Ambient pH signalling in the yeast *Yarrowia lipolytica* involves YIRim23p/PalC, which interacts with Snf7p/Vps32p, but does not require the long C terminus of YIRim9p/PalI

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A conserved ambient pH signal transduction pathway has been evidenced in both ascomycetous yeasts and filamentous fungi, called the Rim or Pal pathway, respectively. However, closely related PalC orthologues are found only in *Yarrowia lipolytica* and in filamentous fungi, where the Rim9p/PalI factor has a much longer C-terminal tail than in other yeasts. We show here that, like *Aspergillus nidulans* *pall* mutants, a *YlrIm9Δ* mutant has a less extreme phenotype than other mutants of the pathway, whereas *rim9* mutants in *Saccharomyces cerevisiae* and *Candida albicans* reportedly exhibit a tight Rim phenotype. Deletion of the long C-terminal tail of YIRim9p/PalI had no phenotypic effect on ambient pH signalling. We also show that the *Y. lipolytica* PalC orthologue, named YIRim23p, is absolutely required for the alkaline pH response. Its only interactant identified in a genome-wide two-hybrid screen is YISnf7/Vps32p, confirming the link between the Rim and the Vps pathways. YIRim13p and YIRim20p both interact with YISnf7/Vps32p but not with YIRim23p. The long C-terminal tail of YIRim9p/PalI interacts neither with YIRim23p nor with YISnf7/Vps32p. These results show that YIRim23p is a bona fide component of the Rim pathway in *Y. lipolytica* and that it participates in the complexes linking pH signalling and endocytosis.

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INTRODUCTION

Extracellular pH regulation plays major roles in the control of many biological processes, including the survival of fungi and bacteria and the virulence of some of these micro-organisms. Environmental pH profoundly affects gene expression in many organisms, especially in those which are able to grow over a wide pH range. Ambient pH-responsive genes include those encoding permeases, plasma-membrane transporters, secreted proteins and enzymes involved in the synthesis of exported metabolites. In the yeast *Yarrowia lipolytica*, transcription of the *XPR2* and *AXP1* genes, encoding alkaline and acidic extracellular proteases, respectively, is tightly controlled by a combination of environmental stimuli, including ambient pH and nutrient availability (Ogrydziak *et al.*, 1977; Ogrydziak, 1993). In *Candida albicans*, the genes encoding the three proteases Sap4p, Sap5p and Sap6p are under the control of extracellular pH, Sap5p being required for E-cadherin degradation during mucosal tissue invasion (Villar *et al.*, 2007).

These genes are regulated by a conserved pH signalling pathway called Pal in filamentous fungi and Rim in yeasts.

This pathway has been intensively investigated in *Aspergillus nidulans* (Penalva & Arst, 2002; Herranz *et al.*, 2005; Hervas-Aguilar *et al.*, 2007; Galindo *et al.*, 2007; Penas *et al.*, 2007; Calcagno-Pizarelli *et al.*, 2007), in *Y. lipolytica* (Lambert *et al.*, 1997; Tréton *et al.*, 2000; Gonzalez-Lopez *et al.*, 2002; Blanchin-Roland *et al.*, 2005), in several other ascomycetes, including *Saccharomyces cerevisiae* (Li & Mitchell, 1997; Xu & Mitchell, 2001; Vincent *et al.*, 2003; Xu *et al.*, 2004; Boysen & Mitchell, 2006), in *C. albicans* (Ramon & Fonzi, 2003; Li *et al.*, 2004; Kullas *et al.*, 2004; Cornet *et al.*, 2005; Barwell *et al.*, 2005; Baek *et al.*, 2006), in the basidiomycete *Ustilago maydis* (Arechiga-Carvajal & Ruiz-Herrera, 2005) and in other fungi (Penalva & Arst, 2002, 2004). At alkaline pH, a cascade of six *pal* or *RIM* genes activates the zinc-finger transcriptional factor PacC/Rim101p through a complex C-terminal proteolytic processing event (see below). Previous reports have established that components of the endocytic trafficking cooperate with the Rim pathway in the recruitment of Rim20p and its targeting to Rim101p. Endosomal sorting complex required for transport (ESCRT)-I, -II and -III are three protein complexes that act sequentially in the sorting of both ubiquitinated membrane protein and biosynthetic cargos

Abbreviation: ESCRT, endosomal sorting complex required for transport.

to the multivesicular body (MVB) vesicles, which are ultimately delivered to the lumen of the vacuole for degradation (Hurley & Emr, 2006). ESCRT-III proteins Snf7/Vps32p and Vps20p and all ESCRT-I and -II components are required for Rim101p activation in *S. cerevisiae* (Xu *et al.*, 2004; Rothfels *et al.*, 2005; Hayashi *et al.*, 2005). This functional link between the Rim and Vps pathways is conserved in *C. albicans* (Xu *et al.*, 2004; Kullas *et al.*, 2004; Cornet *et al.*, 2005) and *Y. lipolytica* (Blanchin-Roland *et al.*, 2005). The PacC/Rim101p truncated form is able to activate alkaline pH-responsive genes and to repress acid-induced genes (for reviews see Penalva & Arst, 2002, 2004). At acidic pH, the Rim/Pal pathway is admittedly inactive, although a specific processing event of Rim101p has been reported in *C. albicans* (Li *et al.*, 2004). In *Y. lipolytica*, the Rim pathway seemed to be indirectly required at acidic pH for the activation of the acid-responsive *AXP1* gene (Gonzalez-Lopez *et al.*, 2002).

Besides its major role in the ambient alkaline pH response and growth, the Rim pathway appears essential for mating and sporulation in *Y. lipolytica* (Lambert *et al.*, 1997) and it controls sporulation in *S. cerevisiae* by activating *IME1* expression (Li & Mitchell, 1997) and corepressing, with Nrg1, *DIT1* expression in mitotic cells (Rothfels *et al.*, 2005). It also regulates cell differentiation during haploid invasive growth and diploid pseudohyphal differentiation in *S. cerevisiae* (Lamb & Mitchell, 2003). It is required for the hyphal formation at alkaline pH in *C. albicans*, in particular by repressing *NRG1* expression in a pH-dependent way (El Barkani *et al.*, 2000; Lotz *et al.*, 2004). Moreover, the resistance of cells to high levels of Ca^{2+} and Li^+ requires activity of the Rim pathway, and the subset of ESCRT components needed for Rim101p processing in *S. cerevisiae* (Lamb & Mitchell, 2003; Platara *et al.*, 2006), in *C. albicans* (Kullas *et al.*, 2007) and in *Fusarium oxysporum* (Caracuel *et al.*, 2003). In addition, the Rim pathway participates directly in cell-wall assembly, and acts in parallel with the protein kinase C (PKC) pathway in *S. cerevisiae* (Castrejon *et al.*, 2006). In *C. albicans*, this pathway regulates *PHR1* and *PHR2*, encoding two isoforms of the cell-wall glycosidases important for adaptation to ambient pH (Muhlschlegel & Fonzi, 1997), and represses the acid-expressed cell-wall gene *RBR1*, encoding a glycosylphosphatidylinositol (GPI) protein required for filamentation (Lotz *et al.*, 2004). Finally, defects in ambient pH response lead to reduced virulence in *C. albicans*, *A. nidulans* and other pathogenic fungi (Penalva & Arst, 2002; Davis, 2003; Bignell *et al.*, 2005; Cornet *et al.*, 2005; Mitchell *et al.*, 2007; Villar *et al.*, 2007).

According to the current model, largely elaborated through studies in *A. nidulans*, the pH signalling pathway is organized into two complexes: a plasma-membrane complex, including Rim21p/PalH, Rim9p/PalI and Rim8p/PalF, and an endosomal-membrane complex, including Rim20p/PalA, Snf7/Vps32p, Rim13p/PalB, to which Rim101p/PacC is recruited for its proteolytic activation. The arrestin-like protein PalF (Rim8p) binds

strongly to two regions within the C-terminal cytosolic tail of the 7TM PalH protein, which is the likely pH sensor (Herranz *et al.*, 2005). At alkaline pH, PalF is phosphorylated and ubiquitinated in a pH- and PalH-dependent manner. These PalF modifications are partially dependent on the 3TM protein PalI, but independent of the three other Pal proteins: PalA, PalB and PalC (Herranz *et al.*, 2005). Recently, PalI (Rim9p) was shown to localize to the plasma membrane where it may assist localization of PalH (Calcagno-Pizarelli *et al.*, 2007). PalF phosphorylation and ubiquitination may lead to the endocytosis of the PalF–PalH complex, transducing the signal to the downstream complex (Herranz *et al.*, 2005). Several reports in *A. nidulans*, *S. cerevisiae* and *C. albicans* clearly show that PalA/Rim20p interacts with both Snf7/Vps32p, a subunit of ESCRT-III (see above), and PacC/Rim101p (Vincent *et al.*, 2003; Xu & Mitchell, 2001). Both Rim20p–Rim101p and Rim20p–Snf7/Vps32p interactions are required for Rim101p processing. An interaction between the calpain-like protease Rim13p/PalB and Snf7/Vps32p was detected during one whole-genome screen of two-hybrid interactants in *S. cerevisiae* (Ito *et al.*, 2001), but no direct interaction between Rim13p/PalB and Rim101p/PacC has been reported. In *A. nidulans*, PalB was recently shown to be the ‘signalling’ protease that catalyses the first, pH-regulated cleavage of PacC (Penas *et al.*, 2007), whereas the second processing step involves the proteasome (Hervas-Aguilar *et al.*, 2007).

PalC was the only Pal protein with no homologue in hemiascomycetous yeasts, except *Y. lipolytica*. However, recently, YGR122w was identified as a likely *S. cerevisiae* orthologue (Galindo *et al.*, 2007). Ygr122wp was previously shown to bind Snf7/Vps32p (Ito *et al.*, 2001; Uetz *et al.*, 2000) and to be required for activation of Rim101p (Rothfels *et al.*, 2005; Barwell *et al.*, 2005), and *ygr122wΔ* results in Li^+ hypersensitivity (Galindo *et al.*, 2007). A mutational analysis of PalC revealed conserved regions, one potentially functional as a BRO1 domain (Tilburn *et al.*, 2005). While this manuscript was in preparation, PalC was shown to localize to cortical structures at alkaline pH and to bind Snf7/Vps32p (Galindo *et al.*, 2007).

In this paper, we show that YIRIM23, the orthologue of PalC, is required for the pH response as well as for mating in *Y. lipolytica*. We also report that YIRim23p binds to YISnf7/Vps32p and confirm that YISnf7/Vps32p binds to both YIRim13p and YIRim20p. We further show that the long C-terminal tail of YIRim9p does not seem to be involved in the pH response.

METHODS

Strains and sequence data. The *Escherichia coli* strain used was Mach1-T1 (Invitrogen). Yeast strains are described in Table 1. Unless otherwise stated, sequence data were obtained from the complete genome assembly of *Y. lipolytica* (<http://cbi.labri.fr/Genolevures>; Dujon *et al.*, 2004).

Table 1. Strains used in this study

Strain	Relevant genotype	Source
AM4	<i>MATB ura3-302 leu2-270 his-1 XPR2 LEU2 XPR2' : lacZ</i>	Lambert <i>et al.</i> (1997)
SY12	<i>MATA ura3-302 leu2-270 lys11-23 XPR2 LEU2 AXP1' : gusC XPR2' : lacZ</i>	Gonzalez-Lopez <i>et al.</i> (2002)
LAM26-03	<i>MATA ura3-302 leu2-270 XPR2 LEU2 XPR2' : lacZ pal1-26/rim13-26</i>	Lambert <i>et al.</i> (1997)
LAM21-03	<i>MATA ura3-302 lys11-23 leu2-270 XPR2::LEU2::XPR2':lacZ pal3-21/rim8-21</i>	Lambert <i>et al.</i> (1997)
SY1359u	<i>MATA ura3-302 leu2-270 lys11-23 XPR2 LEU2 AXP1' : gusC XPR2':lacZ rim23Δ-10</i>	This study
SY1375u	<i>MATA ura3-302 leu2-270 lys11-23 XPR2 LEU2 AXP1' : gusC XPR2':lacZ rim9Δ-13</i>	This study
SY1371	<i>MATA ura3-302 leu2-270 lys11-23 XPR2 LEU2 AXP1' : gusC XPR2' : lacZ rim9Δ-13 RIM9</i>	This study
SY1372	<i>MATA ura3-302 leu2-270 lys11-23 XPR2 LEU2 AXP1' : gusC XPR2' : lacZ rim9Δ-13 RIM9SL</i>	This study
SY1389	<i>MATA ura3-302 leu2-270 lys11-23 XPR2 LEU2 AXP1' : gusC XPR2' : lacZ rim9Δ-13 RIM9SL rim23Δ-1</i>	This study
SY1390	<i>MATA ura3-302 leu2-270 lys11-23 XPR2 LEU2 AXP1' : gusC XPR2' : lacZ rim9Δ-13 rim23Δ-31</i>	This study
PJ69-4A	<i>MATa trp1-901 leu2-3112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ</i>	James <i>et al.</i> (1996)

Culture media and phenotypic tests. Complete YPD medium and derepressing medium (Y) have been described previously (Lambert *et al.*, 1997). Solid and liquid media were buffered at pH 4.0 (with 0.2 M citrate buffer), at pH 7.0 (with 0.2 M sodium phosphate buffer) or at pH 8.0 (with 25 mM Tris/HCl) and supplemented with 0.1 g uracil l⁻¹. The pH of liquid media was stable within 0.3 pH units during growth. Growth at various pHs was assayed as previously described (Blanchin-Roland *et al.*, 2005). Estimation of efficiency of mating for compatible *Y. lipolytica* auxotrophs was done as described by Lambert *et al.* (1997).

DNA and RNA techniques. Standard recombinant DNA techniques were performed essentially as previously described (Gonzalez-Lopez *et al.*, 2002). PCR products were routinely checked by sequencing; sequences were obtained from the DNA sequencing department of

Genome Express. They were assembled and annotated using the GCG package (University of Wisconsin, Madison, WI, USA). All transformation events were checked by colony PCR and confirmed by Southern analysis. Gene expression was determined by real-time quantitative reverse transcriptase-PCR as previously described (Blanchin-Roland *et al.*, 2005).

Deletion of YIRIM23 and YIRIM9. The *rim23Δ::URA3* and the *rim9Δ::URA3* deletion cassettes were constructed according to Fickers *et al.* (2003). The promoter and terminator regions of each gene were amplified using primers RIM23FP1/RIM23P2 + RIM23T1/RIM23HT2 or RIM9CP1/RIM9P2 + RIM9T1/RIM9ET2, respectively (see Table 2 for primer sequences). For each gene, the resulting fragments were annealed and amplified with the flanking primers. These cassettes were cloned into pBSKS+ to generate plasmids BSPTRIM23 and

Table 2. Primers used in this study

Primer	Sequence (5'–3')
RIM23FP1	GGCCTCGACGCACTCCTGTGAGAGCCTCTGCATAG
RIM23P2	ATTACCCTGTTATCCCTAGGTCGTTTAGTTGCAGTCTCGTGCTG
RIM23T1	TAGGGATAACAGGGTAATCTCCATCTGCACACACAGTCAACACAC
RIM23HT2	GATTTCGGCGTCGTCGCAAGGGTCGTCAGTTCACG
RIM23DHNc	GCGGCCATGGGCATATCGTATACCGGCCAG
RIM23DHBa	CGCGGATCCGCTAGTAGTAGAACCCCTGGC
RIM23avalT	GCCACATTGGCTATTGGCAG
RIM9CP1	CCACTTTCATTCTGAAGAGAATGGCTGGGGACGC
RIM9P2	ATTACCCTGTTATCCCTAGAGCGATTATGGGGGGGACTGTTAGC
RIM9T1	TAGGGATAACAGGGTAATACAACCTAATCAGGCACTCTAGAGCCTGG
RIM9ET2	CCTCTACCTGAATTCCATGGCCCGATACGGAAAGTC
RIM91	GGACACAAGCCGCGGCCTAATCAGCCA
RIM92r	GCTTGGAGCCCTGGTATTGACGAGTC
RIM9end5	CCAGCCCCGACTGATAGGACCAGGGTGG
RIM9end3r	CCACCCTGGTCTATCAGTCGGGGCTGG
Rim9avalT	ACTCTATGGCTCTGTGGACC
RIM9CDHNc	GCGGCCATGGGCACTAACAACCTCTCGAGTC
RIM9CDHBa	GCGGGATCCTTCATCTCATGCTGTATGGTCC
RIM8DHSf	ACGCGGGCCATGGAGGCCATGAAATCGGCTTTCAAC
RIM8DHBa	CCGCGGATCCCTAAGAATAAATATCGTCGCTGTCAG
RIM13DHNc	CGCCCCATGGCTCTTCCGGAGGAAATATAC
RIM13DHBa	CGCGGATCCCTAATGACCCAAAGTCACCAAC

BSPTTRIM9, respectively. The loxR-URA3-loxP module (Fickers *et al.*, 2003) was inserted between promoter and terminator sequences at the I-SceI site, generating plasmids pINA1375 and pINA1359, respectively. The cassettes were excised from these plasmids and purified with the QIAquick gel extraction kit (Qiagen). The RIM9 deletion cassette was integrated by double crossover at the YIRIM9 locus of the SY12 strain to give four independent clones of the SY1375 strain (*YlrIm9Δ-13* allele). The RIM23 deletion cassette was similarly used to generate two independent clones of the SY1359 strain (*YlrIm23Δ-10* allele). YIURA3 was excised from the SY1375 strain by using the plasmid pUB4-CRE (Fickers *et al.*, 2003) to give the SY1375u strain.

To delete YIRIM23 in the *YlrIm9Δ-13* background and in the SY1372u strain carrying the YIRIM9SL allele (see below), a deletion cassette was constructed from the loxR-Hph-loxP module (Fickers *et al.*, 2003) and cloned into BSPTTRIM23 at the I-SceI site, to generate plasmid pINA1387. The deletion cassette was excised from pINA1387, purified as above, then integrated by double crossover at the YIRIM23 locus into the SY1372u strain to generate five independent clones of the SY1389 strain (YIRIM9SL, *YlrIm23Δ-31*) and into the SY1375u strain to generate four independent clones of the SY1390 strain (*YlrIm9Δ-13*, *YlrIm23Δ-31*).

Subcloning of the YIRIM9 gene. The complete YIRIM9 ORF flanked by 660 bp of upstream and 544 bp of downstream sequences was rescued from plasmid AW0AA029A08 (AL414126). To increase the size of the upstream sequence, a PCR product was amplified from SY12 genomic DNA using primers RIM91 and RIM92r (Table 2). Both this PCR product and the fragment carrying RIM9 were inserted into the integrative URA3 plasmid pINA300' to give pINA1371.

Construction of the mutant encoding a C-terminally truncated YIRim9p. The YIRIM9SL-1372 allele was obtained by overlapping PCR and contains two mutations, A889T and C894G, which replace the Arg297 and Tyr298 codons by nonsense codons within the RIM9 coding sequence. PCR products were obtained from genomic DNA with primer pairs RIM9CPI-RIM9end3r and RIM9end5-RIM9ET2 (Table 2), mixed and amplified with flanking primers to generate a full-length product containing the internal mutations. This PCR product was inserted into pINA1371 to generate plasmid pINA1372, which was checked by sequencing. The BamHI-digested pINA1372 was targeted to the promoter of RIM9 in the SY1375u strain (*YlrIm9Δ-13* allele) to give three independent clones of the SY1372 strain (YIRIM9SL allele). BamHI-digested pINA1371 was similarly targeted in the SY1375u strain to give three independent clones of the SY1371 strain (YIRIM9 allele). YIURA3 was excised from the SY1372 strain, using pUB4-CRE as above, to give the SY1372u strain.

Yeast two-hybrid assay. *S. cerevisiae* PJ69-4A (Table 1) and plasmids pAS2ΔΔ (TRP1, bla) and pACT2 (LEU2, bla) were used for the two-hybrid analysis (James *et al.*, 1996). Primers were designed to amplify the complete RIM8 nucleotide sequence (RIM8DHSf and RIM8DHBa), the complete RIM13 nucleotide sequence (RIM13DHNC and RIM13DHBa), the complete RIM23 nucleotide sequence (RIM23DHNC and RIM23DHBa) and the C-terminal RIM9 coding sequence from codon 254 to the stop codon 724 (RIM9CDHNC and RIM9CDHBa) (Table 2). The PCR products were digested by NcoI (or SfiI in the case of RIM8) and BamHI to allow in-frame cloning in the multisite of each two-hybrid vector. The recombinant vectors carrying the complete RIM20 nucleotide sequence were constructed by B. Tréton (unpublished). Empty and recombinant two-hybrid vectors were co-transformed into yeast cells and transformants were selected on minimal medium plus histidine, methionine, uracil and adenine for selection of the plasmids. Purified transformants were streaked on YNB plus histidine, methionine and uracil, on YNB plus adenine, methionine, uracil and 5 mM 3-aminotriazole or on YNB plus methionine and uracil for direct selection of the interactants (James *et al.*, 1996).

Two-hybrid library screening. The PJ69-4x strain was transformed with pAS2ΔΔ-RIM23 (see above), and a mid-exponential culture (120 ml) of the resulting transformant was mixed with an aliquot of each of the three *Y. lipolytica* two-hybrid libraries constructed in pGAD-C1 to C3 plasmids in PJ69-4A (Kabani *et al.*, 2000) at a ratio of 3:1 (bait:prey). After mating at 30 °C for 16 h, cells were harvested and plated onto YNB plus adenine, methionine, uracil and 5 mM 3-aminotriazole for selection of His⁺ colonies. The number of diploid and parental cells was determined as previously described (Kabani *et al.*, 2000). The number of diploids obtained (BC1, 9.3×10^7 ; BC2, 4.7×10^9 ; BC3, 7.5×10^5) was sufficient to ensure a representative sampling of the two first pools of the library (10^7 clones per pool). After 7–8 days at 30 °C, His⁺ diploids were replica-plated onto YNB plus histidine, methionine and uracil plates. Ade⁺ colonies were recovered from the third to the eighth day and streaked twice on YNB plus methionine and uracil plates to retain Ade⁺ His⁺ diploids.

RESULTS AND DISCUSSION

Role of YIRIM23 and YLRIM9 in ambient pH response

Previous approaches led us to identify orthologues of all the *A. nidulans* Pal pathway members except one, PalC (Lambert *et al.*, 1997; Gonzalez-Lopez *et al.*, 2002). A putative orthologue (YALIOF17028 g) of palC was identified from the *Y. lipolytica* genome (Dujon *et al.*, 2004; Tilburn *et al.*, 2005). The predicted 441 aa protein was named YIRim23p. A likely PalC orthologue, encoded by YGR122w, was recently identified in *S. cerevisiae* (Galindo *et al.*, 2007). Moreover, PalH has two homologues in *S. cerevisiae* and *C. albicans*, Rim21p and Dfg16p (Barwell *et al.*, 2005), but only one in *Y. lipolytica*, Rim21p. We found two putative paralogues of YIRim21p (YALIOB22814g and YALIOB06710g) in the *Y. lipolytica* genome. Deletion of each of these ORFs had no effect on pH response (not shown). This suggests that the *Y. lipolytica* genome carries only one functional equivalent of PalH/YIRim21 and none of ScDfg16p.

To check if YIRIM23 was involved in ambient pH signalling, the SY1359 strain carrying the null mutation (*YlrIm23Δ-10*) was created (see Methods). We previously observed that the *YlrIm9-D7* insertional mutant had a milder effect than other rim mutants on growth or PHR1 expression at alkaline pH (Gonzalez-Lopez *et al.*, 2002). To check whether the same leaky phenotype was displayed by a null mutant, the SY1375 strain carrying the *YlrIm9Δ-13* allele was constructed (see Methods). The effects of YIRIM23 or YIRIM9 deletion on the transcription of different pH-regulated genes were assessed by real-time quantitative PCR on total RNAs extracted from cultures grown in Y medium at pH 4.0 and pH 8.0, using actin transcript as a reference. As ambient alkaline pH-responding genes, we chose XPR2 and YIPHR1 and as acidic pH-responding gene, YIPHR2 (Blanchin-Roland *et al.*, 2005).

At pH 8.0, both the *YlrIm23Δ-10* and *YlrIm9Δ-13* mutants nearly abolished XPR2 transcription, and the *YlrIm23Δ-10* mutant reduced YIPHR1 expression about 15-fold, whereas the *YlrIm9Δ-13* mutant reduced it about 6-fold (Fig. 1).

These observations were confirmed by measuring the expression of the *XPR2':lacZ* fusion integrated at the *XPR2* locus of these strains: the residual β -galactosidase activity was about 1.4 % for *Ylrin23 Δ -10* and 14.6 % for *Ylrin9 Δ -13*. At pH 4.0, no or very low transcription of these alkaline reporter genes could be detected (Fig. 1). At alkaline pH, in the *Ylrin23 Δ -10* mutant as in the *Ylrin9 Δ -13* mutant, expression of the acid-induced gene *YIPHR2* was derepressed more than 38-fold compared to the wild-type level at the same pH, reaching its level at acidic pH in the reference strain in the case of *Ylrin9 Δ -13* or exceeding it in the case of *Ylrin23 Δ -10* (Fig. 1). Taken together, these results strongly suggest that, like other *YLRIM* genes (*Gonzalez-Lopez et al.*, 2002; *Lambert et al.*, 1997), *YLRIM23* is absolutely required for transcriptional activation of alkaline-induced genes and for repression of acid-induced genes at pH 8.0. The *Ylrin9 Δ -13* mutant, by contrast, led to a somewhat less extreme phenotype, as previously stated (*Gonzalez-Lopez et al.*, 2002).

Ylrin23 Δ -10 induced the characteristic growth defect of Rim mutants, affecting growth at alkaline pH but not at neutral or acidic pH (Fig. 2) (*Gonzalez-Lopez et al.*, 2002). As previously observed for the insertional *rim9-D7* mutant (*Gonzalez-Lopez et al.*, 2002), the *Ylrin9 Δ -13* mutation had a less pronounced effect on growth at alkaline pH.

All these results establish that the *YLRIM23* gene, like other *YLRIM* genes, is required for cellular responses to ambient alkaline pH. The null mutation *Ylrin9 Δ* leads to a less extreme phenotype than other *Ylrin* mutations, as measured by activation of alkaline-induced genes, repression of an acid-induced gene (Fig. 1), growth at alkaline pH (Fig. 2) and efficiency of mating (see below), confirming the data obtained with the insertional mutant described by *Gonzalez-Lopez et al.* (2002). In a similar way, a *pall* null mutation in *A. nidulans* allows partial growth at pH 8.0, whereas all other *pal* mutations completely prevent growth at this pH (*Arst et al.*, 1994; *Denison et al.*, 1998). The phenotype of *rim9* mutants in yeasts has been poorly investigated up to now. In *S. cerevisiae*, *rim9* mutants exhibit a tight defect in sporulation (*Su & Mitchell*, 1993); in *C. albicans*, the *rim9* $-/-$ mutant displays a tight defect in ambient pH response (*M. Cornet*, personal communication).

The long YIRim9p C terminus does not seem to be involved in the pH response

Alignment of *Pall/Rim9p* sequences showed that *YIRim9p* (742 aa) (*Gonzalez-Lopez et al.*, 2006) and its orthologues in filamentous fungi are much longer than their orthologues in other yeasts (239 aa for *ScRim9p*). Strong sequence conservation is restricted to the regions including the

Fig. 1. Expression patterns of alkaline-induced genes (*XPR2* and *PHR1*) and an acid-induced gene (*PHR2*) in *Y. lipolytica* at pH 4.0 and pH 8.0. The strains were: SY12 (WT), SY1359u (*rim23*), SY1375u (*rim9*), SY1371 (*rim9 + RIM9*), SY1372 (*rim9 + RIM9SL*), SY1389 (*rim23 + RIM9SL*), SY1390 (*rim9 + rim23*). *RIM9SL* stands for the *YLRIM9* C-terminally truncated allele (see Methods). Levels of mRNA were measured by quantitative real-time PCR and expressed as a percentage of actin gene expression levels. Results are means $\pm$ SD from four quantifications.

Fig. 2. Effects of the different mutations on sensitivity of *Y. lipolytica* to ambient pH. Droplets of serial dilutions of a late-exponential-phase culture in YPD medium were deposited on solid YPD medium buffered at pH 3.5 with 0.2 M citrate buffer, and at pH 7.0–8.5 with 0.2 M MOPS buffer, and incubated at 28 °C for 40 h. The strains were SY1390 (*rim9+rim23*), SY1389 (*rim23+RIM9SL*), SY1375u (*rim9*), SY1371 (*rim9+RIM9*), SY1372 (*rim9+RIM9SL*), SY1359u (*rim23*), LAM26-03 (*rim13*) and SY12 (WT).

hydrophobic, putative transmembrane segments. The highly hydrophilic C-terminal tails that lie outside the region of homology between all the PalI/Rim9p sequences represent about 75 % of YIRim9p, 66 % of PalI, 82 % of MgPalI and 72 % of NcPalI. We noticed that deletion of a long Rim9p/PalI in *Y. lipolytica* and filamentous fungi results in a leaky phenotype, whereas the short Rim9p appears absolutely required in *S. cerevisiae* and *C. albicans*. Although we failed to detect any structural or functional patterns within these poorly conserved sequences, we wondered whether this large C-terminal tail in YIRim9p played a role in the pH response. To address this question, we established the phenotype of a mutant expressing a C-terminally truncated YIRim9 polypeptide. Point mutations were introduced to replace the adjacent residues R297 and Y298 by nonsense codons

(*YIRIM9SL* allele, see Methods). This construct replaced the wild-type gene at its normal location (SY1372 strain). As a control, the wild-type allele was used to replace the null mutation *Ylrin9Δ-13* (SY1371 strain). The effects of the C-terminally truncated form of YIRim9p on the transcription of different pH-regulated genes were assessed by real-time quantitative PCR at pH 4.0 and pH 8.0. At pH 8.0, identical levels of *XPR2* or *YIPHR1* transcripts were measured in SY1372 (*YIRIM9SL* mutation) and in the isogenic control SY1371. They were reduced by 30 % and 6 % for *XPR2* and *YIPHR1*, respectively, compared to the wild-type strain, reflecting unidentified background differences (Fig. 1). These observations were fully confirmed by measuring the expression of the *XPR2':lacZ* fusion integrated at the *XPR2* locus of SY1372 and SY1371 (72 % of residual β -galactosidase activity for both strains). At alkaline pH, in SY1372 as in SY1371, expression of the acid-induced gene *YIPHR2* was also comparable to wild-type levels at the same pH (Fig. 1). At pH 4.0, the *YIRIM9SL* mutation had no effect on the transcription of these alkaline- or acid-responsive genes (Fig. 1). Taken together, these results strongly suggest that the C-terminal tail of YIRim9p is not required for transcriptional activation of alkaline-induced genes and for repression of acid-induced genes at pH 8.0.

Both *Y. lipolytica* and filamentous fungi carry a conserved *YIRIM23*/PalC gene, besides a long version of *YIRIM9*/PalI. We wondered whether YIRim23p/PalC may counteract a putative negative effect of the YIRim9p/PalI C-terminal tail on pH signal transduction. According to this hypothesis, the null *rim23* mutant in the *RIM9SL* context should behave like the wild-type strain. *RIM23* was also deleted in the *rim9Δ-13* mutant. All clones of SY1389 (*rim23Δ-1, RIM9SL*) and of SY1390 (*rim23Δ-31, rim9Δ-13*) exhibited low levels of *XPR2*-driven β -galactosidase activity at pH 8.0 (about 0.03 % of wild-type levels). Transcription of alkaline- or acidic-responsive genes was affected in a similar way and to the same extent by the double mutations or by the single mutation *Ylrin23Δ-10* (see Fig. 1). The double mutations induced the characteristic growth defect of Rim mutants, drastically crippling growth at alkaline but not at neutral pH (Fig. 2). Taken together, these results show that *YIRIM23* is absolutely required for the *RIM101*-dependent pH response, but does not counteract a putative negative effect of the YIRim9p C-terminal tail on pH signal transduction. This indicates that in *Y. lipolytica* at least, and possibly in filamentous fungi, the long C-terminal tail of YIRim9p/PalI does not play a major role in the pH response and that its presence is not related to that of YIRim23p/PalC.

Mating in *Y. lipolytica* requires YIRim23p and, to a lesser extent, YIRim9p

We previously reported that mating in *Y. lipolytica* requires activation of the Rim pathway in both parents. Mutants affecting *RIM8*, *RIM13*, *RIM20*, or *RIM21* mated less efficiently than the control strains, yielding few *pal*⁺ and even fewer *pal/pal* diploids (Lambert *et al.*, 1997). As shown

Table 3. Effect of RIM status on mating

Strain	Relevant genotype	Status	Mating*
AM4 × SY12	<i>B ura3 his1</i> × <i>A ura3 lys11</i>	+ / +	+++
AM4 × LAM21-03	<i>B ura3 his1</i> × <i>A ura3 lys11 rim8-21†</i>	+ / <i>rim8</i>	+
AM4 × SY1375u	<i>B ura3 his1</i> × <i>A ura3 lys11 rim9Δ-13</i>	+ / <i>rim9Δ</i>	++
AM4 × SY1371	<i>B ura3 his1</i> × <i>A ura3 lys11 rim9Δ-13 RIM9</i>	+ / <i>rim9Δ RIM9</i> ⁺	+++
AM4 × SY1372	<i>B ura3 his1</i> × <i>A ura3 lys11 rim9Δ-13 RIM9SL</i>	+ / <i>rim9Δ RIM9SL</i>	+++
AM4 × SY1359	<i>B ura3 his1</i> × <i>A ura3 lys11 rim23Δ-10</i>	+ / <i>rim23Δ</i>	ε
AM4 × SY1389	<i>B ura3 his1</i> × <i>A ura3 lys11 rim9Δ-13 RIM9SL rim23Δ-1</i>	+ / <i>RIM9SL rim23Δ</i>	—
AM4 × SY1390	<i>B ura3 his1</i> × <i>A ura3 lys11 rim9Δ-13 rim23Δ-31</i>	+ / <i>rim9Δ rim23Δ</i>	—

*Mating was scored on cross-replicas on plates and scored +++ when comparable number of diploids were observed in the control, ++ for about half as many diploids, + for about one-fourth as many diploids, ε for a few diploids and — for no diploids. For each confrontation, crosses were carried out at least eight times.

†Formerly *pal3-21* (Blanchin-Roland *et al.*, 2005).

in Table 3, efficiency of mating was drastically reduced compared to wild-type contexts in + × *rim23Δ-10* crosses, reduced in + × *rim8-21* and slightly reduced in + × *rim9Δ-13*. The RIM9SL C-terminal truncation alone did not affect efficiency of mating whereas no diploids could be obtained from + × *rim23Δ-1*, *RIM9SL* and + × *rim23Δ-31*, *rim9Δ-13* confrontations. This indicates that mating requires Rim23 like other Rim factors indicated above, and to a lesser extent Rim9, but not the C-terminal tail of Rim9p.

Screening of the two-hybrid library for partners of YIRim23p

To investigate the function of YIRim23p, we looked for partners of this factor using the two-hybrid screen (Fields

& Song, 1989). Exhaustive matings against three genomic libraries (Kabani *et al.*, 2000), yielded 19, 175 and 68 His⁺ Ade⁺ diploids, respectively (see Methods). The inserted genomic fragments were directly amplified from yeast colonies by PCR and sequenced. From 132 sequences out of a total of 149 analysed, redundant and overlapping fragments were found for only one ORF, which is a strong argument for a true interaction. This ORF matches the *YISNF7/VPS32* gene (accession number YALI0C16027g), hereafter designated *SNF7* for simplicity. The fusion points were generated by either *TaqI* or *HpaII*, which are the two 'CG' enzymes cutting in the promoter sequence in-frame with the coding sequence, at positions −75 bp and −69 bp relative to the A of ATG, respectively. No fusion point within the coding sequence was generated by the 'CG'

Fig. 3. Two-hybrid interactions within the Rim/Pal pathway. (a) YISnf7p interacts with YIRim23p (A and B) and with YIRim13p (C and D). Co-transformants were grown on medium lacking Trp and Leu (A and C) and on the quadruple selection medium −Leu −Trp −His −Ade (B and D) using strain PJ69-4A. Parts A and B: 1, pAS2ΔΔ + pACT2; 2, pGADSNF7-C2/6 + pAS2ΔΔ; 3, pGADSNF7-C2/47 + pAS2ΔΔ; 4, pAS2ΔΔ-RIM23 + pACT2; 5, pGADSNF7-C2/6 + pAS2ΔΔ-RIM23; 6, pGADSNF7-C2/47 + pAS2ΔΔ-RIM23. Parts C and D: 1, pGADSNF7-C2/6 + pAS2ΔΔ; 2, pAS2ΔΔ-RIM13 + pACT2; 3, pAS2ΔΔ + pACT2; 4, 5 and 6, three independent clones carrying pGADSNF7-C2/6 + pAS2ΔΔ-RIM13. (b) Network of YIRim23p interactions tested in this work. Data were obtained with the −Leu −Trp −His −Ade system with the *S. cerevisiae* PJ69-4A strain. Arrows and T symbols stand for positive or negative two-hybrid interactions for a given prey-to-bait combination, respectively.

enzymes. This suggests that at least the N-terminal region of Snf7 is required for the interaction with Rim23p. No other candidate protein was repeatedly obtained in this screening. Plasmids were purified from one representative clone for each fusion point and co-transformed together with either pAS2ΔΔ or pAS2ΔΔ-RIM23 in the PJ69-4A strain. After checking the expression of the *HIS3* and *ADE2* reporter genes, the clones turned out to be true positives while all the controls were negative (Fig. 3a, A and B). These results indicated no auto-activation of the expression of the reporter genes and reproduced the interaction of Snf7p with Rim23p. This result is in line with recently published data from *A. nidulans* demonstrating by two-hybrid and recombinant protein pull-down assays that PalC binds Snf7p (Galindo *et al.*, 2007). PalC was recently shown to be recruited to plasma-membrane-associated structures in an alkaline pH- and PalH-dependent but PalA-independent manner, which led to the suggestion that PalC can link the putative pH-sensing complex and the endosomal complex (Galindo *et al.*, 2007).

YISnf7p binds to YIRim13p or YIRim20p in two-hybrid interactions, in addition to YIRim23p

The screening of the two-hybrid library indicated only YISnf7p as a possible *in vivo* direct partner of YIRim23p. No two-hybrid interaction could be detected between YIRim23p and YIRim8p, or YIRim13p, or YIRim20p, or the C-terminal tail of YIRim9p. In addition, this region was unable to bind YISnf7p. In contrast, two-hybrid assays confirmed that YISnf7p binds to YIRim20p (data not shown) and also to YIRim13p (Fig. 3a, C and D). Fig. 3(b) summarizes the network of two-hybrid interactions tested. These assays confirm the interactions between Snf7p and Rim20p/PalA/AIP1/Alix already described in *S. cerevisiae*, *A. nidulans* and *C. albicans* (Ito *et al.*, 2001; Vincent *et al.*, 2003) and between Snf7p and Rim13p, revealed only by one whole-genome screen of two-hybrid interactants in *S. cerevisiae* (Ito *et al.*, 2001). They also suggest the absence of interaction between YIRim23p and any of the other Rim factors tested.

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