RESEARCH ARTICLE

Posttranscriptional regulation of *FLO11* upon amino acid starvation in *Saccharomyces cerevisiae*

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Abstract

Various starvation conditions cause adhesive growth of haploid cells or pseudohyphae formation of diploid cells of *Saccharomyces cerevisiae*. For the genetic Σ1278b background, these morphological changes depend on the expression of the gene encoding the cell wall glycoprotein Flo11p, which is increased during nutritional limitations. Deletion of the genes encoding the transcriptional coactivators Rsc1p or Gcn5p impairs *FLO11* transcription, which consequently leads to a loss of both haploid invasive growth and diploid pseudohyphae development upon glucose and nitrogen limitation, respectively. In contrast, amino acid starvation induces *FLO11*-dependent adhesive growth of the *rsc1*Δ and *gcn5*Δ strains although *FLO11* transcription remains very low. The double deletion strain *rsc1*Δ*flo11*Δ, however, does not grow adhesively, suggesting that the adhesion of the *rsc1*Δ strain at amino acid starvation is still *FLO11*-dependent. The $FLO11^{prom}$-*lacZ*-encoded β-galactosidase activities of the *rsc1*Δ and *gcn5*Δ mutant strains increase manifold upon amino acid starvation. It is therefore concluded that low levels of *FLO11* transcripts are essential and sufficient for derepression of *FLO11* expression and adhesive growth during amino acid starvation. A posttranscriptional control is assumed to be behind this phenomenon that permits the increased *FLO11* expression from low *FLO11* transcript abundances.

Introduction

Starvation for nutrients triggers a variety of responses to keep cells alive. Nutrient sensing and adjustment of gene expression are essential to adapt to changing environmental conditions. *Saccharomyces cerevisiae* transmits e.g. information of utilizable glucose by two central signal transduction pathways, namely the Ras/Gpa2p/cyclic AMP (cAMP)/protein kinase A (PKA) and the Snf3p/Rgt2p/Yck pathway. Subsequent adjustment of cellular metabolism and gene expression are achieved by activating as well as repressing cellular processes (reviewed in Santangelo, 2006). During glucose limitation, the Snf1p/AMP-activated protein kinase family of protein kinases plays an essential role in the transcriptional and metabolic carbon catabolite repression and the adaptation to low carbon supply.

Several cellular systems regulate the use of nitrogen compounds in *S. cerevisiae* (Schneper *et al.*, 2004). Ammonium as a nitrogen source is sensed by the high-affinity ammonium permease Mep2p localized at the plasma membrane. Amino acids, both for incorporation into protein and as sources of nitrogen in biosynthesis, are internally sensed by the 'general control of amino acid biosynthesis' (gc) (Hinnebusch, 2005). The nitrogen discrimination pathway is utilized in the absence of favourable ammonium sources such as glutamine or ammonium switching to sources like proline and allantoin. Therefore, mainly the transcription factor Gln3p acts as the master regulator for the respective transcriptome modulation.

Amino acid starvation is sensed in *S. cerevisiae* intracellularly by the kinase Gcn2p recognizing uncharged tRNAs and subsequently phosphorylating the α-subunit of the translation initiation factor eIF2 (Hinnebusch, 2005). Cap-dependent initiation of translation is thereupon reduced and only specific mRNAs become translationally active under such conditions. The *GCN4* mRNA is a well-studied example for

Table 1. Yeast strains used in this study

Strain	Genotype	Source
RH2817	*MATα, ura3-52, trp1::hisG*	This study
RH2656	*MATa/α, ura3-52/ura3-52, trp1::hisG/TRP1*	Braus *et al.* (2003)
RH2661	*MATa/α, ura3-52/ura3-52, trp1::hisG/TRP1, Δflo11::kan^R/Δflo11::kan^R*	Braus *et al.* (2003)
RH2662	*MATa, ura3-52, Δflo11::kan^R*	Braus *et al.* (2003)
RH3184	*MATα, ura3-52, trp1::hisG, Δrsc1::kan^R*	This study
RH3186	*MATa/α, ura3-52/ura3-52, trp1::hisG/TRP1, leu2::hisG/LEU2, Δrsc1::kan^R/Δrsc1::kan^R*	This study
RH3187	*MATα, ura3-52, trp1::hisG, Δisw2::kan^R*	This study
RH3189	*MATa/α, ura3-52/ura3-52, trp1::hisG/TRP1, leu2::hisG/LEU2, Δisw2::kan^R/Δisw2::kan^R*	This study
RH3190	*MATα, ura3-52, trp1::hisG, Δgcn5::kan^R*	This study
RH3192	*MATa/α, ura3-52/ura3-52, trp1::hisG/TRP1, leu2::hisG/LEU2, Δgcn5::kan^R/Δgcn5::kan^R*	This study
RH3214	*MATα, ura3-52, trp1::hisG, Δhtz1::kan^R*	This study
RH3216	*MATa/α, ura3-52/ura3-52, trp1::hisG/TRP1, leu2::hisG/LEU2, Δhtz1::kan^R/Δhtz1::kan^R*	This study
RH3217	*MATα, ura3-52, trp1::hisG, Δssn6::kan^R*	This study
RH3219	*MATa/α, ura3-52/ura3-52, trp1::hisG/TRP1, leu2::hisG/LEU2, Δssn6::kan^R/Δssn6::kan^R*	This study
RH3220	*MATα, ura3-52, trp1::hisG, Δsfl1::kan^R*	This study
RH3222	*MATa/α, ura3-52/ura3-52, trp1::hisG/TRP1, leu2::hisG/LEU2, Δsfl1::kan^R/Δsfl1::kan^R*	This study
RH3265	*MATα, ura3-52, trp1::hisG, Δrsc1::kan^R, Δflo11:: NAT*	This study
RH3275	*MATa, ura3-52, Δflo11::NAT*	This study

its derepression during amino acid starvation. It encodes a transcriptional key regulator modulating large parts of the transcriptome to adjust cells to periods of compromised protein biosynthesis. The target of rapamycin (TOR) pathway is another nutrient sensing system controlling cell growth and proliferation by responding to both cellular nitrogen and amino acid supplies (reviewed in Dann & Thomas, 2006; De Virgilio & Loewith, 2006; Wullschleger *et al.*, 2006). TOR also regulates the gc by regulating the phosphorylation of a specific residue of Gcn2p that in turn determines eIF2α phosphorylation and translational activity.

Different nutrient sensing and signalling pathways have a strong impact on cellular differentiation of *S. cerevisiae*-like diploid pseudohyphae development or haploid adhesive growth (Pan *et al.*, 2000; Cutler *et al.*, 2001; Gagiano *et al.*, 2002; Braus *et al.*, 2003). Various limitations finally activate the transcription of the *FLO11* (also referred to as *MUC1*) gene through their respective signalling pathways. *FLO11* encodes a glycosylphosphatidylinositol-(GPI)-anchored cell wall adhesin that mediates tight cell–cell and cell–surface interactions (Lo & Dranginis, 1996). *FLO11* expression is essential for adhesive as well as for pseudohyphal growth (Lo & Dranginis, 1998), which is reflected by one of *S. cerevisiae*'s largest promoters and a complex transcriptional regulation (Gancedo, 2001).

Here, amino acid starvation-induced *FLO11* expression is compared with glucose or nitrogen starvation-induced *FLO11* expression and surprising differences were found. *Saccharomyces cerevisiae* strains lacking certain transcriptional coregulators as e.g. Rsc1p or Gcn5p are impaired in activating *FLO11* transcription and, therefore, also fail in adhesive growth and pseudohyphae development at glucose and nitrogen limitation, respectively. In contrast, during amino acid starvation, the same strains still show very low *FLO11* transcript levels but they nevertheless grow adhesively. The present data suggest an efficient posttranscriptional control for *FLO11* expression.

Materials and methods

Yeast strains and growth conditions

All the yeast strains used in this study and their genotypes are listed in Table 1. Strains are derivatives of the *S. cerevisiae* Σ1278b genetic strain background (Gimeno *et al.*, 1992). Deletion mutants were generated using the Euroscarf deletion collection's BY strain (Brachmann *et al.*, 1998) for PCR amplification of the respective deletion cassettes. Transformations were carried out using the lithium-acetate yeast transformation method (Ito *et al.*, 1983). Integrations were confirmed by Southern analysis (Southern, 1975) and/or analytical PCR. The *rsc1Δ/flo11Δ* double-mutant strain was constructed by mating RH3184 (*rsc1Δ::kanMX*) and RH3275 (*flo11Δ::NAT*) and following tetrad dissection.

Yeast strains were generally cultivated in YPD complete medium (1% yeast extract, 2% bacto peptone, 2% glucose) at 30 °C. For selection of gene disruption, 200 μg mL^{-1} geneticin/G418 was added. For wash tests, cells were grown on solid YPD medium (2% agar), and alternatively on synthetic minimal medium [0.15% yeast nitrogen base (YNB), 50 mM $(NH_4)_2SO_4$, 200 mM myo-inositol, 2% glucose] supplemented with the appropriate amino acids. 3-aminotriazol was added to a final concentration of 10 mM to induce histidine starvation. Low-ammonium medium

Table 2. Plasmids used in this study

Plasmid	Description	Source
pBIISK(+)	Commercial cloning vector	Stratagene, La Jolla
pME2632	pBIISK(+) with integrated deletion cassette of *RSC1* ORF (kan^R)	This study
pME2626	pBIISK(+) with integrated deletion cassette of *ISW2* ORF (kan^R)	This study
pME2628	pBIISK(+) with integrated deletion cassette of *GCN5* ORF (kan^R)	This study
pME2631	pBIISK(+) with integrated deletion cassette of *HTZ1* ORF (kan^R)	This study
pME2648	pBIISK(+) with integrated deletion cassette of *SSN6* ORF (kan^R)	This study
pME2653	pBIISK(+) with integrated deletion cassette of *SFL1* ORF (kan^R)	This study
B3782 (pME2063)	3 kb *FLO11* promoter fragment in YEp355	Rupp *et al*. (1999)
pME3008	3 kb EcoRI–HindIII $flo11^{prom}$ in pBIISK(+)	This study
pME3009	As pME3008 but *FLO11* uORF-ATG mutated to TTG	This study
pME3010	As B3782 but *FLO11* uORF-ATG mutated to TTG	This study

(SLAD; YNB medium containing 50 μM ammonium sulphate) was used to monitor pseudohyphal formation.

Plasmids

The plasmids used in this study are listed in Table 2. Deletion cassettes were amplified from the respective Euroscarf deletion strains (Brachmann *et al*., 1998), and inserted via TA-cloning (Zhou & Gomez-Sanchez, 2000) into pBlueskript II SK (Stratagene) using the restriction site EcoRV. An $FLO11^{prom}$-*lacZ* reporter with mutated upstream ORF (uORF) was generated by first transferring the EcoRI–HindIII 3-kb fragment of plasmid pME2063 comprising the wild-type *FLO11* promoter into pBlueskript II KS (+), yielding pME3008. Site-directed mutagenesis by PCR with oligonucleotides oHR3 (5′ ccgttctcttctgatgaggtaacctttac 3′) and oHR4 (5′ ggatttttgaggcctacaaaagtatattttaattagaaccacaacaagacgaggg 3′) resulted in a 420-bp product that changes the uORF start codon ATG to TTG. Using the BstEII and StuI restriction sites, this PCR product was cloned into pME3008, yielding pME3009. The mutated *FLO11* promoter was retransferred into the pME2063 backbone using EcoRI and HindIII restriction, yielding the $FLO11^{prom}$-*lacZ* reporter with mutated uORF in plasmid pME3010.

Genetic crosses, sporulation and tetrad dissection

Diploid homozygous *S. cerevisiae* strains were obtained by mating of haploid yeast strains that had been verified by Southern hybridization experiments. Mating, sporulation and tetrad dissection were performed according to Sherman (1991).

Adhesive growth and pseudohyphal formation assays

Adhesive growth of haploids and diploids was performed as described previously (Roberts & Fink, 1994). Cells were patched on the respective medium and incubated for 1–5 days at 30 °C. Plates were photographed to show total growth and washed under a stream of water. The remaining adhesive cells were documented by photographing the washed plate.

Pseudohyphal growth after growing 5 days on SLAD medium at 30 °C was viewed using an Axiovert microscope (Carl Zeiss, Jena, Germany), and photographed with a Kappa DX30 digital camera and the Kappa Image Base software (Kappa Opta-Electronics, Gleichen, Germany).

RNA analysis

Total RNAs were isolated from exponentially growing yeast cells according to the method of Cross & Tinkelenberg (1991). For Northern hybridizations, 20 μg of total RNA was separated on 1.4% agarose gels containing 3% formaldehyde and subsequently transferred onto nylon membranes by capillary blotting. *FLO11* and *ACT1* transcripts were detected using specific ^{32}P-radiolabelled DNA fragments with the Prime-It labelling kit from Stratagene (La Jolla). Signal intensities were visualized and quantified using a BAS-1500 phospho-imaging scanner (Fuji, Tokyo, Japan).

Quantification real-time PCR

Total RNA was isolated from yeast cells that were grown in YNB in the presence and absence of 3-AT. The quality of the RNA was checked using the Bioanalyzer 2100 from Agilent. Then, RNA samples were treated with DNAseI (Roche) at 37 °C for 20 min and, after checking the RNA quality again, cDNA synthesis was carried out using 1 μg RNA and the BioRad iScript cDNA Synthesis Kit according to the manufacturer's instructions. Additionally, the cDNA was purified via Qiagen gel extraction columns. Twenty nanograms of cDNA was used as a template for real-time PCR. The *FLO11* gene was correlated to two reference genes *ACT1* and *CDC28* for normalization. The primers were designed using the Primer3 program (available at http://primer3.sourceforge.net/). The following primers were used: 5′ CTGGTCCAAAA GATACCGTCCAAC 3′ and 5′ ATGCATATTCAGCGGC

ACTACCTT 3′ for *FLO11*; 5′ GAATTGAGAGTTGCCCCA-GAAGAA 3′ and 5′ AGAAACCAGCGTAAATTGGAACGA 3′ for *ACT1;* and 5′ CTTTTGGTGTTCCGTTGAGAGCTT 3′ and 5′ TCGATCTCACTATCGCCACTGAAG 3′ for *CDC28*.

The 16 μL real-time PCRs were performed on the iQ5 Cycler (BioRad) under the iQ SYBR Green Supermix Kit (BioRad). RT Q-PCR amplification was performed in triplicate using the following conditions: initial denaturation for 30 s at 98 °C, followed by 40 × cycle 2: denaturation (1 s at 94 °C), annealing (15 s at 64 °C) and extension (10 s at 72 °C). For melt curve data collection, these two cycles were followed by cycle 3 (15 s at 94 °C), cycle 4 (30 s at 64 °C) and cycle 5 (10 s at 59 °C; 67 × with increasing temperature after each cycle by 0.5 °C).

The specificity of the amplicons was confirmed by generating a melt curve profile of all amplified products. Gene expression was quantified on the basis of the threshold cycle values (C_t) at which a statistically significant increase in fluorescence intensity was first detected. The C_t-value for *FLO11* was normalized to both reference genes within each sample, and the normalized mean values were compared with the sample containing wild-type mRNA without 3AT treatment, generating $\Delta\Delta C_t$ values. The normalized level of target mRNA in the sample relative to the reference (wt-3AT) was expressed as $1 + E^{\Delta\Delta C_t}$, with E being the real PCR efficiency, which has been found to vary between 80% and 100%. The amplification efficiency was calculated using the slope of a standard curve using serial dilutions.

Transcriptional start site (TSS) determination

To determine the TSS of the *FLO11* gene, the method of *r*apid *a*mplification of *c*DNA *e*nds (5′ RACE) was applied. Therefore, the GeneRacer™ kit of Invitrogen (CA) was used comprising SuperScript™ III reverse transcriptase and the TOPO TA cloning® kit for sequencing. Before 5′ RACE, total RNA was treated with RNAse-free DNAse to prevent amplification from any genomic DNA. 5′ RACE experiments were performed according to the supplier's manual using oHR5 (5′ cgttgtaaccgtatagttggacggtacc 3′) and oHR6 (5′ gccaattgaagtctaagttgggacagcc 3′) as the *FLO11*-specific and as the nested *FLO11*-specific reverse primer, respectively. For each strain and condition used, at least six independent clones were sequenced for cDNA end determination to account for the possible selection of alternative TSSs.

β-Galactosidase assay

Strains carrying the *FLO11*^{prom}-*lacZ* reporter (B3782) or the uORF-mut-*flo11*^{prom}-*lacZ* reporter (pME3010) were grown in selective media to the exponential growth phase. Extracts were prepared and assayed for specific β-galactosidase activity as described previously (Rose & Botstein, 1983) and normalized to the total protein, resulting in specific enzyme activities (Bradford, 1976).

Results

Rsc1p and H2A.Z/Htz1p are chromatin-associated coregulators that affect *FLO11* transcription

To study the impact of transcriptional coregulators on starvation-induced adhesive growth on solid surfaces, yeast strains with different gene deletions were used to monitor the expression of the major adhesin-encoding gene in haploid Σ1278b cells, the *FLO11* gene. Among the deletion strains tested, the *rsc1*Δ, *gcn5*Δ, *isw2*Δ and *ssn6*Δ strains showed significantly reduced *FLO11* mRNA levels as determined by Northern hybridizations from total RNA preparations of liquid YPD-cultures (Fig. 1). The compromised *FLO11* transcription is especially pronounced in the *rsc1*Δ strain with only one-tenth of *FLO11* transcript abundance in comparison with the wild-type strain, normalized against the internal *ACT1* mRNA level. *FLO11* mRNA abundance is approximately halved in the *isw2*Δ and *ssn6*Δ strains, and reduced by one-third in the *gcn5*Δ strain. In contrast, the

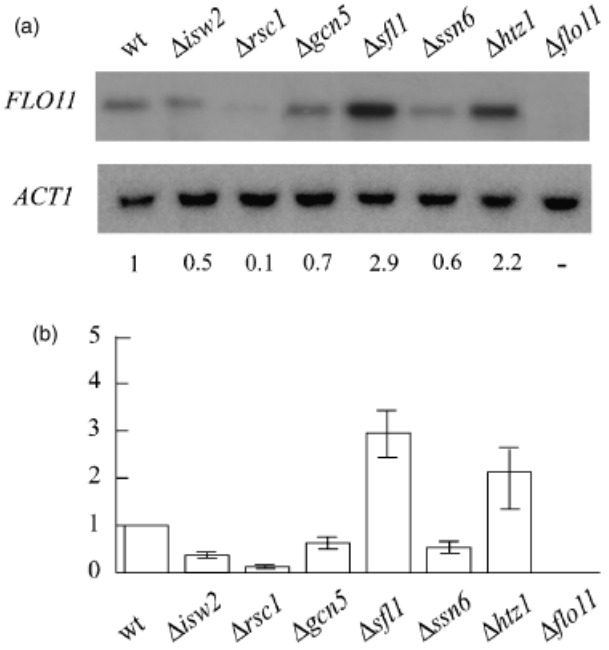

Fig. 1. (a) Northern hybridizations with total RNAs of haploid yeast strains with deletions in coregulator-encoding genes grown in liquid YPD. mRNA levels of *isw2*Δ (RH3187), *rsc1*Δ (RH3184), *gcn5*Δ (RH3190), *ssn6*Δ (RH3217), *sfl1*Δ (RH3220) and *htz1*Δ (RH3214) were compared. Twenty micrograms of total RNAs from each sample were used. ^{32}P-labelled *FLO11* and *ACT1* riboprobes were used for hybridization. Numbers given indicate the relative expression levels of *FLO11* mRNA normalized to the *ACT1* mRNA, averaged from six individual experiments with total RNA derived from three independent cultures. (b) Visualization of quantities by graph.

deletion of the *HTZ1* gene, encoding the histone variant H2A.Z/Htz1p, causes a twofold increase in *FLO11* mRNA levels (Fig. 1). This is similar to the threefold *FLO11* induction in the *sfl1*Δ strain lacking the *FLO11* repressor protein Sfl1p, which serves as a control here (Robertson & Fink, 1998). The *gcn5* deletion strain also serves as a control and has already been described as an *FLO11* compromising background (Laprade *et al*., 2002). Deletion of *SSN6* in DBY747 cells, however, has earlier been shown to induce *FLO11* transcription (Conlan & Tzamarias, 2001). DBY747-derivative strains are of the mating type **a**, whereas the present data derive from haploid Σ1278b strains of mating type α. It could be confirmed that a deletion of *SSN6* in Σ1278b cells of the **a** mating type also causes increased *FLO11* transcript levels by factor 1.5 (data not shown). This suggests that the effect of *SSN6* on *FLO11* transcription is mating type dependent.

In summary, these observations reveal two genes not yet described as players in *FLO11* expression: *RSC1* as an activator and *HTZ1* as a repressor gene for *FLO11* transcription.

RSC1 is essential for glucose limitation-induced adhesive growth and for nitrogen limitation-induced pseudohyphae development

The set of deletion strains with altered *FLO11* expression was then studied for adhesive growth after 5 days of growth on YPD agar medium by wash tests (Fig. 2a). Prolonged growth on rich medium results in the local decline of glucose concentrations, which triggers *FLO11* transcription that causes subsequent adhesive growth (Cullen *et al*., 2000). In contrast to the wild-type strain, the deletion strains *rsc1*Δ, *gcn5*Δ *isw2*Δ and *ssn6*Δ are no longer able to grow adhesively on agar upon glucose limitation (Fig. 2a, *flo11*Δ strain as negative control). This finding correlates with the reduced *FLO11* mRNA levels observed from liquid cultures as described before. The requirement of *GCN5* for *FLO11*-dependent adhesive growth also confirms the results published by Laprade *et al*. (2002). In contrast to the *isw2*Δ adhesion deficiency presented here, Trachtulcova *et al*. (2004) have shown earlier that the deletion of *ISW2* can induce adhesion. However, they also showed that their induction was independent of *FLO11*, which might explain the different results. For cells of the Σ1278b background, the nonadhesiveness of *isw2*Δ cells correlated to lowered *FLO11* mRNA abundance.

Severe de-repression of *FLO11* transcription is known to cause adhesive growth after short growth periods (1 day) on rich solid agar medium. Wild-type cells are nonadhesive after only 1 day of growth, whereas *FLO11*-derepressed cells of the *sfl1*Δ background adhere significantly after short growth periods (Robertson & Fink, 1998). It was found here that the *htz1*Δ strain is also characterized by hyperadhesive growth (Fig. 2b), which corresponds to the increased *FLO11* mRNA levels in this strain (Fig. 1).

Pseudohyphae formation of diploid *S. cerevisiae* cells during nitrogen limitation was then investigated on SLAD medium. Pseudohyphae formation was observable for *isw2*Δ/*isw2*Δ, *ssn6*Δ/*ssn6*Δ and *sfl1*Δ/*sfl1*Δ strains but not for the *rsc1*Δ/*rsc1*Δ and the *gcn5*Δ/*gcn5*Δ strains (Fig. 2c). Therefore, *RSC1* seems to play a similar important role for *FLO11*-dependent yeast morphogenesis as it is known for Gcn5p (Laprade *et al*., 2002). The different impact of *ISW2* and *SSN6* on haploid adhesion and diploid pseudohyphal growth might arise either from the different signal of nutrient limitation, finally activating *FLO11* (glucose vs. nitrogen) or from the difference in ploidity/gene copy number (haploid vs. diploid).

Although adhesive growth upon glucose limitation is not so strong for diploid cells compared with haploid cells, it can also be addressed by wash tests. The diploid set of strains revealed wild-type-like adhesive growth for the *isw2*Δ/*isw2*Δ and *ssn6*Δ/*ssn6*Δ strains but, again, prevented adhesion for the *rsc1*Δ/*rsc1*Δ and *gcn5*Δ/*gcn5*Δ strains (data not shown). This is in accordance to the observations described before for diploid pseudohyphal growth and corroborates the importance of *RSC1* and *GCN5* for *FLO11* expression.

Activated *FLO11* transcription during amino acid starvation depends on *RSC1*

Beyond glucose and nitrogen limitation, also amino acid starvation caused by the addition of histidine or tryptophan analogues is a strong signal for the induction of *FLO11* expression and adhesive growth (Braus *et al*., 2003). The set of deletion strains for *FLO11* transcript abundance at amino acid starvation was then analysed. Cells were therefore cultivated in liquid YNB medium in the presence of 10 mM 3-amino triazole (3-AT), a histidine analogon imposing false feedback inhibition, thereby causing histidine starvation. A fivefold increase in *FLO11* mRNA abundance of wild-type cells observed upon amino acid starvation (Fig. 3, wt+3-AT) confirms earlier data and serves as a control for induction of starvation (Braus *et al*., 2003). Even during amino acid starvation, the *rsc1*Δ, *isw2*Δ and *ssn6*Δ strains express low *FLO11* mRNA levels that are clearly below those of nonstarving wild-type cells (wt − 3-AT). Again, the *rsc1*Δ strain shows the severest decline in *FLO11* transcript abundance, giving rise to only very faint signals. The *gcn5*Δ and *htz1*Δ strains display slightly higher *FLO11* mRNA levels than the nonstarving wild-type cells. From the Northern hybridization data, the induction ratios between growth in the presence and absence of amino acid starvation for each strain were calculated from the relative *ACT1*-normalized *FLO11* intensities (relating to the wild-type strain at nonstarvation) and are given in Table 3. The wild-type strain

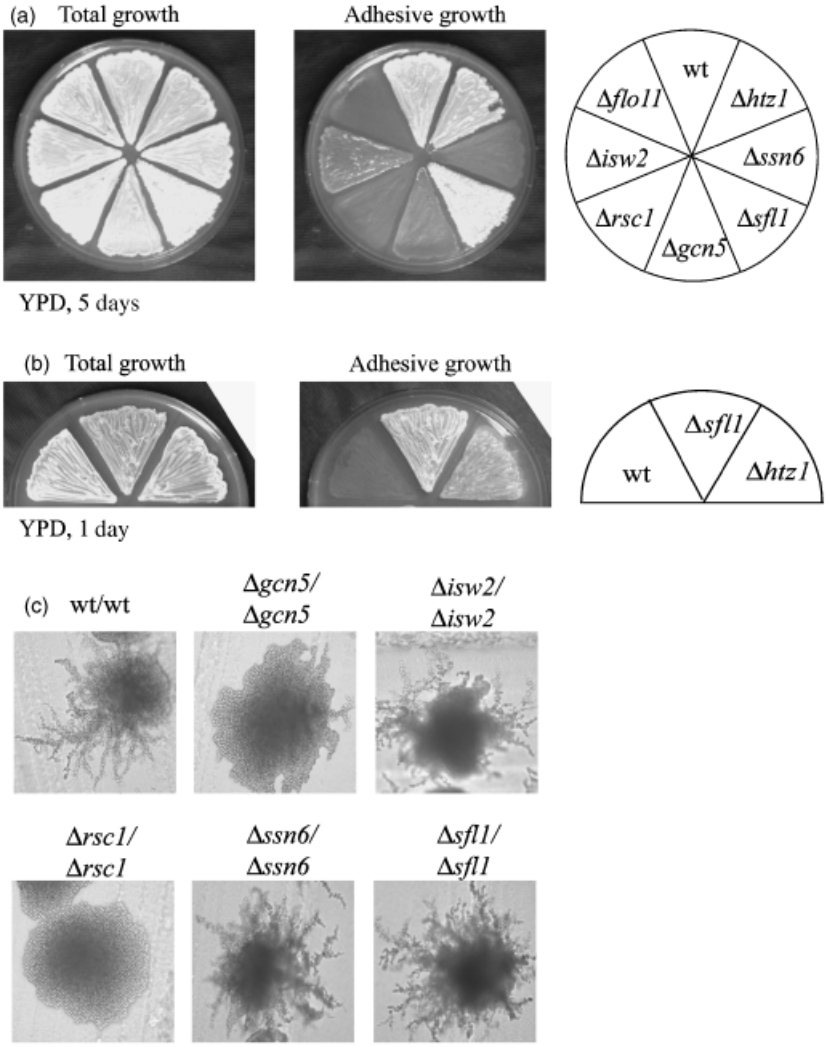

Fig. 2. Analysis of coregulator deletions for *FLO11*-dependent growth phenotypes. (a) Strains were analysed for adhesive growth and patched on YPD medium, incubated and photographed after 5 days of growth (total growth), washed and rephotographed to document adhesive growth. Nonadhesive yeast cells were washed away from the agar surface. The following strains were used: wt (RH2817) and *flo11*Δ (RH2662) as controls, *isw2*Δ (RH3187), *rsc1*Δ (RH3184), *gcn5*Δ (RH3190), *ssn6*Δ (RH3217), *sfl1*Δ (RH3220) and *htz1*Δ (RH3214). (b) Haploid *Saccharomyces cerevisiae* strains with increased *FLO11* mRNA levels were tested for early manifestation of adhesion. Haploid cells of *sfl1*Δ (RH3220), *htz1*Δ (RH3214) and the wt (RH2817) and *flo11*Δ (RH2662) were plated on YPD and cultivated for 1 day. Nonadhesive yeast cells were washed away. (c) Diploid homozygous *S. cerevisiae* strains were streaked out on nitrogen starvation (SLAD) plates to induce pseudohyphal growth and were incubated for 5 days at 30 °C. Colony phenotypes were microscopically examined. wt/wt (RH2656) as control, *isw2*Δ/*isw2*Δ (RH3189), *rsc1*Δ/*rsc1*Δ (RH3186), *gcn5*Δ/*gcn5*Δ (RH3192), *ssn6*Δ/*ssn6*Δ (RH3219) and *sfl1*Δ/*sfl1*Δ (RH3222), respectively, were tested. The *htz1*Δ/*htz1*Δ (RH3216) strain was not included in the assay due to its strong growth defect on SLAD medium.

and the *gcn5*Δ strain increase the *FLO11* mRNA abundance five- and threefold, respectively. However, measurable *FLO11*-induction upon amino acid starvation is prevented in the *rsc1*Δ, *isw2*Δ or *ssn6*Δ strains.

FLO11-dependent adhesive growth at amino acid starvation is independent from *RSC1*

So far, the presented *FLO11* mRNA abundance data obtained from Northern hybridizations correlate with the adhesiveness or the nonadhesiveness of the respective deletion strain. If this generally holds true, one would expect that the *isw2*Δ and the *ssn6*Δ strains, but especially the *rsc1*Δ strain, are also nonadhesive at amino acid starvation, as for these strains the *FLO11* mRNA levels remain very low even in the presence of 3-AT (Fig. 3). Surprisingly, the *rsc1*Δ, *isw2*Δ and *ssn6*Δ strains all showed strong adhesive growth on agar plates with 3-AT after only 1 day of growth (Fig. 4a). It was then tested whether this effect was still *FLO11* dependent. As an *rsc1*Δ*flo11*Δ double-deletion strain is not

adhesive anymore at amino acid starvation, at least low *FLO11* mRNA levels are required for adhesive growth in the *rsc1*Δ strain (Fig. 4b). Similarly, adhesion of diploid cells upon amino acid starvation was very pronounced for the *rsc1*Δ/*rsc1*Δ or *gcn5*Δ/*gcn5*Δ strains (data not shown).

In order to actually rule out a significant transcription-based *FLO11* induction, the low *FLO11* mRNA levels were double-checked in the *rsc1*Δ and *gcn5*Δ strains at amino acid starvation by quantitative PCR (qPCR). The resulting *FLO11* mRNA abundance data were normalized against *ACT1* and *CDC28*, and the numbers were then related to the uninduced wild-type strain (Fig. 5a). The severe *FLO11* mRNA decrease and its poor activation upon amino acid starvation for the *rsc1*Δ and *gcn5*Δ strains as shown by the Northern hybridizations are well confirmed by the qPCR experiments performed with total RNA isolations from new independent cultures. The results suggest that very low levels of *FLO11* mRNA are sufficient but essential to trigger adhesive growth during amino acid starvation, suggesting an efficient posttranscriptional control of *FLO11* expression.

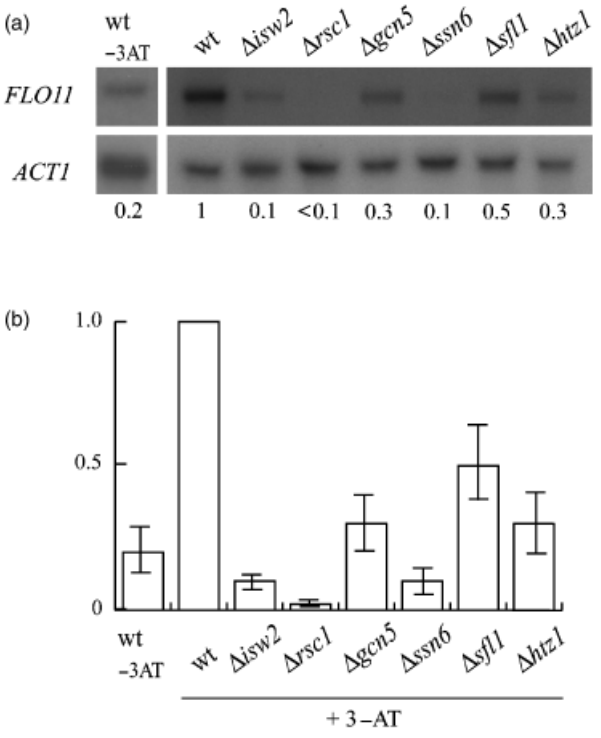

Fig. 3. (a) *FLO11* mRNA abundance during growth at amino acid starvation. Total RNAs were prepared from haploid yeast strains cultivated in liquid YNB supplemented with 10 mM 3-AT. Strains *isw2*Δ (RH3187), *rsc1*Δ (RH3184), *gcn5*Δ (RH3190), *ssn6*Δ (RH3217), *sfl1*Δ (RH3220), *htz1*Δ (RH3214) and the wt strain (RH2817) as control were analysed. Numbers given indicate the relative expression levels of *FLO11* mRNA normalized to the *ACT1* mRNA, averaged from six individual experiments with total RNA derived from three independent cultures. (b) Visualization of quantities by graph.

Amino acid starvation activates *FLO11*^{prom}-*lacZ* expression even in *rsc1*Δ and *gcn5*Δ strains

As a rigid cell wall protein with poor solubility in cell extracts, reliable quantitative assays for the accurate measurement of cellular Flo11 protein levels have, to the authors' knowledge, so far not been established. Alternatively, the β-galactosidase activities of an *FLO11*^{prom}-*lacZ* reporter allele were therefore determined. By virtue of an authentic *FLO11* promoter including its 5′-untranslated region (5′ UTR), the β-galactosidase readout mainly reflects the combined consequences of transcriptional and translational impact on *FLO11-lacZ* expression. The reporter fusion consists of a 3-kb *FLO11* promoter including its 5′-untranslated region (5′ UTR) together with the first three amino acid-encoding codons of the Flo11 protein, followed, in frame, by the *lacZ* encoding sequence. As described earlier (Braus *et al.*, 2003), the thus encoded β-galactosidase activities increase significantly upon amino acid starvation in Σ1278b cells by a factor of *c.* 34 (Fig. 5b). Expressing this reporter allele in the *rsc1*Δ strain at nonstarvation results in less than 10% β-galactosidase activity in comparison with the basal activity in wild-type cells. This was expected from the low *FLO11* mRNA present in Northern hybridizations and the qPCR experiment. However, starting from this low basal *FLO11*^{prom}-*lacZ* expression rate in *rsc1*Δ cells, amino acid starvation causes an *c.* 15-fold increase in β-galactosidase activity, and an *c.* 17-fold increase in *gcn5*Δ cells (Fig. 5b). This β-galactosidase activity increase is about 10-fold higher than the respective *FLO11* mRNA inductions measured by qPCR (Fig. 5). The absolute β-galactosidase activity of *rsc1*Δ cells at amino acid starvation is about threefold higher than that of wild-type cells under nonstarvation conditions, and it is about sevenfold higher in amino acid-starved *gcn5*Δ cells.

Table 3. Factor of transcriptional *FLO11* activation upon amino acid starvation according to the Northern hybridization data

		wt	*isw2*Δ	*rsc1*Δ	*gcn5*Δ	*sfl1*Δ	*ssn6*Δ	*htz1*Δ
Nonstarvation	*FLO11* (norm.)	0.2	0.1	< 0.1	0.1	0.6	0.1	0.4
Amino acid starvation		1	0.1	< 0.1	0.3	0.5	0.1	0.3
Factor	*FLO11* aa-st. *FLO11* n-st.	5	1	ND	3	0.8	1	0.8

aa-st., amino acid starvation; n-st., nonstarvation; norm., normalized against *ACT1* as an internal standard; ND, not determined because of too low intensities.

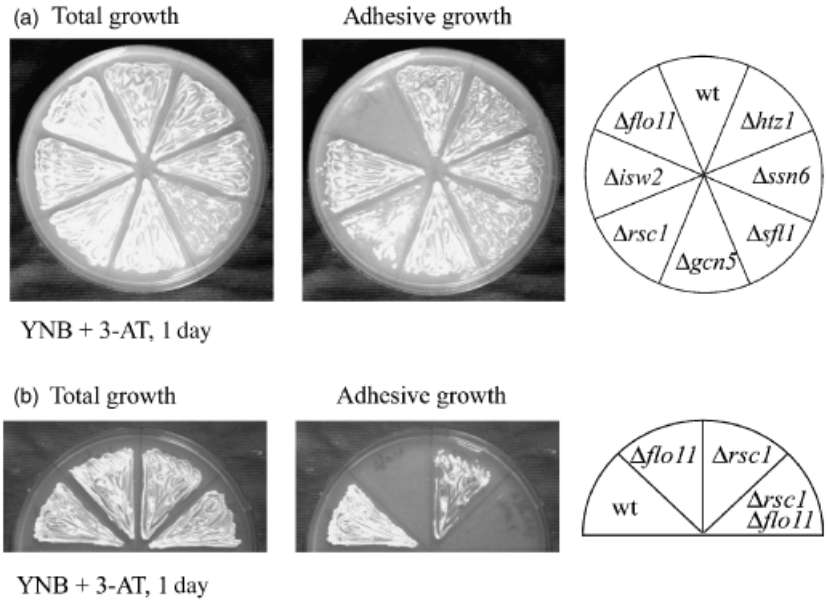

Fig. 4. Adhesive growth of the deletion strains at amino acid starvation. (a) Haploid yeast mutant strains were grown on YNB medium in the presence of 10 mM 3-AT to induce amino acid starvation. The plates were incubated at 30 °C for 1 day and photographed before (total growth) and after (adhesive growth) washing off the nonadhesive cells. As control, a wt (RH2817) and an *flo11* deletion strain (RH2662) were used. The tested deletion strains were *isw2*Δ (RH3187), *rsc1*Δ (RH3184), *gcn5*Δ (RH3190), *ssn6*Δ (RH3217), *sfl1*Δ (RH3220) and *htz1*Δ (RH3214). (b) Haploid yeast strains RH2817 (wt), as well as RH2662 (*flo11*Δ) as controls, RH3184 (*rsc1*Δ) and RH3265 (*rsc1*Δ*flo11*Δ) were patched on YNB medium containing 10 mM 3-AT. The plates were incubated at 30 °C for 1 day and photographed before (total growth) and after (adhesive growth) washing off the nonadhesive cells.

This induction reflects the *FLO11*-dependent adhesiveness of the *rsc1*Δ and *gcn5*Δ strains during growth at amino acid starvation despite low *FLO11* mRNA levels. Interestingly, also the opposite effect, which means high *FLO11* mRNA abundance but abolished adhesive growth, has been observed for a *gcn2*Δ strain earlier. Despite highly activated *FLO11* transcription in *gcn2*Δ cells at amino acid starvation, *FLO11*^{prom}-*lacZ* reporter cells show low β-galactosidase activity, and the *gcn2*Δ strain does not grow adhesively (Braus *et al.*, 2003). These earlier observations, together with the new data, support a posttranscriptional regulation of *FLO11* at amino acid starvation that might function at the level of the mRNA translation.

Mutation of a putative uORF in the *FLO11*-5′ UTR does not significantly change the *FLO11*^{prom}-*lacZ* reporter activity

cis-Elements in 5′ and 3′ UTRs are often involved in the posttranscriptional regulation of mRNAs. For the determination of potential regulatory elements in the 5′ UTR of the *FLO11* mRNA, its TSSs were determined. Using of the *r*apid *a*mplification of *c*DNA 5′ *e*nds approach (5′ RACE), *FLO11* cDNA was generated using a gene-specific reverse primer. PCR amplification of the *FLO11* cDNA and subsequent cloning of the PCR products, followed by DNA sequencing indicate *FLO11* cDNA 5′ ends at positions −7, −11, −13, −17, −28, −30 and −36 for RNA derived from wild-type cells, and at positions −13, −28 and −30 for RNA derived from *rsc1*Δ cells, both strains cultured in both YNB with and without 3-AT (Fig. 6a). Amino acid starvation does not cause a differential use of TSSs. Using a forward primer that anneals upstream of a putative uORF and a gene-specific reverse primer, also a PCR product arises from cDNA, for which subsequent DNA sequencing confirmed the specificity of the *FLO11* 5′ UTR (Fig. 6b, lanes 10–13) in comparison with the controls (lanes 6–9). This hints at a rather small mRNA subpopulation including the putative uORF, which was not found among the 5′ end RACE experiments of more than 24 independent clones. To exclude a possible impact of the putative uORF in *FLO11* expression at amino acid starvation, an *FLO11*^{prom}-*lacZ* reporter plasmid with a mutated uORF-start codon was analysed. β-Galactosidase activities encoded from the uORF-mutated reporter are almost similar to those encoded from the wild-type uORF-*FLO11*^{prom}-*lacZ* reporter in the absence or in the presence of starvation (Fig. 6c). Also, the expression of the uORF-mutated *lacZ*-reporter in the *rsc1*Δ strain is similar to that of the uORF-wild-type reporter. Therefore, the uORF itself does not seem to provide this posttranscriptional *FLO11* mRNA control.

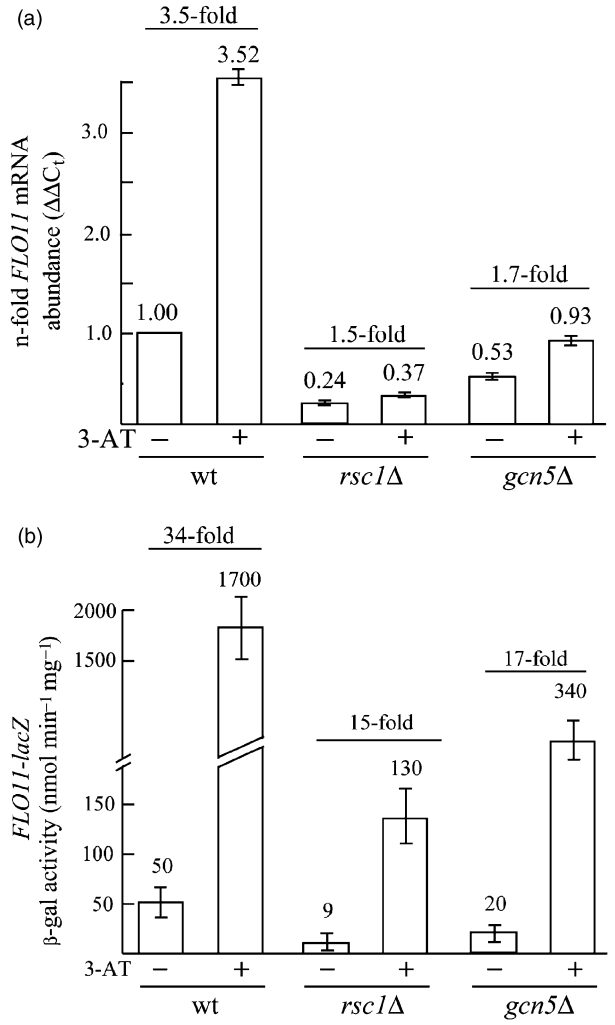

Fig. 5. *FLO11* expression upon 3-AT induction. (a) Relative *FLO11* mRNA abundances as determined by quantitative PCR were measured in haploid yeast strains RH2817 (wt), RH3184 (*rsc1*Δ) and RH3190 (*gcn5*Δ) and normalized against *ACT1* and *CDC28*. Experiments were performed from three independent cultures for each strain and condition. SD are indicated as error bars. $\Delta\Delta C_t$ is the ratio of the normalized threshold cycle values of the samples to be compared. (b) β-galactosidase activities encoded from the *FLO11-lacZ* reporter allele. The expression of *FLO11-lacZ* reporter gene was measured in haploid yeast strains RH2817 (wt), RH3184 (*rsc1*Δ) and RH3190 (*gcn5*Δ) carrying the reporter plasmid B3782. Specific β-galactosidase activities were measured in the absence or in the presence of 10 mM 3-AT. The mean values of six independent cultures are presented, and the SD are indicated as error bars. SD did not exceed 20%.

Discussion

Cellular morphological switches and differentiation programmes depend on the sensing and transduction of important nutritional signals, and subsequent reorganization of gene expression. The corresponding control mechanisms include a wide spectrum of means ranging from the regulation of transcription, of mRNA half-life and export, the regulation of mRNA translation, to posttranslational protein modifications and localizations. The expression of the *FLO11* gene is known to be essential for both haploid adhesive growth and diploid pseudohyphae formation in Σ1278b cells of *S. cerevisiae* during glucose and nitrogen limitation, respectively (Lo & Dranginis, 1998). So far, in the literature, these morphological changes always correlate with an increase in *FLO11* transcription upon those nutritional limitations (Gancedo, 2001; Verstrepen *et al.*, 2003). Also, the epigenetic heritage of an active *FLO11* expression status results in a higher level of gene transcription, and was shown to be dependent on the histone deacetylase activity of Hda1p (Halme *et al.*, 2004). The impact of internal repeats within the *FLO11* ORF and the variability of their number can influence the transcriptional activity of the gene and finally determine the level of *FLO11* mRNA (Voynov *et al.*, 2006).

Amino acid starvation induced by the addition of amino acid analogues strongly induces *FLO11* transcription, *FLO11*-dependent adhesion and *FLO11*^{prom}-*lacZ*-encoded β-galactosidase activities in wild-type Σ1278b cells. In this study, it is shown, that strains with low and almost uninducible *FLO11* mRNA levels, such as the *rsc1*Δ and the *gcn5*Δ strains, still grow adhesively during amino acid starvation. The *FLO11* dependency of this adhesion and the increased β-galactosidase activity encoded from an *FLO11*^{prom}-*lacZ* reporter allele support a posttranscriptional induction of *FLO11* expression upon amino acid starvation. Such a posttranscriptional regulation of *FLO11* could take place at the level of initiation of mRNA translation. The paradigm for regulated initiation of translation in yeast is the derepression of the *GCN4* mRNA during amino acid starvation as part of the *general control of amino acid biosyntheses* (gc) (Hinnebusch, 2005). The gc system senses uncharged tRNAs of the limited amino acid(s) through the sensor kinase Gcn2p, which subsequently phosphorylates the α-subunit of the elongation initiation factor eIF2, thereby changing the translational settings within starving cells by reducing the amount of ternary complex (TC) required for the cap-dependent initiation of translation. Unlike the bulk of repressed mRNAs during starvation, the *GCN4* mRNA is by that means translationally de-repressed, which is specifically conferred by four uORFs in the *GCN4* mRNA 5′ leader sequence. Although the present experiments argue for the existence of a subfraction of *FLO11* mRNAs including the putative uORF, the major transcription start sites exclude this uORF from the *FLO11* transcripts. Also, mutation of this uORF of an *FLO11*^{prom}-*lacZ* reporter construct gave no evidence for an exclusive role of the uORF in *FLO11* expression during amino acid starvation. Remarkably, a *gcn2*Δ strain, characterized by an abolished amino acid starvation response, shows neither *FLO11*-dependent adhesion nor significant *FLO11*^{prom}-*lacZ* expression despite very

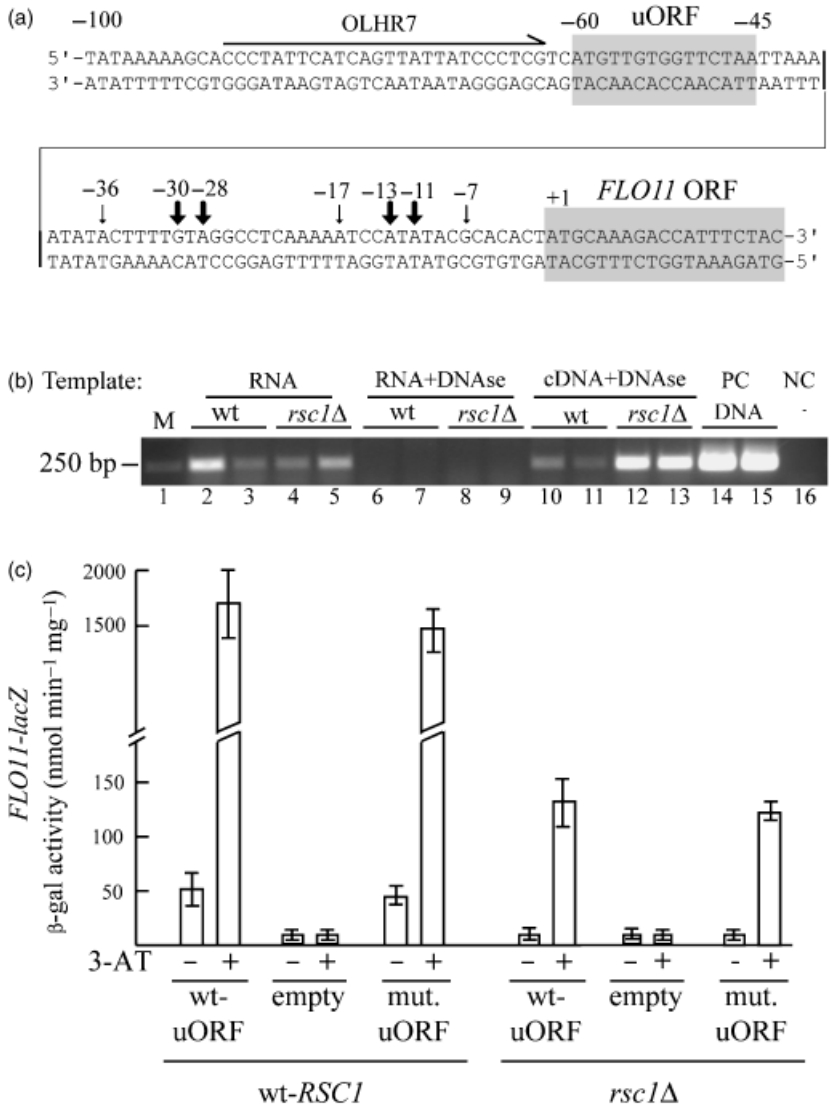

Fig. 6. Transcriptional start site (TSS) determination of the *FLO11* gene and analysis of the putative upstream ORF (uORF). (a) Arrows indicate TSSs as mapped by 5′ RACE relative to the actual ORF translational start site (+1). Bold arrows indicate cDNA 5′ ends found for at least six individual sequenced clones, and lean arrows represent 5′ ends found for only two individual sequenced clones. Oligonucleotide OLHR7 annealed immediately upstream of the putative uORF and was used as a forward primer for cDNA analysis shown in: (b) Each PCR was performed and loaded to the gel in replicate with a reverse primer annealing in the *FLO11* ORF. Lanes 2–5: Total mRNA as a template for testing the RNA preparation for genomic DNA contamination. Lanes 6–9: DNAse-treated total mRNA as a template to ensure the absence of any genomic DNA contamination. Lanes 10–13: cDNA of DNAse-treated total mRNA (used as PCR template in 6–9) as a template. Lanes 14 and 15: positive control with genomic DNA, lane 16: negative control without a template. (c) β-galactosidase activities encoded from a mutated *FLO11-lacZ* reporter allele. Activity was measured for the haploid yeast strains RH2817 (wt), as well as for strain RH3184 (*rsc1*Δ) carrying either the wild-type *FLO11-lacZ* reporter plasmid B3782 (wt-uORF), the plain reporter plasmid YEp355 (empty) or the uORF-mutated reporter plasmid pME3010 (mut. uORF). Specific β-galactosidase activities were measured in the absence or in the presence of 10 mM 3-AT. The mean values of six independent cultures are presented, and the SD are indicated as error bars. SD did not exceed 20%.

high *FLO11* mRNA levels at amino acid starvation (Braus *et al.*, 2003). This suggests that effective *FLO11* mRNA translation is favoured under conditions of generally discriminated cap-dependent mRNA translation that e.g. is amino acid starvation.

The expression of the mammalian cationic amino acid transporter Cat-1 is also elevated upon amino acid starvation. This is the consequence of both increased transcription and translation of the corresponding mRNA (Fernandez *et al.*, 2003). The 5′ UTR-uORF-containing Cat-1 mRNA is suggested to be translationally activated by a mechanism that stalls ribosomes at the uORF, which in turn leads to efficient IRES-mediated translation (Fernandez *et al.*, 2005). It has to be investigated whether mechanisms like ribosome

stalling or IRES apply with the posttranscriptional *FLO11* regulation and what regulatory elements in the mRNA 5′/3′ UTR and/or whether mRNA-binding proteins are involved. The recent publication of Gilbert *et al*. demonstrates that cap-independent translation of mRNAs of genes required for invasive growth (such as *GPR1*, *MSN1* or *FLO8*) is essential for starvation-induced differentiation in yeast (Gilbert *et al*., 2007). It is shown there that IRES elements in the 5′ UTR of these genes account for the cap-independent translation. The investigations of Gilbert *et al*. focus on upstream regulators of *FLO11* expression, whereas this study concentrates on the expression of the adhesin-encoding gene *FLO11* itself. Therefore, it is proposed that not only the upstream regulator genes of *FLO11* but also the *FLO11* mRNA can be subject to a posttranscriptional (and may be translational) regulation. It will be interesting to investigate whether riboswitches, which are an emerging field in the control of mRNA translation from bacteria to eukaryotes (Brantl, 2004; Eisenstein, 2005), might be involved in *FLO11* regulation. Considering cell–tissue adhesion or filamentous growth as initial steps in the pathogenicity of fungi as *Candida albicans* or *Candida glabrata*, such alternative regulation mechanisms might provide further clues for developing strategies to fight fungal infections.

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