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Mss11p Is a Central Element of the Regulatory Network That Controls *FLO11* Expression and Invasive Growth in *Saccharomyces cerevisiae*

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ABSTRACT

The invasive and filamentous growth forms of *Saccharomyces cerevisiae* are adaptations to specific environmental conditions, under particular conditions of limited nutrient availability. Both growth forms are dependent on the expression of the *FLO11* gene, which encodes a cell-wall-associated glycoprotein involved in cellular adhesion. A complex regulatory network consisting of signaling pathways and transcription factors has been associated with the regulation of *FLO11*. Mss11p has been identified as a transcriptional activator of this gene, and here we present an extensive genetic analysis to identify functional relationships between Mss11p and other *FLO11* regulators. The data show that Mss11p is absolutely required for the activation of *FLO11* by most proteins that have previously been shown to affect *FLO11* expression, including the signaling proteins Ras2p, Kss1p, and Tpk2p, the activators Tec1p, Flo8p, and Phd1p, and the repressors Nrg1p, Nrg2p, Sok2p, and Sfl1p. The genetic evidence furthermore suggests that Mss11p activity is not dependent on the presence of any of the above-mentioned factors and that the protein also regulates other genes involved in cellular adhesion phenotypes. Taken together, the data strongly suggest a central role for Mss11p in the regulatory network controlling *FLO11* expression, invasive growth, and pseudohyphal differentiation.

IN *Saccharomyces cerevisiae*, the choice of a specific cellular growth form is frequently governed by nutrient availability. Nutrient-rich environments generally favor yeast-type unicellular multiplication, while nutrient-limited environments tend to support pseudohyphal and/or invasive growth. Nutrient depletion, on the other hand, leads to a different set of adaptations, either entry into the G_0 phase of the cell cycle or meiosis and spore formation, depending on the cell type and on the nutritional composition of the growth substrate. The transition from unicellular to invasive and/or pseudohyphal growth is characterized by important morphological and physiological changes and referred to as a dimorphic switch or as pseudohyphal differentiation. This switch can be triggered by nitrogen source or amino acid limitation (Gimeno *et al.* 1992; Lambrechts *et al.* 1996a; Braus *et al.* 2003). It may also occur in response to carbon limitation, growth on alcohols (Cullen and Sprague 2000; Lorenz *et al.* 2000), or growth on other inefficiently used carbon sources, including starch (Lambrechts *et al.* 1996a). Invasive and pseudohyphal growth is characterized by directional unipolar budding and by cells that remain attached to each other after budding (Gimeno *et al.* 1992). Haploid cells growing as filaments display stronger adhesiveness than diploid cells and the filaments of haploid cells penetrate solid growth substrates more efficiently (Roberts and Fink 1994).

Complex regulatory networks govern the conversion of environmental signals into specific developmental outcomes. The nutrient-responsive regulatory network that controls haploid invasive growth consists of several signal transduction modules, including the nutrient-responsive mitogen-activated protein kinase (MAPK) cascade and the cyclic AMP-dependent protein kinase A (PKA) pathway (reviewed in Lengeler *et al.* 2000; Gancedo 2001; Gagiano *et al.* 2002). The MAP kinases Kss1p and Fus3p and the cAMP-dependent kinase Tpk1-3p activate or inactivate various transcription factors, which in turn control the expression of genes that are responsible for the cellular differentiation process (Cook *et al.* 1997; Madhani *et al.* 1997; Bardwell *et al.* 1998; Robertson and Fink 1998; Pan and Heitman 1999; Breitkreutz *et al.* 2003). Other signaling elements or regulators that have been associated with pseudohyphal differentiation and invasive growth include cell cycle regulators, in particular the G_1 cyclins (Oehlen and Cross 1998; Loeb *et al.* 1999; Ahn *et al.* 2001), the amino-acid-specific response pathway (Braus *et al.* 2003), and the meiosis-specific regulator Rme1p (van Dyk *et al.* 2003).

FLO11/MUC1 and *STA2* are two of the genes controlled by signaling pathways activated in response to the nutrient status of the growth substrate (Gagiano *et al.* 1999a,b; Rupp *et al.* 1999). *FLO11* encodes a glycosyl-phosphatidylinositol-anchored cell surface protein, which is a member

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of the flocculin family, a group of proteins involved in cell-cell adhesion (Caro *et al.* 1997; Guo *et al.* 2000; Verstrepen *et al.* 2003). Apart from its previously reported role in flocculation (Lo and Dranginis 1996), the Flo11p glycoprotein was also shown to be required for invasive growth and pseudohyphal development (Lambrechts *et al.* 1996a; Lo and Dranginis 1998), cell-substrate adhesion (Guo *et al.* 2000), and biofilm formation (Reynolds and Fink 2001). The *STA2* gene encodes a secreted α-glucoamylase, which enables yeast to degrade starch and to grow on starch-containing medium (Lambrechts *et al.* 1996a; Gagiano *et al.* 1999b). The promoters of *FLO11* and *STA2* are almost identical and as a consequence these genes are coregulated by a similar set of transcription factors (Gagiano *et al.* 1999a,b, 2003; van Dyk *et al.* 2003).

A large number of factors with complex functional relationships were shown to act on the ~3-kb promoter of *FLO11*, one of the largest promoters identified in *S. cerevisiae* (reviewed in Gagiano *et al.* 2002; Palecek *et al.* 2002). On the basis of their roles, the regulators can be divided into different subgroups, which, apart from the general transcription machinery (RNA polymerase II and associated subcomplexes), include factors that act downstream of the cAMP and MAP kinase signaling modules. While these factors appear to be generally required for invasive growth, other factors appear to act only in response to the limitation of specific nutrients. Furthermore, *FLO11* regulation has been linked to several chromatin-remodeling proteins, whose association with the above-mentioned signaling network has not yet been clearly established.

Signaling pathway-responsive regulators include the MAPK-controlled proteins Ste12p and Tec1p, which constitute a heterodimeric transcriptional activator that regulates genes involved in filamentous growth, including *FLO11* (Madhani and Fink 1997; Rupp *et al.* 1999; Köhler *et al.* 2002; Zeitlinger *et al.* 2003). The cAMP-dependent PKA pathway regulates the activities of the Flo8p transcriptional activator and the Sfl1p transcriptional repressor (Liu *et al.* 1996; Robertson and Fink 1998; Pan and Heitman 1999, 2002). A 250-nucleotide stretch has been identified in the *FLO11* promoter that is bound by both Flo8p and Sfl1p (Pan and Heitman 2002).

Nutrient-specific transcription factors include Gcn4p, Nrg1p, and Nrg2p. In response to amino acid starvation, Gcn4p upregulates *FLO11* transcription, but does not appear to bind the *FLO11* promoter (Braus *et al.* 2003). Snf1p, a highly conserved protein kinase required for the derepression of genes subject to glucose-repression (reviewed in Carlson 1999), activates *FLO11* transcription by antagonizing the two repressors Nrg1p and Nrg2p (Kuchin *et al.* 2002).

Msn1p and Rme1p, two putative chromatin-remodeling factors (Covitz *et al.* 1994; Sidorova and Breeden 1999), were previously shown to activate *FLO11* transcription when expressed from multi-copy plasmids (Gagiano *et al.* 1999a,b; van Dyk *et al.* 2003). *MSN1* encodes a protein involved in the regulation of several genes, including *HO* and *STA2* (Estruch and Carlson 1990; Lambrechts *et al.* 1996b; Sidorova and Breeden 1999). The regulator of meiosis, Rme1p, acts as a repressor of the early meiosis gene *IME1* (Kassir *et al.* 1988; Covitz and Mitchell 1993) and as an activator of the G_1-cyclin gene *CLN2* (Toone *et al.* 1995) and of *STA2* and *FLO11* (van Dyk *et al.* 2003).

Finally, the activator-encoding gene *PHD1* (Gimeno and Fink 1994) and the associated repressor Sok2p (Ward *et al.* 1995) are also involved in the regulation of *FLO11* transcription. While their mode of action has not been established, activation by Phd1p was shown to be dependent on the presence of *FLO8* (Pan and Heitman 2000).

Mss11p has previously been shown to play a role in invasive growth and starch degradation (Webber *et al.* 1997; Gagiano *et al.* 1999b, 2003). The data indicated that the protein is able to regulate the transcription of *FLO11* and *STA2* directly in response to nutritional signals (Gagiano *et al.* 2003). However, its relationship with other *FLO11* regulators and its position within the *FLO11* regulatory network have not been elucidated. Here we report on the genetic interactions between *MSS11* and all of the genes encoding the aforementioned factors. The results indicate that the pronounced decrease in *FLO11* expression observed in *mss11*Δ strains cannot be suppressed by most of the genes important for *FLO11* regulation. The data show that the presence of *MSS11* is absolutely required for the activation of *FLO11* by hyperactive alleles or multiple copies of *RAS2*, *KSS1*, *TPK2*, *TEC1*, *FLO8*, and *PHD1*, as well as for derepression of the gene in strains deleted for *SFL1*, *SOK2*, *NRG1*, and *NRG2*. In our analysis, the only factors that appear to not depend on Mss11p are Msnp1 and Rme1p, which have been associated with chromatin-remodeling functions. Our data strongly suggest an essential and central role for Mss11p in the transcriptional regulation of *FLO11* and that the protein acts in the very center of the regulatory network that specifically governs *FLO11* transcription.

MATERIALS AND METHODS

Strains, media, and recombinant DNA techniques: The yeast strains used in this study are listed in Table 1. Standard YEPD medium was used to cultivate yeast strains prior to transformation. Plasmids and disruption cassettes were introduced using the lithium acetate method described by Ausubel *et al.* (1994). Strains were cultivated at 30° and synthetic dropout medium containing 2% glucose, 0.67% yeast nitrogen base (Difco Laboratories, Detroit), and essential amino acids was used to propagate transformants (Sherman *et al.* 1991). Geneticin-resistant transformants were selected on YPED medium supplemented with 200 mg/liter geneticin (Sigma-Aldrich, St. Louis). Invasive growth was assessed on 0.2% glucose medium, and 2% potato starch (Sigma-Aldrich) was used as carbon source for starch (SCS) plates. SLAD (synthetic low ammonium dextrose) medium contained 2% glucose and SCGE (synthetic complete glycerol ethanol) medium was prepared using 3% glycerol

TABLE 1

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

Strain	Relevant genotype	Source or reference
YHUM272[a]	*MATα ura3-52 trp1Δ::hisG leu2Δ::hisG his3Δ::hisG*	H.-U. Mösch
Σ1278b*flo8Δ*	*MATα ura3-52 trp1Δ::hisG leu2Δ::hisG his3Δ::hisG flo8Δ::LEU2*	This study
Σ1278b*kss1Δ*	*MATα ura3-52 trp1Δ::hisG leu2Δ::hisG his3Δ::hisG kss1Δ::kanMX4*	This study
Σ1278b*msn1Δ*	*MATα ura3-52 trp1Δ::hisG leu2Δ::hisG his3Δ::hisG msn1Δ::URA3*	This study
Σ1278b*mss11Δ*	*MATα ura3-52 trp1Δ::hisG leu2Δ::hisG his3Δ::hisG mss11Δ::LEU2*	This study
Σ1278b*phd1Δ*	*MATα ura3-52 trp1Δ::hisG leu2Δ::hisG his3Δ::hisG phd1Δ::LEU2*	This study
Σ1278b*sfl1Δ*	*MATα ura3-52 trp1Δ::hisG leu2Δ::hisG his3Δ::hisG sfl1Δ::kanMX4*	This study
Σ1278b*ste12Δ*	*MATα ura3-52 trp1Δ::hisG leu2Δ::hisG his3Δ::hisG ste12Δ::URA3*	This study
Σ1278b*tec1Δ*	*MATα ura3-52 trp1Δ::hisG leu2Δ::hisG his3Δ::hisG tec1Δ::LEU2*	This study
Σ1278b*tpk2Δ*	*MATα ura3-52 trp1Δ::hisG leu2Δ::hisG his3Δ::hisG tpk2Δ::kanMX4*	This study
Σ1278b*flo8Δsfl1Δ*	*MATα ura3-52 trp1Δ::hisG leu2Δ::hisG his3Δ::hisG flo8Δ::LEU2 sfl1Δ::kanMX4*	This study
Σ1278b*msn1Δsfl1Δ*	*MATα ura3-52 trp1Δ::hisG leu2Δ::hisG his3Δ::hisG msn1Δ::URA3 sfl1Δ::kanMX4*	This study
Σ1278b*mss11Δsfl1Δ*	*MATα ura3-52 trp1Δ::hisG leu2Δ::hisG his3Δ::hisG mss11Δ::LEU2 sfl1Δ::kanMX4*	This study
Σ1278b*ste12Δsfl1Δ*	*MATα ura3-52 trp1Δ::hisG leu2Δ::hisG his3Δ::hisG ste12Δ::URA3 sfl1Δ::kanMX4*	This study
Σ1278b*tec1Δsfl1Δ*	*MATα ura3-52 trp1Δ::hisG leu2Δ::hisG his3Δ::hisG tec1Δ::LEU2 sfl1Δ::kanMX4*	This study
Σ1278b*flo11Δ::lacZ*	*MATα ura3-52 trp1Δ::hisG leu2Δ::hisG his3Δ::hisG flo11Δ::lacZ-HIS3*	van Dyk *et al.* (2003)
Σ1278b*flo11Δ::lacZ flo8Δ*	*MATα ura3-52 trp1Δ::hisG leu2Δ::hisG his3Δ::hisG flo11Δ::lacZ-HIS3 flo8Δ::LEU2*	van Dyk *et al.* (2003)
Σ1278b*flo11Δ::lacZ gpa2Δras2Δ*	*MATα ura3-52 trp1Δ::hisG leu2Δ::hisG his3Δ::hisG flo11Δ::lacZ-HIS3 gpa2Δ::LEU2 ras2Δ::kanMX4*	This study
Σ1278b*flo11Δ::lacZ kss1Δ*	*MATα ura3-52 trp1Δ::hisG leu2Δ::hisG his3Δ::hisG flo11Δ::lacZ-HIS3 kss1Δ::kanMX4*	This study
Σ1278b*flo11Δ::lacZ msn1Δ*	*MATα ura3-52 trp1Δ::hisG leu2Δ::hisG his3Δ::hisG flo11Δ::lacZ-HIS3 msn1Δ::URA3*	van Dyk *et al.* (2003)
Σ1278b*flo11Δ::lacZ mss11Δ*	*MATα ura3-52 trp1Δ::hisG leu2Δ::hisG his3Δ::hisG flo11Δ::lacZ-HIS3 mss11Δ::LEU2*	van Dyk *et al.* (2003)
Σ1278b*flo11Δ::lacZ nrg1Δ*	*MATα ura3-52 trp1Δ::hisG leu2Δ::hisG his3Δ::hisG flo11Δ::lacZ-HIS3 nrg1Δ::kanMX4*	van Dyk *et al.* (2003)
Σ1278b*flo11Δ::lacZ nrg2Δ*	*MATα ura3-52 trp1Δ::hisG leu2Δ::hisG his3Δ::hisG flo11Δ::lacZ-HIS3 nrg2Δ::kanMX4*	van Dyk *et al.* (2003)
Σ1278b*flo11Δ::lacZ phd1Δ*	*MATα ura3-52 trp1Δ::hisG leu2Δ::hisG his3Δ::hisG flo11Δ::lacZ-HIS3 phd1Δ::LEU2*	van Dyk *et al.* (2003)
Σ1278b*flo11Δ::lacZ ras2Δ*	*MATα ura3-52 trp1Δ::hisG leu2Δ::hisG his3Δ::hisG flo11Δ::lacZ-HIS3 ras2Δ::LEU2*	van Dyk *et al.* (2003)
Σ1278b*flo11Δ::lacZ sfl1Δ*	*MATα ura3-52 trp1Δ::hisG leu2Δ::hisG his3Δ::hisG flo11Δ::lacZ-HIS3 sfl1Δ::kanMX4*	van Dyk *et al.* (2003)
Σ1278b*flo11Δ::lacZ sok2Δ*	*MATα ura3-52 trp1Δ::hisG leu2Δ::hisG his3Δ::hisG flo11Δ::lacZ-HIS3 sok2Δ::kanMX4*	van Dyk *et al.* (2003)
Σ1278b*flo11Δ::lacZ Δste12*	*MATα ura3-52 trp1Δ::hisG leu2Δ::hisG his3Δ::hisG flo11Δ::lacZ-HIS3 ste12Δ::URA3*	van Dyk *et al.* (2003)
Σ1278b*flo11Δ::lacZ tec1Δ*	*MATα ura3-52 trp1Δ::hisG leu2Δ::hisG his3Δ::hisG flo11Δ::lacZ-HIS3 tec1Δ::LEU2*	van Dyk *et al.* (2003)
Σ1278b*flo11Δ::lacZ tpk2Δ*	*MATα ura3-52 trp1Δ::hisG leu2Δ::hisG his3Δ::hisG flo11Δ::lacZ-HIS3 tpk2Δ::kanMX4*	This study
Σ1278b*flo11Δ::lacZ flo8Δnrg1Δ*	*MATα ura3-52 trp1Δ::hisG leu2Δ::hisG his3Δ::hisG flo11Δ::lacZ-HIS3 flo8Δ::LEU2 nrg1Δ::kanMX4*	This study
Σ1278b*flo11Δ::lacZ flo8Δnrg2Δ*	*MATα ura3-52 trp1Δ::hisG leu2Δ::hisG his3Δ::hisG flo11Δ::lacZ-HIS3 flo8Δ::LEU2 nrg2Δ::kanMX4*	This study
Σ1278b*flo11Δ::lacZ flo8Δsfl1Δ*	*MATα ura3-52 trp1Δ::hisG leu2Δ::hisG his3Δ::hisG flo11Δ::lacZ-HIS3 flo8Δ::LEU2 sfl1Δ::kanMX4*	This study
Σ1278b*flo11Δ::lacZ flo8Δsok2Δ*	*MATα ura3-52 trp1Δ::hisG leu2Δ::hisG his3Δ::hisG flo11Δ::lacZ-HIS3 flo8Δ::LEU2 sok2Δ::kanMX4*	This study

(*continued*)

TABLE 1

(Continued)

Strain	Relevant genotype	Source or reference
Σ1278b*flo11*Δ::*lacZ mss11*Δ*nrg1*Δ	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG flo11*Δ::*lacZ-HIS3 mss11*Δ::*LEU2 nrg1*Δ::*kanMX4*	This study
Σ1278b*flo11*Δ::*lacZ mss11*Δ*nrg2*Δ	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG flo11*Δ::*lacZ-HIS3 mss11*Δ::*LEU2 nrg2*Δ::*kanMX4*	This study
Σ1278b*flo11*Δ::*lacZ mss11*Δ*sfl1*Δ	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG flo11*Δ::*lacZ-HIS3 mss11*Δ::*LEU2 sfl1*Δ::*kanMX4*	This study
Σ1278b*flo11*Δ::*lacZ mss11*Δ*sok2*Δ	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG flo11*Δ::*lacZ-HIS3 mss11*Δ::*LEU2 sok2*Δ::*kanMX4*	This study
Σ1278b*flo11*Δ::*lacZ msn1*Δ*sfl1*Δ	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG flo11*Δ::*lacZ-HIS3 msn1*Δ::*URA3 sfl1*Δ::*kanMX4*	This study
Σ1278b*flo11*Δ::*lacZ ste12*Δ*sfl1*Δ	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG flo11*Δ::*lacZ-HIS3 ste12*Δ::*URA3 sfl1*Δ::*kanMX4*	This study
Σ1278b*flo11*Δ::*lacZ tec1*Δ*sfl1*Δ	*MAT*α *ura3-52 trp1*Δ::*hisG leu2*Δ::*hisG his3*Δ::*hisG flo11*Δ::*lacZ-HIS3 tec1*Δ::*LEU2 sfl1*Δ::*kanMX4*	This study
ISP15	*MAT***a** *his3 leu2 thr1 trp1 ura3 STA2*	This laboratory
ISP15*msn1*Δ *ste12*Δ*tec1*Δ	*MAT***a** *his3 leu2 thr1 trp1 ura3 STA2 msn1*Δ::*ura3*Δ::*kanMX4 ste12*Δ::*URA3 tec1*Δ::*LEU2*	This study
ISP15*flo8*Δ*msn1*Δ *ste12*Δ*tec1*Δ	*MAT***a** *his3 leu2 thr1 trp1 ura3 STA2 flo8*Δ:: *ura3*Δ::*loxP msn1*Δ::*URA3 ste12*Δ::*ura3*Δ::*kanMX4 tec1*Δ::*LEU2*	This study
BY4742[b]	*MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0*	EUROSCARF
BY4742*kss1*Δ	*MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0 kss1*Δ::*kanMX4*	EUROSCARF
BY4742*nrg1*Δ	*MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0 nrg1*Δ::*kanMX4*	EUROSCARF
BY4742*nrg2*Δ	*MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0 nrg2*Δ::*kanMX4*	EUROSCARF
BY4742*ras2*Δ	*MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0 ras2*Δ::*kanMX4*	EUROSCARF
BY4742*sfl1*Δ	*MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0 sfl1*Δ::*kanMX4*	EUROSCARF
BY4742*sok2*Δ	*MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0 sok1*Δ::*kanMX4*	EUROSCARF
BY4742*tpk2*Δ	*MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0 tpk2*Δ::*kanMX4*	EUROSCARF

[a] YHUM272 (10560-6B) is from the Σ1278b background.
[b] BY4742 is from the S288C genetic background (see Brachmann *et al.* 1998).

and 3% ethanol. β-Galactosidase assays were performed on strains pregrown in liquid medium containing 2% glucose. All media were supplemented with 0.67% yeast nitrogen base (YNB) containing preadded ammonium sulfate (Difco), except for the SLAD medium, which contains 0.17% YNB to which 50 μM ammonium sulfate was separately added. In all cases 2% agar (Difco) was used as the solidifying agent for the plate media.

Escherichia coli strain DH5α was used for plasmid amplification (GIBCO BRL/Life Technologies, Rockville, MD). Plasmid-bearing bacterial strains were cultivated at 37° in Luria-Bertani broth. Standard bacterial transformations and plasmid isolation procedures were performed as described by Sambrook *et al.* (1989).

Plasmid construction and primers: The constructs used in this study are listed in Table 2. *KSS1* was excised from pXT1 (kindly provided by D. Engelberg, The Hebrew University of Jerusalem) as a 1647-bp *Eco*RI-*Sph*I fragment and cloned into the corresponding sites of YEplac112 (Gietz and Sugino 1988) to generate YEplac112-KSS1. The *TPK2* gene from the S288c genetic background was amplified with primers TPK2-Fp, 5′-ATA TACGTACACACAATTCCATATCGAG-3′, and TPK2-Rp, 5′-GCA ACGCTTGTTCTTCTCATCTCTT-3′. The resulting 1737-bp PCR product was cloned into pGEM-Teasy (Promega, Madison, WI), subsequently isolated as a *Spe*I-*Sna*BI fragment, and cloned into the *Xba*I-*Sma*I sites of YEplac112 to generate YEplac112-TPK2. The *flo8*Δ::*LEU2* (pΔflo8-L) disruption cassette was constructed by replacing a 760-bp *Pst*I-*Bgl*II fragment of YEplac 112-FLO8 (Gagiano *et al.* 1999a) with a 2000-bp *Pst*I-*Bam*HI fragment containing the *LEU2* marker of pJJ252 (Jones and Prakash 1990). *Mlu*NI and *Sna*BI restriction enzymes were used to remove the 4070-bp *flo8*Δ::*leu2* disruption cassette from pΔflo8-L.

KSS1-Fp, 5′-GTACTTCCAATCTGTAGATATTGCACTTT ATC-3′, and KSS1-Rp, 5′-CCGTTTAGGCAAAGCAGTGA AGA-3′, TPK2-Fp, and TPK2-Rp were used to PCR amplify *kss1*Δ::*kanMX4* and *tpk2*Δ::*kanMX4* from the genomic DNA of the corresponding BY4742 mutant strains.

Yeast strain construction: Invasive growth and β-galactosidase assays were performed on strains derived from 1278b (10560-6B) (see Table 1 and van Dyk *et al.* 2003). The *lacZ* gene of Σ1278b*flo11*Δ::*lacZ* is under the control of the endogenous *FLO11* promoter (van Dyk *et al.* 2003). The *STA2*-bearing strain ISP15 and isogenic mutants were used to assess starch degradation phenotypes (described in Gagiano *et al.* 1999b; van Dyk *et al.* 2003). The S288c-derived BY4742 strain collection (Brachmann *et al.* 1998) was purchased from the European *Saccharomyces cerevisiae* Archive for Functional Analysis (EUROSCARF). Disruption cassettes used to delete copies of *FLO8*, *GPA2*, *MSN1*, *MSS11*, *NRG1*, *NRG2*, *PHD1*, *RAS2*, *SFL1*, *SOK2*, *STE12*, and *TEC1* in the Σ1278b wild-type and Σ1278b-*flo11*Δ::*lacZ* reporter strains were obtained by means of PCR amplification using primers, genomic templates, and the disruption cassettes previously described in van Dyk *et al.* (2003). The genomic DNA of BY4742*kss1*Δ::*kanMX4*, BY4742*ras2*Δ::*kanMX4*, and BY4742*tpk2*Δ::*kanMX4* (EUROSCARF) served as PCR templates for the amplification of the corresponding disruption cassettes.

To generate ISP15*msn1*Δ*ste12*Δ*tec1*Δ and ISP15*flo8*Δ*msn1*Δ-*ste12*Δ*tec1*Δ, *URA3* markers were recovered using the *ura3*Δ::*kanMX* disruption cassette of pΔura3::kan (Gagiano *et al.* 1999a). The *kanMX* gene was subsequently removed with the

TABLE 2

Plasmids used in this study

Plasmid	Relevant genotype	Source or reference
YEplac112	2μ *TRP1*	Gietz and Sugino (1988)
YEplac112-FLO8	2μ *TRP1 FLO8*	Gagiano *et al.* (1999a)
YEplac112-KSS1	2μ *TRP1 KSS1*	This study
YEplac112-MSN1	2μ *TRP1 MSN1*	Gagiano *et al.* (1999b)
YEplac112-MSS11	2μ *TRP1 MSS11*	Gagiano *et al.* (1999b)
YEplac112-PHD1	2μ *TRP1 PHD1*	van Dyk *et al.* (2003)
YEplac112-STE12	2μ *TRP1 STE12*	Gagiano *et al.* (1999b)
YEplac112-TEC1	2μ *TRP1 TEC1*	van Dyk *et al.* (2003)
YEplac112-TPK2	2μ *TRP1 TPK2*	This study
YCplac22-RAS2^{val19}	*CEN4 TRP1 RAS2*^{Val19}	Gagiano *et al.* (1999b)
pXT1[a]	2μ *LEU2 KSS1*	D. Engelberg
pGEM-Teasy		Promega
pGEM-Teasy-TPK2	PCR fragment containing *TPK2*	This study
pUG6[b]	*kanMX*^{R}	J. H. Hegemann
pSH47[b]	*CEN6 URA3*	J. H. Hegemann
pΔura3::kan	*ura3*Δ::*kanMX*	Gagiano *et al.* (1999a)
pJJ252	*LEU2*	Jones and Prakash (1990)
pΔflo8-L	*Flo8*Δ::*LEU2*	This study
pΔflo8	*Flo8*Δ::*URA3*	Gagiano *et al.* (1999a)
pΔgpa2	*gpa2*Δ::*LEU2*	van Dyk *et al.* (2003)
pΔmsn1	*msn1*Δ::*URA3*	Gagiano *et al.* (1999b)
pMSS11-Δ	*mss11*Δ::*LEU2*	Webber *et al.* (1997)
pΔphd1	*phd1*Δ::*LEU2*	van Dyk *et al.* (2003)
pΔste12	*ste12*Δ::*URA3*	Gagiano *et al.* (1999b)
pΔtec1	*tec1*Δ::*LEU2*	van Dyk *et al.* (2003)

[a] pXT1 contains the original *KSS1* isolate cloned in YEp13 (Courchesne *et al.* 1989).
[b] Plasmids pUG6 and pSH47 are described in Güldener *et al.* (1996).

loxP-kanMX-loxP/Cre system as described by Güldener *et al.* (1996).

Invasive growth and starch degradation plate assays: For the invasive growth, plate assay transformants were spotted onto 0.2% glucose medium and incubated for 5 days at 30°. Colonies were removed by vigorous rubbing under a constant stream of running water. The plates were subsequently allowed to dry on the bench before photographs were taken. Only cells of colonies that penetrated the growth medium were present after the washing procedure. The density of the residual cells observed under the surface of the growth medium reflects the ability of a strain to grow invasively.

Starch degradation was observed as transparent zones present around yeast colonies grown on solid starch-containing medium. The ability of strains to utilize starch was assessed by dropping 15 μl of synthetic complete dextrose (SCD) culture suspensions, grown to an optical density (OD_{600}) of ~1.0, onto starch (SCS) plates. The spotted cultures were incubated for 5 days at 30°. Starch precipitation was induced by adding 96% ethanol to the starch plates, after which the plates were incubated at 4°. Clear transparent zones around the colonies were observed within a few minutes of incubation.

β-Galactosidase assays: The composition of the media, culture preparation, and growth conditions were previously described (van Dyk *et al.* 2003). Assays for β-galactosidase activity were performed exactly according to the instructions of Ausubel *et al.* (1994). At least three independent transformants were used for each experiment, and experiments were performed in triplicate. Every data point represents the average of three transformants with a standard deviation of <15%. Activity of β-galactosidase is expressed in Miller units (Ausubel *et al.* 1994).

RNA extraction and Northern analysis: Test tubes containing 5 ml of SCD (2% glucose) selective media were inoculated to an OD_{600} of ~0.05 from overnight precultures. The cultures were grown to an OD_{600} of ~1.0 and cells were harvested from 4 ml of cell suspension. Total RNA was extracted by the glass bead mechanical disruption method (Ausubel *et al.* 1994). Approximately 10 μg of total RNA was separated on 1.2% formaldehyde agarose gels. The RNA was transferred and crosslinked to BioBond-Plus nylon membranes (Sigma-Aldrich).

Probes to detect the mRNA of the *FLO11* and of the actin-encoding *ACT1* genes were generated through PCR with primers FLO11-Fprobe, 5′-TCACGACGGCTATTCCAACC-3′; FLO11-Rprobe, 5′-TTAGAATACAACTGGAAGAGCGAG-3′; ACT1-F probe, 5′-GACGCTCCTCGTGCTGTCTT-3′; and ACT1-Rprobe, 5′-GGAAGATGGAGCCAAAGCGG-3′; they were then labeled using the PCR DIG probe synthesis kit (Roche Diagnostics). The labeled PCR products correspond to the nucleotides +3700–+4104 and +73 and +972 of the *FLO11* and *ACT1* open reading frames (ORFs), respectively. After hybridization, the probe-target hybrids were visualized as described in the digoxigenin (DIG) application manual (Roche Diagnostics).

RESULTS

***MSS11* is required for the regulation of invasive growth and *FLO11* expression by *FLO8*:** The effect of *MSS11* on invasive growth phenotypes and on *FLO11* transcription was investigated in a Σ1278b strain and compared to the effect of *FLO8* (Figure 1A). Previous studies showed

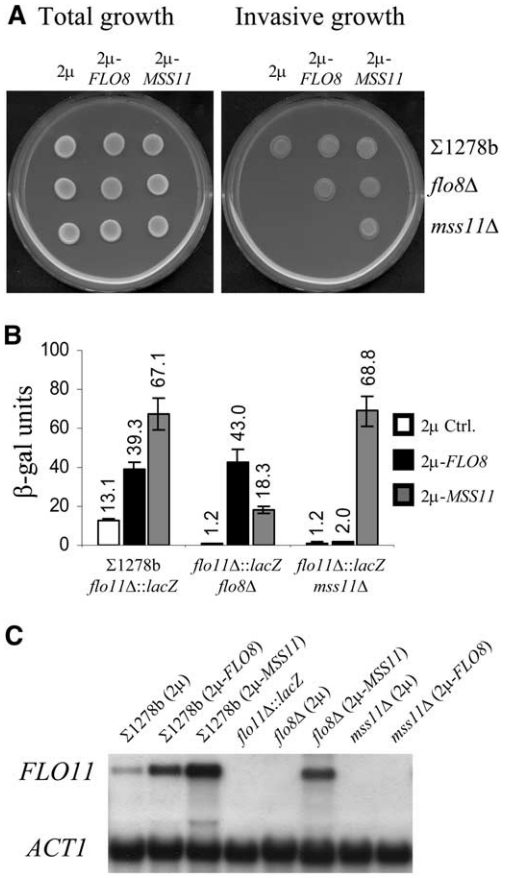

FIGURE 1.—*FLO8* requires *MSS11* for the regulation of invasive growth and P_{FLO1I}-*lacZ* expression. (A) Total growth (before washing) and invasive growth (cells observed after the plate surface was rubbed under running water) phenotypes of isogenic strains Σ1278b wild type, *flo8*Δ, and *mss11*Δ transformed with YEplac112 (2μ), YEplac112-FLO8 (2μ-*FLO8*), or YEplac112-MSS11 (2μ-*MSS11*) on low-glucose (0.2%) medium. Multiple copies of *FLO8* were insufficient to suppress the invasion defect of *mss11*Δ, while 2μ-*MSS11* restored the phenotype of *flo8*Δ. (B) β-Galactosidase activity of isogenic Σ1278b*flo11*Δ::*lacZ* strains grown in SCD (2% glucose) liquid medium. The genomic ORF of *FLO11* was replaced with *lacZ* in the wild-type Σ1278b strain (described in VAN DYK *et al.* 2003), and *FLO8* and *MSS11* deletions were subsequently created in the Σ1278b*flo11*Δ::*lacZ* strain. β-Galactosidase activity is expressed in Miller units (AUSUBEL *et al.* 1994). (C) Northern blot showing the mRNA transcript levels of *FLO11* and *ACT1* in the Σ1278b strains used for the invasive growth plate assay (A). The Σ1278b*flo11*Δ::*lacZ* reporter strain served as a negative control and *ACT1* was used as the internal standard.

that multiple copies of *MSS11* were able to suppress the phenotypic effect of a *FLO8* deletion in a starch-degrading ISP20 strain (GAGIANO *et al.* 1999a). The data presented in Figure 1A show that the same observations can be made in the Σ1278b genetic background. Deletions of either *FLO8* or *MSS11* resulted in loss of ability of Σ1278b to grow invasively. However, while high-copy expression of *MSS11* restored the agar-invasion defect of the *flo8*Δ mutant, the 2μ-*FLO8* plasmid was unable to suppress the invasive growth defect displayed by *mss11*Δ.

To assess whether this effect was directly linked to the transcriptional regulation of *FLO11*, strains in which the endogenous *FLO11* open reading frame had been replaced with a *lacZ* ORF were used (VAN DYK *et al.* 2003). The P_{FLO1I}-*lacZ* expression data presented in Figure 1B are consistent with the invasive growth data (Figure 1A). Compared to the wild type (which shows 13.1 units of β-galactosidase activity), both *flo8*Δ and *mss11*Δ exhibited an 11-fold reduction (1.2 units) in β-galactosidase activity. Introduction of the 2μ-*FLO8* or 2μ-*MSS11* plasmids into the Σ1278b*flo11*Δ::*lacZ* strain led to a 3-fold (from 13.1 to 39.3 units) and a five-fold (from 13.1 to 67.1 units) increase, respectively, and restored *lacZ* expression in the corresponding *flo8*Δ and *mss11*Δ strains. However, when multiple copies of *FLO8* were assessed in the *mss11*Δ strain, the low *lacZ* expression levels remained unaffected, while 2μ-*MSS11* in the *flo8*Δ mutant resulted in a 15-fold induction and a higher *FLO11* expression level than that in the wild-type control strain. These results were further verified by Northern analysis (Figure 1C). The data show an excellent correlation between the concentrations of *FLO11* mRNA and the *lacZ* expression data for all the investigated strains.

***sfl1*Δ-dependent derepression of *FLO11* requires *MSS11* and *FLO8*:** Previous investigations indicated that the activity of the Flo8p transcriptional activator is controlled by the cAMP-dependent PKA pathway, which appears to control *FLO11* transcription by regulating the antagonistic activities of Flo8p and Sfl1p (PAN and HEITMAN 2002). The authors' data suggested that *FLO8* was required for Sfl1p-dependent derepression of *FLO11*. Since our results indicated that Flo8p function depends on the presence of *MSS11*, we assessed whether *FLO11* derepression, caused by a *SFL1* deletion, was also dependent on *MSS11* through phenotype analysis (Figure 2A), P_{FLO1I}-*lacZ* expression (Figure 2, B and C), and Northern blot analysis (Figure 2D).

Deletion of *SFL1* significantly enhanced invasive growth (Figure 2A) and led to very high levels of P_{FLO1I}-*lacZ* expression (430.3 units; Figure 2B). The data show that *MSS11* is required to the same degree as *FLO8* for the hyperinvasive phenotype and the high *FLO11* expression levels displayed by *sfl1*Δ mutant. P_{FLO1I}-*lacZ*-dependent β-galactosidase activity in the *sfl1*Δ*mss11*Δ strain (6.4 units) is indeed similar to the activity found in the *sfl1*Δ*flo8*Δ strain (5.9 units).

However, multiple copies of either *FLO8* or *MSS11* were not able to significantly enhance the high β-galactosidase expression levels in the *sfl1*Δ genetic background. This may suggest that these genes are dependent on the presence of Sfl1p to activate *FLO11* and that Mss11p might act within the cAMP-dependent transcription complex. However, when the 2μ-*MSS11* was transformed into the *flo8*Δ*sfl1*Δ double-mutant strain, an invasive growth phenotype similar to the wild-type strain was observed (Figure 2A). The strain also displayed P_{FLO1I}-lacZ-dependent β-galactosidase expression

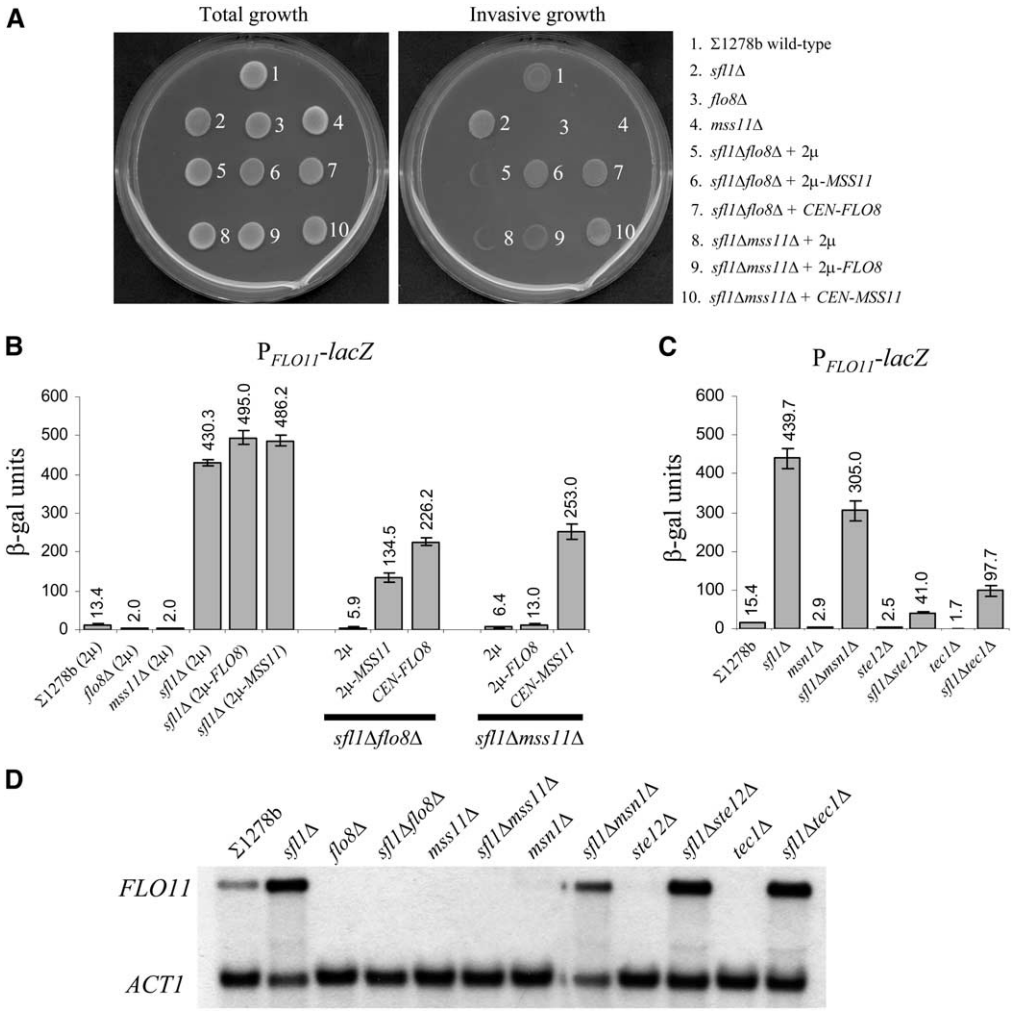

FIGURE 2.—Assessment of *MSS11* and *FLO8* function in strains lacking *SFL1*. The strains listed in A are from a Σ1278b background. Low-copy (CEN) and high-copy (2μ) plasmids were used to either serve as positive controls (the low-copy YCplac22-FLO8 and YCplac22-MSS11 plasmids) or determine the effect of multiple copies (2μ-plasmid, YEplac112) of *FLO8* and *MSS11* on invasive growthin the *sfl1*Δ*flo8*Δ and *sfl1*Δ*mss11*Δ double mutants. *MSS11* (2μ) significantly enhanced agar invasion in *sfl1*Δ *flo8*Δ, whereas *FLO8* (2μ) only slightly increased invasion of *sfl1*Δ*mss11*Δ. (B) β-Galactosidase activities of the corresponding P_{FLO11}-*lacZ* strains as well as a *sfl1*Δ mutant carrying YEplac112-FLO8 or YEplac112-MSS11. The quantitative data are consistent with the phenotypes observed in A. (C) Deletions of *MSN1*, *STE12*, or *TEC1* in the Σ1278b*flo11*Δ::*lacZsfl1*Δ strain do not abolish derepression of *FLO11* in a *sfl1*Δ background. (D) Northern blot analysis of the transcript levels of *FLO11* and *ACT1* in the corresponding Σ1278b (*FLO11*) strains.

levels that were higher (134.5 units) than those observed in the wild-type strain transformed with the same plasmid (67.1 units; Figures 2B and 1B). Multiple copies of *FLO8*, on the other hand, only slightly enhanced the invasive growth phenotype of the *sfl1*Δ*mss11*Δ strain (Figure 2A) and resulted in a twofold increase in P_{FLO11}-*lacZ* expression (Figure 2B). Taken together, the data therefore suggest that Mss11p does not require either *FLO8* or *SFL1* to activate *FLO11*.

Since derepression of P_{FLO11}-*lacZ* in a *sfl1*Δ strain requires both *MSS11* and *FLO8*, other transcriptional activators of *FLO11* were also assessed for their ability to support Sfl1p-dependent derepression. The data show that *MSN1*, *STE12*, and, as previously reported by PAN and HEITMAN (2002), *TEC1* appear to not be required for this purpose, since their absence in a *sfl1*Δ background continued to result in P_{FLO11}-*lacz* expression levels that are significantly above those found in the wild type, with 305, 41, and 97 units, respectively (Figure 2C).

All of the P_{FLO11}-*lacZ* data were further verified through Northern blot analysis (Figure 2D). Again, the mRNA levels confirmed the data generated through measurement of β-galactosidase activity, indicating that these data accurately reflected variations in mRNA concentration.

Deletion of *MSS11* blocks Ras2p- and Tpk2p-dependent regulation of *FLO11*: *TPK2* and $RAS2^{Val19}$, respectively, encode one of the catalytic subunits of the PKA complex and a hyperactive protein that activates the Kss1p-MAPK and PKA pathways (MÖSCH *et al.* 1996, 1999; KÜBLER *et al.* 1997; ROBERTSON and FINK 1998; PAN and HEITMAN 1999). These genes were included in the genetic analysis to further assess the relationship between the PKA pathway and *MSS11*.

As seen in Figure 3, A–C, multiple copies of *TPK2* enhanced the invasive growth phenotype and increased *FLO11* mRNA levels in the wild-type strain and P_{FLO11}-*lacZ* expression three-fold (from 13.1 to 41.5 units) in the P_{FLO11}-*lacZ* strain. The 2μ-*TPK2* plasmid was able to partially suppress the invasive growth defect of the *mss11*Δ and *flo8*Δ strains. However, this suppression was not due to increased *FLO11* transcription since both the P_{FLO11}-*lacZ*-driven β-galactosidase activity (Figure 3B) and the Northern blot (Figure 3C) indicated that *FLO11* transcription remained unaffected by *TPK2* expression levels in these strains. The data therefore suggest that

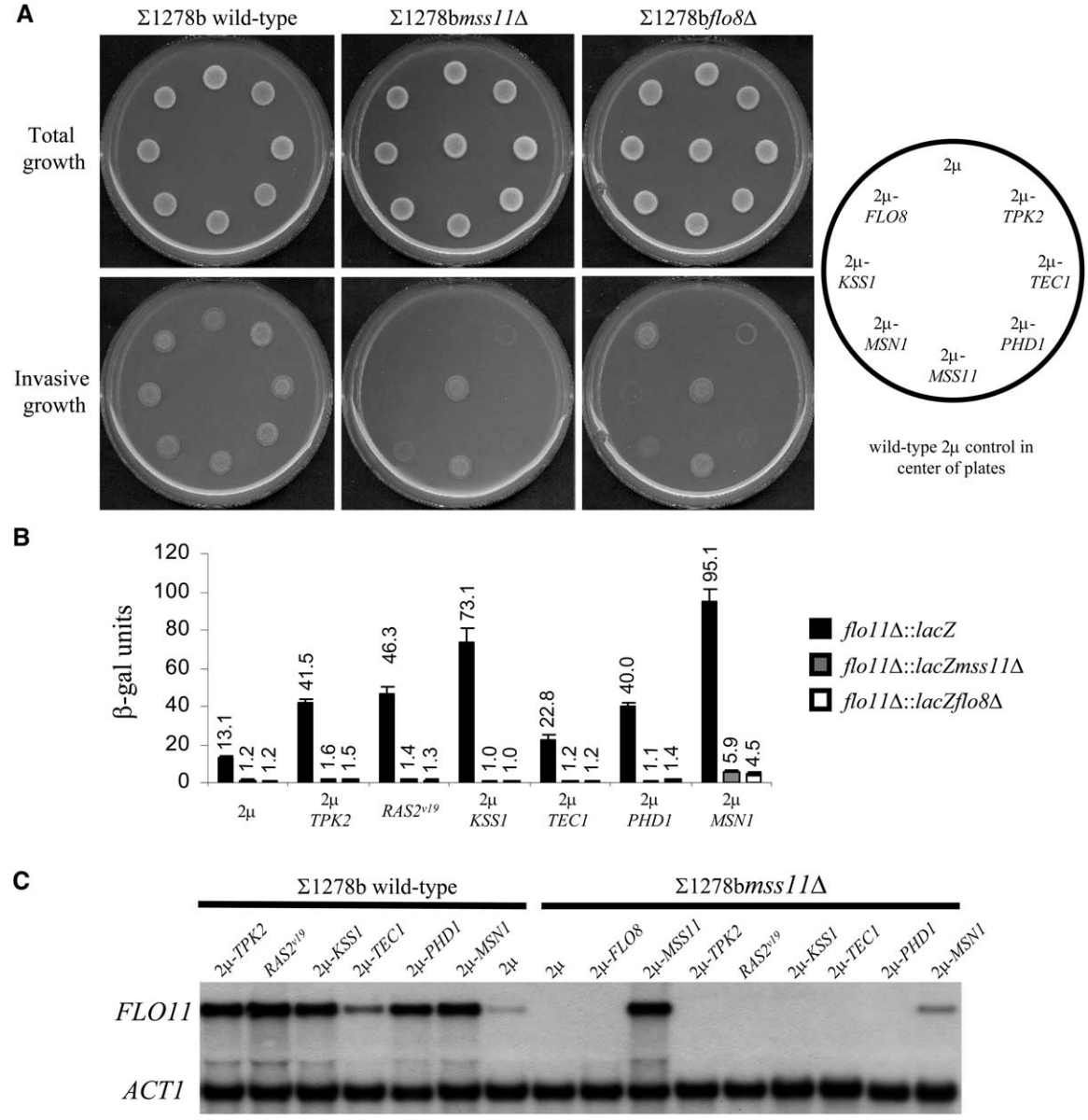

FIGURE 3.—*MSS11* and *FLO8* are required by the Kss1p-MAPK and PKA pathways and *PHD1*, but not by *MSN1*. (A) Invasive growth on 0.2% glucose medium of Σ1278b wild type and Σ1278b*mss11*Δ and Σ1278b*flo8*Δ strains, transformed with YEplac112 (2μ) without insert or with the same plasmid carrying a single copy of *FLO8*, *KSS1*, *MSN1*, *MSS11*, *PHD1*, *TEC1*, and *TPK2*. The central colony in the panels showing the phenotypes of Σ1278b*mss11*Δ and Σ1278b*flo8*Δ is the wild-type strain transformed with YEplac112. (B) β-Galactosidase activity of the corresponding P_{FLO11}-*lacZ* strains, grown in 2% glucose liquid medium. (C) *FLO11* and *ACT1* transcript levels of Σ1278b wild type and Σ1278b*mss11*Δ carrying the aforementioned genes in multiple copies or a single copy of the hyperactive $RAS2^{Val19}$ allele.

Tpk2p can regulate other genes that lead to invasive growth independently of *FLO11* and that this regulation does not require *FLO8* or *MSS11*. Similarly, the introduction of $RAS2^{Val19}$ resulted in a 3.5-fold increase of *FLO11* transcription in the wild-type strain, but failed to induce the β-galactosidase activity of the P_{FLO11}-*lacZ mss11*Δ strain (Figure 3B) or to increase mRNA levels in the *mss11*Δ strain (Figure 3C). To assess whether *MSS11* acted as a general suppressor of $RAS2^{Val19}$-dependent phenotypes, other phenotypes associated with the expression of $RAS2^{Val19}$, including very slow growth on nonfermentable carbon sources and reduced survival in stationary phase, were also assessed. These phenotypes, however, were unaffected by the deletion of *MSS11* (data not shown).

While these data show that the PKA pathway requires *MSS11* and *FLO8* to activate *FLO11* transcription, they do not exclude that a functional PKA pathway may be required for *MSS11*-dependent invasive growth and P_{FLO11}-*lacZ* expression. Deletion of *RAS2* and *GPA2* negatively affects filamentous growth presumably by diminishing the intracellular cAMP levels of the cell (KÜBLER

TABLE 3

Expression of P_{FLO11}-*lacZ* in Σ1278b mutant strains

Relevant genotype	Mean β-galactosidase activity (Miller units ±SD)		
	2μ	2μ-*MSS11*	2μ-*FLO8*
Σ1278b*flo11*Δ::*lacZ*	12.9 ± 0.8	70.7 ± 4.3	37.2 ± 3.7
*gpa2*Δ*ras2*Δ	2.8 ± 0.2	67.1 ± 0.9	15.8 ± 1.3
*tpk2*Δ	2.8 ± 0.1	22.3 ± 1.6	7.7 ± 0.4
*kss1*Δ	4.3 ± 0.4	50.8 ± 4.5	22.1 ± 3.3
*ste12*Δ	2.5 ± 0.1	16.0 ± 0.2	5.5 ± 0.4
*tec1*Δ	1.7 ± 0.2	26.2 ± 0.4	12.2 ± 1.6
*phd1*Δ	5.0 ± 0.6	63.2 ± 1.2	35.1 ± 4.0
*msn1*Δ	2.2 ± 0.2	28.4 ± 3.3	7.1 ± 0.5

The listed mutants are isogenic to the Σ1278b*flo11*Δ::*lacZ* reporter strain. Strains were transformed with YEplac112, YEplac112-MSS11, and YEplac112-FLO8 and assayed following growth in SCD (2% glucose) liquid medium (see MATERIALS AND METHODS). At least three transformants were assayed and the average β-galactosidase activity is presented. The error margins for the presented average data do not exceed 15%. The experiment was performed in triplicate and similar tendencies were observed.

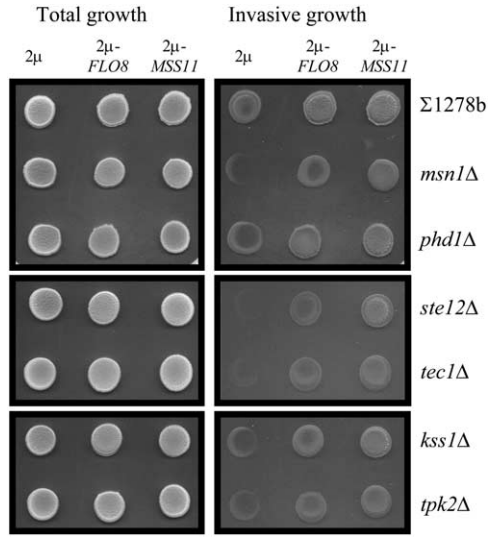

FIGURE 4.—*FLO8* and *MSS11* regulate invasive growth independently of *MSN1*, *PHD1*, and genes of the Kss1p-MAPK and cAMP-PKA pathways. Σ1278b wild type and the isogenic mutant strains *msn1*Δ, *phd1*Δ, *ste12*Δ, *tec1*Δ, *kss1*Δ, and *tpk2*Δ were transformed with YEplac112 (2μ), YEplac112-FLO8 (2μ-*FLO8*), and YEplac112-MSS11 (2μ-*MSS11*) and were subsequently spotted onto 0.2% glucose-containing medium. High-copy expression of *FLO8* and *MSS11* enhanced the invasiveness of all the tested strains.

et al. 1997; LORENZ and HEITMAN 1997). We therefore generated a *gpa2*Δ*ras2*Δ double mutant and a *tpk2*Δ single mutant. Deletion of *GPA2* and *RAS2* resulted in a fivefold decrease in P_{FLO11} *lacZ*-driven β-galactosidase activity (Table 3), but introduction of 2μ-*MSS11* increased the activity to 67.1 units, which is comparable to the 70.7 units observed in the wild-type strain transformed with the same plasmid. Deletion of *TPK2* also resulted in a fivefold decrease in β-galactosidase activity of the P_{FLO11}-*lacZ* strain, but 22.3 units of activity were obtained in the presence of the 2μ-*MSS11* plasmid. In all cases, multiple copies of *MSS11* induced invasive growth significantly (Figure 4).

Introduction of 2μ-*FLO8* into the wild-type P_{FLO11}-*lacZ* strain resulted in 37.2 units of β-galactosidase activity, while the *lacZ* expression levels of the *gpa2*Δ*ras2*Δ and *tpk2*Δ mutants were increased to only 15.8 and 7.7 units, respectively (Table 3). These significantly reduced expression levels are consistent with the direct regulation of Flo8p by the PKA pathway. Mss11p, on the other hand, appears to be much less affected by this pathway. Indeed, the reduced activation observed in the *TPK2* deletion strain can probably be explained by the effect of Tpk2p on Flo8p, since induction by 2μ-*MSS11* is similar in the *tpk2*Δ and *flo8*Δ deletion strain (Figure 1B and Table 3).

Deletion of *TPK2* did not prevent the 2μ-*FLO8* plasmid from enhancing invasive growth (Figure 4), suggesting either that other Tpks can phosphorylate Flo8p in the absence of Tpk2p or that gene dosage and not only PKA-mediated protein modification of Flo8p contributes to *FLO11* expression.

***MSS11* is required by the Kss1p-MAPK pathway:** Figure 3, A–C, shows that the agar-invasion phenotypes, P_{FLO11}-*lacZ* expression levels, and mRNA concentrations of the wild-type strains increased in the presence of multiple copies of *KSS1* and *TEC1*. The P_{FLO11}-*lacZ* expression levels increased five- and twofold in strains transformed with the 2μ-*KSS1* and 2μ-*TEC1* plasmids, respectively. The data show that these increases are entirely dependent on *MSS11* and *FLO8*, except for some invasion observed for the *flo8*Δ strain that carries multiple copies of *KSS1*. This invasive phenotype appears to be clearly independent of *FLO11*, since no increase in expression levels was observed for this gene. High-copy expression of *TEC1* did not suppress the *FLO8* mutation as had previously been reported (PAN and HEITMAN 1999). This discrepancy may be accounted for by differences in *TEC1* expression levels, since in the previous study *TEC1* expression was controlled by the *TDH1* promoter (PAN and HEITMAN 1999), while the episomal plasmid used in this study carries a copy of *TEC1* with its native promoter.

KSS1, *STE12*, or *TEC1* were deleted to ascertain whether the disruption of elements of the MAPK pathway would affect the effects of 2μ-*MSS11* and 2μ-*FLO8*. Compared to the control strain, Σ1278b*flo11*Δ::*lacZ*, the gene deletions decreased *lacZ* expression between three- and sevenfold (Table 3), which is reflected in decreased invasive growth phenotypes of the corresponding *FLO11*-carrying strains (Figure 4). With the introduction

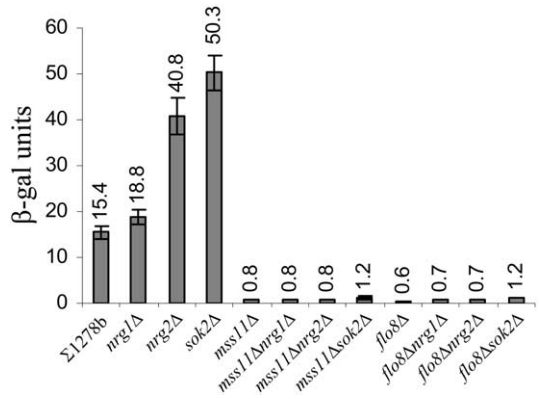

FIGURE 5.—P_{FLO11}-*lacZ* derepression is dependent on *MSS11* and *FLO8*. Deletion of *NRG1*, *NRG2*, or *SOK2* in the Σ1278b-*flo11*Δ::*lacZ* strain resulted in elevated β-galactosidase activity. Increases in activity were abolished when *MSS11* or *FLO8* was deleted in the corresponding repressor mutants.

of 2μ-*MSS11*, the *lacZ* expression levels increased in all three mutants, with the level of induction being at least similar to that observed for 2μ-*MSS11* in the wild-type strain. In the case of the 2μ-*FLO8* plasmid, similar results were observed. In all cases, the *FLO11* expression data and the level of invasiveness paralleled each other.

The dependency of the MAPK pathway elements on the presence of Mss11p appears specific to invasive growth and *FLO11* transcription. Indeed, the *mss11*Δ strain did not display any mating-associated defects, as does, for example, the *ste12*Δ strain (data not shown).

The hyperactive *STE11-4* allele and 2μ-*STE12* were also transformed into the Σ1278b and Σ1278b*flo11*Δ::*lacZ* strains, but severe growth defects were observed. Invasive growth and β-galactosidase activity did not increase in the transformed strains. To verify previous reports (MÖSCH *et al.* 1999; KÖHLER *et al.* 2002) and the functionality of the two constructs, their effect on *FRE*(*Ty*)*::lacZ* expression was assessed. Both plasmids significantly induced this reporter system, and 2μ-*STE12* also restored the ability of a sterile *ste12*Δ mutant to mate (results not shown). The data indicate that the regulation of the FRE element does not reflect *FLO11* transcription.

Relationships among *MSS11*, *FLO8*, and other genes encoding regulators of *FLO11*: It has been reported that the Phd1p-Sok2p activator and repressor module requires *FLO8* to regulate *FLO11* transcription (PAN and HEITMAN 2000). It is therefore not surprising that our data indicate that the same applies to the relation between this module and *MSS11*. As shown in Figure 3, A–C, introduction of 2μ-*PHD1* led to enhanced agar invasion, induced *lacZ* expression, and increased *FLO11* mRNA levels in the Σ1278b and Σ1278b*flo11*Δ::*lacZ* control strains, but not in the *mss11*Δ and *flo8*Δ mutants. Introduction of 2μ-*MSS11* and 2μ-*FLO8* in the *phd1*Δ strains, on the other hand, effectively suppressed the defects in agar invasion (Figure 4) and *lacZ* expression (Table 3). The data obtained with *sok2*Δ strains confirm the observations made for *PHD1* since no derepression is observed in these strains in the absence of *MSS11* or *FLO8* (Figure 5), while both genes activate *FLO11* efficiently in this strain (Table 4). Flo8p and Mss11p function does not depend on Phd1p and Sok2p, while these factors clearly require the presence of both.

To test whether Mss11p and Flo8p might activate gene transcription by antagonizing other identified transcriptional repressors of *FLO11*, the effect of multiple copies of *MSS11* and *FLO8* was assessed in the corresponding mutants. As can be seen in Table 4 and Figure 5, deletion of *NRG2* led to a significant increase in the activity of β-galactosidase, while deletion of *NRG1* resulted in a less severe increase. Expression of both 2μ-*MSS11* and 2μ-*FLO8* in these strains induced P_{FLO11}-*lacZ* expression, with a level of induction similar to that observed in the wild-type strain (Table 4). The absence of these repressors therefore did not prevent multiple copies of *FLO8* and *MSS11* from further enhancing the expression of the *lacZ* reporter gene.

To assess whether derepression in the absence of the repressors is dependent on functional *FLO8* and *MSS11* alleles, deletions of either *MSS11* or *FLO8* were combined with deletions of each of the repressor genes. The data in Figure 5 show that the deletion of *MSS11* and *FLO8* in the *nrg1*Δ and *nrg2*Δ strains abolished the increases in β-galactosidase activities observed in the single mutants. Since similar observations were made when *MSS11* or *FLO8* were deleted in the *sfl1*Δ background (Figure 2A), the data show that *mss11*Δ and *flo8*Δ block *FLO11* derepression in all the repressor mutants that were assessed.

Msn1p does not require *MSS11* or *FLO8* to activate *FLO11*: LAMBRECHTS *et al.* (1996a) previously reported on the ability of multiple copies of *MSN1* to activate *FLO11*. Our data show that 2μ-*MSN1* continued to lead to notable agar-invasion phenotypes in both the *mss11*Δ and the *flo8*Δ mutant strain (Figure 3A), as well as increased *lacZ* expression in the corresponding P_{FLO11}-*lacZ* strains (Figure 3B). Most importantly, the fold induction observed in the presence of multiple copies of *MSN1* is similar in the wild-type strain and the two mutant strains. 2μ-*MSS11* and 2μ-*FLO8*, on the other hand, also suppressed the defects of the Σ1278b*msn1*Δ and Σ1278b *flo11*Δ::*lacZmsn1*Δ strains (Figure 4 and Table 3), indicating that the regulatory role of Msn1p is independent from that of Mss11p and Flo8p. These observations are similar to those made for *RME1* overexpression and deletion (VAN DYK *et al.* 2003).

GAGIANO *et al.* (1999a,b) presented phenotype-based data suggesting that deletion of *MSS11* may block activation by Msn1p. The data here indicate that this apparent suppression was probably due to the very low basal *FLO11* expression levels in the *MSS11* deletion strain, which did not allow Msn1p-dependent induction of invasive growth. Indeed, our data show that the basal

TABLE 4

Expression of P_{FLO11}-*lacZ* in repressor mutants

	Mean β-galactosidase activity (Miller units ±SD)				
Plasmids	Σ1278b	*nrg1*Δ	*nrg2*Δ	*sok2*Δ	*sfl1*Δ
YEplac112	13.1 ± 0.6	16.8 ± 0.4	35.9 ± 4.5	44.7 ± 5.7	434.3 ± 3.9
YEplac112-MSS11	70.7 ± 4.3	96.4 ± 3.9	147.4 ± 4.8	185.5 ± 1.7	486.2 ± 14.1
YEplac112-FLO8	37.2 ± 3.7	36.4 ± 4.2	102.1 ± 7.4	133.5 ± 8.8	495.0 ± 17.7

FLO11 expression levels of the ISP strains are much lower than those in Σ1278b. It is therefore probable that in the ISP *mss11*Δ strains, 2μ-*MSN1* does not raise *FLO11* expression levels efficiently enough to result in invasive growth phenotypes, and hence the misinterpretation.

***SFL1* deletion induces invasive growth independently of *FLO11*:** Previous reports suggest that invasive growth is not solely dependent on *FLO11* (Palecek *et al.* 2000), but can also be induced by overexpression of *FIG2* and *FLO10* (Guo *et al.* 2000; Halme *et al.* 2004). Deletion of *SFL1* in a *flo11*Δ strain also results in agar invasion (Robertson and Fink 1998), which is attributed to the upregulation of *FLO10* (Guo *et al.* 2000; Halme *et al.* 2004). We similarly showed that 2μ-*MSS11* is able to induce invasive growth in a *flo11*Δ background (Gagiano *et al.* 1999b). These observations prompted us to assess the genetic relationships among *FLO8*, *SFL1*, and *MSS11* in *FLO11*-deleted strains.

As can be seen in Figure 6A, the *flo11*Δ and the double-deleted strains *flo11*Δ*flo8*Δ and *flo11*Δ*mss11*Δ, as expected, were unable to grow invasively, unlike the *flo11*Δ*sfl1*Δ strain, confirming the previous report of Guo *et al.* (2000). The residual invasive growth phenotype of *flo11*Δ*sfl1*Δ was abolished by deletions of *FLO8* and *MSS11* (Figure 6B). Multiple copies of *MSS11* significantly enhanced agar invasion in the *flo11*Δ*sfl1*Δ *flo8*Δ strain, but 2μ-*FLO8* did not suppress the defect of the *flo11*Δ*sfl1*Δ*mss11*Δ triple mutant. The genetic relationships among *FLO8*, *SFL1*, and *MSS11* are therefore not merely relevant to *FLO11* expression, but also to invasive growth in general, which implies that other target genes are also regulated by a mechanism that involves Flo8p, Sfl1p, and Mss11p.

The hyperflocculation phenotype of a *sfl1*Δ mutant depends on *MSS11* and *FLO8*, even in the absence of *FLO11*: The introduction of multiple copies of *FLO8* and *MSS11* in the S288c genetic background leads to flocculation (results not shown). This, however, is not the case in the Σ1278b strain, in which all the *FLO* genes, with the exception of *FLO11*, are repressed (Halme *et al.* 2004). In this strain, the role of *FLO8* and *MSS11* in flocculation became apparent only when *SFL1* was deleted. To further establish the involvement of a general regulatory mechanism/complex (Flo8p, Sfl1p, and Mss11p) in the regulation of *FLO11*-unrelated target genes, we assessed the flocculation phenotypes of *flo11*Δ strains.

The *flo11*Δ, *flo11*Δ*flo8*Δ, and *flo11*Δ*mss11*Δ mutants shown in Figure 6C exhibited similar sedimentation

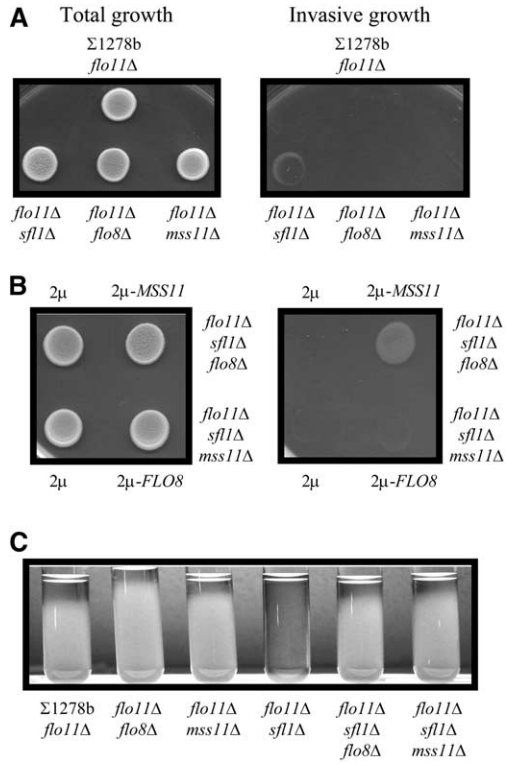

Figure 6.—*MSS11* and *FLO8* controls invasive growth and flocculation independently of *FLO11*. (A and B) Invasive growth phenotypes on 0.2% glucose medium of *FLO11* mutant strains from the Σ1278b genetic background. Deletion of *SFL1* in Σ1278b*flo11*Δ restored agar invasion (A). This phenotype of Σ1278b*flo11*Δ*sfl1*Δ was abolished when *FLO8* or *MSS11* was deleted (B). YEplac112-MSS11 suppressed the invasion defect of Σ1278b*flo11*Δ*sfl1*Δ*flo8*Δ, but YEplac112-FLO8 was unable to complement the Σ1278b*flo11*Δ*sfl1*Δ*mss11*Δ defect. (C) Flocculation phenotypes of the strains used in A and B. Deletion of *SFL1* in Σ1278b*flo11*Δ resulted in flocculation that is dependent on *FLO8* and *MSS11*. Single colonies were inoculated in 5 ml YPED liquid media and allowed to grow for 48 hr at 30° on a rotating wheel. The cultures were vortexed vigorously for 1 min to ensure that flocculating cells are resuspended and were subsequently placed on the bench to allow for cell aggregation and sedimentation. The cultures were photographed after 20 min.

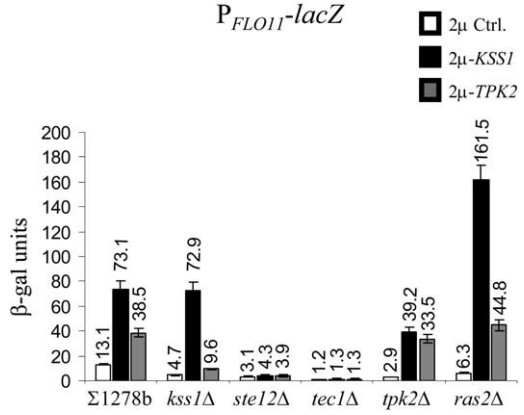

FIGURE 7.—Interaction between the Kss1p-MAPK and cAMP-dependent PKA pathways. YEplac112, YEplac112-KSS1, and YEplac112-TPK2 were introduced to Σ1278b*flo11*Δ::*lacZ* and to five isogenic mutants with deleted copies of *KSS1*, *STE12*, *TEC1*, *TPK2*, and *RAS2*, respectively.

phenotypes as can be deduced from the transparent sections of the corresponding cell cultures. The *flo11*Δ*sfl1*Δ strain, on the other hand, was highly flocculent and the cells settled immediately after agitation. Deletions of *FLO8* and *MSS11* abrogated the hyperflocculation phenotype of the *flo11*Δ*sfl1*Δ strain and the phenotypes of these strains were comparable to those of *flo11*Δ*flo8*Δ and *flo11*Δ*mss11*Δ strains. Mss11p and Flo8p therefore appeared to regulate flocculation independently of *FLO11* when *SFL1* is disrupted. The transcription of other *FLO* genes was shown to be under the control of Sfl1p and Flo8p (KOBAYASHI *et al.* 1999; GUO *et al.* 2000; HALME *et al.* 2004), but our genetic data strongly suggest that Mss11p is also required for this regulation.

Interaction between the Kss1p-MAPK and PKA pathways: High-copy expression of *TPK1*, *TPK2*, and *TPK3* was previously shown to stimulate *FRE*(*Ty*)::*lacZ* expression in a *ras2*Δ mutant (MÖSCH *et al.* 1999). The elevated expression was dependent on *STE12* and *TEC1*, and it was therefore suggested that the Tpk subunits act on the Ste12p-Tec1p transcription factor to induce downstream target genes. This result implies that the Kss1p-MAPK and PKA pathways are interconnected at the level of specific transcription factors. In agreement with this report, we observed that multiple copies of *TPK2* induce P$_{FLO11}$-*lacZ* expression threefold (Figure 7) and that this induction was dependent on the presence of *STE12*, *TEC1*, and to a lesser extent *KSS1*, but not on *RAS2*. High-copy expression of *KSS1* in a *ras2*Δ strain led to increased P$_{FLO11}$-*lacZ*-dependent β-galactosidase activity (161.5 units), when compared to 73.1 units observed for the wild type.

The 5.6-fold increase in reporter gene activity conferred by 2μ-*KSS1* in the wild type was dependent on *STE12* and *TEC1*, a result that was anticipated since Kss1p is an upstream regulator of the Ste12p-Tec1p transcription factor. Reporter gene activity in a strain carrying 2μ-*KSS1* was also dependent on *FLO8* for the induction of P$_{FLO11}$-*lacZ* (Figure 3B), suggesting that the Kss1p-MAPK and PKA pathways are interdependent. On the other hand, Kss1p activity was not dependent on a functional copy of *TPK2*, since a 13.5-fold increase in β-galactosidase activity was obtained when 2μ-*KSS1* was introduced into the P$_{FLO11}$-*lacZ tpk2*Δ strain.

Mss11p is still functional in mutants deleted for several activators: Yeast strains carrying one of the three glucoamylase-encoding *STA* genes are able to utilize starch as a sole carbon source. The *STA1-3* genes and *FLO11* have almost identical promoters and are therefore controlled by a similar set of transcription factors, which include Mss11p, Flo8p, Msn1p, Ste12p, Tec1p, Nrg1p, Sfl1p, and Sok2p (GAGIANO *et al.* 1999a,b; PARK *et al.* 1999; VAN DYK *et al.* 2003; KIM *et al.* 2004). Multiple deletion mutants, including strains *msn1*Δ*ste12*Δ*tec1*Δ and *flo8*Δ*msn1*Δ*ste12*Δ*tec1*Δ, were generated in the *STA2*-bearing ISP15 genetic background to assess whether our data regarding the ability of Mss11p to activate transcription in mutants deleted for individual activator genes may be the result of multiple interactions of the protein with several positive regulators.

As can be seen in Figure 8, A and B, both triple and quadruple mutants were able to grow on starch-containing SCS medium, but displayed significantly reduced glucoamylase activity. The quadruple mutant in particular displays a severe growth defect on this medium, whereas growth on glucose-containing medium was unaffected. High-copy expression of *FLO8* and *MSS11* suppressed the starch degradation defect of the triple mutant (Figure 8C), while introduction of 2μ-*MSS11* in the quadruple mutant restored the strain's ability to degrade starch and also suppressed the severe growth defect (Figure 8B).

We also assessed whether Mss11p activity is linked to glucose repression in the same genetic background. The quadruple mutant was unable to grow invasively on media containing either 2% glucose or 3% glycerol and ethanol (Figure 8C). However, agar invasion was restored under both conditions with the introduction of 2μ-*MSS11*, and similar data were obtained when invasive growth phenotypes were assessed for the quadruple mutant on medium containing limited amounts of nitrogen (Figure 8C).

DISCUSSION

Mss11p is a central element in the regulation of invasive growth: The results presented here strongly suggest a central role for Mss11p in the regulation of *FLO11* expression and invasive growth. At least four arguments can be made in support of this hypothesis:

1. The deletion of *MSS11* completely suppressed the activation of *FLO11* by hyperactive alleles and multiple copies of genes encoding components of the nutrient-responsive MAP kinase cascade and of the cAMP-signaling pathway that activate *FLO11* in the wild type.
2. Similarly, multiple copies of genes encoding transcrip-

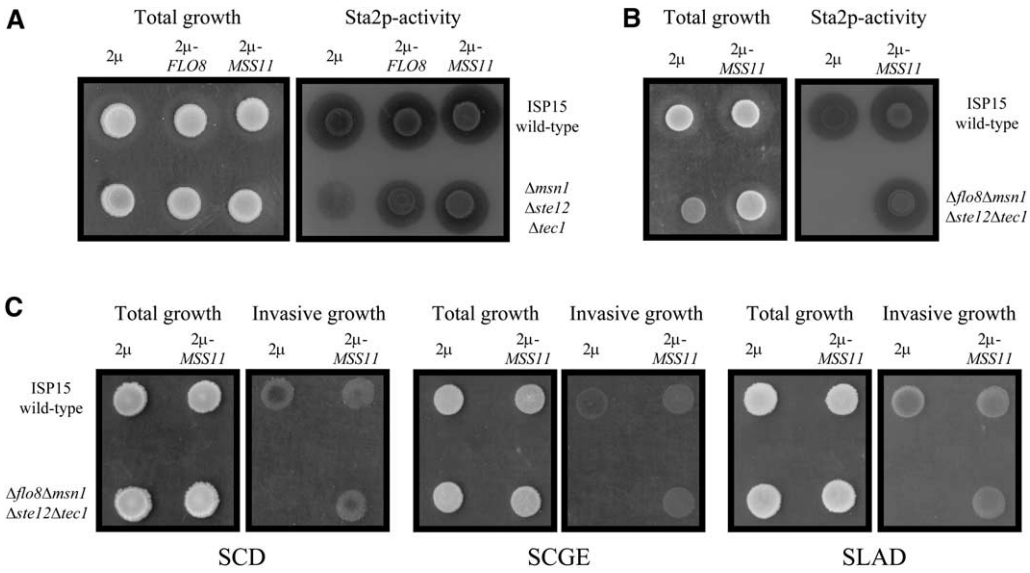

FIGURE 8.—Mss11p regulates starch degradation and invasive growth under different nutritional conditions. Total growth and starch degradation phenotypes of (A) ISP15 wild type and ISP15 *msn1*Δ*ste12*Δ*tec1*Δ and (B) ISP15*flo8*Δ*msn1*Δ*ste12*Δ*tec1*Δ, grown on starch-containing medium at 30° for 6 days. Transparent zones are indicative of Sta2p-glucoamylase activity. YEplac112-FLO8 and YEplac112-MSS11 restored Sta2p-glucoamylase activity in the triple mutant (A), while the 2μ-plasmid carrying *MSS11* also restored growth and starch degradation phenotypes in the quadruple mutant (B). (C) Invasive growth phenotypes of ISP15 wild type and the quadruple mutant on SCD (glucose repressed), SCGE (glucose derepressed), and SLAD (limited nitrogen) media.

tional activators, including *FLO8*, *TEC1*, and *PHD1*, were no longer able to increase *FLO11* expression in the *mss11*Δ background.

3. In the same *mss11*Δ background, no derepression of *FLO11* was observed in strains with deletions of the previously identified *FLO11* repressor-encoding genes *NRG1*, *NRG2*, *SOK2*, and *SFL1*.
4. Multiple copies of *MSS11* activated *FLO11* expression, even in the absence of the above-mentioned individual activators.

The same was true in strains with combinations of deletions in activator-encoding genes. In many regards, the genetic interactions displayed by *MSS11* are very similar to those observed for *FLO8*. However, *MSS11* was clearly epistatic to *FLO8*, since deletion of *MSS11* was not suppressed by multiple copies of *FLO8*, whereas multiple copies of *MSS11* suppressed the effects of a *FLO8* deletion efficiently. Multiple copies of *MSS11* were unable to further enhance *FLO11* transcription in a *sfl1*Δ strain. However, these data probably reflect saturation of the transcription capacity of the promoter in this strain. Indeed, Mss11p is clearly not dependent on the presence of *SFL1*, since it efficiently activates transcription in a *flo8*Δ*sfl1*Δ double mutant.

***MSS11* also affects other genes involved in flocculation:** Our data show that Mss11p and Flo8p are required for Sfl1p-dependent flocculation, even in the absence of *FLO11*. Mss11p, similar to Flo8p and Sfl1p, is required for the expression of genes involved in flocculation. Microarray analysis using *mss11*Δ and *MSS11* multiple copy strains suggests that the dominant flocculation gene *FLO1* might be the specific target gene responsible for these phenotypes (our unpublished data), but additional confirmation of these data is required.

***MSS11* specifically affects flocculation and invasive growth-related phenotypes:** While *MSS11* is essential for *FLO11* expression and affects transcription of other flocculation genes, it does not appear to affect other cellular functions. Indeed, the *mss11*Δ strain displayed only *FLO11* and flocculation-related phenotypes. As reported previously, this strain does not present any morphological or growth defects in a range of conditions, including growth on various carbon or nitrogen sources (GAGIANO *et al.* 1999b). When compared to the wild-type strain, the *mss11*Δ strain did not display significant differences in its viability upon nutrient depletion and in its response to heat stress, osmotic shock, or salt toxicity (GAGIANO *et al.* 1999b and data not shown). Furthermore, while suppressing the effect of the cAMP/PKA pathway and of the MAP kinase cascade on *FLO11* expression, it does not affect any of the other phenotypes that are associated with these pathways. Indeed, a *RAS2*^{Val19} *mss11*Δ double mutant displays *RAS2*^{Val19} phenotypes with regard to viability under starvation conditions, growth on nonfermentable carbon sources, and glycogen accumulation (data not shown). Mating efficiency of *mss11*Δ strains is also similar to wild-type strains, suggesting that the gene does not affect the mating signaling pathway, which shares many elements with the nutrient-responsive MAP kinase cascade. Finally, the microarray analysis

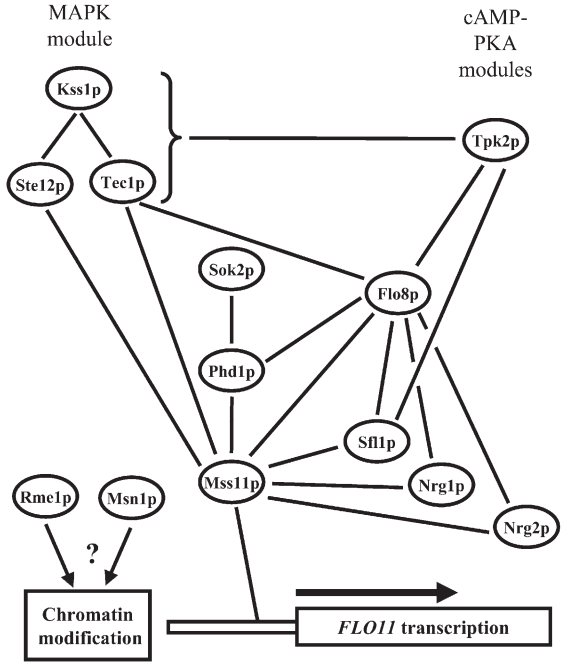

FIGURE 9.—A model summarizing the genetic interactions among Mss11p and other factors implicated in the transcriptional control of *FLO11*. The lines do not imply physical or functional interactions.

of strains deleted for *MSS11* or carrying *MSS11* on a multiple copy plasmid reveal only very few genes whose expression was significantly affected by modified *MSS11* expression levels. Of these genes, *FLO11* is consistently identified as the most significantly affected gene (our unpublished data).

Role of Mss11p in *FLO11* expression: The results of the genetic analysis presented in this article are summarized in Figure 9. The central position of Mss11p and the ability of Mss11p to activate transcription on its own (GAGIANO *et al.* 2003) strongly suggest a direct link between this protein and essential elements of the RNA polymerase II transcription machinery. However, various attempts through immunoprecipitation and several two-hybrid screens using Mss11p or nonactivating domains of Mss11p as bait have failed to yield any information regarding its interaction with other proteins. The genetic analysis suggests a close link of Mss11p with Flo8p and Sfl1p. This is supported by the fact that the only significant homology between Mss11p and any other protein consists of two small domains that are shared by Mss11p and Flo8p (GAGIANO *et al.* 2003). Furthermore, Mss11p appears to have the same range of target genes as Flo8p. However, direct attempts to show interactions between Flo8p and Mss11p in the two-hybrid system have failed (data not shown).

Mss11p does not appear to have any ortholog in other species, including in closely related organisms. No protein with significant homology to Mss11p was identified in the genomes of *Candida albicans*, *Cryptococcus neoformans*, and *Neurospora crassa*. This suggests that *MSS11* might be of relatively recent evolutionary origin. It also raises the question of the nature of interactions between Mss11p and other proteins. It has recently been suggested that proteins with strong and multiple protein interactions are better conserved evolutionarily and therefore have a higher probability of having well-conserved orthologs in other species (PAGEL *et al.* 2004).

In our analysis, of the previously identified genes that encode factors that positively affect *FLO11* transcription, only two were shown to be able to activate *FLO11* in the absence of *MSS11*, *MSN1*, and *RME1*. Indeed, multiple copies of these two genes result in similar fold increases of P_{FLO11}-*lacZ* expression in the wild-type strain and in the *mss11*Δ strain, although the expression levels in the latter strain remain low since they increase from a very low base. Both these factors have been linked to chromatin-related modes of action (COVITZ *et al.* 1994; SIDOROVA and BREEDEN 1999), while the factors whose role in *FLO11* expression is suppressed by *MSS11* have all been associated more directly with the RNA polymerase II transcriptional machinery. It is therefore possible that Msn1p and Rme1p activate *FLO11* transcription through more nonspecific means, for example, by modifying nucleosome positioning and by rendering a general promoter element such as the TATA box more accessible. It is a well-established fact that such elements can be recognized in a nonspecific way by the transcription machinery and can lead to transcriptional activation without the requirement for a specific activator (ROEDER 1996).

The regulation of *FLO11* recently has been shown to be subjected to epigenetic regulation (HALME *et al.* 2004). Sfl1p was identified as one of the essential elements within the epigenetic regulatory network. The suppression of Sfl1p- and Flo8p-dependent regulation of *FLO11* by Mss11p strongly suggests that Mss11p also plays a role in epigenetic regulation.

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