STE11 disruption reveals the central role of a MAPK pathway in dimorphism and mating in *Yarrowia lipolytica*

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Abstract

Yarrowia lipolytica is a dimorphic fungus whose morphology is controlled by several factors such as pH and different compounds. To determine if the *STE11*-mitogen-activated protein kinase (MAPK) pathway plays a role in dimorphism of *Y. lipolytica*, we isolated the gene encoding a Mapkkk. The isolated gene (*STE11*) has an ORF of 2832 bp without introns, encoding a protein of 944 amino acids, with a theoretical M_r of 100.9 kDa, that exhibits high homology to fungal Mapkkks. Disruption of the *STE11* gene was achieved by the pop-in/pop-out procedure. Growth rate and response to osmotic stress or agents affecting wall integrity were unaffected in the deleted mutants, but they lost the capacity to mate and to grow in the mycelial form. Both alterations were reverted by transformation with the wild-type *STE11* gene. The *Y. lipolytica STE11* gene driven by two different promoters was unable to complement *Saccharomyces cerevisiae ste11*Δ mutants, although the gene was transcribed. Also, a wild-type *MAPKKK* gene from *Ustilago maydis* failed to complement *Y. lipolytica* Δ*ste11* mutants. Both negative results were attributed to a failure of the transgenic gene products to interact with the corresponding regulatory and scaffold proteins. This hypothesis was supported by the observation that a truncated version of the *U. maydis MAPKKK* gene reversed mating and dimorphic defects in the mutants. All these results demonstrate that the MAPK pathway is essential for both morphogenesis and mating in *Y. lipolytica*.

Introduction

Yarrowia lipolytica is a nonconventional yeast that is widely studied because of its applications in biotechnological processes that include the degradation of hydrophobic substrates such as alkanes, fats, oils and fatty acids, production of organic acids, and secretion of homologous and heterologous proteins (for reviews, see Barth & Gaillardin, 1996; Domínguez *et al*., 2000; Fickers *et al*., 2005). In addition, *Y. lipolytica* is a useful model for the study of fungal differentiation. In nature, *Y. lipolytica* grows as a mixture of budding yeasts and long hyphae, but under controlled conditions it may be grown as almost homogeneous populations of either yeast-like cells or branched hyphae (Rodríguez & Domínguez, 1984; Guevara-Olvera *et al*., 1993).

This dimorphic capacity, i.e. the ability to grow like a yeast or in the hyphal form, depending on the environmental conditions, is a property of a number of fungal species, independent of their taxonomic position. Dimorphism has important implications because of its association with pathogenicity in different fungi (see review in Vanden Bossche *et al*., 1993), and also because it is a phenomenon useful for understanding fungal cell differentiation. *Yarrowia lipolytica* is an attractive model organism for these studies, since its dimorphic transition can be easily and reproducibly induced under *in vitro* conditions, and is amenable both to classical genetic analysis (Barth & Gaillardin, 1996) and studies involving diverse molecular techniques (Barth & Gaillardin, 1996; Domínguez *et al*., 2000). Also worth considering is that the dimorphic transition of *Y. lipolytica* shares many similarities with that of *Candida albicans*, the most important human pathogenic fungus (see reviews in Liu, 2001, 2002; Dhillon *et al*., 2003), but it offers the advantage of having a sexual cycle that alternates haploid and diploid stages.

Different effectors involved in the dimorphic transition of *Y. lipolytica* have been described. These include the carbon source (Rodríguez & Domínguez, 1984), nitrogen source (Szabo & Stofaníková, 2002; Ruiz-Herrera & Sentandreu, 2002), blood serum (Kim *et al*., 2000; Domínguez *et al*.,

2000), citrate (Ruiz-Herrera & Sentandreu, 2002), and external pH (Ruiz-Herrera & Sentandreu, 2002). The mechanisms by which all these agents exert their effect and how their signals are transmitted to the nucleus remain unknown. In the dimorphic fungi *C. albicans* and *Ustilago maydis*, a mitogen-activated protein kinase (MAPK) pathway has been related to filamentous growth (Schultz *et al.*, 1997; Banuett, 1998; Martínez-Espinoza *et al.*, 2004). MAPK cascades are multifunctional signaling pathways that are evolutionarily well conserved in all eukaryotic cells. A typical MAPK module of phosphorylation involves three types of protein kinase in hierarchical order: a protein kinase (Mapk), a Mapk kinase (Mapkk) and a Mapkk kinase (Mapkkk). Sequential activation of these kinases by phosphorylation lies at the heart of signal transduction through these modules (reviewed in Banuett, 1998; Widmann *et al.*, 1999). In *Saccharomyces cerevisiae*, at least five MAPK pathways have been identified (reviewed in Lengeler *et al.*, 2000). These govern different phenomena in the life cycle of the yeast: mating and invasiveness in haploid strains, pseudohyphal development and spore formation in diploid strains, as well as maintenance of cell wall integrity and cell response to osmotic stress (for reviews see Heinisch *et al.*, 1999; Pan *et al.*, 2000; Saito & Tatebayashi, 2004; Bardwell, 2005). In *C. albicans*, several genes encoding components of the MAPK cascades, such as *CST20* and *HST7*, have been shown to be involved in the regulation of the dimorphic transition (Leberer *et al.*, 1996). The same occurs in the dimorphic basidiomycete *U. maydis*, where genes of the MAPK pathway have been shown to be involved in mycelial growth (Banuett, 1998; Martínez-Espinoza *et al.*, 2002, 2004).

In the present study we have investigated the role of the *STE11*-dependent MAPK pathway in the dimorphic transition of *Y. lipolytica*. To achieve this aim, we have isolated and deleted the gene encoding a Mapkkk, the first member of the phosphorylation module. We chose this gene because of the observations that deletion of the *Ubc4* gene that encodes a Mapkkk in *U. maydis* suppressed filamentous growth of mutants lacking adenylate cyclase (Martínez-Espinoza *et al.*, 2002, 2004), and that in *Aspergillus nidulans* deletion of *steC*, a gene encoding a Mapkkk, severely affects different developmental processes, including sexual differentiation, conidiophore morphogenesis, and heterokaryon formation (Wei *et al.*, 2003).

Materials and methods

Strains and culture conditions

The strains of *Y. lipolytica*, *S. cerevisiae* and *U. maydis* used in this study are described in Table 1. *Escherichia coli* Top10 strain was used routinely for plasmid propagation. Fungal strains were maintained at $-70\,^{\circ}C$ in 50% v/v glycerol.

Table 1. Fungal strains used in this work

Strain	Genotype	Origin
Yarrowia lipolytica		
P01A	MatA, *ura3-52*, *leu 2-270*	INRA
E151	MatB, *his1*, *leu 2-270*	INRA
E121	MatA, *lys 11-23*	INRA
E151-A	MatB, *his1*, pINA240	This work
AC1	MatA, *ura3-52*, *leu2-270*, Δ*ste11*	This work
AC2	MatA, *ura3-52*, *leu2-270*, Δ*ste11*	This work
AC1-A	MatA, *ura3-52*, Δ*ste11*, pINA240	This work
JAC1	MatA, *leu2-270*, pGP-1	This work
JAC2	MatA, *leu2-270*, pGP-1	This work
MC1	MatA, *leu2-270*, pINA444	This work
MC2	MatA, *leu2-270*, pINA444	This work
2Q	MatA, *ura3-52*, pKpp4-Q	This work
4Q	MatA, *ura3-52*, pKpp4-Q1	This work
6Q	MatA, *ura3-52*, pKpp4-Q1	This work
2T	MatA, *ura3-52*, pTs5	This work
5T	MatA, *ura3-52*, pTs5	This work
7T	MatA, *ura3-52*, pTs5	This work
6CAT	MatA, *ura3-52*, pKpp4-CAT2	This work
9CAT	MatA, *ura3-52*, pKpp4-CAT2	This work
Nd1	MatA, *leu2-270* + *pGP-1* MatB + *his1* pINA240	This work
Nd2	MatA, *leu2-270* + *pGP-1* MatB + *his1* pINA240	This work
Nd3	MatA, *leu2-270* + *pGP-1* MatB + *his1* pINA240	This work
Nd4	MatA, *leu2-270* + *pGP-1* MatB + *his1* pINA240	This work
Saccharomyces cerevisiae		
JCY100	MatA, *leu::hisG*, *his3*Δ*::hisG*, *trp::hisG*,	P. Guzman
PHY80	Matα, *ura3-52*, *lys2–*, *ade3*Δ*0*, *his–*, *trp1–*	A. Herrera
15271	Matα, *his3*Δ*1*, *leu2*Δ*0*, *lys2*Δ*0*, *ura3*Δ*0*, Δ*ste11*	Invitrogen
MTS1	Matα, *his3*Δ*1*, *leu2*Δ*0*, *lys2*Δ*0*, pJl-1	This work
MTS2	Matα, *his3*Δ*1*, *leu2*Δ*0*, *lys2*Δ*0*, pJl-1	This work
MTS3	Matα, *his3*Δ*1*, *leu2*Δ*0*, *lys2*Δ*0*, pJl-1	This work
AA1	Matα, *his3*Δ*1*, *leu2*Δ*0*, *lys2*Δ*0*, pAZ2	This work
AA2	Matα, *his3*Δ*1*, *leu2*Δ*0*, *lys2*Δ*0*, pAZ2	This work
AA3	Matα, *his3*Δ*1*, *leu2*Δ*0*, *lys2*Δ0, pAZ2	This work
Ustilago maydis		
FB2	*a2b2*	F. Banuett

When required, *Y. lipolytica* and *S. cerevisiae* were transferred to liquid or solid YPD media (1% yeast extract, 2% peptone, 2% glucose), and incubated at 28 °C (*Y. lipolytica*) or 30 °C (*S. cerevisiae*). The strains were routinely grown in YPD or Yeast Nitrogen Base (YNB) (0.67% YNB without amino acids/ammonium sulfate (Difco, Detroit, MI), 1% glucose, 0.5% ammonium sulfate). Whenever required, YNB medium was supplemented with one or more of the following substances: uracil ($22.4\,mg\,L^{-1}$), leucine ($262\,mg\,L^{-1}$), histidine ($46\,mg\,L^{-1}$), lysine ($180\,mg\,L^{-1}$), tryptophan ($80\,mg\,L^{-1}$) and adenine ($20\,mg\,L^{-1}$). *Ustilago maydis* was recovered and grown as described previously (Ruiz-Herrera *et al.*, 1995). YNB–FOA ((YNB containing 5-fluoroorotic acid (FOA) $1\,g\,L^{-1}$) was used to select *ura3*

mutants. YMC medium (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 0.05% sodium citrate) was used for mating assays of *Y. lipolytica* (Barth & Gaillardin, 1996). *Saccharomyces cerevisiae* mating was carried out in YPD. The cell viability of *Y. lipolytica* was determined by inoculating aliquots of different dilutions of suspensions of known cell density (counted with a hemocytometer) in duplicate plates of complete YPD medium. After 48 h, colonies were counted. The growth rate was measured in liquid media. Cells (10^6 mL^{-1}) were inoculated into 200 μL of YNB or YPD media in 96-well microplates, and shaken (180 r.p.m.) at 28 °C. At intervals, OD at 595 nm was measured with an Ultramark microplate imaging system device (BioRad, Hercules, CA). Top10 *E. coli* was grown at 37 °C in Luria–Bertani medium (1% tryptone, 0.5% yeast extract, 0.5% sodium chloride) with ampicillin (100 μg mL^{-1}) or kanamycin (50 μg mL^{-1}) for plasmid selection.

Induction of dimorphic transition

Dimorphic transition of *Y. lipolytica* was induced basically as reported previously (Ruiz-Herrera & Sentandreu, 2002). Media composition is described for each experiment. Samples were observed with a Leica DMRE microscope, and images were captured using a Spot camera (Diagnostic Instruments, Sterling Heights, MI). At least 500 cells were scored to determine the ratio of yeast and mycelial cells. For each assay, three experiments with duplicate samples were performed.

Stress assays

Different stress conditions were tested on solid YPD or YNB media. Cells were grown in liquid medium at 28 °C until the stationary phase was reached. Cells were collected and washed with sterile distilled water by centrifugation. Different cell numbers, 10^7, 10^6, 10^5 and 10^4, were spotted on agar plates containing different additions. Plates were incubated for 48–72 h at 28 °C, and growth was scored. The effect of glucanase (Lyticase, Sigma, St Louis, MO) was measured as follows. Cells were grown in YPD or YNB media overnight, centrifuged and adjusted to an OD (600 nm) of 1.0 with distilled water. Next, 1-mL samples of cell suspensions were incubated at 25 °C with 50 μL of a suspension containing 1, 0.5 or 0.25 mg of lyticase in 50 mM phosphate buffer, pH 7. The OD of the suspensions (600 nm) was measured at 15-min intervals over 1 h.

Mating assay

The mating assay of *Y. lipolytica* in liquid medium (YMC) was conducted as described by Barth and Gaillardin (Barth & Gaillardin, 1996), but conjugation time was prolonged to 17 days. Mating efficiency was calculated as the proportion of diploids able to grow in plates of selective media in relation to the total viable cells (see above). Three experiments with three samples each were analyzed for each assay. Qualitative mating assays of *S. cerevisiae* on solid media (YPD) were performed basically as previously described (Lamson *et al*., 2002).

Genetic transformation

Genetic transformation of *Y. lipolytica* and *S. cerevisiae* was carried out by the lithium acetate method (Barth & Gaillardin, 1996). *Escherichia coli* transformation was performed by standard techniques (Sambrook & Russell, 1999).

DNA and RNA manipulation techniques

Genomic DNA was isolated as described by Hoffman and Winston (1987), but DNA for sequencing and ligation reactions was purified using the QIAquick Gel extraction Kit (QIAGEN, Valencia, CA). Isolation of *Y. lipolytica* plasmids for *E. coli* transformation was conducted as described by Barth and Gaillardin (1996). Isolation of plasmid DNA from *E. coli* was performed by standard procedures (Sambrook & Russell, 1999). Enzymatic modifications of DNA such as restriction, ligation and dephosphorylation were performed as recommended by the corresponding suppliers of reagents (Invitrogen, Carlsbad, CA; New England Biolabs, Beverly, MA). Total RNA was isolated according to the method described by Jones *et al*. (1985). Southern and Northern hybridizations were performed by standard techniques (Sambrook & Russell, 1999). DNA probes were labeled with [α]-^{32}P-dCTP using the random primer labeling system (Amersham Biosciences, Sunnyvale, CA). Hybridization of ribosomal RNA with a 5.8-kb fragment of human 28S ribosomal DNA was used for loading control.

PCR

The list of oligonucleotides used as primers is shown in Table 2. The standard PCR protocol included an initial cycle of 94 °C for 5 min, followed by 30–40 additional cycles with the following program: 94 °C for 30 s, primer-specific annealing temperature for 60 s, and a Taq DNA polymerase specific extension temperature of 1 min kb^{-1} of target length. For reverse transcriptase-PCR (RT-PCR) (see below), 30 amplification cycles (a value at which the amplification kinetics were still exponential) were programmed. Whenever PCR products were used for cloning, an additional extension cycle of 72 °C for 7 min was applied to the amplification program. Taq DNA polymerase (Invitrogen) was used for routine amplifications. When high fidelity was required, the Expanded High Fidelity PCR System (Roche, Rotkreuz, Switzerland) was used as recommended by the

Table 2. Primers used in this work

Number	Sense	5′ → 3′Sequence	T_m (°C)
1107	Forward	CAATACCACCACTGCACTACCACTACAC	68
330	Forward	GARTAYGTSCCIGGMGGMTCIGTG	60
1108	Forward	GGATGGCCCCCGAGGTTGTCAAGCAG	70
2743	Forward	GGCTATCTCAGTAGC	46
1441	Forward	CAGCATGTCGGAGTATC	54.5
MatDel1	Forward	GGCGAGCTGGACGAAAAGGTAGAC	76
1668	Forward	GGGGTACCTATGAGTGCTGCAACACC	62.5
1682	Forward	CGGTACCTGATGCTTGTATGATGGTATTTA	66
1684	Forward	CCTCGGGGTATTTGTCTGCTTTGGTGC	66
1500	Forward	CGTGTGATATCGGGAGTGCAAGGTCG	69
1658	Forward	GCGTTGAGACTGGTGTGAT	52
2436	Forward	CTTGTTGTCCACCAGCAC	50
003	Reverse	GGTACCATGGTTCCCTCGAAAGCCGCA	62.5
1109	Reverse	GTAGGAGGGCATTTTGGTGGTGAAGAG	70
MatDel2	Reverse	CGAGGGCGAGGAGCGAGATG	68
329	Reverse	KCCRATYTTRAARATIGCYTG	60
1669	Reverse	CAGGTACCGCAGTGAGTCTAAGAATCC	62.5
1106	Reverse	GACGAACGACAGCATGCCAAGGAGAAC	68
1440	Reverse	CAAGTCTCCTCAATGGC	54.5
1683	Reverse	GGATCCCCCCCAGGTCAGAGCCAT	66
1685	Reverse	GGGTACCGCCGTTTACAGAAGAATGGA	66
2957	Reverse	CAAAAACTCGTGGGCCAGCAACTCCG	70
1501	Reverse	GATGATGACCTGGGCAGTG	52
1659	Reverse	GGGGGGATCCGTGTGTAAATATGAATAT	69
2724	Reverse	CGAGAGATCCGAAGG	50

manufacturer. PCR products were cloned into pCR2.1 TOPO vector (Invitrogen).

RT-PCR analyses were used to determine *YlSTE11* transcription; 3 μg of total RNA and an oligo-dT (20-mer) were used for reverse transcription reactions, following the supplier's instructions (Invitrogen). These reactions were followed by PCR (see above), using primers 1108 and 2957 (see Table 2). PCR with primers 1500 and 1501, which amplify a fragment of 180 bp for the highly conserved elongation translation factor (*Efα1*) (designed from tomato sequence of accession number X14449), was used as internal control.

DNA sequencing and sequence analyses

DNA sequencing was performed with an ABI PRISM 377 DNA automated sequencer (Perkin Elmer, Foster City, CA) using double-stranded DNA as template, and universal and reverse as well as some internal sequence-specific primers (2436, 2742 and 2743, see Table 2). Nucleotide and amino acid sequences were analyzed using the DNAStar (DNAStar Inc., Madison, WI), DNASIS (Hitachi, Tokyo, Japan) and DNAStrider (from marck@jonas.saclay.cea.fr) programs. Multiple sequence alignments were done with the CLUSTALW program.

Plasmids

Plasmid pSM-1 is a pBKS(+) vector harboring the partial sequence (5′-UTR and a truncated ORF) of the *STE11* gene in a 5.3-kb *Kpn*I genomic fragment. Plasmid pSM-2 corresponds to a pCR2.1 TOPO vector containing the 3′ end of the ORF and the 3′-UTR of the *STE11* gene in a 2-kb *Sac*I–*Eco*RV genomic fragment. Plasmid pJACC1 harbors the full *STE11* gene, including the promoter and terminator sequences. It was constructed by subcloning an *Eco*RV–*Kpn*I 3.6-kb fragment from pSM-1, ligated to a *Kpn*I–*Not*I 861-bp fragment from pSM-2, at *Eco*RV–*Not*I sites in the pCR2.1 TOPO vector. Subcloning the *Eco*RV–*Eco*RI 1-kb fragment from pJACC1, which contained the 5′-UTR sequence of *STE11* plus the ATG and 54 extra base pairs, into pCR2.1 TOPO gave rise to pJACC2.

Plasmid pSte11Δ1908 was used for disruption of the *STE11* gene. To construct this plasmid, an *Eco*RV–*Kpn*I 1036-bp fragment from plasmid pJACC2 was ligated to a *Kpn*I–*Not*I 864-bp fragment from plasmid pSM-2. The resulting *Eco*RV–*Not*I fragment was subcloned into pBKS(+) at these sites to generate pJACC3. Next, the *Y. lipolytica URA3* gene from plasmid pINA444 (Barth & Gaillardin, 1996) was excised with *Hin*CII and subcloned at the single *Hin*CII site into plasmid pJACC3.

Plasmid pGP-1, an autoreplicative vector, was used to complement the disrupted AC1 and AC2 (Δ*ste11*) strains. It was constructed as follows. The *STE11* gene was PCR-amplified from plasmid pJACC1 with the universal and reverse primers. The resulting 4.4-kb fragment was then subcloned into the pCR2.1 TOPO vector, duplicating in this way the *Bam*HI site located in the polylinker sequence, to generate plasmid pAG1. The *STE11* gene was then excised from pAG1 using *Bam*HI, and subcloned at this site into pINA444.

Plasmid pJl-1 was used to complement the *S. cerevisiae ste11*Δ null mutant. It was constructed as follows. The entire *Y. lipolytica STE11* ORF was PCR-amplified using primers 1441 and 1440 (Table 2), and cloned into the pCR2.1 TOPO vector. Then, the *STE11* ORF was sense subcloned into the pYes2 vector (Invitrogen) at the *Bam*HI–*Not*I sites under the transcriptional control of the inducible *GAL1* promoter. Plasmid pAz2, where the *Y. lipolytica STE11* ORF was under the transcriptional control of the *S. cerevisiae STE11* native promoter, was constructed as follows. The *STE11* promoter (816 bp) was PCR-amplified using primers 1658 and 1659, which harbor a *Bam*HI site (Table 2), and genomic DNA from *S. cerevisiae* strain PHY80 as a template. Next, it was cloned into the pCR2.1 TOPO vector, creating plasmid pAz1. The promoter was then released by digestion with *Bam*HI, and ligated at the *Bam*HI site into plasmid pJl-1.

Plasmid pKpp4-Q1 was obtained as follows. The Kpp4-ORF (1567 amino acids) of the gene encoding a Mapkkk

from *U. maydis* (Müller *et al.*, 2003) was PCR-amplified using genomic DNA from strain FB2 as a template and primers 1668 and 1669, which bear a sequence for a *Kpn*I restriction site (Table 2). The PCR product was cloned into the pCR2.1 TOPO vector, yielding the plasmid pKpp4-ORF. Next, the ORF was subcloned into plasmid pRRQ1 (Richard *et al.*, 2001) at the *Kpn*I site in order to place it under the transcriptional control of the strong synthetic promoter *XPR2* (*Y. lipolytica* extracellular alkaline protease) (Madzak *et al.*, 1999).

Plasmid pTs5 was constructed as follows. Using pJACC1 DNA as a template, the *Y. lipolytica STE11* gene promoter sequence was PCR-amplified using primers 1684 and 1685 (with a *Kpn*I site; see Table 2). The terminator sequence was also amplified by PCR using primers 1682 and 1683 (with *Kpn*I or *Bam*HI sites, respectively; see Table 2). Both promoter (1390 bp) and terminator (694 bp) fragments were cloned into the pCR2.1 TOPO vector, giving plasmids pTs1 and pTs2, respectively. Next, promoter and terminator fragments were excised with *Sac*I–*Kpn*I and *Kpn*I–*Bam*HI from pTs1 and pTs2, respectively, and ligated. The resulting fragment was subcloned into the pUC19 vector at *Sac*I–*Bam*HI sites, giving rise to pTs3. The *Kpp4* ORF from plasmid pKpp4-ORF (see above) was subcloned at the *Kpn*I site of pTs3 to give pTs4. Finally, the chimeric gene was subcloned from pTs4 into the *Y. lipolytica* autoreplicative pINA240 vector (carrying the *LEU2* gene as a selection marker; C. Gaillardin, INRA, Thiverval-Grignon, France) at the *Bam*HI site, yielding pTs5.

Plasmid pKpp4-CAT2, which carries a truncated version of the *Kpp4* ORF (it lacks the region encoding amino acids 1–1002), was constructed as follows. A truncated *Kpp4* ORF (1692 bp) was amplified using genomic DNA from strain FB2 and primers 003 and 1669 (both containing a *Kpn*I site). The 003 forward primer also carried an ATG codon (Table 2). The PCR product was cloned into the pCR2.1 TOPO vector, giving rise to plasmid pKpp4-CAT1. Next, the full *Kpp4* ORF from plasmid pTs5 was eliminated with *Kpn*1, and the truncated *Kpp4* ORF was subcloned at the *Kpn*I site.

All plasmids constructed in this study were verified by restriction analysis and sequencing.

Isolation of the *Y. lipolytica STE11* gene

A fragment of the *Y. lipolytica* gene encoding a Mapkkk was obtained by PCR using genomic DNA and the degenerate primers 329 and 330 (see Table 2). These primers were designed from the amino acid sequences located at the catalytic domain between subdomains IV and X, where the most conserved region from several Mapkkk proteins is found. Two PCR products of 426 and 500 bp, respectively, were obtained. Sequence analysis of the 426-bp fragment revealed an ORF encoding a peptide made of 134 amino acids, with a high degree of similarity to the catalytic domain of several fungal Mapkkk proteins (not shown). Southern blot hybridization of genomic DNA digested with several restriction enzymes, using the 426-bp fragment as a probe, revealed a single hybridization signal (data not shown), suggesting that the gene existed as a single copy in the *Y. lipolytica* genome.

A minigenomic library was constructed using *Kpn*I fragments of size *c.* 5.3 kb. Screening this minigenomic library (3500 colonies) with the 426-bp fragment as a probe identified two positive colonies (28-ste and 72-ste). Since plasmid DNA from both colonies showed the same restriction pattern, only one (named pSM-1) was further analyzed. Its sequence analysis showed it to contain a 5′-UTR sequence followed by a truncated ORF with similarity to fungal genes encoding Mapkkk proteins. In order to clone the missing part of the gene, a second minigenomic library was constructed using *Sac*I–*Eco*RV fragments of about 2 kb in size. After screening of the minigenomic library with the 426-bp fragment (see above), clone 472-ste (pSM-2) was identified. Sequence analysis of plasmid pSM-2 showed it to contain the missing part of the ORF plus 710 bp corresponding to the 3′-UTR sequence of the probable *Y. lipolytica* Mapkkk-encoding gene. Plasmids pSM-1 and pSM-2 were utilized to join physically the gene into plasmid pJACC1 (see above). The sequence of the gene encoding the *Y. lipolytica* Mapkkk (*STE11*) reported here has been submitted to EMBL with accession number AJ577132.

Results

Analysis of the *Y. lipolytica STE11* gene sequence

Analysis of the entire sequence of the gene revealed a continuous ORF of 2832 bp. The translated sequence was predicted to encode a protein of 944 amino acids, with a theoretical M_r of 100.9 kDa. This Mapkkk protein has an average molecular size intermediate for fungal Mapkkk proteins, considering on one side the Kpp4 protein from *U. maydis* (1567 amino acids) and the Ste11α protein from *Cryptococcus neoformans* (1230 amino acids) as the largest ones, and on the other side the small Byr2 protein, formerly named Ste8 (Styrkársdóttir *et al.*, 1992), from *Schizosaccharomyces pombe* (659 amino acids). A search of protein databases showed that similarities of the protein were high for the conserved catalytic domain (Fig. 1). The catalytic domain of this protein shares 64% amino acid identity with ncr-1p from *Neurospora crassa*, 63% with Ste11αp from *Cr. neoformans*, 61% with Mapkkk protein from *C. albicans*, 60% with Byr2p/Ste8p from *Sch. pombe,* 59% with Kpp4p from *U. maydis* and 55% with Ste11p from *S. cerevisiae*. These data demonstrate that the cloned gene corresponded

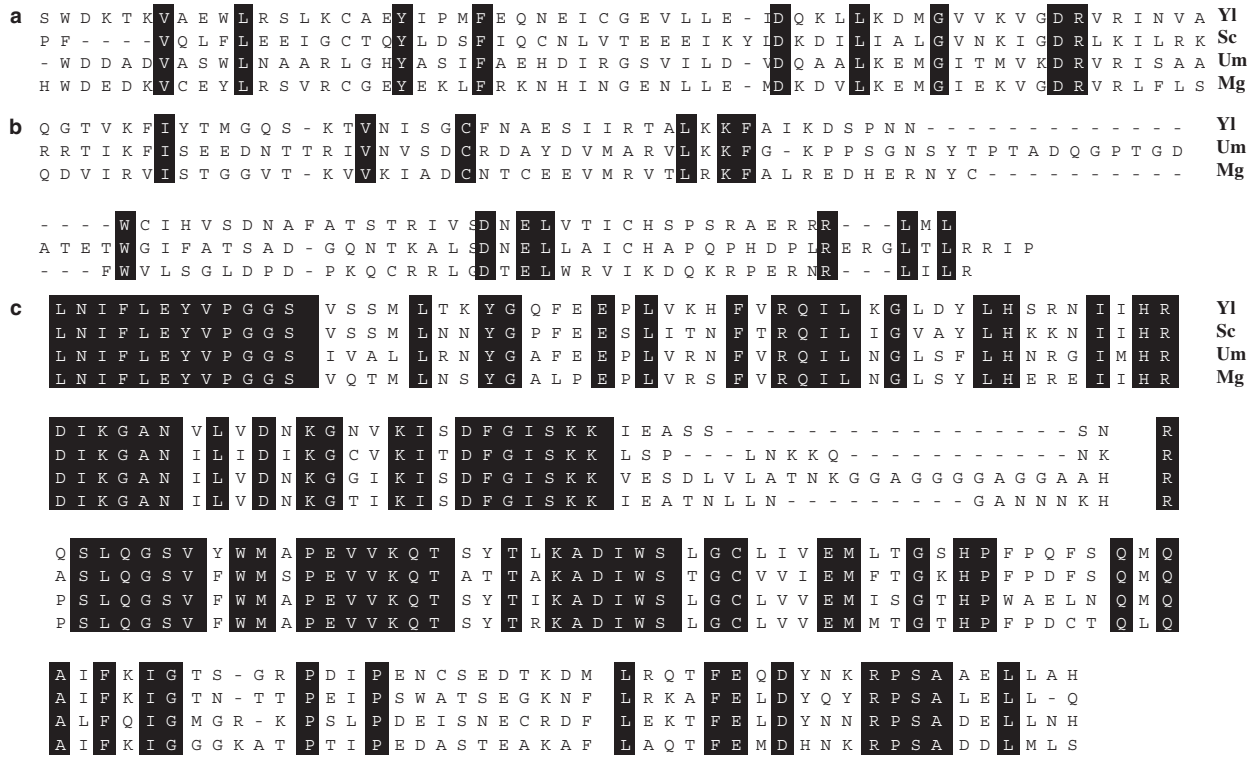

Fig. 1. Comparison of the different domains of *Yarrowia lipolytica* Ste11p and other fungal Mapkkk proteins. Amino acid sequences of the predicted *Y. lipolytica STE11* gene product (AJ577132), *Saccharomyces cerevisiae* (NP013466), *Ustilago maydis* (AAN63948) and *Magnaporthe grisea* (XP369824) were aligned. (a), SAM domain; (b), RA domain; (c), catalytic domain (subdomains IV–X). Dashes indicate gaps in the alignment; identical residues are shown in boxes. Yl, *Y. lipolytica*; Sc. *S. cerevisiae*; Um, *U. maydis*; Mg, *M. grisea*. Note that the RA domain of *S. cerevisiae* has not yet been defined.

to the *Y. lipolytica* homolog, which was named *STE11*. Like other similar enzymes, YlSte11p displays three main domains: a catalytic domain, a sterile α motif (SAM) domain, and a Ras-association (RA) domain. The 3′-terminus of the gene encodes a highly conserved catalytic domain composed of 258 amino acids with similarity to the serine–threonine kinase family, and shows high homology to the catalytic domains from other Mapkkks (Jouannic *et al.*, 1999). Domains of this type are subdivided into 11 subdomains characteristic of this family (Hanks *et al.*, 1988). The SAM domain of 68 amino acids was located at the N-terminus, between amino acids 191 and 250. This domain is found in over 300 described proteins, including regulators of signal transduction and transcription. SAM domains function as protein interaction modules through homodimerization or oligomerization with other SAM domains (Sambrook *et al.*, 1997). The RA domain of 84 amino acid residues could be identified between positions 348 and 432. These two domains, SAM and RA, in contrast to the catalytic domain, show low similarities to those from other Mapkkks (Fig. 1). This point is interesting with regard to the results obtained by transgenic complementation described below. The 5′ regulatory sequence of *Y. lipolytica STE11* shows two putative TATA boxes located at −263 and −295 bp from the possible ATG initiation translation codon. A ′CT′-rich domain, which is believed to play a role in transcriptional regulation (Xuan *et al.*, 1990), was observed in the promoter region of the gene. In the 3′ region, 31 bp downstream of the TGA stop codon, a typical termination consensus sequence for yeast genes (Zaret & Sherman, 1982) was identified, TAG . . . TGA . . . TTT, and a putative poly-A signal (AATAA) was located 62 bp downstream of the termination codon.

Transcription of the *STE11* gene

RT-PCR analyses were used to determine the expression of the *STE11* gene. Strain P01A was grown for 16 h in four different media: (1) YNB medium containing *N*-acetylglucosamine (GlcNAc) and citrate buffer, pH 7 (at which mycelial growth occurs); (2) the same medium, but at pH 3; (3) YPD with pH 7 phosphate buffer; and (4) YNB medium containing glycerol, glutamic acid and phosphate buffer, pH 7. Under these three last conditions, the cells grow in a yeast-like fashion. RNA was isolated and subjected to RT-PCR analysis. The expected 300-bp band was amplified from strain P01A grown only in medium containing

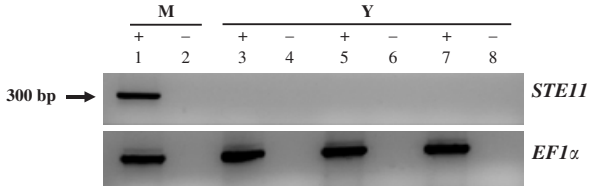

Fig. 2. *YlSTE11* expression under different conditions of cell growth. RNA was obtained from 16-h grown cells, and subjected to RT-PCR analysis. Upper panel: lanes 1 and 2, cells grown in YNB–GlcNAc–citrate medium, pH 7; lanes 3 and 4, cells grown in YNB–GlcNAc–citrate medium, pH 3; lanes 5 and 6, cells grown in YPD–phosphate medium, pH 7; lanes 7 and 8, cells grown in YNB–glycerol–glutamic acid–phosphate medium, pH 7. Reactions incubated with (+) or without (−) reverse transcriptase. Bottom panel: load control (*Ef1α*). M, mycelial cells; Y, yeast cells.

GlcNAc and citrate buffer, pH 7. Cells grown in the other media did not give the corresponding amplification band (Fig. 2).

Deletion of the *STE11* gene and isolation of null mutants

The pop-in/pop-out replacement method (Boeke *et al.*, 1987) was used to eliminate almost all the wild-type gene's ORF (93%) (Fig. 3). To achieve this aim, strain *Y. lipolytica* P01A was transformed with plasmid pSte11Δ1908 (see above) linearized with *Nde*I. Several *Ura*+transformants were recovered as result of the plasmid integration (pop-in) step. To determine whether the integration step had occurred at the correct locus, DNA from 15 transformants was isolated and PCR-analyzed using primer pairs 1108–1109 and 1107–1106 (Table 2). In a legitimate integration process, these primer pairs should amplify fragments of 4.3 or 1.8 kb, respectively. Nine positive transformants were thus identified (data not shown) that were also confirmed by Southern hybridization, using genomic DNA digested with *Kpn*I, and, as a probe, a fragment of the promoter sequence (1 kb). The two expected hybridization signals of 2.7 and 5.3 kb, respectively, for mutant and wild-type loci were observed (see Fig. 3d, lane 1). Two of these transformants were selected to conduct a counterselection on YNB-FOA medium supplemented with leucine and uracil (see Materials and methods). Eleven FOA-resistant and (*ura*–) strains were recovered (AC1–AC11) as a result of this excision (pop-out) step. The nature of the allele (wild-type or mutant) harbored by FOA-resistant colonies was confirmed by Southern hybridization. Genomic DNA was digested with *Kpn*I and hybridized with a promoter fragment (1 kb) as a probe; four strains (37%) conserved the wild-type allele, whereas seven strains (63%) contained the mutant allele of the *STE11* gene. Representative data are shown in Fig. 3d. Since these strains lacked 93% of the *STE11* gene, we consider them to be null mutants.

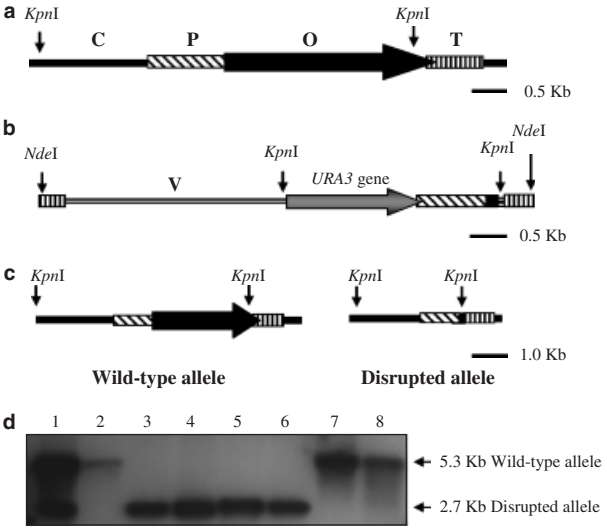

Fig. 3. Disruption of the *STE11* gene. (a) Schematic representation of the *STE11* gene. C, fungal chromosome; P, promoter; O, ORF; T, terminator. (b) Disruption plasmid pSte11Δ1908 linearized with *Nde*1. V, vector DNA. (c) Possible alleles resulting after the pop-out step. (d) Southern hybridization of DNA from some selected transformants digested with *Kpn*I using as a probe a promoter sequence (1 kb). Lane 1, control strain where a legitimate integration step had occurred at the pop-in step. Lanes 2 and 7, strains that conserved the wild-type allele after the pop-out step. Lanes 3–6, strains that conserved the mutant allele after the pop-out step. Lane 8, parental strain P01A.

Expression of the *STE11* gene in null mutants was determined by RT-PCR and Northern blot analyses. Strains P01A and AC1 (a Δ*ste11* mutant, see below) were grown in YNB medium containing GlcNAc and citrate buffer, pH 7. The expected 300-bp band was amplified from strain P01A (Fig. 4a, lane 1), but a sample from the AC1 strain did not show any amplification fragment (Fig. 4a, lane 3). These results were confirmed by Northern blot hybridization with the whole *STE11* ORF as a probe. Results with strain P01A revealed a single transcript of *c.* 3 kb (Fig. 4b, lane 1). In contrast, no transcript was detected in samples obtained from the AC1 and AC2 Δ*ste11* mutant strains that grew at pH 7 in the yeast form (Fig. 4b, lanes 2 and 3).

Phenotypic analysis of Δ*ste11* null mutants

Growth rate

The growth rate of Δ*ste11* mutant strains AC1 and AC2 and the parental strain P01A was scored at 28 °C in YPD and YNB liquid media. In both media, no significant differences in growth rate were observed between the mutants and parental strains (data not shown).

Sensitivity to stress conditions

We studied the behavior of the parental P01A and mutant Δ*ste11* strains AC1, AC2 and AC3 under different stress

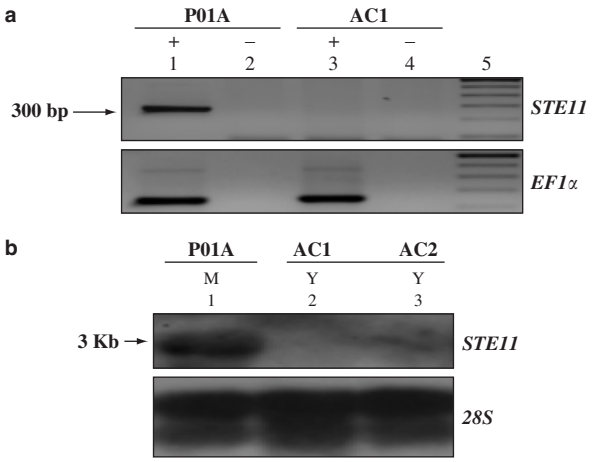

Fig. 4. Determination of *STE11* transcription in Δ*ste11* strains. (a) RT-PCR analysis. RNA was obtained from 16-h cells grown in YNB–GlcNAc, pH 7, with citrate buffer medium. Upper panel, *STE11* analysis, lane 5, 100-bp ladder. Reactions incubated with (+) or without (−) reverse transcriptase. Bottom panel, load control (*Ef1α*). (b) Northern blot hybridization with the whole *STE11* ORF sequence. RNA was obtained as in (a). M, mycelial cells; Y, yeast cells. Bottom panel, load control (human 28S gene).

Table 3. Mating efficiency of wild-type, mutant and transformant strains

Cross	Mating efficiency*
E151 × E121	$136 \times 10^{-5} \pm 25.0 \times 10^{-5}$
E151 × P01A	$54 \times 10^{-5} \pm 6.9 \times 10^{-5}$
E151 × AC1	0 –
E151 × AC2	0 –
E151-A × JAC1	$1.8 \times 10^{-5} \pm 0.15 \times 10^{-5}$
E151-A × JAC2	$1.4 \times 10^{-5} \pm 0.21 \times 10^{-5}$
E151-A × MC1	0 –
E151-A × MC2	0 –

*Mean of diploids per total cell numbers in triplicate experiments ± standard deviations of the mean. P01A, parental strain; E151, *MATB* strain; E121, *MATA* strain; E151-A, *MATB* strain transformed with pINA240; AC1, AC2, Δ*ste11* mutants; JAC1, JAC2, strains transformed with pGP-1 containing *STE11* gene; MC1, MC2, strains transformed with empty pINA 444.

conditions on solid media. Plates of YNB medium were supplemented with 1.5 M sorbitol, 0.4 or 1.25 M NaCl, or Calcofluor white (300 or 500 $\mu g\,mL^{-1}$). Neither of these compounds affected growth of the mutant strains as compared with P01A (data not shown). The effect of glucanase on cell integrity was tested using the mutants AC1 and AC2 compared with parental strain P01A. No differences in the rate of cell lysis, measured as absorbance reduction (see Materials and methods), were detected between parental and mutant strains, regardless of whether strains were grown in YNB or YPD media, or different glucanase concentrations were used.

Mating

We performed mating assays between strains E121 and E151, and P01A and E151 as controls. Mating results obtained from the E121 × E151 cross were basically as reported (Barth & Gaillardin, 1996; Kurischko *et al.*, 1999), showing a mating efficiency of *c.* 1×10^{-3} (Table 3), but mating between strain E151 and strain P01A took a longer conjugation period (17 days), instead of the 4 days reported (Barth & Gaillardin, 1996), and mating efficiency was only *c.* 0.5×10^{-3} (Table 3). The explanation for this discrepancy may be the alterations suffered by strain P01A after the repeated transfers in the laboratory.

The Δ*ste11* strains AC1 and AC2 were unable to mate with the tester strain E151. Not one diploid grew on the selective plates in repeated experiments (Table 3). This result demonstrates that the *STE11* gene is indispensable for mating.

Dimorphism

The effect of several well-documented stimuli that induce the dimorphic transition in *Y. lipolytica* were tested. It has been reported that in acid media *Y. lipolytica* grows in the yeast-like form, whereas in neutral media most of the population shows mycelial growth (Ruiz-Herrera & Sentandreu, 2002). The AC1, AC2 and P01A strains all grew in the yeast form in YNB–glucose, pH3, or GlcNAc media containing citrate. On the other hand, when the pH of the medium was taken to 7 with citrate buffer, the parental P01A strain grew in the form of mycelia, whereas growth of the mutants was yeast-like (Table 4). Blood serum has also been described as a strong inducer of mycelial growth in *Y. lipolytica* (Domínguez *et al.*, 2000; Kim *et al.*, 2000). We analyzed the effect of calf serum addition (5%) to YNB–GlcNAc media, buffered to pH 3 or 7, on dimorphism of AC1, AC2 and

Table 4. Dimorphic transition in *Yarrowia lipolytica* wild-type and Δ*ste11* mutant strains

	P01A		AC1		AC2	
Growth medium	M*	Y†	M	Y	M	Y
YNB–Glc, pH 3	0	100	0	100	0	100
YNB–GlcNAc, pH 3	10	90	0	100	0	100
YNB–Glc, p H7	94	6	1	99	2	98
YNB–GlcNAc, p H7	97	3	2	98	2	98
YNB–GlcNAc, pH 3+Serum	10	90	1	99	1	99
YNB–GlcNAc, pH 7+Serum	99	1	3	97	4	96

*M, mycelial cells;

†Y, yeast cells.

Media contained glucose (Glc) or *N*-acetylglucosamine (GlcNAc). pH was adjusted with citrate buffer, and strains were incubated with shaking at 28 °C for 14 h. P01A, parental strain; AC1, AC2, Δ*ste11* mutants.

Data are expressed as percentage values. Results are averages of three experiments with duplicate samples each. SD did not exceed a value of 2.

P01A. In pH 3 medium, dimorphism was slightly stimulated by serum, but mutant strains grew as almost homogeneous yeast populations, while at pH 7 only the parental strain formed significant amounts of mycelium (Table 4).

Complementation of mutant strains

To determine whether the phenotypic effects observed in strains AC1 and AC2 were due to mutation of the *STE11* gene, both strains were transformed with plasmid pGP-1, which harbors the wild-type gene. Strains transformed with the empty plasmid pINA444 were used as controls. Several *Ura*+transformants were recovered from transformation with either plasmid. Two strains transformed with pGP-1 (JAC1 and JAC2) and two control strains transformed with pINA444 (MC1 and MC2) were further analyzed. Plasmid DNA from all these strains was isolated and used to transform *E. coli*. Plasmid DNA from several of the bacterial transformants obtained was subjected to restriction analysis with several enzymes, showing in all cases a pattern identical to that of the original plasmids pGP-1 or pINA444, depending on the fungal strain from which they came (data not shown). These results confirmed that no rearrangement in plasmid DNA had occurred during transformation.

Phenotypic analysis of transformant strains

Mating competence

The mating competence of transformants JAC1, JAC2, MC1 and MC2 was tested. For these analyses, the following crosses were conducted: JAC1 × E151-A (see Table 1), JAC2 × E151-A, MC1 × E151-A, MC2 × E151-A, and P01A × E151 (control). Crosses JAC1 × E151-A, JAC2 × E151-A and P01A × E151 produced diploid colonies on selective media, whereas, as expected, crosses MC1 × E151-A and MC2 × E151-A were sterile (Table 3). A molecular approach was used to confirm the ploidy state of some putative diploids recovered on selective media. In *Y. lipolytica*, both *MAT* loci (*A* and *B*) lie between homologs of *S. cerevisiae APN2* and *SLA2* (Kurischko *et al.*, 1999; Butler *et al.*, 2004). Accordingly, both loci can be amplified by PCR using the primers MATDel1 and MATDel2 (Table 2), which correspond to sequences from the flanking genes. Using these primers and genomic DNA isolated from the putative diploids Nd1 and Nd2, obtained from the JAC1 × E151-A cross, and Nd3 and Nd4, obtained from the JAC2 × E151-A cross, two PCR products of *c.* 4 and 5 kb that corresponded to *MATA* and *MATB,* respectively, were amplified (Fig. 5). The parental strains of each mating type gave a single band of the corresponding size (Fig. 5). These results confirmed that strains JAC1 and JAC2 had recovered their fertile phenotype through transformation with the *STE11* wild-type gene.

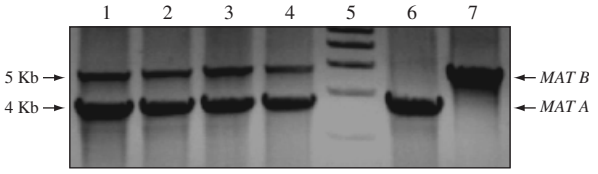

Fig. 5. Molecular analysis of mating loci in diploid and haploid strains by PCR: lanes 1–4, PCR amplification of Nd1–Nd4 diploid DNA; lane 6, PCR amplification of P01A (*MATA*) DNA; lane 7, PCR amplification of E151 (*MATB*) DNA; lane 5, 1-kb DNA ladder. All amplifications carried out with MATDel1 and MATDel2 primers.

Table 5. Dimorphic transition in Δ*ste11* mutants transformed with plasmids carrying or not carrying the wild-type *STE11* gene

	JAC1		JAC2		MC1	
Growth medium	M	Y	M	Y	M	Y
YNB–Glc, pH 7	90	10	87	13	2	98
YNB–Glu, pH 7+Serum	97	3	94	6	1	99

Morphology and expression of data as defined in Table 4.
Growth media and growth conditions as in Table 4.
JAC1, JAC2, strains transformed with pGP-1 containing *STE11* gene; MC1, strain transformed with empty pINA444.
Results are averages of three experiments with duplicate samples each. SD values did not exceed a value of 3.5.

Dimorphic capacity

Strains JAC1, JAC2, MC1 and MC2 were analyzed for their capacity to grow in the mycelial form using induction conditions at neutral pH with or without serum. The inability to grow in the mycelial form observed in the mutants AC1 and AC2 was rescued in the transformant strains JAC1 and JAC2, but not in the negative control strains MC1 and MC2 (representative results are shown in Table 5; see also Fig. 6b). Taken together, these results confirm that the phenotype observed in mutants AC1 and AC2 was due to mutation in *STE11*.

Transformation of a *S. cerevisiae* Δ*ste11* mutant with the *Y. lipolytica* STE11 ORF

We analyzed whether the *Y. lipolytica STE11* ORF was able to complement the sterile phenotype of *S. cerevisiae* strain 15271 (*ste11*Δ). This was transformed with plasmid pJl-1, which harbors the *Y. lipolytica STE11* ORF under the control of the *GAL1* promoter, and the *URA3* gene as selective marker. Several *Ura*+ transformants were recovered, and the identity of the plasmid they carried was confirmed by restriction analysis (data not shown). Transformants MTS-1, MTS-2 and MTS-3 were selected, and mating assays were conducted using JCY100 as partner. Mating between *S. cerevisiae* strains PHY80 and JCY100 was conducted as a control. The *Y. lipolytica STE11* ORF gene failed to rescue the sterile phenotype, regardless of whether the mating assays

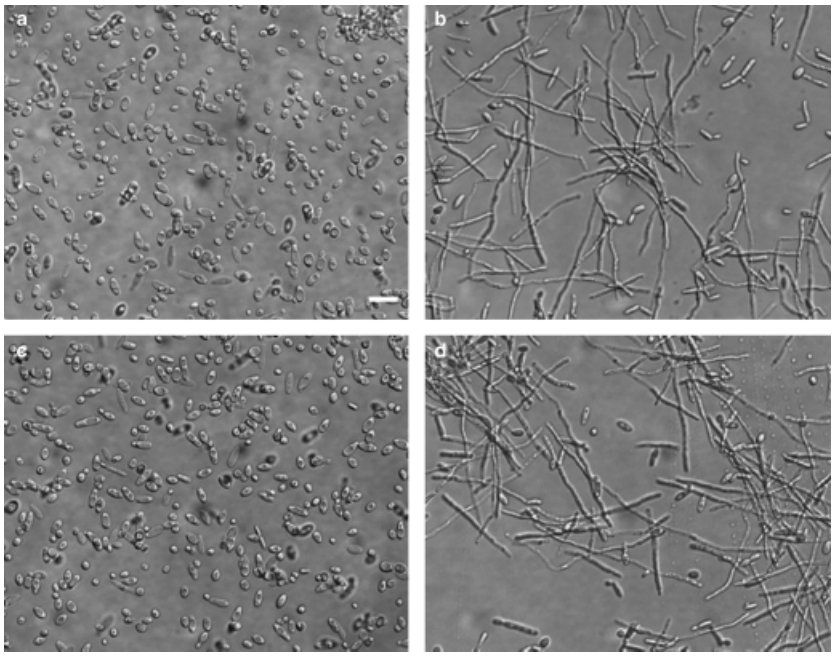

Fig. 6. Dimorphic growth in Δ*ste11 Yarrowia lipolytica* strains, native and transformed with plasmids containing different *MAPKKK* alleles. Strains were grown for 16 h in YNB medium containing citrate buffer, pH 7. (a) AC1 Δ*ste11* mutant. (b) JAC1, transformed with pGP-1 containing the *Y. lipolytica* wild-type gene. (c) 2 T, transformed with pTs5 containing the whole *Ustlago maydis Kpp4* ORF. (d) 6CAT, transformed with pKpp4-CAT2 containing the truncated *U. maydis Kpp4* ORF. Magnification bar, 10 μm.

were conducted in liquid or on solid media, different induction times were tested, or several other variations in the standard mating assay were attempted. This result was not due to failure in transcription of *STE11*. Northern hybridization assays using as a probe the entire *Y. lipolytica STE11* ORF, performed with samples harvested after 12, 24 or 42 h of galactose induction in the MTS-1 strain, showed the presence of the corresponding transcript in significant amounts, but not in glucose-grown cells (Fig. 7). Owing to the complementation failure obtained with the pJl-1 plasmid (see above), a different approach was attempted. Strain 15271 was then transformed with plasmid pAz2, which carries the *Y. lipolytica STE11* ORF under the control of the *S. cerevisiae STE11* gene promoter. Several *Ura*+ transformants were recovered, and mating assays were conducted, using the transformants AA1, AA2 and AA3 with the tester strain JCY100. Again, mating capacity was not restored in any of the transformants assayed. To analyze whether the negative result was due to transcription deficiency, RNA samples from AA1 and AA2 cells collected after 24 or 48 h were subjected to Northern hybridization. In both strains, a signal of the expected size for the transcript was detected (Fig. 7).

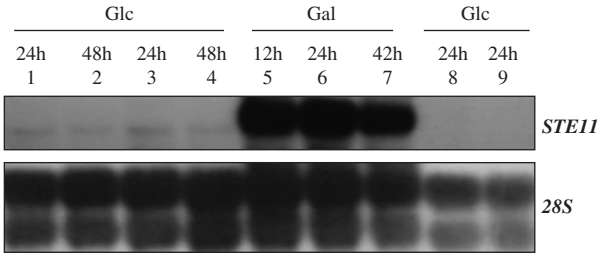

Fig. 7. Transcription of transgenic *Yarrowia lipolytica STE11* ORF in *Saccharomyces cerevisiae*. RNA from *S. cerevisiae* 15271, transformed or not with plasmids containing the *Y. lipolytica STE11* ORF driven by different promoters, and grown for different times, was subjected to Northern hybridization. Lanes 1–4, two strains containing the *Y. lipolytica STE11* ORF driven by the native *S. cerevisiae STE11* promoter (1, 2, AA1 strain; 3, 4, AA2 strain). Lanes 5–8, MTS-1 strain containing the *Y. lipolytica STE11* ORF driven by the inducible *S. cerevisiae GAL1* promoter. Lane 9, untransformed 15271 parental strain. Glc, cells grown with glucose; Gal, cells grown with galactose. Upper panel, hybridization with the whole *Y. lipolytica STE11* ORF. Lower panel, load control (28S hybridization).

Transformation of *Y. lipolytica* Δste11 mutants with the *U. maydis Kpp4* ORF

Yarrowia lipolytica mutants AC1 and AC2 were transformed with plasmids pKpp4-Q1 or pTs5, which carry the *U. maydis Kpp4* ORF under the control of different promoters (see Materials and methods). Several *Leu*+ transformants were recovered. After confirming that strains 2Q, 4Q and 6Q (transformed with pKpp4-Q1) and 2 T, 5 T and 7 T (transformed with pTs5) harbored the correct plasmid, they were used to conduct mating assays, using strain E151 as a tester. Mating between strains E151 and P01A was used as control. The results obtained showed that neither pKpp4-Q1 nor pTs5 was able to rescue the sterile phenotype of Δ*ste11* null mutants, since not a single diploid grew on the test plates (Table 6). We also tested the dimorphic capacity of some transformant strains in YNB medium, pH 7, containing GlcNAc or glucose and citrate buffer. Our results showed that no strain was able to grow in the mycelial form (see Fig.

Table 6. Comparison of mating efficiency between parental strains, mutant strains, and strains transformed with plasmids carrying or not carrying different alleles of *Ustilago maydis Kpp4*

Cross	Mating efficiency*
E151 × P01A	$5.9 \times 10^{-4} \pm 3.90 \times 10^{-5}$
E151 × 2Q	0
E151 × 4Q	0
E151 × 6Q	0
E151 × 2T	0
E151 × 5T	0
E151 × 7T	0
E151 × 6CAT	$4.6 \times 10^{-5} \pm 0.26 \pm 10^{-5}$
E151 × 9CAT	$1.6 \times 10^{-5} \pm 013 \times 10^{-5}$
E151 × AC1	0

*Mating efficiency ± SD as described in Table 3.

P01A, parental strain; AC1, Δ*ste11* mutant; E151, *MATB* strain; 2Q, 4Q, 6Q, strains transformed with pKpp4-Q1 containing *Ustilago maydis Kpp4* ORF driven by *Yarrowia lipolytica XPR2* promoter; 2T, 5T, 7T, strains transformed with pTs5 containing *U. maydis Kpp4* ORF driven by *Y. lipolytica STE11* promoter; 6CAT, 9CAT, strains transformed with pKpp4-CAT2 containing *U. maydis* truncated *Kpp4* ORF driven by *Y. lipolytica STE11* promoter.

6c). These results demonstrate the inability of the *Kpp4* ORF to reverse the effect of the *STE11* mutation in *Y. lipolytica*.

Transformation of *Y. lipolytica* Δ*ste11* mutants with a truncated version of the *U. maydis Kpp4* gene

Given that neither the sterile phenotype nor the inability to grow in the mycelial form shown by AC1 and AC2 mutant strains were reversed using the whole *Kpp4* ORF, another strategy was attempted. In *S. cerevisiae*, it has been reported that specific point mutations in the *STE11* gene give rise to the constitutively active alleles *STE11-1* and *STE11-4* (Stevenson *et al*., 1992). The same thing occurs with deletion of the N-terminal region (*STE11ΔN*; Cairns *et al*., 1992). These alleles give rise to the constitutive activation of the corresponding kinase. As for *STE11-1* and *STE11ΔN*, the corresponding active alleles *kpp4PS* and *kpp4-2* have been described in *U. maydis* (Müller *et al*., 2003). Mutants of either fungus show the morphological alterations displayed by wild-type mating cells, in the absence of the corresponding partner or the opposite pheromone (Cairns *et al*., 1992; Stevenson *et al*., 1992; Müller *et al*., 2003). On the basis of these results, we decided to use a truncated version of the *U. maydis Kpp4* gene that encoded a protein conserving the catalytic, but not the regulatory domain, to try to complement our *Y. lipolytica* Δ*ste11* mutants. The hypothesis was to leave the catalytic domain free to interact with the substrate Mapkk, thus allowing the operation of the MAPK phosphorylation cascade, and the rationale for using the *U. maydis* gene was based on its higher similarity to *YlSTE11* than the yeast gene. To achieve this aim, we constructed a putative functional allele of the *Kpp4* gene (*Kpp4CAT*) by elimination of the N-terminus domain (encoding amino acids 1–1002), which in Mapkkk proteins has regulatory and inhibitory functions, preventing the protein from being active unless it is correctly bound to other proteins (Cairns *et al*., 1992; Müller *et al*., 2003). Mutants AC1 and AC2 were transformed with plasmid pKpp4-CAT2, which carries the truncated form of the *Kpp4* gene. Several *Leu+* transformants were recovered. After confirming that the correct plasmid was harbored by the transformant strains 6CAT and 9CAT, their dimorphic and mating capacities were assayed. The results obtained showed that the *Kpp4CAT* allele was able to reverse the sterile phenotype exhibited by the AC1 and AC2 mutants, when crossed with strain E-151 (Table 6 and Fig. 8). Confirmation of the ploidy nature of several of the diploids obtained was assessed by amplification of the mating-type loci by PCR, using the technique described above (data not shown). The capacity to grow in the mycelial form was also recovered in 6CAT and 9CAT transformants (Table 7 and Fig. 6d). These results are evidence that the phenotypic effects originating from deletion of *STE11* were efficiently relieved by the truncated, but not by the wild-type, *Kpp4* ORF.

Discussion

In fungi, two main signal transduction pathways have been related to dimorphism: the MAPK and protein kinase A (PKA) pathways (reviewed in Banuett, 1998; Lengeler *et al*., 2000). The objective of the present study was to analyze the possible role of the MAPK pathway in the morphogenesis of *Y. lipolytica*. To achieve this aim, we proceeded to the isolation and disruption of the gene encoding the first member of a MAPK cascade. The results obtained by analysis of the sequence of the isolated gene, and the similarities of the encoded protein with Mapkkk proteins from different fungi, led us to conclude that we had indeed identified the *Y. lipolytica* homolog, which was named *STE11* after observing that it was essential for mating. This conclusion was later corroborated through the functional complementation of *ste11*Δ *Y. lipolytica* mutants, using a truncated allele of the homolog *Kpp4* gene from *U. maydis*. The encoded product exhibited typical characteristics of this kind of protein kinase.

The behavior of Δ*ste11* mutants demonstrates that the missing function of the gene did not compromise basic cellular programs such as growth capacity or cell wall integrity. It is interesting to recall that in *S. cerevisiae*, expression of *FKS2* (a gene encoding a β-1,3-glucan synthase) is under the control of *STE11*, and its levels are reduced two- to three-fold in Δ*ste11* mutants (Lee & Elion,

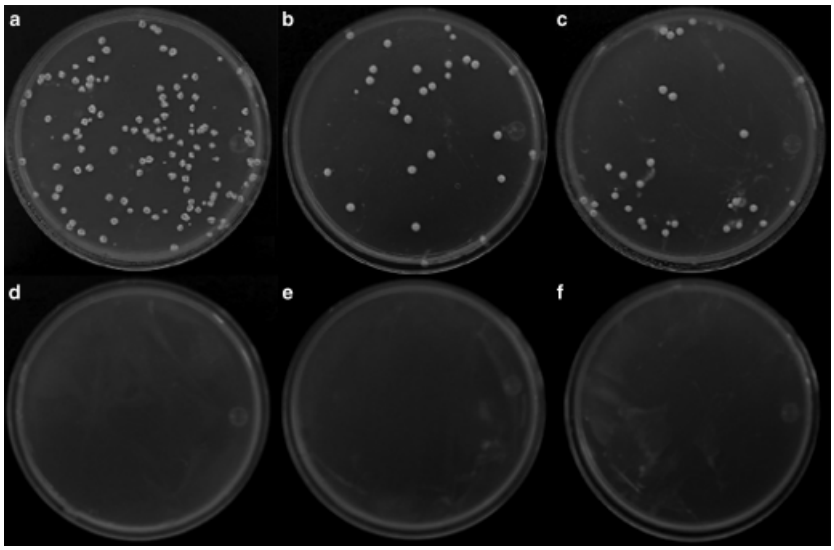

Fig. 8. Mating assays of parental and Δ*ste11* mutants, native and transformed with the *Kpp4CAT* allele. Conjugation was conducted in YMC medium for 17 days. Then diploids were grown on plates of selective YNB media. Mating crosses were: (a) E151 × P01A; (b) E151 × 6CAT; (c) E151 × 9CAT; (d) E151 × AC1; (e) E121 × 6CAT; (f) E121 × 9CAT.

Table 7. Dimorphic transition in Δ*ste11* mutants transformed or not transformed with a plasmid carrying the *Kpp4CAT* allele

	AC1		6CAT		9CAT	
Growth medium	M	Y	M	Y	M	Y
YNB–Glu, pH 7, citrate	3	97	75	25	75	25
YNB–GlcNAc, pH 7, citrate	3	97	63	37	60	40
YNB–Glu, pH 7, PO_4, serum	3	97	73	27	72	28

Morphology as defined for Table 4.
Growth media and growth conditions as defined for Table 4; PO_4, phosphate buffer.
AC1, Δ*ste11* strain; 6CAT, 9CAT, transformed with pKpp4-CAT2 containing the *Ustilago maydis* truncated *Kpp4* gene.
Results are averages of three experiments, each with duplicate samples. SD did not exceed a value of 6.

1999). Additionally, these authors found that *FKS2* expression increased 44-fold in an *STE11-4* strain that expresses a constitutively activated form of Ste11p (Lee & Elion, 1999). These results suggest a role of *STE11* in *S. cerevisiae* cell wall integrity, through regulation of β-1,3-glucan synthesis. *Yarrowia lipolytica* contains a single *FKS* homolog that is essential for viability (León *et al.*, 2002). Accordingly, we tested the effect of some agents that disturb cell wall integrity (Calcofluor white and β-1,3-glucanase) on Δ*ste11* mutants, and observed that the parental and mutant strains were equally sensitive to both agents. These results indicate that, contrary to the situation in *S. cerevisiae*, *STE11* is not involved in a pathway related to the maintenance of cell wall integrity. Growth of Δ*ste11* mutants was also unaffected by cultivation in media containing high concentrations of NaCl or sorbitol. In *S. cerevisiae,* the Hog pathway (involved in osmotic stress protection) is activated not only by Ste11p, but also by Ssk2p and Ssk22p redundant kinases (reviewed in Saito & Tatebayashi, 2004). Probably in *Y. lipolytica* as in *S. cerevisiae*, other Mapkkk may be involved in the response to osmotic stress.

A developmental program in which fungal Mapkkk proteins are known to participate is the regulation of the response to pheromones. These induce important morphological changes in sexually compatible strains that culminate in mating (for reviews see Elion, 2000; Bardwell, 2005). We found that the mating competence of *Y. lipolytica* was totally abolished by the *STE11* mutation. This result agrees with the behavior of *Sch. pombe byr2*/*ste8* mutants, the *STE11* homolog (Styrkársdóttir *et al.*, 1992), *Cr. neoformans* (Clarke *et al.*, 2001) and *S. cerevisiae* Δ*ste11* mutants (Chaleff & Tatchell, 1985), which are also completely sterile. In *A. nidulans* (Wei *et al.*, 2003) and *U. maydis* (Müller *et al.*, 2003), only crosses between two sexually compatible mutant strains are sterile.

Regarding morphogenesis, deletion of *Y. lipolytica STE11* evidenced the active participation of the MAPK pathway in hyphal growth. The Δ*ste11* mutants were unable to respond to neutral pH, citrate or serum addition, effectors that induce the yeast-to-mycelium transition in the parental strain. These results suggest that in *Y. lipolytica*, *STE11* is involved in the single pathway responsible for signal transduction in dimorphism, independently of the several stimuli that induce the yeast-to-hypha dimorphic transition. In this sense, it contrasts with *C. albicans*, in which multiple independent pathways exist to induce hyphal growth (Braun & Johnson, 2000; reviewed in Whiteway & Oberholzer, 2004). Also, in this fungus the PacC/Rim101p transcription factor and its processing pathway (Pal/Rim) are involved in the pH-dependent dimorphic transition (Ramon *et al.*, 1999). In contrast, *Y. lipolytica* mutants affected in genes of the Pal/Rim pathway show no defects in filamentation in response to citrate or serum at neutral pH (González-López *et al.*, 2006). A newly described pH-response pathway

dependent on the *MDS3* gene has been implicated in *C. albicans* morphogenesis. Homozygotic *mds3*/*mds3* mutants are defective in filamentation in response to alkaline pH, but not in response to serum or GlcNAc (Davis *et al*., 2002). The failure of the activated Rim101-405 protein to suppress the filamentation defect of *mds3*/*mds3* homozygous diploids supports the hypothesis that Rim101 and Mds3p act in independent regulatory pathways (Davis *et al*., 2002).

As shown here, in *Y. lipolytica* the MAPK pathway is involved in hyphal growth, whereas previous data suggest that the PKA pathway is related to yeast-like growth, by the observation that addition of cAMP induces yeast development (Ruiz-Herrera & Sentandreu, 2002). In *U. maydis* also, both signal pathways work in opposite directions. cAMP addition induces yeast-like growth, and mutants in any member of the MAPK pathway also grow in a yeast-like way (Martínez-Espinoza *et al*., 2004). Moreover, mutants deficient in the gene encoding adenylate cyclase (*uac1*) grow constitutively in the hyphal form, and this phenotype is efficiently suppressed in double mutants simultaneously deficient in genes belonging to the MAPK pathway (Martínez-Espinoza *et al*., 2002, 2004), as well as in *ubc1* mutants that are deficient in the gene that encodes the regulatory PKA subunit (Gold *et al*., 1994).

Surprisingly, we found that *STE11* was expressed only when cells were grown under conditions that induced mycelial development. Cells grown in the yeast form, either at acid or at neutral pH with different carbon or nitrogen sources that fail to induce the mycelial transition (Ruiz-Herrera & Sentandreu, 2002), did not express the gene at detectable levels. These results suggest the existence of a tight association between Ste11p levels and mycelial growth. This behavior contrasts with the observation that *S. cerevisiae STE11* (Chaleff & Tatchell, 1985) and *Cr. neoformans STE11*α (Clarke *et al*., 2001) are constitutively expressed in rich media, and are not regulated by the mating type locus. In *Sch. pombe*, *BYR2*/*STE8* is expressed in rich media, but can be further induced by nitrogen starvation (Styrkársdóttir *et al*., 1992).

The recovered ability to mate, as well as to grow in the hyphal form in response to mycelial inducers in the *Y. lipolytica* strains JAC1 and JAC2 transformed with the *STE11* wild-type gene, confirmed that the phenotypic characteristics displayed by the AC1 and AC2 mutants were indeed the result of *STE11* disruption.

The observation that the *Y. lipolytica STE11* was unable to rescue the sterile phenotype in a Δ*ste11 S. cerevisiae* mutant (although the gene was expressed), and that the *U. maydis KPP4* gene was also unable to do the same in *Y. lipolytica* Δ*ste11* mutants, regardless of whether their expression was regulated by native, inducible or constitutive promoters, deserves a comment. It is known that in *S. cerevisiae* responses to osmotic stress, pheromone and invasive growth, physical interactions with Ste11p, the adaptor protein Ste50p and kinase Ste20p are indispensable, all of them playing a critical role in the output of each pathway. Also, in the pheromone response, an interaction with the scaffold Ste5 protein must exist (see Bardwell, 2005). Similarly, in *Sch. pombe* Byr2p/Ste8p (Mapkkk), the regulatory domain is necessary and sufficient for bridging the interaction with Ste4p and Ras1p (Tu *et al*., 1997). *Yarrowia lipolytica* Ste11p has a high similarity to the *U. maydis* and *S. cerevisiae* homologs, but only at the catalytic domain. No important similarities are found at those domains responsible for the specific protein interactions. Accordingly, it was hypothesized that this phenomenon makes the transgenic proteins unable to interact correctly at the corresponding complexes and fulfill their necessary functions. Similarly, although *Sch. pombe* Byr2p/Ste8p is highly similar to Ste11p from *S. cerevisiae* (Styrkársdóttir *et al*., 1992), full complementation of mutants in this gene with the *S. cerevisiae* gene was highly variable, occurring in only a few cases (Styrkársdóttir *et al*., 1992).

The success of the strains transformed with the truncated version of the *U. maydis Kpp4* gene in recovering the wild-type phenotype demonstrates that the hypothesis is probably correct. The observation that Δ*ste11* mutants transformed with the *Kpp4* truncated version recovered their mating capacity to similar levels as the wild-type gene did contrasts with *S. cerevisiae*, in which the Ste11p alleles *STE*Δ*SAM* (missing SAM domain region) or *STE*Δ*EE* (missing residues that interact with Ste5p) only partially activated the mating pheromone pathway in an *ssk*2Δ, *ssk*22Δ, Δ*ste11* strain (Wu *et al*., 1999). *Kpp4CAT* also efficiently reversed the defect in the dimorphic transition of Δ*ste11* mutants. This result reinforces our hypothesis, indicating the functionality of the catalytic domain of *Kpp4* in *Y. lipolytica* for both mating and dimorphism. It is important to recall that functionality of Ste11p in this fungus is similar to that in *U. maydis* and *S. cerevisiae*, where the homolog Mapkkks are involved in two processes: mating and dimorphism in *U. maydis* (Martínez-Espinoza *et al*., 2002; Müller *et al*., 2003), and mating and pseudomycelium formation in *S. cerevisiae* (Chaleff & Tatchell, 1985, reviewed in Lengeler *et al*., 2000).

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