The pH-Responsive Transcription Factors YlRim101 and Mhy1 Regulate Alkaline pH-Induced Filamentation in the Dimorphic Yeast Yarrowia lipolytica

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ABSTRACT Environmental pH influences cell growth and differentiation. In the dimorphic yeast Yarrowia lipolytica, neutral-alkaline pH strongly induces the yeast-to-filament transition. However, the regulatory mechanism that governs alkaline pH-induced filamentation has been unclear. Here, we show that the pH-responsive transcription factor Y. lipolytica Rim101 (YlRim101) is a major regulator of alkaline-induced filamentation, since the deletion of YlRIM101 severely impaired filamentation at alkaline pH, whereas the constitutively active YlRIM1011-330 mutant mildly induced filamentation at acidic pH. YlRim101 controls the expression of the majority of alkaline-regulated cell wall protein genes. One of these, the cell surface glycosidase gene YlPHR1, plays a critical role in growth, cell wall function, and filamentation at alkaline pH. This finding suggests that YlRim101 promotes filamentation at alkaline pH via controlling the expression of these genes. We also show that, in addition to YlRim101, the Msn2/Msn4-like transcription factor Mhy1 is highly upregulated at alkaline pH and is essential for filamentation. However, unlike YlRim101, which specifically regulates alkaline-induced filamentation, Mhy1 regulates both alkaline- and glucose-induced filamentation, since the deletion of MHY1 abolished them both, whereas the overexpression of MHY1 induced strong filamentation irrespective of the pH or the presence of glucose. Finally, we show that YlRim101 and Mhy1 positively coregulate seven cell wall protein genes at alkaline pH, including YlPHR1 and five cell surface adhesin-like genes, three of which appear to promote filamentation. Together, these results reveal a conserved role of YlRim101 and a novel role of Mhy1 in the regulation of alkaline-induced filamentation in Y. lipolytica.

IMPORTANCE The regulatory mechanism that governs pH-regulated filamentation is not clear in dimorphic fungi except in Candida albicans. Here, we investigated the regulation of alkaline pH-induced filamentation in Yarrowia lipolytica, a dimorphic yeast distantly related to C. albicans. Our results show that the transcription factor YlRim101 and the Msn2/Msn4-like transcription factor Mhy1 are the major regulators that promote filamentation at alkaline pH. They control the expression of a number of cell wall protein genes important for cell wall organization and filamentation. Our results suggest that the Rim101/PacC homologs play a conserved role in pH-regulated filamentation in dimorphic fungi.

KEYWORDS Rim101, filamentation, dimorphic transition, dimorphism, hyphal growth

Yarrowia lipolytica is a nonconventional yeast species that has been used as a microbial cell factory for the production of multiple industrial and pharmaceutical products (1, 2). Y. lipolytica is also a dimorphic yeast that can switch its cell morphology from the oval-shaped yeast form to pseudohypha or hypha in response to environmental cues (3, 4). The development of filaments in dimorphic fungi is thought to be a foraging behavior that helps the cells to search for nutrients (5). It also plays a role in
the infection of the host by pathogenic fungal species such as the human pathogen *Candida albicans* (6).

Environmental pH influences cell growth and differentiation in both bacteria and fungi. The shift from acidic pH to alkaline pH causes stresses to the cells by affecting the uptake of nutrients such as cations into the cell (7, 8). In fungi, the sensing and adaptation to alkaline pH are primarily carried out by the Rim101/PacC signaling pathway (9, 10). The cell surface proteins Rim21 and Dfg16 of this pathway senses alkaline pH in the environment and elicit a series of signal transduction events that activate Rim101/PacC, a zinc finger transcription factor, via other Rim/Pal proteins. The activation of Rim101/PacC involves the proteolytic removal of the inhibitory C-terminal region, which allows Rim101/PacC to enter the nucleus, bind to the promoters of target genes, and activate or repress their expression. The Rim101/PacC signaling pathway was initially identified in the yeast *Saccharomyces cerevisiae* and the filamentous fungus *Aspergillus nidulans* (11, 12) but was later found to be well conserved in other fungi (9, 13). This pathway controls a number of pH responses, including sporulation and haploid invasive growth in *S. cerevisiae* and the production of alkaline proteases and phosphatases in *A. nidulans*.

pH is an important environmental factor that affects the yeast-to-filament transition in dimorphic fungi. In the dimorphic yeasts *C. albicans* and *Y. lipolytica*, acidic pH promotes yeast-form growth, whereas neutral-alkaline pH induces filamentation (4, 14). However, in other dimorphic fungi, such as *Ustilago maydis* and *Trichosporon cutaneum*, acidic pH induces filamentation, whereas neutral-alkaline pH promotes yeast-form growth (15, 16). The regulatory mechanism that governs pH-regulated filamentation is not well understood except in *C. albicans*. Studies in *C. albicans* showed that the Rim101/PacC homolog, *C. albicans* Rim101 (CaRim101), plays a crucial role in the regulation of pH-regulated filamentation (17). CaRim101 positively regulates the expression of *CaPHR1*, a cell surface glycosidase gene important for growth, cellular morphogenesis, and filamentation at neutral-alkaline pH (18, 19). CaRim101 also positively regulates the expression of cell surface adhesin genes, such as *HWP1* and *HYR1* (8), which are associated with hyphal formation (20, 21).

Although CaRim101 plays an important role in the control of pH-regulated filamentation in *C. albicans*, it is not known whether the Rim101/PacC homologs also control pH-regulated filamentation in other dimorphic fungi. In this study, we show that *Y. lipolytica* Rim101 (YlRim101) shares a conserved function with *C. albicans* CaRim101 in the regulation of pH-regulated filamentation. In addition, we reveal a novel role of the Msn2/Msn4-like transcription factor Mhy1 in the regulation of pH-regulated filamentation.

**RESULTS**

**YlRim101 is a major regulator of alkaline-induced filamentation.** The Rim101/PacC signaling pathway plays an important role in the adaptation to alkaline pH in fungi (9, 13). In the dimorphic yeasts *C. albicans* and *Y. lipolytica*, alkaline pH strongly induces the yeast-to-filament transition (4, 14). While CaRim101 plays an important role in the regulation of filamentation at alkaline pH in *C. albicans* (17), previous studies suggested that YlRim101 is not required for filamentation in *Y. lipolytica* (22, 23). Thus, we deleted the **YlRIM101** (YALI0B13640) gene in the wild-type strain PO1a and reexamined the role of YlRim101 in alkaline-induced filamentation.

*Y. lipolytica* can efficiently utilize both glycerol and glucose. When grown in glycerol medium, the wild-type strain PO1a grew in the oval-shaped yeast form at acidic pH (pH 3.0 to pH 6.0), but the cells became markedly elongated at neutral pH (pH 7.0) and formed long filaments at slightly alkaline pH (pH 7.5) (Fig. 1A, top row). Twenty-eight percent and 85% of wild-type cells were longer than 20 μm at pH 7.0 and pH 7.5, respectively (Fig. 1B). In contrast, the cells of the Ylrim101Δ mutant were just slightly elongated at pH 7.0 but did not form filaments at pH 7.5 (Fig. 1A, second row). Moreover, only 6% of the Ylrim101Δ cells were longer than 20 μm at pH 7.0, and this...
FIG 1 YlRim101 positively regulates alkaline-induced filamentation. (A) Cells of the wild-type (WT) and Ylrim101Δ strains carrying the plasmid vector pINA445 (Vec) were grown in liquid YNBG (glycerol) and YNBD (glucose) media buffered at pHs ranging from 3.0 to 7.5 at 30°C. (B and C) Cells as in panel A were measured for cell length. The percentages of cells longer than 20 μm (B, YNBG medium) and 30 μm (C, YNBD medium) are shown (n > 600 cells). The mother cell and the bud that it carries were counted as one cell. Statistically (Continued on next page)
number did not increase at pH 7.5 (Fig. 1B). This finding suggests that YLRim101 is crucial for alkaline-induced filamentation. Reintroduction of the YLRIM101 gene into the Ylr101Δ mutant restored filament formation at pH 7.5 (see Fig. S1 in the supplemental material), indicating that the filamentation defect was caused by YLRIM101 deletion.

When grown in glucose medium, the wild-type strain displayed a markedly elongated morphology at acidic pH (pH 4.0 to pH 6.0), presumably due to glucose-stimulated weak filamentation (see Discussion). Long filaments were formed at pH 7.0 and pH 7.5 (Fig. 1A, third row). Thirty-two percent and 88% of wild-type cells were longer than 30 μm at pH 7.0 and pH 7.5, respectively (Fig. 1C). In contrast, the Ylr101Δ mutant retained the elongated morphology but did not form long filaments at pH 7.0 and pH 7.5 (Fig. 1A, bottom row). Moreover, only 6% and 14% of the Ylr101Δ cells were longer than 30 μm at pH 7.0 and pH 7.5, respectively (Fig. 1C). This finding supports the view that YLRim101 is crucial for filamentation at alkaline pH. The Ylr101Δ cells still weakly responded to alkaline stimulation, since the percentage of Ylr101Δ cells longer than 30 μm at pH 7.5 was slightly higher than that of pH 7.0 (Fig. 1C). The difference is statistically significant (P < 0.05).

Next, we examined whether the active form of YLRim101 induces filamentation in glycerol medium at acidic pH, where filamentation normally does not occur. Upon upstream signaling at alkaline pH, Rim101/PacC undergoes enzymatic cleavage to remove the inhibitory C-terminal region (9, 13). The C-terminally truncated form of Rim101/PacC is thought to be the active form for function. Although the cleavage site has not been determined for YLRim101, a previous study showed that the C-terminally truncated alleles YLRIM101-1119 (encodes YLRim1011-330) and YLRIM101-5 (encodes YLRim1011-419) induced XPR2-lacZ expression at pH 4.0, whereas wild-type YLRIM101 did not (24), indicating that YLRim1011-330 and YLRim1011-419 are constitutively active. We observed that the expression of YLRim1011-330 and YLRim1011-419 mutants in the Ylr101Δ mutant efficiently restored filament formation in glycerol medium at pH 7.5 (see Fig. S1 in the supplemental material). Thus, they can be used to mimic the activated form of YLRim101.

The expression of the YLRim1011-330 mutant in the Ylr101Δ strain grown in glycerol medium caused cell elongation at pHs from 3.0 to 5.0 and the formation of some long filaments at pH 6.0 (Fig. 1D). Like YLRim1011-330, the YLRim1011-419 mutant displayed a similar effect at acidic pH (data not shown). In contrast, the expression of wild-type YLRim101 in the Ylr101Δ strain did not cause cell elongation at pHs from 3.0 to 6.0 (Fig. 1D). Moreover, the overexpression of the wild-type YLRim101 in the wild-type strain under the control of the strong YITEF1 promoter still did not cause cell elongation in glycerol medium at pH 4.0 (data not shown). This finding indicates that the constitutively active form of YLRim101 is capable of causing mild filamentation at acidic pH.

**YLRim101 controls the expression of the majority of alkaline-regulated cell wall protein genes.** To explore how YLRim101 regulates alkaline-induced filamentation, we wanted to identify the genes that are susceptible to alkaline induction. To this end, we performed transcriptome analysis of the wild-type strain PO1a grown in glycerol medium buffered at acidic pH (pH 4.0) and slightly alkaline pH (pH 7.5) by transcriptome sequencing (RNA-Seq). Our results indicated that a total of 1,593 genes were significantly differentially expressed (≥2-fold, P < 0.05) at pH 7.5 compared to that at pH 4.0 (the full set of data will be published elsewhere). Of these, 621 genes were upregulated, whereas 972 genes were downregulated at pH 7.5. A total of 124 genes were highly upregulated (≥5-fold), whereas 300 genes were highly downregulated (≥5-fold).

The cell wall plays an important role in filamentation. It is required for the maintenance of hyphal growth. Therefore, the cell wall protein genes that were significantly differentially expressed (≥2-fold, P < 0.05) at pH 7.5, particularly those that were upregulated, drew our
attention. A total of 41 cell wall protein genes were upregulated at pH 7.5 (Table 1, Table S1). Fifteen of them were highly upregulated (≥5-fold). These genes encode putative cell wall structural proteins, such as the ones similar to *S. cerevisiae* Cwp1, cell surface enzymes involved in cell wall biosynthesis such as Yiphr1 and Yicrh12, and cell surface proteins similar to *S. cerevisiae* Flo11 or *C. albicans* Hyr1. All these proteins appear to possess a signal peptide. The majority of them are putative glycosylphosphatidylinositol (GPI)-anchored cell surface proteins, whereas several proteins are transmembrane proteins. There were also 26 cell wall protein genes that were downregulated at pH 7.5 (Table 1, Table S1). Sixteen of them were highly downregulated (≥2-fold). These genes encode putative cell wall structural proteins, such as the ones similar to *S. cerevisiae* Pir1 and Cwp1, cell surface enzymes similar to *S. cerevisiae* Yps3 and Dcw1, the cell surface glycosidase Yiphr2, and cell surface proteins similar to *S. cerevisiae* Flo11 or *C. albicans* Hyr1.

We also examined the transcriptome of the *Ylr1* mutant at pH 7.5 by RNA-Seq and compared it with that of the wild-type strain. Our results showed that 22 of the 41 alkaline-upregulated cell wall protein genes (54%) exhibited a significant reduction in read counts (≥2-fold, P < 0.05) in the *Ylr1* mutant, indicating that they were Ylr1 regulated. Among the 15 highly alkaline-upregulated cell wall protein genes, 14 of them (93%) were Ylr1 regulated (Table 1, Table S2; Fig. 2A depicts the top 12 genes). This finding suggests that Ylr1 is important for the upregulation of the majority of alkaline-upregulated cell wall protein genes, particularly the highly alkaline-upregulated ones. We monitored the top 12 alkaline-upregulated cell wall protein genes for their transcriptional activities in the *Ylr1* mutant using *promoter-lacZ* reporters. They were all markedly reduced in the *Ylr1* mutant.

| Direction of regulation | Ylr1-regulated genes | Non-Ylr1-regulated genes |
|-------------------------|----------------------|--------------------------|
| **Up**                  |                      |                          |
| High (≥5-fold), 15 genes | YALI0F26655 (U2), YALI0E22286 (U4), YALI0A00176 (U6), PHR1 (YALI0D4857 U8), YALI0C11165 (U15), YALI0C23452 (U19), YALI0E26125 (U22), YALI0A21373 (U26), YALI0D08358 (U33), YALI0E01210 (U61), YALI0F19030 (U74), YALI0B18194 (U103), YALI0A17919 (U111), YALI0D07248 (U120) | YALI0E19426 (U113) |
| Low (≥2-fold), 26 genes  | CW1P (YALI0E18788, U158), CRH12 (YALI0E24673, U166), CH52 (YALI0B16324, U204), YALI0D07270 (U338), YALI0A11198 (U488), YALI0C08473 (U550), YALI0F09990 (U573), YALI0F10901 (U578) | YALI0D00154 (U138), CWH43 (YALI0E33473, U149), YALI0E02823 (U150), PSA1 (YALI0C06490, U177), YALI0F21428 (U203), DFGS (ALI0F18722, U205), YALI0E20888 (U229), CRH11 (YALI0C09680, U232), UTR2 (YALI0B15510, U320), YALI0F01925 (U322), UAP1 (YALI0E03146, U353), CHSS (YALI0E1670, U372), YALI0B03564 (U376), KRE6 (YALI0C14190, U434), YALI0E10175 (U480), CH53 (YALI0C24354, U482), CWH41 (YALI0F14927, U526), YALI0E22374 (U564) |
| **Down**                |                      |                          |
| High (≥5-fold), 16 genes | PIR2 (YALI0C2981, D9), PIR1 (YALI0B20306, D23), YALI0F18282 (D24), YALI0C22836g (D40), YALI0A13013 (D91), YALI0A20438 (D115), YALI0E31108 (D163), YALI0A02002 (D166), YALI0B07403 (D167), EXG1 (YALI0F05390, D231), PIR2 (YALI0D0639, D32), YALI0C14938 (D247), YALI0C10135 (D251), YALI0C01411 (D260) | YALI0A08800 (D196), YALI0D01331 (D277) |
| Low (≥2-fold), 10 genes  | YALI0E33891 (D720)   |                          |

*Numbers in parentheses indicate each gene’s ranking in the full list of the RNA-Seq data set. Genes that showed at least 2-fold changes (P < 0.05) in the *Ylr1* mutant compared to that in the wild-type strain are defined as *Ylr1* regulated. Genes that encode proteins that share similarities to the cell surface adhesin *S. cerevisiae* Flo11 or *C. albicans* Hyr1 are in boldface. YALI0D09185, which encodes a protein that shares weak similarity to the *S. cerevisiae* adhesin Aga1 but lacks an identifiable GPI modification site, is underlined.

*YALI0E22088 is classified as non-*Ylr1* regulated, because its transcription did not decrease but rather increased in the *Ylr1* mutant, and the extent of the increase is marginal (2.0-fold, P = 0.03).
compared to those in the wild-type strain (Fig. 2B). Remarkably, the expression of the constitutively active YlRim101-330 mutant in the wild-type strain at pH 4.0 significantly upregulated the transcription levels of five of the 12 genes, YALI0E22286 (U4), YIPHR1 (U8), YALI0C23452 (U19), YALI0E26125 (U22), and YALI0E01210 (U61) (Fig. 2C). These results indicate that YlRim101 positively regulates a subset of alkaline-upregulated cell wall protein genes, which may play roles in cell wall organization and filamentation.

Among the 26 alkaline-downregulated cell wall protein genes, 15 of them (58%) were YlRim101 regulated (≥2-fold, \( P < 0.05 \)). Among the 16 highly alkaline-downregulated genes, 14 of them (87%) were YlRim101 regulated (Table 1, Table S2). This finding suggests that YlRim101 is also important for the downregulation of the majority of alkaline-downregulated cell wall protein genes.

We found that all the 41 alkaline-upregulated and the 26 alkaline-downregulated cell wall proteins did not exhibit differential expression (≥2-fold, \( P < 0.05 \)) in the wild-type and Ylrim101Δ strains at pH 4.0 (Table S3). This finding suggests that YlRim101 does not regulate the expression of these genes at acidic pH.

The YlRim101-regulated cell surface glycosidase genes YIPHR1 and YIPHR2 are required for growth, cell wall function, and filamentation. YIPHR1 and its homolog, YIPHR2, encode proteins that are highly similar to S. cerevisiae Gas1 and C. albicans CaPhr1 and CaPhr2, which are GPI-anchored cell surface 1,3-β-glucanosyltransferases necessary for cell wall organization and cellular morphogenesis (18, 25, 26). Previous studies have shown that YIPHR1 and YIPHR2 are pH-responsive genes and are transcriptionally regulated by YlRim101 in an inverted pattern similar to that of C. albicans CaPhr1 and CaPhr2, respectively (27–29). We obtained a similar result by RNA-Seq.
and promoter-lacZ analyses. YlPHR1 (U8) was highly upregulated at pH 7.0 and pH 7.5 (Table 1, Fig. 3A), whereas YlPHR2 was highly downregulated at pH 7.5 (Table 1, Fig. 3B). In addition, Ylrmin101 is required for both the upregulation of YlPHR1 at pH 7.0 and pH 7.5 as well as the downregulation of YlPHR2 at pH 7.5 (Fig. 3A and B).

Since the cellular roles of YlPHR1 and YlPHR2 have not been characterized previously, we generated Ylphr1D, Ylphr2D, and Ylphr1D Ylphr2D mutants and examined the growth and cell morphology. We observed that the Ylphr1D and Ylphr2D mutants exhibited slower growth and increased sensitivity to the cell wall-perturbing agent Congo red compared to the wild-type strain at pH 7.5 and pH 4.0, respectively, whereas the Ylphr1D Ylphr2D double mutant exhibited the same defects at both pH 4.0 and pH 7.5 (Fig. 3C). This finding suggests that YlPHR1 and YlPHR2 are required for normal growth and cell wall function at alkaline pH and acidic pH, respectively, similar to C. albicans CaPHR1 and CaPHR2 (18, 25).

The Ylphr1Δ mutant exhibited normal cell morphology in YNBD medium at pHs ranging from 3.0 to 7.0. However, it failed to form long filaments at pH 7.5 (Fig. 4A, second row), indicating that YlPHR1 is required for cellular morphogenesis specifically at alkaline pH, i.e., alkaline-induced filamentation. In contrast to the Ylphr1Δ mutant, the Ylphr2Δ mutant exhibited normal filamentation at pH 7.0 and pH 7.5. However, at acidic pH (pH 3.0 to pH 6.0), the Ylphr2Δ mutant exhibited a round cell morphology (Fig. 4A, third row), which is different from the elongated cell morphology of the wild-type strain, indicating that YlPHR2 is required for cellular morphogenesis specifically at acidic pH. The Ylphr1Δ Ylphr2Δ mutant did not exhibit the elongated cell morphology at pHs ranging from 3.0 to 6.0 or formed filaments at pH 7.0 and pH 7.5 (Fig. 4A, bottom row), suggesting that YlPHR1 and YlPHR2 are the two major 1,3-β-glucanosyltransferase genes in the cells. We noticed that the Ylphr1Δ mutant failed to form long filaments at pH 7.5 but formed them at pH 7.0 (Fig. 4A, second row). This is likely due to the functional compensation by YlPhr2, which is still highly expressed at pH 7.0.
We showed earlier that the constitutively active YlRim1011-330 mutant induced filamentation at acidic pH (Fig. 1D). This effect can still be seen in the Ylphr1D mutant but was severely impaired in the Ylphr2D mutant at pH 4.0 (Fig. 4B), suggesting that YlPHR2 is important for filamentation at acidic pH. The overexpression of MHY1 induced the formation of long filaments in the wild-type strain. However, this effect was compromised in the Ylphr2Δ mutant at pH 4.0 and in the Ylphr1Δ mutant at pH 7.5 (Fig. 4C), supporting the view that YlPHR1 and YlPHR2 are required for filamentation at alkaline pH and acidic pH, respectively.

**YlRIM101 and MHY1 are the two major transcription factor genes highly upregulated at alkaline pH.** In addition to the cell wall protein genes, there were 25 transcription factor genes that exhibited significant differential expression ($\geq$2-fold, $P < 0.05$) at pH 7.5 compared to that at pH 4.0, as shown by RNA-Seq (Table 2, Table S4). Of these, 11 genes were upregulated, whereas 14 genes were downregulated at pH 7.5. Three
YlRim101 and Mhy1 in pH-Regulated Filamentation

YlRim101-330 mutant at pH 4.0 (Fig. 5C). These results indicate that YlRim101 partially downregulated genes were highly upregulated (≥5-fold), whereas five genes were highly downregulated (≥5-fold).

The two highly alkaline-upregulated transcription factor genes YIRIM101 and MHY1 drew our attention, since they both regulate filamentation. MHY1 encodes an Msn2/Msn4-like zinc finger transcription factor that plays a key role in filamentation (30, 31). It is not surprising to find YIRIM101 on the list, since YIRIM101 is known to be alkaline induced (24). The identification of MHY1 as an alkaline-induced gene is unexpected. YIRIM101 and MHY1 appear to be the two major transcription factor genes upregulated at alkaline pH, since they were among the three highly alkaline-induced transcription factor genes (Fig. 5A). Additionally, they exhibited the highest RNA-Seq read counts at pH 7.5 among all 11 alkaline-upregulated genes (Table S4).

Both RNA-Seq and promoter-lacZ data indicate that MHY1 is an alkaline-induced gene, since its transcription was highly upregulated at alkaline pH (Fig. 5A and B). Our results also showed that the level of MHY1 transcription was reduced about 2-fold in the Ylrim101Δ mutant at pH 7.5 (Fig. 5A and B, and Table S5). Moreover, the level of MHY1 transcription increased 2.5-fold upon the expression of the constitutively active YIRIM101 gene at pH 4.0 (Fig. 5C). These results indicate that YIRIM101 partially regulates MHY1. Since a significant portion of MHY1 upregulation was retained in the Ylrim101Δ mutant at pH 7.5, the upregulation of MHY1 may also involve a YIRIM101-independent mechanism. We found that Mhy1 does not regulate YIRIM101 (Fig. 5D).

In addition to MHY1, YIRIM101 also regulates YIRIM101 itself. The alkaline-induced expression of YIRIM101 was severely impaired in the Ylrim101Δ mutant, as shown by the promoter-lacZ reporter (Fig. 5E), in agreement with a previous report (24).

Mhy1 regulates both alkaline- and glucose-induced filamentation. We observed that the mhy1Δ mutant exhibited an oval-shaped yeast-form morphology at both pH 4.0 and pH 7.5, even in the filamentation-favoring glucose medium. It neither exhibited an elongated morphology at pH 4.0 nor formed any filaments at pH 7.5 (Fig. 6A). The Ylrim101Δ mhy1Δ mutant also resembled the mhy1Δ mutant in cell morphology. The induction of filament formation by the constitutively active YIRIM101 gene at pH 4.0 also completely depends on Mhy1 (Fig. 6B). These results suggest that Mhy1 is essential for both alkaline- and glucose-induced filamentation. On the other hand, Mhy1 overexpression in both the wild-type and Ylrim101Δ strains caused the formation of long filaments in the yeast-form-favoring glycerol medium at pH 4.0 (Fig. 6C, top row). It also caused strong filamentation in the Ylrim101Δ mutant at pH 7.5 (Fig. 6C, bottom row), indicating that Mhy1 is capable of causing filamentation irrespective of the pH or the presence of glucose when its expression increases. These results suggest that Mhy1 is a key positive regulator of both alkaline- and glucose-induced filamentation.

Mhy1 and YIRIM101 positively coregulate five adhesin-like genes, and three of them appear to promote filamentation. Alkaline pH highly upregulates the transcription of 15 cell wall protein genes (Table 1), which may play roles in filamentation. Since 14 of these genes are YIRIM101 regulated, we wanted to know whether Mhy1 also

**TABLE 2** The alkaline-upregulated and alkaline-downregulated transcription factor genes identified by RNA-Seq*  

| Direction of regulation | YIRIM101-regulated genes | Non-YIRIM101-regulated genes |
|-------------------------|--------------------------|------------------------------|
| Up                      |                          |                              |
| High (≥5-fold), 3 genes | MHY1 (YALI0E21582, U46), YALI0A12925 (U49), RIM101 (YALI0B13640, U22) | YALI0E20449 (U131), YALI0B13354 (U218), YALI0E29271 (U321), TEC1 (YALI0F15169, U601) |
| Low (≥2-fold), 8 genes  | ACE2 (YALI0E16973, U127), YALI0F25113 (U165), YALI0C09482 (U220), YALI0C08327 (U300) |                              |
| Down                    |                          |                              |
| High (≥5-fold), 5 genes | YALI0D15664 (D101), YALI0B05478 (D134), YALI0F03157 (D298) | YALI0E18161 (D212), YALI0D20482 (D280) |
| Low (≥2-fold), 9 genes  | YALI0E23518 (D661), PPR1 (YALI0B09713, D822) | YALI0B0660 (D527), YALI0D24167 (D616), YALI0C13178 (D732), YALI0A16841 (D906), YALI0D09625 (D934), YALI0D20394 (D966), YALI0F16599 (D970) |

*Numbers in parentheses indicate each gene’s ranking in the full list of the RNA-Seq data set. Genes that showed at least 2-fold changes (P < 0.05) in the Ylrim101Δ mutant compared to that in the wild-type strain are defined as YIRIM101 regulated.
regulates their expression. To this end, we monitored the transcriptional activities of the 15 cell wall protein genes in the wild-type, Ylr101Δ, and mhy1Δ strains grown in YNBG medium buffered at pH 4.0 and pH 7.5 by promoter-lacZ reporters. The results showed that eight genes exhibited differential expression in the mhy1Δ mutant. Of these, the five genes YALI0A00176 (U6), YALI0C11165 (U15), YALI0C23452 (U19), YALI0D09185 (U33), and YALI0F19030 (U4) exhibited a drastic reduction by more than 3.9-fold in transcription in the mhy1Δ mutant (Fig. 7A), suggesting that Mhy1 is crucial for their expression. The two genes Ylr101Δ and mhy1Δ strains carrying pINA445-PYlr101-lacZ grown in YNBG medium buffered at pH 4.0 and pH 7.5 at 30°C. Statistically significant differences are indicated by the asterisks (**, P < 0.01; ***, P < 0.001). NS, not statistically significant.

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FIG 5 Ylr101Δ and Mhy1 are the two major transcription factor genes upregulated at alkaline pH. (A) RNA-Seq read counts of the transcription factor genes Mhy1, Ylr101, and YALI0A12925. Cells of the wild-type (WT) and Ylr101Δ strains carrying pINA445 were grown in YNBG medium buffered at pH 4.0 and pH 7.5 at 30°C. For YALI0A12925, a blown-up bar graph is shown in the box, because its read counts are too low (less than 40) to be seen. (B) β-Galactosidase activities of Pmhy1-lacZ in cells of the wild-type and Ylr101Δ strains carrying pINA445-Pmhy1-lacZ grown in YNBG medium buffered at pH 4.0 and pH 7.5 at 30°C. (C) β-Galactosidase activities of Pmhy1-lacZ in cells of the wild-type strain carrying pINA445-Pmhy1-lacZ/pINA443 (Vec) or pINA445-Pmhy1-lacZ/pINA443-Ylr101Δ strains grown in YNBG medium buffered at pH 4.0 and pH 7.5 at 30°C. (D) β-Galactosidase activities of Pmhy1-lacZ in cells of the wild-type and mhy1Δ strains carrying pINA445-Pmhy1-lacZ grown in YNBG medium buffered at pH 4.0 and pH 7.5 at 30°C. (E) β-Galactosidase activities of Pmhy1-lacZ in cells of the wild-type and Ylr101Δ strains carrying pINA445-PYlr101-lacZ grown in YNBG medium buffered at pH 4.0 and pH 7.5 at 30°C. Statistically significant differences are indicated by the asterisks (**, P < 0.01; ***, P < 0.001). NS, not statistically significant.

regulates their expression. To this end, we monitored the transcriptional activities of the 15 cell wall protein genes in the wild-type, Ylr101Δ, and mhy1Δ strains grown in glycerol medium at pH 7.5 by promoter-lacZ reporters. The results showed that eight genes exhibited differential expression in the mhy1Δ mutant. Of these, the five genes YALI0A00176 (U6), YALI0C11165 (U15), YALI0C23452 (U19), YALI0D09185 (U33), and YALI0F19030 (U4) exhibited a drastic reduction by more than 3.9-fold in transcription in the mhy1Δ mutant (Fig. 7A), suggesting that Mhy1 is crucial for their expression. The two genes YIPHR1 (U8) and YALI0E01210 (U61) also exhibited a significant reduction but to a lesser extent (1.4-fold and 1.2-fold), suggesting that Mhy1 is partly required for their expression. The remaining one, YALI0E22286 (U4), is the only gene that exhibited an increase, which is about 2-fold, but not a reduction in the mhy1Δ mutant, suggesting that it was negatively regulated by Mhy1. Thus, Ylr101Δ and Mhy1 coregulate eight of the 15 highly alkaline-induced cell wall protein genes and positively coregulate seven of them (Fig. 7B).

To investigate whether the upregulation of alkaline-upregulated cell wall protein genes is sufficient to cause filamentation, we overexpressed 14 of the 15 highly alkaline-upregulated cell wall protein genes, except YALI0E19426 (U113), under the control of the strong YITEF1 promoter. None of these genes, including YIPHR1, enhanced filament formation when overexpressed individually in the wild-type strain grown in glycerol medium at pH 7.0 (data not shown). We then overexpressed the top six of the 14 genes plus two additional Ylr101- and Mhy1-coregulated genes, YALI0D09185 (U33)
and YALI0F19030 (U74), in pairs. The result showed that, among the 28 pairs of genes, only the two pairs YALI0C23452 (U19)-YALI0F19030 (U74) and YALI0E22286 (U4)-YALI0D09185 (U33) markedly increased the cell length upon overexpression in the wild-type strain grown in glycerol medium at pH 7.0 (Fig. 7C and data not shown), suggesting that the four genes promote filament formation. The gene YALI0E22286 encodes a protein that shares similarity to the S. cerevisiae cell wall protein Cwp1. Interestingly, the three genes YALI0C23452, YALI0F19030, and YALI0D09185 encode proteins that share similarities to the S. cerevisiae cell surface adhesins that mediate cell adhesion. Of these, the two genes YALI0C23452 and YALI0F19030 encode proteins that share similarities to the S. cerevisiae flocculin Flo11 with features including a signal peptide at the N terminus, Pro/Ser/Thr-rich repeats in the central region, potential N-glycosylation sites in the central region, and a potential GPI modification site at the C terminus, whereas the gene YALI0D09185 encodes a protein that shares weak similarity to the S. cerevisiae a-agglutinin Aga1. Like Aga1, YALI0D09185 has a signal peptide at the N terminus and a Ser/Thr-rich central region. However, it lacks an identifiable GPI modification site at the C terminus.

Remarkably, the two YIRim101- and Mhy1-coregulated genes YALI0A00176 and YALI0C11165 also encode proteins that share similarities to the yeast adhesins. YALI0A00176 and YALI0F19030 (U74), in pairs. The result showed that, among the 28 pairs of genes, only the two pairs YALI0C23452 (U19)-YALI0F19030 (U74) and YALI0E22286 (U4)-YALI0D09185 (U33) markedly increased the cell length upon overexpression in the wild-type strain grown in glycerol medium at pH 7.0 (Fig. 7C and data not shown), suggesting that the four genes promote filament formation. The gene YALI0E22286 encodes a protein that shares similarity to the S. cerevisiae cell wall protein Cwp1. Interestingly, the three genes YALI0C23452, YALI0F19030, and YALI0D09185 encode proteins that share similarities to the S. cerevisiae cell surface adhesins that mediate cell adhesion. Of these, the two genes YALI0C23452 and YALI0F19030 encode proteins that share similarities to the S. cerevisiae flocculin Flo11 with features including a signal peptide at the N terminus, Pro/Ser/Thr-rich repeats in the central region, potential N-glycosylation sites in the central region, and a potential GPI modification site at the C terminus, whereas the gene YALI0D09185 encodes a protein that shares weak similarity to the S. cerevisiae a-agglutinin Aga1. Like Aga1, YALI0D09185 has a signal peptide at the N terminus and a Ser/Thr-rich central region. However, it lacks an identifiable GPI modification site at the C terminus.

Remarkably, the two YIRim101- and Mhy1-coregulated genes YALI0A00176 and YALI0C11165 also encode proteins that share similarities to the yeast adhesins.
shares similarities to *S. cerevisiae* Flo11, whereas YALI0C11165 shares similarities to the *C. albicans* adhesin Hyr1, with features including a signal peptide at the N terminus, a Ser/Thr-rich region in the central region, a number of NNGS or NGNGS repeats that are potential N-glycosylation sites, and a potential GPI modification site at the C terminus. Together, our

![FIG 7](msphere.asm.org)

**FIG 7** Mhy1 and YlRim101 positively coregulate five adhesin-like genes, and three of them appear to promote filamentation. (A) β-Galactosidase activities of promoter-lacZ for the indicated 15 highly alkaline-upregulated cell wall protein genes in cells of the wild-type, *YlRim101Δ*, and *mhy1Δ* strains carrying pH445-Δα-αlacZ grown in YNBG medium buffered at pH 7.5 and 30°C. Statistically significant differences are indicated by the asterisks (*, P < 0.05; **, P < 0.01; ***, P < 0.001). NS, not statistically significant. (B) A table that lists the genes regulated by *YlRim101*, Mhy1, or both among the 15 highly alkaline-upregulated cell wall protein genes. The number in parentheses indicates the ranking of that gene in the full list of all alkaline-upregulated (U) genes. The genes that encode proteins that share similarities to the cell surface adhesins *S. cerevisiae* Flo11 and *C. albicans* Hyr1 are in boldface. YALI0D09185, which encodes a protein that shares weak similarity to the *S. cerevisiae* adhesin Aga1 but lacks an identifiable GPI modification site, is underlined. *Mhy1 negatively regulates its expression. (C) Cells of the wild-type strain carrying the pair of plasmids pYL13 (Vec)/pYL21 (Vec), pYL13-YALI0E22286/pYL21-YALI0F19030, pYL13-YALI0C23452/pYL21-YALI0D09185, pYL13-YALI0C23452/pYL21, pYL13-YALI0E22286/pYL21, pYL13/pYL21-YALI0F19030, and pYL13/pYL21-YALI0D09185 were grown in YNBG medium buffered at pH 7.0. The percentages of cells longer than 20 μm, 30 μm, and 40 μm are indicated below the pictures (n > 600 cells). Note that the presence of URA3-marked vector (pYL21) decreased the lengths of elongated cells and filaments in the strain carrying LEU2-marked vector due to unknown reasons. Bar, 10 μm.
results indicate that Mhy1 and YlRim101 positively coregulate five adhesin-like genes, three of which appear to promote filamentation.

**DISCUSSION**

Alkaline pH influences a number of cellular processes, such as nutrient uptake and protease production, in fungi. In dimorphic fungi, alkaline pH also affects the yeast-to-filament transition. While a number of cellular responses to alkaline pH are known to be regulated by the Rim101/PacC signaling pathway, the regulatory mechanism that governs alkaline pH-regulated filamentation is not well understood, except in the yeast *C. albicans*. In *C. albicans*, the Rim101/PacC signaling pathway plays an essential role in alkaline-regulated filamentation (17). However, the functional conservation of this signaling pathway is not clear in other dimorphic fungi. In this study, we show that YlRim101 is crucial for alkaline-induced filamentation in *Y. lipolytica*, a yeast species distantly related to *C. albicans*. In *C. albicans*, the Rim101/PacC signaling pathway plays an essential role in alkaline-regulated filamentation (17). However, the functional conservation of this signaling pathway is not clear in other dimorphic fungi. In this study, we show that YlRim101 is crucial for alkaline-induced filamentation in *Y. lipolytica*, a yeast species distantly related to *C. albicans*, suggesting that the Rim101/PacC signaling pathway plays a conserved role in the control of this process in dimorphic fungi. In addition to YlRim101, we identified an Msn2/Msn4-like transcription factor that is also pH responsive and essential for filamentation at alkaline pH, but its role is not shared with the Mhy1 homolog in *C. albicans*, suggesting that additional regulation by other factors also exists in other dimorphic fungi. Furthermore, we identified several cell wall protein genes that are coregulated by YlRim101 and Mhy1 and important for cell wall organization and filamentation.

The role of YlRim101 in the control of alkaline-regulated filamentation is largely conserved in *Y. lipolytica* compared to that of *CaRim101* in *C. albicans*. YlRim101 and CaRim101 both promote alkaline-induced filamentation. In *C. albicans*, CaRim101 positively regulates the cell surface glycosidase genes *CaPHR1* and *CaCRH11* (8, 18). We find that YlRim101 also positively regulates *YlPHR1* and *YlCRH12*, homologs of *C. albicans* *CaPHR1* and *CaCRH11*, respectively. More importantly, the inverted pattern of CaRim101-dependent regulation on a pair of alkaline and acidic glycosidase genes, *CaPHR1* and *CaPHR2*, the requirement for *CaPHR1* in cell wall organization and filamentation, as well as the requirement for *CaPHR2* in cell wall organization in *C. albicans* are well conserved in *Y. lipolytica*. However, there are still some differences in functions between YlRim101 and CaRim101. For example, CaRim101 regulates the alkaline-induced gene *CaKRE6* (8), which encodes a subunit of β-1,6-glucan synthase involved in cell wall biosynthesis (32). In contrast, YlRim101 does not regulate *YlKRE6*, although it is still alkaline induced. The two yeasts also show some differences in the effectors that regulate alkaline-induced filamentation. Apart from YlRim101, the transcription factor Mhy1 plays an additional role in the regulation of alkaline-induced filamentation. However, the *C. albicans* homolog of Mhy1, MnlI, is reported to regulate the response to weak acid. It neither is alkaline induced nor regulates filamentation (33, 34).

*Y. lipolytica* can efficiently utilize both glucose and glycerol. A distinct feature of the *Y. lipolytica* strain PO1a is that the cells display an elongated, rod-like morphology in glucose medium but not in glycerol medium. This phenomenon is not common in other dimorphic yeasts, including *C. albicans*. We propose that the elongated morphology seen in cells grown in glucose medium represent an early state of filament development during the yeast-to-filament transition based on three observations. First, this morphology can be observed in wild-type cells grown in glycerol medium at pH 7.0 (Fig. 1A, top row). When the pH increases to 7.5, long filaments (hyphae) start to form. Moreover, cells expressing the constitutively active YlRim101-1-330 mutant displayed elongated morphology in glycerol medium at acidic pH but formed long filaments at pH 7.0 (Fig. 1D, bottom row). Second, the deletion of *MHY1* abolished this morphology in glucose medium at pH 4.0 (Fig. 6A). Third, we reported previously that the inactivation of the TORC1-Sch9 signaling pathway caused the same elongated morphology and the upregulation of *MHY1* in cells grown in glycerol medium (35). Thus, it appears that glucose can stimulate filamentation, but only weakly. To date, how glucose induces filamentation remains elusive.
YlRim101 is best known for its function in the induction of the alkaline protease gene XPR2 (24, 27). Its role in alkaline-induced filamentation has been investigated before but was underestimated (22, 23). One reason is that the culture media that were utilized to grow the yeast strains in previous studies contain filamentation-stimulating nutrients such as glucose, GlcNAc, or peptone. The Ylrim101D mutant still responded to these stimuli irrespective of the pH and might exhibit an elongated morphology or form short filaments depending on the nutrients utilized (see the bottom two rows of Fig. 1A, for example), which might have obscured the filamentation defect of the Ylrim101D mutant. Another reason appears to be the utilization of culture media buffered at pH 7.0 instead of pH 7.5 in previous studies. Since pH 7.0 induces filamentation less strongly than pH 7.5 does (see Fig. 1A, for example), the defect of the Ylrim101D mutant might be less obvious.

Based on our results, we propose a model to explain how alkaline pH induces filamentation in Y. lipolytica (Fig. 8). We propose that alkaline-induced filamentation is primarily regulated by the transcription factors YlRim101 and Mhy1. Of the two, YlRim101 is the major regulator of this process, since the majority of alkaline-upregulated cell wall protein genes and nearly all 15 highly alkaline-upregulated cell wall protein genes are YlRim101 regulated. In addition, YlRim101 partly regulates the transcription of MHY1. Like C. albicans CaRim101 (8), YlRim101 can also function as a repressor of gene expression, since it is involved in the downregulation of a subset of cell wall protein genes, including YlPHR2 (Table 1). Although we cannot rule out the possibility that some of the YlRim101-downregulated cell wall proteins are important for filamentation, we show that one of these genes, YlPHR2, is not required for filamentation at alkaline pH (pH 7.5). We speculate that the activator role of YlRim101 in the regulation of cell wall protein genes is more important for YlRim101 in promoting filamentation.

Compared to YlRim101, Mhy1 appears to play a less important role in the regulation of alkaline-induced filamentation, since Mhy1 is required for the upregulation of just seven of the 15 highly alkaline-upregulated cell wall protein genes. However, we observed that MHY1 deletion and MHY1 overexpression affected filamentation much more strongly than those of YlRIM101 in glycerol medium, indicating that Mhy1 is more potent than YlRim101 in the regulation of filamentation. These two observations seem to be at odds. We hypothesize that Mhy1 also regulates some other genes that
are important for filamentation but do not exhibit significant upregulation at alkaline pH. This can explain why MHY1 deletion and MHY1 overexpression produce a stronger phenotype. The observation that the deletion of MHY1 abolished filamentation in glucose medium at both pH 4.0 and pH 7.5 supports this possibility. The failure of these Mhy1-regulated genes to become alkaline-upregulated might be due to other regulators of alkaline-induced filamentation that dampen the effect of Mhy1 (see below).

In addition to YLRim101 and Mhy1, other effectors that regulate alkaline-induced filamentation may also exist, because 19 of the 41 alkaline-upregulated cell wall protein genes are non-YLRim101 regulated (Table 1), and one of the 15 highly alkaline-upregulated cell wall protein genes is neither YLRim101 regulated nor Mhy1 regulated (Fig. 7B). These regulators may collectively play a minor role in the regulation of alkaline-induced filamentation. Some of these regulators may even dampen the effect of Mhy1 on the upregulation of filamentation-associated genes via downregulating the transcription levels of these genes, preventing these Mhy1-regulated genes from getting upregulated at alkaline pH. We noticed that the transcription factor gene YITEC1, which is known to repress filamentation (36), was upregulated 2-fold at pH 7.5, as shown by RNA-Seq (see Table S4 in the supplemental material).

At alkaline pH, the conserved Rim/Pal signaling pathway activates YLRim101, which in turn stimulates its own transcription, leading to a rapid accumulation of active YLRim101 in the cells. Like YLRIM101, MHY1 is highly upregulated transcriptionally at alkaline pH. This process involves both YLRim101 and an unknown YLRim101-independent mechanism. It is not known how Mhy1 is activated and by what signal. Mhy1 may need to be activated via a certain type of posttranslational modification, similar to YLRim101. It is interesting that Mhy1 stimulates its own transcription, as does YLRim101 (31). The autoregulation of YLRim101 and Mhy1 may ensure a rapid adaptation to alkaline pH for the cells.

In the dimorphic yeasts S. cerevisiae and C. albicans, the signaling pathways that promote filamentation induce the expression of several genes that encode GPI-anchored cell surface glycoproteins, such as the S. cerevisiae gene FLO11 and the C. albicans genes HYR1 and HWP1 (6, 20, 37). The proteins encoded by these genes lack enzymatic activities. Some of them play an important role in filament formation and/or function as cell surface adhesins that regulate cell adhesion (21, 38–43). In C. albicans, CaRim101 upregulates the adhesin genes HWP1 and HYR1 at alkaline pH (8). We find that, in Y. lipolytica, among the 15 highly alkaline-upregulated cell wall protein genes, YLRim101 and Mhy1 coregulate eight of them, including the five adhesin-like genes and the cell surface glycosidase gene YIPHR1. The cellular roles of these adhesin-like genes have not been established previously. The observation that three of them, YALI0C23452, YALI0F19030, and YALI0D09185, weakly caused cell elongation upon overexpression supports the idea that these adhesin-like genes promote filamentation.

The cell surface glycosidase gene YIPHR1 is required for cell wall organization and filamentation. It may also be required for cell adhesion, since C. albicans CaPHR1 is known to be required for cell adhesion (19). The CRH family genes, which encode another family of GPI-anchored cell surface glycosidases, are also required for cell wall assembly and cell adhesion in C. albicans (44, 45). We find that the three CRH family genes YICRH11, YICRH12, and YIUTR2 are all upregulated at alkaline pH. In addition, YLRim101 positively regulates YICRH12. This finding suggests that the CRH family genes also are required for cell wall organization and cell adhesion in Y. lipolytica.

Mhy1 is reported to bind to the DNA motif WNAGGG (W = A or T; N = A, T, G, or C) (31). We found that all eight YLRim101- and Mhy1-coregulated cell wall protein genes contain this motif (mostly 3 to 7 copies) in the upstream intergenic region. At least one copy of this motif is present within the 1,000-bp sequence upstream of the start codon of each gene, suggesting that Mhy1 directly regulates the expression of these genes. YLRim101 is thought to bind to the DNA sequence with core motif GCCARG (R = A or G) (24), which is identical to the core consensus site of PacC in A. nidulans (9). We found that seven of the eight YLRim101- and Mhy1-coregulated cell wall protein genes,
MATERIALS AND METHODS

Strains and media. The Y. lipolytica strains used in this study are listed in Table S7 in the supplemental material. PO1a (MATa leu2-270 ura3-302) was used as the wild-type strain. Y. lipolytica strains were grown at 30°C in YPD medium (20 g/liter peptone, 10 g/liter yeast extract, 2% glucose), YNBD medium (6.7 g/liter yeast nitrogen base without amino acid, 1% glucose), or YNBG medium (6.7 g/liter yeast nitrogen base without amino acid, 1% glycerol) supplemented with 80 mg/liter leucine, 20 mg/liter uracil, or both, when required. YNBD and YNBG media were buffered after autoclave to pH values ranging from 3.0 to 7.5 with Na2HPO4.

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Staining and detection. DNA was transformed into Y. lipolytica cells by the lithium acetate method (36). YRIM101 was deleted in the wild-type strain PO1a by homologous recombination. Briefly, an ~1.0-kb sequence upstream of the YIRIM101 ORF (RIM101P) and an ~1.0-kb sequence downstream of the ORF (RIM101T) were amplified by PCR from genomic DNA. RIM101P and RIM101T were then inserted into the flanking sites of loxR-YUARA3-locP in pYL8 (36). The resulting RIM101P-locR-YUARA3-loxP-RIM101T deletion cassette was used to transform strain PO1a. Ura+ transformants were examined by PCR to identify the correct YIRIM101::loxR-YUARA3-loxP clones. The YUARA3 marker was later excised by Cre-mediated site-specific DNA recombination between loxR and loxP sites (36), yielding the YIRIM101::lopR mutant strain. The strain YLX497 (mhy1Δ) was described previously (31).

RNA-Seq analysis. Y. lipolytica cells were grown in liquid YNBG medium supplemented with uracil and buffered at pH 7.5 or pH 4.0 at 30°C. Cells were harvested when the optical density at 600 nm (OD600) reached 1.0. Three replicates were performed for each sample. Total RNA was extracted using a yeast RNA kit (Omega, China) by following the manufacturer’s instructions. A total amount of 1 μg RNA per sample was used. Sequencing libraries were generated using a TruSeq RNA library preparation kit (Illumina, USA). Paired 150-bp sequencing was performed on a NovaSeq 6000 (Illumina) at Berry Genomics Corporation (Beijing, China). The RNA-Seq reads were checked for quality by FastQC before cleaning and by Trimomatic after cleaning and were then mapped to the reference genome (Y. lipolytica CIB122) using HISAT2 (v2.0.6) (46). Raw read counts for each gene were calculated using HTSeqCount (47). Read count data were normalized using edgeR (R-3.3.3) (48). Normalized read counts were used to identify differentially expressed genes with adjusted P values of <0.05 and fold change below ~2.0 or above 2.0.

β-Galactosidase assay. The β-galactosidase activity in the cells was determined by the crude cell extract assay with o-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate, as reported previously (36). Crude cell extracts were prepared by vortexing with glass beads. Protein concentration in the
cell extracts was measured by the Bradford method. The specific β-galactosidase activity was normalized by the amount of total protein in each extract and was calculated according to the following formula: $U = (OD_{420} \times 1.7)/0.0045 \times$ protein concentration (mg ml$^{-1}$) × sample volume (ml) × time (min).

The assays were performed in triplicate.

**Microscopy.** Y. lipolytica cells were grown in liquid YNBG or YNBD medium for 16 h at 30°C before differential interference contrast (DIC) images were taken. An Olympus BX51 microscope (Tokyo, Japan) and a Retiga 2000R charge-coupled device (CCD) camera (QImaging Corporation, Canada) were used to visualize cell morphology. The DIC images were acquired using QCapture Suite (QImaging Corporation, Canada).

**Data availability.** RNA-Seq data can be found in the tables in the supplemental material. Full data can be obtained from the authors upon request.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**FIG S1** TIF file, 0.3 MB.

**TABLE S1** XLS file, 0.1 MB.

**TABLE S2** XLS file, 0.1 MB.

**TABLE S3** XLS file, 0.1 MB.

**TABLE S4** XLS file, 0.04 MB.

**TABLE S5** XLS file, 0.04 MB.

**TABLE S6** XLS file, 0.04 MB.

**TABLE S7** DOCX file, 0.02 MB.

**TABLE S8** DOCX file, 0.03 MB.

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We have no conflicts of interest to declare.

**REFERENCES**

1. Groenewald M, Boekhout T, Neveuglise C, Gaillardin C, Van Dijck PWM, Wyss M. 2014. *Yarrowia lipolytica*: safety assessment of an oleaginous yeast with a great industrial potential. Crit Rev Microbiol 40:187–206. https://doi.org/10.3109/1040841X.2013.770386.

2. Miller KE, Alper HS. 2019. *Yarrowia lipolytica*: more than an oleaginous workhorse. Appl Microbiol Biotechnol 103:9251–9262. https://doi.org/10.1007/s00253-019-10200-x.

3. Perez-Campo FM, Dominguez A. 2001. Factors affecting the morphogenetic switch in *Yarrowia lipolytica*. Curr Microbiol 43:429–433. https://doi.org/10.1007/s002840010333.

4. Ruiz-Herrera J, Sentandreu R. 2002. Different effectors of dimorphism in *Yarrowia lipolytica*. Arch Microbiol 178:477–483. https://doi.org/10.1007/s00203-002-0478-3.

5. Gimeno CJ, Ljungdahl PO, Styles CA, Fink GR. 1992. Unipolar cell divisions in *Yarrowia lipolytica*: more than an oleaginous switch. Microbiology 138:843–853. https://doi.org/10.1099/mbr.1992.00415-0.

6. Berman J, Sudbery PE. 2002. *Candida albicans*: a molecular revolution built on lessons from budding yeast. Nat Rev Genet 3:918–930. https://doi.org/10.1038/nrg948.

7. Davis D. 2003. Adaptive to environmental pH in *Candida albicans* and its relation to pathogenesis. Curr Genet 44:1–7. https://doi.org/10.1007/s00294-003-0415-2.

8. Bensen ES, Martin SJ, Li M, Berman J, Davis DA. 2004. Transcriptional profiling in *Candida albicans* reveals new adaptive responses to extracellular pH and functions for Rim101p. Mol Microbiol 54:1335–1351. https://doi.org/10.1111/j.1365-2958.2004.04350.x.

9. Penalva MA, Arst HNJ. 2002. Regulation of gene expression by ambient pH in filamentous fungi and yeasts. Microbiol Mol Biol Rev 66:426–446. https://doi.org/10.1128/mmbr.66.3.426-446.2002.

10. Davis D. 2009. How human pathogenic fungi sense and adapt to pH: the link to virulence. Curr Opin Microbiol 12:365–370. https://doi.org/10.1016/j.mib.2009.05.006.

11. Caddick MX, Brownlee AG, Arst HNJ. 1986. Regulation of gene expression by pH of the growth medium in *Aspergillus nidulans*. Mol Gen Genet 203:346–353. https://doi.org/10.1007/BF00339378.

12. Su SS, Mitchell AP. 1993. Identification of functionally related genes that stimulate early meiotic gene expression in yeast. Genetics 133:67–77. https://doi.org/10.1093/genetics/133.1.67.

13. Comet M, Gaillardin C. 2014. pH signaling in human fungal pathogens: a new target for antifungal strategies. Eur J Cell Biol 13:342–352. https://doi.org/10.1128/EJC.00313-13.

14. Odds FC. 1988. Candida and candidosis, 2nd ed. Bailliere Tindall, London, United Kingdom.

15. Aréchiga-Carvajal ET, Ruiz-Herrera J. 2005. The Rim101/pacC homologue from the basidiomycete *Ustilago maydis* is functional in multiple pH-sensitive phenomena. Eur J Cell Biol 4:999–1008. https://doi.org/10.1128/EC.4.999-1008.2005.

16. Zhu LB, Wang Y, Zhang ZB, Yang HL, Yan RM, Zhu D. 2017. Influence of environmental and nutritional conditions on yeast-mycelial dimorphic transition in *Trichosporon cutaneum*. Biotechnol Biotechnol Equip 31:516–526. https://doi.org/10.1080/13102818.2017.1292149.

17. Davis DA, Wilson RB, Mitchell AP. 2000. Rim101-dependent and -independent pathways govern pH responses in *Candida albicans*. Mol Cell Biol 20:971–978. https://doi.org/10.1128/mcb.20.3.971-978.2000.

18. Saporito-Irwin SM, Birse CE, Sypherd PS, Fonzi WA. 1995. PHR1, a pH-regulated gene of *Candida albicans*, is required for morphogenesis. Mol Cell Biol 15:601–613. https://doi.org/10.1128/mcb.15.2.601.

19. Calderon J, Zavrel M, Ragni E, Fonzi WA, Rupp S, Popolo L. 2010. PHR1, a pH-regulated gene of *Candida albicans* encoding a glucan-remodelling enzyme, is required for adhesion and invasion. Microbiology 156:2484–2494. https://doi.org/10.1099/mic.0.038000-0.

20. Bailey DA, Feldmann PJ, Bovey M, Gow NA, Brown AJ. 1996. The *Candida albicans* HYR1 gene, which is activated in response to hyphal development, belongs to a family encoding yeast cell wall proteins. J Bacteriol 178:5353–5360. https://doi.org/10.1128/jb.178.18.5353-5360.1996.
21. Shockey LL, McNemar MD, Saporito-Irwin SM, Sypherd PS, Fonzi WA. 1999. HPV1 functions in the morphological development of Candida albicans downstream of EFG1, TUP1, and RBF1. J Bacteriol 181:5273–5279. https://doi.org/10.1128/JB.181.7.5273-5279.1999.

22. Szabo R, Stoiankova V. 2002. Presence of organic sources of nitrogen is critical for filament formation and pH-dependent morphogenesis in Yarrowia lipolytica. FEMS Microbiol Lett 206:45–50. https://doi.org/10.1111/j.1574-6968.2002.tb0984x.

23. Gonzalez-Lopez CI, Ortiz-Castellanos L, Ruiz-Herrera J. 2006. The ambient pH response Rim pathway in Yarrowia lipolytica: identification of YIRIM9 and characterization of its role in dimorphism. Curr Microbiol 53:8–12. https://doi.org/10.1007/s00284-005-0070-6.

24. 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. https://doi.org/10.1128/mcb.17.7.3966.

25. Mühlschlegel FA, Fonzi WA. 1997. PHR2 of Candida albicans encodes a functional homolog of the pH-regulated gene PHR1 with an inverted pattern of pH-dependent expression. Mol Cell Biol 17:5960–5967. https://doi.org/10.1128/mcb.17.10.5960.

26. Ragni E, Fontaine T, Gissi C, Latgé JP, Popolo L. 2007. The Gas family of proteins of Saccharomyces cerevisiae: characterization and evolutionary analysis. Yeast 24:297–308. https://doi.org/10.1002/yea.1473.

27. Gonzalez-Lopez CI, Szabo R, Blanchin-Roland S, Gaillardin C. 2002. Genetic control of extracellular protease synthesis in the yeast Yarrowia lipolytica. Genetics 160:417–427. https://doi.org/10.1093/genetics/160.2.417.

28. Blanchin-Roland S, Costa GD, Gaillardin C. 2005. ESCRT-I components of the endocytic machinery are required for Rim101-pseudohyphal ambient pH regulation in the yeast Yarrowia lipolytica. Microbiology 151:3627–3637. https://doi.org/10.1099/micro.0.28196-0.

29. Blanchin-Roland S, Da Costa G, Gaillardin C. 2008. Ambient pH signalling in the yeast Yarrowia lipolytica involves YIRim23p/PalC, which interacts with Snf/Ph/Vps32p, but does not require the long C terminus of YIRim9p/Pal. Microbiology 154:1668–1676. https://doi.org/10.1099/mic.0.2008/017046-0.

30. Hurtado CA, Rachubinski RA. 1999. MHY1 encodes a C3H-type zinc finger protein that promotes dimorphic transition in the yeast Yarrowia lipolytica. J Bacteriol 181:3051–3057. https://doi.org/10.1128/JB.181.10.3051-3057.1999.

31. Wu H, Shu T, Mao Y-S, Gao X-D. 2020. Characterization of the promoter, downstream target genes and recognition DNA sequence of Mhy1, a key filamentation-promoting transcription factor in the dimorphic yeast Yarrowia lipolytica. Curr Genet 66:245–261. https://doi.org/10.1007/s00294-019-01018-1.

32. Mio T, Yamada-Okae T, Yabe T, Nakajima T, Arisawa M, Yamada-Okae H. 1997. Isolation of the Candida albicans homologs of Saccharomyces cerevisiae KRE6 and SKNI: expression and physiological function. J Bacteriol 179:2363–2372. https://doi.org/10.1128/jb.179.7.2363-2372.1997.

33. Nicholls S, Straffon M, Enjalbert S, Nantel A, Macaikill S, Whiteway M, Brown AJ. 2004. Msn2- and Msn4-like transcription factors play no obvious roles in the stress responses of the fungal pathogen Candida albicans. Eukaryot Cell 3:1111–1123. https://doi.org/10.1128/EC.3.5.1111-1123.2004.

34. Ramsdale M, Selway L, Stead D, Walker J, Yin Z, Nicholls SM, Crowe J, Sheils EM, Brown AJ. 2008. MNL1 regulates weak acid-induced stress responses of the fungal pathogen Candida albicans. Mol Biol Cell 19:4393–4403. https://doi.org/10.1091/mbc.e07-09-0946.

35. Liang S-H, Wu H, Wang R-R, Wang Q, Shu T, Gao X-D. 2017. The TORC1-Sch9-Rim15 signaling pathway represses yeast-to-hypha transition in response to glycerol availability in the oleaginous yeast Yarrowia lipolytica. Mol Microbiol 104:553–567. https://doi.org/10.1111/mmi.13645.

36. Zhao X-F, Li M, Li Y-Q, Chen X-D, Gao X-D. 2013. The TEA/ATTS transcription factor YlTe1cp represses the yeast-to-hypha transition in the dimorphic yeast Yarrowia lipolytica. FEMS Yeast Res 13:50–61. https://doi.org/10.1111/1567-3295.12008x.

37. Rupp S, Summers E, Lo HJ, Madhani H, Fink G. 1999. MAP kinase and cAMP filamentation signaling pathways converge on the unusually large promoter of the yeast FLO11 gene. EMBO J 18:1257–1269. https://doi.org/10.1093/emboj/18.5.1257.

38. Lambrechts MG, Bauer FF, Marmur J, Pretorius IS. 1996. Muc1, a mucin-like protein that is regulated by Mss10, is critical for pseudohyphal differentiation in yeast. Proc Natl Acad Sci U S A 93:8419–8424. https://doi.org/10.1073/pnas.93.16.8419.

39. Lo WS, Dranginis AM. 1998. The cell surface flocculin Flo11 is required for pseudohyphal formation and invasion by Saccharomyces cerevisiae. Mol Biol Cell 9:161–171. https://doi.org/10.1091/mbc.9.1.161.

40. Staab JB, Bradway SD, Fidel PL, Sundstrom P. 1999. Adhesive and mammalian transglutaminase substrate properties of Candida albicans Hwp1. Science 283:1535–1538. https://doi.org/10.1126/science.283.5407.1535.

41. Verstrepen KJ, Klis FM. 2006. Flocculation, adhesion and biofilm formation in yeasts. Mol Microbiol 60:5–15. https://doi.org/10.1111/j.1365-2958.2006.05072.x.

42. Dranginis AM, Rauceo JM, Coronado JE, Lipke PN. 2007. A biochemical guide to yeast adhesins: glycoproteins for social and antisoal occasions. Microbiol Mol Biol Rev 71:282–294. https://doi.org/10.1128/MMBR.00037-06.

43. de Groot PWJ, Bader O, de Boer AD, Weig M, Chauhan N. 2013. Adhesins in human fungal pathogens: glues with plenty of stick. Eukaryot Cell 12:470–481. https://doi.org/10.1128/EC.00364-12.

44. Alberti-Segui C, Morales AJ, Xing H, Kessler MM, Willins DA, Weinstock KG, Cottarel G, Fechtel K, Rogers B. 2004. Identification of potential cell-surface proteins in Candida albicans and investigation of the role of a putative cell-surface glycosidase in adhesion and virulence. Yeast 21:285–302. https://doi.org/10.1002/yea.1061.

45. Pardini G, De Groot PW, Coste AT, Karababa M, Klis FM, de Koster CG, Sanglard D. 2006. The CRH family coding for cell wall glycosylphosphatidylinositol proteins with a predicted transglycosidase domain affects cell wall organization and virulence of Candida albicans. J Biol Chem 281:40399–40411. https://doi.org/10.1074/jbc.M606316200.

46. Kim D, Langmead B, Salzberg SL. 2015. HISAT: a fast spliced aligner with low memory requirements. Nat Methods 12:357–360. https://doi.org/10.1038/nmeth.3317.

47. Pardini G, De Groot PW, Coste AT, Karababa M, Klis FM, de Koster CG, Sanglard D. 2006. The CRH family coding for cell wall glycosylphosphatidylinositol proteins with a predicted transglycosidase domain affects cell wall organization and virulence of Candida albicans. J Biol Chem 281:40399–40411. https://doi.org/10.1074/jbc.M606316200.

48. Robinson MD, McCarthy DJ, Smyth GK. 2010. edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26:139–140. https://doi.org/10.1093/bioinformatics/btp616.