Curr Genet (2006) 49: 375–383
DOI 10.1007/s00294-006-0068-z

RESEARCH ARTICLE

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

Received: 31 January 2006 / Revised: 24 February 2006 / Accepted: 27 February 2006 / Published online: 25 March 2006

Abstract The ability of many microorganisms 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.

Communicated by G. Braus

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Keywords *Saccharomyces cerevisiae* · Flocculation · Invasive growth · *MSS11* · *FLO* genes

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, 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 (for a review see Verstrepen et al. 2003). Flocculation requires the presence of Ca^{2+} ions in the medium (Stratford 1989), and is inhibited by the presence of mannose (Stratford and Assinder 1991). The ability to flocculate 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, 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 (for a review see 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 (for review see Gancedo 2001). The transcriptional regulator Mss11p performs a central role regarding the regulation of pseudohyphae formation by regulating *FLO11* expression (Gagiano et al. 1999a, b; 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*.

Materials and methods

Strains, media, and culture conditions

The yeast strains used in this study are listed in Table 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 et al. 1994). 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 et al. 1994). 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.

Plasmid construction and recombinant DNA techniques

Plasmids and constructs used in this study are listed in Table 2. Standard procedures for the isolation and manipulation of DNA were used throughout this study (Ausubel et al. 1994; Sambrook 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

Table 1 Strains used in this study

Strain	Relevant genotype	Source or reference
FY23 *mss11*Δ	*MAT*a *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); EUROSCARF
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 *his3 lys2 ura3 flo8-1*Δ::*FLO8-LEU2*	This study
BY4742 *FLO8 flo1*Δ	*MAT*α *his3 lys2 ura3 flo8-1*Δ::*FLO8-LEU2 flo1*Δ::*KanMX4*	This study
BY4742 *FLO8 flo10*Δ	*MAT*α *his3 lys2 ura3 flo8-1*Δ::*FLO8-LEU2 flo10*Δ::*KanMX4*	This study
BY4742 *FLO8 flo11*Δ	*MAT*α *his3 lys2 ura3 flo8-1*Δ::*FLO8-LEU2 flo11*Δ::*KanMX4*	This study
BY4742 *FLO8 mss11*Δ	*MAT*α *his3 lys2 ura3 flo8-1*Δ::*FLO8-LEU2 mss11*Δ::*KanMX4*	This study

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.

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

Table 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)

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:

$$\text{Flocculation } (\%) = \left(\frac{A - B}{A}\right) \times 100 \quad (1)$$

The standard error of the mean (SEM) was calculated for cultures from at least five individual transformants for the data set used in Fig. 1, whereas three individual transformants were used to generate the data with regard to the expression of *MSS11* truncations.

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 et al. 1994). RNA was separated on 1% agarose gels containing 0,7% formaldehyde and thereafter transferred and cross linked to BioBond-Plus^{TM} 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′-AACAGTAGT CACCTCTTCGC-3′; FLO1r, 5′-AGACACTTAAACC ACTACCG-3′; FLO10probeF, 5′-ATGCCTGTGGCT GCTCGATA-3′; and FLO10probeR, 5′-TGTCGGTA GGTGCATCTGCG-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).

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.

Results

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

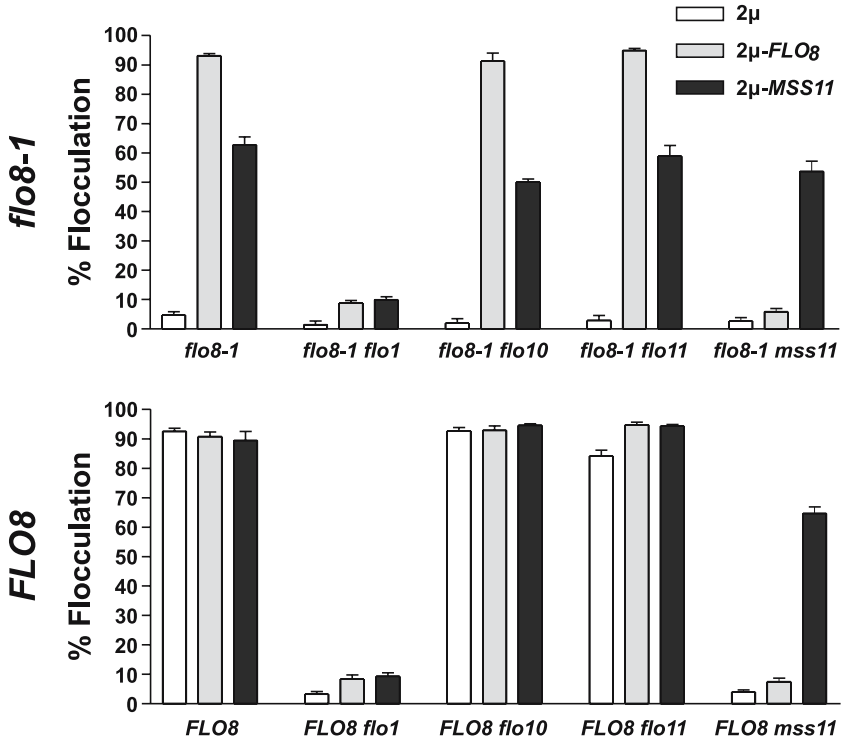

Fig. 1 Relative flocculation levels displayed by the indicated yeast strains. The relevant genetic backgrounds are indicated. *White bars* correspond to yeast carrying vector alone (2µ) while *grey* and *black bars* represent yeast transformed with plasmids YEpLac195-FLO8 (2µ-*FLO8*) and YEpLac195-MSS11 (2µ-*MSS11*), respectively. *Error bars* indicate the standard error of the mean (SEM)

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. 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. 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. 1).

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. 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.

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. 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*.

Role of *FLO* genes in haploid invasive growth

The ability of the same set of strains to invade agar is shown in Fig. 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.

Transcriptional regulation of the *FLO* genes

Figure 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

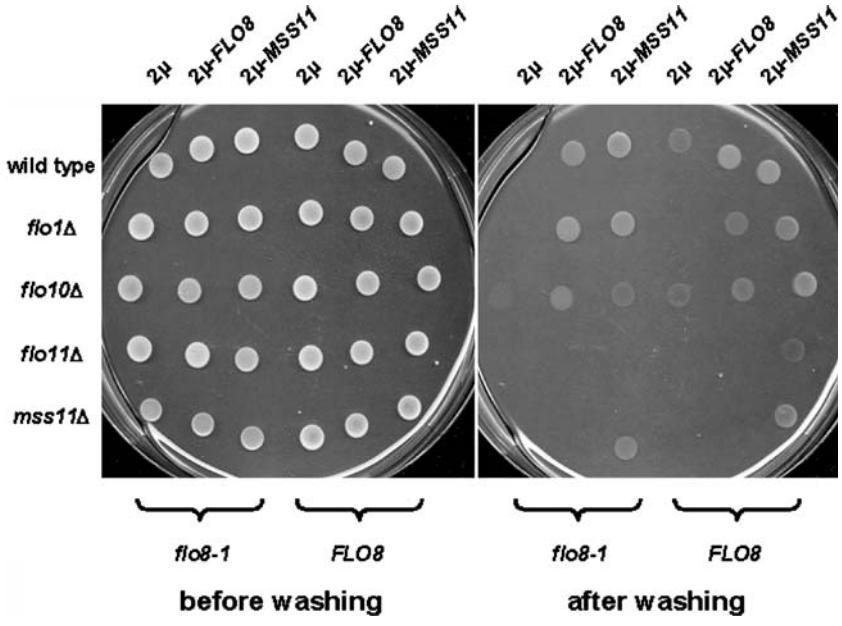

Fig. 2 Haploid invasive growth of yeast strains. The relevant genetic backgrounds are indicated. Liquid cultures of transformants were spotted on solid SLAD media and left to grow for 6 days at 30°C. The image on the *left* shows total growth, whereas the *right-hand* image shows the same plate after the strains have been washed off the agar surface under running water

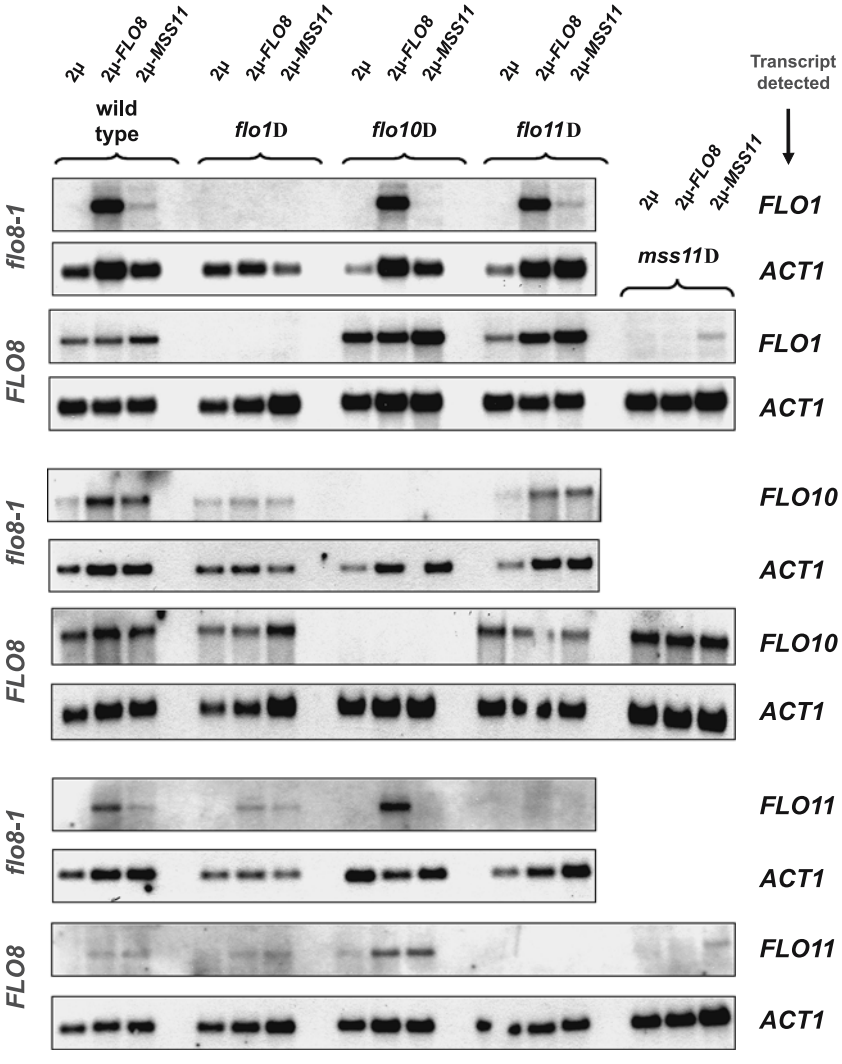

Fig. 3 Northern blot analysis of *FLO1*, *FLO10* and *FLO11* in the various strains used in this study. The relevant genetic background and plasmids are indicated, as well as the specific transcripts probed for

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*.

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 2 μ-*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.

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. 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. 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).

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, b; van Dyk et al. 2005) and the development of pseudohyphae (Lorenz and Heitman 1998; Gagiano et al. 1999b), 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 extracellular 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, b; 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 overexpressing 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* containing multiple copies of *MSS11*. This strain grew

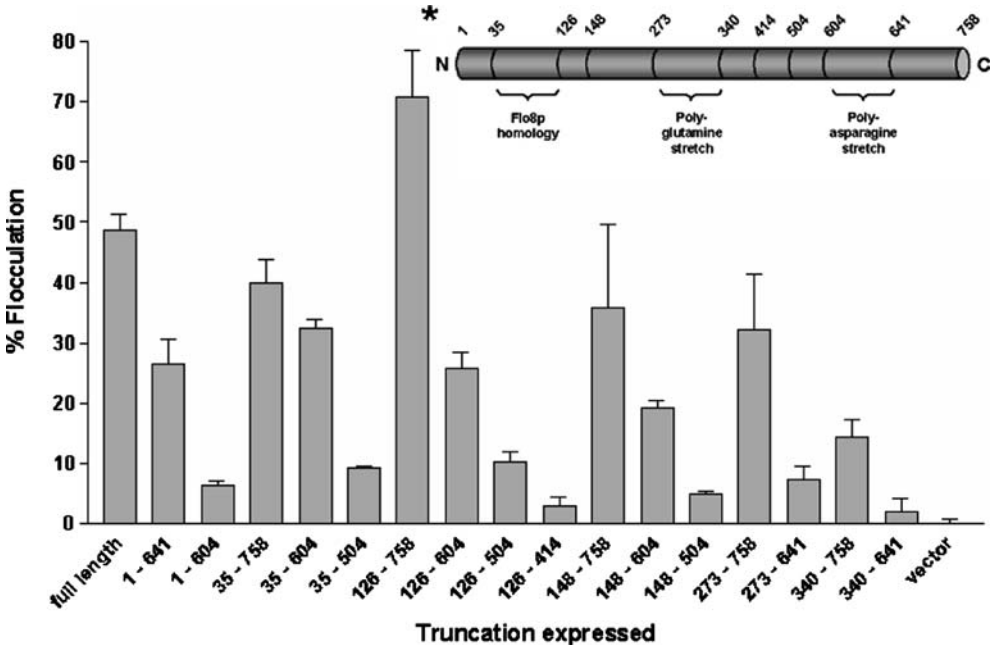

Fig. 4 Relative flocculation levels displayed by yeast *flo8-1 mss11*Δ transformed with plasmids containing various truncated forms of the open reading frame of *MSS11* under transcriptional control of its native promoter. Mss11p is schematically represented, and *numbers* correspond to the amino acids that represent each truncated version. *Error bars* indicate the standard error of the mean (SEM)

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 2 μ 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.

Acknowledgements We thank Marco Gagiano for the construction of the set of plasmids containing the different truncated parts of *MSS11* in vector YEpLac112, and Dewald van Dyk and Klaus Suetterlin for the construction of vectors YCpLac22-FLO8-LEU2 and YEpLac195-FLO8, respectively. This work was supported financially by the National Research Foundation (NRF), the South African Wine Industry (Winetech), and the Department of Trade and Industry's ''Technology and Human Resource for Industry Programme'' (THRIP).

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