Fungal Genetics and Biology 47 (2010) 446–457

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

Functional analysis of the pH responsive pathway Pal/Rim in the phytopathogenic basidiomycete *Ustilago maydis*

Cervantes-Chávez José Antonio [a,1], Ortiz-Castellanos Lucila [a], Tejeda-Sartorius Miriam [a], Gold Scott [b], Ruiz-Herrera José [a,*]

[a] Departamento de Ingeniería Genética, Unidad Irapuato, Centro de Investigación y de Estudios Avanzados del IPN, Mexico
[b] Plant Pathology Department, University of Georgia, Athens, GA 30602-7274, USA

ARTICLE INFO

Article history:
Received 8 December 2009
Accepted 9 February 2010
Available online 12 February 2010

Keywords:
pH
Rim pathway
Signal transduction
Dimorphism
Virulence
Protein secretion
Ustilago maydis

ABSTRACT

The most important mechanism for fungal response to the environmental pH is the Rim or Pal pathway. Details on its operation are known through the analysis of ascomycete fungi. In this study we analyzed whether this pathway is conserved in a basidiomycete, *Ustilago maydis*. We could identify only five homologues of the seven known components of the pathway in the *U. maydis* as well as in other basidiomycete genomes. We determined that only genes encoding Rim20/PalA, Rim13/PalB and Rim23/PalC, that constitute the endosomal membrane complex, and Rim9/PalI of the complex located at the plasma membrane are conserved, but this latter lacked a detectable role in signal transduction. Mutants in this pathway showed a pleiotropic phenotype, but dimorphism and virulence were not affected. Our data reveal that the Rim/Pal pathway is conserved in basidiomycetes, but with notable differences to the ascomycete systems.

© 2010 Elsevier Inc. All rights reserved.

1. Introduction

One of the most important physicochemical factors of the environment that affect cell growth and development is pH. Normally cells grow in a limited and specific range of pH around neutrality, although there are microorganisms that can tolerate extreme acid or alkaline conditions. Fungi are no exception, and depending on the external pH they secrete to the medium different low molecular mass (*Mr*) compounds and proteins that perform important functions in their adaptation to the prevalent environmental conditions. The most important mechanism controlling these functions in fungi is a signal transduction pathway termed by different authors as Rim or Pal (Orejas et al., 1995; Tilburn et al., 1995; Peñalva and Arst, 2002; Arst and Peñalva, 2003), here neutrally denominated as Pal/Rim. This is a signaling cascade that leads to the proteolytic activation of a zinc finger transcription factor named PacC in filamentous fungi or Rim101 in yeasts (Su and Mitchell, 1993; Orejas et al., 1995; Tilburn et al., 1995) that activates or represses transcription of a selected number of genes (reviewed by Peñalva and Arst, 2002; Arst and Peñalva, 2003). In ascomycete models PacC/Rim101 activation depends on the action of six proteins: PalH/Rim21, PalF/Rim8, PalI/Rim9, PalA/Rim20, PalB/Rim13 and PalC/Rim23, as well as some components of the endocytic system (Peñalva and Arst, 2002, 2004; Xu et al., 2004; Cornet et al., 2005; Blanchin-Roland et al., 2005, 2008; Peñalva et al., 2008).

The Pal/Rim pathway has been characterized at the molecular level in several ascomycetes such as *Aspergillus nidulans* and *Saccharomyces cerevisiae*, the human pathogen *Candida albicans* and the non-conventional yeast *Yarrowia lipolytica* (Davis, 2003; Peñalva and Arst, 2004; González-López et al., 2006; Galindo et al., 2007; Calcagno-Pizarelli et al., 2007; Blanchin-Roland et al., 2008; Peñalva et al., 2008; Cornet et al., 2009). Nevertheless, the genes that integrate the signaling cascade and their respective function are largely unknown in other fungal groups. For some time the pathway was considered specific of ascomycetes, until the homologue of Rim101/PacC was identified in the basidiomycete *Ustilago maydis* (Aréchiga-Carvajal and Ruiz-Herrera, 2005).

U. maydis (DC) Cda. is a biotrophic pathogen that causes common smut in maize (*Zea mays* L.) and teozintle (*Z. mays*, subsp. *parviglumis*), a disease that in maize can result in economically significant reduction in yield (reviewed by Christensen, 1963). Although not an obligate parasite, completion of the *U. maydis* life cycle requires invasion of the host. During its life cycle the fungus

* Corresponding author. Address: Departamento de Ingeniería Genética, Centro de Investigación y de Estudios Avanzados del IPN, Unidad Irapuato, Km. 9.6 Libramiento Norte, Carretera Irapuato-León, 36500 Irapuato, Gto. Mexico. Fax: +52 462 624 5849.
E-mail address: jruiz@ira.cinvestav.mx (R.-H. José).
[1] Present address: Pacific Agri-Food Research Centre, 4200 Highway 97, P.O. Box 5000, Summerland, B.C. Canada.

1087-1845/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.fgb.2010.02.004

displays an alternation of a saprophytic stage growing as a budding yeast, and an invasive pathogenic dikaryotic mycelium originating from the mating between two sexually compatible yeast cells that invades the host (reviewed by Martínez-Espinoza et al., 2002; Feldbrügge et al., 2004; Klosterman et al., 2007). Filamentation of the *U. maydis* dikaryon is under the genetic control of the *b* mating type *locus*, and constitutes a virulence factor since yeast-like monomorphic mutants are non-virulent (Martínez-Espinoza et al., 1997). This dimorphic switch can be controlled *in vitro* by nitrogen starvation (Banuett and Herskowitz, 1989), or by the pH of the growth medium (Ruiz-Herrera et al., 1995), as occurs with different fungi pathogenic for man (see Davis, 2009).

In the present study we have made a functional analysis of the Pal/Rim pathway in this basidiomycete fungal model to determine its similarities and differences with the ascomycete pathway. Our data reveal that the basidiomycete model presents similarities, but also important differences with ascomycete systems.

2. Materials and methods

2.1. Strains and culture conditions

The *U. maydis* strains used in this work and their relevant genotypes are shown in Table 1. They were maintained at -70 °C in 50% (v/v) glycerol. When required they were transferred to liquid or solid (2% agar) complex medium (CM; Holliday, 1961) and incubated routinely at 28 °C for variable periods of time. When necessary, carboxin (10 μM) or hygromycin B (300 μg ml^{-1}) were added as selective agents. Yeast-like or filamentous growth was obtained in minimal medium (MM; Holliday, 1961) adjusted respectively to pH 7 or pH 3 (Ruiz-Herrera et al., 1995). Growth in MM or CM media was measured by the Optical Density of the cultures at 600 nm (O.D.600). *Escherichia coli* strain DB3.1 (Invitrogen) was used to propagate plasmid pDONR-Cbx (García-Pedrajas et al., 2008). *E. coli* DH5α (Bethesda Research laboratories) was used for transformations to produce DelsGate constructs (García-Pedrajas et al., 2008; see below), as well as for routine plasmid propagation.

Table 1
Strains used in this work.

Name	Genotype	Source
FB1	*a1b1*	F. Banuett[a]
FB2	*a2b2*	F. Banuett
AC71	*a2b2 Δrim9*, Cbx^{R}[b]	This work
AC81	*a2b2 Δrim9*, Cbx^{R}	This work
AC91	*a1b1 Δrim9*, Cbx^{R}	This work
AC101	*a1b1 Δrim9*, Cbx^{R}	This work
AC121	*a2b2 Δrim20*, Cbx^{R}	This work
AC131	*a2b2 Δrim20*, Cbx^{R}	This work
AC44	*a1b1 Δrim20*, Cbx^{R}	This work
AC53	*a1b1 Δrim20*, Cbx^{R}	This work
AC401	*a2b2 Δrim13*, Cbx^{R}	This work
AC444	*a2b2 Δrim13*, Cbx^{R}	This work
AC1	*a1b1 Δrim13*, Cbx^{R}	This work
AC7	*a1b1 Δrim13*, Cbx^{R}	This work
AC2	*a2b2 Δrim23*, Cbx^{R}	This work
AC6	*a2b2 Δrim23*, Cbx^{R}	This work
AC60	*a1b1 Δrim23*, Cbx^{R}	This work
AC62	*a1b1 Δrim23*, Cbx^{R}	This work
AC01	*a2b2 Δrim20*, Cbx^{R} + pAC601c, Hyg^{R}[b]	This work
AC5	*a2b2 Δrim20*, Cbx^{R} + pAC601c, Hyg^{R}	This work
AC11	*a2b2 Δrim13*, Cbx^{R} + pAC601c, Hyg^{R}	This work
AC13	*a2b2 Δrim13*, Cbx^{R} + pAC601c, Hyg^{R}	This work
AC3	*a2b2 Δrim23*, Cbx^{R} + pAC601c, Hyg^{R}	This work
AC4	*a2b2 Δrim23*, Cbx^{R} + pAC601c, Hyg^{R}	This work
BMA2	*a2b2 Δrim101* Hyg^{R}	Aréchiga-Carvajal and Ruiz-Herrera (2005)

[a] California State University, Long Beach, USA.
[b] Carboxin or hygromycin B resistant.

This strain was grown at 37 °C in Luria–Bertani medium (1% tryptone, 0.5% yeast extract and 0.5% sodium chloride) containing ampicillin (100 μg $m1^{-1}$) or kanamycin (50 μg ml^{-1}) as selective agents.

2.2. Genetic transformation

Protoplasts of *U. maydis* were prepared using *Trichoderma harzianum* lysing enzymes (Sigma–Aldrich, St. Louis, MO), transformed under hypertonic conditions with the corresponding disruption cassettes, and inoculated on plates of selective media, following the protocol described by Wang et al. (1988). *E. coli* transformation was performed by standard procedures (Sambrook and Russell, 1999).

2.3. Techniques for nucleic acids manipulation

Genomic DNA from *U. maydis* was isolated as described by Hoffman and Winston (1987). Total RNA was isolated according to Jones et al. (1985). Southern and Northern hybridization techniques were performed by standard procedures (Sambrook and Russell, 1999). DNA probes were labeled using the random primer labeling system and [α-^{32}P] dCTP (Amersham Biosciences, Buckinghamshire, UK). Ribosomal RNA (rRNA) stained with ethidium bromide was used as loading control. Plasmid DNA was isolated from *E. coli* by standard procedures (Sambrook and Russell, 1999). Plasmid DNA from *U. maydis* complemented mutants was recovered by *E. coli* transformation (Hoffman and Winston, 1987).

DNA enzymatic reactions such as digestion, ligation and vector dephosporylation were performed as recommended by the manufacturers of the reagents used (Invitrogen, New England Biolabs). DNA for sequencing, ligation and random primer labeling reactions was purified using the QIAquick Gel extraction Kit (QIagen, Valencia, CA, USA). DNA sequencing was performed with an ABI PRISM 377 DNA automated sequencer (Perkin Elmer) using double stranded DNA as template and M13F and M13R (Invitrogen) or specific primers (see Table 2).

2.4. Polymerase chain reaction (PCR) conditions

Routine PCR reactions were conducted with Taq DNA polymerase (Invitrogen), using the following general program: an initial cycle of 94 °C for 5 min; amplification (30–35 cycles) at 94 °C for 30 s followed by annealing at primer-specific temperature for 60 s, and polymerization at 72 °C (1 min kb^{-1} of DNA target length). When required, the expanded high fidelity PCR system (Boehringer, Mannheim) was used according to the manufacturer's instructions. An extension period of 7 min at 72 °C was programmed for those PCR products that were cloned into pCR2.1 or pCR4 TOPO vectors (Invitrogen).

2.5. Plasmid constructs

Plasmids pACΔ*rim9* and pACΔ*rim20* harboring the constructions to delete genes *Rim9* and *Rim20* respectively, were prepared by the DelsGate methodology according to García-Pedrajas et al. (2008). Briefly, for each gene 1 kb of their 5′ or 3′ flanking regions were separately amplified by PCR. Primers Rim9a and Rim9b, or Rim20a and Rim20b were used to obtain the 5′ flank from *Rim9* or *Rim20* respectively; "a" primers contained at their 5′ ends the recognition sequence for the *I-Sce*I restriction enzyme in the forward orientation, whereas "b" primers harbored the *attB1* sequence. Primer pairs Rim9c and Rim9d or Rim20c and Rim20d were used to amplify independently the corresponding 3′ flank; at their 5′ end "c" primers and "d" primers contained the *attB2* sequence and the *I-Sce*I recognition sequence in the reverse

Table 2
Primers used in this work.

Primer	Sequence (5′–3′)
AC100F[a]	GACCTGCCCCAAGAACCTCAACCCTG
AC111R[b]	GATGCAGGCAACCAGCTCGAAGGGCG
AC112F	GAGAGTCGTGAGTCGTACCGCGTTGT
AC115F	GGCAGGCAGTATTTGTGACGGCAGAC
AC116R	TGCGGGTTGGATTGTTGCTTCACGGTAC
AC117	GTCTTTGAATCGCTCGGCATGAACTTG
AC118R	CGAGAGCAGATGTATAGTAAGCGAACG
AC119F	AGGCCACTCAATCGTAACAGCATGAC
AC120R	CTATCAAGATCAAACCTCCACCTCGAG
AC121R	GGCACCCTCGAAAATTATCTCTCCAAG
AC122R	CTCTCGATCCTAGCCATGCTGACTGTC
AC132F	CCATCAAGATCATCAGGGCGCAGGAG
AC134F	GAATTCGCGTTACGGCACAAGGGTTT
AC135R	AAGCTTTTAAGCGAGGTGGATGTAAGAG
AC136F	AAGCTTAAACAGAATCCGTGTACTTGTATC
AC137R	GAGCTCTACTTGATGAGTTAGGGCGC
AC138F	GAGGACGAGGCCGAGGCAGCAGTTG
AC139F	GCGTCGTCATCTGGTGTATTTGGGCAT
AC140R	GTAAGAGGGGAGAGCACGACAAGCGG
AC142F	CATGGCTGCGACTCTGCTCCTG
AC143R	TACCCCAGCCGACACCACCCATAG
AC144F	CAACAACGCTACTGCCGAGACGG
AC145R	CTCCATTCACTGCGGCACCCACTA
ActinF	TCACGCGAGTCGAGGGAGAAG
ActinR	ACAGCGCAGGCGTGAAGAG
PHR1F	TTGGCTGTGTCGAGAATGTC
PHR1R	GGGAACCTTGACACTTGCAT
Rim9aF	AAAATAGGGATAACAGGGTAATGGCACGGAAAGGCAGGCAGTAT
Rim9bR	ggggacaagtttgtacaaaaaagcaggctaACTATAAGCGGGGTACGGAGAGC
Rim9cF	ggggaccactttgtacaagaaagctgggtaTCGATCGACGTTTTGTATGTAG
Rim9dR	AAAAATTACCCTGTTATCCCTACGGAGCAGCAGGAGCACCACACGAA
Rim20aF	AAAATAGGGATAACAGGGTAATTGGGCTGTTGCGGTTCTGATTC
Rim20bR	ggggacaagtttgtacaaaaaagcaggctaAGGGAGAAGCAGGAGGGAAAGAT
Rim20cF	ggggaccactttgtacaagaaagctgggtaCCACAGCAACATCAACCGACTC
Rim20dR	AAAAATTACCCTGTTATCCCTAGCTGCACTCGCCAAGGACTCAT
Rim9F	CGGCAGGCGCGGAACTACC
Rim9R	CATTCGATCCCAACCAGCTTC
M13F	GTAAAACGACGGCCAG
M13R	CAGGAAACAGCTATGAC

The underlined text in primers represents the recognition sequence for: *Eco*RI (AC134), *Hind*III (AC135 and AC136), *Sac*I (AC137), *I-Sce*I in the forward orientation in primers (Rim9aF and Rim20aF) or in reverse orientation primers (Rim9dR and Rim20dR). Lowercase in primer pairs (Rim9bR and Rim20bR) or (Rim9cF and Rim20cF) represent the *attB1* and *attB2* sequences, respectively.
[a] F, forward orientation.
[b] R, reverse orientation.

orientation respectively (see Table 2 and García-Pedrajas et al., 2008). Next, the 1 kb fragments belonging to the 5′ or 3′ flanks were purified and recombined into pDONR-Cbx plasmid (see García-Pedrajas et al., 2008), the reaction being conducted independently for each gene using BPII clonase enzyme (Invitrogen) to give rise to plasmids pACΔ*rim*9 and pACΔ*rim*20.

Full *Rim9* (*ca* 3.9 kb) and *Rim20* (*ca* 5 kb) gene sequences were amplified by PCR and cloned into pCR2.1. *Rim9* (5′ flanking region, 1 kb; ORF, 1.9 kb; 3′ flanking region, 1 kb) was amplified using primers Rim9aF and Rim9dR, and *Rim20* (5′ flanking region, 1 kb; ORF, 3 kb; 3′ flanking region, 1 kb) using primers AC119 and AC121, giving rise to plasmids pAC*Rim9* and pAC*Rim20* respectively.

Plasmid pACΔ*rim*13 harboring the disruption cassette to delete *Rim13* was prepared as follows: the *ca* 5 kb full gene (5′ flanking region, 1 kb; ORF, 3 kb; 3′ flanking region, 1 kb) was amplified by PCR using primers AC117 and AC122, and cloned into pCR4 vector generating plasmid pAC*Rim13*. This plasmid was digested with *Sac*I that removed a *ca* 3 kb fragment corresponding to 95% of the ORF and *ca* 0.2 kb of the 3′ flanking sequence. Separately, the Cbx resistance gene was recovered as a 2.3 kb *Sac*I fragment from plasmid pCBX122 (Keon et al., 1991) and cloned into the *Sac*I site of modified plasmid pAC*Rim13* giving rise plasmid to pACΔ*rim13*. The entire disruption cassette (4.3 kb) was amplified by PCR with primers AC117 and AC122 and used for transformation.

Plasmid pACΔ*rim23* harboring the disruption cassette to delete *Rim23* gene was prepared as follows: the full gene (*ca* 3.9 kb; 5′ flanking sequence, 1.25 kb; ORF, 1.83 kb; 3′ flanking sequence, 0.84 kb) was amplified by PCR using primers AC139 and AC140 and cloned into pCR4 vector generating plasmid pAC*Rim23*. Next, a fragment of *ca* 1.8 kb harboring 0.5 kb of the 5′ flanking region and 1.3 kb of the ORF was excised with *Sac*I and replaced by the 2.3 kb *Sac*I fragment harboring the Cbx resistance gene marker from pCBX122 plasmid (see above), giving rise to plasmid pAC-Δ*rim*23. The whole disruption cassette (4.4 kb) was recovered by PCR using primers AC139 and AC140.

A constitutive truncate version of the Rim101/PacC transcription factor, spanning the sequence for the three zinc fingers domain (the first 283 aa of the ORF) was engineered as follows: primers AC134 harboring an *Eco*RI site at its 5′ end, and AC135 containing a *Hind*III sequence and a stop codon at its 5′ end were used to amplify a fragment around 1.6 kb in size (0.81 kb of 5′ flanking region and 0.85 kb of the ORF; Table 2). Next the 3′ flanking sequence of the gene (0.56 kb) was amplified using primers AC136 and AC137 flanked at their 5′ ends by *Hind*III and *Sac*I restriction sites respectively. Both PCR products were cloned separately into pCR2.1 vector giving rise to plasmids pAC601a and pAC601b respectively. Then, the *Eco*RI–*Hind*III and *Hind*III–*Sac*I fragments were recovered from the former plasmids and ligated together into the self-replicative plasmid pNEBU-H harboring the *Hph* gene for hygromycin B selection (R. Kahmann, Max Planck Institute) previously digested with *Eco*RI and *Sac*I. The plasmid harboring the correct insert was identified by restriction pattern analysis and designated as pAC601c.

Probes (5′ flanking region) for Southern hybridization were prepared by PCR with the following primers using genomic DNA as a template: *Rim9*, AC115 and AC116; *Rim20*, AC119 and AC120; *Rim13*, AC117 and AC118 (Table 2), to produce fragments of the following corresponding size respectively: 0.8 kb, 0.9 kb and 1.0 kb. For gene *Rim23* the 1.3 kb *Eco*RI–*Sac*I fragment (3′ flanking region) from plasmid pACΔ*rim23* was used as a probe. The following probes located within the ORF of each gene were used for Northern hybridization: *Rim9*, a 1.5 kb fragment obtained by PCR using primers Rim9F and Rim9R and plasmid pAC*Rim9* as a template; *Rim20*, a 2.3 kb *Xho*I fragment recovered by digestion of plasmid pAC*Rim20*; *Rim13*, a 3 kb *Sac*I fragment from plasmid pAC*Rim13*; *Rim23*, a 0.8 kb *Xho*I–*Pst*I fragment from plasmid pAC*Rim23*.

All the constructs used in this study and all the isolated genes were verified by restriction pattern and by sequencing.

2.6. Deletion of U. maydis Rim genes

Deletion of genes *Rim9* and *Rim20* was accomplished by transformation of protoplasts prepared from *U. maydis* FB1 or FB2 wild-type strains with *I-Sce*I linearized plasmids pACΔ*rim9* or pAC-Δ*rim20* respectively; whereas deletion of *Rim13* and *Rim23* genes involved transformation with disruption cassettes obtained by PCR amplification from plasmids pACΔ*rim13* or pACΔ*rim23* respectively (see above). Transformants were recovered on plates of hypertonic complete medium (CM) containing carboxin as the selective agent. Homologous integration of the disruption cassettes was confirmed by PCR using the indicated primer pairs which amplified the following fragments: AC112 and AC100 (Δ*rim9*), 2.2 kb; AC119 and AC100 (Δ*rim20*), 2.1 kb; AC132 and AC100 (Δ*rim13*), 1.9 kb, and AC138 and AC111 (Δ*rim23*), 1.3 kb (not shown). Confirmation of some PCR-identified mutants was obtained by Southern hybridization using as probes the corresponding 5′ flanking regions, with the exception of *Rim23*, for which a

fragment of the 3′ flanking region was used (see Figs. 1 and 2 in Supplementary material). For each gene, two mutants (both, *a1b1* and *a2b2*) were selected for further analyses (see Table 1).

2.7. Identification and expression analysis of Ena2, Prs1 and Phr1 genes from U. maydis

A blast search conducted in the *U. maydis* database allowed the identification of *Ena2* gene (*um00204*) using as queries Ena1, Ena2 and Ena5 proteins from *S. cerevisiae* and Ena1 from *Fusarium oxysporum*. For Northern hybridization, an ORF fragment of 3.19 kb amplified by PCR with primers AC142 and AC143 from the *um00204* sequence, was used as a probe (see Table 2). Expression of *U. maydis Ena2* was measured in cells incubated for variable time periods with or without LiCl (0.003 M) or NaCl (0.4 M). Briefly, cells were grown on MM pH7 for 18 h at 28 °C under constants shaking. Cells were pelleted by centrifugation, washed twice with sterile distilled water, inoculated into MM containing 100 mM Tris·HCl pH 9 plus LiCl or NaCl, and incubated under the above conditions. Cells were recovered by centrifugation and frozen with liquid nitrogen. Total RNA was isolated from these samples and subjected to Northern analysis.

Homologues of *C. albicans* Pra1 (a pH-regulated cell surface antigen) and Phr1 (a pH regulated GPI-anchored membrane protein) were identified in the *U. maydis* data base using the *C. albicans* corresponding proteins as queries. A single ORF matched with each protein: *um10632* for PRA1 with 42% identity (we designated its homologue as *UmPrs1* to avoid confusion with the *U. maydis Pra1* gene encoding a pheromone receptor), and *um01640* (*UmPhr1*, 35% identity). These sequences were used to design the following primer pairs: AC144 and AC145 (*UmPrs1*) or PHR1F and PHR1R (*UmPhr1*) to amplify ORF fragments of 471 or 620 bp respectively. Total RNA was isolated from FB2 cells grown on MM at pH 3 (100 mM sodium acetate), 7 (100 mM Tris·HCl) or 9 (100 mM Tris·HCl) for 9 h at 28 °C under constant shaking, and gene expression was determined by RT-PCR analysis. As a control a fragment from the constitutive gene *Um actin* was amplified with primers ActinF and ActinR (600 bp).

2.8. Stress assays

The effect of different stress conditions on *U. maydis* was tested on plates of solid MM adjusted to the indicated pH values for each experiment, using 100 mM Tris·HCl for pH 7–12 or 100 mM sodium acetate for pH–6. Cells were grown in liquid MM pH 7 at 28 °C for 18 h, collected by centrifugation and washed twice with sterile distilled water. Cell suspensions were adjusted to contain 10^8 cells ml^{-1} (counted with a Neubauer chamber), 10-fold serial dilutions were prepared, and 10 µl of each were spotted on plates of MM solid medium. For oxidative or acid stress assays, cell suspensions (10^8 cells ml^{-1}) were incubated at 28 °C under shaking conditions with 10 mM H_2O_2 for 2 h or 16 mM acetic acid for 3 h. Cells were recovered by centrifugation, suspended in sterile distilled water, diluted and spotted as above on MM pH 9 solid medium. For heat shock, 10^8 cells ml^{-1} were incubated at 46 °C for 10 min, then diluted and spotted as above. In all cases, plates were incubated at 28 °C for 48 h or 72 h and photographed.

2.9. Mating and virulence tests

Mating was assayed by the fuzz reaction (Banuett and Herskowitz, 1989). For virulence assays 8–14 days-old maize plants *cv* cacahuazintle were inoculated either by injection or by surface application (unpublished) using an aliquot of a mixture of sexually compatible cells (10^5 cells) grown and washed as above. Disease symptoms were evaluated weekly for 1 month or until death of the plant occurred.

2.10. Analysis of protease secretion

Secretion of proteases was assayed on solid medium with gelatin as substrate. Plates of solid (1% agar) MM pH 7 or 9 without glucose and supplemented with 2.5% gelatin, were spot-inoculated with 10^8 *U. maydis* cells and incubated at 28 °C for 48 h. Plates were then stained with PlusOne Coomassie tablets Phastagel R-350 according to the manufacturer's instructions (GE healthcare, Uppsala Sweden), and photographed.

2.11. Assay of sensitivity to lysing enzymes

Yeast cells were grown in MM pH 7 as described above, recovered by centrifugation and washed once with 25 mM Tris·HCl, pH 7.5 containing 25 mM $CaCl_2$. Cells (10^7) were then incubated at 28 °C with 100 µl of lysing enzymes from *T. harzianum* (50 mg ml^{-1}) in a final volume of 0.5 ml of the same buffer solution containing 1 M sorbitol. After 30 min the samples were diluted with 1 M sorbitol and the amount of protoplasts were determined by microscopic observation.

2.12. Polysaccharide secretion

Liquid MM (100 ml) adjusted to pH 9 with 100 mM Tris·HCl buffer was inoculated with the corresponding strains at 0.01 O.D.600. Cultures were incubated for 40 h at 28 °C under shaking conditions. After cell elimination by centrifugation, polysaccharide was recovered, dried and weighed according to Aréchiga-Carvajal and Ruiz-Herrera (2005).

3. Results

3.1. In silico identification of members of the Pal/Rim signaling pathway in basidiomycetes, and their isolation from Ustilago maydis

Using as queries several fungal protein sequences previously identified as members of the Pal/Rim pathway in ascomycetes, we were able to identify the *U. maydis* homologues of only four of the corresponding six Pal genes in an *in silico* search [*Rim101* gene was isolated previously (Aréchiga-Carvajal and Ruiz-Herrera, 2005)]. The databases blasted were: http://www.broadinstitute.org/annotation/genome/ustilago_maydis/, http://mips.helmholtz-muenchen.de/genre/proj/ustilago/ and http://blast.ncbi.nlm.nih.gov/Blast.cgi. The four genes identified were: *PalI/Rim9* (**FN395064**, *um00581*), *PalA/Rim20* (**FN395061**, *um11510*), *PalB/Rim13* (**FN395062**, *um02075*) and *PalC/Rim23* (**FN395063**, *um04392*), here after simply called *Rim9*, *Rim20*, *Rim13* and *Rim23* (altogether Rim genes; bold and underlined characters indicate NCBI accession numbers). Search for *PalF/Rim8*, *PalH/Rim21* and the putative *UmDfg16* [whose role in Rim101 processing was addressed in *S. cerevisiae* and *C. albicans* (Barwell et al., 2005)] homologous genes was unsuccessful, even when using only the most conserved motifs of the corresponding ascomycete proteins. To determine whether this was a characteristic of *U. maydis* in particular or of basidiomycetes in general, we conducted an exhaustive search for members of the pathway in the available genomes of other basidiomycetes. We could identify in all of them only homologues of the same genes identified in *U. maydis* (see the alignments of the Pal/Rim homologues in Figs. 3–6 of the Supplementary material).

With these data, we proceeded to isolate *U. maydis Rim9*, *Rim20*, *Rim13* and *Rim23* genes. The whole open reading frames (ORFs) and their 5′ and 3′ flanking sequences were synthesized by PCR, and cloned. Sequence analysis revealed that none of the coding regions were interrupted by introns. *Rim9* encodes a protein of 639 amino acids in length with three trans-membrane domains and

one signal peptide same as described by Calcagno-Pizarelli et al. (2007) in *A. nidulans*. This protein is larger than the reported homologues from ascomycetes such as *C. albicans* (346 aa) or *S. cerevisiae* (239 aa), but agrees in size with its homologues from the basidiomycetes *Coprinopsis cinereus* (631 aa), *Cryptococcus neoformans* (637 aa) or *Postia placenta* (557 aa), showing an identity around 30%. The TMD domain in ascomycete systems has been suggested to be involved in the formation of one of the signaling complexes located at the plasma membrane together with PalF/Rim21 and PalH/Rim8 proteins (Calcagno-Pizarelli et al., 2007). *Rim20* encodes a protein made of 880 amino acid residues that in common with their homologues from other fungi exhibits a BRO1 domain located at its *N*-terminal end (position 2–432), suggesting involvement in endosomal targeting, as well as in protein–protein interactions. The protein shares low similarity with the respective homologues from *C. neoformans*, *A. nidulans* and *Laccaria bicolor* (40%, 37% and 32% respectively). *Rim13* encodes a putative calcium-activated cysteine protease (calpain-like) made of 1002 amino acids. Its highest level of identity was around 27% with the homologue proteins from *L. bicolor, C. cinereus* and *Neurospora crassa*. *Rim23*, putatively involved in endocytic trafficking, encodes an ORF made of 611 amino acids, showing a BRO1 domain like Rim20, located at its *N*-terminus at position 179–302; its highest identity was observed with proteins from basidiomycete species: *L. bicolor* (39%), *C. cinereus* (39%) and *C. neoformans* (30%).

3.2. Analysis of the expression of Rim genes

Since in other fungi the Pal/Rim pathway is involved in the dimorphic switch, a time course (from 3 to 24 h) of expression patterns of *Rim* genes was determined by Northern hybridization in yeast or mycelial cells. Cells were grown in MM pH 7 for 24 h, inoculated and grown in pH 3 or 7 MM for the indicated periods of time, and total RNA was isolated and probed with a fragment of the corresponding ORF. Cells grown at pH 3, showed very low levels of transcripts, with a small increase after 6 h of incubation. At neutral pH, transcript levels were higher in general, being highest for *Rim20*, reaching a maximum after 9 h of incubation to decrease later on (Fig. 1). *Rim20* was unusual in that it showed earlier induction, with elevated transcript levels maintained from 3 to 9 h of incubation, decreasing at 12 h, and increasing again at 24 h (see Fig. 1). These results were reproducible in two different experiments.

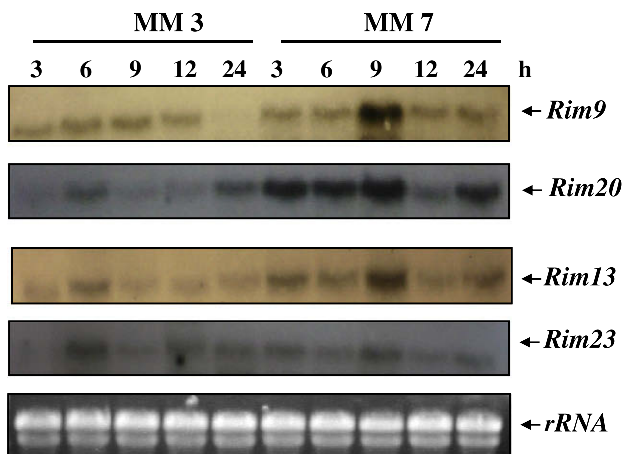

Fig. 1. Transcription analysis of *Rim* genes. FB2 strain was incubated at 28 °C with shaking either in MM pH3 (MM3) or MM pH 7 (MM7), and samples were withdrawn at the indicated times in hours. As probes specific ORF were prepared as indicated in Section 2. Ribosomal RNAs (rRNA) stained with ethidium bromide are shown as loading control.

3.3. Growth, dimorphism, mating and virulence of rim mutants

Rim mutants were obtained as described in Materials and methods, and aspects of the phenotype altered in different ascomycete *rim* mutants were analyzed. The first analyses involved the determination of their general capacity to grow in the yeast and mycelial forms, to mate, and to invade maize plants.

No difference in growth rate in liquid medium (MM or MC) of pH 3 or 7 was observed between mutants representative of each genotype and the parental strains FB1 (*a1b1*) and FB2 (*a2b2*), indicating that function of these genes is dispensable under the experimental conditions used (data not shown). Also, no significant difference in the dimorphic transition was observed when the same mutants were compared to the parental strains, but cell morphology of *rim* mutants resembled that described for Δ*rim101/pacC* (see Aréchiga-Carvajal and Ruiz-Herrera, 2005). Mutants were also unaffected in their mating capacity when analyzed by means of the fuzz reaction using combinations of mutant × wild-type or mutant × mutant strains of compatible mating types, revealing that the Pal/Rim mutant is not involved in *U. maydis* mating.

Virulence of the mutants was tested by inoculation of maize seedlings with the following pairs of sexually compatible mutants: AC71 and AC91 (Δ*rim9*), AC121 and AC53 (Δ*rim20*), AC401 and AC1 (Δ*rim13*), and AC2 and AC60 (Δ*rim23*). As controls, maize seedlings were inoculated with a mixture of the parental strains FB1 and FB2. The same disease symptoms with the same degree of severity were observed in the parental or mutant mixtures, no matter whether plants were injured by the injection needle, or the inoculum was deposited on the leaf surface (Table 3). Teliospore formation and germination were also unaffected (not shown). All these last results contrast with the defects in mating and sporulation of *Y. lipolytica rim* mutants (Lambert et al., 1997; Blanchin-Roland et al., 2008).

3.4. Inhibition of rim mutants growth at alkaline pH

It has been described that *rim*/*pal* mutants from several ascomycetes show difficulties to grow at neutral or alkaline pH (Rollins, 2003; Cornet et al., 2005). On these grounds we investigated the effect of pH on the growth of *U. maydis rim* mutants representative of the different genotypes, and the Δ*rim101* mutant BMA2 previously isolated (Aréchiga-Carvajal and Ruiz-Herrera, 2005), using the protocol described in Materials and methods. Growth of mutants in the range of initial pH from 3 to 10 was indistinguishable from wild-type strains; but when pH was increased to 11, mutants did not grow properly in contrast to the wild-type strains. Finally, growth of Δ*rim20,* Δ*rim13,* Δ*rim23* and Δ*rim101* mutants was totally prevented at pH 12 in contrast to parental strains FB1 or FB2 and Δ*rim9* mutants (see representative data in Fig. 2).

3.5. Effect of different stress conditions on rim mutants

It has been described in ascomycetes that some *rim* mutants are more sensitive to ionic stress than the parental strains (Lamb and

Table 3
Results of virulence assays.

a1b1	Cross	a2b2	Numbers of plants with tumors
FB1	×	FB2	29 ± 1
AC91(Δ*rim9*)	×	AC71 (Δ*rim9*)	28 ± 2
AC53(Δ*rim20*)	×	AC121 (Δ*rim20*)	29 ± 0.9
AC1(Δ*rim13*)	×	AC401 (Δ*rim13*)	28 ± 2
AC60(Δ*rim23*)	×	AC2 (Δ*rim23*)	27 ± 2.3

Thirty *Zea mays* seedlings (14 days old) were inoculated with a mixture of sexually compatible *U. maydis* cells. Results were scored 30 days after inoculation and are the average of three experiments; ± standard deviation.

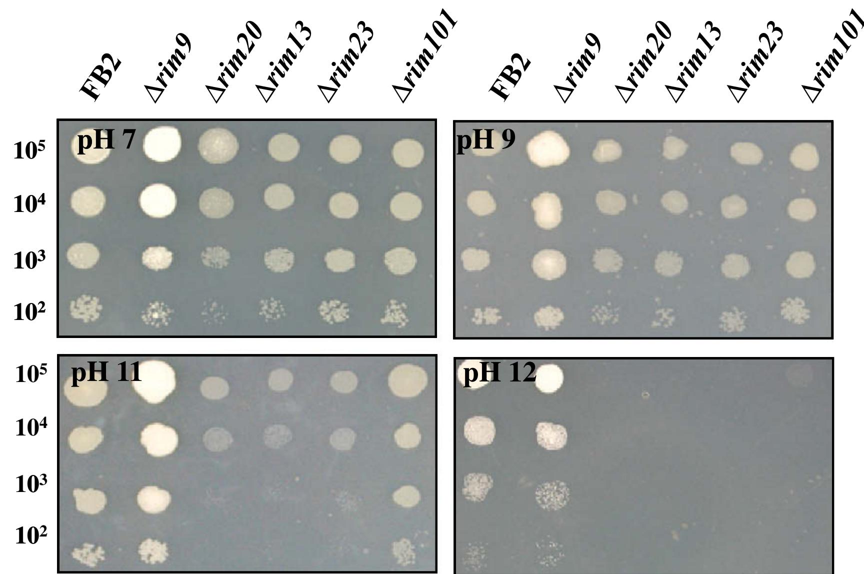

Fig. 2. Effect of pH on *U. maydis* growth. Strains FB2, AC71 (Δ*rim9*), AC121 (Δ*rim20*), AC401 (Δ*rim13*), AC2 (Δ*rim23*) and BMA2 (Δ*rim101*) were grown in MM pH 7 for 18 h at 28 °C with constant shaking. Cells were collected and 10 μl of 10-fold serial dilutions were spotted on plates of solid MM of the indicated initial pH.

Mitchell, 2003; Bensen et al., 2004; Kullas et al., 2007). Accordingly, we tested the sensitivity of *U. maydis rim* mutants to osmotic or saline stress at different pH values. Mutants did not show any growth defect on media made hypertonic with 1.5 M sorbitol either at pH 3, 7 or 9 (data not shown). On the other hand, mutants Δ*rim20*, Δ*rim13* and Δ*rim23* as well as the previously reported Δ*rim101*, barely grew on MM pH 9 supplemented with 0.005 M LiCl or 0.6 M NaCl (see representative data in Fig. 3). In the presence of 1.2 M KCl growth of Δ*rim101* strain was not affected at pH 7, but was completely inhibited at pH 9, whereas Δ*rim20*, Δ*rim13* and Δ*rim23* mutants were unable to grow at either pH (Fig. 3). Interestingly, growth of Δ*rim9* mutants under the above conditions was undistinguishable from the parental strains (data not shown).

Other stress conditions also were more deleterious to *rim* mutants than to the wild-type strains. After oxidative (10 mM H_2O_2, 2 h), acid (16 mM acetic acid, 3 h), or heat (46 °C for 10 min) stresses of representative *rim* mutants, they were cultivated on acid, neutral or alkaline solid medium (see Section 2). An inhibitory effect was observed only when after being subjected to these stress conditions mutants were further grown on MM pH 9 (see representative data in Fig. 4). Notice that growth of Δ*rim9* mutant and the parental strain was unaffected by these treatments.

3.6. Effect of agents that perturb the cell wall and plasmalema on rim mutants

Previous results showed that *rim101* mutants were more sensitive to lysing enzymes than the wild-type strains (Aréchiga-Carvajal

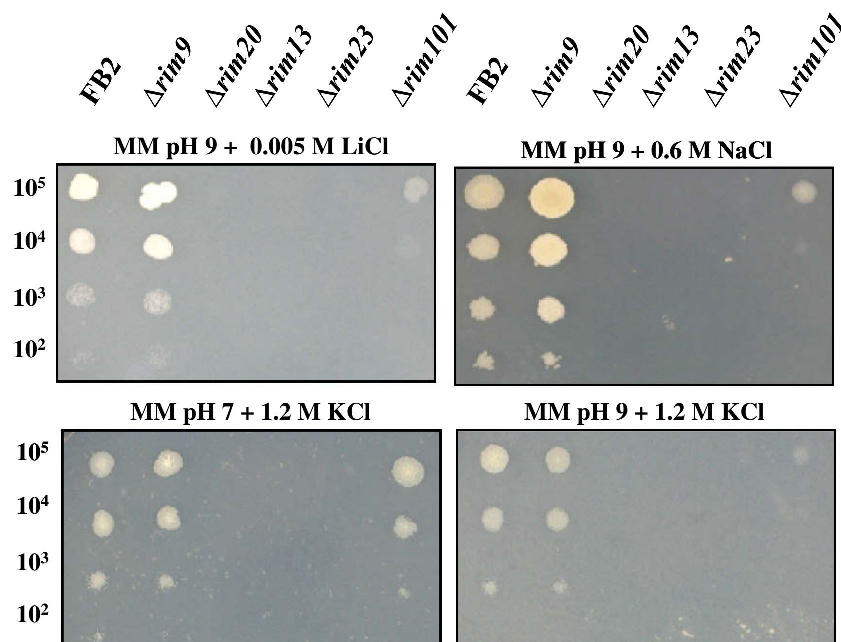

Fig. 3. Effect of cations on *U. maydis* growth. The same strains as in Fig. 2 were grown under similar conditions and spotted as in Fig. 2 on solid MM adjusted at the indicated initial pH (supplemented with 100 mM Tris·HCl), containing the indicated ions. Plates were photographed after 72 h incubation at 28 °C.

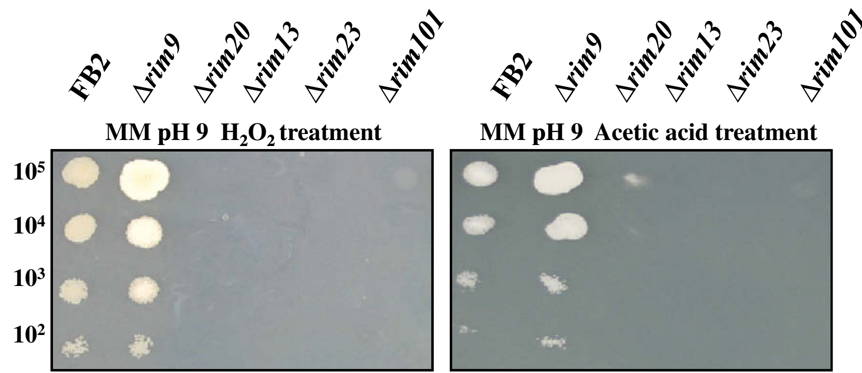

Fig. 4. Effect of oxidative or stress conditions on *U. maydis*. Inocula of the same strains were grown under similar conditions as in Fig. 2. Then 10^8 cells ml^{-1} were incubated with 10 mM H_2O_2 for 2 h or with 16 mM acetic acid for 3 h with shaking at 28 °C, 10-fold serial dilutions were prepared and spotted as in Fig. 2 on MM of initial pH 9 (100 mM Tris·HCl). Plates were photographed after incubation for 72 h at 28 °C.

and Ruiz-Herrera, 2005), revealing alterations in cell wall structure. In this work we analyzed the effect of these enzymes, and Congo red (5 μg ml^{-1}) and SDS (0.017%) on the rest of the *rim* mutants. The deleterious effect of Congo red and SDS was dependent on pH, not being observed at acid or neutral pH, marginal at pH 8, and very strong at pH 9. Interestingly, the effect of Congo red and SDS was additive, and at pH 8 growth of the mutants was completely suppressed when added together (not shown). Again, Δ*rim9* mutants were as resistant to these compounds as the wild-type strains (results at pH 9 are shown in Fig. 5). Analysis of sensitivity to lysing enzymes determined by their rate of protoplast production, showed that *rim* mutants AC121 (Δ*rim20*), AC401 (Δ*rim13*), AC2 (Δ*rim23*) had a more fragile cell wall than the wild-type strain FB2 (Fig. 5). The AC71 (Δ*rim9*) mutant showed a value intermediate between the FB2 strain and the rest of mutants.

3.7. Polysaccharide and protease secretion by rim mutants

Previously we reported that Δ*rim101* mutants turned the growth media viscous due to the secretion of a polysaccharide

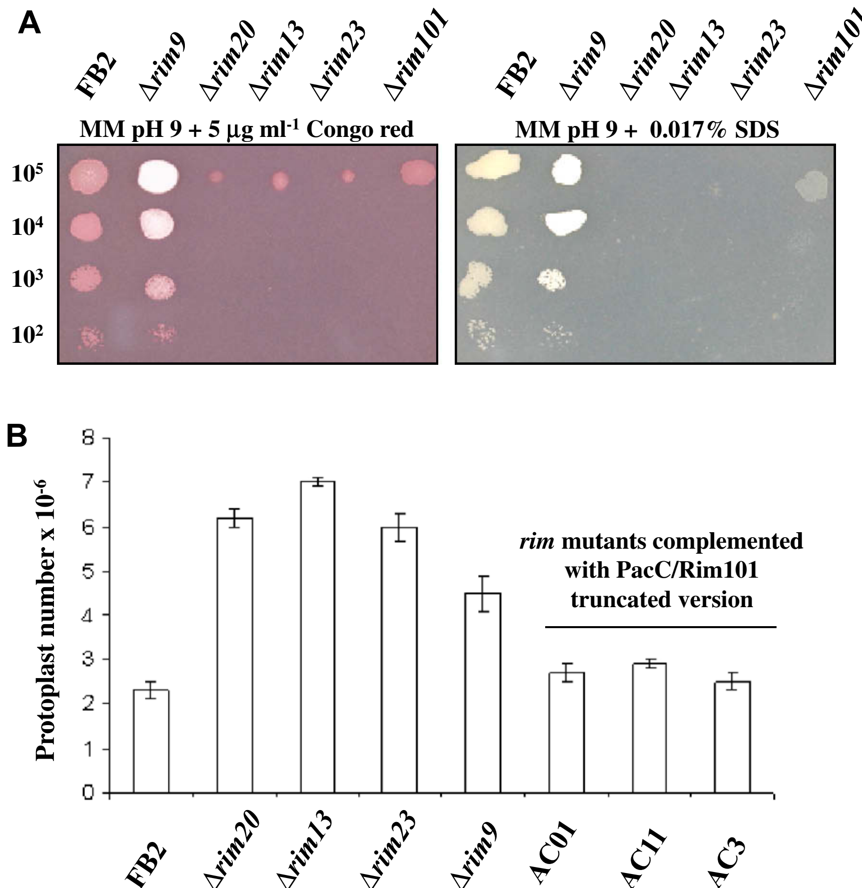

Fig. 5. Effect of agents that affect the cell envelope on *U. maydis*. (Panel A) The same strains as in Fig. 2 were grown under similar conditions and spotted as in Fig. 2 on solid minimal media of initial pH 9 (Tris·HCl) plus 5 μg ml^{-1} Congo red (left), or MM pH 9 plus 0.017% SDS (right). Plates were photographed after incubation for 72 h at 28 °C. (Panel B) Number of protoplasts formed after treatment of 10^7 cells of the strains indicated with 5 mg of lysing enzymes. The results are the average of three experiments. AC01, complemented Δ*rim20*; AC11, complemented Δ*rim23*; AC3, complemented Δ*rim13*.

(Aréchiga-Carvajal and Ruiz-Herrera, 2005). This phenotype was also shown by mutants AC121 (Δ*rim20*), AC401 (Δ*rim13*) and AC2 (Δ*rim23*) that produced significant and similar amounts of the polysaccharide (300, 280, and 350 mg, respectively from 100 ml cultures). Unexpectedly AC71 mutant (Δ*rim9*) also produced this compound although in a lesser amount (65 mg) than the rest of the mutants. In contrast, no precipitate was recovered from cell-free medium of FB2 strain (Fig. 6, panel A).

Mutants AC121 (Δ*rim20*), AC401 (Δ*rim13*) and AC2 (Δ*rim23*) were unable to digest gelatin in agar plates (Fig. 6, panel B), as was previously observed for Δ*rim101* mutants (Aréchiga-Carvajal and Ruiz-Herrera, 2005), but AC71 (Δ*rim9*) formed a proteolytic halo grossly of the same size as the parental FB2 strain (data not shown).

3.8. Analysis of the expression of selected genes

In *S. cerevisiae* the P-Type Na^+ ATPase Ena1p maintains Na^+, Li^+ and K^+ homeostasis (Haro et al., 1991; Benito et al., 1997). Considering the extreme sensitivity of *rim* mutants to Na^+ and Li^+, we analyzed the possibility that the expression of a related gene might be defective in these mutants. Using fungal Ena-like proteins as queries in the *U. maydis* database, we identified gene *um00204* that showed the highest homology. This gene encodes a protein that shares all the structural characteristics exhibited by Ena proteins, and accordingly was isolated [during the course of our study the same gene was identified and denominated as *UmEna2* (Benito et al., 2009)]. Gene expression was evaluated by Northern hybridization in media supplemented with NaCl (0.4 M) or LiCl (0.003 M). After a time as short as 20 min *UmEna2* expression was induced in the wild-type strain, its transcript values increasing after 100 min of incubation. Representative data for cells grown with LiCl are shown in Fig. 7, panel A. No *UmEna2* induction occurred in AC121 (Δ*rim20*), AC401 (Δ*rim13*), AC2 (Δ*rim23*) or BMA2 (Δ*rim101*) mutants incubated with any of those salt solutions (Fig. 7, panel A). This result reveals a positive regulatory role of the Pal/Rim pathway on *Ena2* transcription. In the Δ*rim9* mutant only a faint hybridization signal was detected (Fig. 7, panel A), even though this mutant was not sensitive to Na^+ or Li^+ ions under our experimental conditions (see above).

Genes *PRA1* and *PHR1* from *C. albicans* are up-regulated at alkaline pH through Rim101 (Sentandreu et al., 1998; Bensen et al., 2004 respectively). For this reason we searched the homologue genes in *U. maydis* to determine if they behaved similarly. As observed in *C. albicans* (Sentandreu et al., 1998), expression of *UmPrs1* in the FB2 strain was pH dependent: almost no expression was detected at pH 3, faint expression was observed at pH 7, and strong up-regulation occurred at pH 9. No expression of the gene was detected in strains AC121 (Δ*rim20*), AC401 (Δ*rim13*), AC2 (Δ*rim23*) or BMA2 (Δ*rim101*) at any pH tested (see representative data in Fig. 7, panel B). In contrast, expression in mutant AC71 (Δ*rim9*) was similar to the parental strain at pH 7. These results indicate that as occurs in *C. albicans*, *UmPrs1* is regulated through the Pal/Rim pathway. On the other hand, we observed that expression of *UmPhr1*, a Gas1 homologue, was not regulated by the Pal/Rim

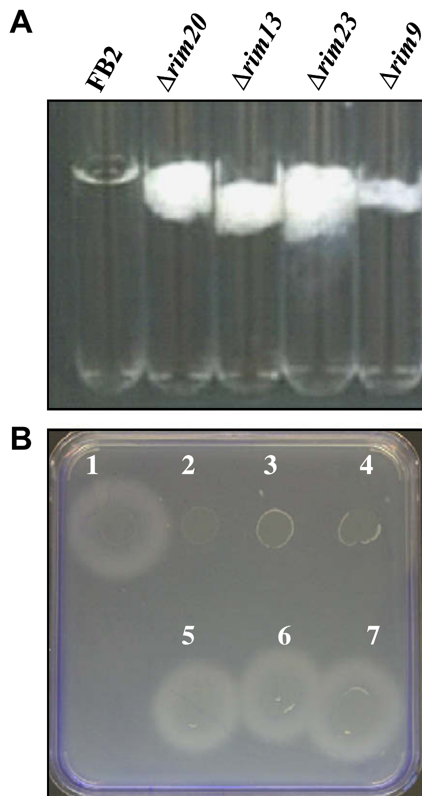

Fig. 6. Polysaccharide and protease secretion in *U. maydis* strains. (Panel A) Polysaccharide secretion. The indicated strains were grown in liquid MM of initial pH 9 (100 ml) and incubated for 40 h at 28 °C in constant shaking. Cells were eliminated by centrifugation, and the polysaccharide present in the medium was precipitated with cold-ethanol. (Panel B) MM pH 9 agar plates containing 2% gelatine were inoculated with a drop containing 10^8 cells and incubated for 48 h at 28 °C. After this time plates were stained with PlusOne Coomassie tablets Phastagel R-350 and photographed. Spot 1, FB2; spot 2, AC121 (Δ*rim20*); spot 3, AC401 (Δ*rim13*); spot 4, AC2 (Δ*rim23*); spot 5, AC01 (Δ*rim20*//pAC601c (truncated version of *Rim101/PacC*); spot 6, AC11 (Δ*rim13*//pAC601c); spot 7, AC3 (Δ*rim23*//pAC601c).

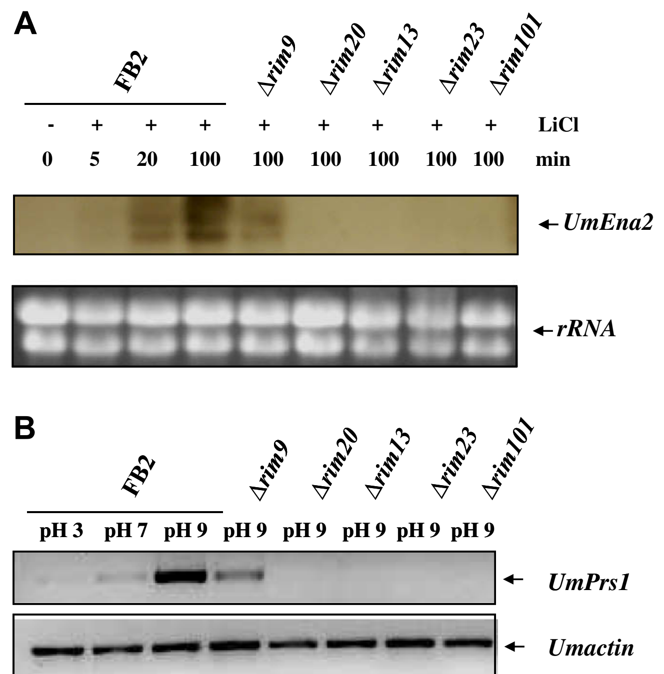

Fig. 7. Expression of selected genes. (Panel A) Effect of LiCl on the expression of *UmEna2*. MM pH 9 (100 mM Tris·HCl) with (+) or without (−) LiCl (0.003 M) was inoculated with the indicated strains and incubated at 28 °C under constant shaking for the period of time indicated in min. Cells were collected by centrifugation, total RNA was purified and used for Northern hybridization. Membrane was probed with the *UmEna2* ORF amplified with primers AC142 and AC143. As a loading control, ribosomal RNAs (*rRNA*) stained with ethidium bromide are shown. (Panel B) Expression analysis of *UmPrs1*. FB2 and mutants strains were grown in MM adjusted to pH 3, 7 or 9. Cultures were incubated for 9 h at 28 °C under constant shaking. Cells were recovered by centrifugation, total RNA was isolated and genes *UmPrs1* and *UmActin* were measured by RT-PCR with primers AC144 and AC145, or ActinF and ActinR respectively. Lane 1, pH 3; lane 2, pH 7; lanes 3–8, pH 9. Lanes 1–3, FB2; lane 4, Δ*rim9*; lane 5, Δ*rim20*; lane 6, Δ*rim13*; lane 7, Δ*rim23*, lane 8, Δ*rim101*.

pathway, since equivalent expression values were detected in all tested strains, and the same expression pattern was observed in strain FB2 under different pH values (3, 7 or 9; not shown).

3.9. Wild-type phenotype recovery using a truncated version of Rim101/PacC

Mutants AC121 (Δ*rim20*), AC401 (Δ*rim13*) and AC2 (Δ*rim23*) were transformed with plasmid pAC601c which harbors a truncated, and putatively constitutive version of *Rim101/PacC* (see Section 2 and Fig. 8, panel A). Transformants were selected on a medium supplemented with hygromycin B and carboxin, and two strains from each mutant genotype were selected for further experiments: AC01 and AC5 (Δ*rim20*), AC11 and AC13 (Δ*rim13*) and AC3 and AC4 (Δ*rim23*) (see Table 1). Plasmid DNA was recovered from this set of strains in *E. coli* to check for their correct identity. Transformed strains were able to grow at an initial pH 12 (Fig. 8), and were not sensitive to 0.005 M LiCl at pH 9 (Fig. 8), 0.6 M NaCl at pH 9 or 1.2 M KCl at pH 9 (not shown). Additionally, these strains were resistant at pH 9 to agents that perturb cell wall or cytoplasm membrane integrity: 0.017% SDS (Fig. 8) or Congo red (5 μg ml^{-1}, not shown). Also, transformants were not hypersensitive to lysing enzymes forming about the same relative numbers of protoplast as strain FB2 after similar treatment (see Fig. 5). In addition transformants lost their increased sensitivity to acid, oxidative and heat stresses (not shown), recovered the capacity to hydrolyze gelatin (see, Fig. 6, panel B), and did not secrete the polysaccharide that turned the medium viscous (not shown).

4. Discussion

The main goal of this study was to determine at the molecular level the functional conservation of the Pal/Rim pH sensing pathway in the basidiomycete *U. maydis*, and its comparison with the better characterized ascomycete systems.

In ascomycetes, Pal/Rim function involves the internalization of the signal through the participation of two active signaling complexes. The first one is physically located at the plasma membrane and involves interaction of the Rim21/PalH, Rim9/PalI and Rim8/PalF proteins. The second one is an endosomal membrane complex, which involves proteins Rim20/PalA, Rim13/PalB and Snf7/Vps32 (ESCRT-III machinery). After both complexes establish physical contact through the interaction of Rim23/PalC (see below), Rim101/PacC is recruited for its proteolytic activation (see Xu and Mitchell, 2001; Peñalva and Arst, 2002; Vincent et al., 2003; Arst and Peñalva, 2003; Galindo et al., 2007; Peñalva et al., 2008; Blanchin-Roland et al., 2008). *In silico* analysis of the *U. maydis* genome database identified the corresponding homologues of *Rim9/PalI*, *Rim20/PalA*, *Rim13/PalB* and *Rim23/PalC*. However, regardless the crucial physiological role exerted by Rim8/PalF and Rim21/PalH proteins in ascomycetes, their corresponding homologues could not be identified in the *U. maydis* genome, nor in other available basidiomycete genomes. It is possible that the function of these genes may be fulfilled in basidiomycetes by genes having no detectable homology. The case of *U. maydis* Δ*rim9* is interesting. Mutants in this gene showed weak or absent mutant phenotype in the conducted assays. In ascomycetes contrasting results have been reported for *rim9* mutants: noticeable defects in *S. cerevisiae* (Li and Mitchell, 1997) and *C. albicans* (Cornet et al., 2009), weak phenotype in *Y. lipolytica* (González-López et al., 2002; Blanchin-Roland et al., 2008) or *A. nidulans* Δ*palI* (Arst et al., 1994; Denison et al., 1998), and the observation that PacC is correctly processed in *A. nidulans* Δ*palI* (*rim9*) mutants (Calcagno-Pizarelli et al., 2007). These results suggest that the role of *Rim9*, required or not, may depend on each species.

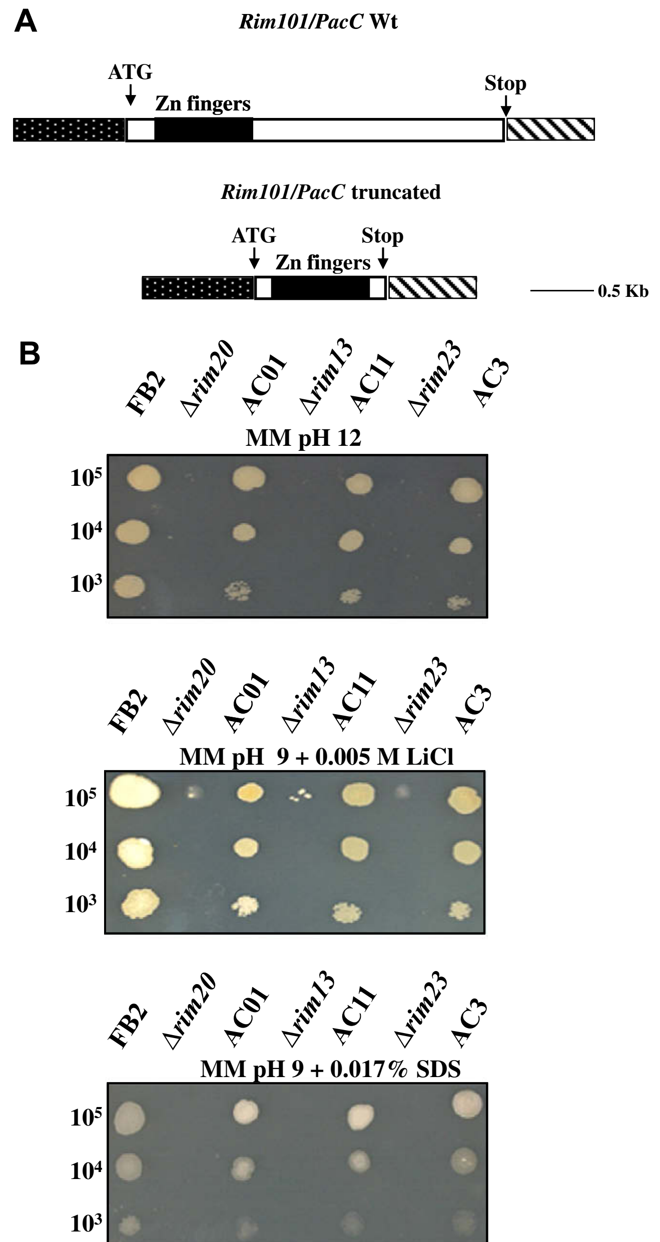

Fig. 8. Wild-type phenotypic recovery of *U. maydis rim* mutants with a truncated version of *Rim101/PacC*. Protoplasts of AC121 (Δ*rim20*), AC401 (Δ*rim13*) and AC2 (Δ*rim23*) strains were transformed with a constitutive form of Rim101/PacC (plasmid pAC601c). (Panel A) Scheme of *Rim101/PacC* wild-type and truncated version. Dotted box 5′ flank sequence, white box ORF, filled box zinc fingers, stripped box 3′ flank sequence. (Panel B) The response of selected transformants was tested to stress conditions following the protocol described in the legend to Fig. 2. Lane 1, FB2; lane 2, AC121 (Δ*rim20*); lane 3, AC01 (Δ*rim20*//pAC601c); lane 4, AC401 (Δ*rim13*); lane 5, AC11 (Δ*rim13*//pAC601c); lane 6, AC2 (Δ*rim23*); lane 7, AC3 (Δ*rim23*//pAC601c).

In contrast to the differences in the plasmalemal complex between the ascomycete and *U. maydis* pathways, functionality of the endosomal complex seems to be similar. In agreement with the members that make up this complex in ascomycetes, the *N*-termini of the *U. maydis* homologues of Rim20 and Rim23 proteins possess characteristic BRO1 adaptor domains. It is known that after Rim20 binds to Snf7/Vps32 (ESCRT-III machinery) protease Rim13 is recruited, while Rim23 through its BRO1 domain allows the interaction between the endosomal and plasma membrane complexes (Ito et al., 2001; Vincent et al., 2003; Tilburn et al.,

2005; Boysen and Mitchell, 2006; Galindo et al., 2007; Blanchin-Roland et al., 2008). In addition, *U. maydis* Rim13 showed the typical calpain-like and calpain-III domains but lacked the *N*-terminus of *A. nidulans* PalB (Rim13). The role of this last domain during the assembly of the signaling complex through the interaction with Vps24 (ESCRT III-like protein) was recently demonstrated (Rodríguez-Galán et al., 2009).

In general, scant information exists about the regulation of the members of the Pal/Rim pathway at the transcriptional level. It has been suggested that the main control mechanism occurs at the post-translational level, although no such mechanism was detected in *A. nidulans* PalB (Peñas et al., 2007). In *U. maydis* it appears that the Pal/Rim pathway is partially regulated at the transcriptional level. Although expression of *Rim23* was essentially constitutive, higher expression levels of *Rim20*, *Rim13* and *Rim9* occurred at neutral as compared to acid pH. The expression pattern observed for *Rim20* agrees with the behavior reported for *A. nidulans PalA*, whose transcript was detected at pH values between 6.5 and 8 (Negrete-Urtasun et al., 1997), whereas expression of *PalB* was not regulated by external pH in this species (Denison et al., 1995).

Mutation of *Rim* genes in *U. maydis* gave rise to a pleiotropic phenotype, as occurs in other systems, but the processes affected did not strictly coincide with *rim* mutant phenotypes in other studied fungal species. For example, *U. maydis rim* mutants were not affected in their capacity to mate or to form sexual spores. In contrast, *Y. lipolytica* Δ*rim8*, Δ*rim13*, Δ*rim20* or Δ*rim21* mutants displayed reduced mating and sporulation efficiencies (Lambert et al., 1997; Blanchin-Roland et al., 2008). Dimorphism was also unaffected in *U. maydis rim* mutants, despite the central role exerted by pH on the *in vitro* dimorphic transition (Ruiz-Herrera et al., 1995). This characteristic contrasts with *C. albicans rim* mutants which are not able to form filaments in response to alkaline pH, although they still do in the presence of serum (Davis et al., 2000), but agrees with the behaviour of Δ*rim9*, Δ*rim20* and Δ*rim13* *Y. lipolytica* mutants which are able to carry out the dimorphic switch (González-López et al., 2006).

Other phenotypic traits of *U. maydis rim* mutants were different or similar to other fungal species. Avirulence of *U. maydis rim* mutants agreed with the observation that virulence of the *rim101* or *rim20* mutants of the basidiomycete human pathogen *C. neoformans* is not impaired (Liu et al., 2008), and that *F. oxysporum PacC*+/− loss of function mutants were more virulent to tomato plants than the wild-type strains (Caracuel et al., 2003a). But on the other hand contrasted with the observation that *RIM101* or *RIM13* are required for development of oropharyngeal candidiasis or candidal keratomycosis respectively (Mitchell et al., 2007; Nobile et al., 2008), or that *PacC* is indispensable for *Scletorinia sclerotiorum* (Rollins, 2003) and *Colletotrichum acutatum* virulence (You and Chung, 2007). It is probable that synthesis of virulence factors at alkaline pH determines whether functionality of the pathway is required for virulence of the different pathogenic species.

Growth inhibition of *U. maydis rim* mutants at alkaline pH agrees with the phenotype of *rim* mutants in other fungi, *e. g. C. albicans* (Cornet et al., 2009), *Y. lipolytica* (González-López et al., 2002; Blanchin-Roland et al., 2008), *S. cerevisiae* (Futai et al., 1999; Castrejon et al., 2006), *A. nidulans* (Arst et al., 1994; Denison et al., 1998), and the phytopathogenic fungi *S. sclerotiotum* and *C. acutatum*, (Rollins and Dickman, 2001; You and Chung, 2007).

U. maydis rim mutants showed to be hypersensitive to alkaline cations, but not to osmotic stress. This is probably due to alterations in cation handling by the cells. In *S. cerevisiae* the Ena system (Ena1–Ena5) is essential to keep ion homeostasis resulting in salt tolerance (Haro et al., 1991). In *U. maydis* ion pumping is carried out by proteins encoded by *UmEna1* and *Ena2* genes (Benito et al., 2009). The missing expression of *UmEna2* after salt addition in our *rim* mutants, in contrast to the wild-type strain, reveals the role of the encoded protein in ion detoxification, no matter that it has been described that UmEna2 is located at the endoplasmic reticulum (Benito et al., 2009), and suggests that it is under the control of the Pal/Rim pathway. These data agree with the observed expression of *ENA1* homologues in *S. cerevisiae*, *F. oxysporum* or *Torulaspora delbrueckii* under salt stress (Lamb et al., 2001; Caracuel et al., 2003b; Hernandez-Lopez et al., 2006), and their absence of expression in *rim101*/*pacC* mutants (Lamb et al., 2001; Caracuel et al., 2003b; Bensen et al., 2004). Search of the Rim101/PacC recognition sequence (G/AGCCAAG) in the regulatory region of *UmEna2* gave negative results, suggesting an indirect regulation of this gene by PacC/Rim101, as described for *Ena1* in *S. cerevisiae* (Ruiz and Ariño, 2007).

Other genes regulated by PacC/Rim101 in *C. albicans* are *PRA1* and *PHR1*. These genes are expressed at alkaline but not at acid pH (Sentandreu et al., 1998; Davis et al., 2000; Bensen et al., 2004), both contain at least two canonical sites for PacC/Rim101 recognition, and are not expressed in Δ*rim8*, Δ*rim20* or Δ*rim101* mutants (Davis et al., 2000; Bensen et al., 2004). The behavior of the *U. maydis* homologue gene *UmPrs1* agrees with these results, except that its promoter lacks related recognition motifs for PacC/Rim101, suggesting an indirect regulation as *Ena2*. The observation that *U. maydis Phr1* expression is independent of pH and the Pal/Rim pathway contrasts with the *C. albicans* or *Pneumocystis carinii* homologue genes (Davis et al., 2000; Kottom et al., 2001), but agrees with the behavior of the *F. oxysporum gas1* homologue whose expression is not affected in *rim* mutants (Caracuel et al., 2005).

One important phenotypic alteration in *rim* mutants was their defect in cell wall construction revealed by their increased sensitivity to Congo red and lytic enzymes. Similarly, growth of Δ*rim13* *C. albicans* mutants was impaired by 10 μM Calcofluor white at pH 8 (Li et al., 2004), and *S. cerevisiae* Δ*rim21* strains were hypersensitive to lytic enzymes (Castrejon et al., 2006). It is feasible that in *U. maydis*, as well as in ascomycete yeasts, there exists cooperation between the Rim/Pal and PKC pathways, the last one described to be involved in maintenance of cell wall integrity through the phosphorylation of the Map kinase Slt2 homologue (Tong et al., 2004; Castrejon et al., 2006).

U. maydis rim mutants were hypersensitive to acid, oxidative, or heat shock stress. It is possible that the effect of stress is additive to the existence of a weak cell wall or altered plasma membrane. It is opportune to recall that adaptation of *S. cerevisiae* to weak acids is managed by the Pal/Rim pathway, mainly through the transcriptional activation of genes involved in the assembling or remodeling of the cell wall (Mira et al., 2009), and that *U. maydis rim* mutants secrete a polysaccharide, suggested to be normally associated to the cell wall.

The inability of *U. maydis rim* mutants to secrete a protease was expected since Δ*rim101* mutants presented this phenotype (Aréchiga-Carvajal and Ruiz-Herrera, 2005). Incapacity of protein secretion is a general characteristic of all fungal mutants defective in the pathway (see reviews by Peñalva and Arst, 2002, 2004 for a discussion)

Gain of function *pac*C^c mutants of *A. nidulans* resulting from truncation of amino acids 100–412 from the *C*-terminus of PacC show an alkalinity mimicking phenotype (Espeso et al., 2000). In agreement with this phenomenon, and considering that *U. maydis Rim20*, *Rim13* and *Rim23* genes act upstream of the transcription factor *Rim101*, all phenotypes displayed by *rim* mutants were alleviated by expression of the truncated version of *RIM101* lacking the *C*-terminus portion. This result also indicates that the mutant phenotype of *rim* strains was due to the missing function of the corresponding genes.

In conclusion, our results evidence that the operation of the Pal/Rim pathway in *U. maydis* as representative of basidiomycetes has

great similarities with the mechanism that operates in ascomycetes, but it also displays noticeable differences regarding the probable absence of homologues of the genes involved in the reception of the pH stimulus. It is apparent that while the plasmalemmal complex of the pathway has diverged during evolution, the endosomal complex is highly conserved.

Acknowledgments

This work and JACCH postdoctoral fellowship were partially supported by CONACYT-México. We are grateful to R. Kahmann for kindly providing plasmid pNEBU H, to Claudia León, Alicia Mireles and Yesenia Ruiz for their skilled technical support, and to Mr. Antonio Cisneros for photographs. We also thank María García and Marina Nadal (Georgia University, USA) for their guidance during DelsGate constructs preparation and the two anonymous reviewers for improving the original version of the manuscript.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2010.02.004.

References

Aréchiga-Carvajal, E.T., Ruiz-Herrera, J., 2005. The *RIM101*/*pacC* homologue from the basidiomycete *Ustilago maydis* is functional in multiple pH-sensitive phenomena. Eukaryot. Cell 4, 999–1008.

Arst, H.N., Peñalva, M.A., 2003. PH regulation in *Aspergillus* and parallels with higher eukaryotic regulatory systems. Trends Genet. 19, 224–231.

Arst Jr., H.N., Bignell, E., Tilburn, J., 1994. Two new genes involved in signalling ambient pH in *Aspergillus nidulans*. Mol. Gen. Genet. 245, 787–790.

Banuett, F., Herskowitz, I., 1989. Different *a* alleles of *Ustilago maydis* are necessary for maintenance of filamentous growth but not for meiosis. Proc. Natl. Acad. Sci. USA 86, 5878–5882.

Barwell, K.J., Boysen, J.H., Xu, W., Mitchell, A.P., 2005. Relationship of DFG16 to the Rim101p pH response pathway in *Saccharomyces cerevisiae* and *Candida albicans*. Eukaryot. Cell 4, 890–899.

Benito, B., Quintero, F.J., Rodríguez-Navarro, A., 1997. Overexpression of the sodium ATPase of *Saccharomyces cerevisiae*: conditions for phosphorylation from ATP and Pi. Biochim. Biophys. Acta 1328, 214–226.

Benito, B., Garciadeblás, B., Pérez-Martín, J., Rodríguez-Navarro, A., 2009. Growth at high pH and sodium and potassium tolerance in above cytoplasmic pH media depend on ENA ATPases in *Ustilago maydis*. Eukaryot. Cell 8, 821–829.

Bensen, E.S., Martin, S.J., Li, M., Berman, J., Davis, D.A., 2004. Transcriptional profiling in *Candida albicans* reveals new adaptive responses to extracellular pH and functions for Rim101p. Mol. Microbiol. 54, 1335–1351.

Blanchin-Roland, S., Da Costa, G., Gaillardin, C., 2005. ESCRT-I components of the endocytic machinery are required for Rim101-dependent ambient pH regulation in the yeast *Yarrowia lipolytica*. Microbiology 151, 3627–3637.

Blanchin-Roland, S., Da Costa, G., Gaillardin, C., 2008. Ambient pH signalling in the yeast *Yarrowia lipolytica* involves YlRim23p/PalC, which interacts with Snf7p/Vps32p, but does not require the long C terminus of YlRim9p/PalI. Microbiology 154, 1668–1676.

Boysen, J.H., Mitchell, A.P., 2006. Control of Bro1-domain protein Rim20 localization by external pH, ESCRT machinery, and the *Saccharomyces cerevisiae* Rim101 pathway. Mol. Biol. Cell 17, 1344–1353.

Calcagno-Pizarelli, A.M., Negrete-Urtasun, S., Denison, S.H., Rudnicka, J.D., Bussink, H.J., Múnera-Huertas, T., Stanton, L., Hervás-Aguilar, A., Espeso, E.A., Tilburn, J., Arst Jr., H.N., Peñalva, M.A., 2007. Establishment of the ambient pH signaling complex in *Aspergillus nidulans*: PalI assists plasma membrane localization of PalH. Eukaryot. Cell 6, 2365–2375.

Caracuel, Z., Roncero, M.I.G., Espeso, E.A., González-Verdejo, C.I., Garcia-Maceira, Fe.I., Di Pietro, A., 2003a. The pH signalling transcription factor PacC controls virulence in the plant pathogen *Fusarium oxisporum*. Mol. Microbiol. 48, 765–779.

Caracuel, Z., Casanova, C., Roncero, M.I.G., Di Pietro, A., Ramos, J., 2003b. PH response transcription factor PacC controls salt stress tolerance and expression of the P-Type Na^+-ATPase Ena1 in *Fusarium oxysporum*. Eukaryot. Cell 2, 1246–1252.

Caracuel, Z., Martinez-Rocha, A.L., Di Pietro, A., Madrid, M.P., Roncero, M.I., 2005. Fusarium oxysporum gas1 encodes a putative β1–3 glucanosyltransferase required for virulence on tomato plants. Mol. Plant Microbe Interact. 18, 1140–1147.

Castrejon, F., Gomez, A., Sanz, M., Duran, A., Roncero, C., 2006. The RIM101 pathway contributes to yeast cell wall assembly and its function becomes essential in the absence of mitogen-activated protein kinase Slt2p. Eukaryot. Cell 5, 507–517.

Christensen, J. J., 1963. Corn smut caused by *Ustilago maydis*. In: American Phytopathological Society Monograph No. 2. American Phytopathological Society. St. Paul, Mn. USA, 41 pp.

Cornet, M., Bidard, F., Schwarz, P., Da Costa, G., Blanchin-Roland, S., Dromer, F., Gaillardin, C., 2005. Deletions of endocytic components *VPS28* and *VPS32* affect growth at alkaline pH and virulence through both *RIM101*-dependent and *RIM101*-independent pathways in *Candida albicans*. Infect. Immun. 73, 7977–7987.

Cornet, M., Richard, M.L., Gaillardin, C., 2009. The homologue of the *Saccharomyces cerevisiae RIM9* gene is required for ambient pH signalling in *Candida albicans*. Res. Microbiol. 160, 219–223.

Davis, D.A., 2003. Adaptation to environmental pH in *Candida albicans* and its relation to pathogenesis. Curr. Genet. 44, 1–7.

Davis, D.A., 2009. How to human pathogenic fungi sense and adapt to pH: the link to virulence. Curr. Opin. Microbiol. 12, 365–370.

Davis, D.A., Wilson, R.B., Mitchell, A.P., 2000. RIM101-dependent and independent pathways govern pH responses in *Candida albicans*. Mol. Cell Biol. 29, 971–978.

Denison, S.H., Orejas, M., Arst Jr., H.N., 1995. Signaling of ambient pH in *Aspergillus* involves a cysteine protease. J. Biol. Chem. 270, 28519–28522.

Denison, S.H., Negrete-Urtasun, S., Mingot, J.M., Tilburn, J., Mayer, W.A., Goel, A., Espeso, E.A., Peñalva, M.A., Arst Jr., H.N., 1998. Putative membrane components of signal transduction pathways for ambient pH regulation in *Aspergillus* and meiosis in *Saccharomyces* are homologous. Mol. Microbiol. 30, 259–264.

Espeso, E.A., Rocal, T., Díez, E., Rainbow, L., Bignell, E., Alvaro, J., Suárez, T., Denison, S.H., Tilburn, J., Arst Jr., H.N., Peñalva, M.A., 2000. On how a transcription factor can avoid its proteolytic activation in the absence of signal transduction. EMBO J. 19, 719–728.

Feldbrügge, M., Kämper, J., Steinberg, G., Kahmann, R., 2004. Regulation of mating and pathogenic development in *Ustilago maydis*. Curr. Opin. Microbiol. 6, 666–672.

Futai, E., Maeda, T., Sorimachi, H., Kitamoto, K., Ishiura, S., Suzuki, K., 1999. The protease activity of a calpain-like cysteine protease in *Saccharomyces cerevisiae* is required for alkaline adaptation and sporulation. Mol. Gen. Genet. 260, 559–568.

Galindo, A., Hervás-Aguilar, A., Rodríguez-Galán, O., Vincent, O., Arst Jr., H.N., Tilburn, J., Peñalva, M.A., 2007. PalC, one of two Bro1 domain proteins in the fungal pH signalling pathway, localizes to cortical structures and binds Vps32. Traffic 8, 1346–1364.

García-Pedrajas, M.D., Nadal, M., Kapa, L.B., Perlin, M.H., Andrews, D.L., Gold, S.E., 2008. DelsGate, a robust and rapid gene deletion construction method. Fungal Genet. Biol. 45, 379–388.

González-López, C.I., Szabo, R., Blanchin-Roland, S., Gaillardin, C., 2002. Genetic control of extracellular protease synthesis in the yeast *Yarrowia lipolytica*. Genetics 160, 417–427.

González-López, C.I., Ortiz-Castellanos, L., Ruiz-Herrera, J., 2006. The ambient pH response Rim pathway in *Yarrowia lipolytic*: identification of *YlRIM9* and characterization of its role in dimorphism. Curr. Microbiol. 53, 8–12.

Haro, R., Garciadeblás, B., Rodríguez-Navarro, A., 1991. A novel P-type ATPase from yeast involved in sodium transport. FEBS Lett. 291, 189–191.

Hernandez-Lopez, M.J., Panadero, J., Prieto, J.A., Randez-Gil, F., 2006. Regulation of salt tolerance by *Torulaspora delbrueckii* calcineurin target Crz1p. Eukaryot. Cell 5, 469–479.

Hoffman, C.S., Winston, F., 1987. A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. Gene 57, 267–272.

Holliday, R., 1961. The genetics of *Ustilago maydis*. Genet. Res. 2, 203–230.

Ito, T., Chiba, T., Ozawa, R., Yoshida, M., Hattori, M., Sakaki, Y., 2001. A comprehensive two-hybrid analysis to explore the yeast protein interactome. Proc. Natl. Acad. Sci. USA 98, 4569–4574.

Jones, J.D.G., Dunsmuir, P., Bedbrook, J., 1985. High level expression of introduced chimeric genes in regenerated transformed plants. EMBO J. 4, 2411–2418.

Keon, J.P.R., White, G.A., Hargreaves, J.A., 1991. Isolation, characterization and sequence of a gene conferring resistance to the systemic fungicide carboxin from the maize smut pathogen, *Ustilago maydis*. Curr. Genet. 19, 475–481.

Klosterman, S.J., Perlin, M.H., García-Pedrajas, M., Covert, S.F., Gold, S.E., 2007. Genetics of morphogenesis and pathogenic development of *Ustilago maydis*. Adv. Genet. 57, 1–47.

Kottom, T.J., Thomas Jr., C.F., Limper, A.H., 2001. Characterization of *Pneumocystis carinii PHR1*, a pH-regulated gene important for cell wall integrity. J. Bacteriol. 183, 6740–6745.

Kullas, A.L., Martin, S.J., Davis, D., 2007. Adaptation to environmental pH: integrating the Rim101 and calcineurin signal transduction pathways. Mol. Microbiol. 66, 858–871.

Lamb, T.M., Mitchell, A.P., 2003. The transcription factor Rim101p governs ion tolerance and cell differentiation by direct repression of the regulatory genes *NRG1* and *SMP1* in *Saccharomyces cerevisiae*. Mol. Cell Biol. 23, 677–686.

Lamb, T.M., Xu, W., Diamond, A., Mitchell, A.P., 2001. Alkaline response genes of *Saccharomyces cerevisiae* and their relationship to the *RIM101* pathway. J. Biol. Chem. 276, 1850–1856.

Lambert, M., Blanchin-Roland, S., Le Louedec, F., Lepingle, A., Gaillardin, C., 1997. Genetic analysis of regulatory mutants affecting synthesis of extracellular proteinases in the yeast *Yarrowia lipolytica*: identification of a *RIM101*/*pacC* homolog. Mol. Cell. Biol. 17, 3966–3976.

Li, W., Mitchell, A.P., 1997. Proteolytic activation of Rim1p, a positive regulator of yeast sporulation and invasive growth. Genetics 145, 63–73.

Li, M., Martin, S.J., Bruno, V.M., Mitchell, A.P., Davis, D.A., 2004. *Candida albicans* Rim13p, a protease required for Rim101p processing at acidic and alkaline pHs. Eukaryot. Cell 3, 741–751.

Liu, O.W., Chun, C.D., Chow, E.D., Chen, C., Madhani, H.D., Noble, S.M., 2008. Systematic genetic analysis of virulence in the human fungal pathogen *Cryptococcus neoformans*. Cell 135, 174–188.

Martínez-Espinoza, A.D., León, C., Elizarraraz, G., Ruiz-Herrera, J., 1997. Monomorphic nonpathogenic mutants of *Ustilago maydis*. Phytopathology 87, 259–265.

Martínez-Espinoza, A.D., García-Pedrajas, M.D., Gold, S.E., 2002. The *Ustilaginales* as plant pests and model systems. Fungal Genet. Biol. 35, 1–20.

Mira, N.P., Lourenço, A.B., Fernandes, A.R., Becker, J.D., Sá-Correia, I., 2009. The RIM101 pathway has a role in *Saccharomyces cerevisiae* adaptive response and resistance to propionic acid and other weak acids. FEMS Yeast Res. 9, 202–216.

Mitchell, B.M., Wu, T.G., Jackson, B.E., Kirk, R.W., 2007. *Candida albicans* strain-depedent virulence and Rim13p-mediated filamentation in experimental keratomycosis. Invest. Opthalmol. & Vis. Sci. 48, 774–780.

Negrete-Urtasun, S., Denison, S.H., Arst Jr., H.N., 1997. Characterization of the pH signal transduction pathway gene *palA* of *Aspegillus nidulans* and identification of possible homologs. J. Bacteriol. 175, 1832–1835.

Nobile, C.J., Solis, N., Myers, C.L., Fay, A.J., Deneault, J.S., Nantel, A., Mitchell, A.P., Filler, S.G., 2008. *Candida albicans* transcription factor Rim101 mediates pathogenic interactions through cell wall functions. Cell Microbiol. 10, 2180–2196.

Orejas, M., Espeso, E.A., Tilburn, J., Sarkar, S., Arst Jr., H.N., Peñalva, M.A., 1995. Activation of the *Aspergillus* PacC transcription factor in response to alkaline ambient pH requires proteolysis of the carboxy-terminal moiety. Genes Dev. 9, 1622–1632.

Peñalva, M.A., Arst Jr., H.N., 2002. Regulation of gene expression by ambient pH in filamentous fungi and yeasts. Microbiol. Mol. Biol. Rev. 66, 426–446.

Peñalva, M.A., Arst Jr., H.N., 2004. Recent advances in the characterization of ambient pH regulation of gene expression in filamentous fungi and yeasts. Annu. Rev. Microbiol. 58, 425–451.

Peñalva, M.A., Tilburn, J., Bignell, E., Arst Jr., H.N., 2008. Ambient pH gene regulation in fungi: making connections. Trends Microbiol. 16, 291–300.

Peñas, M.M., Hervás-Aguilar, A., Múnera-Huertas, T., Reoyo, E., Peñalva, M.A., Arst Jr., H.N., Tilburn, J., 2007. Further characterization of the signaling proteolysis step in the *Aspergillus nidulans* pH signal transduction pathway. Eukaryot. Cell 6, 960–970.

Rodríguez-Galán, O., Galindo, A., Hervás-Aguilar, A., Arst, H.N.Jr., Peñalva, M.A., 2009. Physiological involvement in pH signaling of Vps24-mediated recruitment of *Aspergillus* PalB cysteine protease to ESCRT-III. J. Biol. Chem. 284, 4404–4412.

Rollins, J.A., 2003. The *Sclerotinia sclerotium pac1* gene is required for sclerotial development and virulence. Mol. Plant Microbe Interact. 16, 785–795.

Rollins, J.A., Dickman, M.B., 2001. PH signaling in *Sclerotinia sclerotiorum*: Identification of a *pacC/RIM1* Homolog. Appl. Environ. Microbiol. 67, 75–81.

Ruiz, A., Ariño, J., 2007. Function and regulation of the *Saccharomyces cerevisiae ENA* sodium ATPase system. Eukaryot. Cell 6, 2175–2183.

Ruiz-Herrera, J., León, C., Guevara-Olvera, L., Carabez-Trejo, A., 1995. Yeast-mycelial dimorphism of haploid and diploid strains of *Ustilago maydis*. Microbiology 141, 695–703.

Sambrook, J., Russell, D.W., 1999. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Sentandreu, M., Elorza, M.V., Sentandreu, R., Fonzi, W.A., 1998. Cloning and characterization of *PRA1* a gene encoding a novel pH-regulated antigen of *Candida albicans*. J. Bacteriol. 180, 282–289.

Su, S.S., Mitchell, A.P., 1993. Molecular characterization of the yeast meiotic regulatory gene *RIM1*. Nucleic Acids Res. 21, 3789–3797.

Tilburn, J., Sarkar, S., Widdick, D.A., Espeso, E.A., Orejas, M., Mungroo, J., Peñalva, M.A., Arst, H.N.Jr., 1995. The *Aspergillus* PacC zinc finger transcription factor mediates regulation of both acid- and alkaline-expressed genes by ambient pH. EMBO J. 14, 779–790.

Tilburn, J., Sánchez-Ferrero, J.C., Reoyo, E., Arst Jr., H.N., Peñalva, M.A., 2005. Mutational analysis of the pH signal transduction component PalC of *Aspergillus nidulans* supports distant similarity to BRO1 domain family members. Genetics 171, 393–401.

Tong, A.H., Lesage, G., Bader, G.D., Ding, H., Xu, H., Xin, X., Young, J., Berriz, G.F., Brost, R.L., Chang, M., Chen, Y., Cheng, X., Chua, G., Friesen, H., Goldberg, D.S., Haynes, J., Humphries, C., He, G., Hussein, S., Ke, L., Krogan, N., Li, Z., Levinson, J.N., Lu, H., Ménard, P., Munyana, C., Parsons, A.B., Ryan, O., Tonikian, R., Roberts, T., Sdicu, A.M., Shapiro, J., Sheikh, B., Suter, B., Wong, S.L., Zhang, L.V., Zhu, H., Burd, C.G., Munro, S., Sander, C., Rine, J., Greenblatt, J., Peter, M., Bretscher, A., Bell, G., Roth, F.P., Brown, G.W., Andrews, B., Bussey, H., Boone, C., 2004. Global mapping of the yeast genetic interaction network. Science 303, 808–813.

Vincent, O., Rainbow, L., Tilburn, J., Arst Jr., H.N., Peñalva, M.A., 2003. YPXL/I is a protein interaction motif recognized by *Aspergillus* PalA and its human homologue, AIP1/Alix. Mol. Cell Biol. 23, 1647–1655.

Wang, J., Holden, D.W., Leong, S.A., 1988. Gene transfer system for the phytopathogenic fungus *Ustilago maydis*. Proc. Natl. Acad. Sci. USA 85, 865–869.

Xu, W., Mitchell, A.P., 2001. Yeast PalA/AIP1/Alix homolog Rim20p associates with a PEST-like region and is required for its proteolytic cleavage. J. Bacteriol. 183, 6917–6923.

Xu, W., Smith Jr., F.J., Subaran, R., Mitchell, A.P., 2004. Multivesicular body-ESCRT components function in pH response regulation in *Saccharomyces cerevisiae* and *Candida albicans*. Mol. Biol. Cell 15, 5528–5537.

You, B.J., Chung, K.R., 2007. Phenotypic characterization of mutants of the citrus pathogen *Colletotrichum acutatum* defective in a PacC-mediated pH regulatory pathway. FEMS Microbiol. Lett. 277, 107–114.