and Thorner, 2007). Kss1p acts as the downstream effector of the MAPK pathway and activates the transcription factor Ste12p (Bardwell *et al.*, 1998). Ste12p has many gene targets but specifically bind to the *FLO11* promoter by cooperative action with the transcription factor Tec1p (Madhani and Fink, 1997). cAMP-PKA signalling is activated by the activity of the GTP binding proteins Ras2p and Gpa2p (Tamaki, 2007). They activate Cyr1p, an adenylate cyclase that converts ATP into the signalling molecule cAMP. The PKA protein complex consists of two regulatory subunits of Bcy1p and the catalytic subunits Tpk1/2/3p. cAMP binds to Bcy1p, relieving its inhibitory effect on the Tpk proteins which in turn have positive and negative effects on *FLO11* transcription (Robertson and Fink, 1998). Tpk2p plays the biggest role in transcriptional signalling and controls the activity of the cAMP-PKA downstream transcription factors Flo8p and Sfl1p. Acting on the *FLO11* promoter Flo8p and Sfl1p function in the activation and repression of transcription, respectively. The genetic analysis of the factors acting on *FLO11* transcription shows that all transcriptional activation is blocked by a deletion in *MSS11* (van Dyk *et al.*, 2005). Evidence suggests that Mss11p directly interacts with Flo8p and cooperatively binds DNA, interacting with the central transcription machinery to control gene expression (Kim *et al.*, 2004a). Upstream of the MAPK pathway the Msb2p membrane sensor signals through interaction with Cdc42p (Cullen *et al.*, 2004). The nature of the signal that is transmitted is not known but could involve information on cell wall integrity considering that Msb2p contains a glycosylated extra-cellular domain that may very well interact with cell wall components. Two other membrane sensors, Gpr1p and Mep2p, signal via Gpa2p to the cAMP-PKA pathway (Kubler *et al.*, 1997; Lorenz and Heitman, 1997; Pan and Heitman, 1999). Gpr1p communicates information on carbon- and nitrogen source availability (Lorenz *et al.*, 2000b; Tamaki *et al.*, 2000) and Mep2p is an ammonium membrane transporter that signals under conditions of nitrogen starvation (Lorenz and Heitman, 1998). Ras2p was shown to effect both MAPK and cAMP-PKA signalling, showing that these pathways cross communicate (Mosch *et al.*, 1999). The upstream signals that control Ras2p activity have not been identified to date.

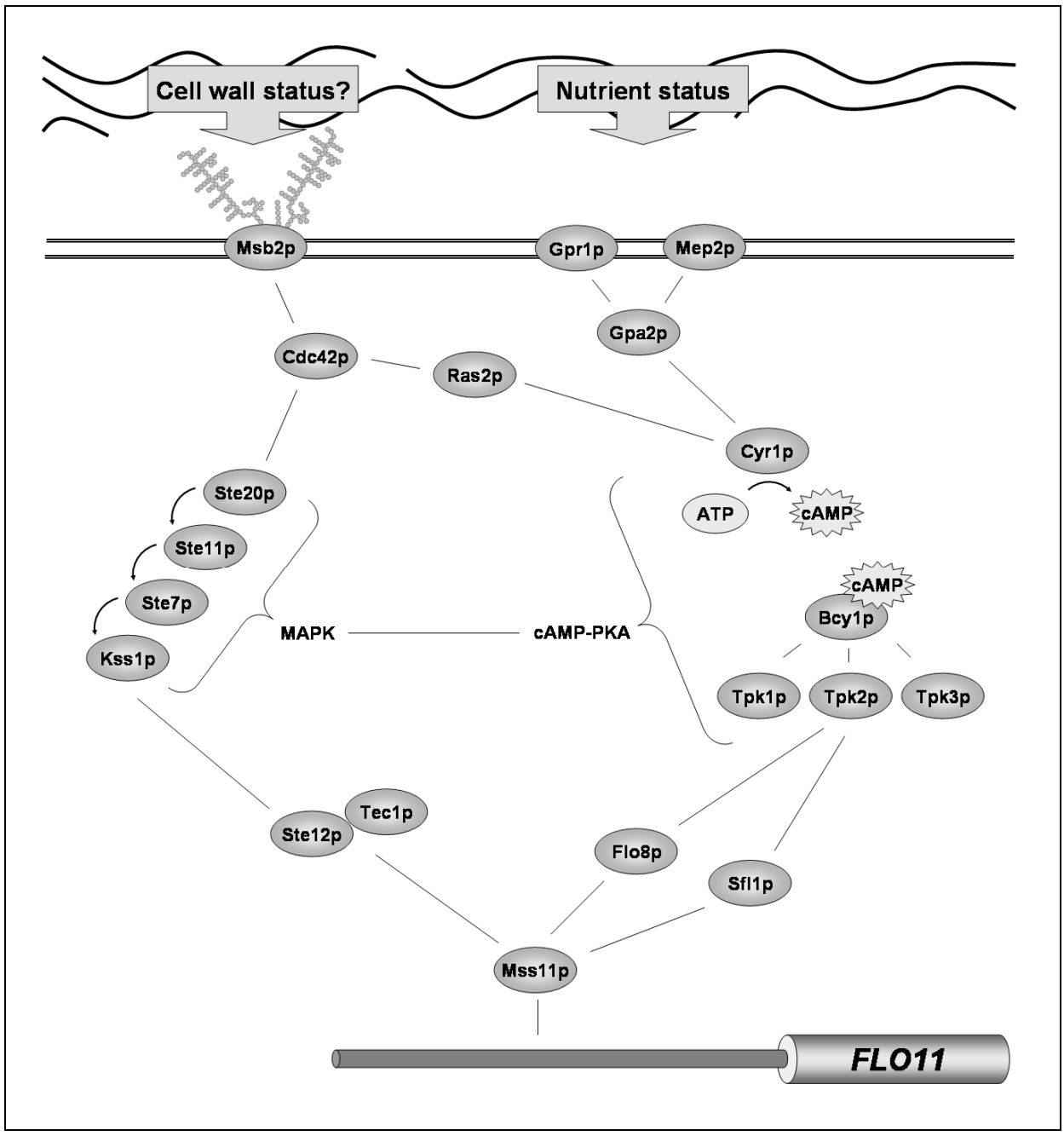

Figure 2.10 The sensing and signalling mechanisms that control *FLO11* expression. Receptor systems, the MAPK and cAMP-PKA pathways as well as relevant downstream effectors are shown. Not shown is other signalling pathways or components that control *FLO11* by epigenetic means. Solid lines indicate the exchange of information between components and do not necessarily imply physical interaction.

2.7.2 Transcriptional regulation of other *FLO* genes

Flo1p-dependent flocculation has been shown to be responsive to the specific nutrient status of the yeast's surroundings (Sampermans *et al.*, 2005). Furthermore the cAMP-PKA signalling pathway, being responsive to such nutrient signals (Gagiano *et al.*, 2002), has been shown to be required for *FLO1* transcription (Fichtner *et al.*, 2007). Evidence shows that it is the cAMP-PKA transcription factor Flo8p that specifically acts as the positive regulator of *FLO1* transcription (Kobayashi *et al.*, 1999) (Figure 2.11). *FLO1* is further regulated by the Swi-Snf co-activator and the Tup1p-Ssn6p co-repressor complexes that function on chromatin level

(Fleming and Pennings, 2001). These same complexes have been shown to also act on the *FLO11* promoter (Conlan and Tzamarias, 2001; Kim *et al.*, 2004a). Furthermore, the Srb8p/Ssn8p protein complex was shown to have a repressive effect on both *FLO* genes (Fichtner *et al.*, 2007; Kim *et al.*, 2004b). Srb8p/Ssn8p interacts with the yeast mediator complex which in turn regulates gene transcription through interaction with RNA polymerase II (Myers and Kornberg, 2000). A mediator component, Cin4p was shown to be required for the repression of *FLO1* but not *FLO11* (Fichtner *et al.*, 2007). On the other hand the same authors showed that the Sfl1p repressor, functioning downstream of the cAMP-PKA pathway, was only required for *FLO11* repression. This is surprising considering that both *FLO1* and *FLO11* are activated by the transcription factor Flo8p and suggests that these genes are differentially regulated by the same signalling pathways.

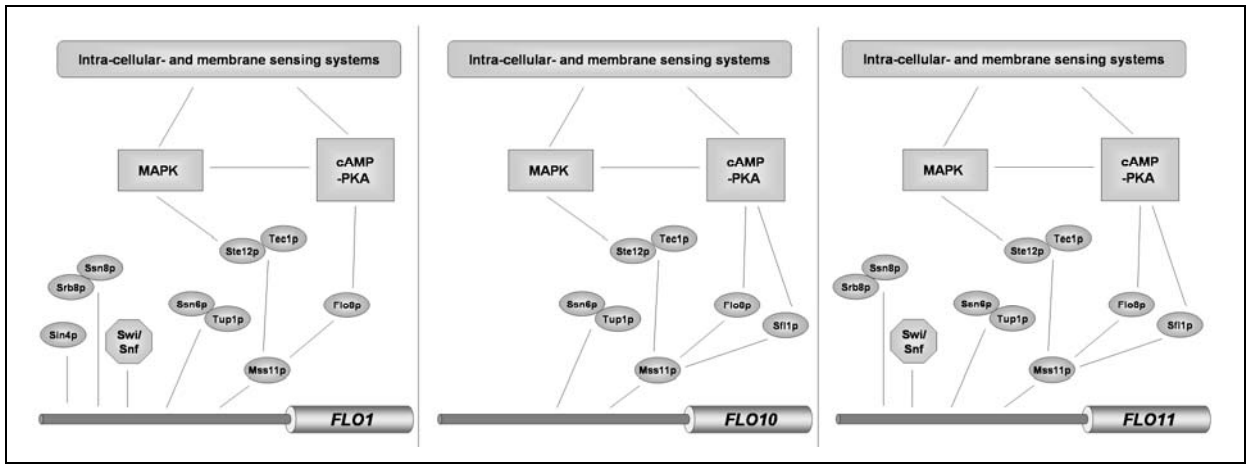

Figure 2.11 A diagrammatic summary of the current information available on the regulation of *FLO* expression. Solid lines indicate exchange of information or inhibitory/activation function but do not necessarily imply physical interaction.

Mss11p is a central factor in the regulation of *FLO11* (van Dyk *et al.*, 2005) and is in addition required for *FLO1* transcription (Bester *et al.*, 2006). The analysis of signalling mutants reveals that Mss11p also performs a very important role in *FLO10* regulation but is not absolutely required as observed for the other *FLO* genes (see Chapter 4). By expressing truncated versions of Mss11p the same protein regions were shown to be important for regulating both flocculation and the transcription of *FLO11* (Bester *et al.*, 2006). With the observation that flocculation is almost completely Flo1p-dependent in this strain this would suggest that Mss11p affects both *FLO1* and *FLO11* transcription by the same molecular mechanism.

Experiments using chromatin immuno-precipitation (ChIP) in combination with DNA micro-arrays (chip) provides a wealth of information on the transcription factors (TF's) that physically bind the promoters of *FLO1*, *FLO10* or *FLO11*. (Borneman *et al.*, 2006; Harbison *et al.*, 2004; Horak *et al.*, 2002; Lee *et al.*, 2002; Lieb *et al.*, 2001; Workman *et al.*, 2006). Figure 2.12 contains a diagram compiled using all the current available data generated by the above referenced workers in order to illustrate exclusive or shared promoter binding of the *FLO* genes by TF's. The analysis suggests that certain transcription factors act only on certain *FLO* members, others act on two, and some regulate all three of them. Some of these TF's have been studied in more detail and put in context of Flop-adhesin phenotypes. Two interesting examples are Mga1p and Phd1p regulating the yeast invasion phenotype. These transcription

factors were shown to act as targets for other TF's in the TF interaction network. This network control among other targets the expression of *FLO* genes (Borneman *et al.*, 2006). Being targets of other TF's and regulating *FLO* expression by means of promoter binding, they act as master regulators of *FLO* expression within this TF interactive regulatory network.

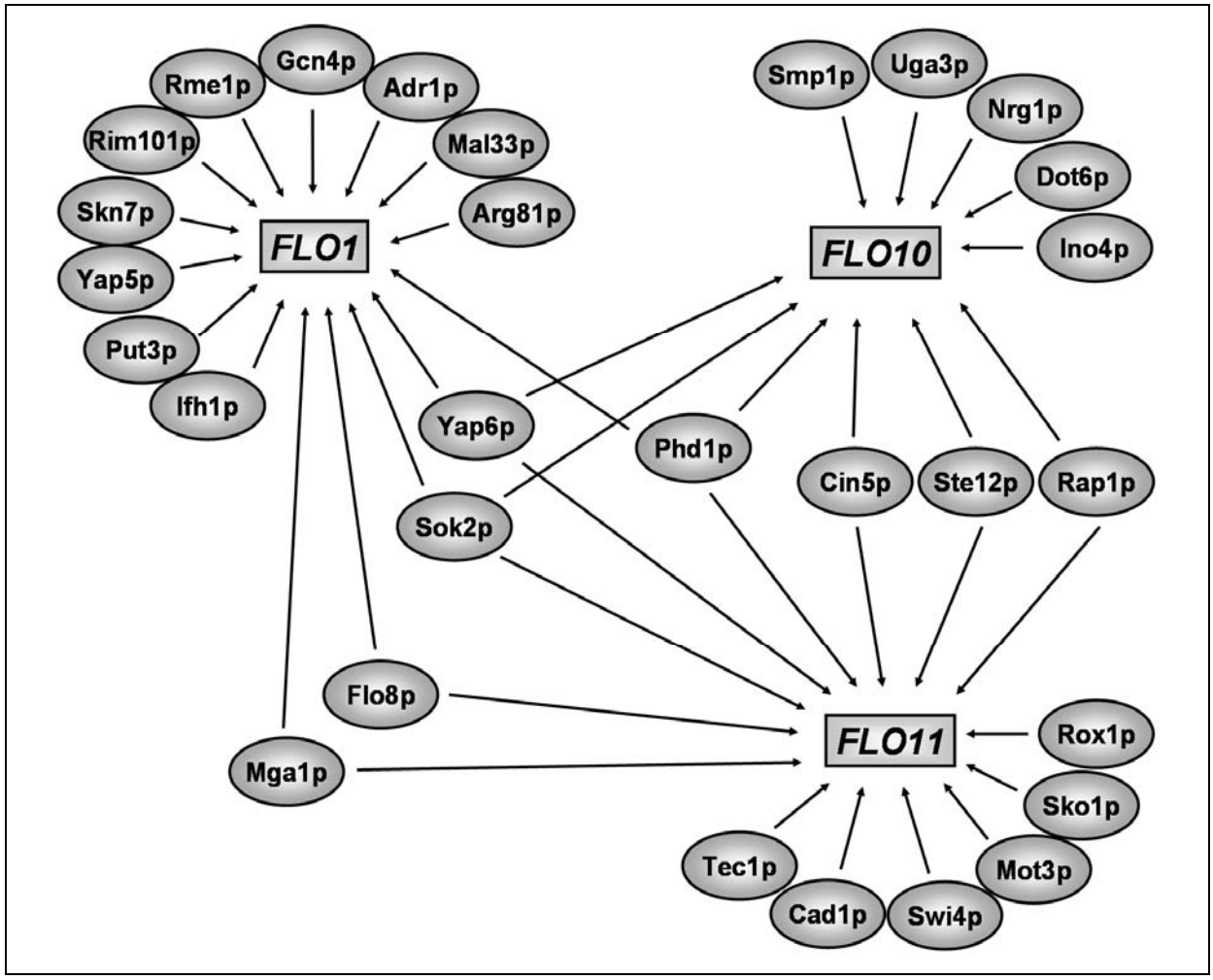

Figure 2.12 A diagrammatic representation of the transcription factors for which direct biochemical evidence show binding to the promoters of *FLO1*, *FLO10* and *FLO11*. Data was obtained from the YEASTRACT online database on September 4th 2009 (Monteiro *et al.*, 2008; Teixeira *et al.*, 2006).

Taken together the above mentioned evidence strongly suggests that the *FLO* gene family is regulated by signalling mechanisms that share components and modules. *FLO* genes are however differentially regulated by (1) showing different dependencies on shared signalling pathways and (2) require different repressor- and transcription mediator factors.

2.8 Industrial importance of mannoproteins with special focus on Flo proteins

The study of Flo proteins and their cell-cell and cell-surface adhesin interactions in laboratory yeast provides useful information that can be extrapolated to the fields of medicine, biotechnology and fermentation technology. As mentioned previously, internal repeat length variability in coding regions as well as the effect of epigenetic silencing of the *FLO* genes control yeast-yeast and yeast-surface interactions. The same interactions and control mechanisms determine the capability of pathogenic yeasts to adhere to medical equipment or host cells as well as their ability to invade tissue. In fact there is a significant correlation between genes in the yeast genome that contain internal Ser/Thr rich repeats, genetic variability and the ability to form pseudohyphae (Bowen and Wheals, 2006). It has been hypothesised that the outer cell wall masks the more immunogenic glucan layer of the cell wall from the host in host-yeast interactions. Furthermore, being able to constantly adjust the cell wall interface by means of repeat variation of cell wall genes, the host defence system can be more effectively evaded (Wheeler and Fink, 2006). The same principle defines the cell-cell interaction of various pathogens that include *Candida albicans*, *Neisseria* species, *Blastomyces dermatitidis*, *Histoplasma capsulatum* and *Coccidiodes posadasii* (Nather and Munro, 2008). Thus the development of drugs that target this protein layer (such as those affecting GPI anchor synthesis) will most probably result in the effective treatment of yeast infections. The sequencing of a *S. cerevisiae* strain isolated from the lung of an AIDS patient suffering from pneumonia reveals that in comparison to the widely used laboratory strain S288c, this strain displays variation in internal repeat regions of cell wall protein encoding genes. They include the adhesin genes *AGA1* and *FLO10* and the GPI-protein encoding *TIR1* and *FIT1* (Wei *et al.*, 2007). Most likely this variation in cell wall composition facilitates this strain's pathogenicity. In another study, genome wide gene copy number was determined for haploid, diploid and polyploid *S. cerevisiae* yeast strains from different geographic origins. The reference laboratory strain S288c, wild yeasts isolated from vineyards and wine cellars in Portugal, commercial wine fermentation yeasts as well as clinical isolates of opportunistic human pathogens were analysed. Sub-telomeric instability was associated with the pathogens, while Ty element insertion regions determined genomic differences of natural wine fermentation strains. Strikingly the majority of the wine yeast strains displayed a diminished copy number for members of the *FLO* gene family *FLO1*, *FLO5* and *FLO9* in comparison to the reference strains S288c (Carreto *et al.*, 2008). In another study it was shown that the domestication of wild yeast involved the marked down regulation of the *FLO11* gene but not other members of the flocculation family, a change that correlated with a Flo11p-dependent colony morphology change from "fluffy" to smooth (Kuthan *et al.*, 2003). These changes are not only the results of genomic rearrangements but also the reversible effect of epigenetic regulation (Halme *et al.*, 2004). Furthermore, a study of various wine yeast strains suggests *FLO11* transcripts of inter-strain length variability (Carstens *et al.*, 1998). Comparing different commercial brewing yeasts it was shown that there is extensive length variation of the internal repeat regions in *FLO1*, *FLO5*, *FLO9*, *FLO5* and *FLO11* (Verstrepen *et al.*, 2005).

Historically, in wine, non-flocculent yeasts were probably selected inadvertently as such yeast displayed more favourable fermentation characteristics. Suspended yeasts are able to efficiently ferment sugars whereas premature yeast flocculation can result in incomplete or sluggish fermentations. Probably the reverse selection procedure was used to select "flor"

yeasts that trap CO_2 gas in cell clumps and rise to the sherry surface to form a thick layer of cells ("velum") (Martinez *et al*., 1997). Flo11p was shown to be critical for "flor" formation (Ishigami *et al.*, 2006) and Flo1p, variants or homologues thereof, the controlling factor in industrial flocculation (Govender *et al.*, 2008; Verstrepen *et al.*, 2001). Controlled over-expression studies in industrial wine yeasts showed that Flo5p also induces Flo1p-like flocculation under wine fermentation conditions (Govender *et al*., 2009). By using promoters for the over-expression of *FLO1*/*FLO5* that respond to conditions prevailing at the end of alcoholic fermentation, wine yeasts were engineered to only flocculate upon the completion of fermentation. Surprisingly *FLO11* expressed in this manner gave the best flocculation results. Data suggests that Flo11p over-expression does not cause Flo1p-like flocculation but rather that it interacts with insoluble wine particles to give a wine with enhanced clarity at the end of fermentation. Apart from saving money from the increased wine volume recovery, fewer particles need to be eliminated from the wine by expensive filtering (Govender, PhD thesis, Stellenbosch University).

Other non Flo mannoproteins of industrial importance are those that prevent the formation of protein haze in bottled white wine. Protein haze has no effect on the organoleptic quality of wine but negatively impacts on the visual appearance of wine. Using a wine yeast strain modified for increased mannoprotein release, wine was produced with increased protein stability (Gonzalez-Ramos *et al* 2008). More specifically, several protein components have been identified that confer this protection and are referred to as haze protection factors (HPF). Hpf1p and Hpf2p have been identified thus far as to function in protection against haze formation (Brown *et al*., 2007; Dupin *et al*., 2000). Furthermore it was shown that the specific glycosylated status of Hpf2p was required for this protective mechanism (Schmidt *et al*., 2009).

Another biotechnological approach has used the Flo1p as a carrier protein to anchor an enzyme on the cell surface. A chimeric version of *FLO1* with its adhesin domain replaced by the coding region for a glucoamylase was expressed and led to successful starch degradation in the extra-cellular environment (Sato *et al.*, 2002). By expressing different truncated versions of this chimeric gene that carries different lengths of the internal repeat region, it was also shown that enzyme activity was dependent on a full length Flo1p backbone structure. This is most probably due to steric inhibition of the cell wall matrix when expressing shorter chimeric proteins. The study shows that is possible to use a cell wall protein to present an enzyme of commercial interest Immobilising the enzyme in this manner makes it possible to readily separate the enzyme from the medium by means of centrifugation, allowing "recycling" of the enzyme for the processing of additional substrate. In the study by Sato *et al.* (2002) the engineered yeast further fermented the glucose formed by starch degradation into ethanol thus forming an integrated processing system that converts starch into ethanol.

Great progress has been made using yeast as a presentation platform for various enzymatic applications. Recent examples include the construction of yeasts that can either convert lactose into an industrially important biopolymer (Li et al., 2009), saccharify and ferment cellulose to ethanol by the use of three enzymes bound to a cell wall scaffold (Tsai et al., 2009), and the development of a system with which the ratio of multiple cell wall displayed enzymes can be varied (Ito et al., 2009).

2.9 Concluding remarks

Cell wall Flo mannoproteins contribute to outer cell wall identity and function in a variety of cellular adhesion interactions. To date evidence shows that Flo1p and Flo11p are the dominant factors with Flo10p performing a supporting role under native conditions (see Chapter 4). Over-expression studies show that diverse mating and non-mating adhesins can complement each other showing that the timing and level of expression of adhesin genes are important for specific function. Furthermore, coding region variation generates surface protein diversity. Rather than being dependent on single factors, adhesion phenotypes appear to be the result of a complex interaction, and balance of, cell wall mannoproteins. Adjusting the expression by signalling or epigenetic means or by coding variability of these cell wall genes, phenotypic outcomes are adjusted to better suit adaptability of yeast to environmental conditions or host organisms.

Not much is know about the specific interplay of cell wall components in these complex phenotypes other than the established role of the Flo proteins. Nevertheless, current knowledge of *FLO* regulatory mechanisms can be used for applications in industry where controlled and modified adhesion phenotypes have much potential. For instance the recent generation of genetically modified yeast with modified flocculation profiles could lead to significant cost saving in the fermentation industry due to better volume recovery and improved filterability of fermented products (Govender *et al.*, 2008).

Thus far the direct analysis of cell wall mannoproteins has proven to be problematic due to the highly glycosylated nature of these proteins. Recently however significant advances have been made in the identification of cell wall mannoproteins with the use of mass spectrometry (Yin *et al*., 2008). In combination with genome transcription analysis, cell wall proteome analysis should start to provide answers on the cellular function of those numerous mannoproteins for which the specific cellular roles are as of yet unknown. Apart from the contribution to the fundamental understanding of cell wall dynamics, such information would prove invaluable for various industrial applications.

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Chapter 3

Research results I

The regulation of *Saccharomyces cerevisiae* *FLO* gene expression and Ca^{2+}-dependent flocculation by Flo8p and Mss11p

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Chapter 3: RESEARCH RESULTS I

The regulation of *Saccharomyces cerevisiae FLO* gene expression and Ca^{2+}-dependent flocculation by Flo8p and Mss11p

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3.1 Abstract

The ability of many micro organisms to modify adhesion-related properties of their cell surface is of importance for many processes, including substrate adhesion, cell–cell adhesion, invasive growth, pathogenic behaviour and biofilm formation. In the yeast *Saccharomyces cerevisiae*, a group of structurally related, cell-wall associated proteins encoded by the *FLO* gene family are directly responsible for many of the cellular adhesion phenotypes displayed by this organism. Previous research has suggested that the differential transcription of *FLO* genes determines specific adhesion phenotypes. However, the transcriptional regulation of most *FLO* genes remains poorly understood. Here we show that the transcriptional activator Mss11p, which has previously been shown to be involved in the regulation of starch degradation, the formation of pseudohyphae and haploid invasive growth, also acts as a strong inducer of flocculation. The data indicate that Mss11p induces flocculation together with Flo8p, and that *FLO1* is the dominant target gene of the two factors in this process. The deletion of *MSS11* leads to a non-flocculent phenotype, and specific domains of Mss11p that are critical for the induction of flocculation are identified. The data clearly show that several essential transcription factors are shared by at least two flocculation genes that control different adhesion phenotypes.

3.2 Introduction

The adhesion properties of *Saccharomyces cerevisiae* cells contribute significantly to the definition of the growth pattern of individual strains in specific environmental conditions. *S. cerevisiae* generally grows in the form of individual, dispersed cells when cultivated under agitation in liquid medium. Vegetative multiplication occurs through bud formation, after which mother and daughter cells separate and individual cells remain dispersed in the growth medium, provided sufficient agitation occurs. Changes in environmental conditions, in particular those leading to stress conditions and nutrient limitation, can however result in modifications of the adhesion properties of the cell wall. Such modifications are reflected in a variety of phenotypes such as flocculation, biofilm formation or substrate adhesion (Braus *et al.*, 2003; Reynolds and Fink, 2001; Sampermans *et al.*, 2005). On solid medium, the modifications contribute to invasive growth and/or the formation of pseudohyphae. The regulation of these processes has received widespread attention. Much of this research has focused on elucidating the regulatory network that controls invasive and pseudohyphal growth as well as biofilm formation (Gagiano *et al.*, 2002;

Gancedo, 2001; Palecek *et al.*, 2002). The regulation of other adhesion-related phenotypes, and in particular of flocculation, has however received significantly less attention. The data presented here show that the transcription factor Mss11p, together with Flo8p, is required for the regulation of flocculation, and that this regulation is dependent on the transcription of the *FLO1* gene. Mss11p and Flo8p have previously been shown to also be central to the transcriptional regulation of the *FLO11* gene (van Dyk *et al.*, 2005), which encodes a protein critical for pseudohyphal development and invasive growth (Guo *et al.*, 2000; Lambrechts *et al.*, 1996; Lo and Dranginis, 1996; Lo and Dranginis, 1998). The data therefore suggest that these transcription factors regulate at least two flocculation genes that control different phenotypes. This finding raises questions regarding the manner in which cells can differentially induce adhesion phenotypes. *FLO1* and *FLO11* are part of a larger family of genes (*FLO* genes). These genes encode structurally related, membrane-anchored and cell-wall associated proteins that were initially identified as being critical for the process of flocculation (Teunissen and Steensma, 1995). Flocculation is defined as the asexual clumping together of cells to form aggregates referred to as 'flocs'. Such aggregates readily sediment to the bottom of liquid cultures in a process referred to as flocculation (Verstrepen *et al.*, 2003). Flocculation requires the presence of Ca^{2+} ions in the medium (Malcolm, 1989) and is inhibited by the presence of mannose (Stratford and Assinder, 1991). The ability to flocculate of different commercial and laboratory strains is highly variable. Laboratory strain S288C appears generally unable to flocculate due to a nonsense point mutation in the *FLO8* gene (Liu *et al.*, 1996), encoding a transcription factor that has been shown to activate the transcription of *FLO1* (Kobayashi *et al.*, 1999). Flo8p has also been shown to act as an activator of *FLO11* transcription (Gagiano *et al.*, 1999a; Kobayashi *et al.*, 1999; Pan and Heitman, 1999; Pan and Heitman, 2002; Rupp *et al.*, 1999). While the different Flo proteins are structurally very similar, different members of this family are responsible for different adhesion phenotypes, suggesting differential regulation of the genes (Verstrepen *et al.*, 2004). The regulation of one of the genes of the family, *FLO11*, has attracted widespread attention because of the specific role of the gene product in cellular adhesion during invasive and pseudohyphal growth (Guo *et al.*, 2000; Lambrechts *et al.*, 1996). Under conditions of limited carbon and/or nitrogen availability yeast might form elongated structures referred to as pseudohyphae. In these structures the axial budding pattern in haploid or the bipolar pattern in diploid cells is replaced by a unipolar budding pattern. Mother and daughter cells remain attached to each other after the completion of budding, and are more elongated in shape (Gancedo, 2001). The transcriptional regulator Mss11p performs a central role regarding the regulation of pseudohyphae formation by regulating *FLO11* expression (Gagiano *et al.*, 1999a; Gagiano *et al.*, 1999b; van Dyk *et al.*, 2005). Mss11p was first identified as a positive regulator of starch metabolism (Webber *et al.*, 1997) and, more recently, has also been implicated in cell cycle control (Stevenson *et al.*, 2001; Yang *et al.*, 2005). Mss11p does not present significant homologies to other proteins but for two short stretches of homology to Flo8p, and contains a stretch of poly-glutamine of 35 amino acids and a stretch of poly-asparagine of 30 amino acids (Gagiano *et al.*, 2003). In this study we show that in the S288c genetic background, Mss11p is required for the induction of Ca^{2+}-dependent flocculent behaviour, and induces flocculation when expressed from a high copy number plasmid. We show that this induction of flocculation is abolished by a deletion of *FLO1*, the dominant flocculation gene, and is not affected by *FLO11*. The data also confirm that flocculation and invasive growth phenotypes are clearly dependent on *FLO1* and *FLO11* expression, respectively, raising the question of how differential regulation of the two genes occurs. In this paper, we furthermore identify inhibitory and activation domains of Mss11p regarding the regulation of flocculation by means of an extensive analysis of truncated forms of *MSS11*.

3.3 Materials and Methods

3.3.1 Strains, media, and culture conditions

The yeast strains used in this study are listed in Table 3.1. As indicated, some strains were purchased from the European *S. cerevisiae* Archive for Functional Analysis (EUROSCARF). All strains are isogenic to the S288c genetic background. Transformations were carried out according to the lithium acetate method (Ausubel, 2004). Yeast cells were cultivated at 30˚C in synthetic media containing 0.67% yeast nitrogen base without amino acids, supplemented with 2% glucose (SCD media) and the required amino acids according to the auxotrophic needs of the relevant strain (Ausubel, 2004). SLAD media is similar to SCD media except that the amount of ammonium sulphate is lowered to 50 mM. For all solid media 2% agar was used.

3.3.2 Plasmid construction and recombinant DNA techniques

Plasmids and constructs used in this study are listed in Table 3.2. Standard procedures for the isolation and manipulation of DNA were used throughout this study (Ausubel, 2004; Maniatis *et al.*, 1989) *Escherichia coli* DH5α (Gibco BRL/Life Technologies) was used for the propagation of all plasmids and was grown in Luria-Bertani broth at 37˚C. All *E. coli* transformations and the isolation of DNA were done according to Ausubel et al. (1994). The *LEU2* auxotrophic marker was isolated as a 1,994 bp fragment from the plasmid pJJ250 (Jones and Prakash, 1990) by means of *Sal*I digestion. This fragment was blunt-ended with the Klenow enzyme and subsequently ligated to *Sna*BI-digested YCpLac22-FLO8 (Gagiano *et al.*, 1999b) to create YCpLac22-FLO8-LEU2, containing the *LEU2* gene inserted downstream of the FLO8 terminator. The *FLO8-LEU2* fusion of 5.2 kb was PCR-amplified using primers IntFLO8f 5'-GCATCTACACGCCGCCGATC-3' and IntFLO8r 5'-TGTGCCGGAATGATTGGTATGG-3', consisting of sequences homologous to the *FLO8* promoter and terminator, respectively. To construct a multiple copy plasmid containing *FLO8* with its own promoter, *FLO8* was isolated as a 3,252 bp *Sph*I–*Eco*RV fragment from plasmid pF415-1 (Kobayashi *et al.*, 1996) and ligated to plasmid YEpLac195 (Gietz and Sugino, 1988), digested with *Sph*I and *Sma*I, to generate plasmid YEpLac195-FLO8. The *FLO8* gene present on plasmid pF415-1 was first cloned from the flocculent strain ATCC60715 (Kobayashi *et al.*, 1996). This copy of *FLO8* does not contain the nonsense point mutation present in *flo8-1* as shown by sequence alignment. In order to express different truncated forms of *MSS11*, various truncated forms of the *MSS11* open reading frame were cloned into the expression vector YEpLac112- MSS11exp (Gagiano *et al.*, 2003) using the same procedure as already described for the construction of YEpLac112- MSS11-OF-OR (Gagiano *et al.*, 2003). All plasmids were sequenced to verify that no mutations were introduced during PCR amplification.

Table 3.1 Strains used in this study.

Strain	Relevant genotype	Source or reference
FY23 *mss11*Δ	*MAT*α *flo8-1 leu2 trp1 ura3 mss11*Δ::*LEU2*	Gagiano *et al.*, 1999b
BY4742	*MAT*α *flo8-1 his3 leu2 lys2 ura3*	Brachmann *et al.*, 1998
BY4742 *flo1*Δ	*MAT*α *flo8-1 his3 leu2 lys2 ura3 flo1*Δ::*kanMX4*	EUROSCARF
BY4742 *flo10*Δ	*MAT*α *flo8-1 his3 leu2 lys2 ura3 flo10*Δ::*kanMX4*	EUROSCARF
BY4742 *flo11*Δ	*MAT*α *flo8-1 his3 leu2 lys2 ura3 flo11*Δ::*kanMX4*	EUROSCARF
BY4742 *mss11*Δ	*MAT*α *flo8-1 his3 leu2 lys2 ura3 mss11*Δ::*kanMX4*	EUROSCARF
BY4742 *FLO8*	*MAT*α *flo8-1 his3 leu2 lys2 ura3 flo8-1*Δ::*FLO8-LEU2*	This study
BY4742 *FLO8 flo1*Δ	*MAT*α *flo8-1 his3 leu2 lys2 ura3 flo8-1*Δ::*FLO8-LEU2 flo1*Δ::*kanMX4*	This study
BY4742 *FLO8 flo10*Δ	*MAT*α *flo8-1 his3 leu2 lys2 ura3 flo8-1*Δ::*FLO8-LEU2 flo10*Δ::*kanMX4*	This study
BY4742 *FLO8 flo11*Δ	*MAT*α *flo8-1 his3 leu2 lys2 ura3 flo8-1*Δ::*FLO8-LEU2 flo11*Δ::*kanMX4*	This study
BY4742 *FLO8 mss11*Δ	*MAT*α *flo8-1 his3 leu2 lys2 ura3 flo8-1*Δ::*FLO8-LEU2 mss11*Δ::*kanMX4*	This study

3.3.3 Ca^{2+}-dependent flocculation assays

Yeast colonies were inoculated in test tubes containing 5 ml of SCD media containing the required amino acids and grown for 2 days to stationary phase. From these cultures, 5 ml of the same medium were inoculated to an initial OD_{600} (optical density measure at 600 nm) of between 0.05 and 0.1. These cultures were grown for 2 days to stationary phase. To determine the extent of Ca^{2+}-dependent flocculation, an aqueous solution of EDTA (pH 8.0) was added to these yeast cultures to a final concentration of 50 mM, followed by vigorous agitation (vortex at maximum speed setting) until the flocs were in homogeneous suspension as determined by visual inspection. The OD_{600} was immediately determined by removing 100 µl of the liquid culture and adding it to 900 µl of a 50 mM EDTA solution (pH 8.0), followed by spectrophotometric measurement (measurement ‘‘A’’) at a wavelength of 600 nm. Ca^{2+}-dependent flocculation was then induced by transferring 1 ml of liquid culture to a micro centrifuge tube, separating the cells from the growth medium by quick centrifugation, removing the supernatant and washing the cells with 1 ml of sterile water followed by the addition of 1 ml of an aqueous solution of 10 mM $CaCl_2$. Micro centrifuge tubes were vigorously agitated (vortex at maximum speed setting) for 10 s and left undisturbed for 60 s. A second spectrophotometric measurement (measurement ‘‘B’’) was performed on a 100 µl sample taken from just below the meniscus in the micro centrifuge tube, as described above. The extent of Ca^{2+}-dependent flocculation was then calculated by the following formula:

$$\text{Flocculation (\%)} = ((A-B)/A) \times 100$$

The standard error of the mean (SEM) was calculated for cultures from at least five individual transformants for the data set used in Fig. 3.1, whereas three individual transformants were used to generate the data with regard to the expression of *MSS11* truncations.

3.3.4 RNA extraction and Northern analysis

Total RNA was isolated from 5 ml yeast cultures grown to stationary phase, as previously described for the flocculation assay, by means of the glass bead disruption method (Ausubel, 2004). RNA was separated on 1% agarose gels containing 0,7% formaldehyde and thereafter transferred and cross linked to BioBond- PlusTM nylon membranes (Sigma-Aldrich). Labelled probes to detect transcripts of the *ACT1* and *FLO11* genes were prepared as previously described (van Dyk *et al.*, 2005). To detect mRNA of the *FLO1* and *FLO10* genes primers FLO1f, 5'-AACAGTAGTCACCTCTTCGC-3'; FLO1r, 5'-AGACACTTAAACCACTACCG-3'; FLO10probeF, 5'-ATGCCTGTGGCTGCTCGATA-3'; and FLO10probeR, 5'-TGTCGGTAGGTGCATCTGCG-3' were used in PCR with genomic DNA from strain BY4742 as template. Detection of hybridised probe was performed as described in the dioxigenin (DIG) manual (Roche Diagnostics).

3.3.5 Invasive growth assays

EDTA was added to cell cultures grown in similar conditions as described for the flocculation assay and RNA preparation, to a final concentration of 20 mM. Test tubes were vigorously agitated to separate flocs and 20 µl of each culture were spotted onto SLAD medium. After 6 days of growth cells were washed off the agar surface by rubbing with a gloved finger under running water, revealing only those cells that have grown into the agar.

Table 3.2 Plasmids used in this study.

Plasmid	Genotype	Source or reference
pF415-1	*CEN4 LEU2 FLO8*	Kobayashi *et al.*, 1996
pJJ250	*LEU2*	Jones and Prakash, 1990
YCpLac22-FLO8	*CEN4 LEU2 FLO8*	Gagiano *et al.*, 1999b
YCpLac22-FLO8-LEU2	*CEN4 LEU2*/*LEU2 FLO8*	This study
YEplac112	2µ *TRP1*	Gietz and Sugino, 1988
YEplac112-MSS11exp	2µ *TRP1* P*MSS11* T*MSS11*	Gagiano *et al.*, 2003
YEplac112-MSS11-OF-OR	2µ *TRP1* $MSS11_{1\text{-}758}$	Gagiano *et al.*, 2003
YEplac112-MSS11-OF-NxR	2µ *TRP1* $MSS11_{1\text{-}641}$	This study
YEplac112-MSS11-OF-NR	2µ *TRP1* $MSS11_{1\text{-}604}$	This study
YEplac112-MSS11-H1F-OR	2µ *TRP1* $MSS11_{35\text{-}758}$	This study
YEplac112-MSS11-H1F-NR	2µ *TRP1* $MSS11_{35\text{-}604}$	This study
YEplac112-MSS11-H1F-ID2R	2µ *TRP1* $MSS11_{35\text{-}504}$	This study
YEplac112-MSS11-H2F-OR	2µ *TRP1* $MSS11_{126\text{-}758}$	This study
YEplac112-MSS11-H2F-NR	2µ *TRP1* $MSS11_{126\text{-}604}$	This study
YEplac112-MSS11-H2F-ID2R	2µ *TRP1* $MSS11_{126\text{-}504}$	This study
YEplac112-MSS11-H2F-ID1R	2µ *TRP1* $MSS11_{126\text{-}414}$	This study
YEplac112-MSS11-PH2F-OR	2µ *TRP1* $MSS11_{148\text{-}758}$	This study
YEplac112-MSS11-PH2F-NR	2µ *TRP1* $MSS11_{148\text{-}604}$	This study
YEplac112-MSS11-PH2F-ID2R	2µ *TRP1* $MSS11_{148\text{-}504}$	This study
YEplac112-MSS11-QxF-OR	2µ *TRP1* $MSS11_{273\text{-}758}$	This study
YEplac112-MSS11-QxF-NR	2µ *TRP1* $MSS11_{273\text{-}604}$	This study
YEplac112-MSS11-QF-OR	2µ *TRP1* $MSS11_{340\text{-}758}$	This study
YEplac112-MSS11-QF-NR	2µ *TRP1* $MSS11_{340\text{-}641}$	This study
YEpLac195	2µ *URA3*	Gietz and Sugino, 1988
YEpLac195-FLO8	2µ *URA3 FLO8*	This study
YEpLac195-MSS11	2µ *URA3 MSS11*	Gagiano *et al.*, 1999b

3.4 Results

3.4.1 Multiple copies of *MSS11* constitutively induce Ca^{2+}-dependent flocculation in S288C

Yeast strains of the S288C genetic background are deficient for flocculation due to a nonsense point mutation in the *FLO8* gene, *flo8-1* (Liu *et al.*, 1996). Introduction of multiple copies of *FLO8* into the strain resulted in strong, constitutive flocculation confirming observations by Liu *et al*. (1996) (Fig. 3.1). *MSS11* has previously been shown to be able to suppress the invasive growth defect of *FLO8* deleted strains (van Dyk *et al.*, 2005). We therefore tested if multiple copies of *MSS11* could also suppress the lack of flocculation ability of the *flo8-1* mutant strain. As shown in Fig. 3.1, the introduction of multiple copies of *MSS11* into this strain also resulted in a constitutive flocculation phenotype, although flocculation is of slightly lower intensity than the phenotype of the *FLO8* restored strain. Thus, multiple copies of *MSS11* suppress the lack of Flo8p with regard to the induction of both flocculation and invasive growth.

To assess flocculation phenotypes in a background with a functional *FLO8* gene, we replaced the chromosomal mutant allele with a wild-type copy of the gene as described in the Materials and methods section. The strains containing a functional chromosomal copy of the *FLO8* gene presented a constitutive, Ca^{2+}-dependent flocculation phenotype indistinguishable from the *FLO8* multiple copy phenotype. Multiple copies of either *FLO8* or *MSS11* in this strain did not result in further increases in flocculation (Fig. 3.1).

3.4.2 *FLO1* is the primary target gene responsible for the flocculation phenotype induced by Flo8p and Mss11p

In order to identify the possible target genes through which *FLO8* and *MSS11* induce flocculation, *FLO8* and *flo8-1* yeast with single deletions in *FLO1*, *FLO10*, and *FLO11* were transformed with the corresponding plasmids and assayed for their ability to flocculate (Fig. 3.1). The data show that the deletion of *FLO1* resulted in strains that lost the ability to flocculate, and multiple copies of *FLO8* and of *MSS11* were not able to induce flocculation to any significant degree in these strains. Deletion of *FLO10* did not affect flocculation significantly, although multiple copies of *MSS11* did not restore flocculation in a *flo8-1* strain to the same degree as in the *FLO10* wild type, suggesting a role for Mss11p in *FLO10* regulation. *FLO11* deletion led to a slight reduction in flocculation levels in the *FLO8*-restored strain (*FLO8 flo11*Δ). This effect is suppressed by multiple copies of *FLO8* or *MSS11*. The data clearly suggest that in the S288C genetic background, *FLO1* is the only relevant target gene responsible for *FLO8* or *MSS11*- dependent flocculation.

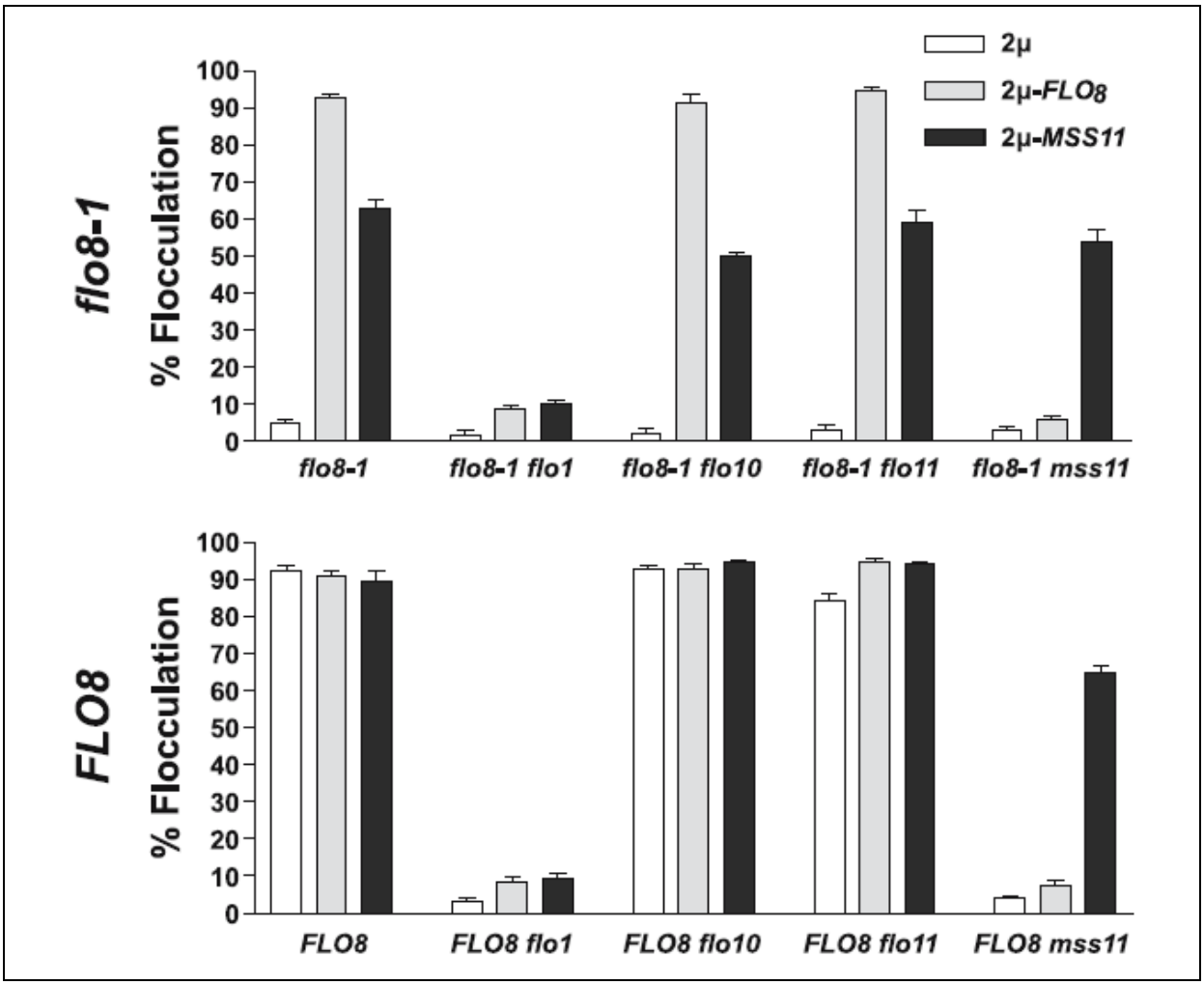

Figure 3.1 Relative flocculation levels displayed by the indicated yeast strains. The relevant genetic backgrounds are indicated. *White bars* correspond to yeast carrying vector alone (2µ) while *grey* and *black* bars represent yeast transformed with plasmids YEpLac195-FLO8 (2µ-*FLO8*) and YEpLac195-MSS11 (2µ-*MSS11*), respectively. Error bars indicate the standard error of the mean (SEM).

3.4.3 *MSS11* is critical for flocculation to occur

Upon deletion of *MSS11* in the strain carrying the wild type copy of *FLO8*, total abolishment of flocculation ability was observed (Fig. 3.1). This effect of *MSS11* deletion could only be marginally suppressed by multiple copies of *FLO8* in both the *flo8-1* and *FLO8* genetic contexts. Multiple copies of *MSS11* could, as expected, induce the flocculation phenotype in this strain. This induction was observed to be to the same degree as observed in strain *flo8-1* transformed with multiple copies of *MSS11*.

3.4.4 Role of *FLO* genes in haploid invasive growth

The ability of the same set of strains to invade agar is shown in Fig. 3.2. The S288c original strain did not present any invasive phenotypes. However, restoration of *FLO8* resulted in observable levels of invasive growth. Multiple copies of *FLO8* and *MSS11* significantly increased the level of invasive growth in both the *flo8-1* and *FLO8* strains. Deletion of *FLO1* reduced invasive growth slightly, most prominently in the *FLO8* restored strain, but no significant differences were observed in this strain when *FLO8* or *MSS11* were present in multiple copies. The deletion of the *FLO11* gene, except in the case of strain *FLO8 flo11*Δ carrying multiple copies of *MSS11*, resulted in the

abolishment of observable invasive growth in our conditions. The same was true for a strain deleted for *MSS11* confirming the data of van Dyk et al. (2005) obtained in the Σ1278b genetic background. Thus we confirm that *FLO11* is the dominant target gene for the process of invasive growth in the S288c genetic background. Restoration of genomic *FLO8* leads to an invasive phenotype, absent in S288c carrying the *flo8-1* mutation.

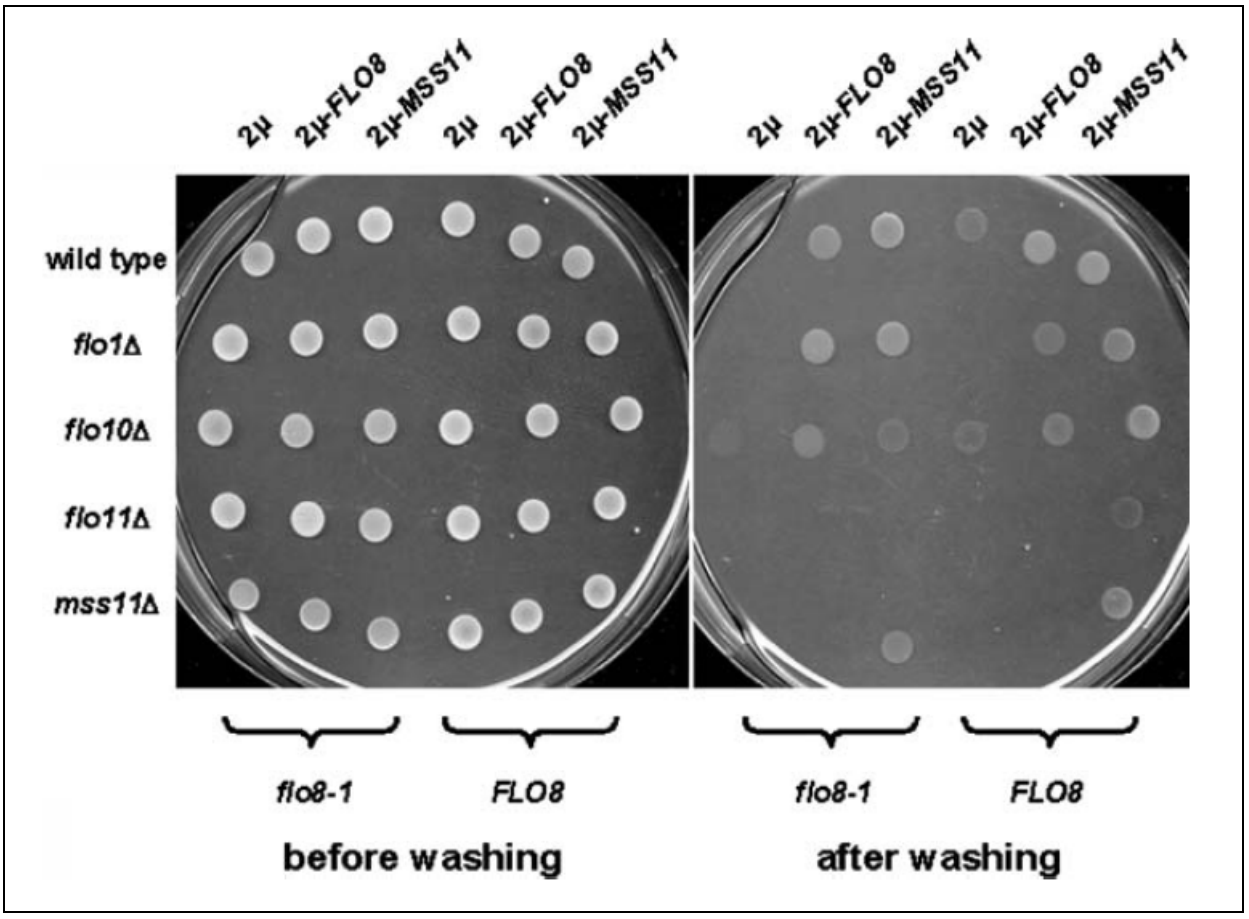

Figure 3.2 Haploid invasive growth of yeast strains. The relevant genetic backgrounds are indicated. Liquid cultures of transformants were spotted on solid SLAD media and left to grow for 6 days at 30°C. The image on the left shows total growth, whereas the right-hand image shows the same plate after the strains have been washed off the agar surface under running water.

3.4.5 Transcriptional regulation of the *FLO* genes

Figure 3.3 shows the data from a Northern blot analysis of *FLO1*, *FLO10*, and *FLO11* expression in the same set of transformants assayed for flocculation ability and invasive growth, with the exception of the *flo8-1 mss11*Δ strain which was omitted from this analysis. While the data allow assessment of the general effect of multiple copies or of non-functional *FLO8* or *MSS11* on *FLO* mRNA levels, exact fold induction or repression data could not be determined since the control strains transformed with the multiple copy plasmid without insert do not show any or very low transcription of *FLO1* and *FLO11*. Only transcription of *FLO10* could be detected in the original S288c strain, whereas upon restoration of *FLO8* significant transcription of *FLO1*, but not *FLO11*, could also be detected. In the context of the *flo8-1* mutation, *FLO1* transcription levels were significantly increased by multiple copies of *FLO8*, and, to a lesser degree, *MSS11*. While this suggests regulation of *FLO1* by Flo8p and Mss11p, multiple copies of the two genes could not further increase *FLO1* expression in the *FLO8* restored strain. This correlates with the flocculation data which showed maximum flocculation in the *FLO8*-restored strain, without further increases in

the presence of multiple copies of *FLO8* or *MSS11*. The importance of Mss11p for *FLO1* regulation is confirmed by the data obtained in the *FLO8 mss11*Δ strain, where no *FLO1* transcript can be detected, even in the presence of multiple copies of *FLO8*. This clearly demonstrates that Mss11p is essential for proper regulation of *FLO1*.

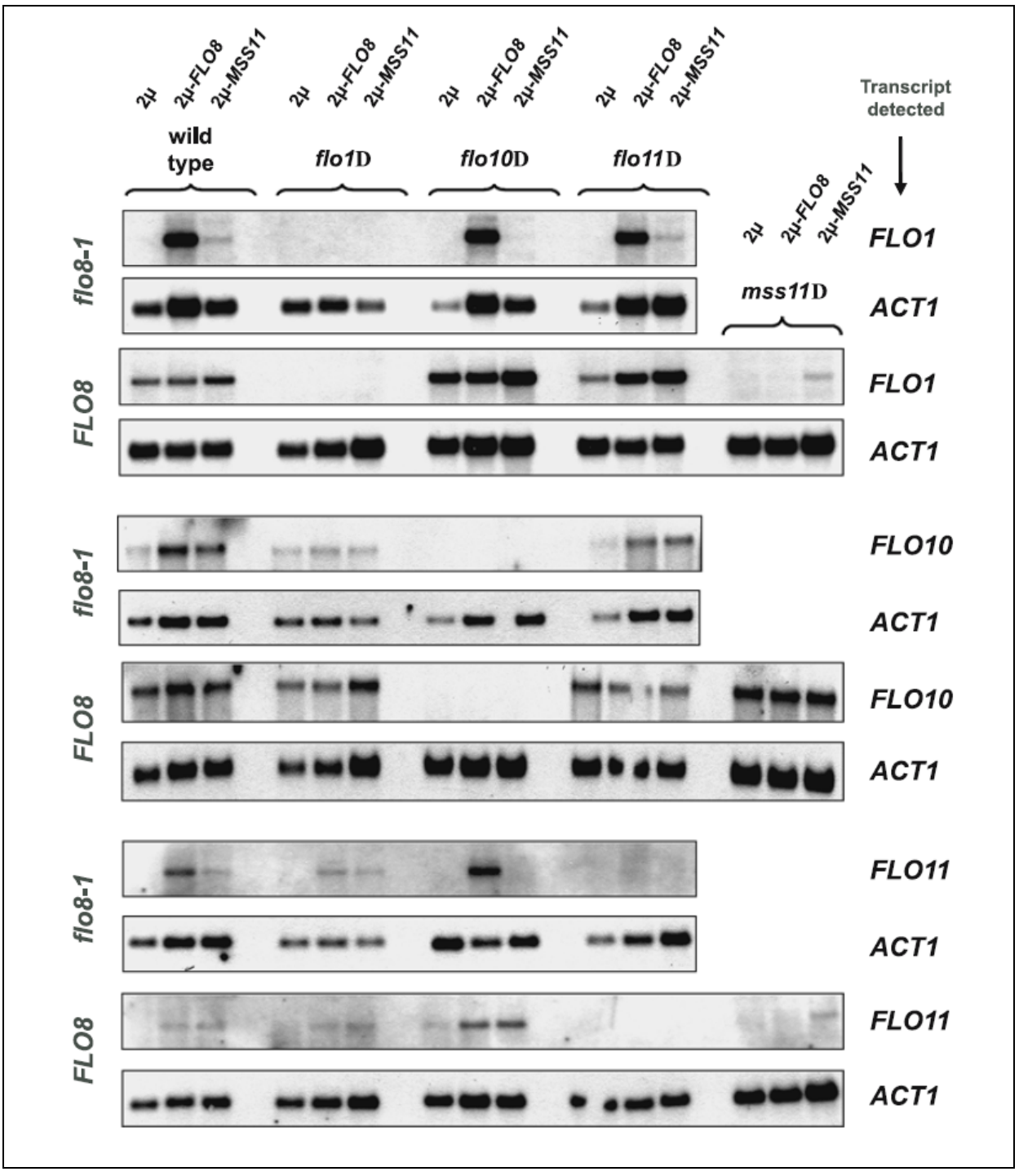

Figure 3.3 Northern blot analysis of *FLO1*, *FLO10* and *FLO11* in the various strains used in this study. The relevant genetic background and plasmids are indicated, as well as the specific transcripts probed for.

FLO10 transcription remained largely unaffected by multiple copies of *FLO8* or *MSS11* or by the deletion of these genes. The observed minor variation in the expression profile of *FLO10* could be the result of differences introduced by the experimental handling of flocculent versus non-flocculent strains. Indeed, one major experimental problem affecting the quality of the data was to ensure that all RNA-extractions were made from cells in similar physiological conditions and at similar stages of growth. However, it is clear that the growth behaviour and physiology of strongly flocculent strains will differ significantly from one of the non-flocculent strains.

The introduction of multiple copies of *FLO8* and *MSS11* resulted in significantly increased transcript levels of *FLO11* in most of the strains. The only exceptions were when 2µ-*FLO8* was transformed into the *mss11*Δ strain, which was expected since it has been described previously for the Σ1278b genetic background (van Dyk *et al.*, 2005), and the rather surprising absence of detectable *FLO11* mRNA in the *flo10*Δ strain containing multiple copies of *MSS11*. Repeated Northern blots confirmed this result, and we are currently investigating whether the presence of *FLO10* can influence the expression of other *FLO* genes.

3.4.6 Specific regions of Mss11p that are required for the induction and repression of flocculation

A set of truncated forms of *MSS11* was constructed (Gagiano *et al.*, 2003) in order to characterise possible regions within Mss11p that are essential for the induction of flocculation and invasive growth (Fig. 3.4). The domain from amino acids 35–126 contains two regions that display homology to Flo8p. Furthermore, the domains 273–340 and 604–641 contain long repeats of glutamine and asparagine residues, respectively. Truncated forms of *MSS11* were expressed from the same multiple copy expression system as mentioned before and in the *flo8-1 mss11*Δ genetic context. Clear differences of an elevated or diminished degree of flocculation were observed for the different expressed truncations in comparison to full-length *MSS11* as shown in Fig. 3.4. The data suggest that the region containing the first 148 amino acids serves an inhibitory function, due to the fact that removal of this region leads to increased flocculation. On the contrary, two regions, stretching from amino acids 148 to 340 and from amino acid 604 to the C-terminus, appear to be necessary for the proper induction of flocculation. When cells expressing the above-mentioned set of truncations were examined for the ability to grow invasively into agar, the same tendency regarding the function of internal regions of *MSS11* was observed (data not shown).

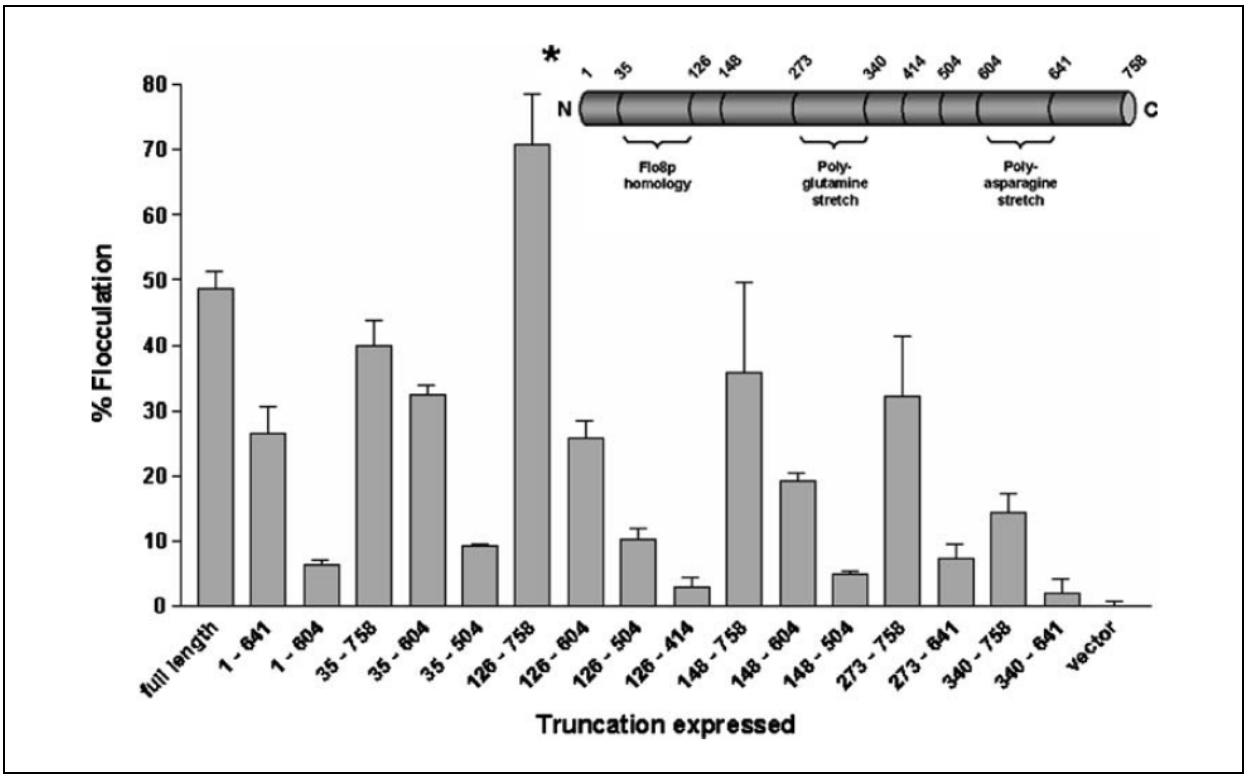

Figure 3.4 Relative flocculation levels displayed by yeast *flo8-1 mss11*Δ transformed with plasmids containing various truncated forms of the open reading frame of *MSS11* under transcriptional control of its native promoter. Mss11p is schematically represented, and numbers correspond to the amino acids that represent each truncated version. Error bars indicate the standard error of the mean (SEM).

3.5 Discussion

MSS11 has previously been shown to be involved in the regulation of starch degradation (Webber *et al.*, 1997), haploid invasive growth (Gagiano *et al.*, 1999a; Gagiano *et al.*, 1999b; van Dyk *et al.*, 2005) and the development of pseudohyphae (Gagiano *et al.*, 1999b; Lorenz and Heitman, 1998), and recent evidence suggests a role for *MSS11* in the regulation of the cell cycle (Stevenson *et al.*, 2001; Yang *et al.*, 2005) In this study we directly implicate Mss11p in the regulation of Ca^{2+}-dependent flocculation. Although it has previously been reported that multiple copies of *MSS11* lead to an increase in flocculent behaviour in comparison to wild type, as determined by visual inspection (Gagiano *et al.*, 1999b), this is the first report that employs a specific assay for Ca^{2+}-dependent flocculation to support the above-mentioned observation.

Flocculation occurs by means of many different mechanisms, of which the most prominent depends on Flo1p and the presence of Ca^{2+} ions in the growth medium, and is inhibited by the presence of mannose (Verstrepen *et al.*, 2003). Our observations show that the flocculation process that is regulated by *FLO8* and *MSS11* is dependant on Ca^{2+}-ions in the extra cellular environment, as well as the presence of the *FLO1* gene. We show that both the *FLO8* and *MSS11* genes are critical for this process to occur. Furthermore, the *flo8-1* mutation is suppressed by multiple copies of *MSS11*. The flocculation assay and Northern blot data clearly identify *FLO1* as the main target gene of Flo8p and Mss11p with regard to flocculation, and the levels of *FLO1* transcription can in all cases be broadly correlated with the levels of flocculation.

FLO1 deletion leads to a near-complete absence of flocculation. This phenotype is only slightly suppressed by the introduction of multiple copies of *FLO8* and *MSS11*, indicating that *FLO11* or other genes that may be regulated by these factors only perform a minor role in flocculation.

Previously Mss11p was shown to be a central role player in the regulation of the process of haploid invasive growth (Gagiano *et al.*, 1999a; Gagiano *et al.*, 1999b; van Dyk *et al.*, 2005), and *FLO11* was identified as the relevant target gene in this process (Lambrechts *et al.*, 1996; Lo and Dranginis, 1998). This study confirms these data, but surprisingly shows that the deletion of *FLO10* may modulate *FLO11* expression. In strains *flo8-1*, *flo8-1 flo1*Δ, *FLO8* and *FLO8 flo1*Δ over expressing either *FLO8* or *MSS11*, *FLO11* transcripts were always clearly detectable, and the invasive growth of strains carrying multiple copies of *FLO8* and *MSS11* could be correlated with the intensity of *FLO11* transcript signals. However, no *FLO11* signal could be detected in strain *flo8-1 flo10*Δ containing multiple copies of *MSS11*. This strain grew invasively into the agar medium, but indeed showed the weakest invasive phenotype of all the strains containing multiple copies of *MSS11*, with the expected exception of the *FLO11* or *MSS11* deletion strains. These data were confirmed with three independently obtained transformants and require further investigation.

Strain *FLO8 flo11*Δ transformed with multiple copies of *MSS11* displays stronger invasive growth than the isogenic strain transformed with the 2μ plasmid alone, suggesting that other Mss11p target genes that influence invasive phenotypes exist in the S288c background, thus emphasising the fact that invasive growth, like flocculation, is not dependent on only one single gene product.

The expression of truncations of *MSS11* in the same high copy number expression system used to express full-length *MSS11* led to the identification of specific regions in Mss11p that are important for the induction and repression of flocculation. The N-terminal region up to the H2 domain clearly has a repressive effect on protein function, while regions stretching from the H2 to the poly-glutamine domain and from the poly-asparagine domain to the C-terminus are required for flocculation induction. This analysis indicates that the regulation of flocculation and of *FLO1* by Mss11p involves the same domains that are required for the regulation of *FLO11* transcription.

The data suggest that Flo8p and Mss11p regulate the transcription of *FLO1* and *FLO11* through similar mechanisms. In both cases, the presence of Mss11p is required for Flo8p to be able to activate the target gene, whereas multiple copies of *MSS11* can support a level of transcription which induces the relevant phenotypes (flocculation or invasion) in the absence of functional Flo8p. Kim et al. (2004) presented evidence that Flo8p and Mss11p function cooperatively to activate the transcription of *STA1*, a gene encoding glucoamylase, whose promoter is nearly identical to the *FLO11* promoter (99% identity over 3 kb). The data suggest that a palindromic sequence, situated more than 1.7 kb upstream of the *STA1* ATG, TTTGC-n-GCAAA (n=97), is responsive to Flo8p and Mss11p, and that both factors may bind to this element. The corresponding sequence in the *FLO11* promoter differs by one nucleotide from the *STA1* sequence, TTTGC-n-CCAAA (n=97), but is still responsive to both factors, albeit with significantly reduced efficiency. Our analysis of the 3 kb of nucleotide sequence upstream of the *FLO1* ATG translation start site revealed no perfect match to the potential binding sites in the *STA1* or *FLO11* promoters. However, a closely related palindromic sequence (TTTGG-n-CCAAA; n=97) is present

in a very similar position (nucleotides -1,669 to -1,775 upstream of the ATG) in the *FLO1* promoter. Whether this sequence can act as a binding site for Mss11p and Flo8p will need to be investigated.

Besides Flo8p and Mss11p, the only other factors that have been linked to *FLO1* regulation are those involved in chromatin remodelling, including the Swi-Snf co-activator and the Tup1p-Ssn6p co-repressor complexes (Fleming and Pennings, 2001). These same complexes have also been linked to *FLO11* regulation (Conlan and Tzamarias, 2001; Kim *et al.*, 2004). While the functions of Flo1p and Flo11p can therefore be clearly separated, their regulation appears to show significant similarities. It is, however, too early to speculate on the degree of similarity since many of the factors affecting *FLO11* expression have not been assessed for their role in *FLO1* regulation. Sequence alignments between the two promoter regions also do not reveal any significant similarities (data not shown). It is indeed likely that cells can modulate expression of the two genes differentially to be able to modulate the adhesion properties of the cell wall according to specific environmental signals.

3.6 Acknowledgements

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Chapter 4

Research results II

Mss11p regulates *Saccharomyces cerevisiae* cell wall properties and Flo1p, Flo10p and Flo11p - dependent adhesion phenotypes

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Chapter 4: RESEARCH RESULTS II

Mss11p regulates *Saccharomyces cerevisiae* cell wall properties and Flo1p, Flo10p and Flo11p -dependent adhesion phenotypes

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4.1 Abstract

The outer cell wall of the yeast *Saccharomyces cerevisiae* serves as the interface with the surrounding environment and defines cell-cell and cell-surface interactions. Many of these interactions are facilitated by specific adhesins that belong to the Flo protein family. This family of mannoproteins has been implicated in phenotypes such as flocculation and substrate adhesion as well as the switch from unicellular growth to pseudohyphal growth. Genetic data strongly suggest that individual Flo proteins are primarily responsible for specific cell wall adhesion phenotypes. *FLO* gene expression is controlled by complex signalling networks that include the mitogen activated protein kinase (MAPK) and cyclic AMP responsive protein kinase A (cAMP-PKA) signalling pathways. Mss11p has been shown to be a central element of *FLO1* and *FLO11* regulation and appears to act together with the cAMP-PKA-dependent transcription factor Flo8p. However, it is unclear how the regulation of and by these transcription factors contributes to phenotypic cell wall plasticity. Here we use genome wide transcript analysis to identify genes that are directly or indirectly regulated by Mss11p. Interestingly, many of these genes encode for cell wall mannoproteins, in particular members of the *FLO*, *TIR* and *DAN* families. To assess whether these genes play a role in the phenotypic changes associated with Mss11p expression, deletion mutants of these genes were assessed in wild type and *flo11*Δ genetic backgrounds. The data show that only *FLO* genes, in particular *FLO1*/*10*/*11* appear to significantly impact on cell wall related phenotypes. Thus all adhesion-related phenotypes appear primarily dependent on the balance of *FLO* gene expression.

4.2 Introduction

Yeast cells are enclosed by a rigid but dynamic cell wall structure that forms the physical barrier to the extra-cellular environment. The cell wall is composed of interlinked polysaccharides of mainly *β*-glucan and to a lesser extent chitin. This layer acts as the supporting scaffold for a group of highly glycosylated mannoproteins which form the outer part of the cell wall and interact with the extra-cellular environment. Mannoproteins are polypeptides that are extensively modified by means of covalently bonded branched polymers of mannose residues (Lesage and Bussey, 2006). Cell-wall bound mannoproteins therefore appear to define the outer physical profile of yeast cells that is presented to the extra cellular surroundings. Various roles have been attributed to these proteins. One family of cell wall proteins, referred to as Flo proteins, has been shown to function in cell-cell as well as cell-substrate recognition and adhesion (Dranginis *et al.*, 2007), and are referred

to as yeast adhesins. Adhesin mediated phenotypes include flocculation (Guo *et al.*, 2000; Verstrepen and Klis, 2006), agar adhesion and/or invasion (Guo *et al.*, 2000; Verstrepen and Klis, 2006), the formation of pseudohyphae (Lambrechts *et al.*, 1996; Lo and Dranginis, 1996; Lo and Dranginis, 1998) or biofilms (Purevdorj-Gage *et al.*, 2007; Reynolds and Fink, 2001), the adherence to plastic surfaces (Mortensen *et al.*, 2007), colony morphology (Kuthan *et al.*, 2003) as well as "flor"/"velum" formation that occurs during the ageing of sherry (Fidalgo *et al.*, 2006; Ishigami *et al.*, 2006). Adhesin-encoding genes typically contain internal tandem repeats that may expand or contract by means of recombination (Verstrepen *et al.*, 2005). Verstrepen *et al* (2005) constructed a collection of strains that each contained different length internal repeats in the adhesin gene *FLO1.* The results show that an increase in repeat length can be directly correlated with the increase in Flo1p-dependent phenotypes such as flocculation and plastic adherence. *FLO* gene expression and Flop structure have also been correlated with changes in the general physical-chemical properties of the cell wall. In particular, the expression of individual *FLO* genes has been shown to strongly and differentially impact on cell wall hydrophobicity (Govender *et al.*, 2008).

The ability to modify cell wall mannoprotein composition forms part of the adaptive response of yeast to environmental changes. These programs are regulated by intracellular signal transduction pathways modulating the expression of mannoprotein encoding genes. Signalling pathways typically sense environmental status by means of cell membrane receptor systems that relay information by means of interacting transduction pathways to the transcription machinery (Gagiano *et al.*, 2002; Gancedo, 2001; Verstrepen and Klis, 2006) leading to appropriate cellular adaptation (Schneper *et al.*, 2004).

Information regarding the regulation of genes responsible for cell-wall dependent phenotypes however remains limited with the exception of *FLO11*, a member of the flocculation gene family (Chen and Thorner, 2007). It is the only *FLO* family member not having a sub-telomeric chromosome location and thus is not subjected to telomere silencing. However, the gene has been shown to be under epigenetic control (Halme *et al.*, 2004). Flo11p is required for and/or contributes to the formation of pseudohyphae (Lambrechts *et al.*, 1996; Lo and Dranginis, 1996; Lo and Dranginis, 1998), "flor" formation (Ishigami *et al.*, 2006), "mat" formation ("biofilm formation"; "yeast sliding motility") (Reynolds and Fink, 2001) as well as flocculation in *S. cerevisiae* var. *diastaticus* (Bayly *et al.*, 2005). While the over-expression of other adhesin encoding genes can compensate for the absence of Flo11p, as has been shown for *FIG2* and *FLO10*, whose over-expression can support pseudohyphal development in yeast carrying a *FLO11* deletion (Guo *et al.*, 2000), the biological relevance of such artificially generated phenotypes remains questionable. *FLO1* encodes a dominant flocculation factor, and appears exclusively required for processes involving cell-cell adhesion. Over-expression of the silent *FLO1* homologues *FLO5* and *FLO9* induces flocculation similar to Flo1p. While the expression of specific adhesins leads therefore to very different phenotypic outcomes, it remains unclear how adhesins are differentially regulated to facilitate specific phenotypic outcomes that would be appropriate in specific environmental conditions. Furthermore, it remains to be clarified whether other proteins that may be co-regulated with these genes are contributing to specific cell-wall related changes.

Mss11p, identified initially as a regulator of starch utilisation (Webber *et al.*, 1997) and of invasive growth (Gagiano *et al.*, 1999b) as well as flocculation (Bester *et al.*, 2006), performs a central role in the regulatory mechanisms controlling *FLO11* expression (van Dyk *et al.*, 2005).

Indeed it has been shown by several authors that various phenotypes which include colony morphology (Barrales *et al.*, 2008) and invasive growth (Gagiano *et al.*, 1999b) are Mss11p-dependent. Epistatic analysis shows that Mss11p is required for both the filamentous growth (FG) specific mitogen activated protein kinase (MAPK) pathway and the cyclic AMP responsive protein kinase A (cAMP-PKA) pathway to exert their effects on *FLO11* transcriptional regulation (van Dyk *et al.*, 2005). Mss11p is also involved in the control of flocculation through activating the transcription of *FLO1* (Bester *et al.*, 2006).

Since Mss11p appears to be a central regulator of cell wall dependent phenotypes, we here identify novel genes whose transcription is significantly altered through over-expression and/or deletion of *MSS11*. For this purpose, two commonly used and phenotypically diverging laboratory strains, S288c and Σ1278b were investigated. Σ1278b is commonly used to investigate the formation of pseudohyphae as well as the ability of yeast to grow invasively into agar containing media. S288c, on the other hand, is the most commonly employed laboratory yeast, but is unable to form pseudohyphae or to grow invasively, an inability that has been linked to a non-sense point mutation (*flo8-1*) in the transcriptional activator Flo8p (Liu *et al.*, 1996). Flo8p acts as a transcriptional activator of *FLO1* (Kobayashi *et al.*, 1999) and *FLO11* (Gagiano *et al.*, 1999a; Pan and Heitman, 1999; Rupp et *al.*, 1999), and restoration of the genomic copy of *FLO8* leads to the reestablishment of both flocculation and invasive growth in this strain (Bester *et al.*, 2006; Liu *et al.*, 1996). Most genome wide expression studies to date have used this strain, which allows better comparison of newly generated data sets with those available in the literature.

The analysis shows that many of the genes identified as being strongly affected by changed concentrations of Mss11p encode other cell wall mannoproteins, suggesting that Mss11p is indeed primarily involved in the modulation of cell wall properties. A genetic analysis of these novel targets genes however reveals that none appears to directly contribute in any significant way to phenotypes that depend on *FLO* gene expression. Indeed, only members of the Flo protein family appear to significantly impact on adhesion-related properties of yeast. We also provide evidence that the transcriptional regulation of *FLO10* and *FLO11* shares common regulators, but that the genes remain differentially regulated.

The data clearly suggest that adhesion phenotypes are entirely dependent on the balance of Flo protein expression, and that the individual role of other co-regulated cell wall proteins appears negligible.

4.3 Materials and Methods

4.3.1 Plasmids, strains, media and culture conditions

Plasmids used in this study are listed in Table 4.1. Table 4.2 lists the *Saccharomyces cerevisiae* yeast strains employed in this study. All strains are isogenic to either the S288c or Σ1278b genetic backgrounds. *FLO8* replacement in strains carrying the *flo8-1* allele was performed as described previously (Bester *et al.*, 2006). Gene deletion cassettes containing the *KanMX4* selection marker was PCR amplified using primers listed in Table 4.3 and genomic DNA from the corresponding BY4742 single deletion strains obtained from the European *Saccharomyces cerevisiae* Archive for Functional Analysis (EUROSCARF). These deletion cassettes were subsequently used to generate deletions in the BY4742 *flo8-1*Δ::*FLO8-LEU* and Σ1278b genetic backgrounds. Yeast transformations were carried out according to the lithium acetate method (Ausubel, 2004). Yeast cultures were grown at 30˚C except for the assessment of "mat" formation (see later). Yeast peptone dextrose (YPD) was used as rich media. Minimal media contained 0.67% yeast nitrogen base with pre-added ammonium sulphate but without amino acids, supplemented with 2% glucose (w/v) and the required amino acids (SCD media) according to the auxotrophic growth requirements of the relevant strain. Low nitrogen (SLAD) media was prepared similar to SCD except that 0.17% yeast nitrogen base without amino acids or ammonium sulphate was used with the addition of ammonium sulphate to a final concentration of 50μM. Selection for the *KanMX4* marker was performed on YPD media supplemented with 200 mg/L Geneticin (G418) (Sigma-Aldrich, South Africa).

Table 4.1 Plasmids used in this study

Plasmid	Genotype	Source or reference
YEpLac195	*2μ URA3*	Gietz and Sugino, 1988
YEpLac195-MSS11	*2μ URA3 MSS11*	Gagiano *et al.*, 1999b

4.3.2 Preparation of yeast total RNA

Yeast cultures were grown in 5ml SCD media from an optical density of 0,1 to between 1 and 2 as determined by spectrophotometric absorbance at a wavelength of 600 nm. Cells were harvested, washed with ice-cold H_2O and re-suspended in ice-cold AE buffer (50 mM sodium acetate, 10 mM EDTA, pH 5.0). Total RNA was extracted as described previously (Schmitt et *al.*, 1990). For transcript analysis total RNA from two independent biological repeats were analysed.

Table 4.2 *S. cerevisiae* strains used in this study.

Strain	Relevant genotype	Source or reference
BY4742	*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0	Brachmann *et al.*, 1998
BY4742 *aqy2*Δ	*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *aqy2*Δ::*KanMX4*	EUROSCARF
BY4742 *dan1*Δ	*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *dan1*Δ::*KanMX4*	EUROSCARF
BY4742 *fig1*Δ	*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *fig1*Δ::*KanMX4*	EUROSCARF
BY4742 *fig2*Δ	*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *fig2*Δ::*KanMX4*	EUROSCARF
BY4742 *flo1*Δ	*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *flo1*Δ::*KanMX4*	EUROSCARF
BY4742 *flo10*Δ	*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *flo10*Δ::*KanMX4*	EUROSCARF
BY4742 *hpf1*Δ	*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *hpf1*Δ::*KanMX4*	EUROSCARF
BY4742 *mss11*Δ	*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura*3Δ0 *mss11*Δ::*KanMX4*	EUROSCARF
BY4742 *nca3*Δ	*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *nca3*Δ::*KanMX4*	EUROSCARF
BY4742 *tir1*Δ	*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *tir1*Δ::*KanMX4*	EUROSCARF
BY4742 *tir2*Δ	*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *tir2*Δ::*KanMX4*	EUROSCARF
BY4742 *tir3*Δ	*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *tir3*Δ::*KanMX4*	EUROSCARF
BY4742 *tir4*Δ	*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *tir4*Δ::*KanMX4*	EUROSCARF
BY4742	*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *flo8-1*Δ::*FLO8-LEU2*	Bester *et al.*, 2006
BY4742 *FLO8 dan1*Δ	*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *dan1*Δ::*KanMX4* *flo8-1*Δ::*FLO8-LEU2*	This study
BY4742 *FLO8 fig1*Δ	*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *fig1*Δ::*KanMX4* *flo8-1*Δ::*FLO8-LEU2*	This study
BY4742 *FLO8 flo1*Δ	*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *flo8-1*Δ::*FLO8-LEU2* *flo1*Δ::*KanMX4*	Bester *et al.*, 2006
BY4742 *FLO8 flo10*Δ	*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *flo8-1*Δ::*FLO8-LEU2* *flo10*Δ::*KanMX4*	Bester *et al.*, 2006
BY4742 *FLO8 flo11*Δ	*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *flo8-1*Δ::*FLO8-LEU2* *flo11*Δ::*lacZ-HIS3*	Bester *et al.*, 2006
BY4742 *FLO8 tir1*Δ	*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *tir1*Δ::*KanMX4* *flo8-1*Δ::*FLO8-LEU2*	This study
BY4742 *FLO8 tir2*Δ	*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *tir2*Δ::*KanMX4* *flo8-1*Δ::*FLO8-LEU2*	This study
BY4742 *FLO8 tir3*Δ	*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *tir3*Δ::*KanMX4* *flo8-1*Δ::*FLO8-LEU2*	This study
BY4742 *FLO8 tir4*Δ	*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *tir4*Δ::*KanMX4* *flo8-1*Δ::*FLO8-LEU2*	This study
BY4742 *FLO8 aqy2*Δ *flo11*Δ::*lacZ*	*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0; *ura3*Δ0 *aqy2*Δ::*KanMX4* *flo8-1*Δ::*FLO8-LEU2 flo11*Δ::*lacZ-HIS3*	This study
BY4742 *FLO8 dan1*Δ *flo11*Δ::*lacZ*	*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0; *ura3*Δ0 *dan1*Δ::*KanMX4* *flo8-1*Δ::*FLO8-LEU2 flo11*Δ::*lacZ-HIS3*	This study
BY4742 *FLO8 fig1*Δ *flo11*Δ::*lacZ*	*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0; *ura3*Δ0 *fig1*Δ::*KanMX4* *flo8-1*Δ::*FLO8-LEU2 flo11*Δ::*lacZ-HIS3*	This study
BY4742 *FLO8 fig2*Δ *flo11*Δ::*lacZ*	*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0; *ura3*Δ0 *fig2*Δ::*KanMX4* *flo8-1*Δ::*FLO8-LEU2 flo11*Δ::*lacZ-HIS3*	This study

Strain	Relevant genotype	Source or reference
BY4742 *FLO8 flo1*Δ *flo11*Δ::*lacZ*	*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0; *ura3*Δ0 *flo1*Δ::*KanMX4 flo8-1*Δ::*FLO8-LEU2 flo11*Δ::*lacZ-HIS3*	This study
BY4742 *FLO8 flo10*Δ *flo11*Δ::*lacZ*	*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0; *ura3*Δ0 *flo10*Δ::*KanMX4 flo8-1*Δ::*FLO8-LEU2 flo11*Δ::*lacZ-HIS3*	This study
BY4742 *FLO8 flo11*Δ::*lacZ hpf1*Δ	*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0; *ura3*Δ0 *hpf1*Δ::*KanMX4 flo8-1*Δ::*FLO8-LEU2 flo11*Δ::*lacZ-HIS3*	This study
BY4742 *FLO8 flo11*Δ::*lacZ nca3*Δ	*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0; *ura3*Δ0 *nca3*Δ::*KanMX4 flo8-1*Δ::*FLO8-LEU2 flo11*Δ::*lacZ-HIS3*	This study
BY4742 *FLO8 flo11*Δ::*lacZ tir1*Δ	*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0; *ura3*Δ0 *tir1*Δ::*KanMX4 flo8-1*Δ::*FLO8-LEU2 flo11*Δ::*lacZ-HIS3*	This study
BY4742 *FLO8 flo11*Δ::*lacZ tir2*Δ	*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0; *ura3*Δ0 *tir2*Δ::*KanMX4 flo8-1*Δ::*FLO8-LEU2 flo11*Δ::*lacZ-HIS3*	This study
BY4742 *FLO8 flo11*Δ::*lacZ tir3*Δ	*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0; *ura3*Δ0 *tir3*Δ::*KanMX4 flo8-1*Δ::*FLO8-LEU2 flo11*Δ::*lacZ-HIS3*	This study
BY4742 *FLO8 flo11*Δ::*lacZ tir4*Δ	*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0; *ura3*Δ0 *tir4*Δ::*KanMX4 flo8-1*Δ::*FLO8-LEU2 flo11*Δ::*lacZ-HIS3*	This study
YHUM272	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG*	H.-U. Mösch
Σ1278b *flo8*	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG flo8*Δ::*LEU2*	van Dyk *et al.*, 2005
Σ1278b *flo11*	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG flo11*Δ::*lacZ-HIS3*	van Dyk *et al.*, 2003
Σ1278b *mss11*	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG mss11*Δ::*LEU2*	van Dyk *et al.*, 2005
Σ1278b *sfl1*	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG sfl1*Δ::*KanMX4*	van Dyk *et al.*, 2005
Σ1278b *ste12*	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG ste12*Δ::*URA3*	van Dyk *et al.*, 2005
Σ1278b *tec1*	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG tec1*Δ::*LEU2*	van Dyk *et al.*, 2005
Σ1278b *sfl1 flo8*	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG sfl1*Δ::*KanMX4 flo8*Δ::*LEU2*	van Dyk *et al.*, 2005
Σ1278b *sfl1 mss11*	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG sfl1*Δ::*KanMX4 mss11*Δ::*LEU2*	van Dyk *et al.*, 2005
Σ1278b *sfl1 ste12*	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG sfl1*Δ::*KanMX4 ste12*Δ::*URA3*	van Dyk *et al.*, 2005
Σ1278b *sfl1 tec1*	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG sfl1*Δ::*KanMX4 tec1*Δ::*LEU2*	van Dyk *et al.*, 2005
Σ1278b *aqy2*Δ *flo11*Δ::*lacZ*	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG aqy2*Δ::*KanMX4 flo11*Δ::*lacZ-HIS3*	This study
Σ1278b *dan1*Δ *flo11*Δ::*lacZ*	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG dan1*Δ::*KanMX4 flo11*Δ::*lacZ-HIS3*	This study
Σ1278b *fig1*Δ *flo11*Δ::*lacZ*	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG fig*Δ::*KanMX4 flo11*Δ::*lacZ-HIS3*	This study
Σ1278b *fig2*Δ *flo11*Δ::*lacZ*	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG fig2*Δ::*KanMX4 flo11*Δ::*lacZ-HIS3*	This study

Strain	Relevant genotype	Source or reference
Σ1278b *flo1*Δ *flo11*Δ::*lacZ*	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG flo1*Δ::*KanMX4 flo11*Δ::*lacZ-HIS3*	This study
Σ1278b *flo10*Δ *flo11*Δ::*lacZ*	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG flo10*Δ::*KanMX4 flo11*Δ::*lacZ-HIS3*	This study
Σ1278b *flo11*Δ::*lacZ hpf1*Δ	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG hpf1*Δ::*KanMX4 flo11*Δ::*lacZ-HIS3*	This study
Σ1278b *flo11*Δ::*lacZ nca3*Δ	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG nca3*Δ::*KanMX4 flo11*Δ::*lacZ-HIS3*	This study
Σ1278b *flo11*Δ::*lacZ tir1*Δ	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG tir1*Δ::*KanMX4 flo11*Δ::*lacZ-HIS3*	This study
Σ1278b *flo11*Δ::*lacZ tir2*Δ	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG tir2*Δ::*KanMX4 flo11*Δ::*lacZ-HIS3*	This study
Σ1278b *flo11*Δ::*lacZ tir3*Δ	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG tir3*Δ::*KanMX4 flo11*Δ::*lacZ-HIS3*	This study
Σ1278b *flo11*Δ::*lacZ tir4*Δ	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG tir4*Δ::*KanMX4 flo11*Δ::*lacZ-HIS3*	This study

Table 4.3 Primers used for generating gene disruption cassettes.

Primer name	Primer sequence (5'→3')
AQY2kanMX4-F	CTTTCATTAACGAATTAGAGCGC
AQY2kanMX4-R	TCAAAGCCATGTGAGCCATG
DAN1kanMX4-F	CTCCGTAGACGCTCCTCTGAA
DAN1kanMX4-R	TGTTAAGCTGTCTGCAATAAGGAAT
FIG1kanMX4-F	GATGGTTTCATGTATGTGTCAGTTAAA
FIG1kanMX4-R	GTCGCTCATCAAGGTGACAGTAA
FIG2kanMX4-F	GGTACATGGTGCTTTCCTTATGC
FIG2kanMX4-R	GCCTTGATTGCAGAGGTTGTG
FLO1-Fp-Conf	CGATAGGGAGGCATCATGGTACTACCG
FLO1-Rp-Conf	AAGAAGCGCAAGAATTATCATTTAGTCAAT
FLO10-F	AAATGGGCTCCTGCCTGAAT
FLO10-R	CTAGCTCATCCGTTGCCGCT
HPF1(YOL155c)kanMX4-F	GCAGAAGTGCCCGTAGGAGA
HPF1(YOL155c)kanMX4-R	GTGGACAATAAGTGAAATAAGTGTCCTT
NCA3kanMX4-F	GTGGCAAAGCGGACAGCT
NCA3kanMX4-R	CCGTAATGCAGAGTACACCTTGA
TIR1kanMX4-F	GAACTGCGTTTGTATGCAACTGT
TIR1kanMX4-R	GTATCCAACAGACAGTAGTGCCAAC
tir2kof	GCTGAGCATCATGTGAGTAA
tir2kor	ATGTGGGCAGGAAGACATGC
tir3kof	GCCCTAGAATGGATTGCAGA
tir3kor	TGCTCTTGCTCGAACATTCC
TIR4kanMX4-F	GCATTTCTAACAAGTAGGATAGTCCAA
TIR4kanMX4-R	GATGATGCTAGGATAGGCACCTT

4.3.3 Microarray hybridisation, data acquisition and -analysis

Probe preparation and -hybridization to Affymetrix Genechip® microarrays were performed according to Affymetrix instructions, starting with 6 µg of total RNA extracts. Results for each strain were derived from two independent culture replicates. Quality of total RNA, cDNA, cRNA and fragmented cRNA were analysed using the Agilent Bioanalyzer 2100. Probe hybridisation to GeneChip® Yeast Genome 2.0 Arrays was performed on the integrated Affymetrix GeneChip® 3000 platform. The GeneChip® Yeast Genome 2.0 Array contains probe sets to detect transcripts from both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Chip scanning and data collection was performed using the Affymetrix GeneChip® Operating Software (GCOS) version 1.4. (http://www.affymetrix.com/support/technical/manuels.affx) All arrays were scaled to a target value of 500 using the average signal from all gene features using GCOS. All arrays in the experimental set were normalised with regard to each other. Data sets are available from the gene expression omnibus (GEO) website under the series record GSE17716.

4.3.4 Quantitative real-time PCR analysis (qPCR)

DNA contamination in total RNA samples was eliminated by DNase I (Roche diagnostics) treatment. One µg total RNA was used as template for cDNA synthesis using the ImProm-II™ reverse transcription system according to the manufacturer instructions (Promega). cDNA samples were diluted 50 times with H_2O before qPCR analysis. Primers and hydrolysis probes used for detection and quantification of cDNA were designed using Primer Express ver. 3 (Applied Biosystems) and listed in Table 4.4. Detection reagents were purchased from Applied Biosystems and Kapa Biosystems (Cape Town, South Africa). qPCR runs and collection of spectral data were performed with a 7500 cycler (Applied Biosystems). Except for cDNA corresponding to transcripts of *FLO1*, *FLO5* and *FLO9*, amplicon formation was monitored with SYBR Green fluorescence with individual primer concentration of 100 nM. Specific labelled hydrolysis probes (Taqman) and primers were designed to differentiate between the cDNA species corresponding to the highly homologous *FLO1*, *FLO5* and *FLO9* genes. Hydrolysis probes were modified by the addition of a 3' minor groove binding (MGB) protein and non-fluorescent quencher, as well as the 5' attachment of fluorescent dyes as described before for the *FLO1* and *FLO5* specific hydrolysis probes and primer sets (Govender *et al.*, 2008). The hydrolysis probe (Applied Biosystems) and primer set used for *FLO9* cDNA detection are listed in Table 4. Hydrolysis probe and primer concentrations were kept at 250 nM and 900 nM respectively for reactions containing probe primer combinations. Cycling conditions during qPCR was as follows: 50˚C for 2 minutes, 95˚C for 10 minutes, 40 cycles of 95˚C for 15 seconds followed by 60˚C for 1 minute. When using SYBR Green for amplicon quantification a dissociation curve analysis was included following the cycling programme to verify amplicon authenticity. Preliminary data analyses were performed with Signal Detection Software (SDS) ver 1.3.1. (Applied Biosystems). Individual qPCR reaction runs were performed at least in duplicate. The relative expression value for each sample was defined as $2^{-Ct}_{(target)}$ where $Ct_{(target)}$ represents the cycle number at which a sample reaches a predetermined threshold signal value for the specific target gene. Relative expression data was normalized to the relative expression value of the reference gene *PDA1* (Wenzel *et al.*, 1995) in each respective sample thus giving normalized relative expression for a target gene as $2^{-Ct}_{(target)}/2^{-Ct}_{(PDA1)}$.

4.3.5 Data analysis

Statistical analyses of data sets were performed by means of the standard T-test. Fold change is defined from sample "A" to "B" as: (B-A)/(minimum value from either A or B) + 1 (if B>A) or -1 (if A>B). In order to identify groupings in gene targets based on gene classification the online application FunSpec was used (Robinson *et al.*, 2002). Cluster analysis of gene targets was performed and visualised with Cluster ver 3.0 and Treeview ver 1.6 respectively (Eisen *et al.*, 1998). In order to identify groupings in gene targets based on gene ontology (GO) classification the online application FunSpec was used (Robinson *et al.*, 2002). The following sources were used for GO classification with a p-value of ≤ 0.001 for the identification of categories: MIPS functional classification, GO molecular function, GO biological process and GO cellular component. Furthermore the following categories were not considered because their descriptions were viewed as of a too general nature (source in brackets): unclassified proteins (MIPS Functional Classification), molecular function (GO Molecular Function), biological process (GO Biological Process) and cellular component (GO Cellular Component). In the case of enriched groups with similar function and containing near identical gene groups, only one representative group was chosen. It should be noted that categorised groups often contain overlapping genes.

Table 4.4 Primers and hydrolysis probes used for qPCR analysis.

Primer/probe name	Primer/probe sequence (5'→3')	Modifications
FLO9-F (TaqmanMGB)	TGTACAATAAAAGCCCCAAAAATG	none
FLO9-R (TaqmanMGB)	GCAATGTGACGATGGCTAGTAGTAA	none
FLO9-probe	CTCTGGCACATTATT	NED dye 5' label , 3' Minor Groove Binder/ Non-Fluorescent Quencher
AQY2-F-(rt-PCR)	GGACCCGACCGGTGTTG	none
AQY2-R-(rt-PCR)	TTAAAACGCGAATGCTTCGTT	none
DAN1-F-(rt-PCR)	GCTTTCCAGGCTTTGCATAAGA	none
DAN1-R-(rt-PCR)	TCGCCACCGGCAAAAA	none
DAN4-F-(rt-PCR)	GCCACTACATCGAACAATGCA	none
DAN4-R-(rt-PCR)	GGCACCCGCAGAGCAA	none
FIG1-F-(rt-PCR)	TCCCTTATACAGAGACTTGGAAATTCA	none
FIG1-R-(rt-PCR)	AATTGGGCTAACTTCAAAATGTTCA	none
FIG2-F-(rt-PCR)	CTTCTGATACTTTTTCTTCATACTCTGATATCT	none
FIG2-R-(rt-PCR)	TGTCCTATGAGGTTGTGCAGTTG	none
FLO11-F-(QRT-PCR)	CCTCCGAAGGAACTAGCTGTAATT	none
FLO11-R-(QRT-PCR)	AGTCACATCCAAAGTATACTGCATGAT	none
HPF1(YIL169c)-F-(rt-PCR)	CTAAGGACATACACTACTGCCACTGGT	none
HPF1(YIL169c)-R-(rt-PCR)	ACTAGTTGCGTGACGGTTGAAGTAG	none
HPF1(YOL155c)-F-(rt-PCR)	CGGTTCATCTTCTGCCACAGA	none
HPF1(YOL155c)-R-(rt-PCR)	GTTCATCTTCTGCCACAGAATCAG	none
NCA3-F-(rt-PCR)	TGGTGGATGGGCCTCTGT	none
NCA3-R-(rt-PCR)	GACATTCCAGGTTCACATGCA	none
PDA1-F-QRT-PCR	GGAATTTGCCCGTCGTGTT	none
PDA1-R-QRT-PCR	GCGGCGGTACCCATACC	none
TIR1-F-(rt-PCR)	TCCAAGCTACCAAGGCTGTTTC	none
TIR1-R-(rt-PCR)	ACCCATACCAACAAAGGCCTTA	none
TIR2-F-(QRT-PCR)	CTCCGCCATTTCTCAAATCAGT	none
TIR2-R-(QRT-PCR)	CCATGACACCAGCACCCATA	none
TIR3-F-(QRT-PCR)	TTTGACGCTATTTTGGCTGATG	none
TIR3-R-(QRT-PCR)	TCTGGATTATTCATTGCCAAGGA	none
TIR4-F-(rt-PCR)	TGCCGACTACATCACCCTATCC	none
TIR4-R-(rt-PCR)	GGCATTTGGTCCAAGGAAAA	none

4.3.6 Internal tandem repeat analysis

Primers (Table 4.5) binding sequences flanking the internal repeat region of *FLO1* were used to determine repeat length as described before (Verstrepen *et al.*, 2005).

Table 4.5 Primers used in *FLO* gene internal tandem repeat analysis.

Primer	Sequence (5'→3')	Reference
FLO1-reps-F	CTAAGTCAATCTAACTGTACTGTCCCTGA	Verstrepen *et al.*, 2005
FLO1-reps-R	GATAGAGCTGGTGATTTGTCCTGAA	Verstrepen *et al.*, 2005

4.3.7 Flocculation assay

Ca^{2+}-dependent flocculation of yeast cultures was determined by a method based on the Helm's sedimentation test. EDTA (pH 8.0) was added to yeast cultures to a final concentration of 50mM and vigorously vortexed until cells were in homogeneous suspension as determined by visual inspection. The spectrophotometric absorbance of the sample was determined at a wavelength of 600 nm (measurement A), whereupon 1 ml cell culture was transferred to a micro centrifuge tube, washed with H_2O and re-suspended in 1 ml aqueous solution of $CaCl_2$ (10mM). Samples were vortex-mixed vigorously for 10 s and left stationary on the bench for 1 min, whereupon the spectrophotometric absorbance of 100 µl removed from just below the meniscus was determined at a wavelength of 600 nm (measurement B). Percentage Ca^{2+}-dependent flocculation was defined as [(A-B)/A] x 100. All measurements were done at least in triplicate.

4.3.8 Hydrophobicity assay

Yeast hydrophobicity was measured by assaying the partitioning of yeast cells between an aqueous and hydrophobic hydrocarbon phase following vigorous mixing (Rosenberg, 2006). Yeast cultures were de-flocculated by EDTA addition after which the spectrophotometric absorbance was determined at a wavelength of 600 nm (measurement A) as described in the flocculation protocol. 1 ml of yeast culture was transferred to a micro centrifuge tube, washed and re-suspended in phosphate, urea, magnesium (PUM) buffer consisting of 127,45 mM K_2HPO_4, 53,35 mM KH_2PO_4, 30mM urea and 0,8 mM $MgSO_4$ (Hinchcliffe *et al.*, 1985). Finally 100 µl *p*-Xylene (1,4-Dimethylbenzene) was added. Samples were vortex-mixed vigorously for 30 s and left to stand for 15 min, whereupon the spectrophotometric absorbance of the aqueous phase was determined at a wavelength of 600 nm (measurement B). The hydrophobicity index (HI) was defined as 1 - (B/A), where higher values reflect a yeast population of an increased hydrophobic nature.

4.3.9 Invasive growth determination

In order to investigate the ability of yeast cultures to grow invasively into agar-containing medium 10µl of yeast suspensions grown overnight to stationary phase were deposited on 2% agar plates with various media composition as indicated for each specific experiment. Flocs in flocculating cultures were disrupted by repetitive pipetting and a sample was immediately removed of which the OD_{600} was determined similarly to as described in the flocculation determination protocol. Cultures were adjusted so as to contain the same concentration of cells, washed with water and spotted on plates. Spotted macro colonies from flocculating cultures have a granular appearance due to cells that re-form flocs on the plate after spotting. After allowing for yeast growth at 30˚C for times depending on specific experiments as indicated, cells were washed off the agar surface by vigorous rubbing with a gloved finger under running water, revealing only those cells that have grown into the medium.

4.3.10 Mat formation

The ability of yeast strains to form spreading growth mats (also referred to as "biofilm" formation or "sliding motility") on plates was determined as described previously (Reynolds and Fink, 2001). 10 µl of a yeast suspension grown overnight in liquid media as described above was deposited in the centre of an YPD plate containing 0,3 % w/v agar and incubated at room temperature (20-25°C). "Mat" formation was monitored by measuring the diameter of growth of at least three independent biological repeats. Measurements were always taken using the same reference point on the plate.

4.3.11 Polystyrene adherence assay

To measure the ability of yeast cells to adhere to polystyrene plastic surfaces, liquid cultures (100µl) was incubated at room temperature in flat bottom polystyrene 96 well plates (Sterilin). After incubation (~2 hours) an equal volume of a solution of 1% (w/v) crystal violet was added to the cells followed by further incubation for 15 min at room temperature. The wells were repeatedly washed with H_20 and assessed for stained cells remaining attached inside the wells.

4.4 Results

4.4.1 Transcription profiles of strains with modified *MSS11* expression levels

Σ1278b and S288C wild type strains transformed by 2µ-*MSS11,* as well as the corresponding wild type strains and the Σ1278b *MSS11* deletion strain were used to evaluate genome wide expression levels by DNA micro-array analysis. The deletion strain and the two wild type strains were transformed with the same multicopy shuttle vector without the *MSS11* gene to compensate for any effects the vector itself might have on gene regulation. All transformants were grown in minimal media to the mid-exponential growth phase before harvesting total RNA for transcriptome analysis. Wild type yeast strains were used as reference to determine fold change in gene expression. Thus the effect of *MSS11* deletion in Σ1278b was determined by the analysis of change in gene expression when comparing strain Σ1278b (+2µ) with Σ1278b *mss11*Δ (+2µ). In the same way the effect of *MSS11* over-expression was determined by the comparison of transformants Σ1278b (+2µ) with Σ1278b (+2µ-*MSS11*), and S288c (+2µ) with S288c (+2µ-*MSS11*).

The analysis confirmed that *MSS11* was expressed as expected in the different strains. No *MSS11* transcript could be detected in Σ1278b carrying the deletion cassette *mss11*Δ::*LEU2* (data not shown), while the transformants with 2µ-*MSS11* displayed an 8 and 26 fold up-regulation in S288c and Σ1278b respectively in comparison to strains transformed with only 2µ. Previous studies had already shown that this system leads to Mss11p related phenotype induction and also to the up-regulation of Mss11p specific target genes such as *FLO1* and *FLO11* (Bester *et al.*, 2006; Gagiano *et al.*, 2003; van Dyk *et al.*, 2005).

Whole genome analysis shows that altered *MSS11* expression affects gene expression to varying degrees in the different strains tested. Determining the total amount of genes affected in each strain by the use of a range of fold change cut-off values (1,5 to 3,0) provides us with a broad indication of the effects of varying *MSS11* mRNA levels (Figure 4.1 A). The wide range of cut-off values was used in order to eliminate, as far as possible, the effect of noise (transcriptional-, experimental- and data processing related) on such observations. Clearly visible from this

representation is the greater impact that *MSS11* over-expression has on the amount of genes affected compared with the corresponding deletion strain. Integrating data from across all data points show that this difference is on average 4,7 fold (Standard deviation (STDEV): 0,57). Furthermore *MSS11* over-expression appears to have a stronger impact in S288C, since for each cut-off value, on average of 2,2 times (STDEV: 0.34) more genes where affected in this strain compared to the Σ1278b over-expression strain. This might reflect the absence of a functional Flo8p in this strain.

The same approach was used to assess the overall impact of Mss11p over-expression and deletion in each individual strain. The data are shown in Figure 4.1. This analysis indicates that *MSS11* deletion predominantly causes the down-regulation of genes, while *MSS11* over-expression predominantly leads to gene up-regulation in both tested strains.

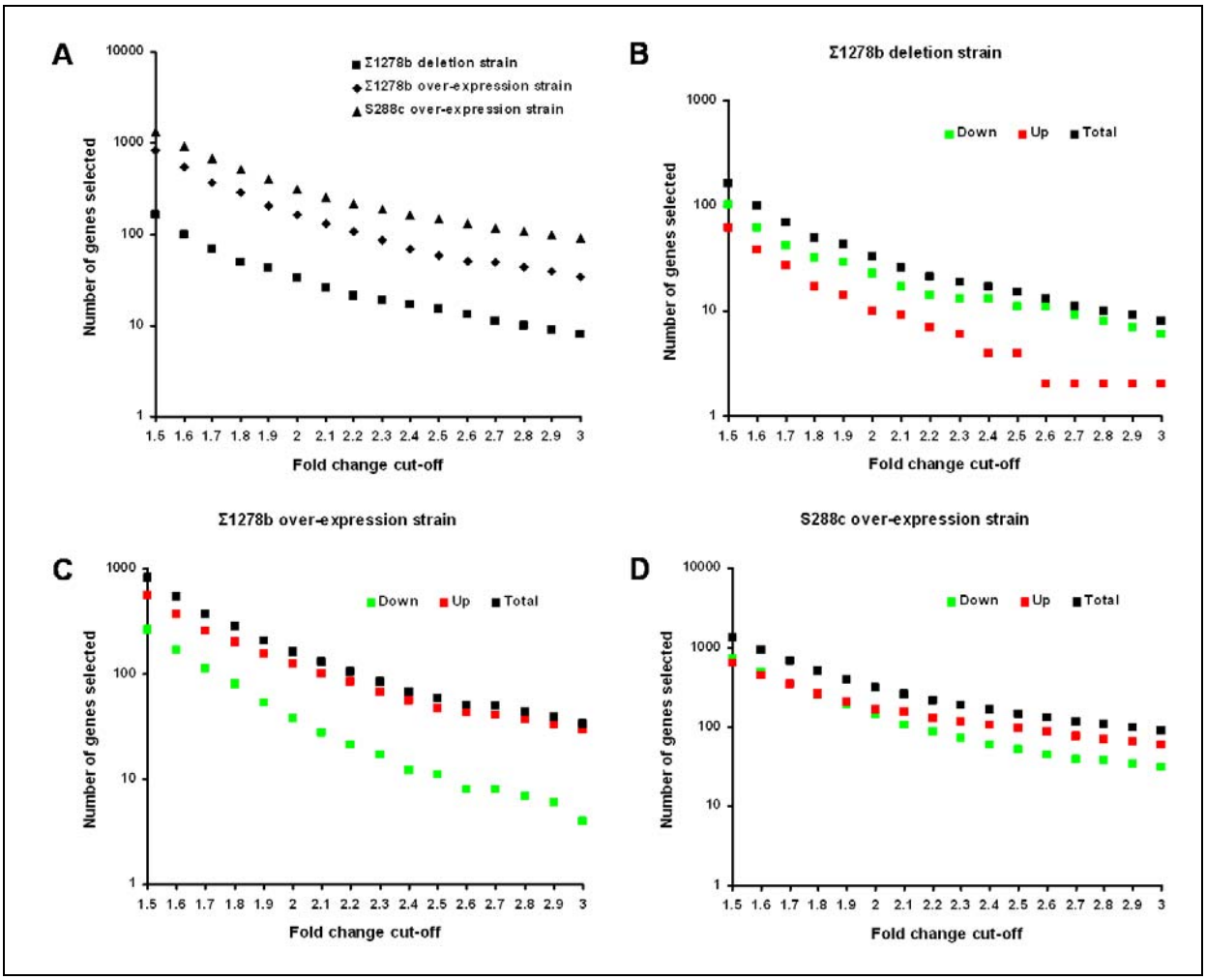

Figure 4.1 The amount of genes affected by *MSS11* over-expression and deletion represented as a function of fold-change cut-off values (ranging from 1,5 to 3,0). The figure shows the combined number of genes (up and down regulated) in all three pair wise comparisons (A), as well as the breakdown in terms of individual strains, with Mss11 deletion in Σ1278b shown in (B), *MSS11* over-expression in Σ1278b in (C), and *MSS11* over-expression in S288c in (D).

Plotting whole genome expression data obtained from *MSS11* over-expression in Σ1278b and S288c reveals the degree of similarity and difference of *MSS11*-dependent gene regulation in the two strains (Figure 4.2). The majority of genes that display a fold change of greater than five falls into the quadrant of the plot representing genes up-regulated in both Σ1278b and S288c. Using a fold cut-off of ≥2 and ≥1.5 for the S288c and Σ1278b data sets respectively, gene ontology (GO) enrichment was performed on the genes that fall in each respective quadrant of the plot in Figure 4.2 (Table 4.6). Enrichment analysis of communally up-regulated genes shows that they are frequently cell wall related or membrane associated genes (GO categories: cell-cell adhesion; stress response; transporter activity; fungal-type cell wall). These genes encode for proteins that function in the transport of sugars such as the *HXT* gene family (*HXT2/4/5/15*)) (Ozcan and Johnston, 1999), as well as others that are activated upon cold stress and hypoxia such as members from the Srp1p/Tip1p family (*DAN1,TIR1/2/3/4*) (Abramova *et al.*, 2001a; Abramova *et al.*, 2001b; Sertil *et al.*, 1997; Tai *et al.*, 2005; ter Linde *et al.*, 1999) and genes from the *FLO* gene family (*FLO1/9/10/11*). Genes down-regulated in Σ1278b and up-regulated in S288c show enrichment for the transport of a variety of amino acids (Bajmoczi *et al.*, 1998; Kosugi *et al.*, 2001; Regenberg *et al.*, 1999; Schmidt *et al.*, 1994) and the metabolism of glycine, methionine and serine (Kastanos *et al.*, 1997; McNeil *et al.*, 1994; Ullrich *et al.*, 2001) (GO categories: biosynthesis of serine; amino acid transmembrane transporter activity; methionine metabolic process). Enrichment of genes up-regulated in Σ1278b and down-regulated in S288c suggests a role in metabolism, specifically that of allantoin (Yoo and Cooper, 1991), carnitine (Swiegers *et al.*, 2001; van Roermund *et al.*, 1999) and malate (Fernandez *et al.*, 1993) (GO categories: catabolism of nitrogenous compounds; carnitine metabolic process). Furthermore genes from this quadrant enriched for processes involved with yeast mating (Bardwell, 2005; Chen *et al.*, 2007) (GO categories: response to pheromone; sexual reproduction).

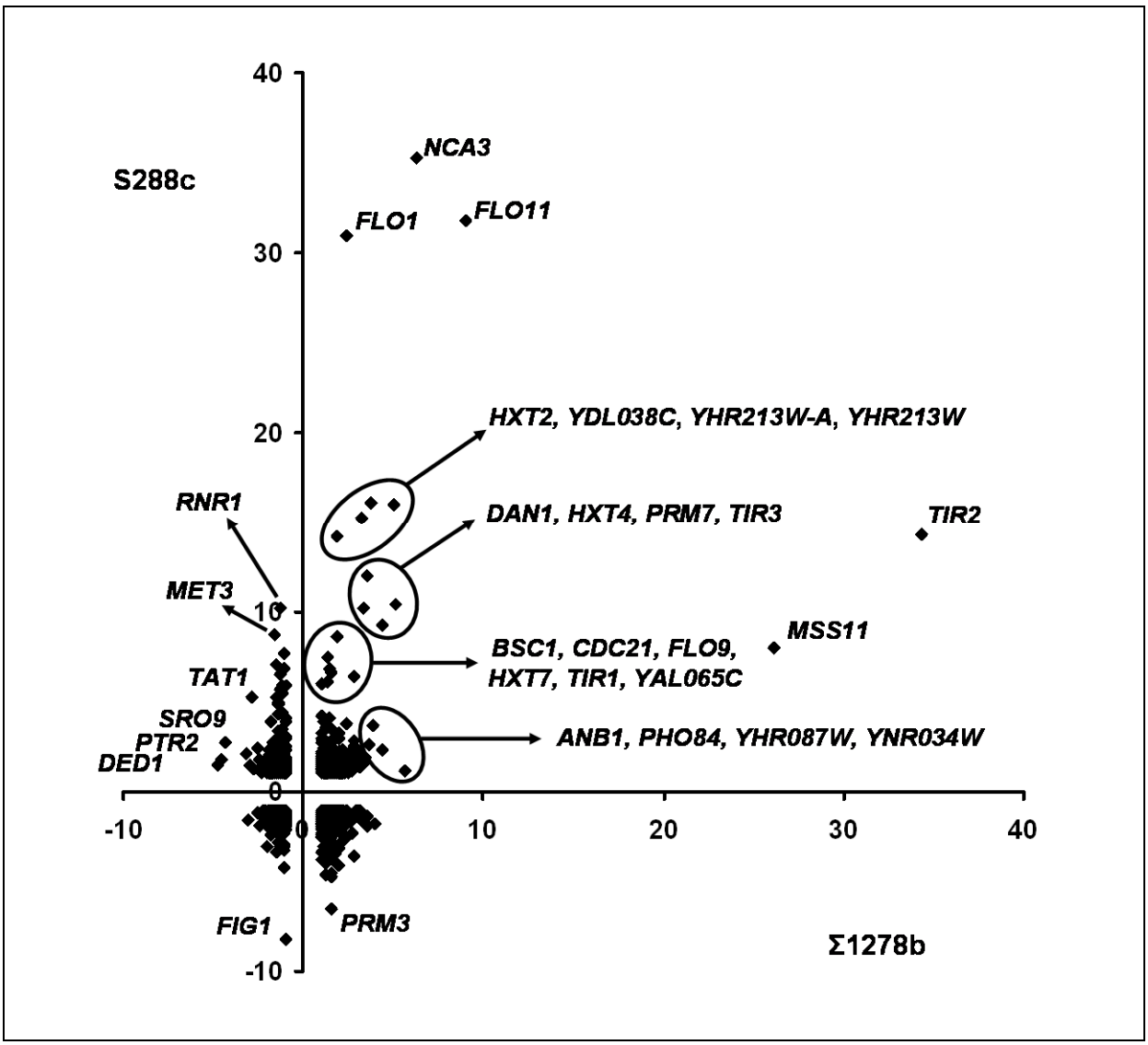

Figure 4.2 Fold change of the yeast transcriptome in response to *MSS11* over-expression as observed for the strains S288c and Σ1278b. Wild type gene expression levels were used as reference condition. Data for strain Σ1278b is shown on the x-axis and that for S288c on the y-axis. The up-regulation of genes is indicated by positive values and down-regulation by negative values respectively. Some data points are annotated with their respective gene or open reading frame names.

Table 4.6 GO enrichment of genes transcriptionally affected by *MSS11* over-expression in both the strains Σ1278b and S288c. Considered only for this analysis were genes that showed ≥ 2 fold change in S288c and ≥ 1,5 fold change in Σ1278b respectively. Preceding enrichment genes were categorised into four groups according to their respective up (↑) or down (↓) regulation in both strains as indicated. For each subgroup the amount of genes preceding GO enrichment is indicated in brackets.

Category	Code	Genes	p-value	Source
Σ1278b↑/S288c↑ (41)				
cell-cell adhesion	[34.07.01]	*FLO9 FLO1 FLO11 FLO10*	2.62×10^{-7}	1
stress response	[32.01]	*YRO2 TIR1 MGA1 TIR3 YGP1 TIR4 TIR2 HSP82*	5.30×10^{-6}	1
transporter activity	[GO:0005215]	*SUL1 HXT15 STL1 HXT4 HXT5 PHO84 HXT2*	1.32×10^{-5}	2
fungal-type cell wall	[GO:0009277]	*FLO9 FLO1 TIR1 TIR3 DAN1 FLO10 YGP1 TIR4 TIR2*	3.19×10^{-8}	4
Σ1278b↓/S288c↓ (5)		(No significant enrichment)		
Σ1278b↑/S288c↓ (28)				
catabolism of nitrogenous compounds	[01.02.02.09]	*DAL7 DAL3*	1.72×10^{-4}	1
response to pheromone	[GO:0019236]	*STE2 AGA2 PRM8 PRM5 PRM6 PRM1 PRM4 PRM3*	1.41×10^{-11}	3
sexual reproduction	[GO:0019953]	*PCL2 STE2 AGA2 PRM6 FUS2 PRM1*	3.15×10^{-10}	3
carnitine metabolic process	[GO:0009437]	*YAT2 CAT2*	5.19×10^{-5}	3
Σ1278b↓/S288c↑ (21)				
biosynthesis of serine	[01.01.09.02.01]	*SER2 SHM2*	2.0×10^{-4}	1
amino acid transmembrane transporter activity	[GO:0015171]	*TAT1 BAP3 MUP1*	1.56×10^{-4}	2
methionine metabolic process	[GO:0006555]	*MET3 MET14*	8.57×10^{-4}	3

1 - MIPS Functional Classification; 2 - GO Molecular Function; 3 - GO Biological Process; 4 - GO Cellular Component

We further plotted whole genome transcriptome data from Σ1278b either deleted in, or over-expressing *MSS11* (Figure 4.3). The most regulated genes in terms of magnitude fold difference correspond to those that are up-regulated in response to *MSS11* over-expression, and down-regulated in response to *MSS11* deletion. With the exception of *HMS1*, all the most significantly affected genes encode for cell wall or membrane proteins and include the adhesin encoding *FLO11*, the Srp1p/Tip1p family members *TIR2/3*, and *HXT2*, encoding a high affinity glucose transporter (Ozcan and Johnston, 1999). *HMS1* encodes a possible transcription factor that causes filamentation when over-expressed (Lorenz and Heitman, 1998). Using a cut-off of 1,5 fold all genes regulated similar to the above mentioned genes enriched exclusively for various stress related categories (GO categories: stress response; oxidative stress response, osmotic and salt response, response to stress) (Table 4.7). Genes up-regulated in response to *MSS11* deletion, as well as down-regulated in response to *MSS11* over-expression, enriched for proteins located in the plasma membrane (GO category: plasma membrane). Two of the genes falling in this category, *AQY2* and *PTR2*, were regulated to a significant degree and encode for plasma membrane transporters of water and di-/tri-peptides respectively (Carbrey *et al*., 2001; Island *et al*., 1991). Analysis of the genes that display up-regulation in both the over-expression and deletion strains did not enrich for any GO categories. The same was observed for genes up-regulated in both strains.

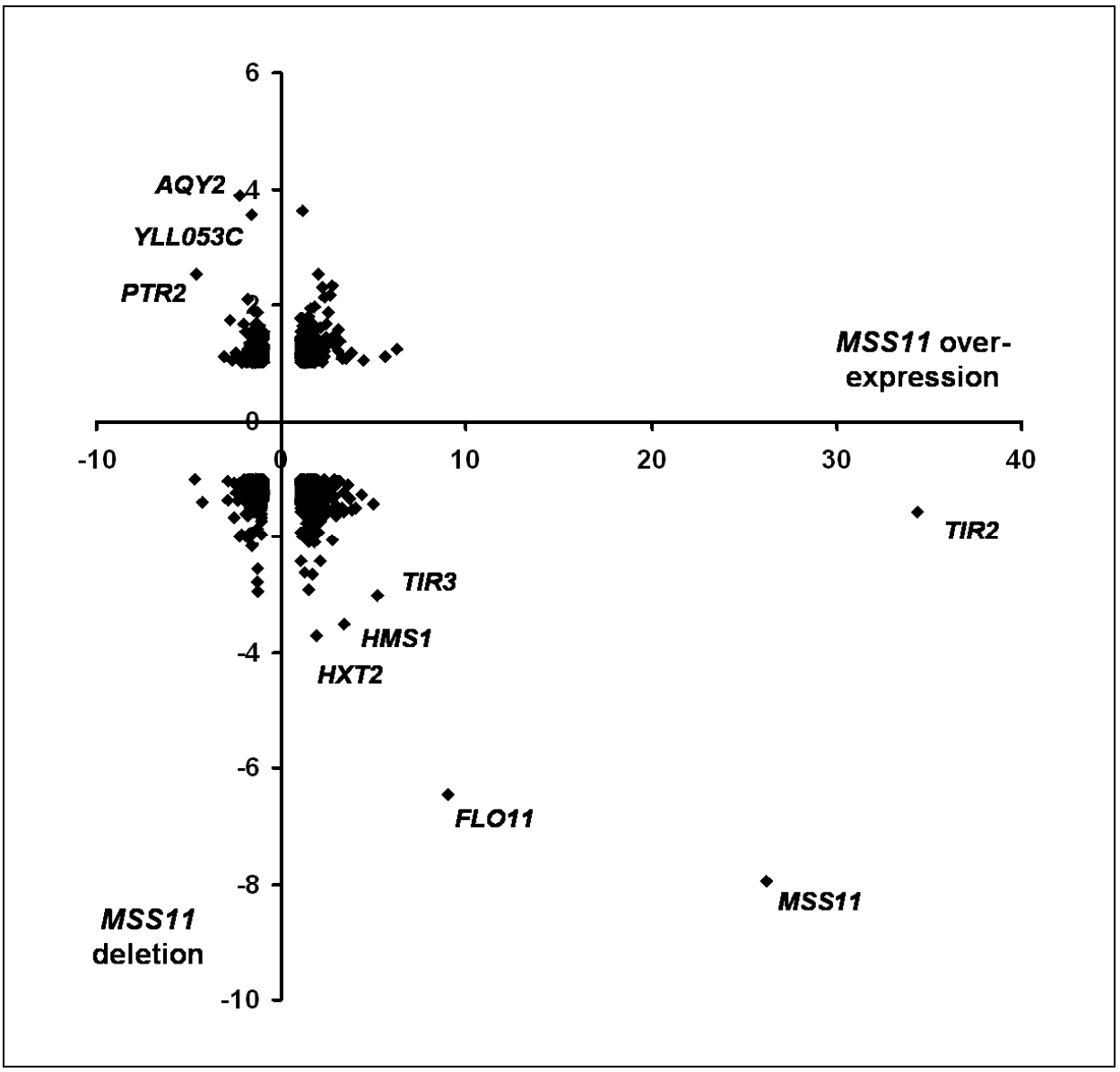

Figure 4.3 Fold change of the yeast transcriptome in response to *MSS11* deletion and over-expression in Σ1278b with wild type gene expression levels as reference condition. The up-regulation of genes is indicated by positive values and down-regulation by negative values respectively. Some data points are annotated with their respective gene or open reading frame names. Note that the maximum value of the y axis (fold change in response to *MSS11* deletion) is four times less than that of the x axis (fold change in response to *MSS11* over-expression).

Table 4.7 GO enrichment of genes transcriptionally affected by *MSS11* deletion (*mss11*Δ) and over-expression (*MSS11* over-exp) in the strain Σ1278b. Considered only for this analysis were genes that showed ≥ 1.5 fold change. Preceding enrichment genes were categorised into four groups according to their respective up (↑) or down (↓) regulation in both strains as indicated. For each subgroup the amount of genes preceding GO enrichment is indicated in brackets.

Category	Code	Genes	p-value	Source
***MSS11* over-exp↑/*mss11*Δ↑ (19)**		(No significant enrichment)		
***MSS11* over-exp↓/*mss11*Δ↓ (11)**		(No significant enrichment)		
cellular iron ion homeostasis	[GO:0006879]	*PCA1 ARN2 TIS11*	8.15×10^{-5}	3
***MSS11* over-exp↑/*mss11*Δ↓ (54)**				
stress response	[32.01]	*TIR3 XBP1 SDP1 YJL144W TSL1 ALD3 TIR2 ATH1*	4.40×10^{-6}	1
oxidative stress response	[32.01.01]	*FRT2 HSP12 SRX1 GAD1 GRE1*	7.73×10^{-5}	1
osmotic and salt stress response	[32.01.03]	*FRT2 HSP12 SIP18 CIN5 GRE1*	1.08×10^{-4}	1
response to stress	[GO:0006950]	*FRT2 HSP12 TIR3 XBP1 TSL1 ALD3 TIR2 GRE1 ATH1*	2.49×10^{-5}	3
***MSS11* over-exp↓/*mss11*Δ↑ (10)**				
plasma membrane	[GO:0005886]	*FUI1 TAT1 PTR2 AQY2 YLL053C FET4*	1.87×10^{-5}	4

1 - MIPS Functional Classification; 2 - GO Molecular Function; 3 - GO Biological Process; 4 - GO Cellular Component

4.4.2 qPCR analysis of selected gene targets

Targets of *MSS11* over-expression in both strains, and the genes inversely affected by *MSS11* deletion and over-expression separately enrich for genes encoding cell wall proteins important for adhesion, stress protection and transport. The data suggest a broader role for Mss11 in the regulation of cell wall protein composition and cell wall properties. To further assess such a role, all genes falling in this category and significantly affected by Mss11p concentration were further investigated for phenotypic impacts (see Results section 4.4.4). The genes selected for further analysis were *AQY2, DAN1, FIG1, FLO1, FLO9, FLO11, TIR1, TIR2,* and *TIR3*. To this selection, the related cell wall genes *DAN4, FIG2, FLO5, HPF1, TIR4, YIL169c* were added. The only non-cell wall encoding gene included was *NCA3* which encodes for a regulator of mitochondrial protein machinery expression, more specifically the subunits 6 (Atp6p) and 8 (Atp8p) of the Fo-F1 ATP synthase (Pelissier *et al.*, 1995). *NCA3* was included because it displayed a very significant induction in the over-expression strains (7- and 45 fold in the Σ1278b and S288c backgrounds respectively). All these genes are listed in Table 4.8 with brief descriptions of their reported cellular function.

Table 4.8 Genes (or gene families) for whom change in transcriptional regulation were confirmed by qPCR. Brief descriptions on these gene(s) are given and the corresponding source material.

Gene name(s)	Brief description	References
AQY2	encodes a plasma membrane protein forming a channel mediating and controlling bidirectional water transport	Carbrey *et al*., 2001
DAN1/4; and *TIR1/2/3/4*	Srp1p/Tip1p related; induced under anaerobic conditions and repressed in the presence of oxygen, although some members are up-regulated in response to aerobic cold stress	Abramova *et al*., 2001a; Abramova *et al*., 2001b; Sertil *et al*., 1997; Tai *et al*., 2005; ter Linde *et al*., 1999
FIG1	Involved with yeast mating; membrane protein required for efficient cell-cell fusion during mating	Aquilar *et al*., 2007; Erdman *et al*., 1998; Muller et al., 2003
FIG2	Involved with yeast mating; glycosylphosphatidylinositol (GPI) anchored protein necessary for maintaining cellular integrity during mating	Erdman *et al*., 1998; Van der Vaart *et al*., 1997; Zhang *et al*., 2002
FLO1/5/9/10/11	Cell wall proteins involved in mating unrelated cell-cell and cell-surface adhesion interactions	Dranginis *et al*., 2007; Verstrepen and Klis, 2006
HPF1	Cell wall protein reported to inhibit the formation of protein haze in white wine	Brown *et al*., 2007
NCA3	regulator of mitochondrial protein machinery expression	Pelissier *et al*., 1995
YIL169c	High sequence similarity to *HPF1*	This study; data not shown

qPCR was used to confirm the micro-array data set. The results broadly confirm the findings of the micro-array analysis. (Figure 4.4; selected data in Figure 4.5). Transcription tendencies for most gene targets follow the same profiles with some differences in the magnitude of fold change. However some discrepancies were observed for a few of the selected genes.

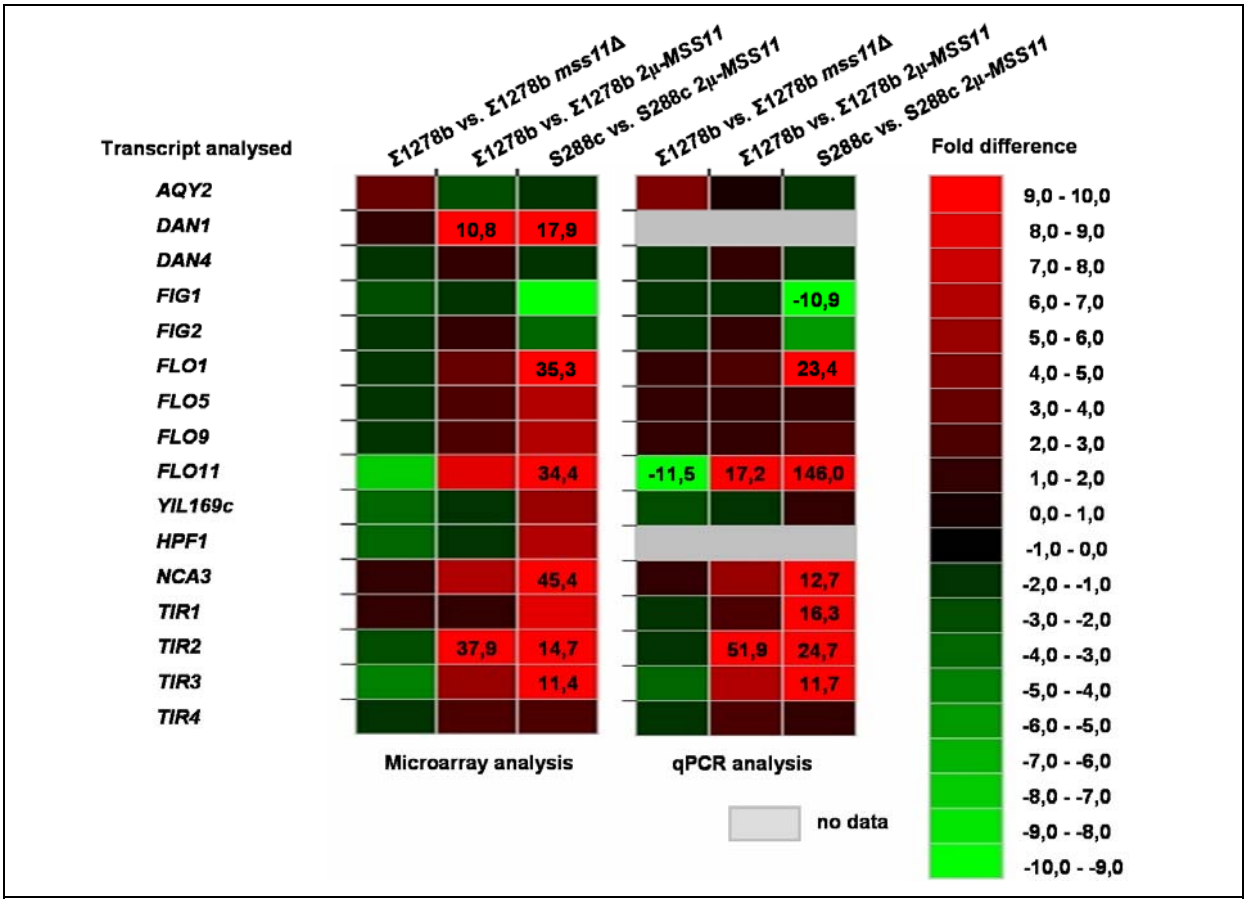

Figure 4.4 Genes most affected by *MSS11* deletion and/or -over-expression. Expression fold change as indicated in the scale with red indicating up-regulation and green down-regulation respectively. Fold changes falling outside the range of the indicated scale is represented as a numerical value displayed on a saturated colour background. Colour map generated by JColorGrid ver1.860 (Joachimiak *et al.*, 2006).

TIR and *FLO* gene members are up-regulated in response to *MSS11* over-expression and down-regulated upon *MSS11* deletion whereas the *DAN* genes only show an up-regulated response in the over-expression strains. In contrast, *FIG* family members are significantly down-regulated in response to *MSS11* over-expression in the S288c genetic background. The micro-array analysis in fact shows that 14 other mating related genes appear also significantly down-regulated in the same strain (listed in Table 4.9). In fact, of the 31 genes that are down-regulated more than 3 fold, half are mating related. They include seven of ten pheromone regulated (*PRM*) genes as well as genes that encode for factors involved in mating related pheromone signalling, cell-cell adhesion and the resulting membrane fusion (Bardwell, 2005; Chen *et al.*, 2007; White and Rose, 2001). *PRM7* was the only mating gene up-regulated (12 fold) in this strain (Table 4.9). This is in contrast to the initial observation that all *PRM* genes are regulated in a similar manner (Heiman and Walter, 2000). Taken together, these results show that Mss11p over-expression up-regulates a variety of non-sexual adhesins while strongly repressing mating-related genes.

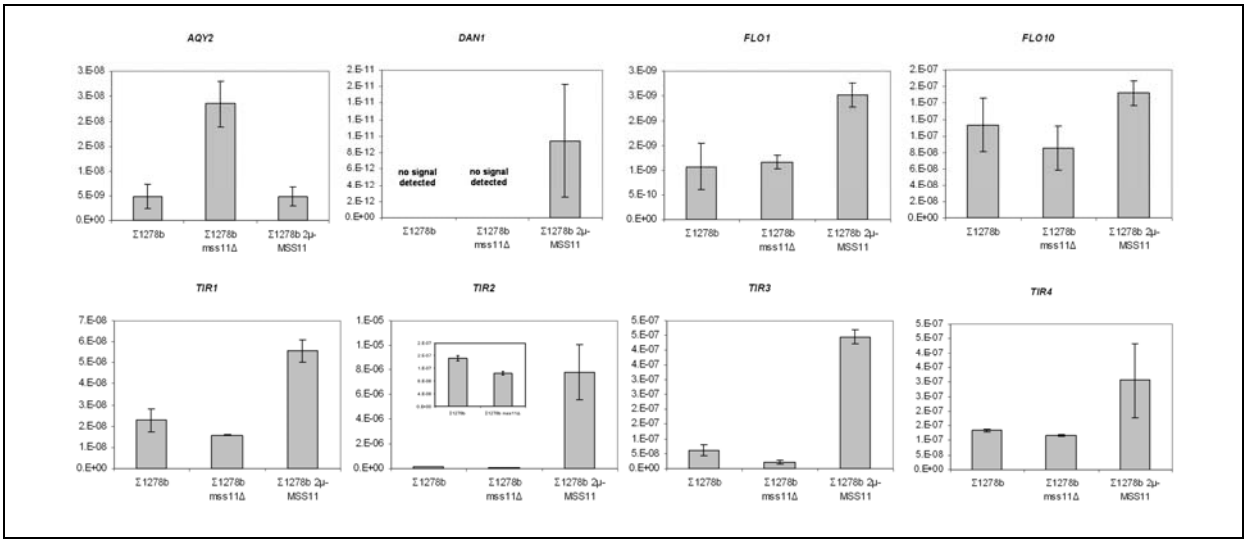

Figure 4.5 qPCR analyses for *AQY2*, *DAN1*, *FLO1*, *FLO10*, *TIR1*, *TIR2*, *TIR3*, *TIR4* gene expression in Σ1278b. Relative expression values determined as described in Materials and methods.

AQY2, in contrast to *FLO*/*TIR* regulation, is up-regulated in the deletion strain and down-regulated in the over-expressing strains. *HPF1* and its homologue *YIL169c* are down-regulated in the deletion strain and only up-regulated in the S288c over-expressing strain. Lastly *NCA3* transcript levels show no change in response to *MSS11* deletion but are significantly induced in both the over-expressing strains.

qPCR analysis shows that *FLO5* and *FLO9* are not regulated as the micro-array analysis suggests. This difference is most probably due to the inability of the Affymetrix Genechip® probe sets to efficiently differentiate between *FLO1*, *FLO5* and *FLO9* signals. These *FLO* genes display very high sequence homology making it extremely difficult to differentiate between their corresponding transcript species. qPCR confirm *FLO1* up-regulation 2 and 23 fold in the over-expression Σ1278b and S288c strains respectively. Furthermore qPCR analysis showed no signal for *HPF1* and generated only data for *YIL169c*, whereas micro-array analysis detected identical signal output for these two genes. Most likely this is again due to the Affymetrix platform's inability to distinguish between these two open reading frames, as these genes are also highly homologues. Surprisingly qPCR shows that *YIL169c* is down-regulated in both the Σ1278b deletion and over-expression strains but up-regulated in the S288c over-expressing strain. No qPCR signal for *DAN1* was observed in either the wild type or deletion strains thus not confirming the >10 fold *DAN1* activation as shown by micro-array analysis. Only in over-expression strains we could detect a signal of large variability (only shown for Σ1278b; Figure 4.4). This suggests that *MSS11* over-expression leads to the induction of *DAN1*, the transcription of which is either below detection levels or silenced in wild type. The 1,6 fold induction of the other *DAN* gene family member, *DAN4* was confirmed in the Σ1278b over-expressing strain but results from either the deletion- and S288c over-expressing strains were inconclusive.

The remainder of the qPCR analysis broadly confirms the micro-array analysis.

Table 4.9 List of mating process related genes affected (3 fold cut-off) by *MSS11* over-expression in strain S288c. Gene descriptions obtained from the Saccharomyces genome database (SGD; http://www.yeastgenome.org/)

Name	Gene description	Fold change	p-value
FIG1	Integral membrane protein required for efficient mating	-8.2	0.007
PRM3	Pheromone-regulated protein required for karyogamy	-6.6	0.019
PRM6	Pheromone-regulated protein	-4.7	0.093
ASG7	Protein that regulates signalling from a G protein beta subunit Ste4p and its relocalization within the cell; specific to a-cells and induced by alpha-factor	-4.6	0.073
PRM1	Pheromone-regulated multispanning membrane protein involved in membrane fusion during mating	-4.5	0.104
AGA2	Adhesion subunit of a-agglutinin of a-cells, C-terminal sequence acts as a ligand for alpha-agglutinin (Sag1p) during agglutination	-4.1	0.134
MFA1	Mating pheromone a-factor, made by a cells	-4.0	0.162
STE2	Receptor for alpha-factor pheromone	-3.9	0.191
BAR1	Aspartyl protease secreted into the periplasmic space of mating type a cells, helps cells find mating partners, cleaves and inactivates alpha factor allowing cells to recover from alpha-factor-induced cell cycle arrest	-3.8	0.124
PRM8	Pheromone-regulated protein	-3.8	0.147
MFA2	Mating pheromone a-factor, made by a cells	-3.7	0.177
FIG2	Cell wall adhesin, expressed specifically during mating; may be involved in maintenance of cell wall integrity during mating	-3.7	0.096
KAR5	Protein required for nuclear membrane fusion during karyogamy	-3.3	0.153
PRM2	Pheromone-regulated protein	-3.3	0.071
PRM4	Pheromone-regulated protein	-3.2	0.126
PRM7	Pheromone-regulated protein	12.0	0.243

4.4.3 Adhesion phenotypes of strains and transformants used for transcription analysis

Yeast strains from both Σ1278b and S288c genetic backgrounds were analysed for cell-cell and cell-surface adhesion phenotypes. In addition, a S288c strain carrying a reconstituted copy of *FLO8* (S288c (*FLO8*)) was included in this analysis (Figure 4.6 and 4.7).

Σ1278b wild type yeast form a very distinctive growth pattern when grown on 0,3% agar YPD which is alternatively referred to as “sliding motility”, “biofilm formation” or “mat” formation (Reynolds and Fink, 2001) (Figure 4.6A and B). Furthermore cultures of this strain grown to stationary phase adhere to wells of polystyrene plates (Figure 4.6C), likely due to high cell wall hydrophobicity (Figure 4.6E), but do not flocculate (Figure 4.6D). All of these phenotypes have been reported to require Flo11p (Barrales *et al.*, 2008; Reynolds and Fink, 2001) with the exception of flocculation for which Flo1p activity is required (Verstrepen and Klis, 2006). *FLO1* has been reported to be silenced in Σ1278b, the over-expression of which causes flocculation in this strain (Guo *et al.*, 2000). S288c wild type is unable to undergo “mat” formation or flocculate. Upon

restoration of *FLO8* “mat” formation and flocculation is restored but these phenotypes remain dependent on the presence of *MSS11*. “Mat” formation by S288c (*FLO8*) appears uniquely different from that formed by Σ1278b. S288c (*FLO8*) only forms fully developed growth “mats” after an extended incubation of three weeks with morphology that display extensive variation between biological repeats. In addition *FLO8* replacement increased cell wall hydrophobicity and cells displayed an increased ability to adhere to polystyrene surfaces. Irrespective of genetic background, an abolishment of all the above mentioned phenotypes is observed upon *MSS11* deletion.

A similar phenotype analysis was performed on transformed strains (Σ1278b and S288c genetic backgrounds) used for the transcriptome analysis (Figure 4.7). Again Mss11p was shown to be absolutely required for all the phenotypes and affects cell wall hydrophobicity. Σ1278b *mss11*Δ is unable to form “mats” (Figure 4.7 A and B) or invade agar plates (Figure 4.7C) and shows a decrease in cell wall hydrophobicity (Figure 4.7E). Remarkably Σ1278b displays very low levels of flocculation (~5%) under these growth conditions which is further lowered upon *MSS11* deletion. *MSS11* over-expression restores invasive capability and flocculation in S288c and leads to increased invasion and floc formation (~12%) in Σ1278b. Both over-expressing strains display increased cell hydrophobicity. Interestingly *MSS11* over-expression could not suppress *flo8-1* in S288c with regard to the lack of “mat” formation, even after extending the incubation period (data not shown). Furthermore the Σ1278b over-expressing strain formed “mats” of a smaller diameter in comparison to wild type.

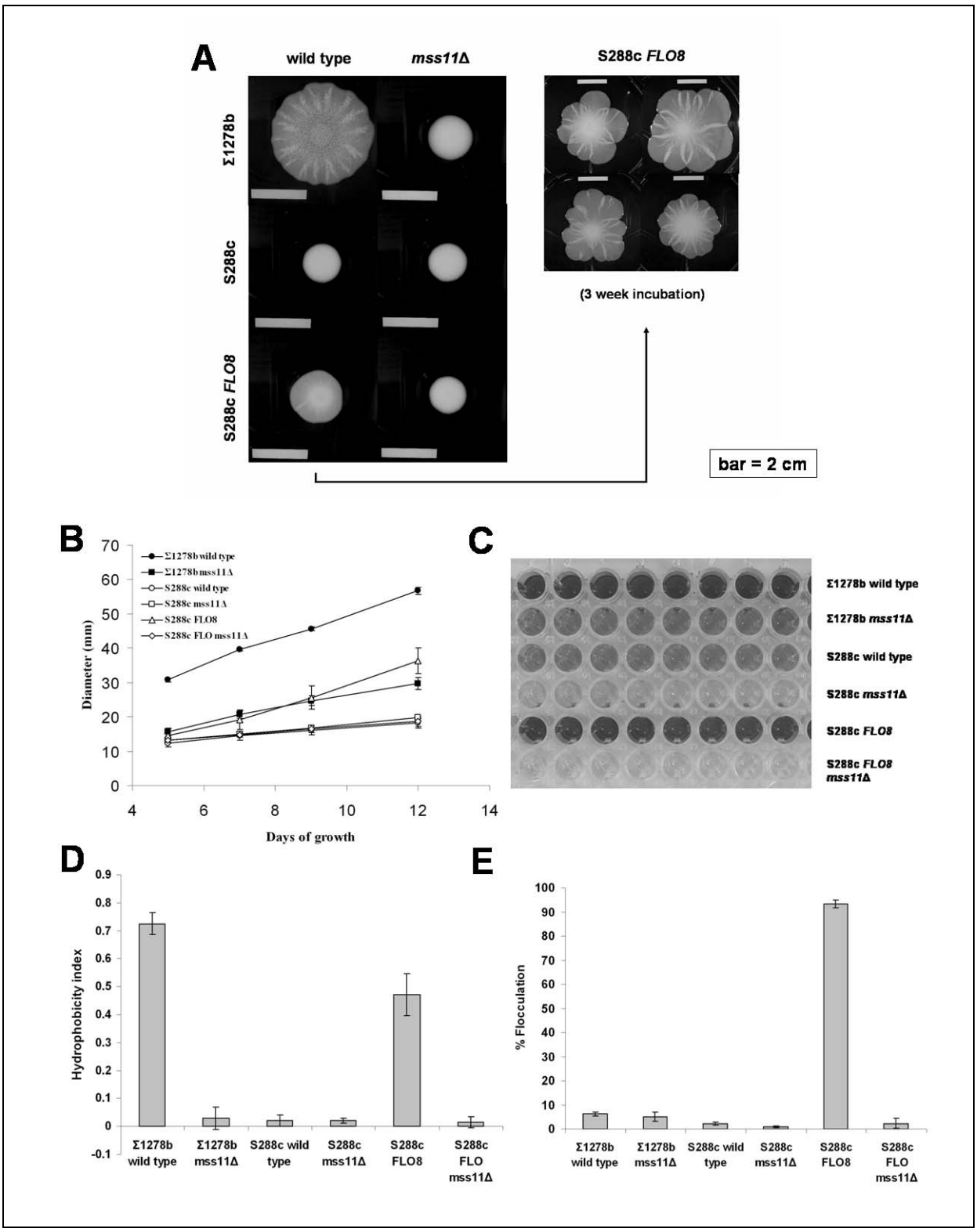

Figure 4.6 Phenotype analysis of Σ1278b, S288c and S288c (*FLO8*) respectively. Various cell-cell and cell-substrate interactions were investigated. (A) "Mat" formation on 0,3% YPD agar after 9 days of growth (left panel). Strain S288c (*FLO8*) was further incubated for ~2 weeks to allow for fully developed "mats" (right panel). Growth was measured up to 12 days post seeding as described in materials and methods (B). Strains grown to stationary phase in YPD were assayed for their ability to adhere to polystyrene (C), to flocculate (D) and for their degree of hydrophobicity (E).

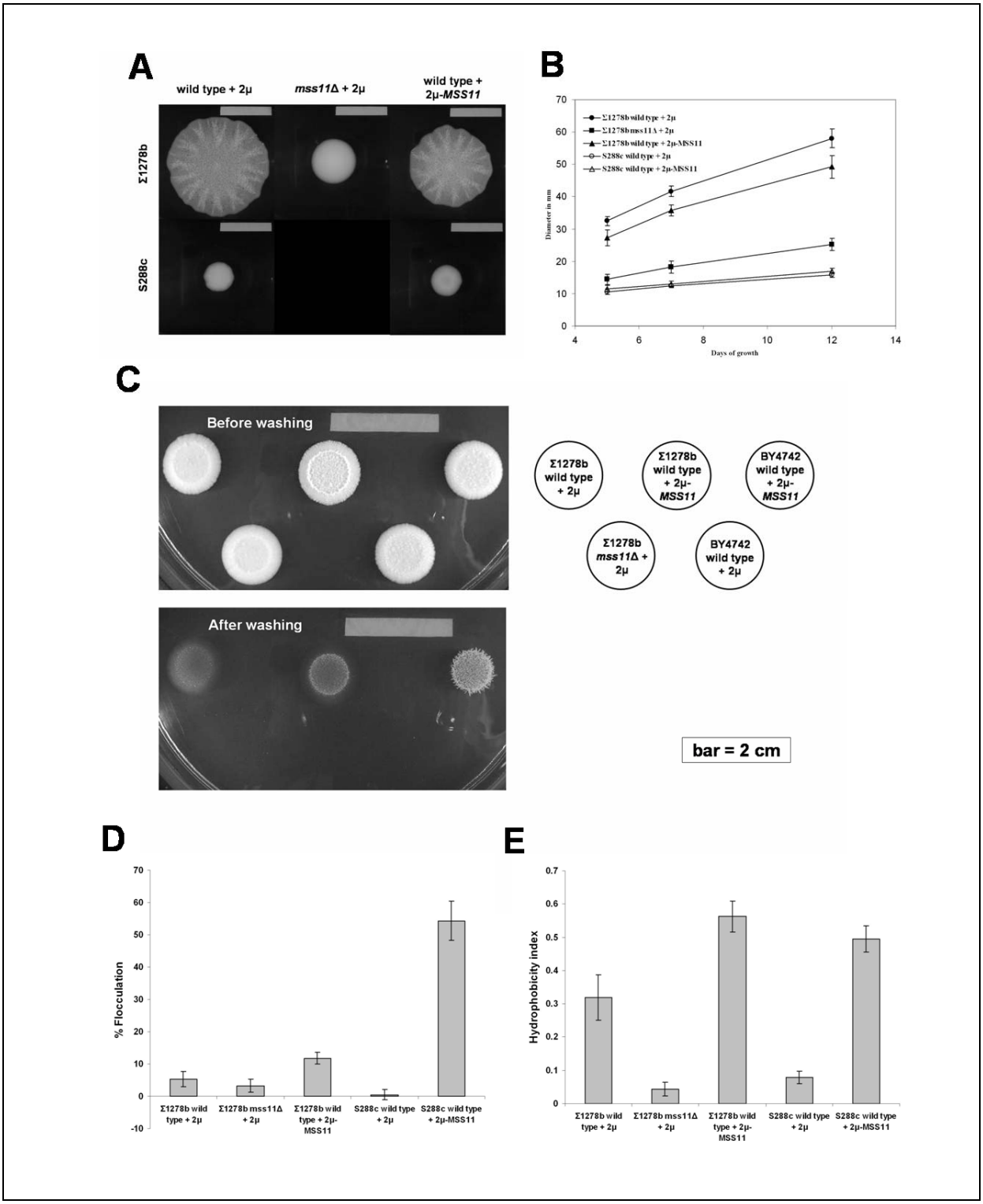

Figure 4.7 Phenotype analysis of the same Σ1278b and S288c transformants used in the transcriptome analysis. (A) "Mat" formation on 0,3% YPD agar after 7 days of growth. (B) Measurement of growth at day 5, 7 and 12 respectively. (C) Invasive growth of transformants. Transformants were grown in selective media and spotted on YPD plates. Shown is the total growth after 6 days incubation (top panel) and invaded cells revealed following subsequent plate washing (bottom panel). Transformants were spotted as indicated. Transformants grown to stationary phase in liquid minimal media (SCD) assayed for (D) their ability to flocculate and (E) the degree of culture hydrophobicity.

4.4.4 Adhesion phenotype screen of single and double *flo11*Δ deletion strains

Previously we have reported that *MSS11* over-expression partially suppress the non-invasive phenotype of the S288c (*FLO8*) strain deleted in *FLO11* (Figure 4.8) (Bester *et al.*, 2006). We further investigated the possibility that any of the genes identified in the transcriptome analysis could be involved in Flo11p independent invasion. Double deletion strains were constructed of *FLO11* and either *AQY2*, *DAN1*, *FIG1*, *FIG2*, *FLO1*, *FLO10*, *YIL169C*, *NCA3*, *TIR1*, *TIR2*, *TIR3* or *TIR4* in both the Σ1278b and S288c (*FLO8*) genetic backgrounds. Together with the *FLO11* single deletion strains the double deletion strains were transformed with either empty vector or 2μ-*MSS11* and spotted on low nitrogen media (SLAD) in order to investigate invasion. SLAD media have been reported to simulate nitrogen starvation conditions that in turn lead to enhanced filamentous growth and agar invasion (Gimeno *et al.*, 1992). Plates were incubated for 6 days after which macro-colonies were washed of the plate surface to reveal invaded cells. Shown in Figure 4.8 is only S288c strains *flo11*Δ, *fig1*Δ *flo11*Δ and *flo10*Δ *flo11*Δ transformed with 2μ-*MSS11* and *flo11*Δ transformed with vector alone. The granular nature of the macro-colonies is caused by flocculation in the cell suspensions after being dropped on the plate. Plate washing reveals that invasion in *flo11*Δ strains is dependent on the over-expression of *MSS11* as S288c (*FLO8*) *flo11*Δ transformed with vector alone does not invade This same requirement of 2μ-*MSS11* for invasion of a *flo11*Δ strain was observed in Σ1278bΔ (data not shown) and strongly suggest that cell wall factors other than Flo11p are involved in agar invasion.

Invasion in the over-expressing *flo11*Δ *fig1*Δ strain is representative of the degree of invasion observed in all the other double deletion strains assessed (data not shown) with the exception of *flo10*Δ *flo11*Δ. The data clearly show that the deletion of any of the selected genes does not impact at all on the ability of these strains to invade. Only the *FLO10* mutation very clearly blocks any invasion caused by *MSS11* over-expression in the *flo11*Δ genetic context. This identifies Flo10p as the adhesin required for invasion in the absence of Flo11p. Although *MSS11* over-expression causes invasion in Σ1278b *flo11*Δ a similar screen did not identify any genes other than *FLO11* that could account for this invasion (data not shown). Furthermore we tested the double deletion strains in S288c (*FLO8*) transformed with vector and 2μ-*MSS11* for their flocculation ability. Only strains carrying a deletion in the dominant flocculation gene *FLO1* displayed a lack of floc formation (data not shown).

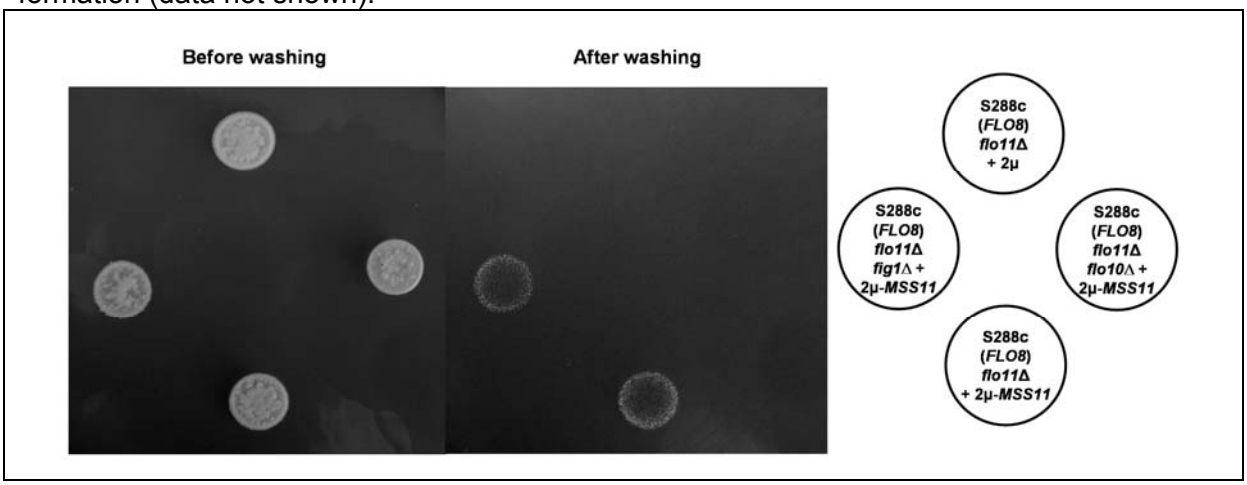

Figure 4.8 Invasion of *flo11*Δ single and double mutants. Shown is total growth after 6 days on SLAD plates (left panel) as well as cells that invaded the agar medium revealed by washing the plate (right panel). Strain S288c (*FLO8*) *flo11*Δ *fig1*Δ transformed with 2μ-*MSS11* is representative of all the deletion strains transformed with the same construct with regard to the ability to grow invasively with the exception of strain S288c (*FLO8*) *flo11*Δ *flo10*Δ.

We further tested if single deletions of *DAN1*, *FIG1*, *FLO1*, *FLO10*, *FLO11*, *MSS11*, *TIR1*, *TIR2*, *TIR3*, and *TIR4* affect flocculation and cell hydrophobicity in S288c (*FLO8*) (Figure 4.9 A and B). Flocculation ability of strains grown to stationary phase was tested in YPD. Strains carrying deletions in *FLO1* and *MSS11* show no flocculation as previously reported. We further identify the significant requirement of Flo10p as flocculation factor as *FLO10* deletion leads to near total abolishment of flocculation. This requirement was not observed for *flo10*Δ strains grown in minimal media (data not shown) thus this effect is dependent on media composition. Surprisingly *FLO11* and *TIR4* deletions led to increased flocculation in comparison to wild type.

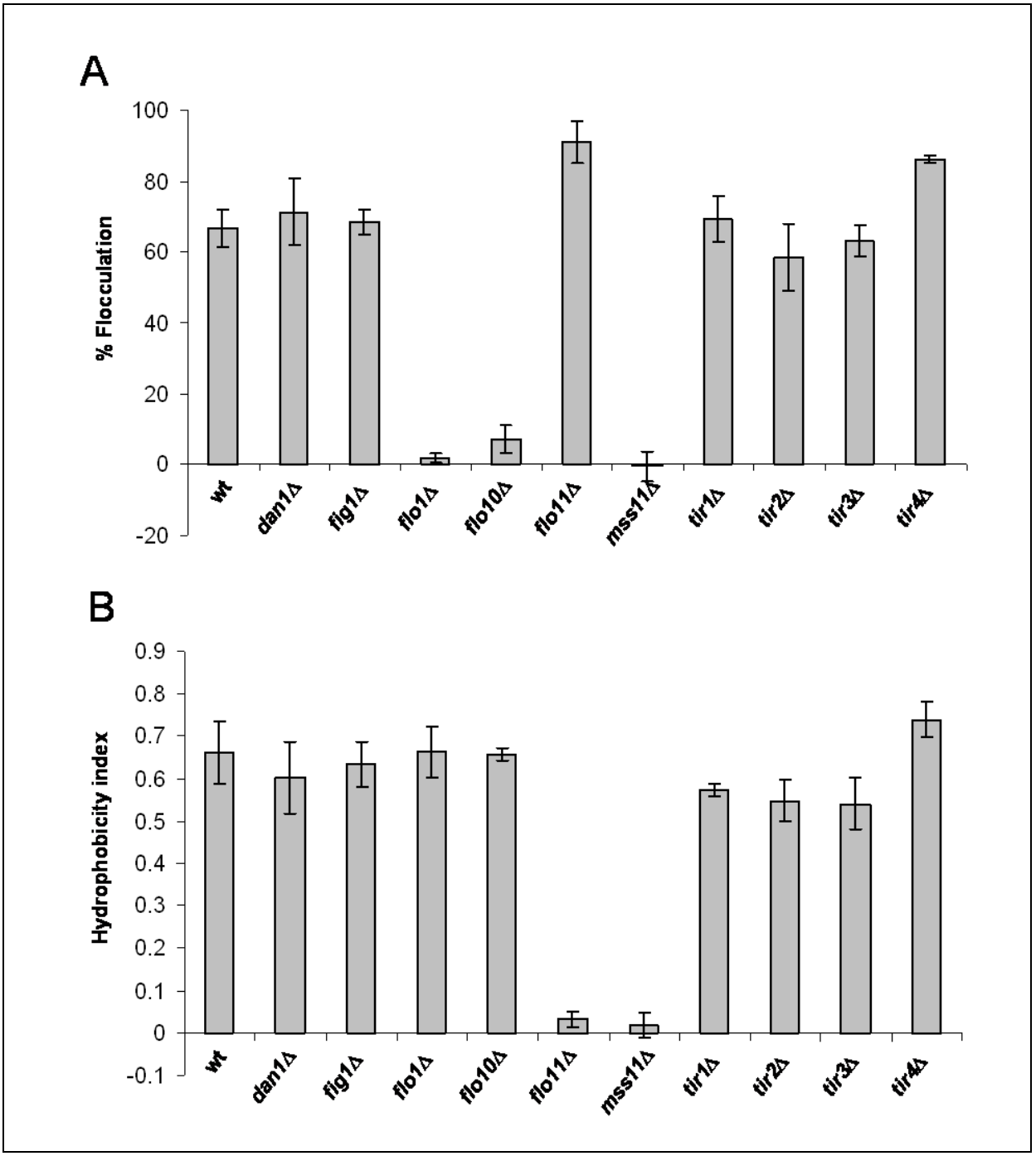

Figure 4.9 Degree of flocculation (A) and hydrophobicity (B) of S288c (*FLO8*) single deletion mutants grown to stationary phase in liquid YPD.

The cell wall hydrophobicity of the same strains is shown in Figure 4.9B. Deletion in both *FLO11* and *MSS11* resulted in hydrophilic yeast which supports previous findings (Barrales *et al.*, 2008). In addition *TIR* deletion affected hydrophobicity. Strains *tir1*Δ, *tir2*Δ and *tir3*Δ are less hydrophobic compared to wild type, whereas *TIR4* deletion leads to increased hydrophobicity.

FLO proteins contain internal tandem repeats that display length variation between different strains and directly affect adhesin phenotypes (Verstrepen *et al.*, 2005). For instance the increase or decrease in length of these repeats in *FLO1* can be directly correlated with the respective increase or decrease in flocculation ability. Furthermore yeast progeny from a common parental strain show great variation in flocculation (Smukalla *et al.*, 2008) that could be due to variation in the *FLO1* coding region. To rule out the possibility that *FLO1* repeat variability is responsible for the discrepancies in flocculation observed in Figure 4.9 we amplified the repeat lengths from genomic DNA from the same strains used for the phenotype assay (Figure 4.10). All strains, with the exception of the *flo1*Δ strains, have copies of *FLO1* with the same size as compared to the wild type strain S288c (*FLO8*).

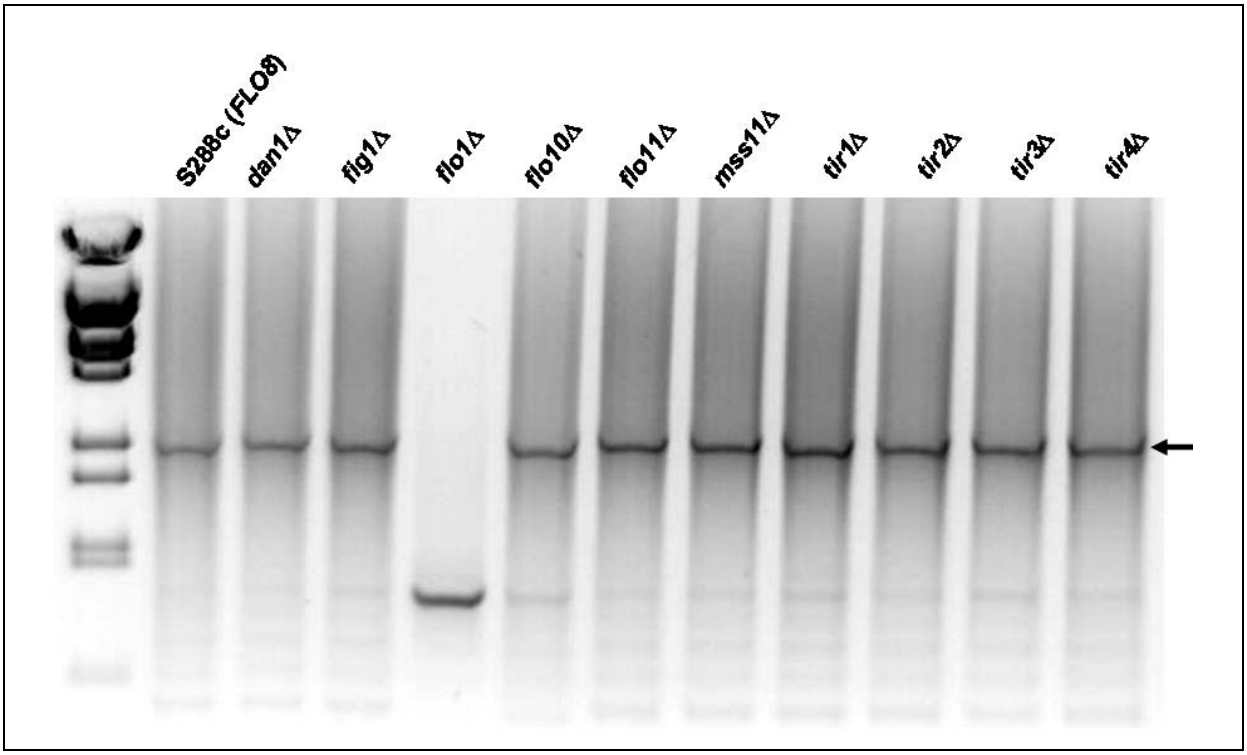

Figure 4.10 *FLO1* intragenic tandem repeat analysis showing repeats are of similar size in the single deletion strain set. *FLO1* repeats were PCR amplified using primers listed in Table 4 and as described before (Verstrepen *et al.*, 2005). DNA marker is lamda DNA digested with the restriction endo-nuclease *Bst*EII.

4.4.5 Differential regulation of *FLO10* and *FLO11*

As mentioned previously the MAPK and cAMP-PKA signalling pathways both act to regulate *FLO11* transcription (Gagiano *et al.*, 1999a; Rupp *et al.*, 1999) with Mss11p as the central regulator (van Dyk *et al.*, 2005). Although *FLO11* transcription shows a response to input of both of these pathways, the MAPK pathway is not absolutely required for transcription (van Dyk *et al.*, 2005). We further investigated the possibility of *FLO10* being regulated similarly. Σ1278b strains carrying single and double deletions in genes encoding for *FLO11* transcriptional control components were analysed for *FLO10* and *FLO11* transcripts by means of qPCR analysis (Figure 4.11). In the study performed by van Dyk *et al* (2005) it was shown that the absence of the Sfl1p repressor leads to the induction of *FLO11* transcription. This is blocked by a deletion of *FLO8*, acting down-stream of the cAMP-PKA pathway, but only partially in yeast deleted for *STE12* or *TEC1*, which function downstream of the MAPK pathway. Our analysis confirms these findings for *FLO11* regulation and we provide data for *FLO10* transcripts using cDNA from the same set of yeast strains (Figure 4.11). Native levels of *FLO10* transcript are lower compared to *FLO11* and the gene appears also repressed by Sfl1p. It is partially dependent on the cAMP-PKA pathway as can be seen from the signal still present in the *sfl1*Δ *flo8*Δ double mutant. No transcription could be detected in the *sfl1*Δ *ste12*Δ double mutant showing that *FLO10* transcription requires MAPK signalling. Transcript is still detected in the *sfl1*Δ *tec1*Δ strain suggesting very specific roles of these MAPK pathway components in the regulation of *FLO10*. Transcription levels in *sfl1*Δ *mss11*Δ were the same as for the *sfl1*Δ *flo8*Δ strain. Clearly other pathways exist that confer *sfl1*Δ up-regulation.

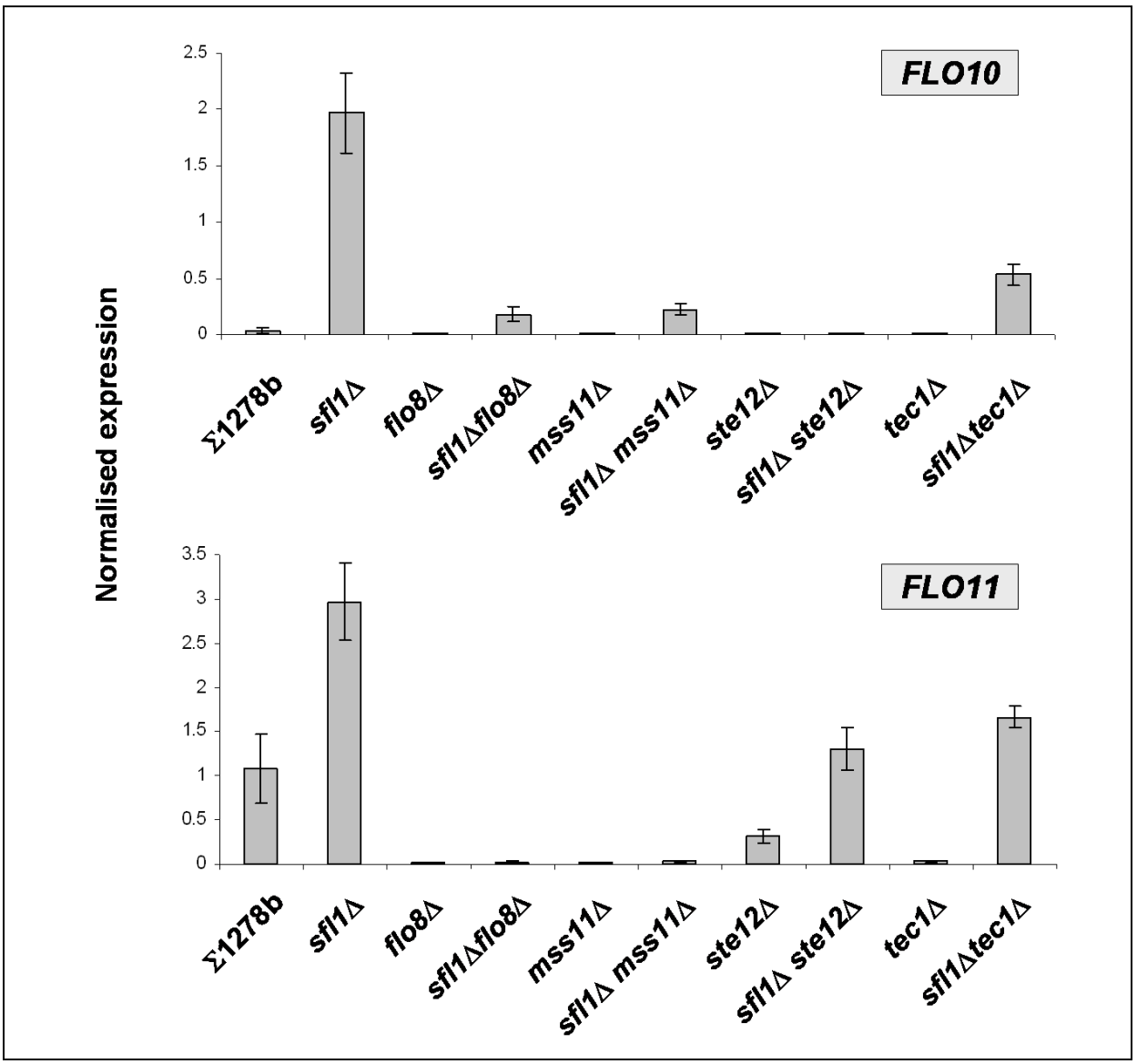

Figure 4.11 *FLO10* and *FLO11* are differentially regulated by components of the MAPK and cAMP-PKA signalling pathways. Error bars represent standard deviation of at least three independent biological replicates.

4.5 Discussion

4.5.1 Mss11p affects multiple cell wall encoding targets

In this study we show that *MSS11* expression levels predominantly impact on an array of genes encoding for cell wall and -membrane proteins. This assessment holds true for two phenotypically different strains, suggesting that Mss11p function is indeed specifically related to cell wall remodelling in response to environmental cues. The data also clearly indicate that only *FLO1*, *FLO10* and *FLO11* appear to be directly involved in Mss11p related phenotypes that were assessed here.

Interestingly, while significantly up-regulating several cell wall proteins, *MSS11* over-expression also had a significant repressive impact on many mating related genes such as Fig1p, which has been shown to be a integral membrane protein required for efficient mating (Aguilar *et al.*, 2007; Muller *et al.*, 2003). It is noteworthy that Fig1p may also play a role in mating-unrelated polarized growth since it was shown that a *FIG1* transposon insertion mutant displayed decreased filamentation in response to 1-butanol (Lorenz *et al.*, 2000). The data therefore clearly suggest that Mss11p plays an important role in directing cellular differentiation towards non-sexual adhesive phenotypes while repressing mating. It is possible that such a function is of some relevance in an evolutionary framework. In a nutrient-poor environment, mating may be undesirable even in the presence of mating partners since it certainly represents an energetically demanding and potentially risky exercise in such unfavourable conditions. Rather, cellular efforts may be directed towards accessing additional nutritional resources. Such an interpretation is reinforced by the fact that *MSS11* over-expression activates specifically the high affinity hexose transporter Hxt2p. The remodelling of mitochondrial structures associated with Nca3p, another significant target of Mss11p, may also support such an interpretation. Mss11p also up-regulates the glucoamylase-encoding *STA* genes that are found in some strains of *S. cerevisiae* (Gagiano *et al.*, 1999a; Webber *et al.*, 1997). Thus Mss11p may be responsible for controlling the switch between the mating and the adhesive or invasive growth forms of *S. cerevisiae*. The role of transcription factors as switches between different developmental pathways is not uncommon, and has for example been reported for Fus3p (Bao *et al.*, 2004; Chou *et al.*, 2004; Chou *et al.*, 2006; Chou *et al.*, 2008) and Rme1p (van Dyk *et al.* 2003). The response of diploid strains, in which mating-related genes are strongly repressed, to *MSS11* over-expression would be of some interest in this regard.

Besides the *FLO* gene family, the *TIR* genes appear to be the second most *MSS11*-affected gene family. Indeed, several members of the group clearly and strongly respond to *MSS11* expression levels. However, our data do not add significantly to a better understanding of these genes. Indeed, their role in the investigated phenotypes appears minor at best, and, but for the rather minor impact on cellular hydrophobicity, no relevant functions can be derived. Since the specific function of these genes remains unknown, it is likely that other conditions will need to be investigated to find Mss11p-dependent observable phenotypes associated with these genes.

Broadly speaking, the data clearly suggest that Mss11p forms part of the cellular control mechanisms that maintain a balanced cell wall composition.

4.5.2 The impact of strain genetic background on gene expression profiles

Apart from the above mentioned similarities, the differences in gene expression profiles between the two over-expression strains concerned in particular genes involved in metabolism. Genes down-regulated in Σ1278b but up-regulated in S288c were enriched for the transport and metabolism of amino acids, while allantoin-, carnitine- and malate metabolism dominated enrichment of genes up-regulated in Σ1278b and down-regulated in S288c. The significance of these findings is difficult to assess. As discussed, one of the major differences between the two strains, in particular with regard to he phenotypes assessed here, is the presence or absence of functional Flo8p. However, we do not have enough knowledge on Flo8p function to assess the relevance in this context.

4.5.3 Mss11p controlled adhesion phenotypes are Flo protein dependent

Our results show that the magnitude of specific phenotypes depends on more than one adhesin. In the strain S288c (*FLO8*), Flo1p is clearly the dominant flocculation protein. In addition Flo10p performs a minor role and the absence of Flo11p even leads to enhanced floc formation. We also observe the phenotype of invasion to be dependent on both *FLO10* and *FLO11*. Flo11p is the dominant factor required for this behaviour but Flo10p clearly contributes to a lesser degree. This observation is strain dependent as neither Flo10p, nor indeed any of the other factors tested, were shown to be responsible for invasion in the Σ1278b *flo11*Δ *MSS11* over-expressing strain (data not shown). We however did not test if this holds true for mat formation and polystyrene adhesion.

Previous work has highlighted the level of functional overlap between Flo proteins by means of controlled or over-expression studies (Govender *et al*., 2008; Guo *et al*., 2000; Van Mulders *et al*., 2009), although the question remains how these different proteins function cooperatively to control phenotypes in the wild type genetic context. Results from this study strongly suggest that such cooperation control the balance of cell wall adhesins to control specific cellular behaviour.

4.5.4 Significance of non-*FLO* targets

This study provides evidence that the Flo adhesins are the only factors directly involved in Mss11p mediated changes in phenotypic behaviour. The question thus remains why so many other cell wall or plasma membrane encoding genes are regulated by Mss11p. One possible explanation is that altered Flop content of the cell wall may induce a secondary cellular response to compensate for the altered cell wall composition. The *DAN*/*TIR* genes have been shown to be up regulated in response to high hydrostatic pressure (Abe, 2007) and the *TIR* genes additionally to cold shock (Abramova *et al.*, 2001a). These genes also respond to a switch to anaerobic growth conditions (Sertil *et al.*, 2007; Tai *et al.*, 2005; ter Linde *et al.*, 1999). Under conditions of anaerobic growth yeast is not able to synthesise sterols *de novo*. Dan1p has been reported to be involved with sterol uptake (Alimardani *et al.*, 2004; Wilcox *et al.*, 2002). Sterols determine the fluidity of the plasma membrane which indirectly determine the movement and activity of membrane proteins (van der Rest *et al.*, 1995). Thus these genes are induced under conditions of stress possibly adjusting cell wall composition in order to compensate for environmental changes. It is very probable that the induction of high levels of Flop by *MSS11* over-expression results in dramatic changes of cell wall composition. In compensation for this the cell may then up-regulate Danp and Tirp production to restore the required balance of cell wall components. This could also be the case in the up-regulation of the *FIG* genes in response to *MSS11* deletion. Such a hypothesis is supported by a

recent study shows that *DAN1* is up-regulated in response to *FLO1* expression (Smukalla *et al.*, 2008). Because of Flo1p being present in the cell wall, cells flocculate and form flocs. Smukalla *et al*. reasons that cells inside flocs are subjected to localised anaerobic conditions, thus *DAN1* is up-regulated to aid in sterol import (Smukalla *et al.*, 2008). On the other hand, this hypothesis is somewhat undermined by the data presented in the following chapter of this dissertation, since *FLO8* over-expression, while resulting in close to indistinguishable phenotypes with regard to adhesion, leads to the up- and down-regulation of a very different set of genes (unpublished data; see Chapter 5). These data may therefore suggest that most of the observations described here are indeed directly due to Mss11p-dependent gene regulation.

4.5.5 The role of Mss11p in the differential regulation of *FLO1*, *FLO10* and *FLO11*

In this study we affirm Mss11p as transcriptional regulator of the *FLO* genes. Mss11p is absolutely required for *FLO11* expression as shown before (van Dyk *et al.*, 2005). *FLO10* displays a similar dependency on Mss11p, but not to the same degree than for *FLO11*. Thus we conclude that one or more pathways exists that function independently of Mss11p in regulating *FLO10* expression. *FLO10* responds to similar signalling pathways as *FLO11*. *FLO11* transcription machinery responds to both cAMP-PKA and MAPK signalling with Mss11p as central regulator (van Dyk *et al.*, 2005). *FLO10* transcription also responds to both of these signalling pathways but is more dependent on MAPK signalling. In fact no signal can bypass the MAPK transcription factor Ste12p showing that *FLO10* transcription is absolutely dependent on MAPK signalling. Previous studies have shown that Ste12p and Tec1p act on the *FLO11* promoter ($FLO11_P$) (Lo and Dranginis, 1998; Madhani and Fink, 1997). These transcription factors were shown to act in concert in the activation of transcription with Ste12p as the general MAPK signalling component and Tec1p as specific filamentous growth transcription factor (Bardwell *et al.*, 1998; Madhani and Fink, 1997). We show that these factors have different roles and requirements in the regulation of the *FLO10* promoter. Transcription is totally dependent on Ste12p but in the *sfl1*Δ *tec1*Δ mutant low levels of transcription is still detected. Thus *FLO10* transcriptional activation requires MAPK signalling but not the filamentous growth specific MAPK component Tec1p.

No information on pathway regulation could be obtained for *FLO1* in Σ1278b as it is silenced in this strain. In S288c it was shown that the Sfl1p repressor, a characterised repressor of *FLO11* transcription (Conlan and Tzamarias, 2001), was not required for *FLO1* repression (Fichtner *et al.*, 2007). Furthermore the same authors showed that a deletion in *SIN4*, encoding a mediator component required for Sfl1p function (Conlan and Tzamarias, 2001), de-repressed *FLO1* but not *FLO11*. It still remains an open question if *FLO1* and *FLO11* share signalling components for transcriptional regulation as is the case with *FLO10* and *FLO11*. Clearly the regulatory mechanisms controlling transcription of all these *FLO* genes share similar components and/or pathways. Furthermore *FLO1* and *FLO11*, and to a lesser extent *FLO10*, require Mss11p for transcriptional activation. Thus differential *FLO* regulation controlled by overlapping pathways and shared components controls the balance of Flo proteins in the cell wall. It is this specific control of cell wall composition that governs adhesion properties of the outer cell wall. Adhesion phenotypes very likely are not the result of individual or unique populations of adhesin proteins but probably the result of a dynamic and complex interaction of different cell wall proteins between themselves and with the extra-cellular environment.

4.6 Acknowledgements

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Chapter 5

Research results III

Cooperative and differential gene regulation by Flo8p and Mss11p as revealed by yeast transcriptome analysis

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Chapter 5: RESEARCH RESULTS III

Cooperative and differential gene regulation by Flo8p and Mss11p as revealed by yeast transcriptome analysis

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5.1 Abstract

Cooperative action between transcription regulators contributes considerably to the level of complexity in gene regulation. In this work we use whole genome expression data to investigate joint as well as differential transcriptome regulation by Flo8p and Mss11p. Both of these factors control the expression of adhesin encoding flocculation (*FLO*) genes, and in particular of *FLO1* and *FLO11*. Over-expression or deletion of *MSS11* or of *FLO8* result in very similar or indistinguishable phenotypes with regard to cell wall properties, yet information about the specific role of each factor and their interaction is limited. Here we use global transcript analysis using strains carrying deletions, or multiple copies of *FLO8* and of *MSS11* to investigate differences and similarities between the impacts of the two proteins. The data show that, apart from the previously identified *FLO* genes, Flo8p and Mss11p appear to co-regulate the expression of the cell wall protein encoding *TIR3* gene, as well as the membrane water channel gene *AQY2*. Besides this overlap, both factors impact on a significant number of non-shared target genes, suggesting that, but for cell wall-related properties, they fulfil significantly different roles in the regulation of cellular adaptation. Projecting transcriptome data from this study on known transcription factor (TF) interaction networks shows that Flo8p and Mss11p impact on other transcription factors, and pinpoints the specific importance of a network interaction between Cin5p, Flo8p, Mga1p and Mss11p. We propose a mode of action for Mss11p as a "controlling switch" in the transcription network.

5.2 Introduction

The ability of the yeast *Saccharomyces cerevisiae* to rapidly respond to changes in its environment, and in particular the ability to optimise the utilisation of often limited resources, is essential for its survival. Cell wall receptor systems sense and translate information regarding extra-cellular conditions via signalling pathways to the nucleus, ultimately affecting gene transcription. Some of these sensing systems have been well characterised and reviewed, for example the extra-cellular nutrient- (Gagiano *et al.*, 2002) and osmolarity sensing pathways (Hohmann *et al.*, 2007). These signalling pathways often share protein components and interact with each other to form complex and integrated control systems. This control is in particular exerted through transcription factor (TF) networks that act downstream of signalling pathways and consists of transcription factors and -effectors that interact as well as regulate each other. It has been shown that such interactions between TF components significantly contribute to the complexity in eukaryotic gene regulation (Wang *et al.*, 2009).

In this study we investigate the relationship between the gene regulation networks of the transcriptional regulators Flo8p and Mss11p. Initially Mss11p was identified as a regulator of starch utilisation in yeast carrying one or more copies of the glucoamylase encoding *STA* genes (Webber *et al.*, 1997), and was later shown to also be required for agar invasion (Gagiano *et al.*, 1999) and flocculation (Bester *et al.*, 2006). Flo8p was first identified as a general flocculation factor (Teunissen and Steensma, 1995) and was shown to specifically function as a transcriptional activator for *FLO* expression (Kobayashi *et al.*, 1999) while also controlling the cellular phenotypes of invasive growth and pseudohyphal development (Liu *et al.*, 1996). Since then, both proteins have been shown to co-regulate the expression of members of the *FLO* gene family, in particular of *FLO1*, *FLO10* and *FLO11* (also *MUC1*) (Bester et al. 2006), as well as of *STA1*. *FLO* genes encode for cell wall proteins that have been shown to participate in various cell-cell and cell-substrate adhesion interactions required for flocculation, biofilm formation, agar/polystyrene adhesion, agar invasion and the formation of pseudohyphae (Verstrepen *et al.,* 2005; Govender *et al.,* 2008; Dranginis *et al.*, 2007). These data sets suggest a close cooperation between the two factors with regard to the regulation of cell wall related phenotypes. Genomic deletion of *FLO8* or *MSS11* results in the near total abolishment of each of these different cellular behaviours, highlighting a critical requirement for both genes. Data show complete absence of *FLO11* expression in the deletion strains, a phenotype that can not be suppressed by any of the other factors that control *FLO11* expression. Genetic analysis however shows that *MSS11* over-expression can suppress a *flo8* deletion in terms of *FLO11* activation and Flo11p-associated phenotypes, but that *FLO8* over-expression are not able to suppress Δ*mss11* in the same manner (van Dyk *et al.*, 2005). Interestingly, this is contrary to recent findings in *Candida albicans*, where the functional homologues of each factor appear to show the opposite epistatic relationship (Su *et al.* 2009). In *S. cerevisiae* and *C. albicans*, the two factors have been shown to physically interact with each other. In *S. cerevisiae*, the data show that the two proteins cooperatively bind to the *STA1* promoter - a promoter sharing 99% sequence homology with that of the *FLO11* promoter (Vivier *et al.*, 1997) - and together promote the association with the RNA polymerase II holoenzyme (Kim *et al.*, 2004). Other data indicate that Flo8p regulates *FLO11* transcription specifically in response to, and downstream from, the cAMP-PKA pathway (Pan and Heitman, 1999; Rupp *et al.*, 1999).

While Mss11p is essential for *FLO11* expression, and interacts with Flo8p (van Dyk *et al.*, 2005), its specific role in the control of these phenotypes remains elusive. In order to better understand the nature of Mss11p contribution, we here use a comparative analysis of the impact of *FLO8* and *MSS11* deletion and over-expression on genome-wide gene expression in the laboratory strain Σ1278b. Σ1278b is preferentially used as a model system to study yeast adhesin phenotypes as it contains a functional copy of *FLO8*, contrarily to most laboratory strains that carry the non-functional *flo8-1* allele (Liu *et al.*, 1996). By sorting differentially expressed target genes according to their functional classification, and performing cluster analysis of their regulation in the deletion and over-expression strains we identify both common and unique gene target groups for these factors. Furthermore we extrapolate our data sets on known transcription factor interaction networks and model Flo8p and Mss11p function in context of transcription factor (TF) regulatory systems.

5.3 Materials and Methods

5.3.1 Strains, plasmids, and growth conditions

S. cerevisiae strains used in this study are all isogenic to the Σ1278b genetic background (Table 5.1). Plasmids are listed in Table 5.2 and were transformed into yeast using the standard lithium acetate method (Ausubel, 2004). Yeast peptone dextrose (YPD) was used as rich media. Minimal media (SCD) contained 0.67% yeast nitrogen base with pre-added ammonium sulphate but without amino acids and were further supplemented with 2% glucose (w/v) and the required amino acids according to the auxotrophic growth requirements of the relevant strain. Except for the determination of "mat" formation, yeast was cultivated at 30°C and on plates containing 2% agarose.

Table 5.1 *S. cerevisiae* strains used in this study.

Strain	Relevant genotype	Source or reference
YHUM272[a]	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG*	H.-U. Mösch
Σ1278b *flo8*	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG flo8*Δ::*LEU2*	van Dyk *et al.*, 2005
Σ1278b *mss11*	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG mss11*Δ::*LEU2*	van Dyk *et al.*, 2005

[a]YHUM272 is isogenic to Σ1278b

Table 5.2 Plasmids used in this study.

Plasmid	Genotype	Source or reference
YEpLac195	2μ *URA3*	Gietz and Sugino, 1988
YEpLac195-FLO8	2μ *URA3 FLO8*	Bester *et al.*, 2006
YEpLac195-MSS11	2μ *URA3 MSS11*	Gagiano *et al.*, 1999

5.3.2 Total RNA extraction

Yeast cultures were grown in 5ml SCD media from an optical density of 0,1 to between 1 and 2 as determined by spectrophotometric absorbance at a wavelength of 600 nm. Cells were harvested, washed with ice-cold H_2O and re-suspended in ice-cold AE buffer (50 mM sodium acetate, 10 mM EDTA, pH 5.0). Total RNA was extracted as described previously (Schmitt *et al.*, 1990). For transcript analysis total RNA from two independent biological repeats were analysed.

5.3.3 Microarray hybridisation and data acquisition

Probe preparation and -hybridization to Affymetrix Genechip® microarrays were performed according to Affymetrix instructions, starting with 6 μg of total RNA extracts. Results for each strain were derived from two independent culture replicates. Quality of total RNA, cDNA, cRNA and fragmented cRNA were analysed using the Agilent Bioanalyzer 2100. Probe hybridisation to GeneChip® Yeast Genome 2.0 Arrays was performed on the integrated Affymetrix GeneChip®

3000 platform. Chip scanning and data collection was performed using the Affymetrix GeneChip® Operating Software (GCOS) version 1.4.

5.3.4 Data analysis

Affymetrix GeneChip® data normalisation was performed using GC-RMA (Wu and Irizarry, 2004). In Chapter 4 normalisation was performed with the GCOS software. This information was used as a basis of the targeted genetic screen to identify genes involved in Mss11p-dependent phenotypes. The GC-RMA normalisation method proved to be more sensitive for change in gene expression over a broader range that provided more suitable data sets for this study. Fold change in gene expression as consequence of either gene deletion or over-expression was calculated with wild type as reference condition. In order to identify groupings in gene targets based on gene ontology (GO) classification the online application FunSpec was used (Robinson *et al.*, 2002). The following sources were used for GO classification with a p-value of 0.001 for the identification of categories: MIPS functional classification, GO molecular function, GO biological process and GO cellular component. Furthermore the following categories were not considered because their descriptions were viewed as of a too general nature (source in brackets): unclassified proteins (MIPS Functional Classification), molecular function (GO Molecular Function), biological process (GO Biological Process) and cellular component (GO Cellular Component). In the case of enriched groups with similar function and containing near identical gene groups, only one representative group were chosen. It should be noted that categorised groups often contain overlapping genes. Cluster analysis of gene targets was performed and visualised with Cluster ver 3.0 and Treeview ver 1.6 respectively (Eisen *et al.*, 1998). Information regarding TF networks was obtained from the online application YEASTRACT on the 2^{d} of Sept 2009 (Monteiro *et al.*, 2008; Teixeira *et al.*, 2006). The open-source network visualization and analysis software Cytoscape ver 2.6.2 was used to extrapolate known transcription network data onto expression data generated in this study.

5.3.5. Phenotype assessment

To ability of yeast strains to form spreading growth mats (also referred to as “biofilm” formation or “sliding motility”) on plates was determined as described previously (Reynolds and Fink, 2001). 10 µl of a yeast suspension grown overnight in liquid media was dropped in the centre of an YPD plate containing 0,3 % w/v agar and incubated at room temperature (20-25°C). Alternatively yeast was seeded in the centre of the plate with a toothpick from a streaked out culture. In order to investigate the ability of yeast cultures to grow invasively into agar-containing medium 10µl yeast suspensions were dropped on plates containing 2% agar. After allowing for yeast growth at 30˚C, cells were washed off the agar surface by vigorous rubbing with a gloved finger under running water, revealing only those cells that have grown into the medium.

5.3.6 Hydrophobicity assay

Yeast hydrophobicity was measured by assaying the partitioning of yeast cells between an aqueous and hydrophobic hydrocarbon phase following vigorous mixing (Rosenberg, 2006). Yeast cultures were de-flocculated by EDTA addition (50 mM) final concentration) after which the spectrophotometric absorbance was determined at a wavelength of 600 nm (measurement A). 1 ml of yeast culture was transferred to a micro centrifuge tube, washed and

re-suspended in phosphate, urea, magnesium (PUM) buffer consisting of 127,45 mM K_2HPO_4, 53,35 mM KH_2PO_4, 30mM urea and 0,8 mM $MgSO_4$ (Hinchcliffe *et al.*, 1985). Finally 100 µl *p*-Xylene (1,4-Dimethylbenzene) was added. Samples were vortex-mixed vigorously for 30 s and left to stand for 15 min, whereupon the spectrophotometric absorbance of the aqueous phase was determined at a wavelength of 600 nm (measurement B). The hydrophobicity index (HI) was defined as 1 - (B/A), where higher values reflect a yeast population of an increased hydrophobic nature. Finally all hydrophobicity index data was divided through the mean wt hydrophobicity to give relative hydrophobicity values.

5.4 Results

5.4.1 Yeast transformants used for this study

Σ1278b strains deleted in, or over-expressing either *FLO8* or *MSS11* were analysed in this study. Using the wild type strain as biological reference, we analysed these modified strains for altered adhesion phenotypes as well as for change in their transcriptome profiles. To over-express genes the following strategy was used: *FLO8* or *MSS11*, containing their native regulatory regions (promoter; terminator) were cloned into a yeast multicopy shuttle vector (2µ) and transformed into wild type yeast. These plasmids are maintained in high copy number in the cell thus resulting in increased dosage of the genes they carry. Increased dosage of *FLO8* and *MSS11* is assumed to lead to over-expression of these respective genes. This assumption is confirmed in this study since an increase of the corresponding transcript is observed for both genes. Similarly we observed the absence of transcript for both *FLO8* and *MSS11* in strains carrying deletions in these respective genes. For all experiments the wild type reference and deletion strains were transformed with empty multicopy vector (2µ), in order to compensate for possible artifactual effects on transcription caused by the introduced by this vector into yeast.

5.4.2 Cell wall associated hydrophobicity and -phenotypes of transformants used for transcription analysis

Σ1278b yeast seeded on low percentage agar plates display a characteristic growth pattern referred to as “mat” formation (Reynolds and Fink, 2001). Typically this growth form resembles a central “hub” with outwards pointing “spoke” structures. Strains unable to grow in this manner form smooth circular giant colonies of a smaller diameter than that of “mats”. Thus apart from having a distinct morphological appearance, “mat” formation enables yeast to rapidly grow across the plate surface (“sliding motility”). In light of the fact that all the strains in this study are isogenic to Σ1278b, we tested all transformants for the ability to develop “mats” (Figure 5.1 A and B). We were able to reproduce this phenotype in wild type Σ1278b, as previously reported (Reynolds and Fink, 2001). Deletions in either *FLO8* or *MSS11* resulted in the total abolishment of “mat” formation (Figures 5.1 A and B respectively), as has been previously reported for *MSS11* (Chapter 4). The over-expression of *FLO8* or *MSS11* did not appear to have a major effect on “mat” morphology, but reduced mat diameter to approximately 80% of wild type, as confirmed by at least three biological replicates (data not shown).

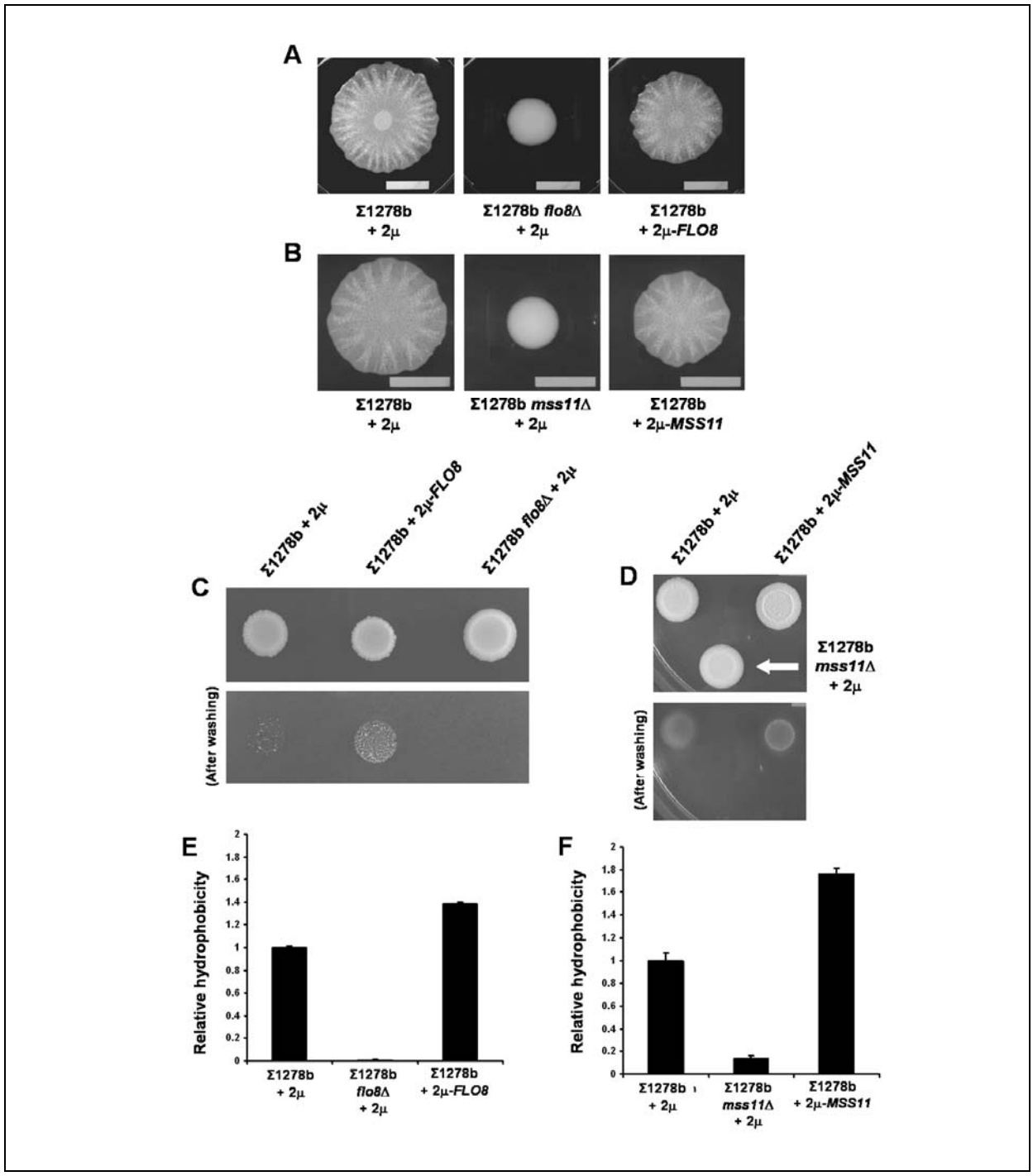

Figure 5.1 Phenotype assessments and the determination of cell wall hydrophobicity of wild type, deletion- and over-expression strains analysed in the transcriptome analysis. Shown is wild type and deletion strains that are transformed with empty vector (2μ) or wild type transformed with over-expression constructs (2μ-*FLO8* and 2μ-*MSS11*) (A and B) "Mat" forming ability of transformants. 10 μl yeast suspensions of the different transformed strains as indicated were dropped on 0,3% YPD agar plates and incubated for 10 days (A) and 7 days (B) at room temperature (Bar = 2cm). (C and D) Transformants show varying abilities to invade agar containing medium. 10 μl yeast suspensions of the different transformed strains as indicated were dropped on 2% YPD agar plates and incubated for 6 days at 30°C after which the plates were washed. Top panel shows total yeast growth and bottom panel invading cells as revealed by plate washing. Media consists of (C) SLAD and (D) YPD. (E and F) Cell hydrophobicity (as indicated by the specific hydrophobicity index) determined for transformants grown overnight to stationary phase at 30°C in liquid SCD media.

Apart from "mat" formation Σ1278b has been historically used as a laboratory yeast model system for agar invasion as well as the formation of pseudohyphae. The requirements for either Flo8p or Mss11p in invasive growth have been documented before (Gagiano *et al.*, 1999; Liu *et al.*, 1996), and here we confirm those findings. Similar to "mat" formation, deletion in either *FLO8* or *MSS11* leads to the total abolishment of wild type invasiveness (Figure 5.1 C and D). When over-expressed, both these genes confer a significantly enhanced invasive phenotype compared to wild type. As was observed previously, the increases and decreases in invasiveness appear very similar for the strains with modified *FLO8* or *MSS11* expression levels (Bester *et al*., 2006).

Finally the transformants were tested for their degree of cell wall hydrophobicity. Σ1278b is known to have a high degree of hydrophobicity in comparison to other strains such as S288c (Chapter 4). The respective gene deletions of either *FLO8* or *MSS11* lead to a significant reduction of hydrophobicity while their over-expression leads to a significant increase (Figure 5.1 E and F respectively). Furthermore the order of magnitude of *FLO8*- and *MSS11*-induced changes is very similar.

5.4.3 Whole genome transcription analysis of transformants

All yeast transformants were grown to mid exponential phase followed by total RNA extraction for transcriptome analysis. This was done in order to minimise inter-strain transcriptome differences due to the effect of growth phase discrepancies. In all cases the change in fold expression due to gene deletion or over-expression was determined using wild type data as reference. Four comparisons were performed in this study: *FLO8* deletion, *FLO8* over-expression, *MSS11* deletion and *MSS11* over-expression. The number of genes affected ≥1,5 fold in each of these comparisons is listed in Table 5.3.

Table 5.3 The total number of genes that display ≥1,5 fold expression change in response to either *FLO8* or *MSS11* deletion or -over-expression.

Strain comparison	Number of genes that display ≥1,5 fold change in expression		
	Down regulated	Up regulated	Total
FLO8 deletion	115	193	308
FLO8 over-expression	46	89	135
MSS11 deletion	197	55	252
MSS11 over-expression	621	334	955

5.4.4 *FLO* regulation

We investigated the effect of Flo8p and Mss11p on *FLO* expression in context of the existing knowledge that these factors regulate *FLO1* and *FLO11* expression. Shown in Table 5.4 is the expression data for *FLO* gene family members. *FLO1* is reported to be silent in the Σ1278b genetic background and indeed does not appear to be significantly regulated in any of the strains tested except for the clear exception of the *MSS11* over-expression strain. The combined expression data for *FLO1*, *FLO5* and *FLO11* obviously cannot be seen as a reflection of the expression of any of the individual genes as the Affymetrix® system is not able to differentiate sufficiently between these highly homologous genes. Furthermore no noteworthy results were obtained for *FLO10* regulation. Its transcription was induced in both *flo8*Δ and 2μ-

FLO8 strains, and fold change in the *mss11*Δ and 2μ-*MSS11* strains were not of significant magnitude. Only *FLO11* is significantly regulated in all four strains. In both deletion strains *FLO11* was repressed and similarly the gene was induced in both over-expression strains. Interestingly *FLO8* deletion leads to increased *FLO11* repression in comparison to the *mss11*Δ strain (~13 fold), whereas *MSS11* over-expression results in ~8 fold higher levels of *FLO11* transcripts when compared to the 2μ-*FLO8* strain.

Table 5.4 Fold change in *FLO* expression levels as the result of either *FLO8* or *MSS11* deletion or -over-expression. Positive values indicate induction and negative values repression. P-values are indicated in brackets.

Gene	Change in fold expression			
	***flo8*Δ**	**2μ-*FLO8***	***mss11*Δ**	**2μ-*MSS11***
FLO1	1.0 (0.411)	1.0 (0.229)	-1.0 (0.218)	6.4 (0.102)
FLO1/5/9[*]	1.2[*] (0.251)	1.0[*] (0.701)	-1.0[*] (0.653)	2.9[*] (0.019)
FLO10	1.6 (0.257)	1.0 (0.635)	-1.3 (0.516)	1.4 (0.361)
FLO11	-299.4 (0.068)	1.6 (0.067)	-23.2 (0.293)	12.4 (0.016)

[*]The Affymetrix® yeast 2.0 platform can not efficiently differentiate between transcripts corresponding to the highly homologous *FLO1*, *FLO5* and *FLO9* genes. The data shown is thus an uneven integration of the expression levels of all three genes, of which *FLO1* expression constitutes the largest part as previously shown by qPCR confirmation of the *MSS11* micro-array data (Chapter 4).

5.4.5 Gene ontology (GO) enrichment of genes that display ≥1,5 fold regulation in deletion and over-expression strains

In order to identify trends in gene function of the target gene groups listed in Table 5.3, these groups were enriched for GO functional categories as described in the Materials and Methods section (Table 5.5). Genes down-regulated in the *FLO8* deletion strain enriched for the biosynthesis of leucine (MIPS:01.01.11.04.01) as well as for transcription factor activity (GO:0003700). In the same deletion strain genes up-regulated enriched for the following metabolism related categories: the oxidation of fatty acids (MIPS:02.25), energy reserve metabolism (GO:0006112) and general metabolic function (GO:0008152). Furthermore enrichment was found for the response to stress (MIPS: 32.01; GO:0006950) and genes encoding plasma membrane- (GO:0005886) and cell wall components (GO:0009277).

Genes down-regulated in response to *FLO8* over-expression showed no significant enrichment. Up-regulated genes enriched for membrane transport (GO:0055085), stress response (MIPS:32.01; GO:0006950) and various metabolic process categories concerning energy reserves (MIPS: 02.19), catalytic activity (GO:0003824), carbohydrates (GO:0003824), and general metabolism (GO:0008152). Note that genes up-regulated in the *flo8*Δ and 2μ-*FLO8* strains both enrich for the stress categories MIPS:32.01 and GO:0006950 of which the following genes, *DDR2*, *HSP12*, *HSP42*, *PIR3*, *SSA1*, *STF2*, *TSL1* and *YGP1*, are up-regulated in both strains.

Repressed genes in the *mss11*Δ strain enriched for factors involved with the catalysis of metabolic processes (GO:0003824) as well as those involved with stress response (MIPS:32.01; GO:0006950), whereas genes up-regulated enriched for aldehyde metabolism (GO:0006081) or being components of the plasma membrane (GO:0005886).

Genes repressed upon *MSS11* over-expression form the largest subgroup and are particularly enriched for GO categories related to ribosome components, assembly and translation. Categories containing the vast majority of targets are defined for ribosomal proteins (MIPS:12.01.01), the binding of nucleic acid (GO:0003676) and RNA (GO:0003723), ribosome biogenesis (GO:0042254), the regulation of translation (GO:0006417) and for components that localise to the nucleolus (GO:0005730). Other categories enriched from this subgroup are for the modification of rRNA (11.06.01) and tRNA (11.06.02) as well as the metabolism of tRNA (GO:0006399). Note that in contrast with the high level of GO enrichment obtained for down-regulated genes in the 2μ-*MSS11* strain, no enrichment was observed for genes induced in the *FLO8* over-expressing strain.

The total number of genes induced in response to *MSS11* over-expression (334) is about half of the number that is repressed. These up-regulated genes are enriched for general metabolism (GO:0008152), carbohydrate metabolism (MIPS: 01.05) and the transport of sugars into the cell (GO:0015146). Furthermore categories were identified for the response to stress (MIPS: 32.01; GO:0006950) and pheromone (GO:0019236) as well as for components of the cell wall (GO:0009277).

Genes down-regulated in *mss11*Δ and up-regulated in 2μ-*MSS11* both enriched for the stress categories MIPS:32.01 and GO:0006950. The following genes from these categories were regulated in both strains: *ALD3*, *ATH1*, *GRE1*, *MGA1*, *PAI3*, *RTA1*, *TIR3*, *TIR4*, *TSL1*, *VHS3* and *XBP1*.

Table 5.5 GO enrichment of genes transcriptionally regulated ≥1,5 fold by either the single deletion or over-expression of *FLO8* and *MSS11*. Table sub-groupings indicate the different strain comparisons (gene deletion or over-expression) and the resulting effect on gene up (↑) or down (↓) regulation. In each case the total number of genes affected is indicated in brackets.

Category	Genes in category	p-value	Source
***FLO8* deletion ↓ (115)**			
biosynthesis of leucine	*LEU2 LEU1 BAT1 LEU4 LEU9*	7.89×10^{-8}	1
transcription factor activity	*TEC1 YAP6 GAT1 SUT1 MGA1 MET28 HMS2 PHD1 DAL80 ARG80 YNR063W CIN5 HMS1 CUP9*	1.46×10^{-5}	2
***FLO8* deletion ↑ (193)**			
oxidation of fatty acids	*POX1 POT1 TES1 SPS19*	7.95×10^{-5}	1
stress response	*NTG1 PAU7 YRO2 SSE2 HSP30 MRH1 CYC7 PAU2 PAU5 STF2 PIR3 TSL1 YGP1 DDR2*	2.56×10^{-4}	1
energy reserve metabolic process	*VPS8 SSA1 GIP4 ICS2 STP4 BDF2 MSH5 MTH1 HXT4 CWP1 CAT8 YNL144C YPL014W*	1.52×10^{-6}	3
metabolic process	*NTG1 BDH1 BDH2 GDH3 ETR1 YCR102C YEF1 FAA2 PNC1 POX1 CRG1 IMD2 POT1 TDH1 BAT2 YLR460C CYB2 YML131W SPS19 DSE4 PLB3 GRE2 YPL113C OYE3*	4.21×10^{-4}	3
response to stress	*SSA1 FRT2 PAU7 SSE2 HSP30 HSP42 HSP78 HSP31 PAU2 HSP12 PAU5 TSL1 YGP1 DDR2 GRE2*	5.76×10^{-4}	3
fungal-type cell wall	*SSA1 KNH1 DSE2 TDH1 PRY3 CWP1 PIR3 FLO10 SUN4 YGP1 DSE4 HPF1*	7.89×10^{-4}	4
plasma membrane	*GPB2 UIP3 PRM9 MST28 HSP30 MRH1 HSP12 SIP2 MEP1 TPO2 HXT4 QDR1 CWP1 PTR2 AQY2 YLL053C YPS3 FET4 FRE4 PLB3 PHM7 PNS1 TPO4 MCH5 TPO3 AQY1*	8.50×10^{-4}	4
***FLO8* over-expression ↓ (46)**			
	No significant enrichment		
***FLO8* over-expression ↑ (89)**			
stress response	*YRO2 STF2 DOG2 TIR3 XBP1 PIR3 HSP104 TSL1 ALD3 PAI3 YGP1 DDR2 HSP82*	2.02×10^{-7}	1
metabolism of energy reserves (e.g. glycogen, trehalose)	*GSY1 GSY2 TSL1 PGM2 HSP82 GPH1 GDB1*	8.64×10^{-6}	1
catalytic activity	*TKL2 GSY1 HXK1 PNC1 AMS1 NQM1 DOG2 FAA3 POT1 TDH1 YJR149W GSY2 CAR2 TSL1 YMR090W YMR196W GAD1 DCS2 GDB1*	2.20×10^{-5}	2
response to stress	*SSA1 HSP26 HSP42 HSP12 CTT1 WSC4 DOG2 TIR3 XBP1 HSP104 TSL1 ALD3 YGP1 DDR2 GRE1 HSP82*	5.18×10^{-9}	3
carbohydrate metabolic process	*SSA1 AMS1 NQM1 WSC4 HXT4 PGU1 PGM2 YNR034W-A GPH1 GDB1*	1.26×10^{-5}	3
metabolic process	*TKL2 PNC1 AMS1 NQM1 DOG2 CRG1 FAA3 POT1 TDH1 YJR149W PGU1 YMR090W ALD3 GDB1 HPA2*	2.04×10^{-4}	3
trans-membrane transport	*HXT7 STL1 HXT4 HXT5*	6.47×10^{-4}	3

Category	Genes in category	p-value	Source
***MSS11* deletion ↓ (197)**			
stress response	*MRK1 PAU2 MGA1 TIR3 XBP1 SDP1 YJL144W MSN4 TSL1 ALD3 PAI3 TIR4 VHS3 PTP2 ATH1*	8.89×10^{-5}	1
catalytic activity	*ACH1 ZTA1 TKL2 PCA1 GPM2 LPP1 DSF1 AGX1 GSY1 HXK1 AMS1 CPD1 GND2 DOG1 ATG7 SGA1 PFK26 POT1 YIR007W CAR2 TSL1 YMR196W GAD1 MLS1 BIO3 VHS3 PMA2 ATH1*	6.34×10^{-4}	2
response to stress	*FRT2 HSP26 MRK1 PAU2 HSP12 TOS3 RTA1 WSC4 TIR3 XBP1 MSN4 TSL1 ALD3 TIR4 GRE1 HAL1 ATH1*	6.87×10^{-5}	3
***MSS11* deletion ↑ (55)**			
aldehyde metabolic process	*DUR1,2 LEU2 YKL071W YLR460C*	1.47×10^{-4}	3
plasma membrane	*PRM9 MST28 FUI1 TAT1 HSP30 PTR2 AQY2 YLL053C FET4 PNS1 MCH5 SSU1*	3.28×10^{-4}	4
***MSS11* over-expression ↓ (621)**			
ribosomal proteins	*MAK5 MRPL32 IMG2 MRPL11 MAK21 YDR115W SSF2 MRPS28 RML2 SPB4 RPL22B MNP1 DBP3 MET13 RPL9A MRPL25 MRP13 NSR1 RSM27 MRP4 NMD3 RPS24B RPS14B RSM26 MRPL13 DBP7 MRPL20 DRS1 RPS0B DBP9 MRPL15 RPL6B MRPL39 MRPL33 RPS7B IPI3 MRPL19 RPL18B RSM19 DBP6 BRX1 NOP8 MRPL23 MRPL40 RRP15*	7.81×10^{-6}	1
rRNA modification	*SPB1 GAR1 IMP3 MPP10 CBF5 SIK1 IMP4 NOP58 RRP9*	1.21×10^{-5}	1
tRNA modification	*NCL1 TRM7 SLM3 TRM8 TRM1 PUS6 TRM5 TIS11 DUS3 DUS4 DUS1 SMM1 TRM112 TRM11*	2.17×10^{-5}	1
nucleic acid binding	*PIN4 YBL111C MAK5 DHH1 NRP1 FAL1 SWI5 MSS116 YDR514C MIG3 SPB4 DBP3 ROK1 NSR1 PXR1 NAM8 DBP8 AIR1 STH1 HCA4 NUC1 RPA12 DHR2 DBP7 DRS1 REX2 MSL5 TIS11 DBP9 DUS3 RGM1 HAS1 RNH201 MSK1 DBP2 WHI3 DBP6 BRE5 NOP12 REX4 TRM11 NOP8 RRP6 AZF1 DED1 DBP1 NAB3 MRD1 PZF1*	6.06×10^{-7}	2
RNA binding	*CCR4 NCL1 HEK2 PIN4 MAK5 SRO9 DHH1 NRP1 FAL1 TRM1 MSS116 RRP45 MRPS28 PUF6 SLF1 RML2 EDC2 LSM5 SPB4 DBP3 ROK1 EDC1 NSR1 PUS6 SKI6 SRB2 NAM8 GAR1 IMP3 DBP8 NMD3 NOP9 HCA4 UTP18 RPS14B DHR2 DBP7 DRS1 PUF3 MSL5 CBF5 DBP9 MRPL15 RPL6B HAS1 RLP7 RNH201 DBP2 WHI3 MPP6 DBP6 BRE5 ESF2 NOP12 TRM11 NOP8 RRP6 UTP23 PNO1 DED1 RRP12 DBP1 NAB3 MRD1 SMX3 PZF1*	3.58×10^{-4}	2
ribosome biogenesis	*MAK16 NCL1 FUI1 YBL054W YBL081W FUR4 HMT1 TAT1 YBR141C MAK5 MCM7 YBR238C ENP1 REI1 YBR271W CTP1 SRO9 SPB1 YCR016W YCR051W PWP2 RSA4 TSR1 YDL063C YDL129W NOP14 NRP1 TRM8 DAS2 FAL1 MAK21 RRP8 ARX1 TRM1 ATC1 PHM6 SSF2 UTP4 ATO3 UTP5 RRP17 DOT1 APT2 UTP6 RMT2 PUF6 YDR514C NSA2 FTR1 SPB4 RPL22B LOC1 SAD1 RSC8 DBP3 LSG1 NSA1 FLC3 SUA5 ROK1 NOP7 PPT1 UTP8 ENP2 ATF2 HGH1 SER2 ZPR1 SDA1 PXR1 YGR283C CIC1 TRM5 STE12 IPI1 GAR1 IMP3 DBP8 NMD3 RIX1 YIL064W RPS24B AIR1 ICE2 DPH1*	9.27×10^{-14}	3

Category	Genes in category	p-value	Source
	HCA4 UTP18 TRL1 SAP185 ALB1 RPA34 FAR1 RPS14B NUC1 MPP10 RPA12 LIA1 MRT4 MAE1 DHR2 LTV1 TPK3 DPH2 DBP7 MTD1 SRP40 DRS1 RIX7 PAM18 RLP24 YLR063W YLR073C DIP2 ZRT2 CBF5 SIK1 CDC123 UTP13 DBP9 DUS3 YLR407W UTP21 FPR4 LEU3 YML082W BUD22 ERB1 RRB1 YMR209C UBP8 TMA23 HAS1 ADE4 NIP1 YMR310C RLP7 YNL024C AQR1 IMP4 RPS7B DBP2 NCS2 AAH1 IPI3 RPA49 URK1 SMM1 DBP6 BRE5 NOG2 ESF2 NOP12 SPE2 BRX1 TRM11 TRM13 PPM2 NOP8 RRP6 UTP23 RKI1 PNO1 DED1 YTM1 RRS1 MCH5 SNU66 NOP58 RPA43 GDS1 YOR390W RRP12 NOG1 NOP53 TGS1 NEW1 YPL279C RPA135 RRP9 RRP15 NOC4 PZF1		
regulation of translation	*CCR4 HEK2 PIN4 AST1 YBR028C AAC3 LYS2 CTP1 NFS1 SIT4 DHH1 HEM3 LCB2 MSS116 HEM1 GPI8 SAC7 ERD1 SLF1 URA3 GLY1 PET122 OXA1 RPL22B SAD1 RSC8 MCM6 HXK2 GCD2 ZPR1 SRB2 AAP1 FSH1 FLX1 MRS3 FAR1 SPE1 YLL054C REX2 MSL5 NMA1 KAP95 SST2 ERG12 HDA1 YNL035C WHI3 ADE12 CAF40 MCK1 LYS9 BRE5 YOL014W DED1 ODC2 TIM18 GDS1 PMA2 TIM50 POC4 KES1 PPQ1 NHP6A*	4.56×10^{-5}	3
tRNA metabolic process	*AST1 FUR4 YBR197C RIF1 BUD31 YDL129W HEM3 ADE8 MTO1 ZPR1 AAP1 TIM54 TPK3 YLL054C NMA1 YNL035C BRE5 ARG8*	6.85×10^{-5}	3
nucleolus	*MAK16 ECM1 YBR141C MAK5 ENP1 SPB1 YCR016W PWP2 RSA4 LUG1 TSR1 NOP14 FAL1 RRP8 BFR2 SSF2 UTP4 UTP5 RRP17 UTP6 PUF6 LSM5 SPB4 LOC1 DBP3 NSA1 ROK1 NOP7 UTP8 ENP2 NSR1 PXR1 YGR283C CIC1 GAR1 DBP8 AIR1 NOP9 HCA4 UTP18 RPA34 MPP10 MRT4 DHR2 DBP7 SRP40 DRS1 RIX7 RLP24 FYV7 DIP2 CBF5 PWP1 SIK1 UTP13 DBP9 UTP21 FPR4 BUD22 ERB1 RRB1 TMA23 HAS1 RLP7 KRE33 DBP6 TRM112 NOG2 ESF2 NOP12 BRX1 REX4 NOP8 UTP23 PNO1 YTM1 NOP58 NOG1 NOP53 TGS1 TIF6 MRD1 RRP9 RRP15 NOC4*	1.00×10^{-14}	4
***MSS11* over-expression ↑ (334)**			
C-compound and carbohydrate metabolism	*PSK1 BDH2 PHO11 ACH1 YBR056W CSH1 RBK1 GPD1 DIA3 YDL124W AAD4 TPS2 GLO2 YAT2 ICL1 HSP12 HXK1 OCH1 UGA1 DOG2 DOG1 YHR210C MUC1 KTR2 PCK1 FMS1 ALD3 ERR3 IDP3 MLS1 GOR1 GCY1 SPR1 YPL088W PDH1*	2.92×10^{-9}	1
stress response	*NTH2 YRO2 NTH1 TPS2 PAM1 CYC7 TIR1 STF2 MGA1 DOG2 TIR3 XBP1 YJL144W PIR3 HSP104 PDR8 TSL1 ALD3 DDR48 PAI3 YGP1 TIR4 TIR2 VHS3 HSP82 ATH1*	1.19×10^{-7}	1
protease inhibitor	*YHR138C TFS1 PAI3 PBI2*	3.09×10^{-5}	1
pentose transmembrane transporter activity	*HXT7 HXT4 HXT2*	4.94×10^{-4}	2
response to stress	*FRT2 PAU8 GPD1 AAD4 NTH1 TPS2 HSP42 HSP78 HSP31 TIR1 SSA4 HSP12 CTT1 RTA1 DOG2 GRE3 TIR3 XBP1 PAU14 DAN1 DAN4 HSP104 PDR8 TSL1 ALD3 DDR48 YGP1 TIR4 TIR2 GRE1 HSP82 ATH1*	2.45×10^{-9}	3
metabolic process	*BDH2 GDH3 NTH2 YBR056W TKL2 DUR1,2 GPD1 NUS1 NTH1 YDR018C TPS2 URH1 PHM8 ICL1 AGX1 PNC1 AMS1 YGL185C NQM1 CRH1 DOG2 DOG1 CRG1 YKL107W URA1 PCK1 YMR084W YMR085W*	6.74×10^{-5}	3

Category	Genes in category	p-value	Source
	YMR090W ALD3 ADH2 YMR315W IDP3 GOR1 SPR1 YPL033C ATH1 HPA2		
response to pheromone	*PRM7 PRR2 STE2 AGA2 PRM8 PRM5 PRM10 PRM6 PRM4*	6.63×10^{-4}	3
fungal-type cell wall	*FLO9 FLO1 DIA3 TIR1 SPI1 AGA2 CRH1 TIR3 DAN1 DAN4 PIR3 YLR194C YGP1 TIR4 TIR2 SPR1 ATH1*	8.96×10^{-5}	4

1 - MIPS Functional Classification; 2 - GO Molecular Function; 3 - GO Biological Process; 4 - GO Cellular Component

5.4.6 Comparison of deletion- and over-expression strains

We further investigated how gene expression is affected by gene deletion and over-expression of *FLO8* and *MSS11.* Expression data from the deletion strains were plotted against those of the over-expression strains (Figure 5.2 and 5.3). In order to better visualise the data, genes whose expression has changed by a factor of above 10 are shown separately (5.2.A. and 5.3 A) from those that are less strongly affected (5.2 B and 5.3 B). In this representation, data points in the second quadrant correspond to genes up-regulated in response to over-expression and down-regulated in the deletion strains, whereas those in the fourth quadrant represents genes that are down-regulated in over-expression strains and up-regulated in deletion strains. Data from these sectors are of most obvious relevance since they display the most commonly expected direct response to the presence or absence of a transcriptional regulator. These genes were further analysed by means of GO enrichment, the results of which is listed in Table 5.6.

Genes down-regulated in response to *FLO8* deletion and up-regulated in response to *FLO8* over-expression, corresponding to the category that should most likely contain the genes that are directly controlled by Flo8p (assuming that the activity of this protein is limited to transcriptional activation) did not enrich for any GO categories. However several genes in this sector are regulated more than 10 fold. *CAR2* (↓18;↑1,7 fold) encodes for the enzyme L-ornithine transaminase which function in the metabolisms of arginine (Degols *et al.*, 1987). *HMS1* (↓70;↑1,4 fold), predicted to encode a helix-loop-helix transcription factor, induces elevated levels of pseudohyphae formation when over-expressed (Lorenz and Heitman, 1998). The other >10 fold regulated genes in this quadrant encode for cell wall components. *FLO11* (↓300;↑1,6 fold) encodes for the already mentioned cell wall adhesin and *TIR3* (↓11;↑2 fold) for a member of the Srp1p/Tip1p protein family with an as of yet undetermined cell wall function (Abramova *et al.*, 2001; Cohen *et al.*, 2001).

Genes down-regulated in response to *FLO8* over-expression and up-regulated when *FLO8* is deleted do not enrich for any GO categories. Included in this data set are *AQY2* and *YLL053c*. *AQY2* encodes a plasma membrane aquaporin that regulates cellular water homeostasis. In S288c, the strain used for annotation of the yeast genome, *AQY2* and *YLL053c* are described as two separate open reading frames adjacent to each other and transcribed in the same direction. In Σ1278b these two genes have been shown to form a single ORF (Carbrey *et al.*, 2001) explaining the identical expression patterns. Hence from here onwards we will only refer to this open reading frame as *AQY2*. Furthermore this quadrant contains two genes regulated more than 10 fold: *POX1* (↑22;1,3↓ fold), encoding an enzyme involved in fatty acid catabolism (Hiltunen *et al.*, 2003), and *HSP30* (↑34;1,3↓ fold), coding a plasma membrane component responsive to various environmental stresses as well as acting as a regulator of the

plasma membrane proton pump (H(+)-ATPase) (Piper *et al.*, 1997; Regnacq and Boucherie, 1993).

Genes down-regulated by *MSS11* deletion and up-regulated by over-expression enriched for the metabolism of energy reserves (MIPS: 02.19) as well as for various stress response categories (MIPS:32.01; MIPS:32.01.01; GO:0006950). Similar to *FLO8*, *TIR3* (↓8;↑8 fold), *FLO11* (↓23;↑12 fold) and *HMS1* (↓12;↑4 fold) were also falling in this category (Figure 5.4). Furthermore *TIR2*, another Srp1p/Tip1p protein family member, was down-regulated 1,4 fold in response to *MSS11* deletion and up-regulated 100 fold when *MSS11* was over-expressed.

Data from the fourth quadrant in Figure 5.3 B notably contains *AQY2* as well as the highly regulated *PTR2* (↑3;↓16 fold), which encodes a integral membrane situated peptide transporter (Island *et al.*, 1991).

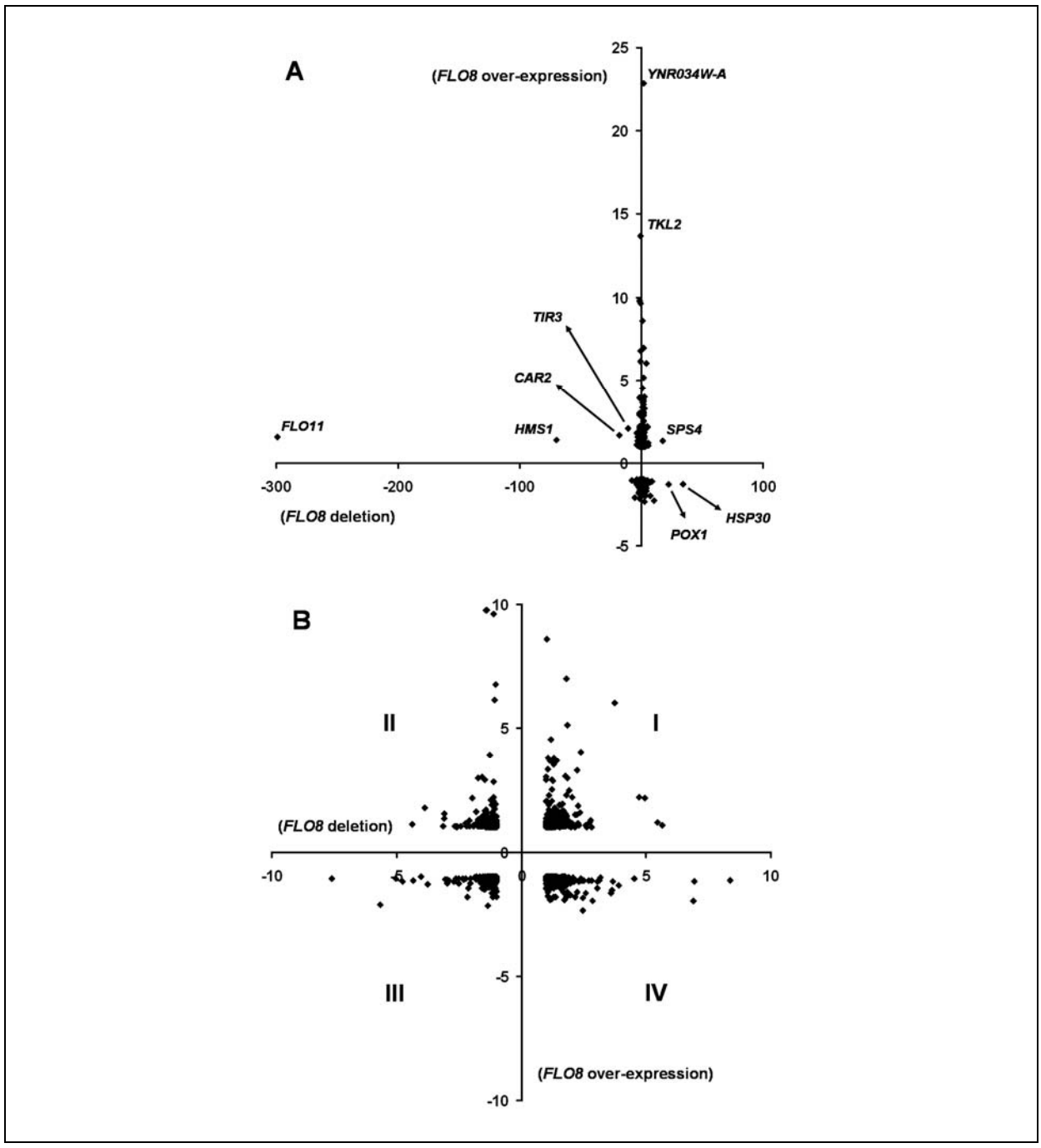

Figure 5.2 Scatter plot analyses of fold change in gene expression in response to *FLO8* deletion and -over-expression. Shown are either all data points (A) or those with a fold change of more than 10 fold excluded (B). Quadrants are indicated in (B) that correspond to genes up-regulated in both comparisons (I), down-regulated in both (III), or dissimilar regulated (II and IV). Genes up-regulated more than ten fold in at least one comparison are annotated with their respective gene names (A).

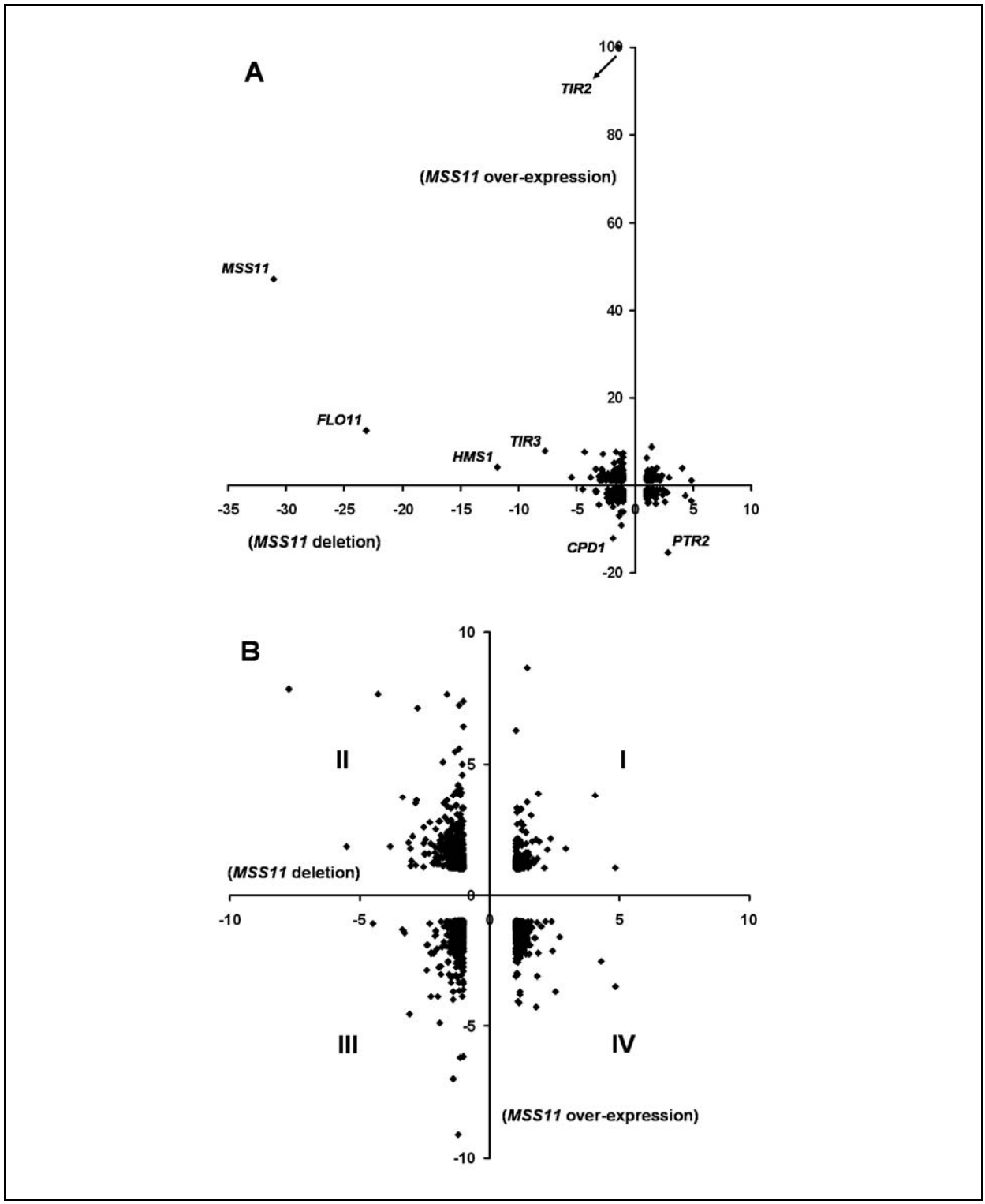

Figure 5.3 Scatter plot analyses of fold change in gene expression in response to *MSS11* deletion and -over-expression. Shown are either all data points (A) or those with a fold change of more than 10 fold excluded (B). Quadrants are indicated in (B) that correspond to genes up-regulated in both comparisons (I), down-regulated in both (III), or dissimilar regulated (II and IV). Genes up-regulated more than ten fold in at least one comparison (with the exception of *TIR3*; -8 and 8 fold change) are annotated with their respective gene names (A).

Table 5.6 GO enrichment of genes regulated ≥ 1,5 fold in response to the single deletion or over-expression of *FLO8* and *MSS11*. Indicated are the strains comparisons tested (gene deletion or over-expression) and the resulting effect in the form of gene up (↑) or down (↓) regulation. In each case the total number of genes affected is indicated in brackets.

Category	Genes in category	p-value	Source
***FLO8* deletion/over-expression ↓/↑ (11)**	(No significant enrichment)		
***FLO8* deletion/over-expression ↑/↓ (20)**	(No significant enrichment)		
***MSS11* deletion/over-expression ↓/↑ (91)**			
stress response	*MGA1 TIR3 XBP1 YJL144W TSL1 ALD3 PAI3 TIR4 VHS3 ATH1*	$6.95x10^{-5}$	1
metabolism of energy reserves(glycogen/trehalose)	*GIP2 GSY1 TSL1 PGM2 ATH1*	$3.06x10^{-4}$	1
oxidative stress response	*FRT2 TSA2 HSP12 GAD1 GRE1*	$9.0x10^{-4}$	1
response to stress	*FRT2 HSP12 RTA1 TIR3 XBP1 TSL1 ALD3 TIR4 GRE1 ATH1*	$3.35x10^{-4}$	3
***MSS11* deletion/over-expression ↑/↓ (18)**			
plasma membrane	*MST28 FUI1 TAT1 PTR2 AQY2 YLL053C FET4 MCH5 SSU1*	$1.02x10^{-6}$	4

1 - MIPS Functional Classification; 2 - GO Molecular Function; 3 - GO Biological Process; 4 - GO Cellular Component

5.4.7 Similarities and discrepancies in Flo8p and Mss11p mediated transcriptional effects

In order to compare the effects of the altered expression of both *FLO8* and *MSS11* on global transcription their respective deletion (Figure 5.4) or over-expression (Figure 5.5) data sets were plotted against each other. Thus for this analysis, data in quadrants I and III represent genes regulated in a similar manner (together regulated up or down) by the two factors, whereas quadrants II and IV contain data of genes regulated in an opposite manner. For the gene deletion plot quadrants containing data for communal down (III) or up (I) regulation show several genes significantly regulated. When comparing over-expressing strains, most regulated data points group in the sector for the up-regulation of genes in both strains (I). All quadrant data were GO enriched and the results are presented in Table 5.7. No significant enrichment was obtained for any quadrant that contained data of genes with dissimilar (e.g. ↓ and ↑) fold regulation (data not shown).

GO enrichment analysis identify genes down-regulated in both deletion strains as to contain genes that encode for transcription factors (GO:0003700), of which *HMS1* was the most regulated with 70 and 12 fold repression in response to *FLO8* and *MSS11* deletion respectively. *CAR2* (↓18;↓2 fold), *FLO11* (↓300;↓23 fold) and *TIR3* (↓11;↓8 fold) also displayed regulation more than 10 fold. Up-regulated genes in the two deletion strains were enriched for plasma

membrane components (GO:0005886), and the most strongly regulated genes were *AQY2* (↑7;↑4 fold), *HSP30* (↑10;↑5 fold) and *YLL053C* (↑34;↑5 fold). Also present in this sector were *POX1* (↑22;↑1,3 fold) as well as a gene reported to be specifically activated during the process of sporolation *SPS4* (↑17;↑4 fold) (Garber and Segall, 1986; Hepworth *et al.*, 1995).

Gene targets identified as to be down-regulated more than 1,5 fold in response to both *FLO8* and *MSS11* over-expression were *AQY2*, *CPD1* (↓1,3;↓12 fold) and *PTR2* (↓1;↓16 fold). *CPD1* encodes a cyclic nucleotide phosphodiesterase that process products of tRNA splicing (Nasr and Filipowicz, 2000).

Genes up-regulated in both over-expression strains enriched for stress response (MIPS:32.01), and more specifically the oxidative stress response (MIPS:32.01.01). Further categories identified were the pentose-phosphate metabolism pathway (MIPS:02.07) and sugar transport (GO:0055085). The following genes were regulated more than 10 fold in at least one of the strain comparisons: *FLO11* (↑1,6;↑12 fold), *TIR2* (↑1; 100 fold), *TKL2* (↑14;↑4 fold) and *YNR034W-A* (↑23;↑5 fold). *TKL2* encodes a transketolase involved in the pentose phosphate pathway (Schaaff-Gerstenschlager *et al.*, 1993). *YNR034W-A* is uncharacterised gene that gets activated by the stress transcription factors Msn2p and Msn4p (Lai *et al.*, 2005).

Table 5.7 GO enrichment of genes regulated ≥ 1,5 fold in response to *FLO8*/*MSS11* deletion or over-expression. The table contains sub-groupings indicating the genetic condition tested (gene deletion or over-expression) and the resulting effect in the form of gene up (↑) or down (↓) regulation. In each case the total number of genes affected is indicated in brackets.

Category	Genes in category	p-value	Source
***FLO8*/*MSS11* deletion ↓/↓ (38)**			
transcription factor activity	*YAP6 MGA1 PHD1 ARG80 CIN5 HMS1 CUP9*	1.62×10^{-4}	2
***FLO8*/*MSS11* deletion ↑/↑ (30)**			
plasma membrane	*PRM9 MST28 HSP30 PTR2 AQY2 YLL053C FET4 PNS1 MCH5*	1.41×10^{-4}	4
***FLO8*/*MSS11* over-expression ↓/↓ (17)**	(No significant enrichment)		
***FLO8*/*MSS11* over-expression ↑/↑ (61)**			
stress response	*YRO2 STF2 DOG2 TIR3 XBP1 PIR3 HSP104 TSL1 ALD3 PAI3 YGP1 HSP82*	1.95×10^{-8}	1
pentose-phosphate pathway	*TKL2 NQM1 SOL4 PGM2*	7.21×10^{-5}	1
oxidative stress response	*TSA2 HSP12 CTT1 GAD1 GRE1*	1.39×10^{-4}	1
transmembrane transport	*HXT7 STL1 HXT4 HXT5*	1.51×10^{-4}	3

1 - MIPS Functional Classification; 2 - GO Molecular Function; 3 - GO Biological Process; 4 - GO Cellular Component

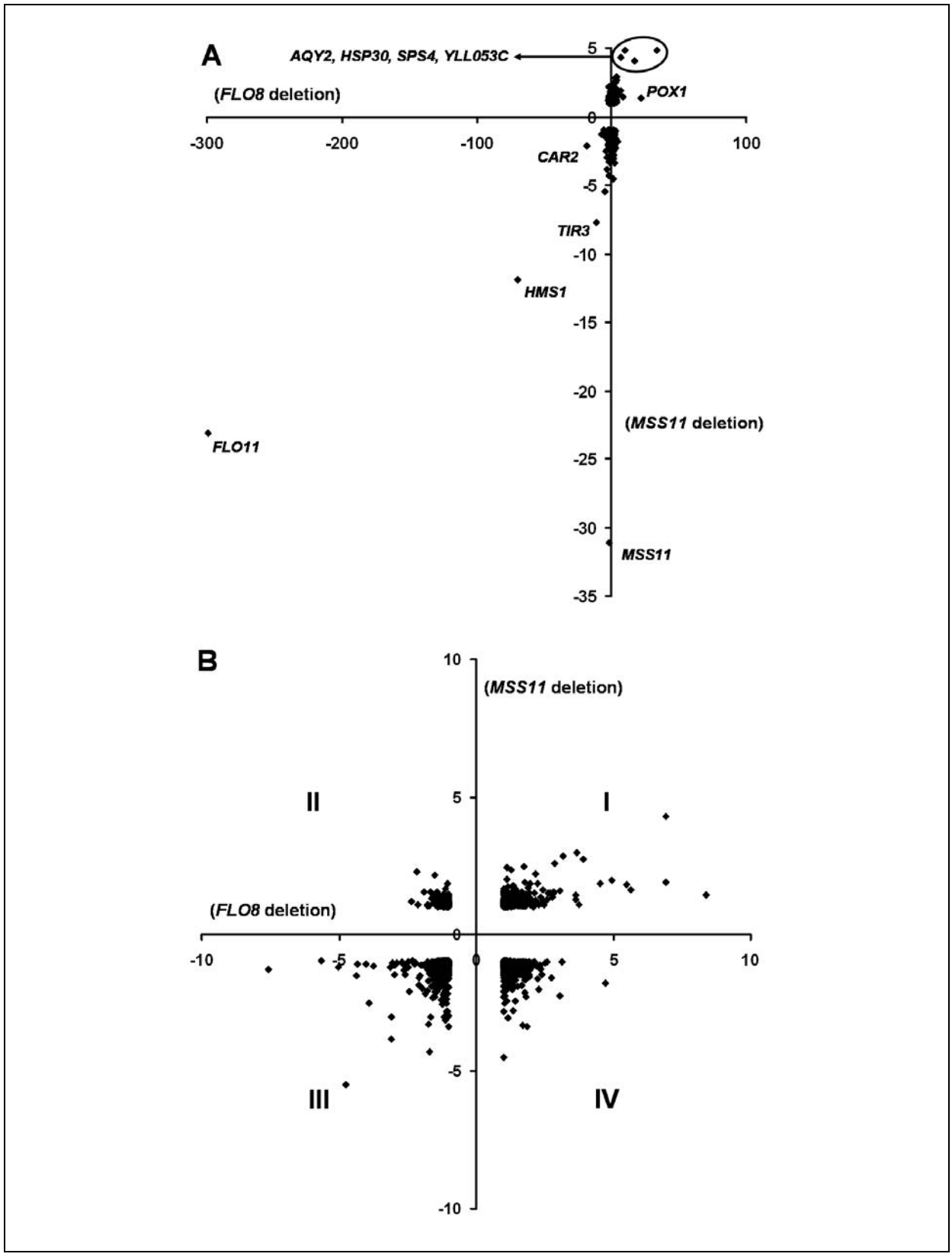

Figure 5.4 Scatter plot analyses of fold change in gene expression in response to either *FLO8* or *MSS11* deletion. Shown are either all data points (A) or those with a fold change of more than 10 fold excluded (B). Quadrants are indicated in (B) that correspond to genes up-regulated in both comparisons (I), down-regulated in both (III), or dissimilar regulated (II and IV). Genes up-regulated more than ten fold in at least one comparison are annotated with their respective gene names (A).

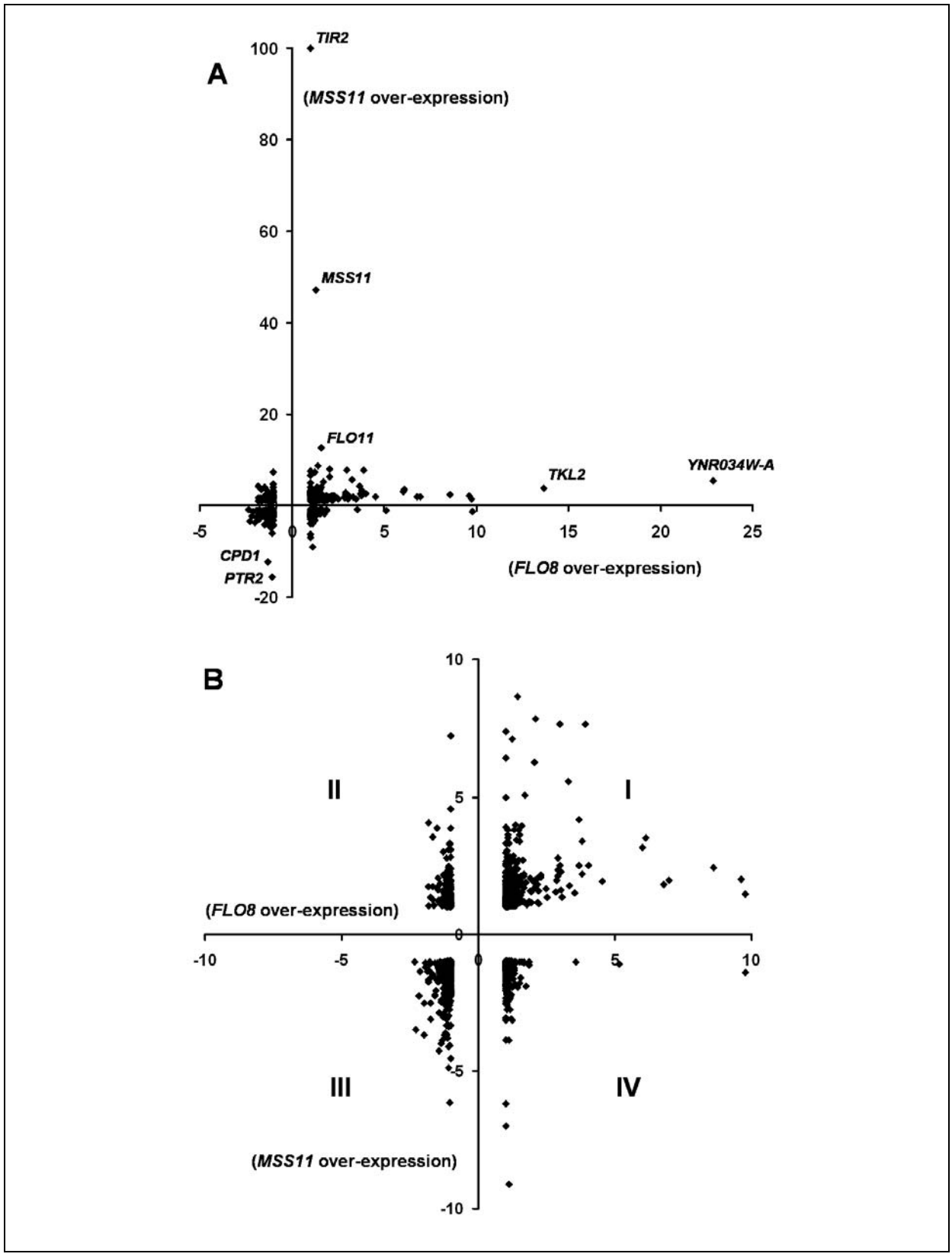

Figure 5.5 Scatter plot analyses of fold change in gene expression in response to either *FLO8* or *MSS11* over-expression. Shown are either all data points (A) or those with a fold change of more than 10 fold excluded (B). Quadrants are indicated in (B) that correspond to genes up-regulated in both comparisons (I), down-regulated in both (III), or dissimilar regulated (II and IV). Genes up-regulated more than ten fold in at least one comparison are annotated with their respective gene names (A).

5.4.8 Genes regulated in a similar manner by both *FLO8* and *MSS11* deletion and over-expression

Figure 5.6 contains a colour representation of genes that either show down-regulation in both deletion strains together with up-regulation in both over-expression strains (A) or up-regulation in both deletion strains with down-regulation in both over-expression strains (B). Note that this data set contains genes for which fold change was > 1,5 fold in only three of the four comparisons. Only the genes *YDR034W-B, TSA2, SNO4, ECM34, FLO11, TIR3* from the left panel and *KNH1, YDL129W, FET4, AQY2, YLL053C* from the right panel displayed more than 1,5 fold regulation in all four of the comparisons. Considering the magnitude of fold change *FLO11, TIR3* and *HMS1* are the most strongly co-regulated genes. This group also contains several transcription factors, in particular *YAP6, MGA1, XBP1, CIN5, HMS1* and *CUP9* (http://www.yeastgenome.org/). From Group B *AQY2* appears to be the most regulated.

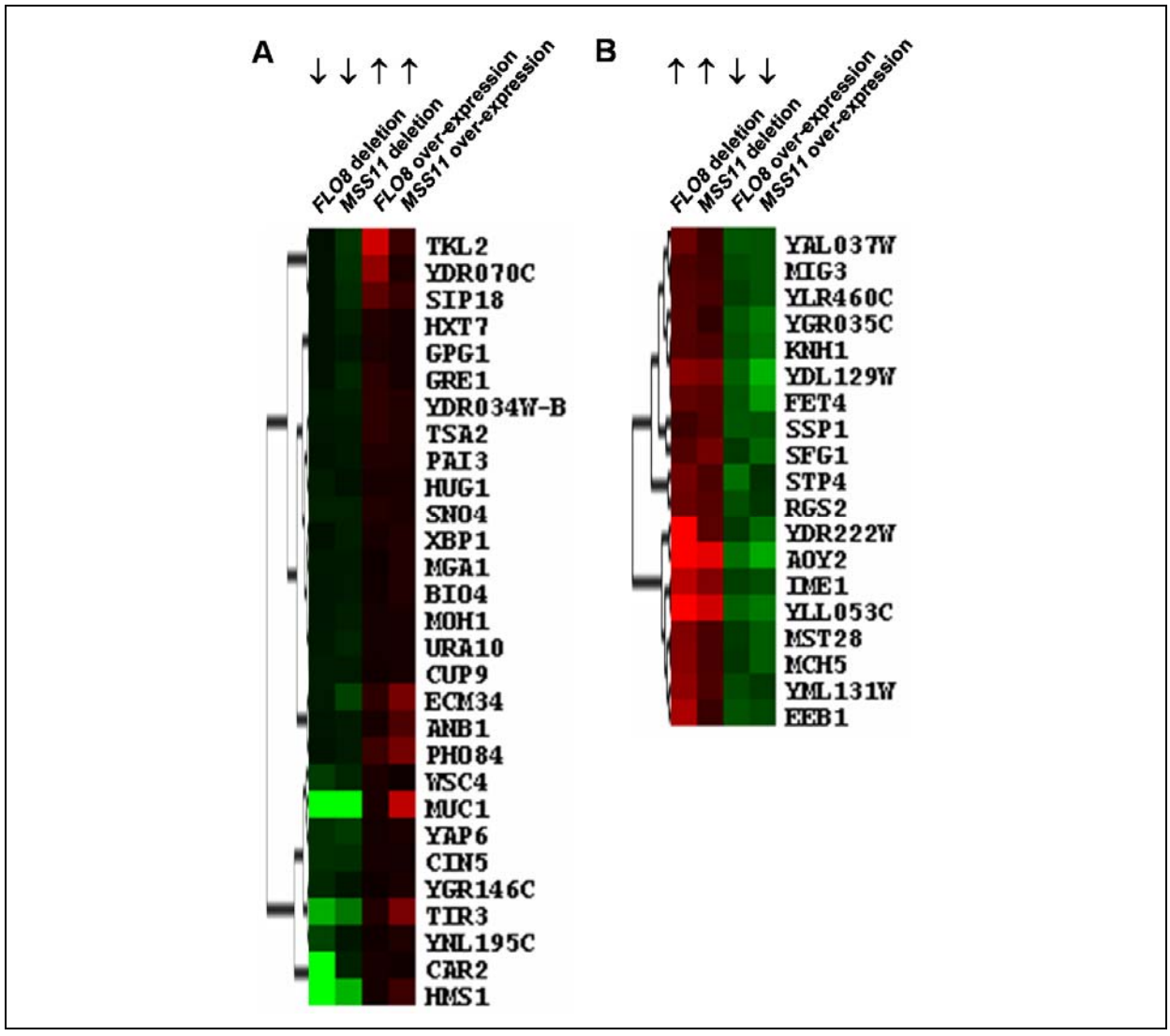

Figure 5.6 Colour display of genes down-regulated in the deletion strains and up-regulated in the over-expression strains (A) or induced in deletion strains and repressed in over-expression strains (B). The genes indicated display more than 1,5 fold regulation in at least three of the four comparisons. The degree of fold change in each panel (A or B) is normalised for the maximum and minimum values from the corresponding data set.

5.4.9 Superimposing the yeast transcription factor (TF) network on transcriptome changes identified in this study

Following the observation that genes down-regulated and up-regulated in *FLO8* and *MSS11* deletion and over-expression strains enrich for transcription factors, we further investigated the interrelationship between Flo8p and Mss11p and the already characterised transcription factor (TF) networks. For this purpose, groups of genes regulated ≥1,5 fold as a result of either gene deletion or over-expression were superimposed on these TF networks. All four transcriptome comparisons were processed in this manner, and the results is represented in Figures 5.7 (*FLO8* deletion), 5.8 (*FLO8* over-expression), 5.9 (*MSS11* deletion) and 5.10 (*MSS11* over-expression). *FLO8* and *MSS11* are included in all the projections in order to highlight their particular positions within the TF networks. Note that targets of regulation in these networks can either be transcription factors themselves or genes of other function. Genes with only a primary (direct) network linkage to either *FLO8* or *MSS11* were also included in this analysis. Figures indicate the interrelationship between genes as well as their respective fold regulation in response to the respective strain comparison.

This analysis identifies TF components that either exclusively interact with Flo8p or Mss11p, or interact with both of them. Shared factors that display significant regulation in at least one of the data sets include the transcription factors Cin5p, Mga1p, Ste12p and Tec1p, the cell wall adhesins Flo1p and Flo11p and Rmi1p, a protein involved with genome stability and that interacts with the RecQ helicase DNA complex (Chang *et al.*, 2005; Chen and Brill, 2007; Mullen *et al.*, 2005). *FLO11* is significantly regulated in all four comparisons, *CIN5* and *MGA1* in three of them, and *FLO1*, *RMI1*, *STE12* and *TEC1* in only one instance. The transcription factor Ste12p functions in both mating and invasive growth signalling pathways (Gancedo, 2001; Leberer *et al.*, 1997) with Tec1p acting cooperatively with Ste12p exclusively in the invasion pathway (Gavrias *et al.*, 1996; Madhani and Fink, 1997). Cin5p belongs to the Yap protein family of transcription factors (Fernandes *et al.*, 1997) and is involved in protein degradation (Sollner *et al.*, 2009), salt tolerance (Mendizabal *et al.*, 1998; Ni *et al.*, 2009), and various stress responses (Nevitt *et al.*, 2004b) such as for osmotic stress (Nevitt *et al.*, 2004a) and in response to exposure to the DNA cross linking drug cisplatin (Furuchi *et al.*, 2001) and the cell wall perturbing agent chitostan (Zakrzewska *et al.*, 2005). Mga1p shows similarity to heat shock transcription factors (Feroli *et al.*, 1997) and when over-expressed cause elevated levels of filamentation (Lorenz and Heitman, 1998).

With regard to unshared components this analysis further shows that Flo8p is connected to more components of the TF network than Mss11p across all four strain comparisons. Furthermore definite trends in gene function can be observed for genes with direct network connection to either Flo8p or Mss11p. As shown before *MSS11* over-expression leads to the suppression of genes involved in ribosome assembly (for review see Venema and Tollervey, 1999). Shown in Figure 5.10 are the genes that are regulated in this strain that have direct network connection to Mss11p. Of these genes the majority are down-regulated and reported to be involved with the process of ribosome assembly. They are the genes *DBP8* (Daugeron and Linder, 2001), *NSR1* (Lee *et al*., 1992), *NOP7* (Miles *et al*., 2005), *ENP1* (Chen *et al*., 2003), *MRD1* (Jin *et al*., 2002), and *REX4* (Eppens *et al*., 2002). Four Flo8p network connected genes are significantly regulated in all four strain comparisons and are either plasma or cell wall related: *AQY2*, *TIR3*, *KNH1*, encoding a protein involved in cell wall beta 1,6-glucan synthesis (Dijkgraaf *et al*., 1996) and *FET4*, which encode a low affinity Fe (II)

transporter (Dix *et al*., 1994). With the exception of *TIR3*, these genes are all up-regulated in deletion strains and down-regulated in over-expression strains. Furthermore Flo8p linked genes from Figure 5.10 enrich for the GO category "plasma membrane" (GO:0005886; p-value: 9.72×10^{-4}) containing *ATO3, FTR1, AQY2, SUR7, HXT2, FET3, FET4, RHO5* and *AQY1*. This group in addition contain genes that encode transcriptional repressors, *SFL1* (Conlan and Tzamarias, 2001) and *NRG2* (Kuchin *et al*., 2002), as well as putative and confirmed transcription factors, *HMS1* (Lorenz and Heitman, 1998), *YAP6* (Fernandes et al., 1997) and *PHO4* (Komeili and O'Shea, 1999).

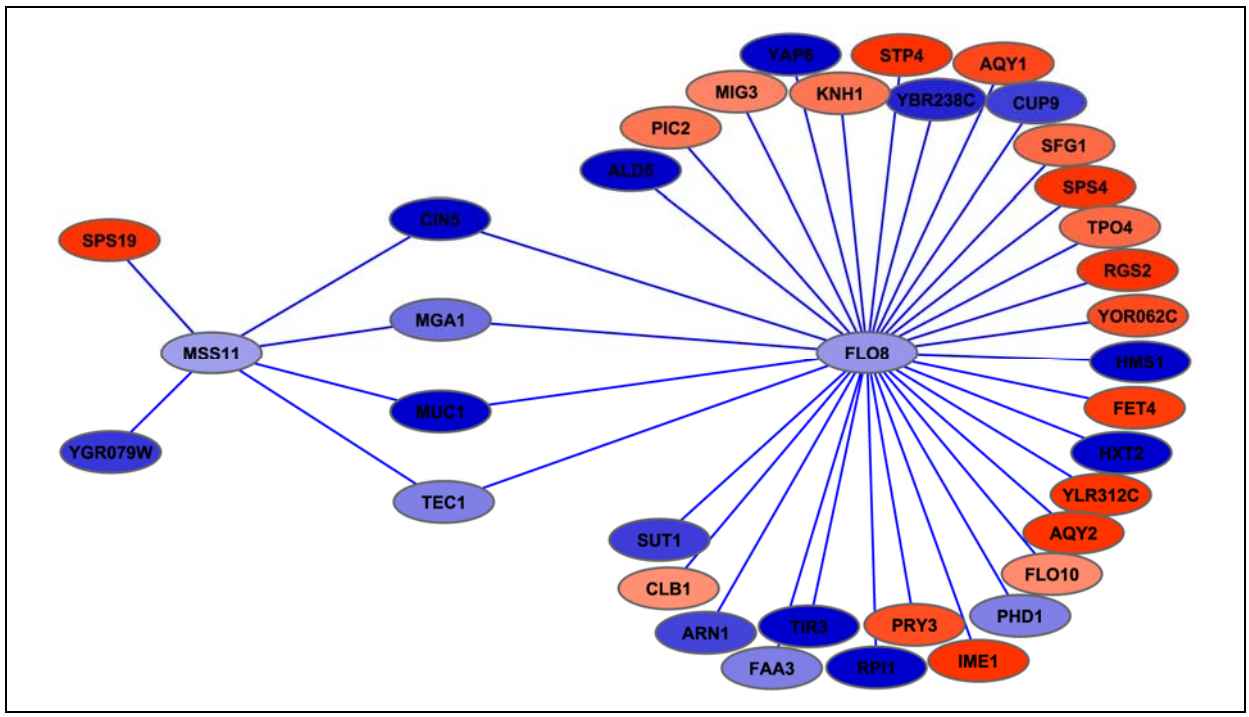

Figure 5.7 Genes forming part of the TF network that are regulated more than 1,5 fold in response to *FLO8* deletion. Lines indicate transcriptional regulation between transcription factors or their respective targets. Genes in red represent induction and those in blue repression. Note that *FLO11* is annotated as *MUC1* in this diagram.

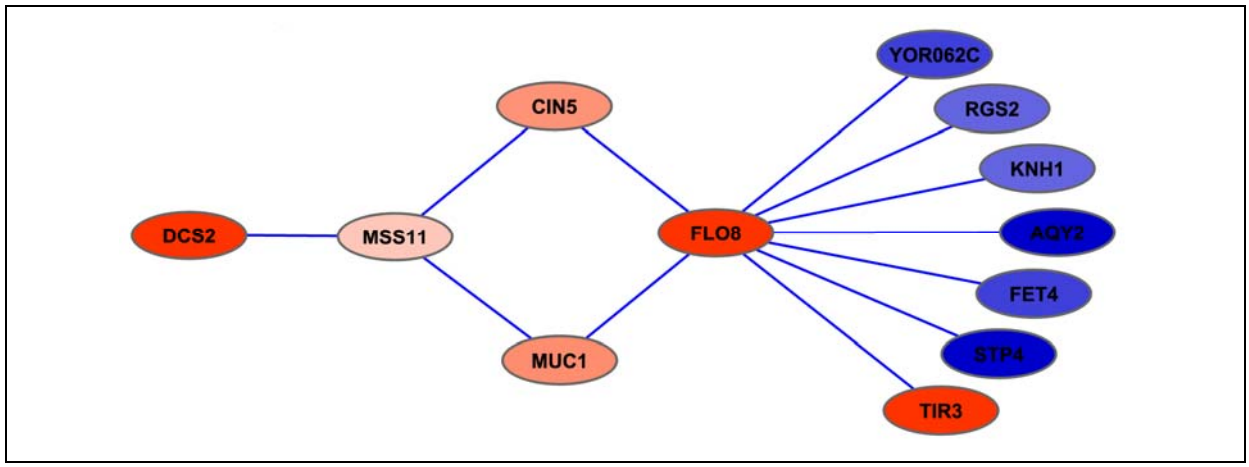

Figure 5.8 Genes forming part of the TF network that are regulated more than 1,5 fold in response to *FLO8* over-expression. Lines indicate transcriptional regulation between transcription factors or their respective targets. Genes in red represent induction and those in blue repression. Note that *FLO11* is annotated as *MUC1* in this diagram.

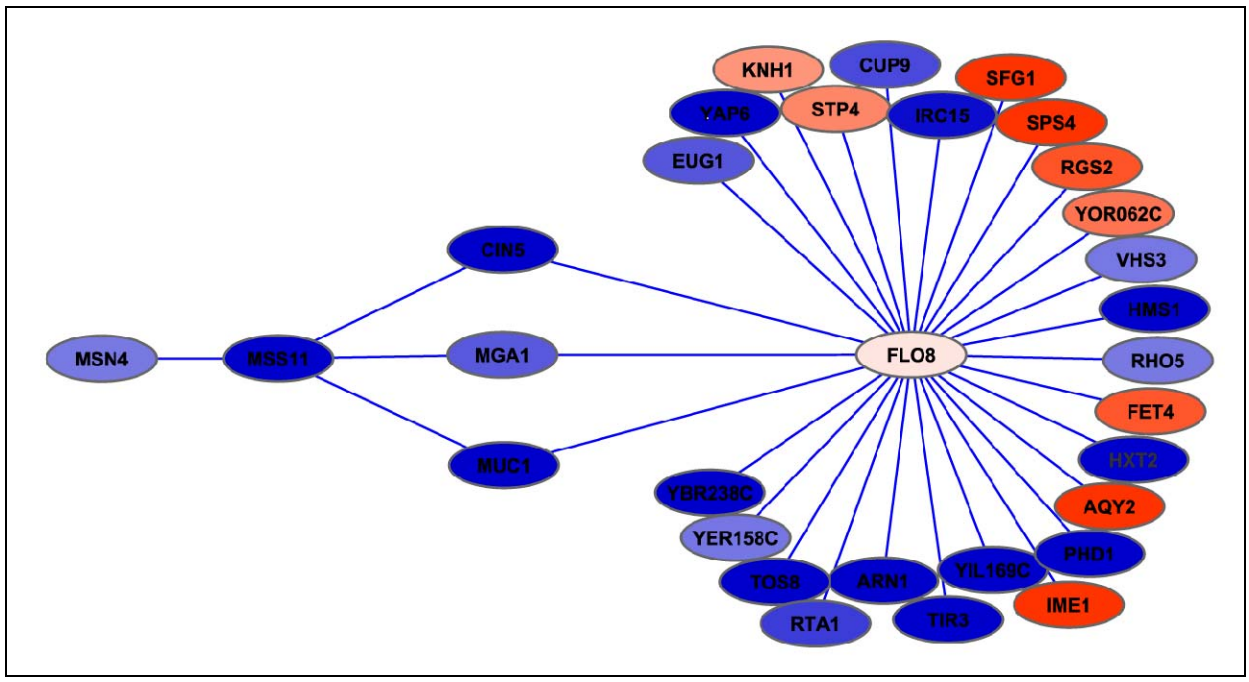

Figure 5.9 Genes forming part of the TF network that are regulated more than 1,5 fold in response to *MSS11* deletion. Lines indicate transcriptional regulation between transcription factors or their respective targets. Genes in red represent induction and those in blue repression. Note that *FLO11* is annotated as *MUC1* in this diagram.

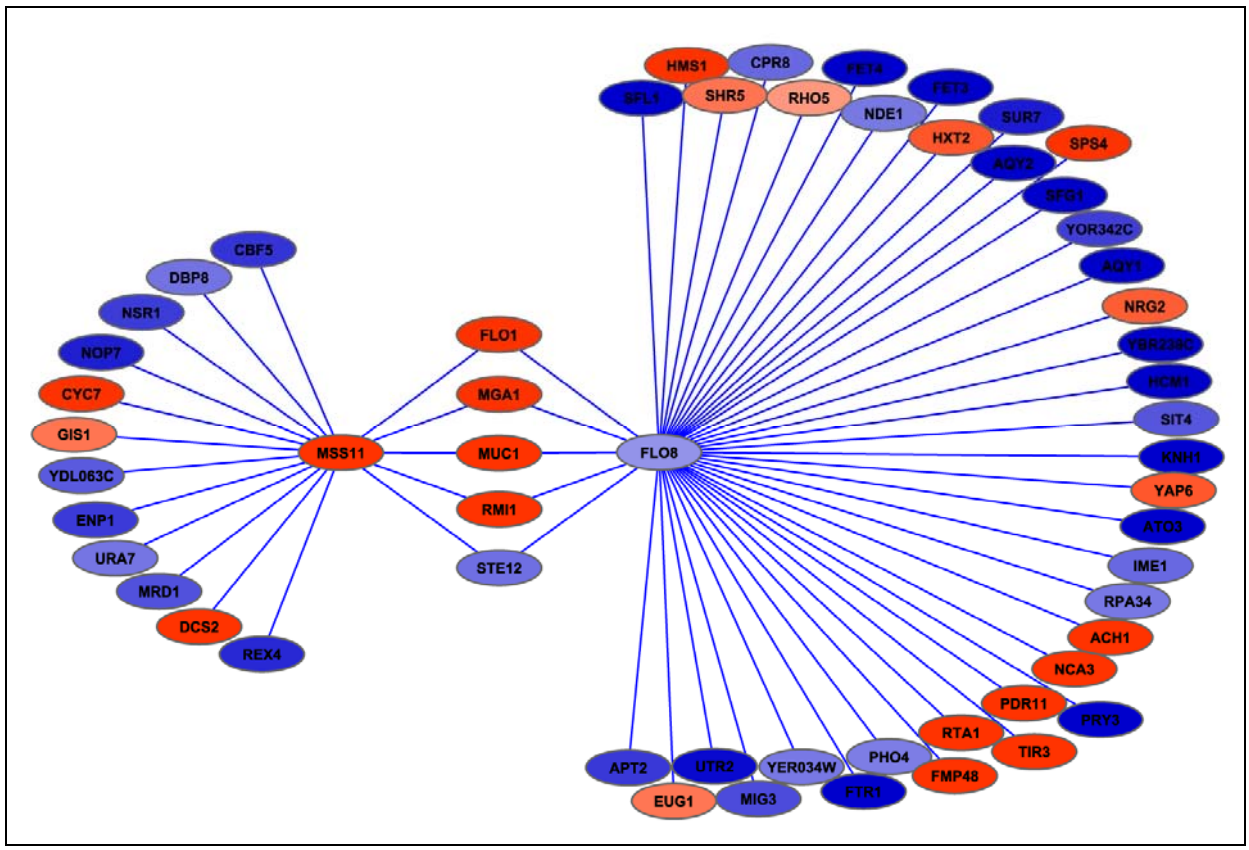

Figure 5.10 Genes forming part of the TF network that are regulated more than 1,5 fold in response to *MSS11* over-expression. Lines indicate transcriptional regulation between transcription factors or their respective targets. Genes in red represent induction and those in blue repression. Note that *FLO11* is annotated as *MUC1* in this diagram.

5.4.10 A regulatory system formed by Flo8p, Mss11p and shared TF network components

TF pathway projections on transcription data show that Flo8p and Mss11p share the transcription factors Cin5p, Mga1p, Rmi1p, Ste12p and Tec1p. TF network data on direct promoter binding shows that these transcription factors form a complex inter-regulatory network with both Flo8p and Mss11p (Figure 5.11). Many of these factors not only regulate the expression of other transcription factors, but also auto-regulate their own expression (Cin5p, Mga1p, Ste12p and Tec1p). Furthermore some TF components serve predominantly as targets for other factors (Cin5p, Mga1p, Mss11p and Rmi1p), while others function in the activation of other factors (Ste12p and Tec1p). Flo8p appears to fall in both these categories.

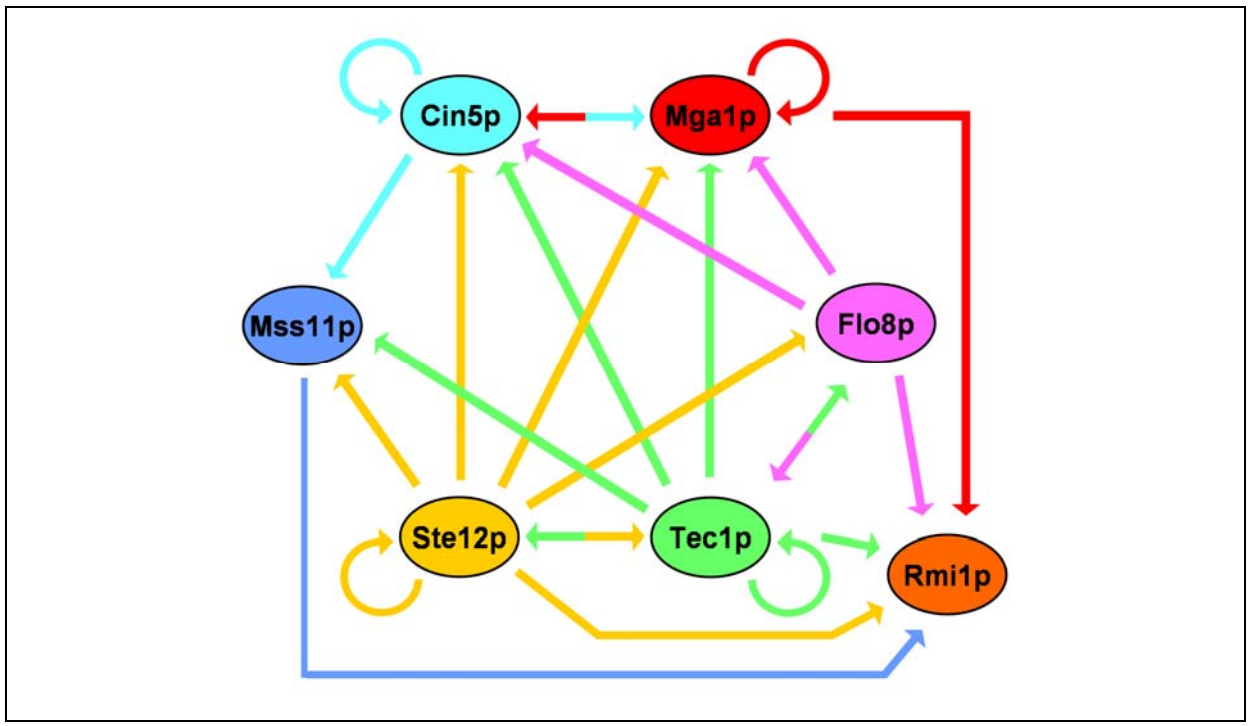

Figure 5.11 The transcription network between Cin5p, Flo8p, Mga1p, Mss11p, Ste12p and Tec1p. Arrows represent transcriptional action on target gene(s) as shown by direct biochemical published findings. Data was obtained from the online application YEASTRACT on Sept 02, 2009 (Monteiro *et al.*, 2008; Teixeira *et al.*, 2006).

5.5 Discussion

5.5.1 Flo8p and Mss11p regulate Σ1278b cell wall phenotypes in a similar manner with *FLO11* as the main target

The phenotypes flocculation, "mat" formation and agar invasion are defined by cell wall protein composition and associated cell wall characteristics such as outer cell wall hydrophobicity. Deletion and over-expression studies of *FLO8* and *MSS11* show that they encode for factors that (1) are required for such cell wall phenotypes to be displayed, (2) enhance or otherwise modify these processes and (3) appear to affect these phenotypes in a similar, nearly identical manner.

Gene deletion leads to a decrease in hydrophobicity and over-expression to an respective increase. Furthermore it is interesting to note that the over-expression of these genes results in a hyper-invasive phenotype while causing the extent of "mat" motility to be partially inhibited. The Flo protein family member Flo11p is the dominant factor required for both "mat" formation (Reynolds and Fink, 2001) and invasive growth (Lambrechts *et al.*, 1996; Lo and Dranginis, 1998). Cell wall hydrophobicity has also been reported to be dependent on Flo11p (Barrales *et al.*, 2008), although later research showed that other Flo proteins can influence hydrophobicity as well (Govender *et al.*, 2008; Van Mulders *et al.*, 2009). Recent evidence shows that invasive growth displays an additional lesser requirement for another flocculation protein, Flo10p, and that the deletion of certain *TIR* genes leads to a slight decrease in cell hydrophobicity (Chapter 4). However Flo11p still appear to be the dominant adhesin controlling these cell wall phenotypes and -characteristics. Furthermore transcriptome analysis shows that *FLO11* is the most regulated *FLO* gene and target gene for both Flo8p and Mss11p. Although other cell wall genes are also significantly regulated by both these factors, such as *TIR3*, they do not appear to significantly impact on the tested phenotypes (Chapter 4). The data also show that *FLO1*, which has been reported to be silenced in Σ1278b, is expressed in the *MSS11* over-expression strain, suggesting that high levels of Mss11p are able to suppress this repression.

5.5.2 Similarities and discrepancies in transcriptome changes mediated by Flo8p and Mss11p

While the impact of Flo8p and Mss11p on *FLO11* has been extensively characterised previously, the aim of this study was to contribute to our understanding of specific roles these proteins play in transcriptional activation. Although high levels of Mss11p thus appear to have the most drastic effect on global transcription, few of these targets appear to be linked to Mss11p itself via direct or indirect TF network interactions. In previous transcription factor network analyses (see Materials and Methods) Flo8p has been reported to bind to the promoters of 227 genes, whereas the number of genes for MSS11p has been estimated to be 65. GO enrichment of these Flo8p-target genes shows that they encode for cell wall (GO:0005618; p-value: 1.68×10^{-5}) and plasma membrane (GO:0005886; p-value: 2.34×10^{-6}) components, facilitate hexose transport (GO:0008645; p-value: 9.27×10^{-6}) and are involved in various metabolic processes involving carbohydrates (GO:0005975; p-value: 2.21×10^{-4}), phosphorus compounds (GO:0006793; p-value: 1.82×10^{-5}) and energy reserves (GO:0006112; p-value:2.14×10^{-4}). The 65 genes the promoters of which is reported to be bound by Mss11p

enrich for location in the nucleolus (GO:0005730; p-value: $1.10x10^{-4}$) and for the processes of ribosome biogenesis (MIPS:12.01) and rRNA processing (MIPS:11.04.01).

These observations appear to in some degree confirm our analysis. Indeed, similar enrichment trends were observed in performing GO enrichment on the sets of genes affected in the transcriptome analysis. Overall, the modification of *FLO8* and *MSS11* expression led to changes in stress response, metabolism and cell wall component genes with the exception of *MSS11* over-expression which resulted in the very significant repression of ribosome biogenesis and translation-related genes. Both these factors repress *AQY2* when over-expressed and induce *AQY2* transcription when deleted. Mss11p also appears to regulate stress related gene expression.

Furthermore, *FLO11*, *HMS1* and *TIR3* are down-regulated in both the *flo8*Δ and *mss11*Δ strains, while these genes are up-regulated in the respective over-expression strains. It is remarkable that both these factors regulate these three genes in a similar manner, all of which encode for proteins either shown or hypothesised to affect cell wall related phenotypes. As mentioned before Flo11p is the dominant adhesin involved in the phenotypes tested in this study, whereas no function has been yet attributed to Tir3p. Hms1p has been shown to control the Flo11p-phenotype of pseudohyphae formation (Lorenz and Heitman, 1998) and is hypothesised to function as a DNA binding transcriptional activator (Robinson *et al*., 2000). This would suggest that Flo8p and Mss11p regulate adhesion phenotypes by regulating multiple cell wall gene targets in a similar manner.

Using less stringent data selection, in which genes were selected to have a fold regulation of ≥1.5 fold in only three out of the four strain comparisons, Flo8p and Mss11p together appear to regulate various transcription factors in the same above mentioned manner. The combination of TF network and expression data sets further affirms the role of Mss11p in the negative regulation of ribosome biogenesis. This analysis further shows that regardless of strain comparison, Flo8p is the direct interacting partner for regulated genes with functions related to the cell membrane, cell wall and transcriptional repression or activation.

5.5.3 Possible cooperative action of Flo8p and Mss11p within the TF network

Transcript analyses from this study show that both the deletion of *FLO8* and *MSS11* leads to the down-regulation of *CIN5* and *MGA1*. As can be seen from Figure 5.11 Flo8p could accomplish this by direct binding to the respective promoters of these genes in order to repress them, whereas there are no existing data suggesting that Mss11p is able to act via the same direct mechanism. Alternatively Flo8p and Mss11p mediated repression of *CIN5* and *MGA1* could be mediated through more elaborate TF network interactions or even mediated through the action of more general cellular interaction networks.

5.5.4 Hypothesised mode of action for Mss11p in regulating gene expression

Clearly Flo8p and Mss11p regulate common targets of which the most notable are *AQY2*, *FLO11*, *HMS1* and *TIR3*. The mode of action of this regulation is likely mediated by complex interactions of these factors within the TF network. Our transcriptome analysis may suggest that Flo8p functions as a transcription factor, whereas Mss11p appears to function either as a TF or as a target of transcription activation systems. Also, although it cannot be ruled out as a Mss11p

specific pleiotropic effect, this study provides evidence that Mss11p affects ribosome biogenesis and translation. Various previous findings support a possible link between the regulation of ribosomes, translation and *FLO11* expression. The ribosomal components Asc1p (Valerius *et al.*, 2007) as well as RPS2A and RPS2B (Strittmatter *et al.*, 2006) was shown to be required for efficient transcription and translation of *FLO11*. Furthermore the translation machinery components Caf20 and Dhh1 were shown to be required for the efficient translation of *STE12* mRNA, in turn ultimately affecting *FLO11* expression (Ka *et al.*, 2008; Park *et al.*, 2006). Prinz *et al* (2007) showed that the mRNA binding protein, Mpt5p, represses the mRNA levels of *STE7* and *TEC1*, encoding for components that function in the MAPK signalling pathway in part controlling *FLO11* expression. Using the same over-expression system as described in this study, GFP-tagged Mss11p localizes to distinct and multiple spots within the nuclear interior (data not shown) (Bester, 2003). This tagged version of Mss11p conferred similar invasion as the untagged version, suggesting that the tagged version acts with the same mechanism in at least the activation of *FLO11* (data not shown). This specific intra-nuclear localization pattern was never shown to correlate with nucleolar location. Nonetheless, if Mss11p location overlaps with the nucleolus, the site of the initiation of ribosome assembly (Fromont-Racine *et al.*, 2003), it would correlate with the effect of *MSS11* over-expression on multiple ribosomal assembly and -component encoding genes. More likely the specific localisation pattern may correlate with various sites on the genome where telomere silencing controls gene expression. In contrast to tagged Mss11p, GFP tagged Flo8p localizes throughout the nuclear interior, more typical for a transcription factor (Bester, 2003; Pan and Heitman, 2002).

5.6 Acknowledgements

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Chapter 6

Research results IV

"Mat" formation by an industrial wine yeast strain as modulated by controlled *FLO11* expression

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Chapter 6: RESEARCH RESULTS IV

"Mat" formation by an industrial wine yeast strain as modulated by controlled *FLO11* expression

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6.1 Abstract

Saccharomyces cerevisiae has for centuries been associated with the food and beverage industry. Through repeated historic selection practices numerous "wild" yeast strains have become "domesticated", and industrial strains today appear phenotypically significantly different from the original wild yeast strains found in natural environments. Several of such changes appear to have affected cell wall related properties of strains, and in particular the ability to switch to filamentous growth and to form multi-cellular communities. In its "natural" environment wild yeast, often challenged by rapidly changing nutrient availability, form multi-cellular structures thought to aid in yeast survival and the search for nutrients (Gemino *et al*., 1992; Palkova and Vachova, 2006). Multi-cellular structures however come with a metabolic expense. Thus the constant cultivation of yeast in a nutrient rich environment typically results in the loss of multi-cellular growth behaviours, which are correlated with the silencing, modification or loss of cell adhesion encoding genes. Here we report on the ability of industrial wine yeast strains to form biofilm-like structures or "mats". The data show that the strain BM45 has retained its ability to form such structures on low agarose plates. "Mat" formation has been reported to be dependent on the Flo11p adhesin, a cell wall anchored, highly glycosylated protein, involved with a variety of cell wall associated phenotypes. We show that "mat" morphology is indeed responsive to different expression levels of *FLO11* in this industrial wine yeast strain. BM45 has been reported to confer a superior "mouth-feel" to wine, possibly due to the release of mannose rich glycoproteins (mannoproteins) into the surroundings during alcoholic fermentation. The screening of wine yeast for the ability to form "mats" may serve as a screening tool for the large scale identification of yeasts with similar oenological potential.

6.2 Introduction

The yeast *Saccharomyces cerevisiae* has traditionally been viewed as a single cell organism. However substantial evidence suggests that *S. cerevisiae* strains, when not growing under conditions of nutrient abundance and preferred physical parameters such as temperature and humidity, form multi-cellular structures with distinct recognisable features (Kuthan *et al.*, 2003). Many lines of evidence suggest that these structures contain differentiated forms of yeasts, providing some community members with a better chance of survival while others are sacrificed in the process (Palkova and Vachova, 2006). "Domesticated yeasts" are being cultivated under favourable conditions for long periods of time and have typically lost the ability to form such multi-cellular structures unlike their "wild yeast" counterparts isolated directly from nature. However,

yeast strains can frequently recover this ability, and such recovery appears to be associated with sustained cultivation under nutrient limiting conditions (Gimeno *et al.*, 1992). It has also been shown that such events are regulated on a genome level by epigenetic regulation (Halme *et al.*, 2004) and genomic recombination events (Verstrepen *et al.*, 2005).

Various multi-cellular growth forms, which appear to be linked to specific cell wall-related adhesion phenotypes, have been identified. Historically, the first such recognised process was flocculation, defined as the asexual, reversible and calcium-dependent aggregation of yeast cells to form flocs (Bony *et al.*, 1997). Studies in laboratory yeast show that flocculation is mainly dependent on the flocculation protein family member Flo1p (Teunissen and Steensma, 1995). Furthermore the expression of the normally silenced *FLO5* and *FLO9* genes also induces flocculent behaviour (Govender *et al.*, 2008; Van Mulders *et al.*, 2009). A variety of other closely related adhesion phenotypes have been attributed to yet another Flo protein, Flo11p. They include the formation of multi-cellular pseudohyphae (Lambrechts *et al.*, 1996), the ability to invade solid agar media (Lambrechts *et al.*, 1996) and the formation of distinct rough ("fluffy") colonies on plates (Kuthan *et al.*, 2003). Furthermore Flo11p is required for biofilm-like phenotypes such as "Mat"- (Reynolds and Fink, 2001) and "flor" formation (Ishigami *et al.*, 2004; Ishigami *et al.*, 2006) as well as the adhesion to surfaces such as glass in a model flow cell system (Purevdorj-Gage *et al.*, 2007) or to polystyrene in microtiter plates (Reynolds and Fink, 2001). "Flor" formation, associated with the maturation process in sherry production, involve the clumping together of hydrophobic yeasts that capture respiratory gasses and rise to the surface to form a thick yeast layer ("velum") at the air-liquid interface (Martinez *et al.*, 1997). The observation of "Mat" formation has thus far been limited to laboratory strains and -conditions and is observed as the distinct growth pattern of yeast seeded on low percentage agar plates. These "Mat" forming yeast strains rapidly grow outwards resembling a central "hub" with outwards pointing "spokes" that appear distinctly different from control yeast that form smooth macro-colonies of a smaller diameter.

All of the above mentioned adhesion growth forms are thought to impact on yeast survival and / or to enable nutrient scavenging under limiting conditions. These behaviours are however energetically expensive for the cell and require the activation of specialised cellular programmes. Thus in yeast being cultivated under more favourable growth conditions these programmes are suppressed or silenced.

S. cerevisiae wine yeast strains are primarily selected for their ability to rapidly ferment grape must to dryness in order to produce wine of good and reliable organoleptic quality. Any process that would result in the premature ending or delay (referred to as "stuck" and "sluggish" fermentations respectively) in fermentation can have a serious negative impact on this process. Premature yeast flocculation is viewed as one of the factors contributing to the abovementioned problem fermentations (Verstrepen *et al.*, 2003). Such flocs sediment more quickly when compared to free suspended single cells and tend to settle into a dense yeast layer at the bottom of the fermentation vessel. This creates conditions where the majority of cells do not have efficient access to fermentable sugars. Thus due to historical strain selection strategies that aimed to avoid such risks, virtually all commercial wine yeast strains are non-flocculating strains.

The yeast cell wall contains an outer layer of mannose rich glycoproteins (mannoproteins) that may be released into wine during fermentation by wine yeasts. Released mannoproteins have been reported to make various positive contributions to wine quality such as improved "mouth feel"

and the protection against the formation of unwanted protein haze or tartaric acid crystallisation (Caridi, 2006). Indeed Gonzalez-Ramos *et al* (2008) showed that by the use of a wine yeast strain genetically modified for increased mannoprotein release, wine with increased protein stability can be produced.

Here we analyse three non-flocculent strains, the laboratory strain FY23 and two commercial wine yeast strains, BM45 and VIN13, for their ability to form "mats" under laboratory conditions. Only BM45 displays typical "mat" growth with the other two strains forming smaller and smooth colonies. Further investigation reveals that this phenotype in BM45 is significantly altered by modified *FLO11* expression. We further explore the possibility of using "mat" formation as a screening tool for the first level identification of industrial yeasts with high mannoprotein release.

6.3 Materials and Methods

6.3.1 Strains and growth conditions

Saccharomyces cerevisiae strains used in this are listed in Table 6.1. FY23 is a commonly used haploid laboratory strain isogenic to the S288c background, whereas VIN13 and BM45 are diploid wine yeast strains with uncharacterised genotypes. Included in this analysis is BM45 strains with *FLO1*, *FLO5* and *FLO11* placed under transcriptional control of either the alcohol dehydrogenase II promoter ($ADH2_p$) or that from the heat shock protein 30 ($HSP30_p$). For instance strain "BM45-F1A" refers to the BM45 strain in which the native *FLO1* promoter has been replaced by the *ADH2* promoter. These strains were constructed by a promoter replacement strategy as described previously (Govender *et al.*, 2008; Govender et al., 2009). Strains were cultivated at 30°C on YPD plates consisting of 1% (wt/vol) yeast extract, 2% (wt/vol) peptone and 2% (wt/vol) glucose.

Table 6.1 *S. cerevisiae* strains used in this study.

Strain	Relevant genotype	Source or reference
BM45	Commercial wine yeast strain (unknown genotype)	Lallemand, Canada
BM45-F1A	$FLO1_P$Δ::*SMR1-ADH2*$_P$	Govender *et al*., 2009
BM45-F1H	$FLO1_P$Δ::*SMR1-HSP30*$_P$	Govender *et al*., 2009
BM45-F5A	$FLO5_P$Δ::*SMR1-ADH2*$_P$	Govender *et al*., 2009
BM45-F5H	$FLO5_P$Δ::*SMR1-HSP30*$_P$	Govender *et al*., 2009
BM45-F11A	$FLO11_P$Δ::*SMR1-ADH2*$_P$	Govender *et al*., 2009
BM45-F11H	$FLO11_P$Δ::*SMR1-HSP30*$_P$	Govender *et al*., 2009
FY23[a]	MATa leu2 trp1 ura3 flo8-1	Winston *et al.*, 1995)
VIN13	Commercial wine yeast strain (unknown genotype)	Anchor Yeast, South Africa

[a]FY23 is isogenic to S288c

6.3.2 Assessment of "Mat" formation

To ability of yeast strains to form spreading growth mats (also referred to as "biofilm" formation or "sliding motility") on plates was determined as described previously (Reynolds and Fink, 2001). Yeast grown on YPD plates were seeded with a sterile toothpick in the centre of YPD plates containing 0,3 % w/v agar and incubated at room temperature (20-25°C). Plates of either 65 mm or 90 mm diameter were used in this study as indicated. The rate of "Mat" formation was determined by measuring the diameter of "mats" of at least three independent biological repeats using the same reference point on the plate.

6.4 Results

6.4.1 Different inherent "Mat" forming abilities of two industrial wine yeast strains

The growth on low percentage agar of the laboratory yeast strain FY23 and two industrial wine yeast strains, BM45 and VIN13, was assessed. FY23 is a commonly used haploid laboratory strain isogenic to the S288c genetic background and carries a nonsense point mutation in its copy of the *FLO8* gene (referred to as the *flo8-1* allele). Flo8p acts as a transcriptional activator of *FLO11* explaining the inability of this strain to form "mats" as reported before (see Chapter 4). Thus FY23 was used as a negative control for "mat" formation in this analysis. Figure 6.1 displays the results of this analysis. Only the growth morphology of the BM45 strain were in accordance with the reported visual appearance of "mat" formation, as it formed the rapidly outwards growing central "hub" with outwards pointing "spoke" structures. VIN13 grew as smaller smooth and circular macro-colonies, very similar in appearance to the colonies formed by FY23. The only difference was the slightly larger size of Vin13 colonies, which is most likely due to different growth speeds of these two strains. Indeed it is generally known that laboratory yeasts show slower growth in comparison to industrial yeast strains which have been selected for efficient fermentation processes. In addition, diploid strains tend to grow more vigorously than haploids.

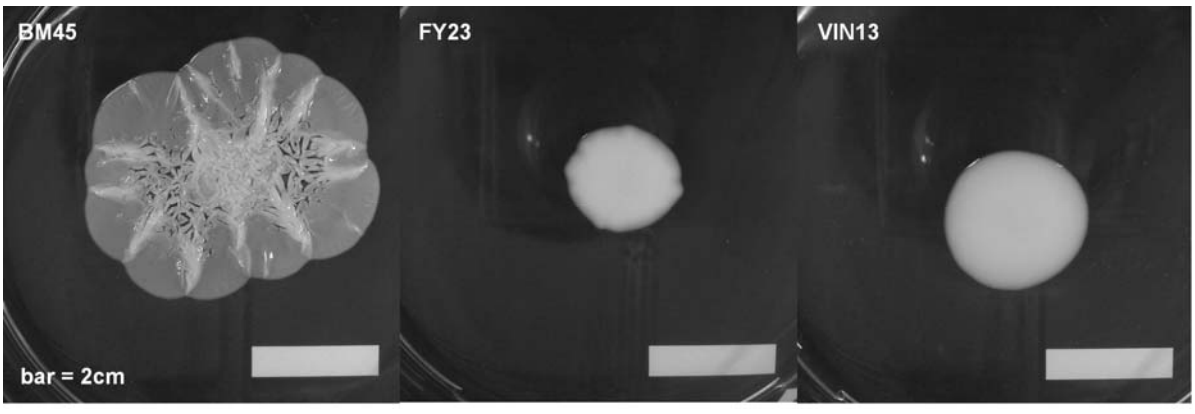

Figure 6.1 "Mat" forming ability of BM45, FY23 and VIN13 parental strains seeded on YPD (0,3% agar) after growth of 7 days. Plates used are 65 mm in diameter and bar corresponds to 2 cm.

6.4.2 Different *FLO11* expression profiles leads to distinct "Mat" formation phenotypes in BM45

We tested the effect of controlled *FLO* expression on the "mat" forming ability of BM45. For this purpose strains in which the flocculation genes *FLO1*, *FLO5* and *FLO11* are placed under control of either the *ADH2* or *HSP30* promoters were tested. These strains, with the parental strain as reference, were seeded on low percentage agar plates and incubated for 14 days (Figure 6.2). The rate at which "mat" formation occurred was measured for at least three independent biological replicates up to 12 days post-seeding (Figure 6.3).

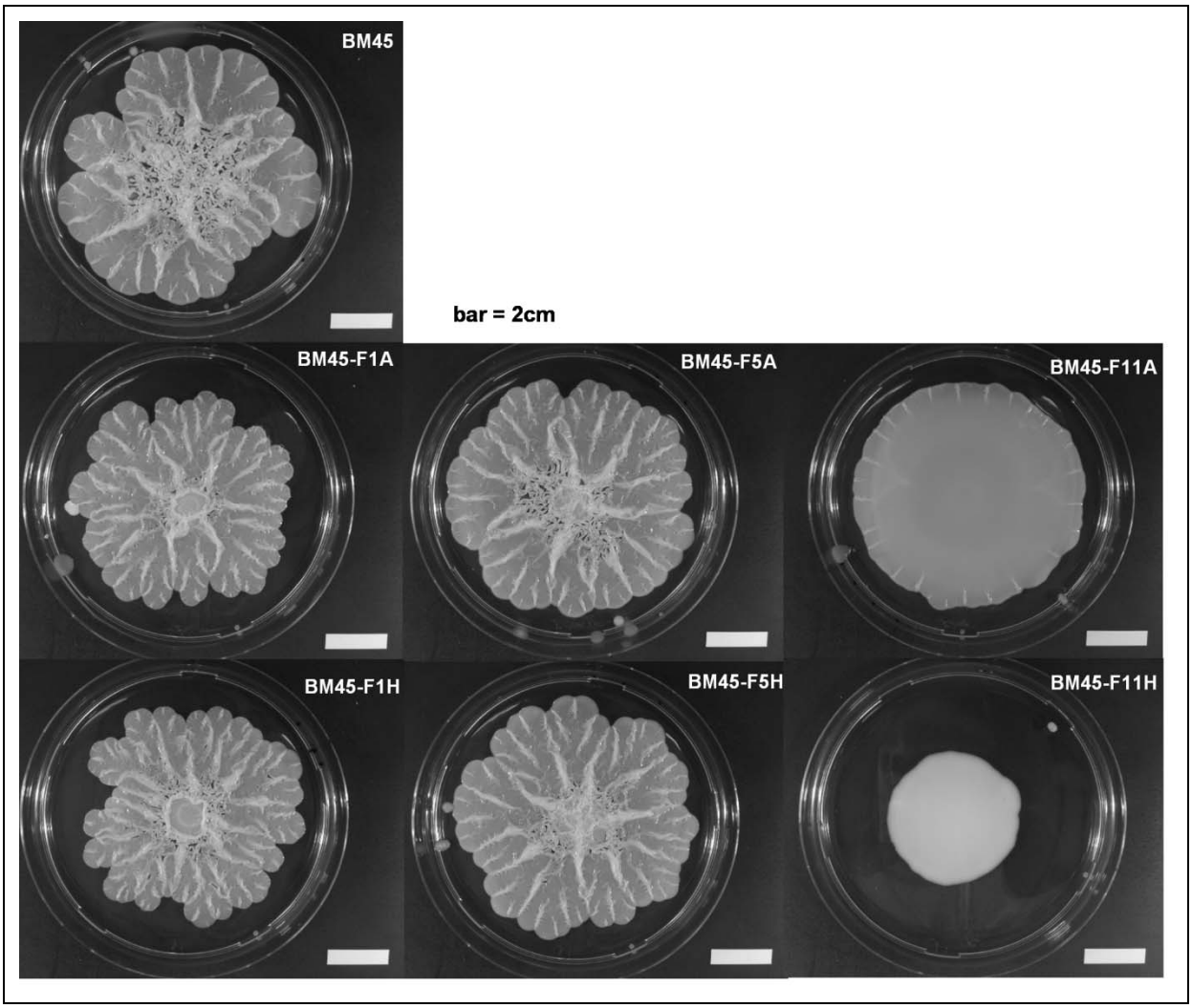

Figure 6.2 "Mat" formatting ability of BM45 parental and modified strains seeded on YPD (0,3% agar) after growth of 14 days. Plates used are 90 mm in diameter and bar corresponds to 2 cm.

With regard to morphology as well as size, "mats" formed by strains BM45-F1A, BM45-F1H, BM45-F5A and BM45-F5H appeared nearly identical to wild type BM45. The small differences observed between these strains, especially with regard to the outer "mat" appearance, are most probably negligible as this area displays a high level of variation between biological repeats of the same strains (data not shown). Furthermore no significant differences in "mat" size are observed as shown by the measurement of growth diameter (Figure 6.3).

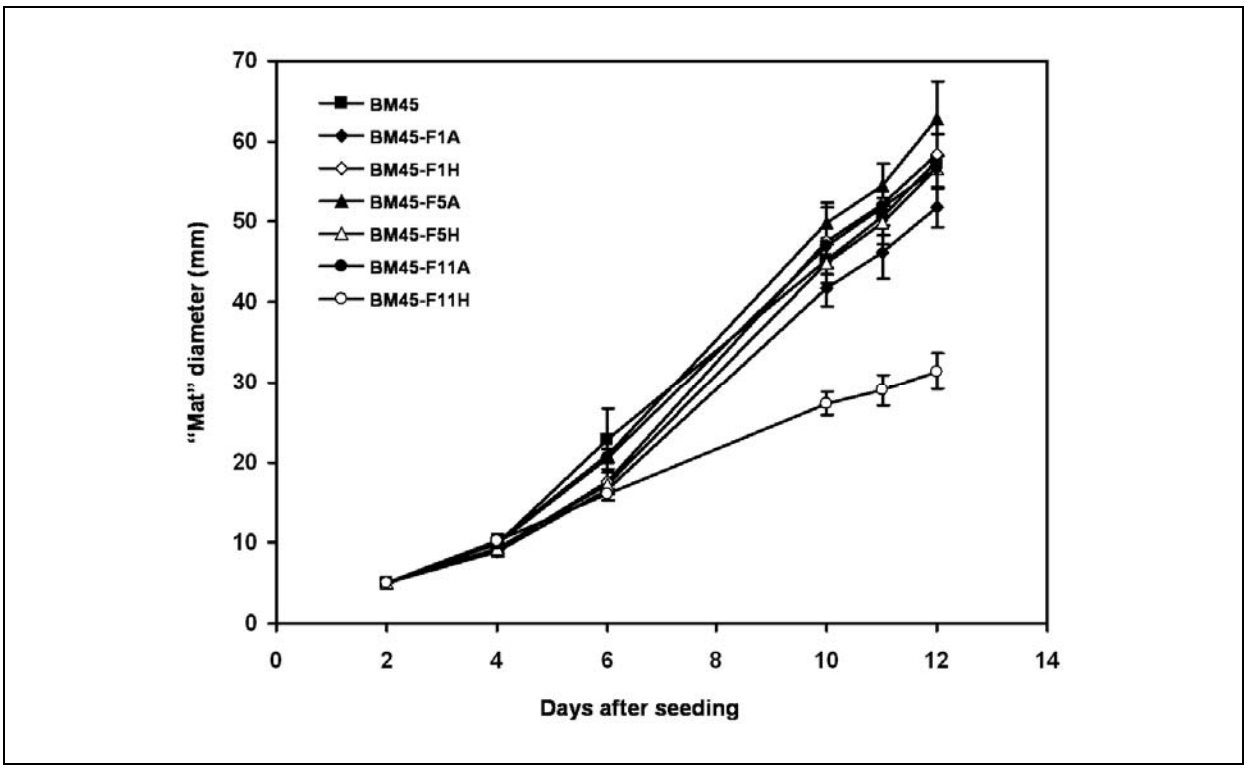

Figure 6.3 Measurement of "Mat" formatting ability of BM45 parental and modified strains seeded on YPD (0,3% agar) over time as indicated. Data show the average of at least three independent biological replicates and the error bars represent the standard deviation (STDEV).

Strains BM45-F11A and BM45-F11H grow distinctly different when tested under the same growth conditions (Figure 6.4). In the case of BM45-F11H the growth is smooth with a significantly smaller diameter, the same appearance as yeast unable to form "mats". On the other hand BM45-F11A forms a fully developed "mat" in terms of size diameter, but its growth mostly lacks the distinct "hub with spokes" morphology typical of wild type growth. However undulations resembling "spokes" on the very outside rim of the "mat" are observed on closer inspection. Even upon prolonged incubation (~1month) strain BM45-F11H was unable to form any structures that resemble "mat" formation (data not shown).

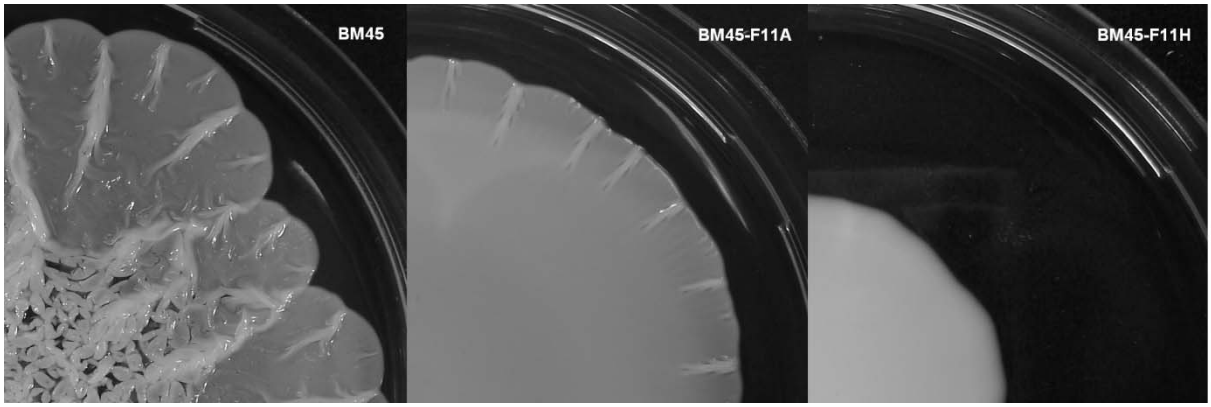

Figure 6.4 Magnified images of "mats" formed by parental BM45, BM45-F11A and BM45-F11H after 14 days of growth on YPD (0,3% agar). Shown is a quarter of the plate to highlight morphological detail of "mat" formation.

6.5 Discussion

Some wild yeast strains could loose their ability to participate in adhesin related phenotypes when adapting to sustained conditions of nutrient excess. This phenomenon has been illustrated for a wild strain repeatedly cultivated on rich media in the laboratory (Kuthan *et al*., 2003). Through repeated cultivation and selection under these conditions these strains thus become increasingly "domesticated". For instance, the repeated fermentation of high sugar containing grape must by the same indigenous vineyard yeasts probably gave rise to the selection of strains better suited for fermentation conditions.

This is the first report of a non-flocculent industrial wine yeast strain that displays the ability to from multi-cellular "biofilm"-like structures. BM45 forms fully developed "mats" albeit morphologically slightly different from that of the laboratory yeast strain Σ1278b (data not shown) (Reynolds and Fink, 2001). Here we show that only the altered expression of *FLO11*, and none of the other *FLO* genes tested, modifies the "mat" morphology in BM45. This corroborates the previous finding that Flo11p is required for "mat" formation (Reynolds and Fink, 2001). $ADH2_p$ driven Flo11p expression does not affect yeast "mat" motility but leads to the near total absence of the "hub" and radial "spoke" structures typically observed in wild type "mats". Surprisingly we observed a total lack of "mat" formation in strain BM45-F11H. Previously it was shown that this strain did display Flo11p-dependent phenotypic characteristics such as model "flor" formation and increased hydrophobicity (Govender *et al.*, 2009). Furthermore these expression constructs were shown to be transcriptionally active in both laboratory- and wine yeasts, especially towards the end of fermentation (Govender *et al.*, 2009; Govender *et al.*, 2008). Thus these modified expression systems are functional and were shown to induce Flo-related phenotypes (Govender *et al.*, 2009; Govender *et al.*, 2008). However on plates BM45-F11H cells were found to be non-invasive, which is unexpected behaviour for a strain shown to express *FLO11* at high levels (Govender *et al*., 2009). Co-incidentally the same behaviour was observed for the strain FY23-F11H (Govender *et al.*, 2008). Govender *et al* speculated that this construct, albeit induced in liquid cultures, was not active under plate growing conditions. In this study plates contain lower levels of agar as compared to the above mentioned studies, but may very well also create conditions which cause *HSP30* promoter driven expression of *FLO11* to be insufficient for supporting Flo11p-dependent phenotypes. This could explain the absence of "mat" formation in BM45-F11H.

"Mats" are composed of a complex three dimensional structure, especially in the "hub" area (Figure 6.4 "BM45"). Clearly observable are sections without any cells and other areas dominated by organised yeast structures. How they are formed is not known but likely the result of directional cell growth and targeted apoptosis within the yeast community. These "open" areas could serve as channels providing the community with nutrients. Cell death possibly provides additional nutrients for renewed cell growth in the "hub" region after nutrient depletion, further supporting "mat" development. Yeast communities forming these secondary structures for increased survival have been observed in biofilms as well as colonies formed on 2% agar plates. In this study we observe a morphological distinct growth pattern for yeast expressing *FLO11* under control of the *ADH2* promoter. When this strain is grown in synthetic media simulating grape must (MS300) *FLO11* mRNA levels are 1.5, 17.3 and 6.1 fold higher than wild type in exponential, early stationary and late stationary growth phases respectively (data not shown from Govender *et al*., 2009). "Mats" contain cells that are dormant, dying, or resuming growth and thus possibly in any three of the aforementioned growth phases. Thus *FLO11* is likely differentially expressed in "mats" from

BM45-F11A. Note that this differential expression should very likely be the case for wild type, as native *FLO11* expression levels have been shown to be responsive to nutrient availability (Gagiano *et al.*, 2002; Gimeno *et al.*, 1992) of which micro-gradients likely exist inside “mats”. *FLO11* mRNA levels has been shown to be the highest in the “mat” outer rim region, and the lowest in the “hub” area (Reynolds *et al.*, 2008). How the altered expression of *FLO11* in BM45-F11A affect specific areas in the “mat” is not clear from this analysis, although it is safe to conclude that the different growth phenotype is probably the result of an overall increase in Flo11p levels. Alternatively, this could be the result of the abolishment of epigenetic control over *FLO11* expression as a result of the promoter replacement strategy. As previously mentioned *FLO11* is under epigenetic control (Halme *et al*., 2004), a phenomenon as of yet not reported for *ADH2*. *FLO11* is likely expressed in only a specific proportion of cells within wild type "mats", while populations of BM45-F11A express *FLO11* in a more homogenous manner. Thus, even with possible similar overall (mean) *FLO11* expression levels in wild type and BM45-F11A strains, these very different expression patterns could account for the clear morphological differences of "mats" formed by these strains. This may further affect internal organisation which in turn impact on yeast survival within these communities. However in this study we did not test if any such differences in viability exist between these strains.

In comparison, BM45 and VIN13 display various oenological and morphological differences. VIN13 is reported to be a strong fermenting yeast giving a wine product with favourable aroma characteristics (http://www.anchorwineyeast.com/envin13.html), whereas BM45 ferments must at a comparatively slower rate but also release high levels of mannoproteins during wine making. (http://www.lallemandwine.com/catalog/products/view/1578). Mannoproteins are reported to have a variety of beneficial effects on wine and wine production (Caridi, 2006). Furthermore differences in colony morphology are observed for Vin13 and BM45. BM45 has a wrinkled (“fluffy”) appearance whereas VIN13 grows as smooth colonies (data not shown). Here we further show that VIN13 is unable to from “mats”. Thus far no direct correlation has been made between these phenotypes and increased mannoprotein release although it would seem likely as Flo11p is itself a mannoprotein and required for the mentioned phenotypes. Hence we propose that colony morphology and “mat” formation may serve as indicator (marker) phenotype for the identification of mannoprotein releasing strains. Current methods for the detection of mannoproteins are expensive, time intensive and require specialised equipment. Thus phenotype screening can serve as a high throughput, cheap and rapid alternative for the first level identification from large strain collections of potential mannoprotein secreting strains.

6.6 Acknowledgements

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Chapter 7

General discussion and conclusions

Chapter 7: GENERAL DISCUSSION AND CONCLUSIONS

7.1 General discussion and conclusions

S. cerevisiae yeast is routinely used as a model organism. Compared to other model organisms, yeast is easily genetically manipulated and well established techniques have been developed for quick and efficient genetic, molecular and phenotypic analysis. Extensive information has become available on the environmental sensing systems of yeast, the signalling pathways that relay this information, and the specialised cellular programmes that get activated through specific transcription profiles. From this, well established models have been developed for the cellular response to physical stress conditions (such as a sudden change in osmolarity of the environment) or limited nutrient availability (sugar- and nitrogen limitation). These programmes ultimately affect adaptive cellular mechanisms and the activation of specific gene expression profiles.

Genes activated in this manner may encode cell wall proteins that function in providing the cell wall with the specific adjusted characteristics that form part of a greater cellular response. In this study we place specific focus on the cell wall proteins involved in the asexual recognition and subsequent adherence between individual cells, or those involved in the interaction between cells and solid substrates. These adhesion proteins control growth phenotypes such as the switch from “yeast form” to “hyphal” growth and the formation of multi-cellular “flocs”, “flor” and “mats”. “Hyphal” growth possibly supports access to nutrients under limiting conditions, whereas the different multi-cellular phenotypes likely aid in the altruistic protection of selected cells within the context of a given community.

Members of the Flo adhesin family, most notably Flo1p and Flo11p, control all of the above mentioned phenotypes (Verstrepen and Klis, 2006). These glycosylated proteins are anchored in the cell wall and extent into the extra-cellular surroundings where they participate in various adhesion interactions. Somewhat misnamed, since not part of this group of proteins, Flo8p has been shown to be a transcriptional activator of *FLO* genes (Kobayashi *et al.*, 1999; Pan and Heitman, 1999; Rupp *et al.*, 1999). Furthermore Flo8p was shown to interact with Mss11p (Kim *et al*., 2004), a central regulator of *FLO11* transcription (van Dyk *et al.*, 2005).

This study shows that Mss11p, together with Flo8p, also controls *FLO1* expression. As with *FLO11* transcription Mss11p acts as the central downstream regulator of *FLO1* transcription. Flo1p functions as the dominant adhesin in controlling “floc” formation, whereas Flo11p acts as the primary controller of “hyphal” growth and the formation of “flor” and “mats”. Thus our results show that Flo8p and Mss1p together regulate the expression of multiple Flo members with the associated diversity in phenotypic outcomes. Further work in this study shows that Flo10p performs a contributing role in adhesion phenotypes, with Flo1p and Flo11p remaining the main role players. Thus it would appear that the Flo protein family exclusively controls all non-sexual adhesion phenotypes of yeast. Furthermore, adhesion phenotypes often seem to be not wholly dependent on one adhesin, but rather defined by the specific balance of Flo proteins in the cell wall. *FLO10* transcription does not display an absolute requirement for Mss11p, such as *FLO1* and *FLO11*, but still greatly depends on it. This strongly suggests that Mss11p, in cooperation with Flo8p, functions as the main transcriptional regulator of non-sexual adhesive phenotypes.

Whole genome transcriptome analysis shows that Flo8p and Mss11p have communal as well as unique gene targets groups. Apart from the *FLO* genes, Mss11p activates other cell wall genes, specifically those from the *DAN* and *TIR* gene families. Further analysis shows that these genes do not however participate in adhesion interactions. Furthermore we find that Flo8p and Mss11p show identical trends in the regulation of the cell wall and membrane associated genes *AQY2*, *HMS1* and *TIR3*, and of other transcription factors regulating adhesion. The integration of transcriptome data with known transcription factor (TF) interaction networks suggests that Flo8p functions as a transcription factor, whereas Mss11p appears to behave more like a transcriptional activation switch. Also, the observation that *MSS11* over-expression repressed genes involved in processes ranging from ribosome biogenesis, rDNA modification and the regulation of translation, strongly suggests that Mss11p may also affect transcription via more indirect mechanisms.

What is very clear from this study is that the over-expression or deletion of either *FLO8* or *MSS11* leads to fold change in *FLO11* of relatively large magnitudes. No other gene target could be identified that were regulated to the same extent by both factors. We hypothesise that at least some of the gene targets that appear shared by Flo8p and Mss11p could in fact be artefacts related to pleiotropic effects of high *FLO11* expression levels (Figure 7.1). While regulating various adhesion phenotypes, Flo11p directly affects the characteristics of the cell wall to an extent that may lead to feed-back regulation and cellular compensation and the activation of other transcriptional programmes. How the cell physically senses such changes in the cell wall is not known but could be mediated by plasma membrane sensors with extra-cellular glycosylated domains. Glycosylated domains, showing properties similar to other cell wall glycosylated components, may be able to sense cell wall properties by direct interaction. Two such sensors, Hkr1p and Msb2p, have been characterised and affect signalling involved with osmo-regulation and filamentation (Cullen *et al.*, 2004; Pitoniak *et al.*, 2009; Yabe *et al.*, 1996). To verify whether such a feed-back hypothesis might explain at least parts of the observed transcriptional changes, we will carry out a transcriptome analysis of strains over-expressing *MSS11*, but with deletions of some or all of the *FLO* genes. In addition, such analysis will be carried out with a strain over-expressing *FLO11*.

Recent evidence shows that Flo8p and Mss11p are required for the expression of adhesins critical for hyphal development and virulence in the human pathogens *Candida glabrata* (Mundy and Cormack, 2009) and *Candida albicans* (Cao *et al.*, 2006; Su *et al.*, 2009). Interestingly, Su *et al.* showed that Flo8p was able to induce hyphal formation in *C. albicans* in the absence of Mss11p, but that Mss11p was not able to suppress a *FLO8* deletion. In this dissertation, the opposite observation is made in *S. cerevisiae*. It would be interesting to see if these factors also display cooperative and distinct effects on the transcriptional programmes of these pathogens.

Many questions regarding Mss11p remain unanswered. We do not fully understand its specific molecular function, its interactions with other cellular components, or the signals that it responds to. We do know that it functions in the transcriptional expression of cell wall genes, specifically those involved in cellular adhesion interactions. Furthermore Mss11p appears to regulate genes that either encode for components, or for the assembly, of ribosomes. Also many cell wall genes are co-regulated with Flo8p, and evidence exist that this involves the physical interaction between Flo8p and Mss11p (Kim *et al.*, 2004). Current knowledge suggests that Flo8p is functionally more strongly connected within the transcription factor regulatory network than is the case for Mss11p. We still do not have sufficient evidence to conclude whether or not Mss11p functions as a

transcription factor. It would rather appear that Mss11p functions as a switch for the activation of certain target groups of genes. Previous searches by our laboratory and by this researcher for Mss11p binding partners have been unsuccessful. Nevertheless the biochemical determination of cellular components interacting with Mss11p will be essential in the understanding of the specific cellular role of Mss11p.

Current information on Mss11p serves as a basis for building a hypothesis on the function of this protein in the broader cellular context. Mss11p positively regulates the high affinity hexose transporter Hxt2p, as well as starch utilisation in strains having one or more of the *STA* genes. It also functions in the morphological switch from vegetative to hyphal growth and the adherence of cells to surfaces. While repressing ribosome biogenesis and mating, but activating other cellular differentiation programmes such as the formation of pseudohyphae, Mss11p may prohibit the cell from entering energetically expensive and possibly unsuccessful programmes, when conditions rather favour the utilisation of scarce nutrients (low glucose concentration; starch) or the directed growth towards better nutrient sources. Thus we hypothesise that Mss11p may act as a “switch” that activates cellular processes associated with nutrient limitation, while inhibiting those associated with nutrient excess.

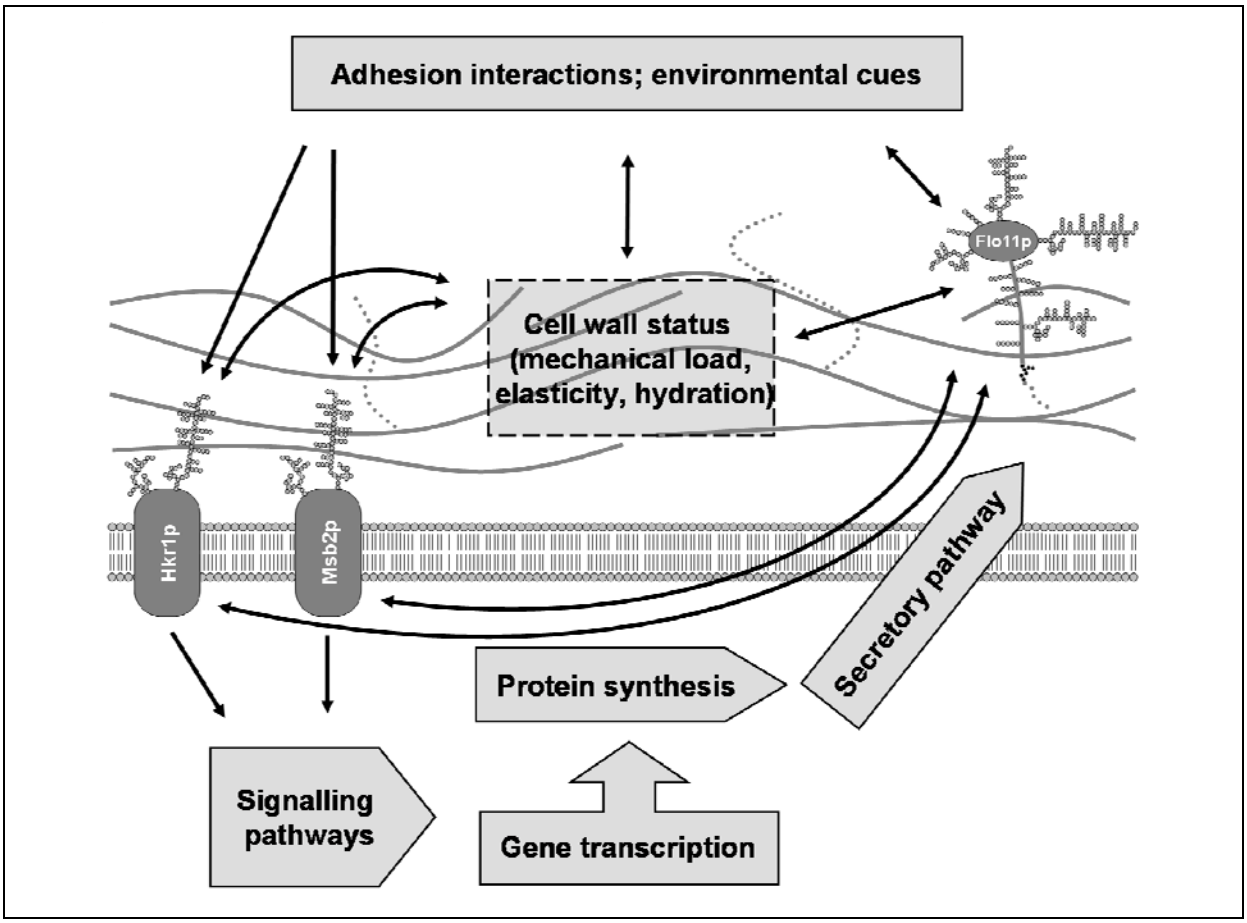

Figure 7.1 Diagram depicting the hypothesised interactions between the various components of the cell wall (cell wall matrix), cell wall linked Flo11p and the two plasma membrane signalling mannoproteins, Hkr1p and Msb2p. Black arrows indicate directionality of interaction, for which the evidence is strictly hypothetical. Also shown is the proposed interaction of this system with the extra-cellular environment as well as with intracellular machinery.

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Addendum A

Controlled expression of the dominant flocculation genes *FLO1*, *FLO5*, and *FLO11* in *Saccharomyces cerevisiae*

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ADDENDUM A

Controlled Expression of the Dominant Flocculation Genes *FLO1*, *FLO5*, and *FLO11* in *Saccharomyces cerevisiae*

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Abstract

In many industrial fermentation processes, the *Saccharomyces cerevisiae* yeast should ideally meet two partially conflicting demands. During fermentation, a high suspended yeast count is required to maintain a satisfactory rate of fermentation, while at completion, efficient settling is desired to enhance product clarification and recovery. In most fermentation industries, currently used starter cultures do not satisfy this ideal, probably because non-flocculent yeast strains were selected to avoid fermentation problems. In this paper, we assess molecular strategies to optimize the flocculation behaviour of S. cerevisiae. For this purpose, the chromosomal copies of three dominant flocculation genes, *FLO1*, *FLO5*, and *FLO11*, of the haploid non-flocculent, non-invasive, and non-flor-forming *S. cerevisiae* FY23 strain were placed under the transcriptional control of the promoters of the *ADH2* and *HSP30* genes. All six promoter-gene combinations resulted in specific flocculation behaviours in terms of timing and intensity. The strategy resulted in stable expression patterns providing a platform for the direct comparison and assessment of the specific impact of the expression of individual dominant *FLO* genes with regard to cell wall characteristics, such as hydrophobicity, biofilm formation, and substrate adhesion properties. The data also clearly demonstrate that the flocculation behaviour of yeast strains can be tightly controlled and fine-tuned to satisfy specific industrial requirements.

Introduction

Industrial fermentations for the production of bioethanol, wine, beer, and other alcoholic beverages are performed in batch processes. At the end of fermentation, the suspended *Saccharomyces cerevisiae* yeast cells must be removed prior to further processing of the fermentation product. The separation of suspended yeast cells may have to be achieved by centrifugation or filtration, which are time-consuming and expensive procedures.

Alternatively, clarification can be achieved by natural settling of the yeast. While single yeast cells tend to settle over time, natural settling becomes a viable option in industrial processes only when cells aggregate, a process also referred to as flocculation. Flocculation is defined as the asexual, reversible, and calcium-dependent aggregation of yeast cells to form flocs containing large numbers of cells that rapidly sediment to the bottom of the liquid growth substrate

(Bony *et al.*, 1997; Stratford, 1989). Although flocculation could provide a seemingly ideal solution to the removal of yeast cells after primary fermentation, it should not occur before the fermentation is completed. As a matter of fact, early flocculation may result in sluggish or stuck fermentation and final products with high residual sugars and unsatisfactory aromatic characteristics (Verstrepen *et al.*, 2001).

Flocculation in S. cerevisiae is mediated by specific cell surface lectins (or flocculins) that are capable of binding directly to mannose residues of mannan molecules on adjacent cells (Miki *et al.*, 1982; Stratford, 1989). This interaction leads to cellular aggregation and finally settling. In some specific cases, cellular aggregation does not lead to settling, but to yeast cells rising to the surface of the substrate and forming an air-liquid interfacial biofilm. This behaviour is also referred to as flotation or flor formation (Pretorius, 2000; Zara *et al.*, 2005).

In S. cerevisiae, two distinct flocculation phenotypes have been characterized on the basis of their sensitivity to sugar inhibition, namely, Flo1 (mannose sensitive) and NewFlo (mannose and glucose sensitive) (Stratford and Assinder, 1991). Most brewer's yeast strains are of the NewFlo phenotype, and flocculation in these strains is initiated after the end of exponential respiro-fermentative phase of growth (Sampermans *et al.*, 2005). The late onset of flocculation in yeast cells with the NewFlo phenotype makes them ideally suited to their task by aiding separation of biomass from the brew.

The genetic basis of flocculation has been the object of several investigations. These studies suggest that a family of subtelomeric genes, *FLO1*, *FLO5*, *FLO9*, and *FLO10*, encode specific lectins that are responsible for flocculation (Teunissen and Steensma, 1995). A non-subtelomeric gene, *FLO11*/*MUC1* (Lambrechts *et al.*, 1996; Lo and Dranginis, 1996), on the other hand, encodes a protein that has been associated with flocculation, flor formation, invasive growth, and substrate adhesion (Bayly *et al.*, 2005; Guo *et al.*, 2000; Ishigami *et al.*, 2004; Lo and Dranginis, 1996; Zara *et al.*, 2005). All Flo proteins are glycosylphosphatidylinositol (GPI) -linked glycoproteins that share a common three-domain structure consisting of an N-terminal lectin domain, a central domain of highly repeated sequences rich in serine and threonine residues, and a carboxyl-terminal domain containing a GPI-anchoring sequence (Verstrepen and Klis, 2006). In recent studies (Liu *et al.*, 2007a; Liu *et al.*, 2007b), evidence was presented that the difference between the NewFlo and Flo1 flocculation phenotypes may be at least partially due to variations in the number of repeat sequences within the *FLO1* coding sequence.

The regulation of *FLO* gene expression is complex, and in particular, the promoter of *FLO11* has been intensively studied. *FLO11* expression is tightly controlled by environmental factors, and several signalling cascades, including the Ras-cyclic AMP-dependent kinase complex, the filamentous-growth-controlling mitogen-activated protein kinase, and the main glucose repression pathways have been directly linked to *FLO11* regulation (Verstrepen and Klis, 2006). Two transcriptional regulators, Mss11p and Flo8p, have been shown to play a central role in the control of flocculation and flotation phenotypes (Bester *et al.*, 2006; Liu *et al.*, 1996; van Dyk *et al.*, 2005). These investigations have shown that *FLO11* transcriptional regulation is particularly dependent on the nutritional status and specific composition of the growth environment. Less information is available regarding the regulation of other *FLO* genes, although it has been shown that *FLO1* expression is also controlled by nutritional status signals, such as carbon and/or nitrogen starvation

(Sampermans *et al.*, 2005), and other environmental indicators, such as pH (Soares and Seynaeve, 2000) and ionic strength (Jin and Speers, 2000).

In addition to this transcriptional regulation, *FLO* gene activity has been shown to be modulated by other regulatory systems. In particular, data suggest that these genes are often under promoter-specific epigenetic control allowing *S. cerevisiae* cells in a homogenous population to reversibly switch between active *FLO* gene expression and silent modes (Halme *et al.*, 2004). Furthermore, sequence analysis reveals that several DNA motifs in the central domain are conserved among different *FLO* genes, promoting diversity of adhesins by frequent intragenic recombination events (Verstrepen *et al.*, 2005).

Considering the complexity of *FLO* gene regulation, it is evident that manipulation of both physiological and environmental factors offers winemakers and brewers limited avenues to control or alter flocculation during fermentations. Therefore, it is not surprising that industrial yeast strains generally possess a less than optimal flocculation profile (Carstens *et al.*, 1998; Verstrepen *et al.*, 2003). For this reason, replacement of the native promoters of these genes with less complex promoters conferring expression patterns that would be better adapted to industrial needs may result in yeast strains that display improved flocculation behaviour for specific industrial purposes.

In previous attempts to modify flocculation behaviour, the flocculation genes *FLO1* and *FLO5* were introduced into non-flocculent *S. cerevisiae* brewing yeast strains (Barney *et al.*, 1990; Ishida-Fujii *et al.*, 1998; Watari *et al.*, 1990; Watari *et al.*, 1994; Watari *et al.*, 1991). However, the resultant modified yeast strains flocculated constitutively and displayed reduced fermentation performance or increased fermentation times. In an approach similar to the one described here, Verstrepen *et al*. (2001) brought the chromosomal *FLO1* gene of the haploid non-flocculent *S. cerevisiae* FY23 laboratory strain under the transcriptional control of the *HSP30* stationary-phase promoter. The resulting strain showed strong flocculation toward the end of fermentation, resulting in a distinctly clearer beer than the beer obtained with wild-type cells (Verstrepen *et al.*, 2001).

In this study, we assess the suitability of six genome-integrated promoter-gene combinations to control stationary-phase-specific flocculation. For this purpose, the native promoters of the dominant flocculation genes *FLO1*, *FLO5*, and *FLO11* in the haploid *S. cerevisiae* FY23 strain were replaced with the inducible promoters *ADH2* and *HSP30*. The *ADH2* promoter is subjected to carbon catabolite repression and has been shown to be repressed several hundredfold during growth on glucose (Gancedo, 1998; Price *et al.*, 1990). De-repression of the *ADH2* promoter generally coincides with transition to growth on ethanol (Noronha *et al.*, 1998). The *HSP30* promoter, on the other hand, has been shown to be induced during entry into the stationary phase of growth, which coincides with the depletion of glucose from the medium, which is found under low-stress nutrient-rich wort and wine fermentation conditions (Donalies and Stahl, 2001; Regnacq and Boucherie, 1993; Riou *et al.*, 1997). In addition, the *HSP30* promoter is activated by several stress factors, including heat shock and sudden exposure to either ethanol or sorbate (Piper *et al.*, 1994; Regnacq and Boucherie, 1993; Seymour and Piper, 1999). Unlike *ADH2* regulation, which is reasonably well understood (Di Mauro *et al.*, 2000; Verdone *et al.*, 2002; Young *et al.*, 1998), the mechanism by which *HSP30* is induced in response to stress remains unclear.

Since the dominant *FLO* genes are transcriptionally silent in the *S. cerevisiae* FY23 strain due to a nonsense mutation in the *FLO8* gene (Liu *et al.*, 1996; Verstrepen *et al.*, 2005; Winston *et al.*, 1995), expression regulated by either the *ADH2* or *HSP30* promoter constructs allows both assessments of the phenotypic consequences of the expression of a particular Flo protein and of the transcriptional character of a promoter in the same genetic background. Indeed, it is difficult to compare reports on flocculation in the literature due to the numerous techniques employed and the variations therein, coupled with the different yeast strain genetic backgrounds (Jin and Speers, 2000). Therefore, the inducible expression of three dominant flocculation genes, *FLO1*, *FLO5*, and *FLO11*, in the haploid laboratory strain *S. cerevisiae* FY23 strain reported in this study presents a unique opportunity to compare the adhesion characteristics (flocculation, invasive growth, and flor formation) of the aforementioned flocculation genes.

Our data show that each promoter-open reading frame (ORF) combination leads to specific flocculation and adhesion behaviours and results in additional important changes in cell surface properties, including hydrophobicity. The data indicate that highly specific flocculation behaviour can be stably conferred to individual yeast strains.

Materials and Methods

Strains

The yeast strains employed in this study are listed in Table 1. All strains were derived from *Saccharomyces cerevisiae* strain FY23 (Liu *et al.*, 1996; Winston *et al.*, 1995). *Escherichia coli* DH5α (Gibco BRL/Life Technologies, Rockville, MD) was used as a host for all plasmid amplifications.

Table 1 *S. cerevisiae* strains employed in this study.

Strain	Genotype	Reference
FY23	*MAT*a *leu2 trp1 ura3 flo8-1*	Winston *et al.*, 1995
FY23-F1A	*MAT*a *leu2 trp1 ura3 flo8-1 FLO1*::*SMR1-ADH2*	This study
FY23-F1H	*MAT*a *leu2 trp1 ura3 flo8-1 FLO1*::*SMR1-HSP30*	This study
FY23-F5A	*MAT*a *leu2 trp1 ura3 flo8-1 FLO5*::*SMR1-ADH2*	This study
FY23-F5h	*MAT*a *leu2 trp1 ura3 flo8-1 FLO5*::*SMR1-HSP30*	This study
FY23-F11A	*MAT*a *leu2 trp1 ura3 flo8-1 FLO11*::*SMR1-ADH2*	This study
FY23-F11H	*MAT*a *leu2 trp1 ura3 flo8-1 FLO11*::*SMR1-HSP30*	This study

Media and cultivation conditions

Yeast strains were routinely cultivated at 30°C in rich YEPD medium, containing 1% (wt/vol) yeast extract, 2% (wt/vol) peptone, and 2% (wt/vol) glucose. Single yeast colonies from 3-day-old YEPD plates were used to inoculate starter cultures in 40 ml YEPD broth contained in 250-ml Erlenmeyer flasks, which were incubated at 30°C with shaking (160 rpm) for 18 h. These starter cultures were used to inoculate pre-cultures at an initial cell density of 5 x 10^5 cells/ml which were incubated at 30°C with shaking (160 rpm) for 18 h. Thereafter, yeast cells for inoculation of experimental cultures were routinely prepared as follows using ice-cold reagents. Yeast cells from pre-cultures

were harvested by centrifugation (4,000 rpm, 5 min), washed once with 100 mM EDTA (pH 7) to ensure de-flocculation, washed once with 30 mM EDTA (pH 7), and finally resuspended in 30 mM EDTA (pH 7). To determine the onset of flocculation, flocculent ability, glucose utilization, and growth rate of yeast in nutrient-rich medium, experimental cultures were seeded at an initial cell density of 5 x 10^5 cells/ml into 40 ml YEPD broth and incubated at 30°C with shaking (160 rpm). At 2-h intervals, for a period of 24 h and a 48 h time point, cell populations were harvested and deflocculated as described previously. The flocculation ability of FY23-F11A and FY23-F11H strains was also assessed in media with a composition identical to that of YEPD medium but with an alternative carbon source, namely, YEPE (3% [vol/vol] ethanol) and YEPGE (3% [vol/vol] ethanol together with 3% [vol/vol] glycerol) were used. In addition, flocculation and invasive growth tendencies were also assessed in chemically defined synthetic complete (SC) medium containing 0.67% (wt/vol) yeast nitrogen base (YNB) without amino acids (Difco, Detroit, MI) supplemented with all nutrients (50) and with the following as the sole carbon source: 2% (wt/vol) glucose (SCD medium), 0.2% (wt/vol) glucose (SCLD medium), 3% (vol/vol) ethanol (SCE medium), and 3% (vol/vol) ethanol with 3% (vol/vol) glycerol (SCGE medium). In addition to the above media, medium containing only the auxotrophic requirements (50) of strain FY23 (leucine, uracil, and tryptophan) was also used (SCDLUT and SCLDLUT media). Flor medium containing 0.67% YNB and 3% (vol/vol) ethanol adjusted to pH 3.5 (24) containing all nutritional requirements was used to assess flor formation. For selection of sulfometuron methyl (SM)-resistant yeast transformants, SC medium containing 0.67% YNB and 2% (wt/vol) glucose was supplemented with amino acids for strain FY23 and 80 to 100 µg/ml SM (DuPont Agricultural Products, France). *E. coli* was grown at 37°C in Luria-Bertani (LB) medium (1% [wt/vol] Bacto tryptone, 0.5% [wt/ vol] yeast extract, and 1% [wt/vol] NaCl), and bacterial transformants were selected using LB medium containing 100 mg/liter ampicillin. In this study, 2% agar (Difco) was used for all solid media. Bacterial and yeast strains were stored in LB medium containing 40% (vol/vol) glycerol and YPD supplemented with 15% (vol/vol) glycerol, respectively (Ausubel, 2004).

DNA manipulation, construction of promoter replacement cassettes, and yeast transformations.

Restriction enzymes and T4 DNA ligase were purchased from Roche Diagnostics GmbH (Mannheim, Germany). Expand High Fidelity PCR system (Roche Diagnostics GmbH) was employed for PCR amplifications. The amplification products were purified from agarose gels and cloned into pGEM-T Easy vector (Promega Corporation, Madison, WI) according to the specifications of the manufacturer. Standard procedures for bacterial transformations and plasmid isolation from *E. coli* were performed (Maniatis *et al.*, 1989). Standard procedures for isolation and manipulation of DNA were employed in all other aspects of this study (Ausubel, 2004). The strategy for construction of promoter replacement cassettes was adapted from the strategy used as described previously (Verstrepen and Thevelein, 2004). The *ADH2* promoter region bearing a *FLO1* promoter (FLO1p) homologous sequence was amplified from pDLG5 plasmid (la Grange *et al.*, 1996) by PCR with ADH2-F and ADH2::FLO1-R primers (Table 2). The SM resistance yielding *SMR1-410* (*SMR1*) marker gene inclusive of promoter and terminator sequences was PCR amplified from plasmid pWX509 (Casey *et al.*, 1988) with the SMR1-R and FLO1::SMR1-F primer pair. The ADH2- FLO1p 834-bp fragment in pGEM-T Easy was recovered by double restriction digestion with *Bgl*II and *Spe*I, while the FLO1p-SMR1 insert (2,962 base pairs [bp]) was released by triple digestion with Alw441, *Bam*HI, and *Sph*I. Both fragments were subsequently ligated. The FLO1p-SMR1-ADH2-FLO1p vector was PCR amplified by using shorter primers FLO1-F and FLO1-R and ligation reaction mixture as the template. The integrating *FLO1*

promoter replacement cassette (3,764 bp) was extracted from agarose gels and purified. A similar strategy was employed for the construction and synthesis of FLO1p-SMR1- HSP30-FLO1p, FLO5p-SMR1-ADH2-FLO5p, FLO5p-SMR1-HSP30-FLO5p, FLO11p-SMR1-ADH2-FLO11p, and FLO11p-SMR1-HSP30-FLO11p integrating promoter replacement cassettes. The primer pairs for different ORFs are as follows: primers FLO5-F and FLO5-R for *FLO5* and primers FLO11-F and FLO11-R for *FLO11*. Note that the *HSP30* promoter (*HSP30*p)-containing region was amplified using FY23 chromosomal DNA as the template. Yeast transformation with 5 µg of DNA was performed according to the lithium acetate method described by Gietz and Schiestl (Gietz and Schiestl, 1995). Chromosomal integration was achieved by a double-crossover homologous recombination event in which the *FLO1*, *FLO5*, or *FLO11* gene was placed under transcriptional control of either the *ADH2* or *HSP30* promoter. The deletion of native promoters was confirmed by PCR using homologous primer sets. The primer pairs for transgenic strains were as follows: primers FLO1-F and FLO1-R for strains FY23-F1A and FY23-F1H, primers FLO5-F and FLO5-R for strains FY23-F5A and FY23-F5H, and primers FLO11-F and FLO11-R for strains FY23-F11A and FY23-F11H. In addition, the integration of promoter replacement cassettes in transformed yeast was further confirmed by PCR using heterologous primer sets that contained a forward primer from outside the region of integration and genomic DNA isolated from transformants. The primer pairs for different transformants were as follows: primers FLO1-F2 and ADH2-R for strain FY23-F1A, primers FLO1-F2 and HSP30-R for strain FY23-F1H, primers FLO5-F2 and ADH2-R for strain FY23-F5A, primers FLO5-F2 and HSP30-R for strain FY23-F5H, primers FLO11-F2 and ADH2-R for strain FY23-F11A, and primers FLO11-F2 and HSP30-R for strain FY23-F11H. The wild-type FY23 strain served as a control in these confirmation experiments.

Growth and enumeration of yeast populations

The cell density of suitably diluted yeast suspensions in 30 mM EDTA (pH 7) was determined either by direct cell counting with a haemocytometer or alternatively by measuring absorbance at 600 nm in a Cary 50 UV-visible spectrophotometer (Varian Inc., CA) using a standard curve as the reference.

Glucose determination

Cells were pelleted from 1-ml samples of experimental cultures by microcentrifugation (10,000 rpm, 1 min). The cell extracts were subsequently filtered through a 0.22-µm cellulose acetate filter and stored at -20°C until glucose analysis. The concentration of glucose in the culture medium was determined using a GAGO-20 glucose assay kit (Sigma, St. Louis, MO) according to the specifications of the manufacturer, using a Biotek 800ELX microplate reader (Biotek Instruments Inc., Winooski, VT).

Table 2 Primers used in this study.

Primer[a]	Sequence (5'→3')[b]	Underlined sequence[c]
FLO1::SMR1-F	TGCGTCACTTTTCCTACGGTGCCTCGCACATGAATGTTATCCGGC GCACGGGTACCGGCTTGGCTTCAGTTGCTG	*FLO1* nucleotides (nt) -813 to -764
FLO5::SMR1-F	GCAATAAACCACATGGCTACCGCACTTCTTGTCACTATCCGGTACC GGCTTGGCTTCAGTTGCTG	*FLO5* nt -1995 to -1956
FLO11::SMR1-F	TCACTGCACTTCAACTATGCCTTATAGCAACCAAGAAGCTAGAAAA TGCCAACTATTAAAAAGATAACCTCTCGGTACCGGCTTGGCTTCAG TTGCTG	*FLO11* nt -2710 to -2639
SMR1-R	CATGGGATCCAGCTTGCAATTTTTGACGGCCCC	*Bam*HI site
ADH2-F	TGACAGATCTAACTCGTTCCAGTCAGGATTG	*Bgl*II site
ADH2-R	TGATAGTTGATTGTATGCTTTTTGTAGC	
ADH2::FLO1-R	CTGCCAAAAACATATAGCGATGAGGCATTGTCATTTTTGGATGTTC TGTTTACTGGTGACTGATAGTTGATTGTATGCTTTTTGTAGC	*FLO1* nt -26 to +34
ADH2::FLO5-R	GCTAATCAATTTAAAGAAAATCAATTGCGGAATTTACTGCAGAGCT GATAGTTGATTGTATGCTTTTTGTAGC	*FLO5* nt -58 to -14
ADH2::FLO11-R	GGACCAAATAAGCGAGTAGAAATGGTCTTTGCATAGTGTGCGTAT ATGGATTTTTGAGGCTGATAGTTGATTGTATGCTTTTTGTAGC	*FLO11* nt -26 to +34
HSP30-F	CATGAGATCTGATGGCATTGCACTCAAG	*Bgl*II site
HSP30-R	TATTAAAGTCTCAAACTTGTTGTTTTG	
HSP30::FLO1-R	GCGATGAGGCATTGTCATTTTTGGATGTTCTGTTTACTGGTGACAA AAGATATTAAAGTCTCAAACTTG	*FLO1* nt -26 to +18
HSP30::FLO5-R	GCTAATCAATTTAAAGAAAATCAATTGCGGAATTTACTGCAGAGCT ATTAAAGTCTCAAACTTG	*FLO5* nt -58 to -14
HSP30::FLO11-R	GGACCAAATAAGCGAGTAGAAATGGTCTTTGCATAGTGTGCGTAT ATGGATTTTTGAGGCAAAAGATATTAAAGTCTCAAACTTGTTG	*FLO11* nt -26 to +34
FLO1-F	AAGTGTGCGTCACTTTTCCTACGGT	
FLO1-F2	ATGGCACTAGTCGATCGAGG	
FLO1-R	AGCGATGAGGCATTGTCATTT	
FLO5-F	GCAATAAACCACATGGCTACC	
FLO5-F2	GGTTGTGTTCTAGGACTTTCTGACG	
FLO5-R	AGTGGTGCTAATCAATTTAAAGAA	
FLO11-F	CCTCTCACTGCACTTCAACTATGC	
FLO11-F2	TTACGGCCTAATGTCGAGAC	
FLO11-R	GGACCAAATAAGCGAGTAGA	

[a]The presence of a F or R at the end of the primer name indicates that the primer is a forward or reverse primer, respectively.
[b]Non-underlined sequences correspond to *ADH2*, *HSP30*, and SMR1-410 or *FLO* gene sequences as denoted by the primer name.
[c]Nucleotide numbering has been done by assigning the A in the ATG start codon of the open reading frame as base 1.

Flocculation assays

The flocculent ability of yeast strains was established using the modified Helm's assay as described by D'Hautcourt and Smart (D'Hautcourt and Smart, 1999). The percentage of flocculation reported in this paper represents the arithmetic mean of three independent determinations. To assess the influence of pH on flocculation, a composite suspension buffer with a very wide buffering range was adapted from the buffer used by Stratford (Stratford, 1996) to replace the buffer employed in the above protocol. This buffer contained 10 mM calcium chloride, 50 mM Tris base, 50 mM succinic acid, 100 mM potassium hydroxide, and 4% (vol/vol) ethanol. The pH of the composite suspension buffer was adjusted with 5 M HCl, and flocculation was assessed as described above. To investigate sugar inhibition of *FLO1* and *FLO5* flocculation phenotypes, either mannose or glucose was added at various concentrations to both the washing and suspension buffers that are employed in the modified Helm's assay (D'Hautcourt and Smart, 1999).

RNA extraction and cDNA synthesis

FY23, FY23-F11A, and FY23-F11A strains were pre-cultured and treated as described above. Experimental batch cultures were inoculated in triplicate at an initial density of 5 x 10^5 cells/ml into 100 ml YEPD broth and incubated at 30°C with shaking (160 rpm) for 12, 16, and 48 h. To investigate the transcription of *FLO* genes, samples from batch cultures were washed with ice-cold H_2O, pelleted, and resuspended in ice-cold AE buffer (50 mM sodium acetate, 10 mM EDTA [pH 5.0]). Total RNA was isolated as previously described (Schmitt *et al.*, 1990). DNA contamination was eliminated by DNase I (Roche Diagnostics) treatment. One microgram of total RNA was used as the template for cDNA synthesis using the ImProm-II reverse transcription system according to the manufacturer's instructions (Promega). cDNA samples were diluted 50 times with H_2O before real-time PCR analysis.

QRT-PCR analysis

Primers and probes used for quantitative real-time PCR (QRT-PCR) analysis are listed in Table 3 and were designed using Primer Express software version 3 (Applied Biosystems, CA). Reagents were purchased from Applied Biosystems and Kapa Biosystems (Cape Town, South Africa). QRT-PCR runs and collection of spectral data were performed with the 7500 cycler (Applied Biosystems). SYBR green was used for the detection of *PDA1* and *FLO11* amplicons with final primer concentrations of 100 nM. Specific probes and primers were designed to differentiate between the cDNA species corresponding to the extensively homologous *FLO1* and *FLO5* genes. Probes were modified by the addition of a 3' minor groove binder and non-fluorescent quencher, as well as the 5' attachment of fluorescent dyes as indicated in Table 3 (Applied Biosystems). Probe and primer concentrations were 250 nM and 900 nM, respectively, in QRT-PCRs. Cycling conditions during QRT-PCR were as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s followed by 60°C for 1 min. When using SYBR green, a dissociation curve analysis was included to verify amplicon authenticity. Preliminary data analyses were performed with Signal Detection Software (SDS) version 1.3.1. (Applied Biosystems). Individual QRT-PCR runs were performed at least in duplicate. The relative expression value for each sample was defined as $2^{-Ct(target)}$ where Ct(target) represents the cycle number at which a sample reaches a predetermined threshold signal value for the specific target gene. Relative expression data were normalized to the

relative expression value of the housekeeping gene *PDA1* in each respective sample, thus giving normalized relative expression for a target gene as $2^{-Ct(target)}/2^{-Ct(PDA1)}$.

Table 3 Real-time PCR primers and probes used in this study.

Primer or probe[a]	Sequence (5'→3')	Modification[b]
Primers		
FLO1-F (Taqman MGB)	ATGCCTCATCGCTATATGTTTTTG	None
FLO1-R (Taqman MGB)	GCTCCTGAGGCCACACTAGTTAG	None
FLO5-F (Taqman MGB)	AGCACCACTAAAAAAAATGACAATTG	None
FLO5-R (Taqman MGB)	GCCAGAAAGGCCAAGATTACC	None
Probes		
FLO1-probe	CAGTCTTTACACTTCTGGC	6-FAM 5' label, 3' minor groove binder/non-fluorescent quencher
FLO5-probe	ACCACTGCATATTTT	Vic dye 5' label, 3' minor groove binder/non-fluorescent quencher
FLO11-F-(QRT-PCR)	CCTCCGAAGGAACTAGCTGTAATT	None
FLO11-R-(QRT-PCR)	AGTCACATCCAAAGTATACTGCATGAT	None
PDA1-F-QRT-PCR	GGAATTTGCCCGTCGTGTT	None
PDA1-R-QRT-PCR	GCGGCGGTACCCATACC	None

[a]The presence of a F or R at the end of the primer or probe name indicates that the primer or probe is a forward or reverse primer or probe, respectively.

[b]6-FAM, 6-carboxyfluorescein.

Determination of hydrophobicity of yeast cell surfaces

The hydrophobicity of yeast cell surfaces was determined by measuring the distribution ratio of yeast cells in a biphasic system consisting of a buffered solution and an organic solvent (Hinchcliffe *et al.*, 1985). Cultures in YEPD broth were incubated at 30°C for 48 h with shaking (160 rpm). The harvested cells from an experimental culture were deflocculated, washed, and diluted to a density of 5×10^6 cells ml^{-1} in 30 mM EDTA (pH 7). Yeast cells from a 20-ml aliquot of this suspension were washed twice and resuspended in 20 ml of phosphate-urea-magnesium (PUM) buffer (pH 7.1) (Hinchcliffe *et al.*, 1985). The absorbance of this suspension (I) was determined at 660 nm. Aliquots of 2.4 ml (three replicates) were dispensed into borosilicate glass tubes (15 by 75 mm), and 200 µl xylene was layered over the yeast suspension. The tubes were capped with rubber; samples were vortexed at maximum speed for 30 s and allowed to stand undisturbed for 15 min. The absorbance of the residual buffer layer (F) at 660 nm was determined. The average modified hydrophobic index (MHI) for a sample was calculated using the equation: MHI = 1 - (F/I).

Invasive growth plate assays

Yeast cultures processed as described above were adjusted to an optical density (measured at a wavelength of 600 nm) of 1.0, and 10-µl aliquots were dropped onto SCLD and $SCLD_{LUT}$ plates without piercing the agar surface and incubated for 5 days at 30°C. Using a gloved finger, superficial growth of yeast colonies was physically removed by washing the plates under a steady stream of water. Plates were allowed to air dry, and cells that invaded the agar were photographed.

Flor formation and buoyant cell density

Cells were pre-cultured in YEPD medium, deflocculated, and washed as described above. Subsequently, 3 x 10^8 cells were recovered by microcentrifugation (10,000 rpm, 1 min), washed once, resuspended in 1 ml flor medium (pH 3.5), and added to test tubes (16 by 165 mm) containing 4 ml flor medium. Biofilm formation was photographed in natural light after 5 days of static incubation at 30°C. Alternatively, the cultures were incubated statically at 30°C for 60 h, after which 1-ml samples were withdrawn from just below the meniscus. The optical density of samples was determined spectrophotometrically at 600 nm.

Analysis of stress-induced expression of *FLO1*- and *FLO5*-encoded flocculins

FY23, FY23-F5H, and FY23-F1H strains were pre-cultured and treated as described earlier. Experimental cultures were inoculated at an initial density of 5 x 10^5 cells/ml into 40 ml YEPD broth and incubated at 30°C with shaking (160 rpm) for 10 h. The incubation of untreated cells was extended for another 45 min at 30°C, whereas other cultures were exposed to the following stress treatments: heat shock for 30 min at 42°C, heat shock for 45 min at 42°C, 3% (vol/vol) ethanol for 30 min at 30°C, 6% (vol/vol) ethanol for 30 min at 30°C, and 6% (vol/vol) ethanol and heat shock for 30 min at 42°C. Ethanol (100%) was added directly to culture medium to yield a final concentration of 6% (vol/vol), and cultures were incubated at defined temperatures with shaking at 160 rpm. All cultures were placed on ice before flocculation was determined using the modified Helm's assay.

Results

Yeast transformation

Following initial selection on SC plates containing SM, putative transformants were inoculated individually into YEPD broth and cultivated for 48 h at 30°C with shaking (160 rpm). A majority of strains transformed with the combinations of FLO1p-SMR1-ADH2-FLO1p, FLO1p-SMR1-HSP30-FLO1p, FLO5p-SMR1-ADH2-FLO5p, and FLO5p-SMR1-HSP30-FLO5p visually displayed strong flocculent phenotypes, suggesting that integration had occurred at the desired loci (Fig. 1A). Three independent transformants of each strain were selected for further analysis. No flocculent phenotype was detectable for putative transformants of strains FY23-F11A and FY23-F11H. A visual assessment of biofilm formation on flor medium was therefore used for the initial screen of putative *FLO11* transformants, and three independent flor-forming strains were retained for further analysis. For each of the selected strains, the deletion of native promoters was confirmed by PCR using homologous primer sets (Fig. 1B). In addition, integration at the correct gene locus was also confirmed by PCR (Fig. 1C) using primers in which the upstream primer was located outside the region of the inserted promoter replacement cassette.

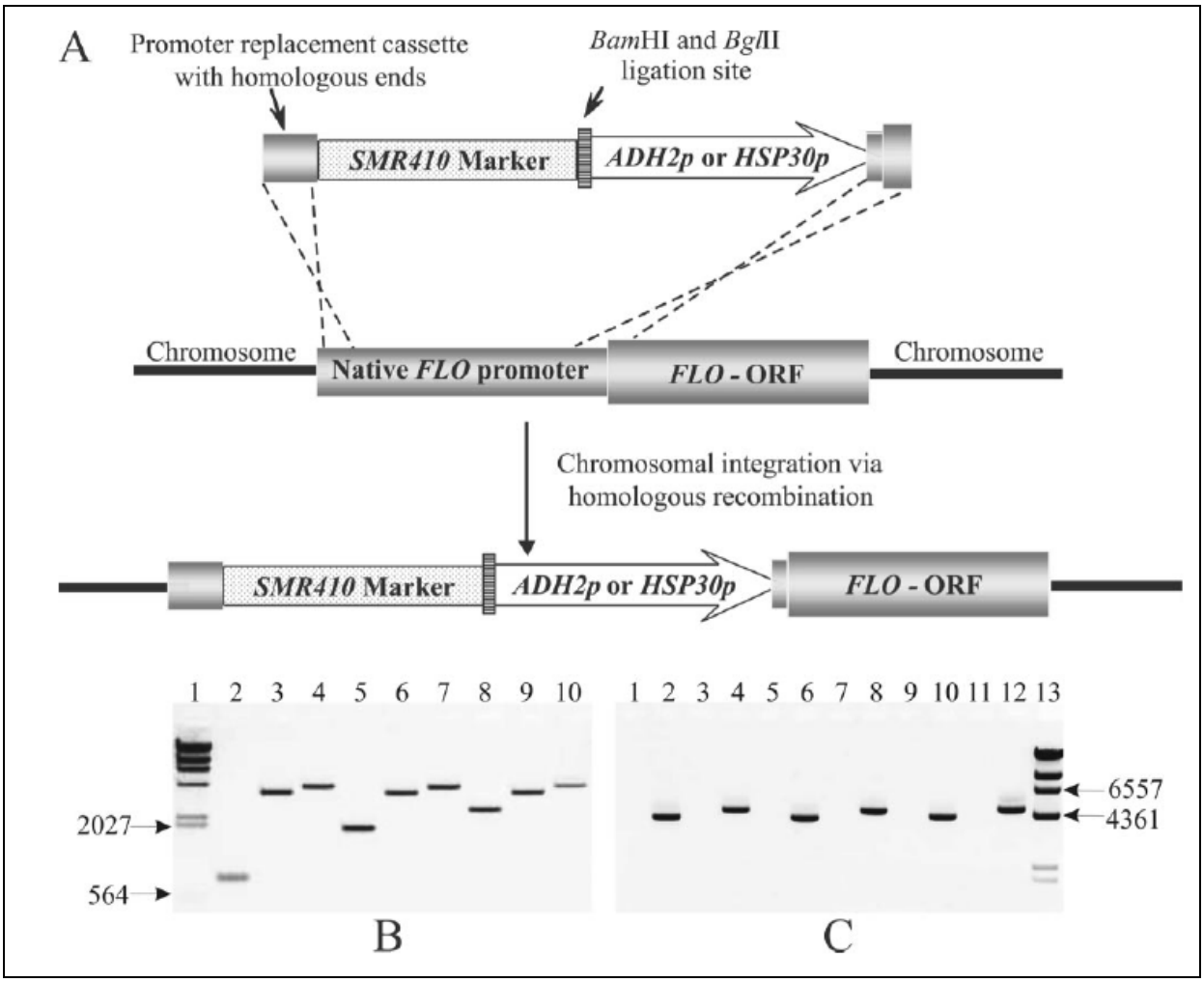

Figure 1 Chromosomal integration of either the *ADH2* or *HSP30* promoter upstream of a dominant *FLO* gene in *S. cerevisiae* strain FY23. (A) Promoter replacement strategy. (B) The deletion of native promoters was confirmed by PCR using homologous primer pairs described in Materials and Methods. The amplification of the native promoter sequence was observed only in the wild-type FY23 strain (FLO1p [837 bp] [lane 2]), FLO5p [1,988 bp] [lane 5], and FLO11p [2,748 bp] [lane 8]), while only the integration cassette was amplified in strains FY23-F1A (FLO1p-SMR1-ADH2-FLO1p, 3,719 bp, lane 3), FY23-F1H (FLO1p-SMR1-HSP30-FLO1p, 4,198 bp, lane 4), FY23-F5A (FLO5p-SMR1-ADH2-FLO5p, 3,701 bp, lane 6), FY23-F5H (FLO5p-SMR1-HSP30-FLO5p, 4,180 bp, lane 7), FY23-F11A (FLO11p-SMR1-ADH2-FLO11p, 3,737 bp, lane 9), and FY23-F11H (FLO11p-SMR1-HSP30-FLO11p, 4,276 bp, lane 10). Lane 1 contained DNA molecular weight markers (phage lambda DNA restricted with *Hin*dIII). (C) The integration of promoter replacement cassettes were confirmed by PCR using heterologous primer sets that contained a forward primer from outside the region of integration and genomic DNA as the template as described in Materials and Methods. The amplification of FLO1p-SMR1-ADH2p (4,191 bp, strain FY23-F1A, lane 2), FLO1p-SMR1-HSP30p (4,670 bp, strain FY23-F1H, lane 4), FLO5p-SMR1- ADH2p (4,098 bp, strain FY23-F5A, lane 6), FLO5p-SMR1-HSP30p (4,577 bp, strain FY23-F5H, lane 8), FLO11p-SMR1-ADH2p (4,333 bp, strain FY23-F11A, lane 10), FLO11p-SMR1-HSP30p (4,812 bp, strain FY23-F11H, lane 12) is evident only in transformants, while lacking in the wild-type FY23 strain with corresponding primer pairs (lanes 1, 3, 5, 7, 9, and 11). Lane 13 contained DNA molecular weight markers (phage lambda DNA restricted with *Hin*dIII).

Stability, growth rates, glucose consumption, and flocculation

To assess the stability of the integrated promoter constructs, the selected transformed strains were cultivated in rich, non-selective medium in repeated batch cultures for more than 100 generations. For each strain, 20 individual colonies were then assessed for their flocculation behaviour (*FLO1* and *FLO5* constructs) and flor-forming behaviour (*FLO11* constructs). All tested colonies displayed the relevant phenotypes. The timing and intensity of the phenotypes were in all cases similar to those observed during the initial screen, indicating that the integration and resulting expression patterns are stable.

The growth rates and sugar utilization capabilities of the wild-type strain FY23 and its six transformants were assessed in YEPD medium containing 2% glucose at 2-hour intervals (Fig. 2 and 3). No significant differences between the wild-type FY23 strain and the transformants regarding biomass growth, cell number, and sugar utilization capability were observed. As seen during the initial screen, strains transformed with combinations involving *FLO1* and *FLO5* ORFs showed flocculent behaviour. Maximal flocculent ability of these strains was displayed 2 to 4 h after glucose depletion (Fig. 2B and 3B). In the ADH2p-FLO1 and ADH2p-FLO5 transformants, flocculation was observed approximately 2 h after glucose exhaustion, while maximum flocculation potential was evident after an additional 4 h (Fig. 2B).

After 48 h of growth in YEPD medium containing 2% (wt/ vol) glucose, *FLO1* transformants (FY23-F1A [98% ± 1%] and FY23-F1H [97% ± 1%]) were more flocculent than the corresponding *FLO5* transgenic yeast strains (FY23-F5A [84% ± 2%] and FY23-F5H [79% ± 3%]) (Fig. 2B and 3B). This also suggests that the *ADH2* promoter (*ADH2*p)-controlled *FLO1* and *FLO5* phenotypes are slightly more flocculent than *HSP30*p regulated phenotypes. This difference was obvious with macroscopic evaluation, where it was evident that *ADH2*p-induced *FLO1* and *FLO5* flocculent phenotypes are markedly stronger than *HSP30*p-mediated *FLO1* and *FLO5* flocculation phenotypes. *ADH2*p-*FLO1* flocs also formed larger clumps that remained at the bottom of the flasks even when agitated at 200 rpm (Fig. 4).

Interestingly, *FLO1* and *FLO5* transformants displayed decreased flocculation capacities in minimal media (data not shown). Under these conditions, the FY23-F1H and FY23-F5H strains, when cultivated in SCD medium containing all nutritional requirements or SCD_{LUT} medium that contained only the auxotrophic requirements of the strains displayed significantly higher flocculation abilities than the FY23-F1Aand FY23-F5A strains did, with the latter strains not flocculating at all in SCD_{LUT} medium. *FLO11* expression mediated by either the *ADH2* or *HSP30* promoter in nutrient-rich YEPD medium (2% [wt/vol] glucose) (Fig. 2B and 3B), YEPE medium (3% [vol/vol] ethanol), or YEPGE medium (3% [vol/vol] ethanol and 3% [vol/vol] glycerol) and minimal media including SCD and SCD_{LUT} media did not yield a flocculent phenotype (results not shown).

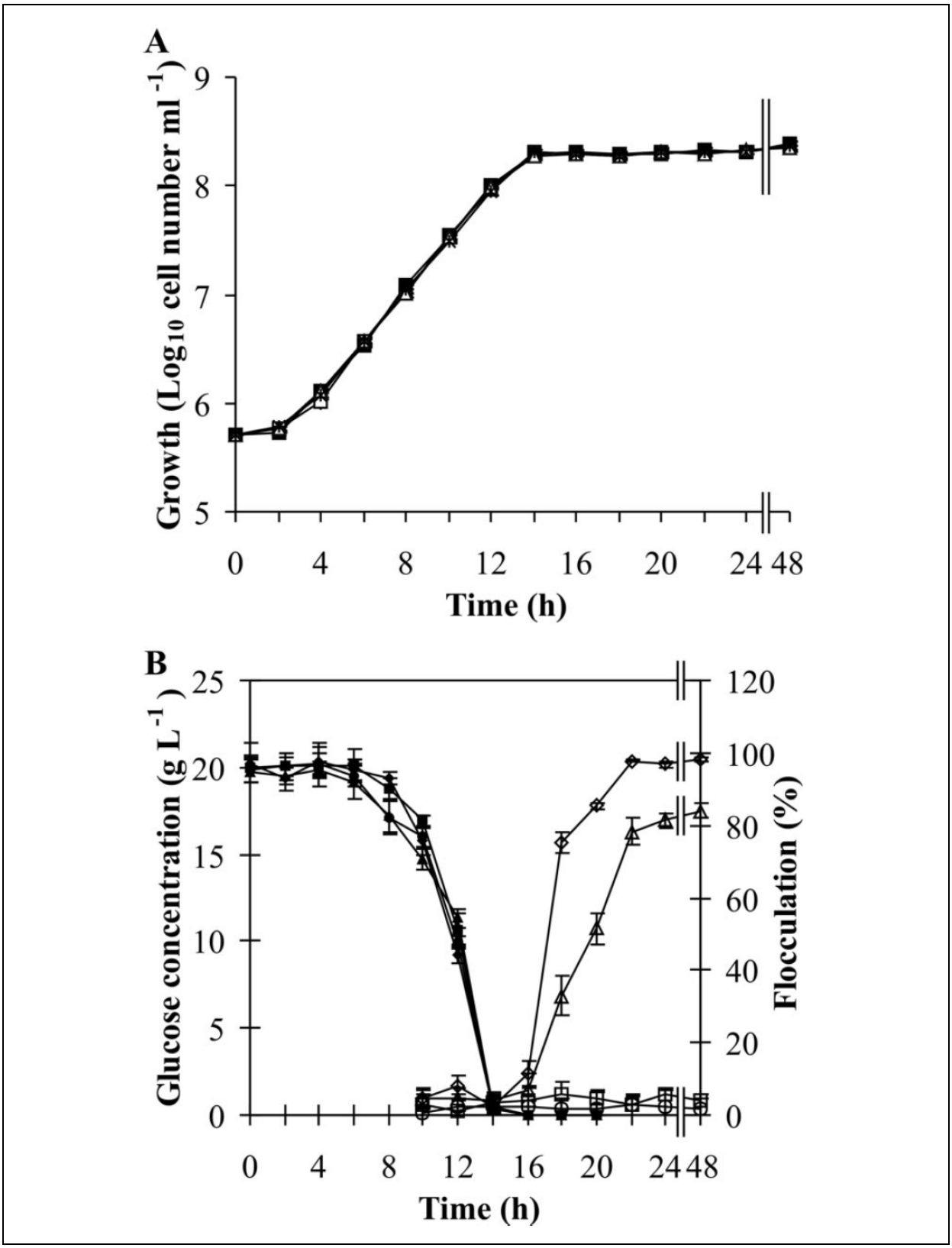

Figure 2 (A) Growth of FY23 (wild type) (□), FY23-F1A (■), FY23-F5A (△), and FY23-F11A (∗) strains. (B) Glucose utilization of FY23 (wild type) (■), FY23-F1A (◆), FY23-F5A (▲), and FY23-F11A (●) strains and flocculation profiles of FY23 (wild type) (□), FY23-F1A (◇), FY23-F5A (△), and FY23-F11A (○) strains. Yeast strains were cultivated in YEPD broth containing 2% glucose at 30°C with shaking (160 rpm). Values represent the means of experiments performed in triplicate, and error bars represent standard deviations.

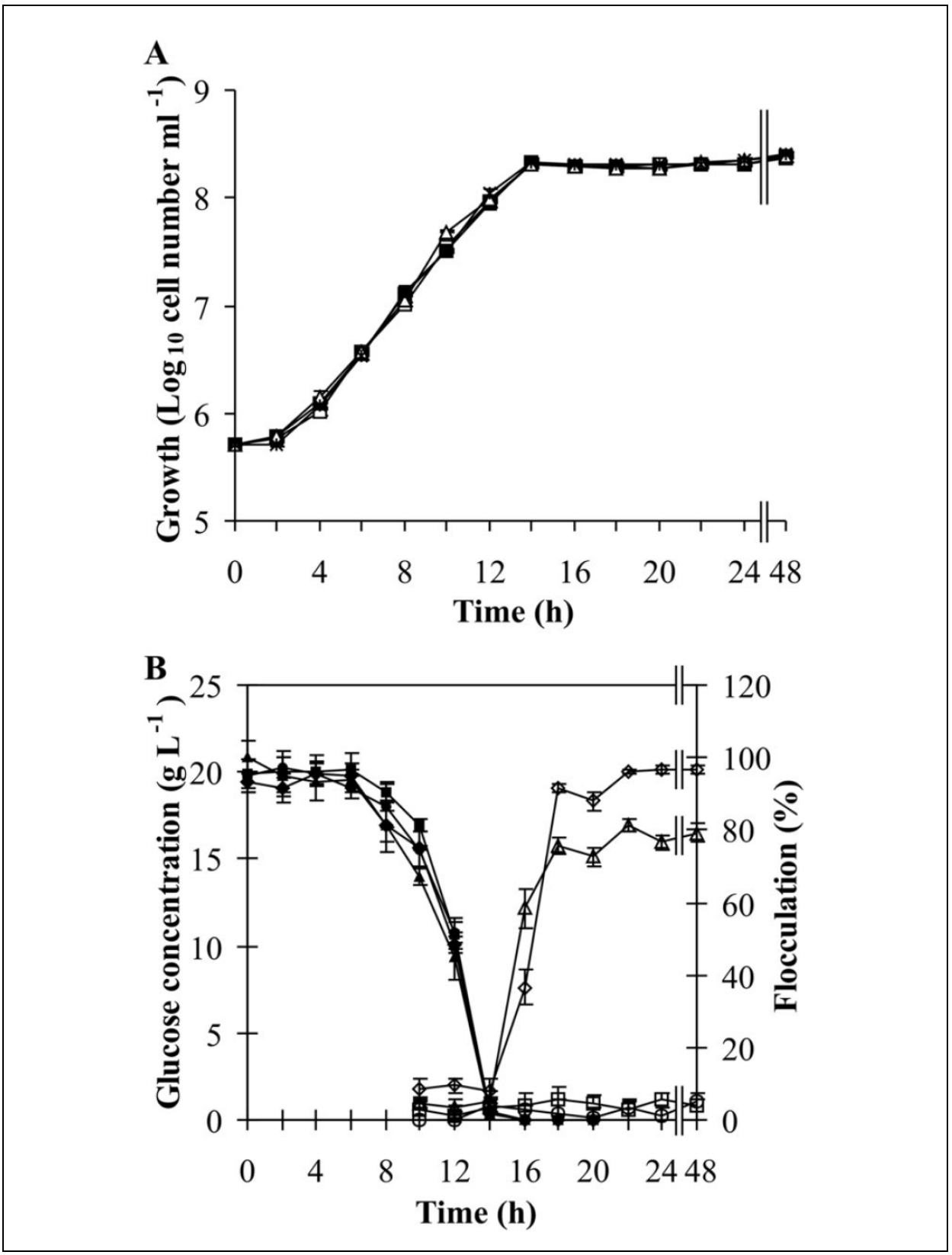

Figure 3 (A) Growth of FY23 (wild type) (□), FY23-F1H (■), FY23-F5H (△), and FY23-F11H (∗) strains. (B) Glucose utilization of FY23 (wild type) (■), FY23-F1H (◆), FY23-F5H (▲), and FY23-F11H (●) strains and flocculation profiles of FY23 (wild type) (□), FY23-F1H (◇), FY23-F5H (△), and FY23-F11H (○) strains. Yeast strains were cultivated in YEPD broth containing 2% glucose at 30°C with shaking (160 rpm). Each point represents the mean of experiments performed in triplicate, and error bars represent standard deviations.

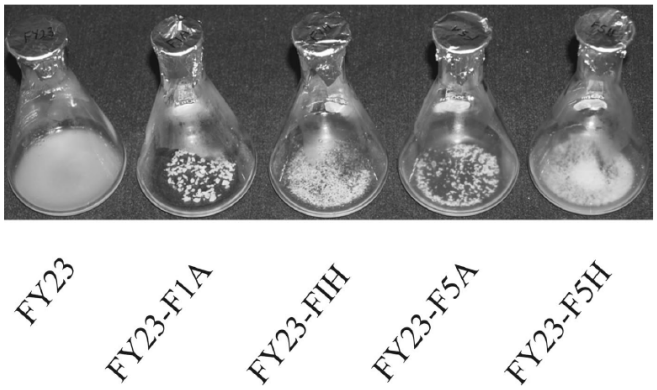

Figure 4 Floc formation by FY23 (wild type), FY23-F1A, FY23-F1H, FY23-F5A, and FY23-F5H strains. Yeast strains were cultivated for 48 h in YEPD broth containing 2% glucose at 30°C with shaking (160 rpm) and photographed in situ.

The flocculent abilities of the wild-type FY23 strain and six transformed yeast strains were studied over a broad pH range (Fig. 5). The FY23-F11A and FY23-F11H strains and the wild-type FY23 strain displayed no significant flocculation ability over the entire pH range. The FY23-F1A and FY23-F1H strains displayed relatively stable flocculation between pH 2 and 10, whereas flocculation was reduced by nearly 40% at pH 1. In contrast, flocculation exhibited by the FY23-F5A and FY23-F5H strains was stable between pH 3 and 10, while flocculation was reduced by approximately 20% at pH 2 and completely abolished at pH 1. This supports previous findings which reported that Flo1-type flocculation displays a broad tolerance to pH (Stratford, 1996), while a significantly reduced range (pH 4 to 5) was observed for NewFlo-type flocculation (Smit *et al.*, 1992).

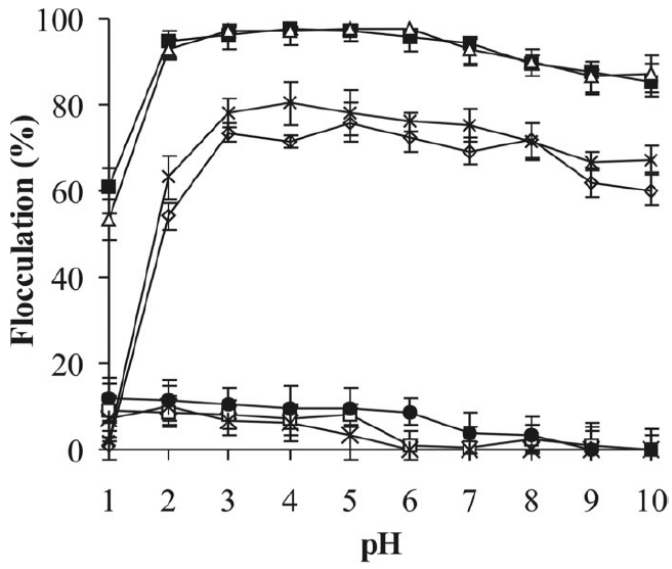

Figure 5 Effect of pH on flocculation of FY23 (wild type) (□), FY23-F1A (■), FY23-F1H (△), FY23-F5A (×), FY23-F5H (◇), FY23-F11A (●), and FY23-F11H (∗) strains. Yeast strains were grown for 48 h in YEPD broth containing 2% glucose at 30°C with shaking (160 rpm). Flocculation was determined using a modified Helm's assay as described by D'Hautcourt and Smart (D'Hautcourt and Smart, 1999) that incorporated a composite suspension buffer with a very wide buffering range from Stratford (1996). Each point represents the mean of experiments performed in triplicate, and error bars represent standard deviations.

The relationship between sugar concentration and inhibition of flocculation in *FLO1* and *FLO5* transformants was also assessed (Fig. 6). Increasing concentrations of mannose were shown to have a progressively inhibitory effect on the flocculation of all these transformants, and flocculation was completely inhibited at 900 mM mannose (Fig. 6A). In contrast, no inhibitory effect was evident in the presence of glucose (Fig. 6B). Although Kobayashi et al. (Kobayashi *et al.*, 1998) reported residual flocculation of 22% at 10 mM mannose for a *FLO1*-expressing *S. cerevisiae* strain displaying Flo1-type flocculation, the overall mannose inhibitory profile reported is similar to this finding. It can be suggested that the concentration of mannose required for complete inhibition of Flo1-type flocculation is variable and strain dependent. This may simply be a consequence of Flo1p concentrations within the cell wall, with higher concentrations of Flo1p requiring a higher level of mannose to achieve inhibition. In addition, changes in *FLO1* sequences between different strains may be responsible for the difference. Since NewFlo-type flocculation is inhibited by both mannose and glucose, while Flo1-type flocculation is exclusively inhibited by mannose (Stratford and Assinder, 1991), this result clearly demonstrates that *FLO1*- and *FLO5*-encoded flocculins exhibit Flo1-type flocculation.

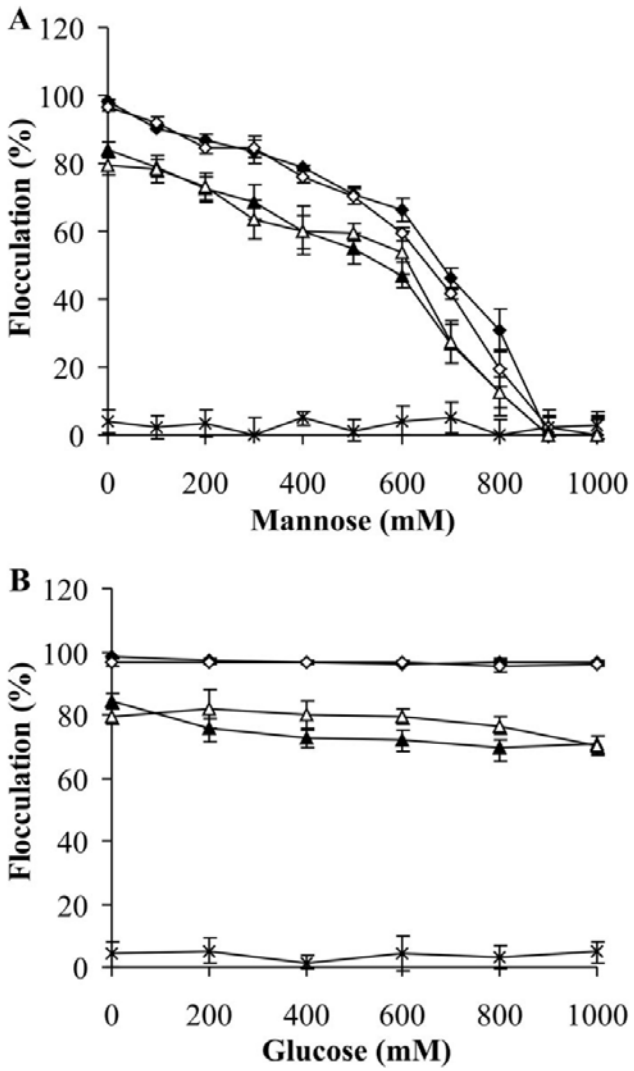

Figure 6 Effects of mannose (A) and glucose (B) on flocculation of FY23 (wild type) (×), FY23-F1A (◆), FY23-F1H (◇), FY23-F5A (▲), and FY23-F5H (△) strains. Yeast strains were grown for 48 h in YEPD broth containing 2% glucose at 30°C with shaking (160 rpm). Flocculation was determined using a modified Helm's assay as described in Materials and Methods. Values represent the means of experiments performed in triplicate, and error bars represent standard deviations.

QRT-PCR analysis

In order to verify whether *ADH2*- or *HSP30*-mediated *FLO* gene expression is similar to the reported expression patterns of these two promoters, total RNA from FY23, FY23-F11A, and FY23-F11H cultures was processed from different growth phases after 12 h (exponential), 16 h (entry/early stationary), and 48 h (late stationary), and QRT-PCR was performed. It is clearly evident (Fig. 7) that both *ADH2* and *HSP30* are tightly repressed in the presence of glucose at 12 h. Entry into stationary phase shows strong induction. RNA levels, while slightly decreased in the late stationary phase, are maintained at high levels. Similar data were observed for the *FLO1* and *FLO5* constructs (data not shown). These transcription levels are strongly correlated with the onset of flocculation and adhesion phenotypes in all strains (Fig. 2 and 3). Moreover, the data clearly suggest that only the *FLO* gene carrying a modified promoter is activated and that the two other genes that were monitored appear not to contribute to the observed phenotypes.

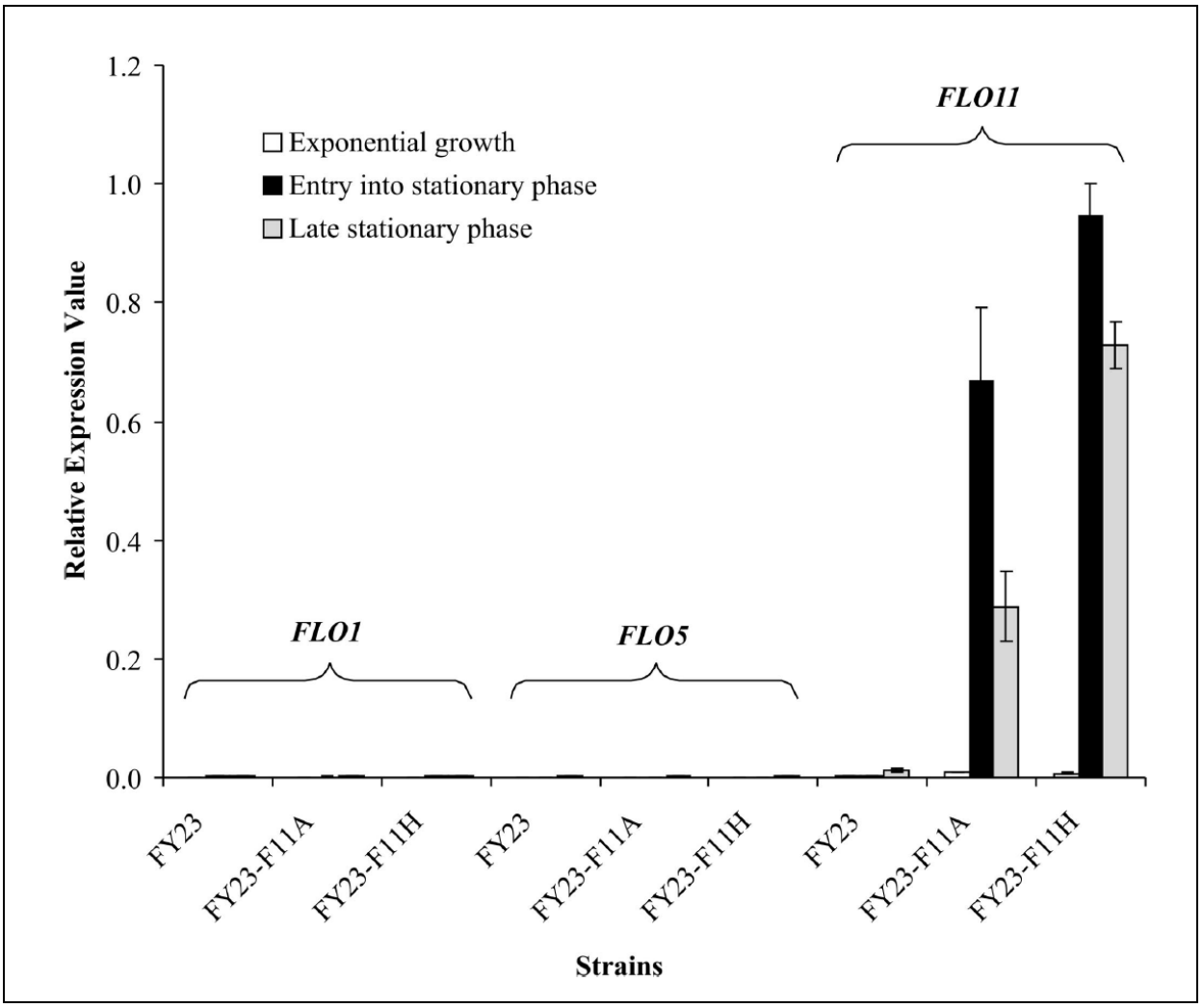

Figure 7 Relative QRT-PCR expression of *FLO1*, *FLO5*, and *FLO11* transcripts in FY23 (wild type), FY23-F11A, and FY23-F11H strains. Samples were taken from sampling points corresponding to exponential growth phase, entry into stationary growth phase, and upon completion of fermentation. As indicated, a bracket denotes the expression of a particular *FLO* gene. The relative expression value for each sample was defined as $2^{-Ct(target)}$ where $Ct_{(target)}$ represents the cycle number at which a sample reaches a predetermined threshold signal value for the specific target gene. Relative expression data were normalized to the relative expression value of the housekeeping gene *PDA1* in each respective sample, thus giving normalized relative expression for a target gene as $2^{-Ct(target)}/2^{-Ct(PDA1)}$. The highest mRNA expression level was arbitrarily set at 1.0. Values represent the means of experiments performed in triplicate, and error bars represent standard deviations.

Heat shock and/or ethanol stress induction of flocculation in FY23-F1H and FY23-F5H strains

Both heat shock treatment and exposure to ethanol were reported as suitable induction conditions for the *HSP30* promoter (Piper *et al.*, 1994; Seymour and Piper, 1999). Thus, it was probable that flocculent phenotypes conferred on transformed strains FY23-F1H and FY23-F5H under transcriptional regulation of *HSP30*p could be triggered when desired in response to these stress conditions. To assess these possibilities, only non-flocculent exponentially growing cell populations of these strains (determined from Fig. 2 and 3) were subjected to heat shock treatments and/or exposure to differing ethanol concentrations. The results clearly indicate that heat shock treatment for 45 min at 42°C elicited the strongest induction of flocculation in both FY23-F1H (94%) and FY23-F5H (65%) strains (Fig. 8). On the other hand, exposure to 3% (vol/vol) ethanol induced flocculation to a lesser extent in both FY23-F1H (70%) and FY23-F5H (28%) transformants. Both strains displayed similar flocculent abilities (approximately 10%) when exposed to 6% (vol/vol) ethanol, while no induction was evident for an ethanol/heat shock combination treatment.

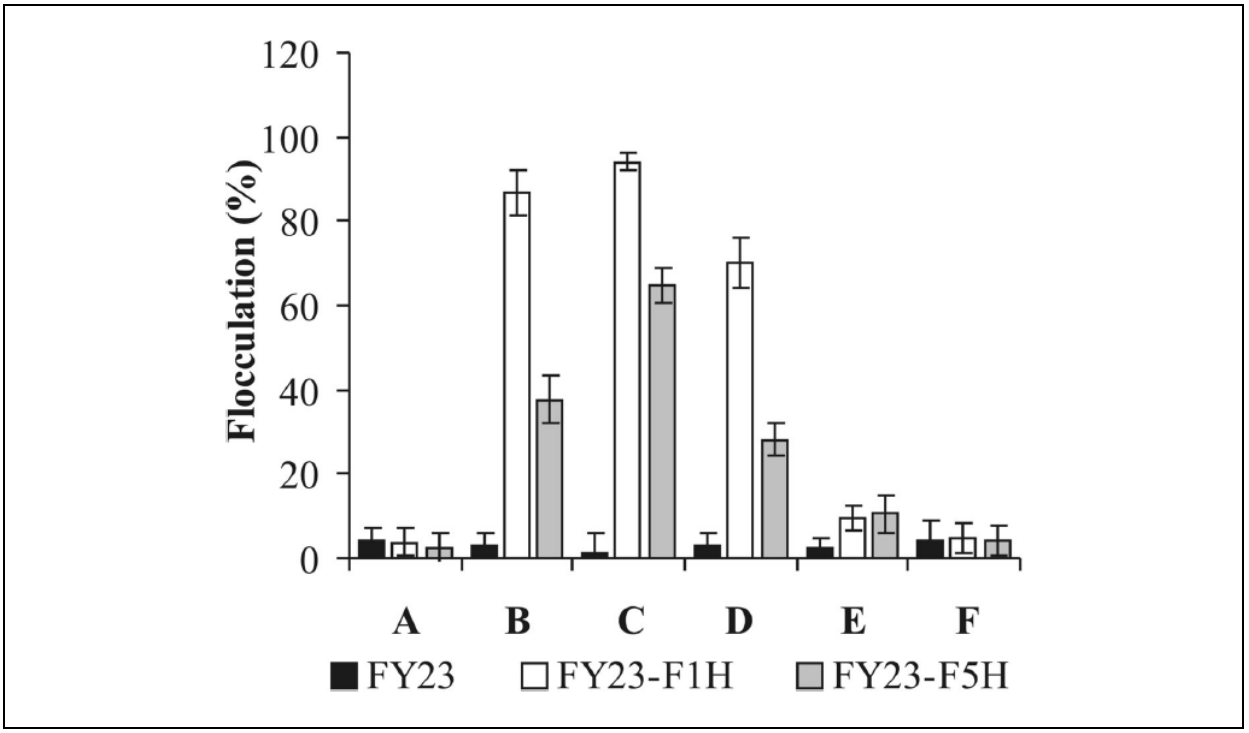

Figure 8 Stress-induced expression of *FLO1*- and *FLO5*-encoded flocculins in *HSP30* transformants. Yeast strains cultivated for 10 h in YEPD were subjected to the following treatments: A, untreated (45 min at 30°C); B, heat shock for 30 min at 42°C; C, heat shock for 45 min at 42°C; D, 3% (vol/vol) ethanol for 30 min at 30°C; E, 6% (vol/vol) ethanol for 30 min at 30°C; F, 6% (vol/vol) ethanol and heat shock for 30 min at 42°C. The results are averages of three independent determinations, and error bars represent standard deviations.

Flor formation and invasive growth

As shown in Fig. 9A, only transgenic yeast FY23-F11A and FY23-F11H strains formed a biofilm after 5 days in flor medium at 30°C under static conditions. The FY23-F11A strain produced a distinctly thicker biofilm (Fig. 9A) and displayed threefold-higher suspended cell densities when evaluated 60 h post-inoculation (Fig. 9B).

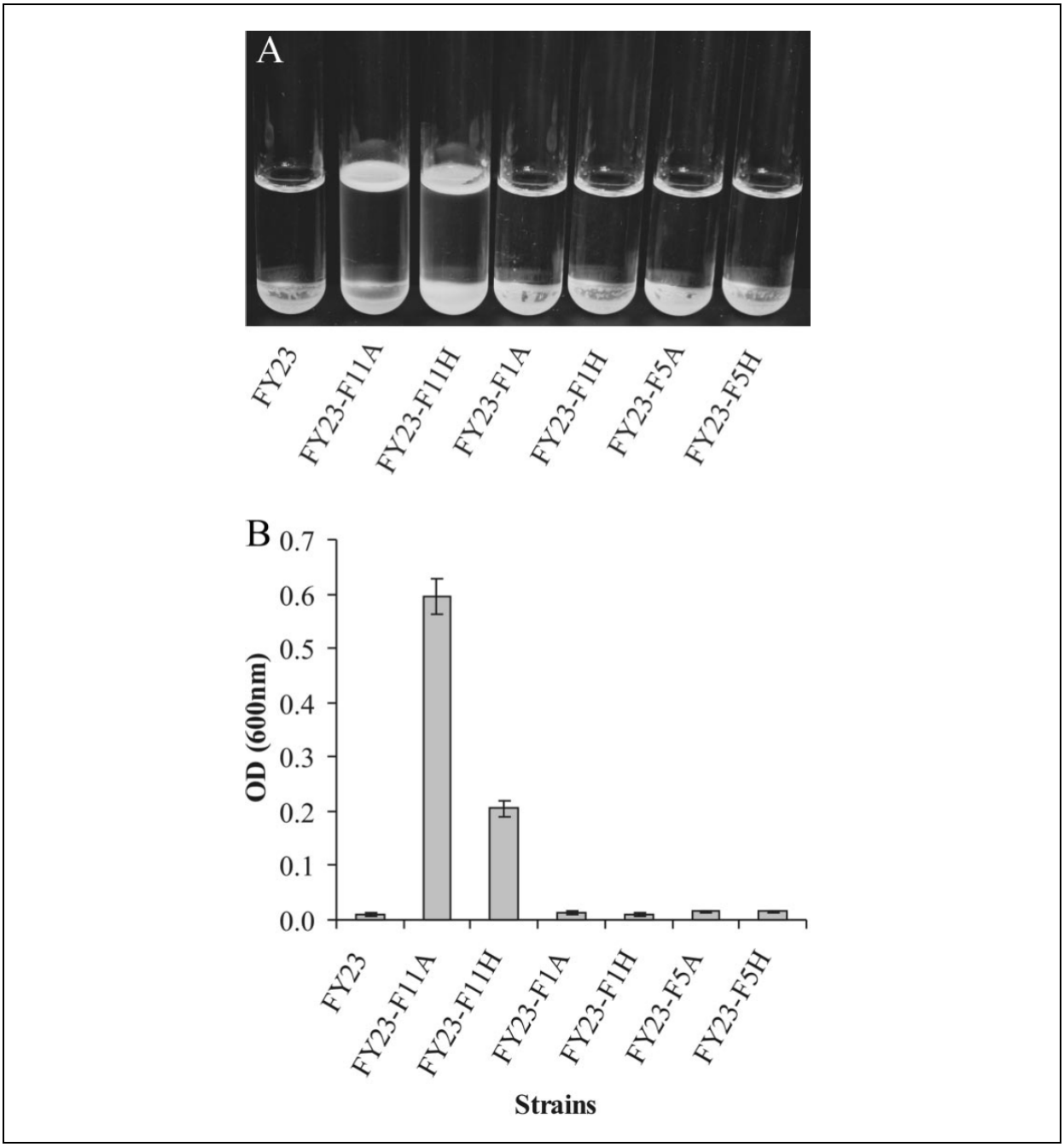

Figure 9 (A) Biofilm formation. Cells were pre-cultured in YEPD broth and recovered by centrifugation, washed once with flor medium, and resuspended at a density of 6 x 10^7 cells ml^{-1} in 5 ml flor medium contained in glass test tubes (16 by 165 mm). The tubes were photographed after 5 days of static incubation at 30°C. (B) Buoyant cell density determinations. The cultures were incubated statically at 30°C for 60 h, after which 1-ml samples were withdrawn from just below the meniscus. The optical density (OD) of samples was determined spectrophotometrically at 600 nm. The results are averages of three independent determinations, and error bars represent standard deviations.

The ability of the wild-type FY23 strain and its six transformants to invade agar is shown in Fig. 10. Only *ADH2*-promoted *FLO11* expression resulted in an invasive growth phenotype in SCLD and $SCLD_{LUT}$ agar media. Moreover, the FY23-F11A strain grew as a larger colony on SCLD agar, and it displayed more aggressive invasive growth behaviour on SCLD agar plates than on $SCLD_{LUT}$ agar plates.

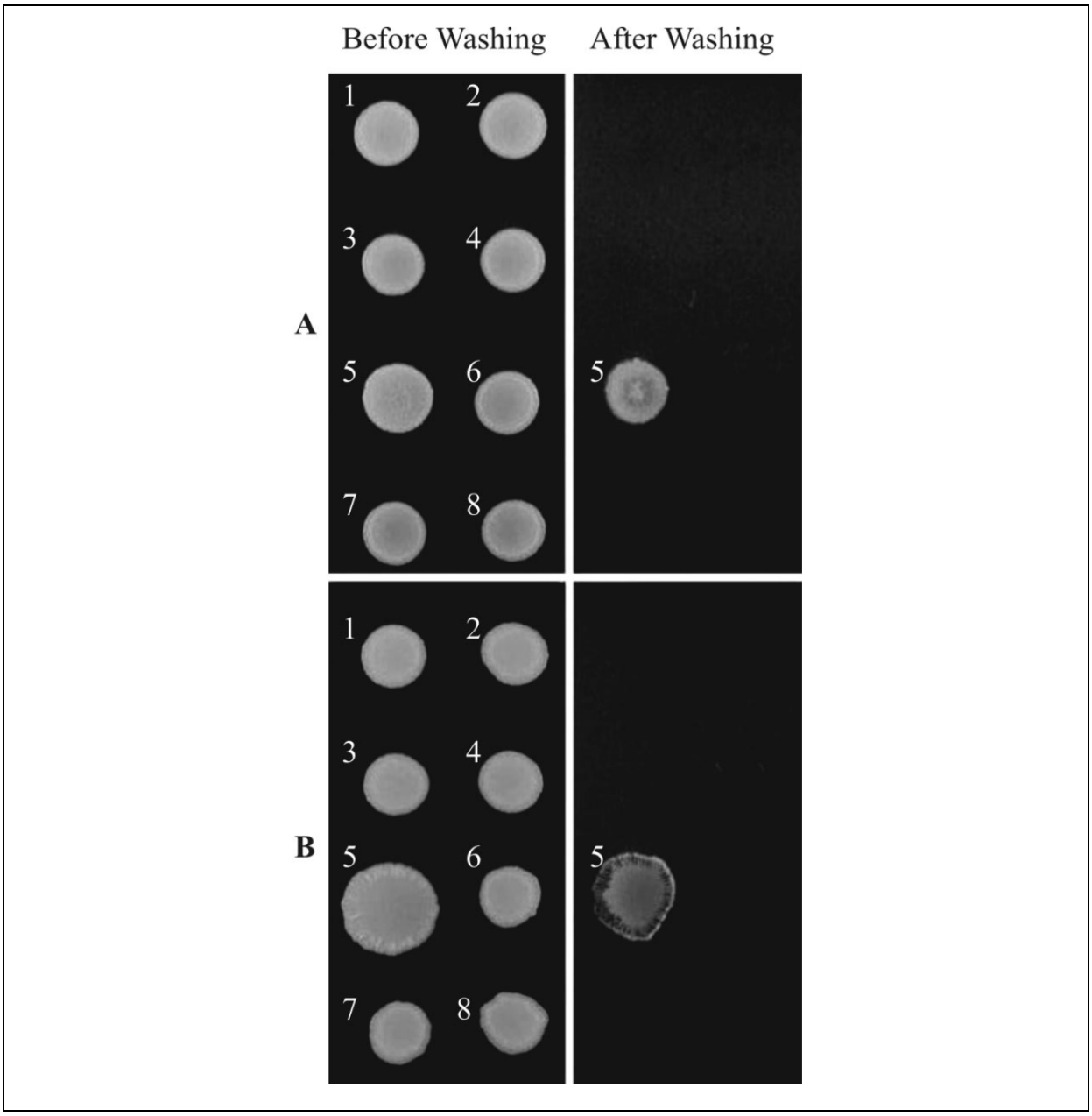

Figure 10 Haploid invasive growth of FY23-F5A (colony 1), FY23- F5H (colony 2), FY23-F1A (colony 3), FY23-F1H (colony 4), FY23- F11A (colony 5), FY23-F11H (colony 6), and FY23 (wild type) (colonies 7 and 8) strains after 5 days growth at 30°C on $SCLD_{LUT}$ (A) and SCLD (B) media.

Effect of *FLO* gene expression on cell surface hydrophobicity

The hydrophobicity of yeast cell surfaces (Fig. 11) from yeast populations grown in YEPD medium for 48 h was determined by measuring the distribution ratio of yeast cells in a biphasic system consisting of a buffered solution and an organic solvent. The order of enhancement in terms of the MHI is FY23-F11A (0.83) > FY23-F11H (0.79) > FY23-F1A (0.64) > FY23-F1H (0.61) > FY23-F5A (0.29) > FY23-F5H (0.26) > FY23 (wild type) (0). Thus, it may be concluded that insertion of *FLO* gene-encoded glycoproteins Flo1p, Flo5p, and Flo11p into the yeast cell wall is responsible for increased cell surface hydrophobicity.

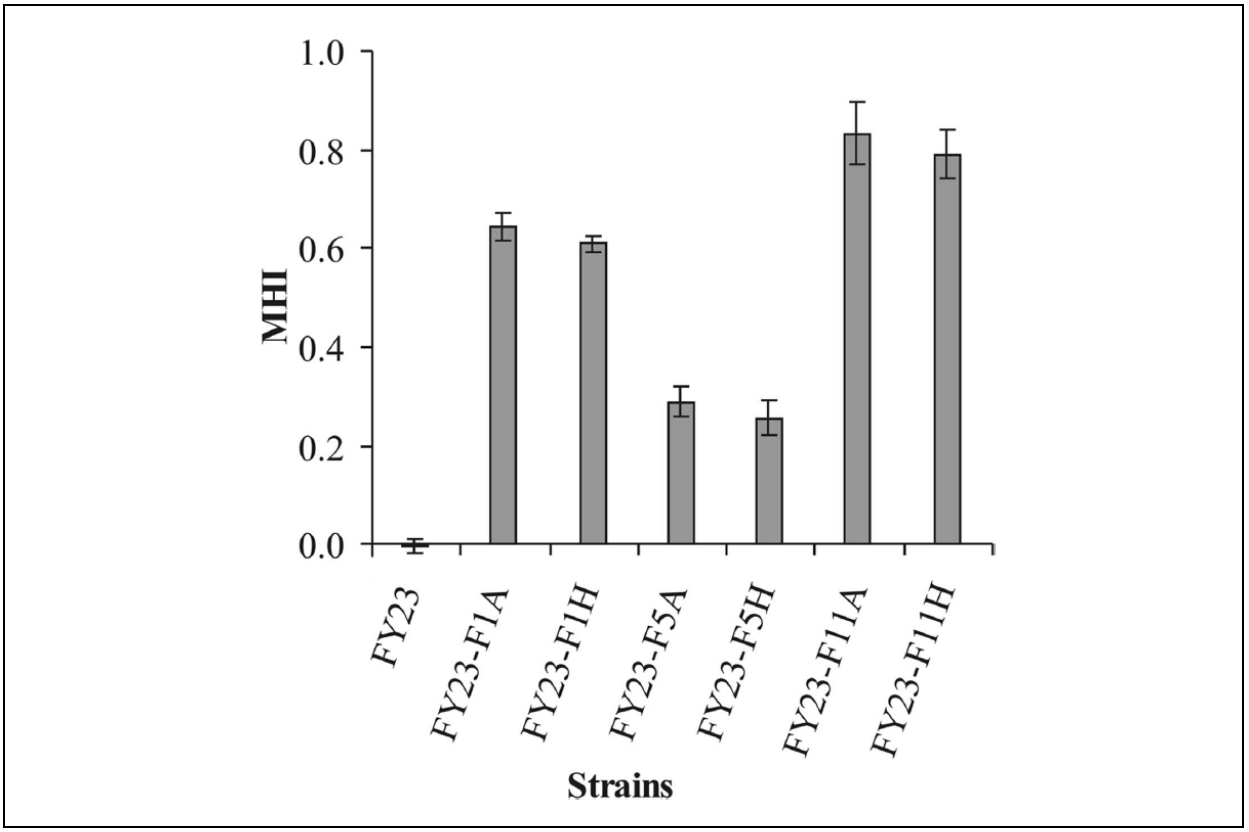

Figure 11 Impact of *ADH2* and *HSP30* expression of *FLO* genes on cell surface hydrophobicity. The wild-type FY23 strain and transformants were cultivated for 48 h in YEPD broth containing 2% glucose at 30°C with shaking (160 rpm). The MHI was determined according to the biphasic-solvent partition assay described by Hinchcliffe et al. (Hinchcliffe *et al.*, 1985). The results are averages of three independent determinations, and error bars represent standard deviations.

Discussion

This is the first report that uses genome-integrated promoter-ORF combinations to compare the impact of various flocculation gene and promoter combinations on cell surface properties and cell surface-dependent phenotypes. The data show that integration confers stable (both in timing and intensity) expression properties to the targeted genes and demonstrate the possibility of adjusting flocculation and flor-forming behaviour to specific industrial requirements. Importantly, all the engineered yeast strains displayed vegetative growth and fermentation properties that are comparable to those of the host strain, indicating that those industrially relevant characteristics were not compromised by modified *FLO* gene expression.

In this study, the genomic *FLO1*, *FLO5*, and *FLO11* ORFs were brought under the transcriptional control of promoters of the *ADH2* and *HSP30* genes by replacement of their native promoter sequences. The distinct advantage of the cloning strategy employed here over those used by other research groups (Chambers *et al.*, 2004; Cunha *et al.*, 2006) is that no sub-cloning of the *FLO* genes is required. Furthermore, expression levels are independent of plasmid-related artefacts, such as variable copy numbers and the increased risk of intragenic recombinations. Indeed, *FLO* genes contain intragenic tandem repetitive sequences that have been previously reported as difficult to clone or even as "unclonable" sequences (Teunissen *et al.*, 1993). Our data therefore provide reliable baseline information regarding the intrinsic ability of the three *FLO* genes to induce adhesion-related phenotypes.

The data show that *FLO1*-based constructs induce flocculation most efficiently, while *FLO5*-based constructs, while still leading to significant flocculation, are less efficient. *FLO11*-based constructs, on the other hand, induce flocculation only weakly. These constructs, however, strongly induced flor formation and cell adhesion, phenotypes that were not observed with *FLO1* or *FLO5*. Strains expressing *FLO11* also presented the highest cell surface hydrophobicity. Hydrophobicity was significantly lower in strains expressing *FLO5*, while strains expressing *FLO1* presented intermediate hydrophobicity levels. These data suggest that hydrophobicity per se is not a major determinant of adhesion-related phenotypes but that the specific sequences of the *FLO* genes are mainly responsible for phenotype specificity.

The observed flocculation patterns were in all cases consistent with the reported and measured expression patterns conferred by the two promoters. In the case of *HSP30*p-*FLO1* and *HSP30*p-*FLO5* transformants, the onset of flocculation occurred toward the end of the respiro-fermentative exponential growth phase and was concomitant with the depletion of glucose from the medium. This is consistent with a previous study which showed in particular that the levels of *HSP30* mRNA increased before glucose exhaustion and climaxes with glucose exhaustion (Regnacq and Boucherie, 1993). The study also confirms the stress-inducible nature of *HSP30*p-controlled expression of *FLO1* and *FLO5* genes to yield flocculent phenotypes in response to specific stress conditions that include heat shock or exposure to ethanol. Although an ethanol concentration of 6% (vol/vol) is recommended for maximal induction of *HSP30*p, it is possible that this concentration brings about a toxic effect in the laboratory strains, which could be responsible for the absence of flocculation in these cells (Claro *et al.*, 2007; Piper *et al.*,1994).

Other groups have previously engineered the expression of individual *FLO* genes. The *FLO1* gene was constitutively expressed, thereby creating transgenic yeast strains that exhibited a

constitutive flocculation property irrespective of the growth phase (Barney *et al.*, 1990; Ishida-Fujii *et al.*, 1998; Watari *et al.*, 1994; Watari *et al.*, 1991). However, efficient fermentation requires a high suspended cell count, and constitutively flocculating yeast may lead to sluggish or stuck fermentations. These transgenic yeast strains are therefore not ideally suited for industrial batch-wise fermentation processes. Cunha and co-workers (Cunha *et al.*, 2006) reported controlled expression of the *FLO5* gene by employing a modified *ADH2* promoter. However, the native core promoter and ORF sequences of the *FLO5* gene used by Cunha et al. (Cunha *et al.*, 2006) were sourced from the YEp-FLO5 plasmid. This plasmid was originally created by Bidard and co-workers (Bidard *et al.*, 1994) and was reported to contain the *FLO5* gene from the *S*. *cerevisiae* 17-13D strain. However, later studies by this research team retracted and confirmed that the *FLO5* gene used in the initial study was in fact identical to the *FLO1* gene sequence (Bidard *et al.*, 1995). We therefore assume that Cunha *et al*. (2006) used the *FLO1* gene in their studies. This implies that our research study is the first to report inducible promoter-controlled *FLO5* and *FLO11* gene expression.

Cunha *et al*. (2006) employed a multicopy plasmid-based strategy fusing the poly (T), UAS1, and UAS2 regions of the *ADH2* promoter upstream of the native core promoter and ORF of the *FLO1* gene for expression in the laboratory yeast strain W303-1a. The same modified promoter was also employed to control *FLO1* gene expression by cloning an integrative cassette to disrupt the *CAN1* gene in a commercial baking yeast strain (Fleischmann). Similar to our study, the strains were reported to flocculate after glucose exhaustion in nutrient-rich medium (Cunha et al., 2006). However, when using the native *ADH2* promoter, the onset of flocculation observed for *ADH2*p-*FLO1* and *ADH2*p-*FLO5* transformants in our study is in line with data published by Lee and DaSilva (Lee and DaSilva, 2005) who reported a similar native *ADH2* promoter-mediated expression pattern for -galactosidase in *S*. *cerevisiae* transformed with a chromosomally integrated *ADH2*p-*lacZ* cassette. Moreover, the native *ADH2* promoter on multicopy plasmids was shown to drive -xylanase production only after glucose exhaustion (Kealey *et al.*,1998; Luttig *et al.*, 1997), clearly suggesting that modification of the native *ADH2* promoter as suggested by Cunha *et al*. (2006) is not necessary. Chambers et al. (Chambers *et al.*, 2004) employed the glucose-repressible *S. cerevisiae JEN1* promoter to regulate *FLO1* gene-mediated flocculation. However, the FY23-F1A and FY23-F5A strains reported here display a much later onset of flocculation in comparison to their *JEN1-FLO1* transgenic *S. cerevisiae* strain W303. These observations are clearly significant, as an early onset of flocculation might lead to a “stuck” or “hanging” fermentation because of insufficient contact between settled yeast cells and the medium. Some authors have reported non-detectable to significant decreases in ethanol production when converting non-flocculent yeast strains into flocculent strains (Cunha *et al.*, 2006; Hinchcliffe *et al.*, 1985; Verstrepen *et al.*, 2001; Watari *et al.*, 1990; Watari *et al.*, 1994; Watari *et al.*, 1991). Although decreased ethanol production will not meet the requirements of bioethanol production, it may be attractive to the alcoholic beverage industries that are currently faced with a growing consumer demand for lower-alcohol beers and wines (Heux *et al.*, 2006; Nevoigt *et al.*, 2002).

The decreased flocculation abilities observed for all strains in chemically defined minimal media may be attributed to starvation for auxotrophically required nutrients, as recent studies by Pronk (2002) recommend increased supplementation of auxotrophic nutrients in comparison to those used in this study as prescribed by Sherman *et al*. (1991). Lee and DaSilva (2005) reported 10-fold-lower-galactosidase activities for transgenic *S*. *cerevisiae* strains expressing lacZ under transcriptional control of the *ADH2* promoter when grown in minimal medium containing 2%

glucose (wt/vol), which further supports these findings. Comparison of the relative promoter strengths of *ADH2*p and *HSP30*p for *FLO* gene expression in minimal media seems to suggest an increased nutritional demand for assimilable nitrogen by *ADH2*p. Although this study shows that *ADH2*p is responsible for later induction of flocculation and stronger flocculent phenotypes in nutrient-rich medium than *HSP30*p, it is most probable that *ADH2*p-controlled flocculation may not be suitable for certain industrial batch fermentation processes, such as winemaking because grape musts are sometimes deficient in assimilable nitrogen compounds (Henschke and Jiranek, 1993).

Although no observable adhesion phenotype was evident for the *FLO11* transformants used in this study, Bayly et al. (Bayly *et al.*, 2005) presented evidence that *FLO11*-encoded flocculin yielded a strongly flocculent Flo1 phenotype in untransformed *S. cerevisiae* strain YIY345. However, it was also reported that *FLO11* over-expression in *S. cerevisiae* strain Σ1278b promotes very weak calcium-independent flocculation, while over-expression in *S. cerevisiae* strain S288C does not promote cell-to-cell adhesion (Guo *et al.*, 2000; Verstrepen and Klis, 2006). It is possible that the flocculent ability of FY23-F11A and FY23-F11H strains may be too weak to be assessed by the modified Helm's assay employed in this study.

As mentioned previously, a nonsense mutation in the *FLO8* gene ensures that the dominant *FLO* genes are transcriptionally silent in the S. cerevisiae FY23 strain employed in this study (Liu *et al.*, 1996; Verstrepen *et al.*, 2005; Winston *et al.*, 1995). Thus, it is possible to eliminate contributions by other dominant *FLO* genes and exclusively assess the phenotypic consequences of *FLO11* expression. Therefore, it may be concluded that *ADH2*- and *HSP30*-facilitated *FLO11* expression is sufficiently responsible for flor formation. This finding is further supported by earlier reports that identified *FLO11* as a primary factor for flor formation in other *S. cerevisiae* strain types (Ishigami *et al.*, 2004; Ishigami *et al.*, 2006; Zara *et al.*, 2005).

It has been proposed that flor wine yeast begins to form flor via a *FLO11*-mediated mechanism only when glucose repression of *FLO11* transcription is eliminated due to depletion of grape sugar after alcoholic fermentation (Ishigami *et al.*, 2004) . Based on the findings of this study, it can be suggested that the *ADH2* or *HSP30* promoter can be utilized to induce flor formation in non-flor wine yeast in a manner that will mimic natural flor wine yeast. The fact that the FY23-11A strain displayed decreased invasive growth in minimal agar that contained only auxotrophic nutritional requirements in comparison to complete nutrient supplementation further supports the previously mentioned notion that the *ADH2* promoter displays an increased demand for assimilable nitrogen. Surprisingly, no invasive growth phenotype was associated with the FY23-F11H strain. This suggests that growth on solid media is not an ideal induction condition for the *HSP30* promoter.

This study highlights that specific adhesion properties appear to be defined primarily by the properties of specific flocculins and not by general cell wall properties, such as hydrophobicity. Each *FLO* gene leads to specific phenotypes and phenotype intensities, with *FLO1* and *FLO5* resulting in cell aggregation and flocculation, whereas *FLO11* expression leads to invasive growth and flor formation. Clearly, the timing and intensities of the phenotypes are entirely dependent on the transcriptional regulation of each individual *FLO* gene.

The data clearly demonstrate that the flocculation behaviour of industrial yeast can be fine-tuned to optimize specific production processes. The modified yeast strains used in this study contain only yeast-derived DNA sequences and can be regarded as self-cloned strains. Such

modified strains are generally recognized as safe and may be approved more readily for industrial exploitation (Verstrepen *et al.*, 2003). The bioengineering of *S. cerevisiae* strains capable of controlled flocculation reported in this study may also benefit downstream processing in the pharmaceutical and nutraceutical industries which employ *S. cerevisiae* in batch-wise fermentations for the biosynthesis of high-value natural products, such as isoprenoids, flavanoids, and longchain polyunsaturated fatty acids. We are currently investigating the impact of the same constructs in industrial wine yeast strains.

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Addendum B

FLO gene dependent phenotypes in industrial wine yeast strains

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ADDENDUM B

FLO gene dependent phenotypes in industrial wine yeast strains

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Abstract

Most commercial yeast strains are non-flocculent. However, controlled flocculation phenotypes could provide significant benefits to many fermentation-based industries. In nonflocculent laboratory strains, it has been demonstrated that it is possible to adjust flocculation and adhesion phenotypes to desired specifications by altering expression of the otherwise silent but dominant flocculation (*FLO*) genes. However, *FLO* genes are characterized by high allele heterogeneity and are subjected to epigenetic regulation. Extrapolation of data obtained in laboratory strains to industrial strains may therefore not always be applicable. Here we assess the adhesion phenotypes that are associated with the expression of a chromosomal copy of the *FLO1*, *FLO5* or *FLO11* open reading frame in two non-flocculent commercial wine yeast strains, BM45 and VIN13. The chromosomal promoters of these genes were replaced with stationary phase inducible promoters of the *HSP30* and *ADH2* genes. Under standard laboratory and wine making conditions, the strategy resulted in expected and stable expression patterns of these genes in both strains. However, the specific impact of the expression of individual *FLO* genes showed significant differences between the two wine strains and with corresponding phenotypes in laboratory strains. The data suggest that optimization of the flocculation pattern of individual commercial strains will have to be based on a strain-by-strain approach.

Introduction

Due to its ability to efficiently ferment the hexoses glucose, fructose and maltose from natural raw materials such as rice, wheat, barley, corn and grape juice, the yeast *Saccharomyces cerevisiae* has traditionally been employed in many food production processes, most prominently in the production of alcoholic beverages and in the baking industry. More recently, the relative ease and availability of genetic tools has resulted in S. cerevisiae increasingly being utilized as a cell factory for the production of various enzymes or metabolites such as insulin (Kjeldsen 2000), L-lactic acid (Saitoh *et al*. 2005) and others (Kealey *et al*. 1998; Maury *et al*. 2005). On completion of most, if not all of these industrial processes, yeast cells must be removed prior to further processing of the product. In the case of wine fermentation, removal processes involve settling, filtration and other clarification strategies. Such processes can be costly and may result in reduced quality of the final product (Pretorius and Bauer 2002). Considering the global trend in food production towards less interventionist, less energy consuming and “greener” processes, wine makers would prefer to reduce or, if possible, entirely eliminate the need for such interventions. A phenotype of particular

interest in this regard is referred to as flocculation. While the phenotype “flocculation” is relatively easily and precisely defined as the asexual, reversible and calcium-dependent aggregation of yeast cells to form flocs containing large numbers of cells that rapidly sediment to the bottom of the liquid growth substrate (Stratford 1989; Bony *et al*. 1997), the exact biological relevance of this phenotype remains poorly understood. It has recently been suggested that flocculation may be a means to protect the cells that are present in the center of a floc from environmental stress or serve as a means of passive transport away from the stress (Verstrepen and Klis 2006; Smukalla *et al*. 2008). The self-clearing of beers at the end of the fermentation by the flocculation and settling of ale yeast is a highly desirable characteristic of brewing yeast strains. Such specific flocculation or adhesion phenotypes could be beneficial for many other industrial processes. However, flocculation must not occur before alcoholic fermentation has been completed since flocculent strains do not ferment 1 efficiently and early flocculation may result in sluggish or stuck fermentations. Structural and functional analysis of the genomic sequence of a laboratory strain (S288C)of *S. cerevisiae* reveals that this yeast strain contains five distinct *FLO* genes which are *FLO1*, *FLO5*, *FLO9*, *FLO10* and *FLO11* (Teunissen and Steensma 1995; Verstrepen *et al*. 2004). Mature Flo mannoproteins are components of the outermost layer of the cell wall and are collectively referred to as adhesins or flocculins. They possess a common modular organization that consists of three domains. An amino-terminal domain that is proposed to harbour the binding site to carbohydrate receptors (mannan) which confers adhesion (Kobayashi *et al*. 1998), a central domain that is rich in serine and threonine residues (Caro *et al*. 1997) and a carboxyl terminal region. The latter region is glycosidically linked via a lipidless remnant of its glycosyl phosphatidylinositol (GPI) anchor moiety to the inner cell wall polysaccharide skeletal network (Caro *et al*. 1997; Hamada *et al*. 1998; Lipke and Ovalle 1998; De Groot *et al*. 2003). Studies have indicated that expression of specific *FLO* genes in *S. cerevisiae* laboratory strains is responsible for specific cell-wall dependent phenotypes, including flocculation and other types of cell-cell adhesion, substrate adhesion, surface hydrophobicity, biofilm formation and the ability to invade a growth substrate (Guo *et al*. 2000; Cunha *et al*. 2006; Verstrepen and Klis 2006; Govender *et al*. 2008; Wang *et al*. 2008; Van Mulders *et al*. 2009). In previous attempts to modify flocculation behaviour of laboratory yeast strains, a dominant *FLO* gene from donor *S. cerevisiae* strains was employed to convert non-flocculent yeast strains into flocculent strains (Watari *et al*. 1991; Chambers *et al*. 2004; Cunha *et al*. 2006; Wang *et al*. 2008). On the other hand, Govender *et al*. (2008), Van Mulders *et al*. (2009) and Verstrepen *et al*. (2001) employed a promoter replacement strategy. A distinct advantage of the this strategy over the use donor-derived *FLO* genes, is that no sub-cloning of the *FLO* genes is required; the modified yeast strains contain only yeast-derived DNA sequences and can be regarded as self-cloned strains, and expression levels are independent of plasmid-related artefacts such as variable copy-numbers. To induce stationary phase specific expression of the *FLO* genes, the native promoters of the flocculation genes *FLO1*, *FLO5*, or *FLO11* in the haploid non-flocculent, non-invasive and non-flor forming *S. cerevisiae* FY23 strain were replaced with inducible promoters of the *ADH2* and *HSP30* genes. The data obtained from these studies suggested the suitability of the promoter-*FLO*-gene constructs to induce desirable phenotypes (Verstrepen *et al*. 2001; Govender *et al*. 2008). Commercial wine yeast strains that have been selected in the last century from natural spontaneous wine fermentations on the basis of their desirable oenological properties are known to be significantly different from the haploid laboratory strain (Pretorius 2000). The aim of the present study was therefore to compare the phenotypes observed in transgenic laboratory strains with those generated in recombinant wine yeast strains, and to assess the fermentative and flocculation potential of transgenic BM45 and VIN13 wine yeast strains under wine-making conditions. The

data presented here confirm that inducible expression of the native *FLO1* and *FLO5* open reading frames, albeit to varying degrees, are responsible for a quantifiable cell-cell adhesion phenotype, whereas inducible expression of the *FLO11* ORF resulted in biofilm/flor formation and invasive growth phenotypes. Irrespective of the promoter involved and contrarily to observations in the laboratory strain, *FLO5*-based constructs were observed to induce flocculation more efficiently than *FLO1*-based constructs in the wine yeast strains. When assessed in standard laboratory culture conditions, ADH2p-controlled *FLO1* and *FLO5* phenotypes of transgenic wine yeast strains are distinctly more flocculent than comparable HSP30p regulated phenotypes. However, data also show that the *ADH2* promoter appears unsuitable for the purpose of driving *FLO* gene expression under wine-making conditions, whereas HSP30p wine yeast transformants showed industrially desirable properties.

Materials and Methods

Strains

All yeast strains used in this study are listed in Table 1. *Escherichia coli* DH5α (Gibco BRL/Life Technologies, Rockville, MD) was used as a host for all plasmid amplifications.

Table 1 *S. cerevisiae* strains employed in this study.

Strain	Genotype	Reference
BM45	Industrial wine yeast strain (unknown genotype)	Lallemand Inc., Canada
BM45-F1A	*FLO1p::SMR1-ADH2p*	This study
BM45-F1H	*FLO1p::SMR1-HSP30p*	This study
BM45-F5A	*FLO5p::SMR1-ADH2p*	This study
BM45-F5H	*FLO5p::SMR1-HSP30p*	This study
BM45-F11A	*FLO11p::SMR1-ADH2p*	This study
BM45-F11H	*FLO11p::SMR1-HSP30p*	This study
EC1118	Industrial wine yeast strain (unknown genotype)	Lallemand Inc., Canada
FY23	*MAT*a *leu2 trp1 ura3 flo8-1*	Winston *et al*., 1995
FY23-F1A	*MAT*a *leu2 trp1 ura3 flo8-1 FLO1p::SMR1-ADH2p*	Govender *et al*., 2008
FY23-F1H	*MAT*a *leu2 trp1 ura3 flo8-1 FLO1p::SMR1-HSP30p*	Govender *et al*., 2008
FY23-F5A	*MAT*a *leu2 trp1 ura3 flo8-1 FLO5p::SMR1-ADH2p*	Govender *et al*., 2008
FY23-F5H	*MAT*a *leu2 trp1 ura3 flo8-1 FLO5p::SMR1-HSP30p*	Govender *et al*., 2008
FY23-F11A	*MAT*a *leu2 trp1 ura3 flo8-1 FLO11p::SMR1-ADH2p*	Govender *et al*., 2008
FY23-F11H	*MAT*a *leu2 trp1 ura3 flo8-1 FLO11p::SMR1-HSP30p*	Govender *et al*., 2008
NT50	Industrial wine yeast strain (unknown genotype)	Anchor Yeast, South Africa
VIN13	Industrial wine yeast strain (unknown genotype)	Anchor Yeast, South Africa
VIN13-F1A	*FLO1p::SMR1-ADH2p*	This study
VIN13-F1H	*FLO1p::SMR1-HSP30p*	This study
VIN13-F5A	*FLO5p::SMR1-ADH2p*	This study
VIN13-F5H	*FLO5p::SMR1-HSP30p*	This study
VIN13-F11A	*FLO11p::SMR1-ADH2p*	This study
VIN13-F11H	*FLO11p::SMR1-HSP30p*	This study
WE372	Industrial wine yeast strain (unknown genotype)	Anchor Yeast, South Africa

Standard laboratory media and culture conditions

Yeast strains were routinely cultivated at 30°C in rich YEPD medium, containing 1% (wt/vol) yeast extract, 2% (wt/vol) peptone and 2% (wt/vol) glucose. Inoculums for experimental yeast cultures were prepared as previously described (Govender *et al*., 2008). To determine the onset of flocculation, flocculent ability, glucose utilisation and growth rate of yeast in nutrient rich medium, experimental cultures were seeded at an initial cell density of 5 x 10^5 cells mL^{-1} into 40 mL YEPD contained in 250 mL Erlenmeyer flasks, and incubated at 30°C with shaking (160 rpm). At 2 h intervals, for a period of 24 h and at a 48 h time point, cell populations were harvested and deflocculated as described previously (Govender *et al*., 2008). In addition, the flocculation potential of *FLO11* wine yeast transformants was assessed in chemically defined synthetic complete (SC) media containing 0.67% (wt/vol) yeast nitrogen base (YNB) without amino acids (Difco, Detroit, MI, USA); 3% (vol/vol) ethanol (SCE) and 3% (vol/vol) ethanol with 3% (vol/vol) glycerol (SCGE) as non-fermentable carbon sources. Invasive growth was assessed in chemically defined synthetic complete (SC) media containing 0.67% (wt/vol) yeast nitrogen base (YNB) without amino acids and 0.2% (wt/vol) glucose (SCLD) as previously described (Govender *et al*., 2008). Flor medium (Ishigami *et al*., 2004) containing 0.67% YNB without amino acids and 3% (vol/vol) ethanol adjusted to pH 3.5 was used to assess flor formation as previously described (Govender *et al*., 2008). For selection of sulphometuron methyl (SM) resistant BM45 and VIN13 transformants, SC medium containing 0.67% YNB and 2% (wt/vol) glucose was supplemented with 280 and 300 μg mL^{-1} SM (DuPont Agricultural Products, France) respectively. Cultivation of *E*. *coli* and selection of bacterial transformants were performed as previously described (39). Bacterial and yeast strains were cryopreserved in LB containing 40% (vol/vol) glycerol and YEPD supplemented with 15% (vol/vol) glycerol respectively (Ausubel *et al*., 1995).

DNA manipulation and construction of promoter-replacement cassettes

To ensure high fidelity amplification, *Pyrobest*™ 2 DNA Polymerase PCR system (Takara Bio Inc., Otsu, Japan) was employed in all amplification reactions in which the amplicon was to be used as a DNA template in a subsequent PCR amplification or as a vector cassette for yeast transformation. All other PCR reactions, were performed using *Takara Ex Taq*™ PCR system (Takara Bio Inc., Otsu, Japan). All primers employed in this study are listed in Table 2. Procedures for bacterial transformations and plasmid isolation from *E. coli* were performed as described by Sambrook *et al*. (1989). Standard procedures for isolation and manipulation of DNA were employed in all other aspects of this study (Ausubel *et al*. 1995). The *FLO5* and *FLO11* promoter replacement cassettes containing either the ADH2p or HSP30p and bearing extensive 5' and 3' *FLOp* homologous tail regions (ranging from 437 to 672 bp), were amplified by PCR from the previously reported FY23 transgenic yeast strains (Govender *et al*. 2008). The *FLO5* and *FLO11* cassettes were amplified using genomic DNA that was isolated from FY23- F5A, FY23-F5H, FY23-F11A and FY23-F11H yeast strains, respectively, as templates. The primer pair employed for the *FLO5* cassettes was FLO5-F2 and FLO5-R2, whilst the FLO11-F2 and FLO11-R2 primer set was employed for the *FLO11* cassettes.

Table 2 Oligonucleotide primers used in this study.

Primer name[a]	Primer sequence (5'→3')
δ-F	CAAAATTCACCTATWTCTCA
δ-R	GTGGATTTTTATTCCAACA
ADH2-R	TGATAGTTGATTGTATGCTTTTTGTAGC
*BVFLO1-F	GCTTCGCAGACGAATGTTTTCCG
*BVFLO1::SMR1-F	GCTTCGCAGACGAATGTTTTCCGACACATGATACTTATCACCGAAAAACC TTATTTCACGGAAAAAACCTTATTTACATTAAAGTTTGAAAAATTTTCTTCT TTTCCGCAATATGGTGGGGCCTGGCTTGGCTTCAGTTGCTGATCTCG
FLO1-F	AAGTGTGCGTCACTTTTCCTACGGT
FLO1-F2	ATGGCACTAGTCGATCGAGG
FLO1-F3	AGTGTTATGCTAGCCAGTTTCAGG
FLO1-F4	GCACATGCCAATTGTGTGCATAGC
FLO1-R	AGCGATGAGGCATTGTCATTT
FLO1-R2	GTGCCAGAAGTGTAAAGACTGC
FLO1-reps-F	CTAAGTCAATCTAACTGTACTGTCCCTGA
FLO1-reps-R	GATAGAGCTGGTGATTTGTCCTGAA
FLO5-F2	GGTTGTGTTCTAGGACTTTCTGACG
FLO5-F3	ACTCAATTTGGACACTCGGTTCGG
FLO5-R	AGTGGTGCTAATCAATTTAAAGAA
FLO5-R2	TGTGCACAACATTCGAACGC
FLO5-reps-F	AAGGGTACGTTTACTCTTTTGACGATGACC
FLO5-reps-R	ACTGAAGAAGAAATTACTGAGGAGGAAATC
FLO11-F2	TTACGGCCTAATGTCGAGAC
FLO11-F3	GCTGCTTGTCTCACATCTAAACTTCG
FLO11-R	GGACCAAATAAGCGAGTAGA
FLO11-R2	CTGGGAAATCCGTTTGAGAG
FLO11-reps-F	TAGTGCCGCTCAATATGCAAGCTCCTGGCA
FLO11-reps-R	TGTTTGACTGCCAGGGTATTTGGATGATGA
HSP30-R	TATTAAAGTCTCAAACTTGTTGTTTTG
SMR1-F	GGCTTGGCTTCAGTTGCTGATCTCG
SMR1-F2	GTGTTTTAACTCGTCGGACACG

[a] F, forward primer and R, reverse primer. Non-underlined sequences correspond to *ADH2*, *HSP30*, *and SMR1-410* or *FLO* gene sequences as denoted by the primer name. *Corresponds to a homologous *FLO1* promoter region sequenced from BM45 and VIN13 industrial wine strains.

The sequence of the *FLO1* promoter region (spanning -1290 to -818 nucleotides) in FY23 was found to differ from that of BM45 and VIN13 wine yeast strains. The corresponding region in wine yeast strains were sequenced and a consensus sequence consisting of 287 nucleotides was located in the *FLO1p* region of BM45 and VIN13 wild type strains and deposited in GenBank (BM45, accession no. FJ238617 and VIN13, accession no. FJ238616). Interestingly Fichtner *et al*. (2007) also observed a similar sequence difference between haploid laboratory *S. cerevisiae* strains Σ1278b and S288C (isogenic to FY23). A 124 nucleotide sequence from this consensus sequence was used in the design of the BVFLO1::SMR1-F primer. The partial promoter replacement cassettes corresponding to *SMR1-ADH2-FLO1p* and *SMR1-HSP30- FLO1p*

cassettes were amplified using genomic DNA isolated from the FY23-F1A and FY23-F1H yeast strains, respectively, as templates. The SMR1-F and FLO1-R2 primer pair was used in the preparation of the aforesaid cassettes. The *FLO1p-SMR1-ADH2-FLO1p* and *FLO1p-SMR1-HSP30-FLO1p* promoter replacement vectors were amplified using the corresponding partial cassette as template DNA and the BVFLO1::SMR1-F and FLO1-R2 primer pair. Promoter replacement cassettes were isolated from 1% (w/v) agarose gels and column purified.

Wine yeast transformations and strain verification

Yeast transformation was performed with 10 µg of DNA according to the electroporation protocol described by Ausubel and coworkers (1995). Electroporation of yeast was performed with a Bio-Rad MicroPulser™ 10 (Bio-Rad Laboratories, CA, USA) using the instrument's pre-programmed setting for *S. cerevisiae* (Sc2) and HiMax electroporation cuvettes (Cell Projects Ltd., Kent, UK) with a 0.2 cm electrode gap.

The deletion of native promoters was assessed by PCR using homologous primer sets that contained a forward primer from outside the region of integration and genomic DNA isolated from transformants as template. The primer pairs for transgenic wine yeast strains were: F1A and F1H (FLO1-F and FLO1-R2); F5A and F5H (FLO5-F3 and FLO5-R2); F11A and F11H (FLO11-F3 and FLO11-R2). In addition, the integration of promoter replacement cassettes in transformed wine yeast was further confirmed by PCR using heterologous primer sets that contained a forward primer from outside the region of integration and genomic DNA isolated from transformants as template. The primer pairs for transgenic strains were: F1A, FLO1-F and ADH2-R; F1H, FLO1-F and HSP30-R; F5A, FLO5-F3 and ADH2-R; F5H, FLO5-F3 and HSP30-R; F11A, FLO11-F3 and ADH2-R; and F11H, FLO11-F3 and HSP30-R. The wild type BM45 and VIN13 strains served as a control in the above confirmation experiments. To verify the integrity of the *ADH2p* and *HSP30p* elements driving *FLO* gene expression, the integrated promoter sequences were amplified from transgenic yeast strains using heterologous primer sets: F1A and F1H (SMR1-F2 and FLO1-R2); F5A and F5H (SMR1-F2 and FLO5-R); F11A and F11H (SMR1-F2 and FLO11-R). Amplicons corresponding to the promoter elements were recovered from 1% (w/v) agarose gels, column purified, cloned into pGEM□-T Easy vector and sequenced.

The parental lineage of BM45 and VIN13 transgenic yeast strains was evaluated using primers (δ-F and δ-R) that are specific for delta (δ) sequences as described by Ness and coworkers (1993). The BM45, EC1118, NT50, VIN13 and WE372 industrial wine yeast wild type strains served as controls in these experiments. Intragenic repetitive domain polymorphism located within *FLO* genes of wine yeast was evaluated using primers sets designed by Verstrepen *et al*. (2005) i.e. *FLO1* (FLO1-reps-F and FLO1-reps-R); *FLO5* (FLO5-reps-F and FLO5-reps-R) and *FLO11* (FLO11-reps-F and FLO11-reps-R).

Enumeration of yeast populations

The cell density of suitably diluted yeast suspensions was determined as previously described (Govender *et al*., 2008).

Stability of the integrated promoter replacement constructs

Single yeast colonies representing selected transformed strains from 3 day old YEPD plates were used to inoculate 20 mL YEPD broth contained in 100 mL Erlenmeyer flasks, which were

incubated at 30°C with shaking (160 rpm) for 24 h. The cultures were then deflocculated with the addition of 50 µL sterile 400mM EDTA (pH 7). These deflocculated cultures were employed to inoculate a fresh batch of 20 mL YEPD broth contained in 100 mL Erlenmeyer flasks at an initial cell density of 5×10^5 cells mL^{-1}, which were incubated at 30°C with shaking (160 rpm) for 24 h. This batch culturing process was repeated for more than 100 generations. Final cultures were suitably diluted and spread on YEPD plates and incubated at 30°C for 48 h. Subsequently 50 colonies of each transformed strain were assessed for their resistance to SM, flocculation ability (*FLO1* and *FLO5* constructs), increased invasiveness (*ADH2p-FLO11* transformants) or lack of invasiveness (*HSP30p-FLO11* transformants) in SCLD plates.

Glucose determination

The concentration of glucose in cell-free extracts of YEPD culture medium was determined as previously described (Govender *et al*., 2008).

Flocculation and cell surface hydrophobicity (CSH) assays

The flocculation and CSH of yeast populations were determined as previously described (Hinchcliffe *et al*. 1985; D'Hautcourt and Smart 1999; Govender *et al*. 2008). To assess sugar inhibition of *FLO1* and *FLO5* flocculation phenotypes, either 1 M glucose or 1 M mannose was added to both the washing and suspension buffers that are employed in the modified Helm's assay (1999)

Defined synthetic grape must (MS300) fermentations

The defined medium (MS300) simulating standard grape juice contained 10% (wt/vol) glucose and 10% (wt/vol) fructose, resulting in 20% (wt/vol) total sugar, with a total nitrogen concentration of 300 mg L^{-1} supplied as amino acids and ammonia, and was prepared as previously described (Bely *et al*., 1990). The fermentative potential of BM45 and VIN13 wild type strains and their 12 transgenic derivatives were assessed in triplicate. Yeast precultures in YEPD were prepared as described above and yeast was harvested by centrifugation (4000 rpm, 5 min) and resuspended in MS300 medium. Batch fermentations (200 mL) of MS300 medium contained in 250 mL Schott bottles equipped with fermentation airlocks were performed by the inoculation of precultured cells at a density of 2×10^6 cells mL^{-1} and were performed at room temperature. To determine the progress of fermentations, carbon dioxide release was monitored on a daily basis by measurement of fermentor weight loss. Samples were withdrawn for analysis under aseptic conditions as swiftly as possible to limit the fermentations exposure to oxygen. The flocculation potential of yeast strains was also assessed in aerobic shake-flask experiments and in anaerobic fermentor vessels using MS300 medium that contained either pectin (1 g L^{-1}) or diatomaceous earth (1 g L^{-1}).

Quantitative real-time PCR analysis (QRT-PCR)

Samples from MS300 fermentations corresponding to the exponential yeast growth phase, entry into stationary yeast growth phase and late stationary yeast growth phase were withdrawn for analysis. Thereafter, samples were processed as previously described (Govender *et al*. 2008). cDNA samples were diluted 50 times with H_2O before real-time PCR analysis. Primers and probes used for QRT-PCR analysis, QRT-PCR runs, collection of spectral data and data analyses were performed as previously described (Govender *et al*. 2008).

Analytical methods

MS300 samples were centrifuged and filtered (0.22 µm cellulose acetate) before analysis. Glucose, fructose, glycerol and ethanol was analyzed via high-pressure liquid chromatography, as previously described and the gas chromatographic analysis of major volatile components was performed as previously described (Rossouw *et al*. 2008).

Biomass determination

Dry cell weight of MS300 batch fermentations was determined by filtering under vacuum 5 mL of culture through a pre-dried (350 W for 4 min in a microwave oven) and pre-weighed 0.45 µm Supor® membrane disc filter (Pall Corporation, NY, USA). The filter was reweighed after being washed with three volumes of distilled water and dried in a microwave oven at 350 W for 8 min. The dry weights of sample replicates were determined in duplicate.

Statistical Analysis

In this study, paired t tests or one-way ANOVA were employed to statistically compare data obtained for BM45 and VIN13 wild type strains to that of transgenic yeast strains. Statistical tests were performed using GraphPad InStat version 3.05 32 bit for Windows 95/NT (GraphPad Software, San Diego California)

Results

Wine yeast transformation

Employing the transformation strategy presented in Govender *et al*. (2008) and using the homology regions derived from the S288C genetic background, SM resistant colonies were obtained for *FLO5* and *FLO11* transformations. A majority of putative BM45 and VIN13 transgenic strains transformed with the combinations *FLO5p-SMR1-ADH2p-FLO5p* and *FLO5p-SMR1-HSP30p-FLO5p* visually displayed strong flocculent phenotypes. Putative BM45-F11A, BM45-F11H, VIN13-F11A and VIN13-F11H transformants displayed no detectable flocculent phenotype, but *ADH2p-FLO11* transformants displayed increased invasiveness on SCLD plates.

As mentioned earlier, due to sequence differences between the laboratory *S. cerevisiae* FY23 strain and commercial wine yeast strains employed in this study, the length of homologous flanking sequences for the *FLO1* promoter replacement cassettes are smaller than the *FLO5* and *FLO11* cassettes. Only a small proportion (3 to 5%) of putative BM45 and VIN13 transgenic strains, transformed with the two combinations *FLO1p-SMR1-ADH2p-FLO1p* and *FLO1p- SMR1-HSP30p-FLO1p*, visually displayed flocculent phenotypes. This percentage is significantly lower than what had been observed in the case of *FLO5p* and *FLO11p* transformants, where more than 50% of SM-resistant colonies had shown the expected flocculation or adhesion phenotypes. This observation is in line with previously published data which suggest that, although strain dependant, an increase in the length of flanking homology sequences can drastically increase the efficiency of DNA fragment transplacement efficiencies (Manthey *et al*. 2004).

Three independent transformants of each strain were selected for further analysis. For each of the selected strains, the integration of promoter replacement cassettes at specific loci were verified by PCR using heterologous primer sets that contained a forward primer from outside the region of

integration and genomic DNA as template. Additionally, the deletion of native promoters for at least one allele was confirmed by PCR using homologous primer pairs that contained a forward primer from outside the site of integration. As reported later in this study, the above verifications are also supported by QRT-PCR analysis. Although there is extensive sequence identity between *FLO1* and *FLO5* genes, specific 3' minor groove binder (MGB) probes and primers designed by Govender and co-workers (2008) clearly differentiated between their cDNA species. We found a surprisingly significant number of strains were both copies of the *FLO*-gene promoters had been replaced by the new constructs. In order to eliminate any possible variability that may be associated with copy number, all results for transgenic wine yeast strains presented in this study were obtained with single copy integrants.

Transgenic yeast strain-typing and genetic stability

Since industrial strains of *S. cerevisiae* are not easy to differentiate from each other, the lineage of all transformants was verified. For this purpose, primers that are specific for delta (δ) sequences as described by Ness *et al*. (1993) were used for all transgenic wine yeast strains. All strains were confirmed to be genetic descendants of BM45 and VIN13 wild type wine yeast strains. The stability of the integrated promoter constructs was assessed after repeated batch culturing in nutrient-rich, non-selective medium for more than 100 generations. Thereafter, all tested colonies displayed resistance to SM, indicating stable integration of the promoter replacement vectors containing the SMR1 marker gene. Flocculation phenotypes of these strains were assessed, and 2% of BM45-F1A and 6% of VIN13-FIH colonies showed no visible flocculation. All other tested colonies of *FLO1* and *FLO5* transformants displayed the relevant phenotypes. In some instances, slight differences in flocculation intensities were observed. This is in contrast to the data reported for the laboratory strain, were the phenotype proved stable and reproducible over the same number of generations. Since the non-flocculent descendants still contained the modified promoter-ORF construct, phenotypic differences observed may be attributed to genetic variations in the open reading frames of *FLO1* and *FLO5* as suggested by other studies (Verstrepen *et al*. 2004; Verstrepen and Klis 2006; Smukalla *et al*. 2008).

Growth rates, glucose consumption, flocculation and CSH

The growth rate and sugar utilization capabilities BM45 and VIN13 strains and their transgenic descendants were evaluated in YEPD at 2-hourly intervals. No significant differences were observed between the wild-type strains and the *ADH2p-FLO5* and *HSP30p-FLO5* transformants regarding biomass growth, cell numbers and sugar utilization capabilities (Fig. 1 and 2). Similar trends in these parameters were observed for other transgenic strains reported in this study. In both BM45 and VIN13 *ADH2p-FLO5* transformants, an onset of flocculation was observed approximately 2 h after glucose exhaustion, while maximum flocculation 1 potential was reached after an additional 6 h (Fig. 1 B and 2 B). Although in terms of onset of flocculation, there is parity with respect to *ADH2p*-mediated *FLO5* flocculation, appreciably different *HSP30p*-regulated *FLO5* flocculation onset profiles were observed in BM45-F5H and VIN13-F5H transformants. The onset of flocculation in BM45-F5H seemed to coincide with glucose depletion, and maximal flocculent ability was achieved after an additional 4 hours (Fig. 1 B). Whilst the commencement of flocculation was considerably delayed in VIN13-F5H and full flocculation potential was only attained after 48 h (Fig. 2 B).

After 48 h growth in YEPD containing 2% glucose, *ADH2p*-based *FLO5* transgenic wine yeast strains [BM45-F5A (72.1 ± 3.9%), VIN13-F5A (59.4 ± 2.7%)] generated flocculent phenotypes that were significantly more flocculent than their HSP30p-based counterparts [BM45-F5H (50.8 ± 2.9%), VIN13-F5H (30.3 ± 2.5%)] (Fig. 3). A similar flocculation tendency was evident for the two promoters controlling *FLO1* expression in BM45 and VIN13 transformants (Fig. 3) that is BM45-F1A (49.4 ± 1.6%) and VIN13-F1A (39.8 ± 2.8%) versus BM45-F1H (21.0 ± 2.5%) and VIN13-F1H (9.0 ± 1.3%). The above relationships illustrate that *FLO5* wine yeast transformants are more flocculent than the corresponding *FLO1* transgenic strains. The flocculent phenotypes displayed by both *FLO1* and *FLO5* wine yeast transformants after 48 h growth in YEPD were completely abolished on exposure to 1 M mannose. On the contrary, no substantial inhibitory effect was evident in the presence of 1 M glucose. Since Flo1-type flocculation is exclusively inhibited by mannose, while NewFlo-type flocculation is inhibited by both mannose and glucose (Stratford and Assinder 1991), this result demonstrates that *FLO1* and *FLO5* transgenic wine yeast encoded flocculins exhibit Flo1-type flocculation.

Expression of *FLO11* in both wine yeast strains, mediated by either the *ADH2* or *HSP30* promoter in nutrient rich YEPD medium (Fig. 3) and minimal media including SCE and SCGE with non-fermentable carbon sources (results not shown) did not yield a flocculent phenotype.

The hydrophobicity of yeast cell surfaces (data not shown) from yeast populations grown in YEPD for 48 h was determined by measuring the distribution ratio of yeast cells in a biphasic system consisting of a buffered solution and an organic solvent. The VIN13 wild type yeast strain showed very low natural hydrophobicity, whilst the BM45 wild type strain has a hydrophobic cell surface. In general it was observed that expression of any *FLO* gene caused a significant increase in cell surface hydrophobicity in comparison to their parental strains. A statistically insignificant ($p > 0.05$) modified hydrophobic index (MHI) was only observed for VIN13-FIH, in comparison to the VIN13 wild type strain.

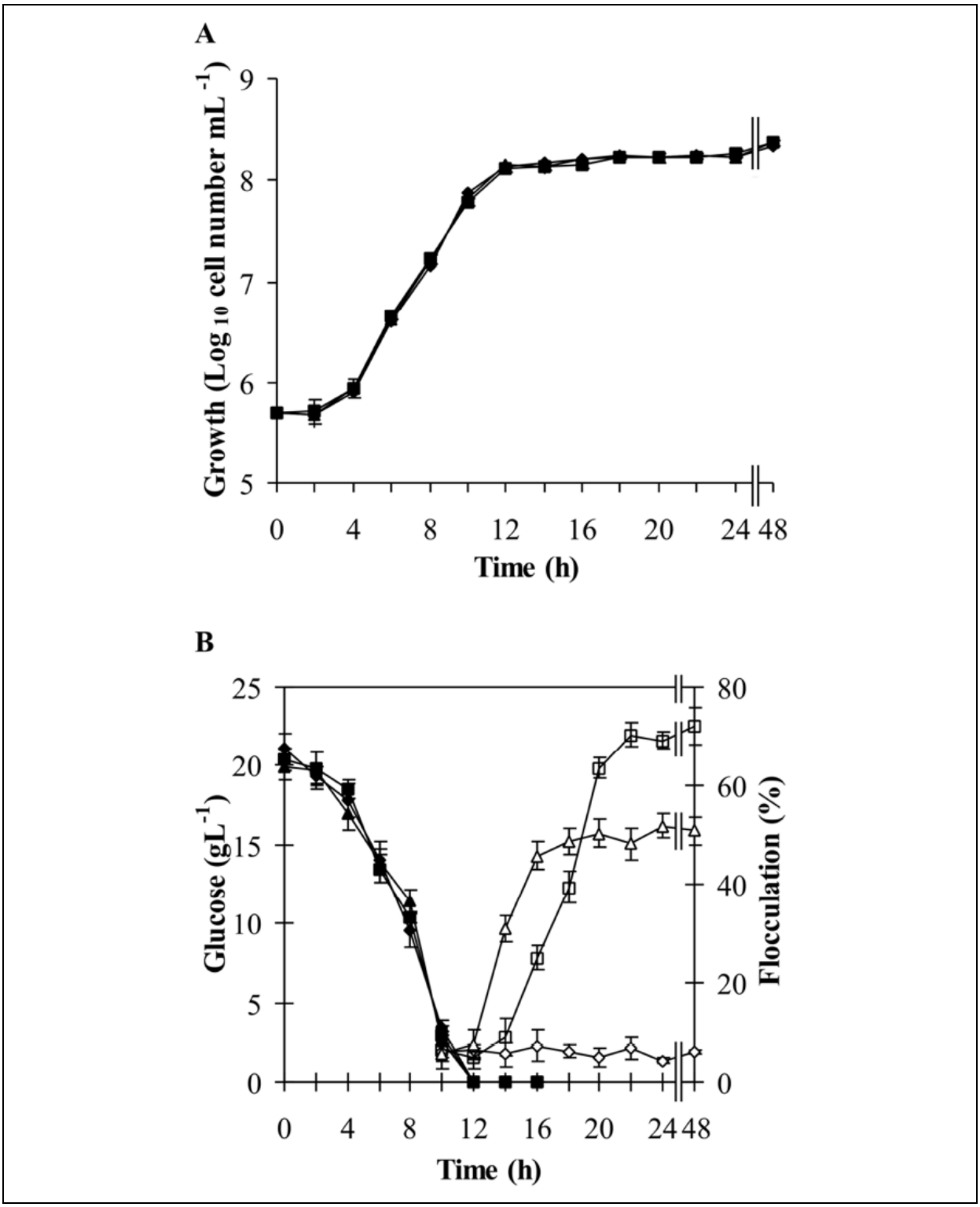

Figure 1 (A) Growth of BM45 wild type (◆); BM45-F5A (■) and BM45-F5H (▲) strains. (B) Glucose utilization of BM45 wild type (◆); BM45-F5A (■) and BM45-F5H (▲) strains. Flocculation profile of BM45 wild type (◇); BM45-F5A (□) and BM45-F5H (△) strains. Yeast strains were cultivated in YEPD containing 2% glucose at 30°C with shaking (160 rpm). Values reflect the mean of experiments performed in triplicate and error bars represent standard deviations.

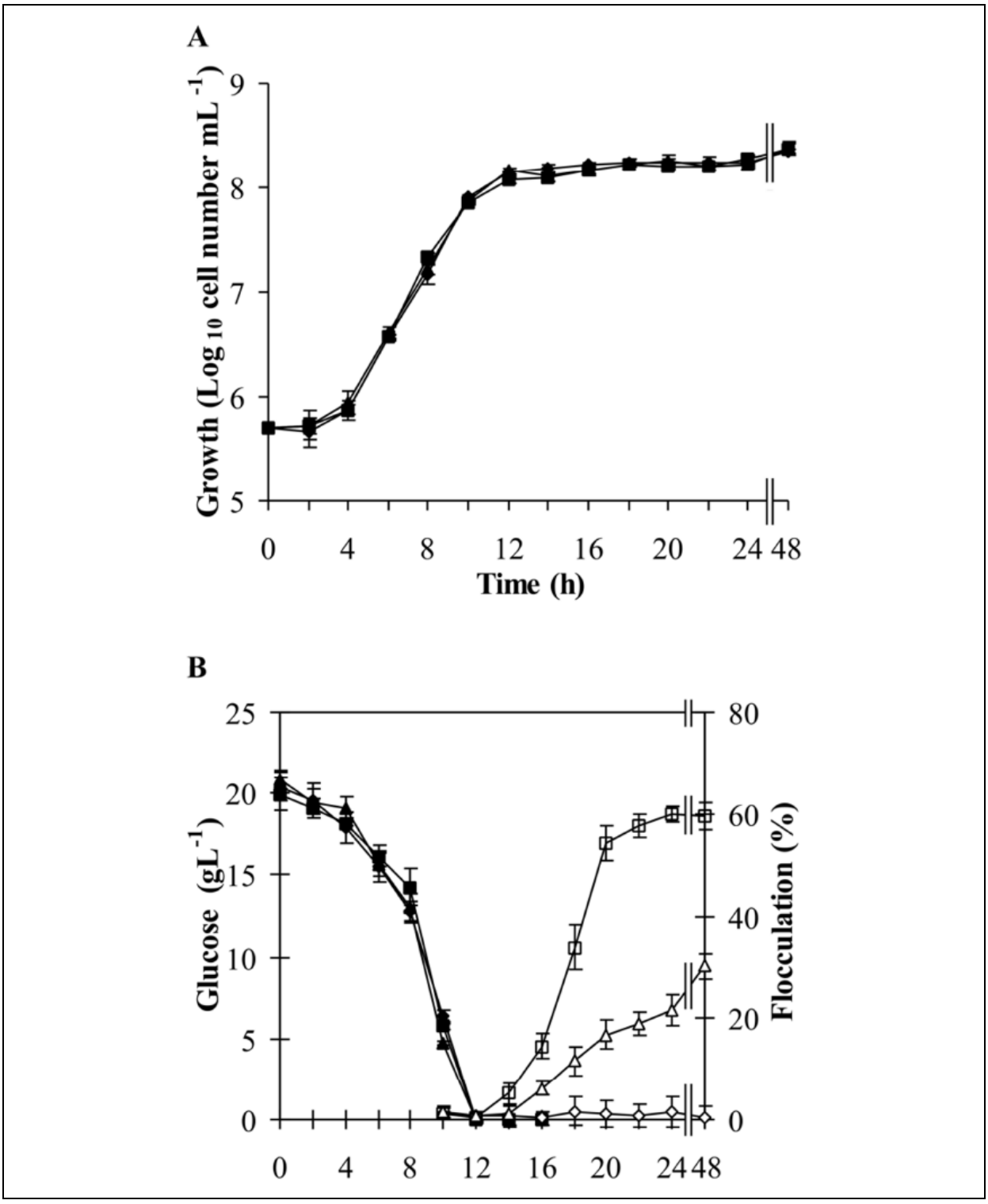

Figure 2 (A) Growth of VIN13 wild type (◆); VIN13-F5A (■) and VIN13-F5H (▲) strains. (B) Glucose utilization of VIN13 wild type (◆); VIN13-F5A (■) and VIN13-F5H (▲) strains. Flocculation profile of VIN13 wild type (◇); VIN13-F5A (□) and VIN13-F5H (△) strains. Yeast strains were cultivated in YEPD containing 2% glucose at 30°C with shaking (160 rpm). Values reflect the mean of experiments performed in triplicate and error bars represent standard deviations.

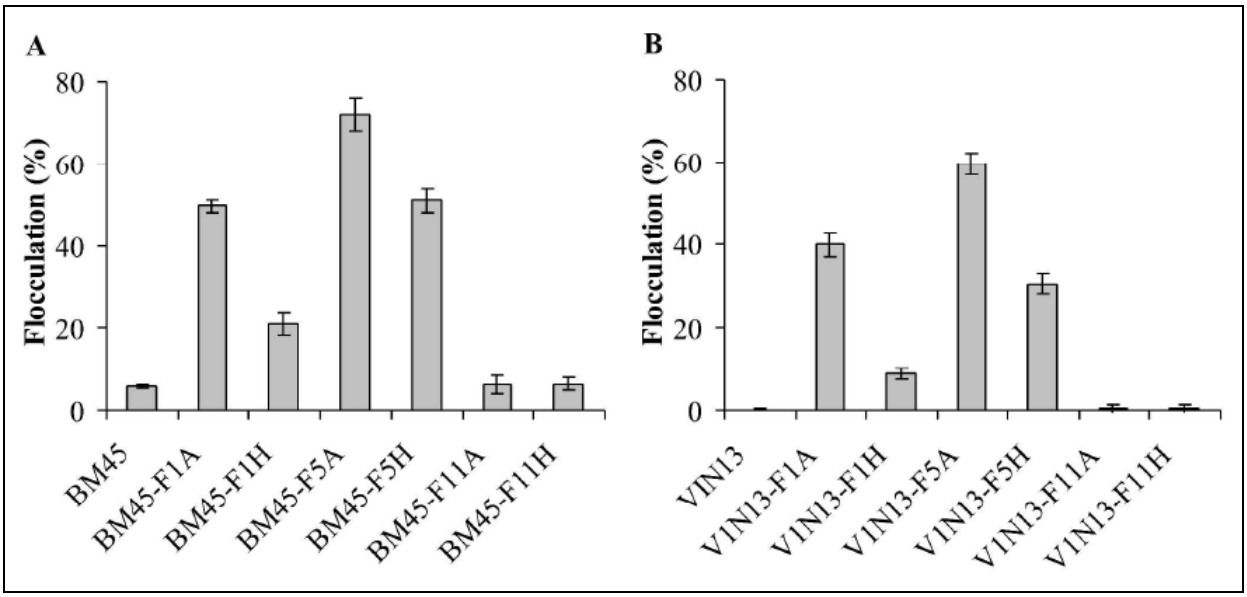

Figure 3 Flocculation of (A) BM45 and (B) VIN13 wild types and their transgenic strains. Yeast strains were cultivated in nutrient-rich YEPD (2% glucose) for 48h at 30°C with shaking (160 rpm). Values reflect the mean of experiments performed in triplicate and error bars represent standard deviations.

Evaluation of the intragenic repetitive domains of *FLO1*, *FLO5* and *FLO11*

Each of the repetitive regions located within the three *FLO* genes from BM45, VIN13, EC1118 and NT50 wild type wine yeast were amplified using PCR and compared to corresponding amplicons obtained from the haploid FY23 laboratory strain that is isogenic to S288C (Fig. 4). With the exception of the BM45 *FLO5* repeat region amplicon, all other wine yeast intragenic repetitive domains displayed decreased lengths when compared to the corresponding amplicons obtained from FY23. No repeat region amplicon was evident for *FLO11* from the BM45 wild type strain. This latter result is not entirely surprising since Fidalgo and coworkers (2006) recently reported the presence of rearrangements within the central tandem repeat domain of the *FLO11* ORF from a flor-forming *S. cerevisiae* wine yeast strain. In addition, they found the *FLO11* coding region contained several point mutations and deletions (Fidalgo *et al*. 2006). Given the innate flor-forming character of the BM45 wild type strain, it is possible that either one or both *FLO11* repetitive region primers employed in this study lacked specificity. It is also interesting to note in terms of the wild type wine yeast strains used in this study, that VIN13 consistently yielded smaller repeat region amplicons than BM45 for *FLO1* and *FLO5*

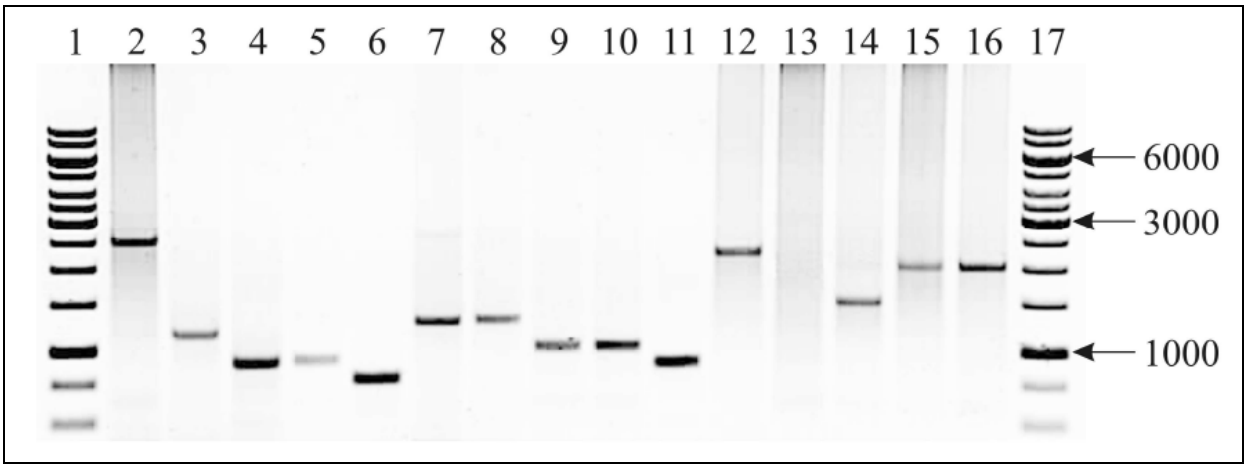

Figure 4 Evaluation of *FLO* intragenic repetitive domain polymorphisms using primers designed by Verstrepen and coworkers (2005) in *FLO1*, *FLO5* and *FLO11* ORFs in five wild type yeast strains. As indicated, a bracket denotes amplicons of a particular *FLO* gene. Lanes 2 (2529 bp), 7 (1288 bp) and 12 (2260 bp): FY23; lanes 3, 8 and 13: BM45; lanes 4, 9 and 14: VIN13; lanes 5, 10 and 15: EC1118; lanes 6, 11 and 16: NT50.

Flor formation and invasive growth

The BM45 wild type wine yeast strain displays natural flor forming ability after 5 days in flor medium at 30°C under static conditions (Fig. 5A). It is visibly apparent that both BM45-F11A and BM45-F11H transgenic strains formed thicker biofilms. Interestingly, the newly acquired flocculation phenotypes displayed by BM45-F1A, BM45-F1H, BM45-F5A and BM45-F5H transformants seemed to have no effect on the inherited flor forming ability of these transgenic strains. As shown in Fig. 5B, only transgenic yeast VIN13-F11A and VIN13-F11H strains formed biofilms with the latter strain producing a more noticeable biofilm.

The ability of the wild-type BM45 and VIN13 strains and their 1 transgenic descendants to invade agar is shown in Fig. 6. The BM45 and VIN13 wild type yeast strains exhibited natural invasiveness. In contrast to all other transgenic wine yeast strains only BM45-F11A and VIN13-F11A grew as distinctly larger-sized colonies and presented different colony morphology on SCLD agar in comparison to their wild type parental strains. Moreover they displayed more extensive invasive growth behaviour in comparison to their wild type parental strains.

Flocculation and fermentation profiles in MS300 medium

The chemically defined medium, MS300 that is employed in this aspect of the study mimics industrial grape must (Bely et al. 1990). Since it was reported that Adh2 was found in aerobically grown yeast cells (Wills 1976; Thomson et al. 2005; Cunha et al. 2006), the flocculent potential of BM45-F1A, BM45-F5A, VIN13-F1A and VIN13-F5A in MS300 medium was assessed under aerobic growth conditions using shake-flask experiments. However, no flocculent phenotypes were displayed by BM45 and VIN13 transgenic yeast strains (data not shown). This is quite surprising, especially since BM45-F1A, BM45-F5A, VIN13-F1A and VIN13-F5A yielded distinctly stronger flocculent phenotypes in nutrient-rich YEPD (Fig. 3) than their comparable HSP30p wine yeast transformants.

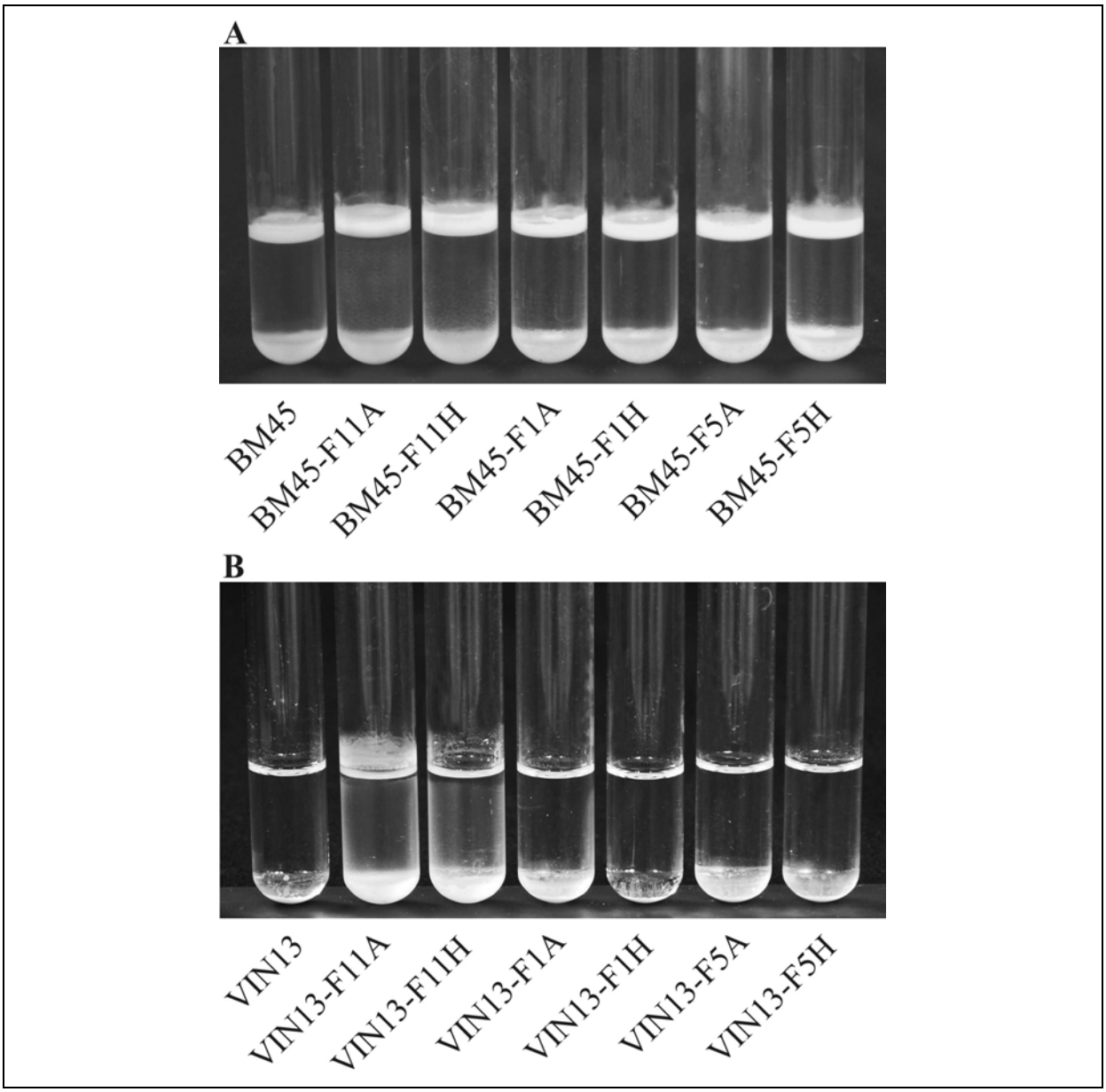

Figure 5 Biofilm formations by (A) BM45 and (B) VIN13 wild types and their transgenic derivatives. Cells were pre-cultured in YEPD broth and recovered by centrifugation, washed once with flor medium and resuspended at a density of 6 × 107 cells mL−1 15 in 5 mL flor medium contained in 16 x 165 mm glass test tubes. The tubes were photographed after 5 days of static incubation at 30°C.

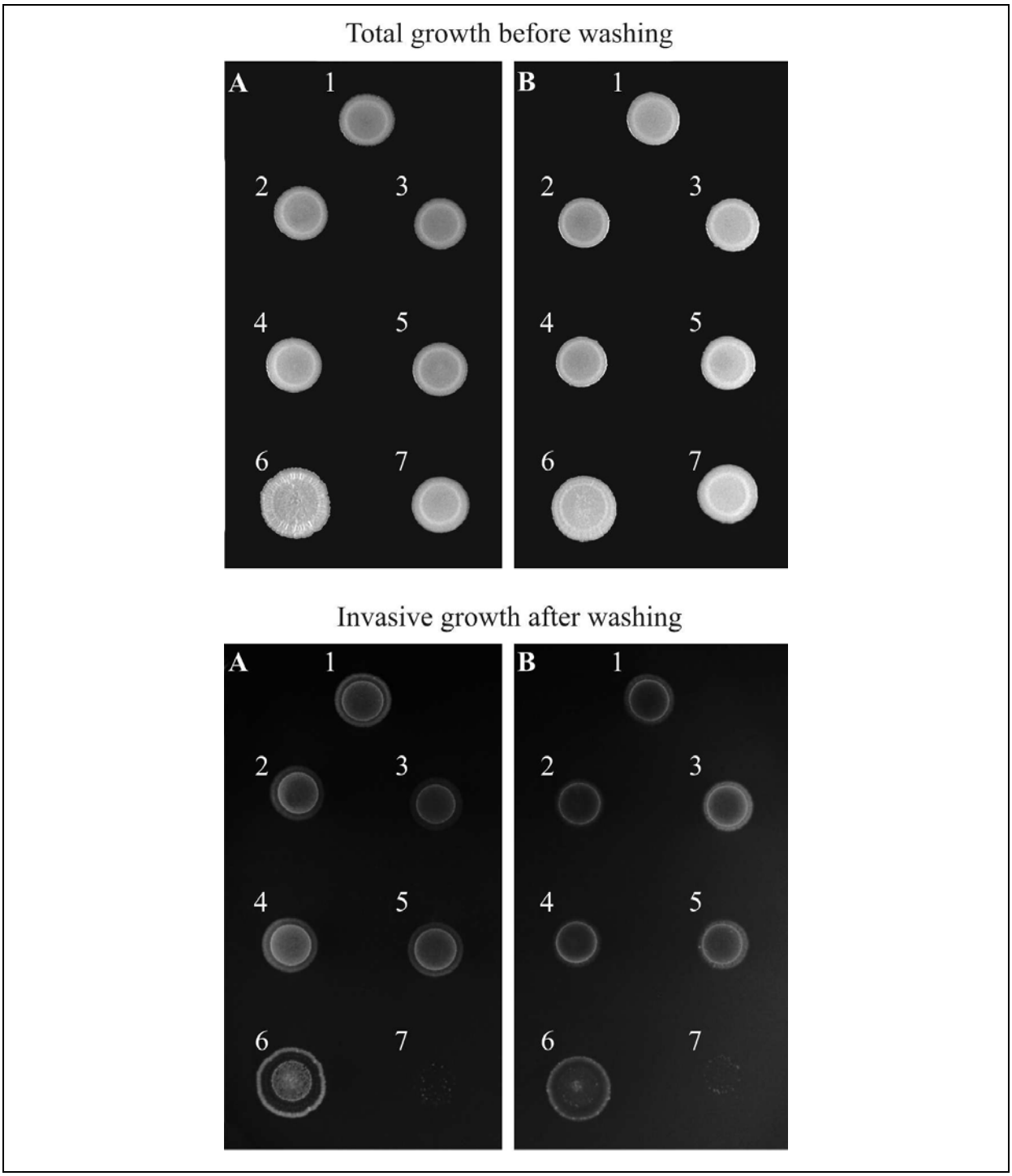

Figure 6 Invasive growth of (A) BM45 wild type (1); BM45-F1A (2); BM45-F1H (3); BM45-F5A (4); BM45-F5H (5); BM45-F11A (6) and BM45-F11H (7) and (B) VIN13 wild type (1); VIN13- F1A (2); VIN13-F1H (3); VIN13-F5A (4); VIN13-F5H (5); VIN13-F11A (6) and VIN13-F11H (7) strains after 5 days growth at 30°C on SCLD medium.

Also at the end of microaerophilic MS300 batch fermentations, the flocculent ability of BM45 and VIN13 wild type wine yeast strains and their transgenic derivatives were determined (Fig. 7). The results clearly illustrate that only *HSP30p* driven expression of *FLO1* and *FLO5* in transgenic wine yeast strains yielded flocculent phenotypes. The flocculent phenotypes produced by BM45-F1H, BM45-F5H, VIN13-F1H and VIN13-F5H transformants in MS300 were similar to those described earlier in nutrient-rich YEPD medium (Fig. 3). The above reinforces our earlier findings in this study that *FLO5* wine yeast transformants are more flocculent than their corresponding *FLO1* transgenic wine yeast strains. Transgenic wine yeast strains containing *FLO1* or *FLO5* under the transcriptional control of *ADH2p* failed to generate flocculent phenotypes under these cultivation conditions (Fig. 7).

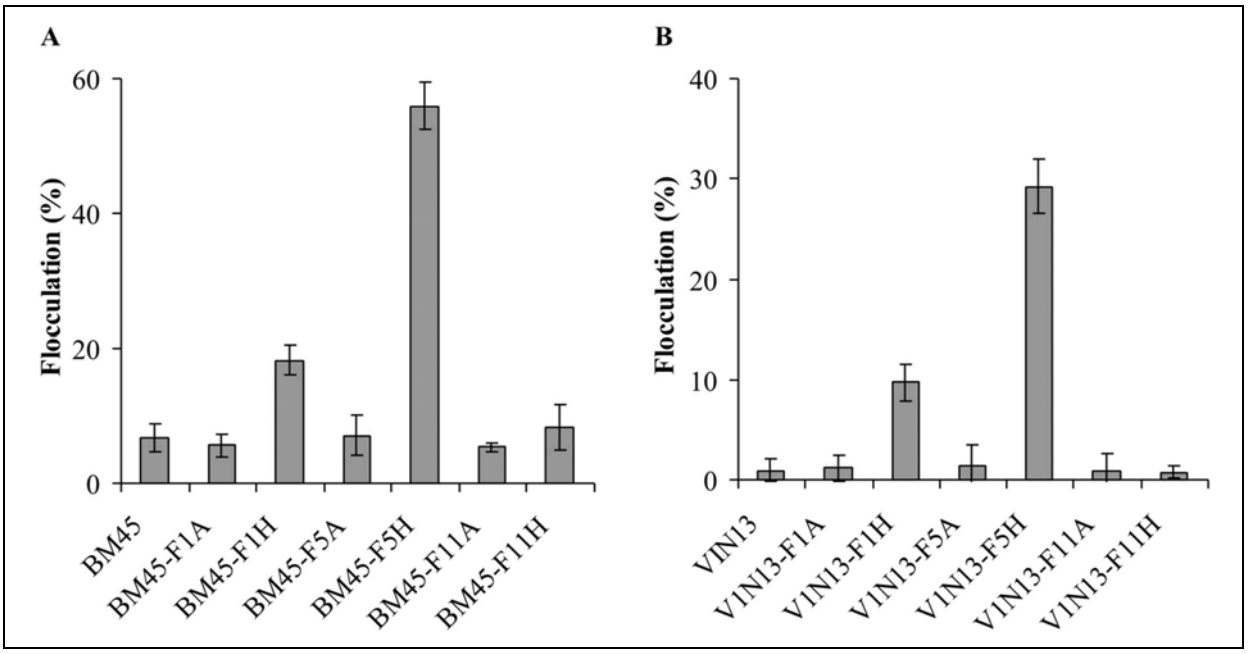

Figure 7 Flocculation of (A) BM45 and (B) VIN13 wild types and their transgenic strains on completion of fermentation in synthetic defined medium (MS300). Values reflect the mean of experiments performed in triplicate and error bars represent standard deviations.

Congruent to earlier observations (Fig. 3), *ADH2p* or *HSP30p* regulated expression of *FLO11* in BM45 and VIN13 transgenic strains (Fig. 7) did not yield flocculent phenotypes under MS300 fermentation conditions. To further simulate natural grape musts that contains suspended amorphous insoluble components such as pectin and diatomaceous earth, chemically defined MS300 medium was also supplemented with these components. The transgenic BM45-F11H and VIN13-F11H strains displayed no flocculent phenotype when cultivated either aerobically or in fermentation vessels equipped with airlocks using MS300 medium containing either pectin or diatomaceous earth (data not shown). This seems to indicate transgenic strains over-expressing *FLO11*-encoded flocculins not interact with amorphous solid components so as to affect faster sedimentation rates.

Since *HSP30p* wine yeast transformants exclusively displayed flocculent phenotypes, only data pertaining to their MS300 fermentation profiles are presented. There were no significant differences ($p > 0.05$) observed in sugar utilization abilities of BM45 and VIN13 wild type wine yeast strains in contrast their *HSP30p* transgenic descendants (Fig. 8). The fermentation profiles in terms of CO_2 released (fermentor weight loss) were similar for all strains that were evaluated (data not shown). Moreover, no significant differences ($p > 0.05$) were observed in the abilities of BM45 and VIN13 wild type wine yeast strains in comparison to their HSP30p transgenic descendants to produce ethanol (Fig. 8), glycerol [BM45 and its transgenic strains (~7.57 gL-1); VIN13 and its transgenic strains (~5.99 gL-116)] and biomass (Fig. 9).

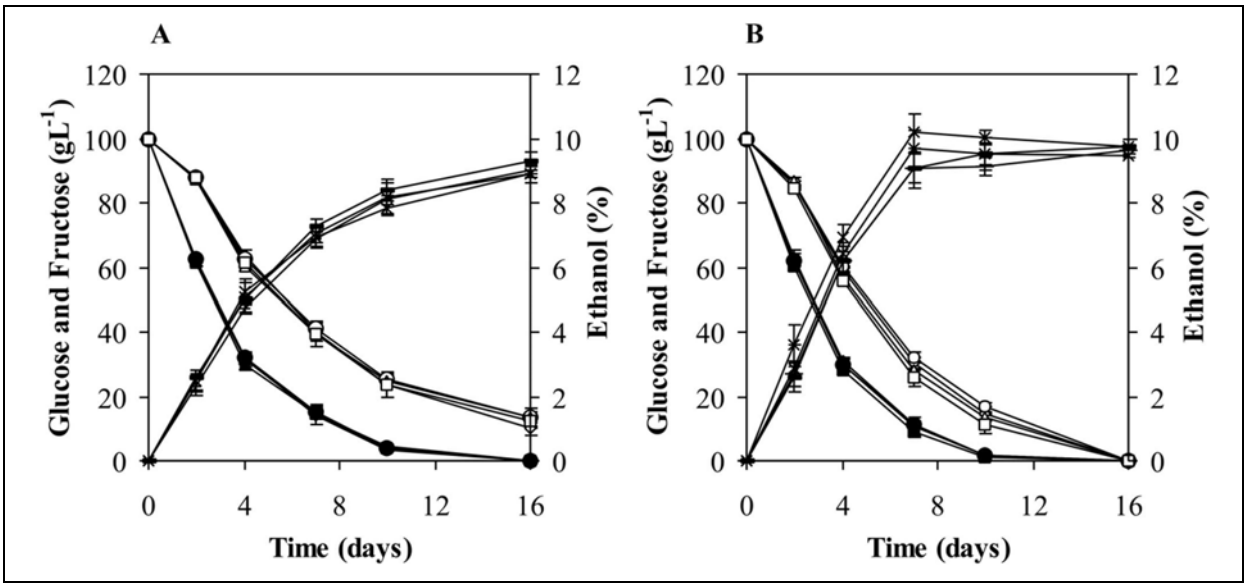

Figure 8 Fermentation profile of (A) BM45 and (B) VIN13 wild types and their 1 transgenic HSP30p descendants in defined synthetic must (MS300). Glucose utilization by BM45 and VIN13 wild types (◆); BM45-F1H and VIN13-F1H (●); BM45-F5H and VIN13-F5H (▲); BM45-F11H and VIN13-F11H (■) strains. Fructose consumption by BM45 and VIN13 wild types (◇); BM45-F1H and VIN13-F1H (Ó); BM45-F5H and VIN13-F5H (△); BM45-F11H and VIN13-F11H (□) strains. Ethanol production by BM45 and VIN13 wild types (–); BM45-F1H and VIN13-F1H (I); BM45-F5H and VIN13-F5H (∗), BM45-F11H and VIN13-F11H (×) strains.

Furthermore, GC monitoring of volatile components at the end of MS300 batch fermentations also revealed no significant ($p > 0.05$) differences in all components analyzed for BM45 and VIN13 wild type wine yeast strains in comparison to their HSP30p transgenic derivatives (Table 3 and 4).

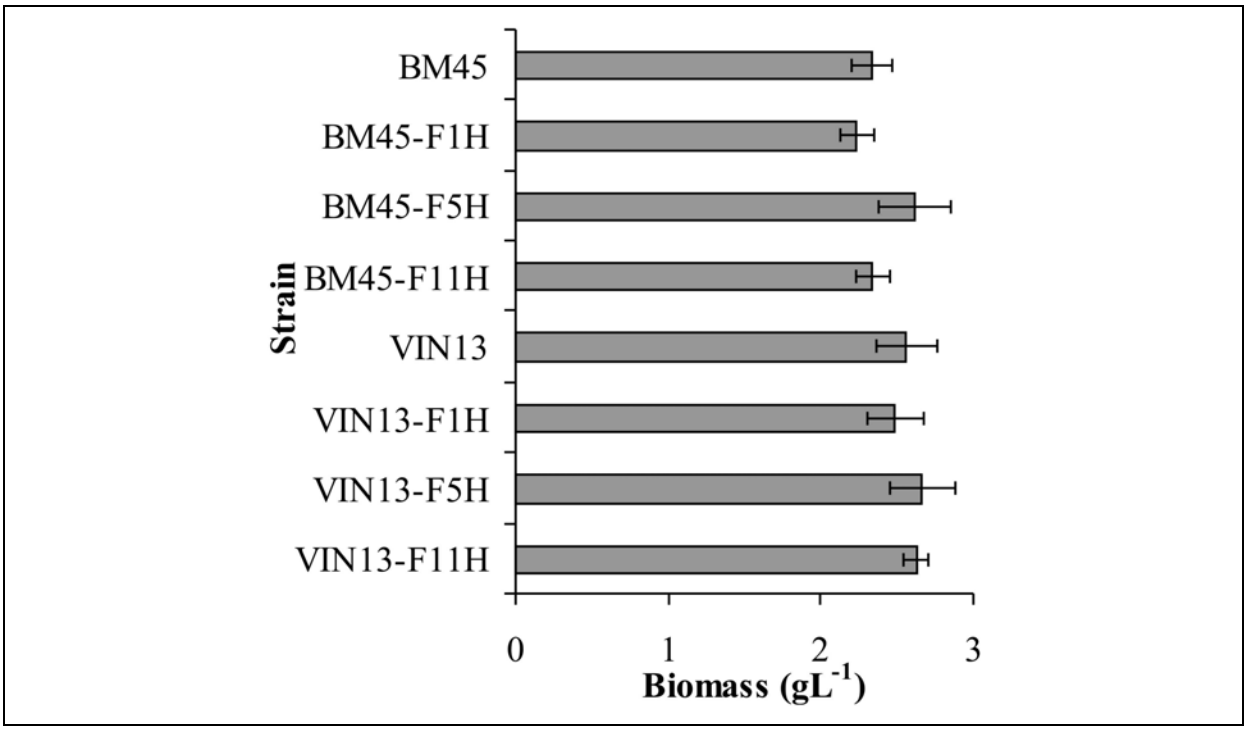

Figure 9 Biomass produced by BM45 and VIN13 wild types and their *HSP30p* transgenic strains on completion of fermentation using in defined synthetic must (MS300). The results are averages of three independent determinations, and error bars represent standard deviations.

Table 3 Volatile components in wines produced from chemically defined synthetic grape must (MS300) with BM45 wild type strain and its transgenic descendants.

Volatile Component	**Strain**			
(mg.L^{-1})	**BM45**	**BM45-F1H**	**BM45-F5H**	**BM45-F11H**
2-Phenyl Ethanol	12.10	11.28	11.57	11.27
2-Phenylethyl Acetate	0.35	0.28	0.36	0.30
Acetic Acid	1744.89	1752.31	1716.66	1666.59
Decanoic Acid	1.82	1.94	1.58	1.78
Diethyl Succinate	0.04	0.02	0.01	0.03
Ethyl Acetate	104.93	104.18	102.65	95.81
Ethyl Caprate	0.35	0.30	0.29	0.26
Ethyl Hexanoate	1.42	1.42	0.84	0.00
Ethyl Lactate	7.96	7.81	6.83	7.24
Hexanoic Acid	0.84	0.84	0.59	0.81
Isoamyl Acetate	0.39	0.38	0.33	0.33
Isoamyl alcohol	53.75	53.96	50.96	51.13
Isobutanol	36.52	48.26	43.65	44.22
Iso-Butyric Acid	0.73	0.77	0.76	0.63
Octanoic Acid	0.07	0.05	0.35	0.05
Propanol	22.82	18.45	19.58	20.09
Propionic Acid	3.56	3.08	3.04	3.54

No statistically significant differences (p>0.05) were observed for all components in comparison to the parental BM45 wild type strain.

Table 4 Volatile components in wines produced from chemically defined synthetic grape must (MS300) with VIN13 wild type strain and its transgenic descendants.

Volatile Component ($mg.L^{-1}$)	Strain			
	VIN13	**VIN13-F1H**	**VIN13-F5H**	**VIN13-F11H**
2-Phenyl Ethanol	10.33	11.19	11.25	9.71
2-Phenylethyl Acetate	0.03	0.12	0.02	0.04
Acetic Acid	1094.84	992.08	1071.18	1096.94
Butyric Acid	0.26	0.29	0.25	0.27
Decanoic Acid	2.09	2.35	1.78	1.95
Diethyl Succinate	0.11	0.15	0.18	0.15
Ethyl Acetate	87.71	84.97	102.21	100.15
Ethyl Caprate	0.25	0.27	0.16	0.27
Ethyl Caprylate	0.02	0.03	0.02	0.03
Ethyl Hexanoate	1.45	1.45	1.34	1.47
Ethyl Lactate	8.36	7.83	9.86	9.17
Hexanoic Acid	1.04	1.06	0.96	1.10
Isoamyl Acetate	0.37	0.38	0.44	0.43
Isoamyl alcohol	48.13	54.08	60.03	64.08
Isobutanol	29.14	29.60	26.55	32.19
Iso-Butyric Acid	0.39	0.53	0.41	0.63
Octanoic Acid	0.53	0.62	0.44	0.55

No statistically significant differences (p>0.05) were observed for all components in comparison the parental VIN13 wild type strain.

FLO gene transcription in MS300 medium

The *FLO* gene expression profiles as mediated by either *ADH2p* or *HSP30p* in synthetic MS300 wine fermentations were evaluated. The total RNA from BM45 and its transformants were processed from different growth phases corresponding to the exponential phase (Day 2), entry into stationary phase (Day 4) and late stationary phase (Day 10) and quantitative real-time PCR was performed. It is evident (Fig. 10) that HSP30p is tightly repressed in the exponential phase of growth, whilst some expression is observed on entry into the stationary and strongly increased RNA levels in late stationary phases. Interestingly, an approximately 10-fold higher *HSP30p* induction of *FLO11* is observed in the late stationary phase of yeast growth in comparison to *FLO5*, whilst the intensity of *FLO5* transcripts was approximately 1 10-fold higher than *HSP30p* induced *FLO1* transcripts. Moreover, the data confirm that only the *FLO* gene carrying a modified promoter is activated, and that the other two genes that were monitored in parallel, do not contribute to the observed phenotypes. In contrast, the activity of *ADH2p* seems to be tightly repressed at all phases of growth. These transcription levels are well aligned with the adhesion phenotypes observed in these strains (Fig. 7A).

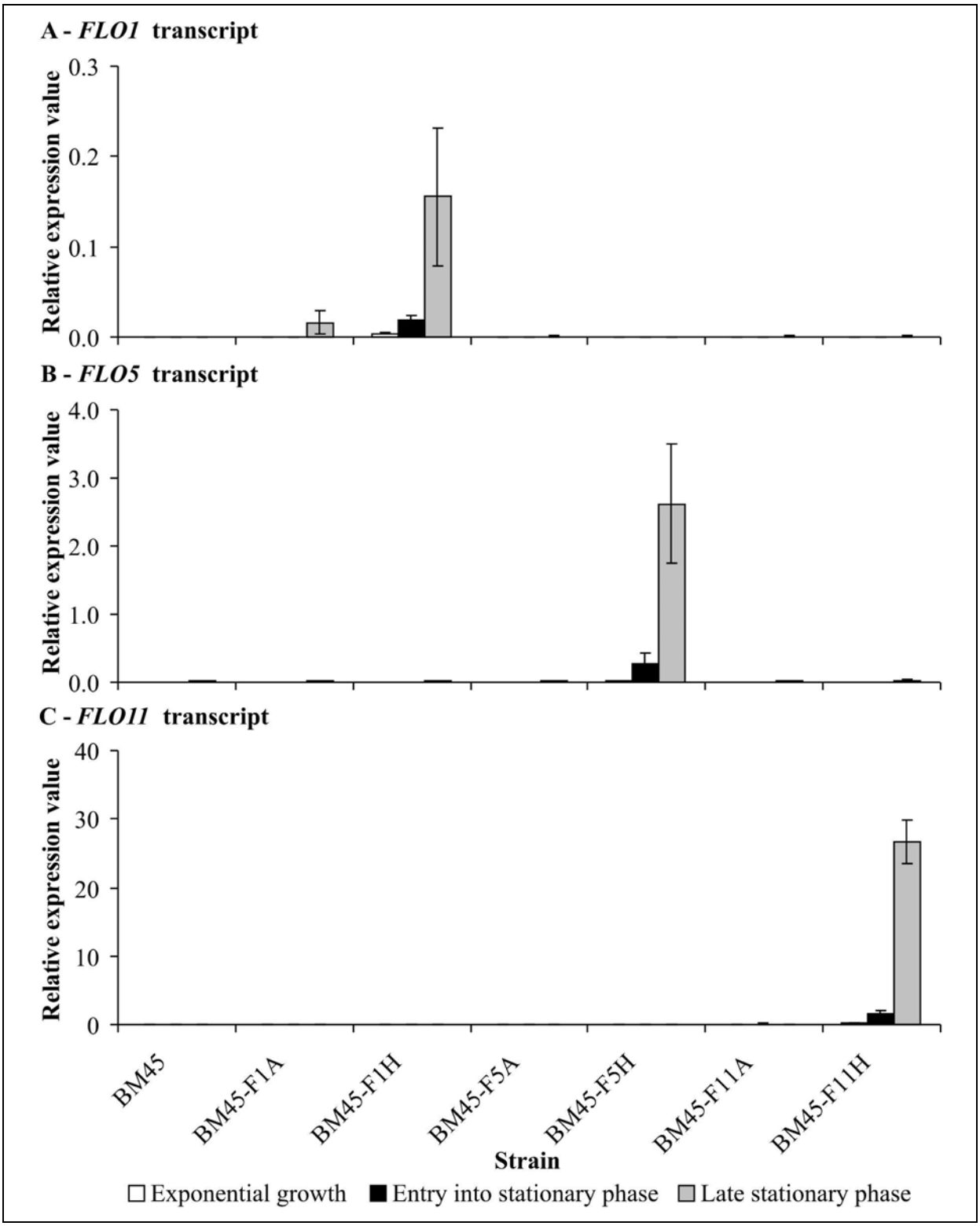

Figure 10 QRT-PCR relative expression of (A) FLO1, (B) FLO5 and (C) FLO11 genes in BM45 wild type and its HSP30p transformants at different stages of wine fermentation on defined synthetic must (MS300). Samples were taken from sampling points corresponding to exponential growth phase (white), entry into stationary growth phase (black) and late stationary phase (grey). Relative expression data was normalized to the relative expression value of the housekeeping gene PDA (Govender *et al*. 2008). Values reflect the mean of experiments performed in triplicate and error bars represent standard deviations.

The effect of initial glucose concentrations on *ADH2p* controlled expression of *FLO5* encoded flocculins.

Cunha and coworkers (2006) showed that a commercial baking yeast strain (Fleischmann) transformed with an integrative cassette containing the *FLO1* ORF under transcriptional control of a modified *ADH2* promoter was capable of conditional flocculation that coincided with thedepletion of glucose when cultivated in YEPD containing 200 gL-1 13 glucose. In addition, protein expression mediated by *ADH2p* in complex nutrient-rich medium was reported to be stronger than that observed in selective medium (Lee and DaSilva 2005; Govender *et al*. 2008). Considering the aforementioned research studies, the flocculent potential of BM45-F5A and VIN13-F5A transgenic strains was evaluated aerobically in shake-flask experiments using YEPD containing increasing glucose substrate concentrations. As shown in Fig. 11, the flocculation ability of the VIN13-F5A transformant was consistently strong up to 30 gL-1 19 glucose and until 50 gL-1 20 glucose for the BM45-F5A transformant. Thereafter the results clearly demonstrate that the flocculent potential of both transformants drastically decreases as the initial glucose substrate concentration increases. The VIN13-F5A transformant displayed no flocculence from 150 gL-1 glucose onwards, whilst the BM45-F5A transformant showed no flocculation at 200 gL-1 glucose. These observations are surprising and contradictory to that of Cunha *et al*. (2006) and may suggest strain-specific transcriptional regulation of *ADH2p*. Although Cunha and coworkers (2006) modified the native *ADH2* promoter to eliminate significant basal expression in the presence of higher glucose concentrations, they may have inadvertently fine-tuned the *ADH2* promoter to operate efficiently under high glucose concentrations.

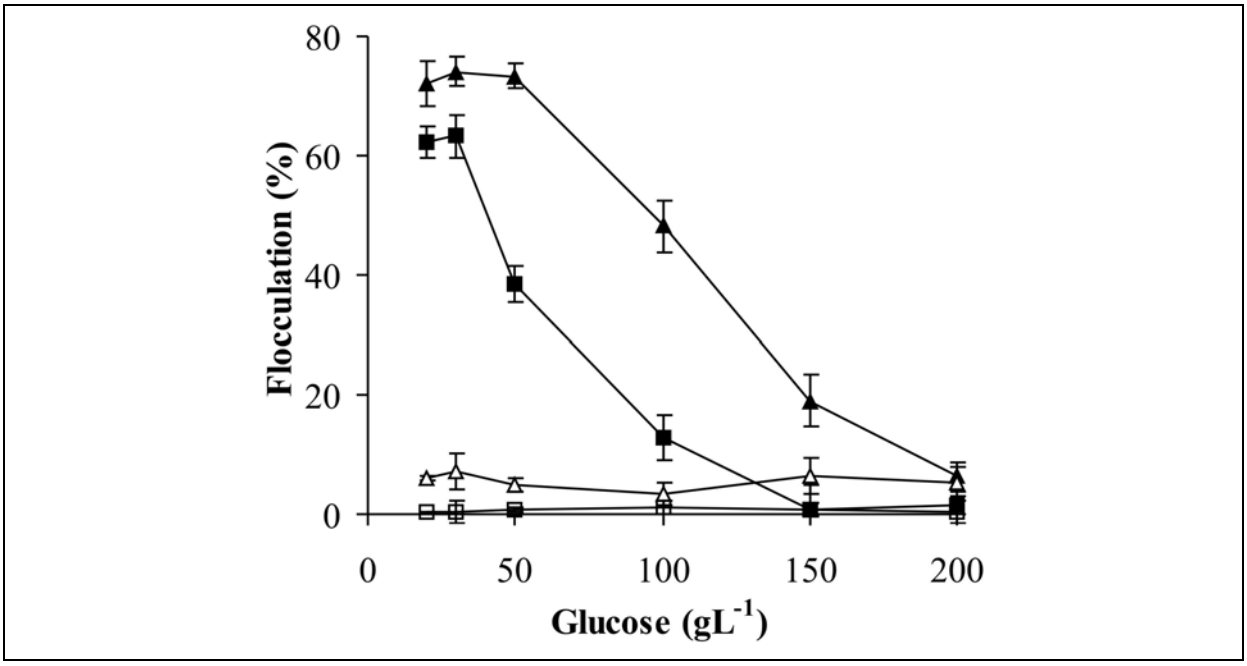

Figure 11 The effect of initial glucose substrate concentration on the flocculent ability of BM45-F5A and VIN13-F5A transgenic yeast strains. Flocculation of BM45 wild type (△); BM45-F5A (▲); VIN13 wild type (□) and VIN13-F5A (■) strains. Yeast strains were aerobically grown in YEPD containing increasing glucose concentrations (20, 30, 50, 100, 150 and 200 gL-124) at 30°C with shaking (160 rpm) until 24 h post-glucose exhaustion. Values reflect the mean of experiments performed in triplicate and error bars represent standard deviations.

Discussion

While the role of *FLO* genes in shaping adhesion properties of yeast has been studied in laboratory yeast, only limited data regarding the role and functionality of native *FLO1*, *FLO5* and *FLO11* open reading frames and of the inherent flocculation or adhesion potential of industrial strains is available. The data presented here clearly demonstrate the potential of these native genes to impart desirable adhesion properties in two non-flocculent commercial wine yeast strains, BM45 and VIN13. The data broadly confirm the specific impacts of the expression of each of these genes as established in laboratory yeast backgrounds, but also highlight important and industrially relevant differences. In particular, the data indicate that while significantly impacting on the adhesion and flocculation properties of the strains, such transgenic wine yeast strains display vegetative growth and fermentation properties that are comparable to that of their parental wild type strains under industrial conditions. In addition, the fermented musts showed almost identical volatile and aroma component profiles. These observations indicate that the genetic modification strategy to impart late fermentation flocculation did not compromise the desirable oenological properties of original non-flocculent host wine yeast strains. Several specific responses of wine yeast strains were different from those observed in the laboratory strain (Teunissen and Steensma 1995; Guo *et al*. 2000; Govender *et al*. 2008). Firstly, the specific flocculation ability of the industrial strains was always lower than of laboratory strain transformed with the same construct. Secondly, and irrespective of the promoter involved, *FLO5*-based constructs induced flocculation more efficiently than *FLO1*-based constructs in these strains. Both of these observations may at least in part be due to significant variations of the size of intragenic repeat regions of the *FLO* genes. Indeed, with the exception of the BM45 *FLO5* repeat region amplicon, all other wine yeast intragenic repetitive domains displayed decreased lengths in contrast to corresponding amplicons obtained from FY23. Verstrepen and co-authors (2005) have shown that an increase in the size of the intragenic repeat region of the *FLO1* gene results in a quantitative increase in *FLO1* mediated phenotypes 1 (e.g. adhesion and flocculation) and vice versa. Furthermore, Fidalgo *et al*. (2006) found the number of repeated sequences in the central domain of *FLO11* from a flor yeast strain to be greatly increased and correlated this to the superior flor forming ability of the strain. The phenotypic analysis of recombinant *FLO11* wine yeast strains confirmed the findings of our previous study that *FLO11*-based constructs were incapable of promoting a flocculent phenotype. Indeed, *FLO11* constructs strongly induced flor formation in both BM45 and VIN13. The same observations were made regarding the ability of *ADH2p-FLO11* strains to invade agarose. Although both BM45 and VIN13 host strains displayed native invasiveness, the *ADH2p-FLO11* derivatives displayed more extensive invasive growth phenotypes. The BM45- F11H and VIN13-F11H transformants were non-invasive thereby corroborating an earlier suggestion that growth on solid media is not an ideal induction condition for the HSP30 promoter (Govender *et al*. 2008). It should be noted that insertion of our *FLO11* replacement cassettes effectively deleted 2612 bp of the native *FLO11* promoter region. Thus, the non invasiveness of *HSP30p-FLO11* wine yeast transformants confirms the effectiveness of our expression strategy in that insertion of our promoter-replacement cassettes reduced native BM45 and VIN13 host strain *FLO11* promoters. A comparison of *ADH2p*-mediated *FLO* gene dependent phenotypes in transgenic laboratory strains (Govender *et al*. 2008) with those generated in recombinant commercial wine yeasts strains under standard laboratory media conditions is presented in Table 3. The data in YEPD medium clearly indicate that *ADH2p* controlled *FLO1* and *FLO5* phenotypes of transgenic wine yeast strains are distinctly more flocculent than comparable *HSP30p* regulated phenotypes. This divide is in contrast to some of the findings in the laboratory yeast strain FY23 (Govender *et al*. 2008). However,

ADH2p-based *FLO1* and *FLO5* constructs failed to promote flocculent phenotypes under both aerobic and microaerophilic MS300 batch fermentation conditions. This is surprising, especially since the previously reported derepression profile of the *ADH2* promoter (Ciriacy 1997; Noronha *et al*. 1998) strongly suggests that significant upregulation of *FLO* gene expression, mediated by *ADH2p* should have at least been observed in the late stationary phase of yeast growth, which corresponds with glucose depletion and high ethanol levels. However, a recent transcriptome study of the commercial wine yeast strain *S. cerevisiae* EC1118 under wine-making conditions using MS300 also demonstrated that there was no change in the expression profile of *ADH2* during all phases of yeast growth (Varela *et al*. 2005). Furthermore gene expression analysis of a bottom-fermenting industrial lager *Saccharomyces* yeast strain under experimental brewing conditions revealed that transcript levels of alcohol dehydrogenases (Adh1, Adh2, Adh3 and Adh5) with the exception of Adh4 are reduced in the late stationary phase of yeast growth (James *et al*. 2002). The promoter was also shown to be subject to transcriptional repression by extracellular ethanol in a strain dependent manner (2001). Although both ADH2p and HSP30p are endogenous *S. cerevisiae* promoters, these data suggest that they may be subject to different regulation modalities in different strains (Nevoigt *et al*. 2006). While the native *ADH2* promoter utilized in this study is not an ideal candidate to drive FLO gene expression under wine-making conditions, it must be noted that *S. cerevisiae* is increasingly being utilized as a cell factory as illustrated in the production of insulin (Kjeldsen 2000), L-lactic acid (Saitoh *et al*. 2005) and polyketides (Kealey *et al*. 1998; Maury *et al*. 2005). Batch fermentations with substantially lower initial glucose substrate concentrations than those employed in wine fermentations are employed for the production of these industrially important compounds. Thus the *ADH2p* based *FLO1* and *FLO5* transformants created in this study that possess the capacity for controlled flocculation may be of benefit to the downstream processing technologies employed in these industries.

Acknowledgements

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