Candida glabrata Yap6 Recruits Med2 To Alter Glycerophospholipid Composition and Develop Acid pH Stress Resistance

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ABSTRACT Candida glabrata is a high-performance microbial cell factory for the production of organic acids. To elucidate the role of the C. glabrata Mediator tail subunit Med2 (CgMed2) at pH 2.0, we deleted or overexpressed CgMed2 and used transcriptome analysis to identify genes that are regulated by CgMed2. At pH 2.0, the deletion of CgMed2 resulted in a cell growth decrease of 26.1% and a survival decrease of 32.3%. Overexpression of CgMed2 increased cell growth by 12.4% and cell survival by 5.9% compared to the wild-type strain. Transcriptome and phenotypic analyses identified CgYap6 as a transcription factor involved in acid pH stress tolerance. Deletion of CgYap6 caused growth defects, whereas its overexpression enhanced cell growth at pH 2.0. Furthermore, total glycerophospholipid content and membrane integrity decreased by 33.4% and 21.8%, respectively, in the CgMed2Δ strain; however, overexpression of CgMed2 increased the total glycerophospholipid content and membrane integrity by 24.7% and 12.1%, respectively, compared with those of the wild-type strain at pH 2.0. These results demonstrated that under acid pH stress, CgMed2 physically interacts with CgYap6, which translocates from the cytoplasm to the nucleus after being phosphorylated by the protein kinase CgYak1. Once in the nucleus, CgYap6 recruits CgMed2 to express glycerophospholipid-related genes. Our study elucidated the function of CgMed2 under acid pH stress and provides a potential strategy to equip Candida glabrata with low-pH resistance during organic acid fermentation.

IMPORTANCE This study investigated the function of the Mediator tail subunit CgMed2 in C. glabrata under low-pH stress. The protein kinase CgYak1 activates CgYap6 for the recruitment of CgMed2, which in turn increases glycerophospholipid content and membrane integrity to confer low-pH stress tolerance. This study establishes a new link between the Mediator tail subunit and transcription factors. Overall, these findings indicate that CgMed2 is a novel target to induce the low-pH stress response in C. glabrata.

KEYWORDS Candida glabrata, Mediator subunit Med2, glycerophospholipid, low-pH stress, transcriptome

Organic acids have become increasingly important in biotechnology, with main applications in the food, pharmaceutical, and textile industries (1). Among the several different microorganisms used to produce organic acids, Candida glabrata is a high-performance yeast used to produce malic acid (2), fumaric acid (3), and α-ketoglutaric acid (4). However, during the production, the accumulation of extracellular acids considerably decreases the pH of the fermentation broth, thereby inhibiting...
cell growth and ultimately reducing the production of the target acid (5). Although exogenous addition of alkaline reagents, such as NaOH and CaCO₃, helps stabilize the correct working pH, it increases the osmotic pressure and the cost of downstream separation and purification (6). Therefore, it is urgent to find efficient strategies to improve the tolerance of C. glabrata to complex industrial environmental conditions.

Many researchers have proposed different methods to increase yeast tolerance to acid stress, such as adaptive laboratory evolution (ALE), transporter engineering, transcription factor engineering, modification of specific genes, and Mediator complex engineering (7, 8). ALE strategies have been proven effective in the production of organic acid (9, 10). For example, ALE has been used to explore the tolerance mechanisms of Saccharomycyes cerevisiae in the presence of inhibiting concentrations of dicarboxylic acids. Moreover, reverse metabolic engineering amplification of Qdr3 (a transporter associated with multidrug resistance) conferred tolerance to dicarboxylic acids while enhancing the production of muconic acid in engineered S. cerevisiae (10). Furthermore, transporter engineering has been used to manipulate the expression level of proton pumps, including plasma membrane H⁺ antiporter Aqr1, proton pump Pma1, and vacuolar proton pumping Pep3, thereby improving acid stress tolerance in yeast (11–13). Transcription factors, such as Haa1, Msn2, Asg1, and Hal9, were found to be involved in the response to acid stress (14–16). For example, under acidic conditions, Asg1 regulates the expression of several genes related to the plasma membrane, cell wall organization, mitogen-activated protein kinase (MAPK) signaling pathway, and trehalose accumulation (14). Moreover, modification of specific genes increases the tolerance to acid stress in yeast (17, 18). For example, deletion of Atg22 in S. cerevisiae resulted in morphology changes that enhanced cell protection against acidic environments and increased the production of intracellular amino acids to respond to amino acid starvation (18). To resist environmental stresses, cells require alteration of the expression level of several different genes, which is difficult to achieve through continuous multigene modification (19). However, the Mediator complex can be engineered to reprogram the gene expression network to improve cell tolerance to low-pH stress (20, 21). For example, overexpression of CgMed3 increases the expression of genes related to lipid metabolism, enhancing the levels of C₁₈:₀, C₁₈:₁, and ergosterol and, consequently, membrane integrity and pyruvate production (20). Therefore, Mediator complex engineering offers a potential strategy to increase acid tolerance and organic acid production in C. glabrata.

The Mediator complex is an evolutionarily conserved, multiprotein complex required for RNA polymerase II-driven transcription; transcription factors recruit Mediator in the upstream activation sequence of the target gene to regulate gene function (22). In S. cerevisiae, the Mediator complex consists of 25 subunits and is organized in four modules designated head, middle, tail, and kinase (23). The tail module comprises five subunits: Med2, Med3, Med5, Med15, and Med16 (24). The main function of the tail module is to integrate the transcriptional regulatory signals from sequence-specific transcription factors (24, 25). Therefore, the tail module can respond to a variety of physiological processes (26). For example, in C. glabrata, Med3 regulates cell growth by coordinating the homeostasis of cellular acetyl coenzyme A (acetyl-CoA) metabolism and the cell cycle cyclin Cln3 (27). Adaptive evolution and integrated systems biology studies on S. cerevisiae revealed that, in addition to abolishing the Crabtree effect (i.e., the ability to rapidly consume glucose and produce ethanol with antiseptic properties), Med2 mutation caused global redistribution of yeast cell metabolism (28). Moreover, the tail module is involved in the response to several environmental stresses (29). In C. glabrata, Med3 and Med15 regulate the expression of genes related to lipid metabolism, specifically increasing the rigidity of the cell membrane and improving the viability of the yeast under acid stress (20, 21). Furthermore, in Candida spp., Med2 not only regulates cell viability under diverse stress conditions but also facilitates filamentous growth (30). However, the mechanism by which Med2 controls cell survival in adverse environment remains unclear.

Here, we used transcriptome sequencing and metabolomics to elucidate the role of
C. glabrata Med2 (CgMed2) in low-pH perturbation response. We discovered that the protein kinase CgYak1 activates the transcription factor CgYap6 inside the nucleus by recruiting the mediator CgMed2, which activates glycerophospholipid genes that resist low-pH stress. This study provides insight into cellular reprogramming in response to low pH and establishes a regulatory circuitry among CgMed2, CgYak1, and CgYap6.

**RESULTS**

CgMed2 facilitates yeast growth at pH 2.0. To investigate whether CgMed2 was necessary for C. glabrata growth at pH 2.0, the wild-type, CgMed2Δ, and CgMed2Δ/CgMED2 (the MED2 overexpression strain was constructed with plasmid pY26, and the level of CgMed2 overexpression was 4.3-fold than that of wild-type strain as determined by qRT-PCR [see Fig. S1 in the supplemental material]) strains were spotted and grown on yeast nitrogen base (YNB) plates at pH 5.5 and pH 2.0. The deletion of CgMed2 caused significant growth decline at pH 2.0, whereas its overexpression enhanced growth compared to that of the wild-type strain (Fig. 1A and B). Comparison of the growth curves of the three strains at pH 5.5 and pH 2.0, showed that the final biomass of CgMed2Δ decreased by 7.58% compared to that of the wild-type strain at pH 5.5. At pH 2.0, the final biomass of the CgMed2Δ and CgMed2Δ/CgMED2 strains was 26.1% lower and 12.4% higher, respectively, than that of the wild-type strain (Fig. 1C and D). Furthermore, at pH 2.0, the wild-type, CgMed2Δ, and CgMed2Δ/CgMED2 cell survival was 78.6%, 46.3%, and 84.5%, respectively (Fig. 1E). Finally, the half-maximal inhibitory concentration (IC50) values for the wild-type, CgMed2Δ, and CgMed2Δ/CgMED2 strains were 16.59, 12.06, and 18.53 mM HCl, respectively (Fig. 1F). These results suggest that CgMed2 plays an important role in the growth of C. glabrata at pH 2.0.

Global transcriptome analysis of the wild-type and CgMed2Δ strains at pH 2.0. To further describe the role of CgMed2 in C. glabrata, transcriptome sequencing (RNA-seq) was conducted to compare global gene expression in the wild-type and CgMed2Δ strains at pH 5.5 and 2.0. Restrictive thresholds (|log2(fold change)| ≥ 2.0; false-discovery rate (FDR) < 0.05) of significantly expressed genes were used for screening. First, the differentially expressed genes (DEGs) between strains grown at pH 2.0 and the normal condition (pH 5.5) were identified in both the wild-type and CgMed2Δ mutant strains (Fig. 2A). Transcriptional profiling and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis revealed that signal transduction and amino acid metabolism were the pathways most significantly affected by low pH in the wild-type strain, whereas signal transduction and transport were the most significantly affected pathways in CgMed2Δ mutant strains. In addition, 299 DEGs were common between wild-type and CgMed2Δ mutant strains, including 143 upregulated and 86 downregulated genes. The commonly upregulated genes were involved in amino acid metabolism, lipid metabolism, energy metabolism, and cofactor metabolism, whereas the commonly downregulated genes were enriched in processes such as carbohydrate metabolism, meiosis, and transport.

We next directly compared the DEGs between the CgMed2Δ mutant and wild-type strains at each pH condition (Fig. 2B). At pH 5.5, DEGs between the strains were involved mainly in carbohydrate metabolism and translation; however, at pH 2.0, the DEGs were involved in signal transduction and catabolism. A total of 77 common DEGs were identified between the two pH conditions, including 57 upregulated and 20 downregulated genes. KEGG analysis showed that the commonly upregulated genes were involved in glycan metabolism, amino acid metabolism, lipid metabolism, and DNA repair, whereas the commonly downregulated genes were enriched in processes such as membrane transport, protein folding and degradation, and meiosis.

To investigate whether transcription factors are involved in regulating these perturbed metabolic pathways, the differentially expressed transcription factors in the CgMed2Δ strain relative to the wild-type strain were screened. Among these, CgYap6, CgHap5, CgCom2, CgSut1, CgAft1, and CgYap5 were the most significantly differentially expressed transcription factors at pH 5.5 and pH 2.0 (Table 1 and Fig. 2C and D).
Therefore, we further assessed the resistance of *C. glabrata* to low pH (2.0) upon deletion or overexpression of these transcription factors. Interestingly, deletion of *CgYap6* and *CgAft1* led to evident growth defects, whereas only overexpression of *CgYap6* conferred resistance to acidic pH (Fig. 2E and F). *Med2*, a subunit of the tail module, interacts with transcription factors to regulate the transcription of nearly all RNA polymerase II-dependent genes in yeast (24). Hence, we hypothesized that the interaction between *CgYap6* and *CgMed2* might play an important role in yeast resistance at pH 2.0.
CgMed2 interacts with transcription factors at pH 2.0. At pH 5.5, CgYap6 fused with enhanced green fluorescent protein (CgYap6-eGFP) localized in both the nucleus and cytoplasm, whereas at pH 2.0, it was detected mostly in the nucleus. In contrast, CgMed2-eGFP was located in the nucleus at both pH 5.5 and pH 2.0 (Fig. 3A). These results indicated that the distribution of CgYap6-eGFP in the nucleus increases at pH 2.0, suggesting that CgMed2 and CgYap6 may cooperate in the nucleus in response to acid pH stress.

To confirm this hypothesis, the genetic interaction between CgMed2 and CgYap6 was evaluated using spot assays, which revealed that the phenotype of the CgMed2Δ/CgYap6Δ double mutant was similar to those of the CgMed2Δ and CgYap6Δ single mutants (Fig. 3B). Moreover, the CgMed2Δ/CgYap6Δ double mutant showed 42.3%
Cg survival, whereas the CgMed2Δ and CgYap6Δ single mutants exhibited 44.6% and 47.5% survival, respectively (Table 2). These results suggest a mechanism by which CgMed2 and CgYap6 work together either in the same pathway or as part of the same protein complex to promote growth under a condition of low pH.

Next, the physical interaction between CgMed2 and CgYap6 was determined at pH 5.5 (see Fig. S2 in the supplemental material) and pH 2.0 (Fig. 3C). Yeast two-hybrid (Y2H) analysis revealed a physical interaction between Gal4-BD (DNA-binding domain)-CgMed2 and Gal4-AD (activation domain)-CgYap6 at pH 2.0 (Fig. 3C). This interaction was also confirmed by coimmunoprecipitation experiment (Fig. 3D; see Fig. S3 in the supplemental material), which showed that CgMed2-Myc coprecipitated with CgYap6-His and vice versa at pH 2.0 (Fig. 3D).

These observations suggest that CgMed2 interacts with CgYap6 at pH 2.0.

**CgYak1 is required for the pH-induced phosphorylation of CgYap6.** Based on the results presented above, we thought that transcription factors may undergo posttranslational modifications at pH 2.0 and then enter the nucleus to recruit the mediator CgMed2 for transcriptional regulation. The protein kinase Yak1, which plays a central role in the regulation of many biological aspects in eukaryotic organisms (31), was differentially expressed in the CgMed2Δ strain compared with the wild-type strain (Table 1). Therefore, we investigated whether CgYak1 phosphorylated CgYap6 at pH 2.0. According to gel electrophoresis results, CgYap6-His was expressed with the expected molecular mass of 31 kDa in the wild-type strain at pH 5.5; however, its weight shifted at pH 2.0 (Fig. 4A). Treatment with alkaline phosphatase showed that the band corresponding to CgYap6-His appeared at 31 kDa, whereas it shifted again when alkaline phosphatase inhibitor was added (Fig. 4B). These data support the hypothesis that the shift of the CgYap6-His band observed at pH 2.0 is the result of phosphorylation.

Additionally, for the CgYak1Δ strain, a CgYap6-His molecular weight change was not observed, indicating that the phosphorylation of CgYap6-His detected at pH 2.0 depended on the presence of CgYak1. To further verify these results, the phosphorylation of CgYap6 by CgYak1 was analyzed in both wild-type and CgYak1Δ strains by reaction with an anti-phosphoserine/threonine antibody, which is used to detect the phosphorylation of Ser-Pro/Thr-Pro (SP/TP) sites (Yak1 consensus phosphorylation sites). At pH 5.5, a weak CgYap6-His phosphorylation band was detected in both strains.
however, at pH 2.0, the phosphorylation level of CgYap6-His increased obviously in the wild-type strain but not in the CgYak1Δ strain (Fig. 4C and D).

Transcriptome and untargeted metabolomics analysis of the wild-type and CgMed2Δ strains at pH 5.5 and pH 2.0. Functional enrichment analysis was performed on up- and downregulated differentially enriched KEGG pathways. Transcrip-

TABLE 2 Survival of various strains at pH 5.5 and pH 2.0

| Strain                | % survival at pH: |
|-----------------------|-------------------|
|                       | 5.5               | 2.0               |
| Wild type             | 100               | 77.4 (2.23)<sup>a</sup> |
| CgMed2Δ               | 98.3 (1.34)       | 44.6 (3.41)<sup>a</sup> |
| CgYap6Δ               | 99.6 (1.62)       | 47.5 (2.57)<sup>a</sup> |
| CgMed2Δ/CgYap6Δ       | 96.4 (1.47)       | 42.3 (1.95)<sup>a</sup> |

<sup>a</sup>Survival rates are expressed relative to those of wild-type cells. Results are the averages from three experiments, with standard deviations in parentheses.

<sup>b</sup><sup>P</sup> ≤ 0.01 versus wild type.
tion, posttranslational modifications, lipid metabolism, signal transduction mechanisms, and carbohydrate metabolism were the five most highly enriched pathways (as mapped in the KEGG), accounting for 22.1%, 19.9%, 10.9%, 8.4%, and 8.2%, respectively, of all differentially expressed genes in the CgMed2Δ strain compared to the wild-type strain at pH 2.0 (Fig. 5A). Specifically, lipid metabolism was the module exhibiting the largest variation of intracellular metabolic pathways. Consequently, the expression level of genes related to lipid metabolism was compared between the wild-type and CgMed2Δ strains at pH 2.0 (Fig. 5B). The mRNA levels of genes involved in glycerophospholipid metabolism were analyzed by quantitative reverse transcription-PCR (qRT-PCR). At pH 5.5, the mRNA levels of glycerol-3-phosphate-O-acyltransferase (SCT1), phosphatidate cytidylyltransferase (CSD1), lysophospholipid acyltransferase (ALE1), CDP-diacylglycerol-serine-O-phosphatidylyltransferase (CHO1), phosphatidylserine decarboxylase (PSD1), and phosphatidyl-N-dimethylethanolamine-N-methyltransferase (OPI3) were 1.3-, 2.1-, 3.2-, 2.7-, 1.6-, and 2.3-fold lower in the CgMed2Δ strain than in the wild-type strain, respectively, whereas the mRNA level of phosphatidate phosphatase (PAH1) remained unchanged (Fig. 5C). Conversely, at pH 2.0, the mRNA levels of those genes were 2.5-, 3.1-, 1.7-, 2.3-, 3.4-, 2.5-, and 3.6-fold lower in the CgMed2Δ strain than in the wild-type strain (Fig. 5D). These data suggested that the expression of genes involved in glycerophospholipid biosynthesis strongly depends on the presence of CgMed2.

To explore the intracellular metabolism of the wild-type and CgMed2Δ strains, we performed untargeted metabolomics for both the wild-type and CgMed2Δ strains growing at pH 5.5 and pH 2.0. The results showed 24 and 20 differentially perturbed metabolic pathways in the wild-type strain and CgMed2Δ strain, respectively. Among those, 17 were common to both the wild-type and CgMed2Δ strains. Those pathways were mostly related to amino acid metabolism, pyrimidine metabolism, amino sugar and nucleotide sugar metabolism, glycerolipid metabolism, and respiration (i.e., tricarboxylic acid [TCA] cycle and glyoxylate cycle). Moreover, untargeted metabolomics of wild-type and CgMed2Δ strains under pH 2.0 stress were compared (see Table S1 in the supplemental material). At pH 5.5 and pH 2.0, 17 and 13 pathways, respectively, were
perturbed in CgMed2Δ compared with the wild-type strain. Among those perturbed pathways, 7 pathways were common in both the wild-type and CgMed2Δ strains. These pathways were mostly related to amino acid metabolism, purine metabolism, glutathione metabolism, and glycerolipid metabolism. Although several of those perturbed pathways were related to amino acid metabolism and carbohydrate metabolism, metabolomics analysis identified glycerolipid metabolism as the only lipid pathway considerably perturbed in CgMed2Δ under all conditions compared to the wild-type strain at pH 5.5 (Fig. 6A).

Based on the metabolomics results, the glycerophospholipid composition in the wild-type, CgMed2Δ, and CgMed2Δ/CgMED2 strains was analyzed. At pH 5.5, the
content of phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphocholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidyglycerol (PG) decreased by 4.8%, 30.3%, 20.4%, 10.8%, 20.3%, and 29.6% in the CgMed2Δ strain compared with those in the wild-type strain. In the CgMed2Δ/CgMED2 strain, the PE, PC, PS, PG, and PI content increased by 6.1%, 30.9%, 6.2%, 28.4%, and 11.2%, respectively, whereas the PA content remained unchanged (Fig. 6B). At pH 2.0, the content of

FIG 6 Untargeted metabolomics analysis of the wild-type strain and mutant CgMed2Δ. (A) The results were combined from hydrophilic metabolomics extraction by LC-MS (including negative ionization and positive ionization). (B and C) Glycerophospholipid composition changes in the wild-type (wt), CgMed2Δ, and CgMed2Δ/CgMED2 strains at pH 5.5 and pH 2.0. All data are presented as mean values from three independent experiments. Error bars indicate the standard deviations. *, P < 0.05; **, P < 0.01; ***, P < 0.001 (compared to the corresponding wild-type strain, as determined by a t test).
PA, PE, PC, PS, PI, and PG decreased by 41.0%, 26.1%, 23.2%, 21.4%, 18.2%, and 33.8%, respectively, in the CgMed2Δ strain compared with those in the wild-type strain. However, in the CgMed2Δ/CgMED2 strain, the PA, PE, PC, PS, PI, and PG content increased by 27.8%, 32.2%, 18.7%, 6.5%, 27.3%, and 12.6%, respectively, compared with those in wild-type strain (Fig. 6C). These results suggest that CgMed2 may be critical for regulating glycerophospholipid composition at pH 2.0.

**CgYap6 regulates glycerophospholipid genes in a CgYak1-dependent manner.**

To reveal the regulatory circuitry among CgMed2, CgYak1, and CgYap6, we assessed whether the phosphorylation of CgYap6 is required for its function in regulating genes involved in glycerophospholipid metabolism. A chromatin immunoprecipitation (ChIP) assay combined with qRT-PCR was performed to detect the binding of CgYap6 to glycerophospholipid genes in the wild-type and CgYak1Δ strains. The qRT-PCR analysis revealed that at pH 5.5, the levels of binding of CgYap6 to the promoter regions of CSD1, CHO1, PSD1, OPI3, and PIS1 were not significantly different between the wild-type and CgYak1Δ strains (Fig. 7A), whereas the binding levels were, respectively, 47.3%, 67.6%, 45.5%, 49.3%, and 48.6% lower in the CgYak1Δ strain than in the wild-type strain at pH 2.0 (Fig. 7B). These data demonstrate that the CgYak1 target transcription factor CgYap6 promotes the binding of CgYap6 to target promoters at pH 2.0. Moreover, the transcription levels of CSD1, CHO1, PSD1, OPI3, and PIS1 were 1.8-, 2.3-, 1.2-, 2.9-, and...
2.1-fold lower in the CgYak1Δ strain than in the wild-type strain at pH 5.5 (Fig. 7C) and were 2.4-, 2.8-, 2.7-, 3.4-, and 2.0-fold lower in the CgYak1Δ strain than in the wild-type strain at pH 2.0, respectively (Fig. 7D). These results suggest that the CgYak1 target transcription factor CgYap6 is indeed important for enhancing the transcription levels of glycerophospholipid genes.

CgMed2 affects membrane integrity at pH 2.0. To describe the role of CgMed2 in membrane integrity, cells of the wild-type, CgMed2Δ, and CgMed2Δ/CgMED2 strains were cultivated at both pH 5.5 and 2.0 for 3 h, followed by propidium iodide uptake analysis. At pH 5.5, approximately 3.8%, 4.4%, and 4.7% of the wild-type, CgMed2Δ, and CgMed2Δ/CgMED2 cells were stained, respectively. At pH 2.0, the proportion of stained cells increased to 20.8% in the CgMed2Δ strain but decreased by 5.9% in the CgMed2Δ/CgMED2 strain compared with those in the wild-type strain (Fig. 8A; see Fig. S8 in the supplemental material). These results suggest that CgMed2 contributes to increasing membrane integrity at pH 2.0.

To visually observe membrane integrity, the wild-type, CgMed2Δ, and CgMed2Δ/CgMED2 strains were cultivated at both pH 5.5 and 2.0 for 3 h, followed by SYTOX green and FM4-64 uptake analysis observed with confocal laser scanning microscopy observations. At pH 5.5, almost all cells of the three strains had integral membranes (Fig. 8B). At pH 2.0, some cells of the wild-type, CgMed2Δ, and CgMed2Δ/CgMED2 strains displayed damaged membranes, although the damage was the least apparent for the CgMed2Δ/CgMED2 strain, followed by the wild-type strain, and the CgMed2Δ strain showed the highest number of cells with damaged membranes (Fig. 8C). These results suggested that CgMed2 plays an important role in membrane integrity.


**DISCUSSION**

In this study, we investigated the role of $Cg$Med2 in the response to pH 2.0 stress. Our results suggest that this stress response prompts $Cg$Yak1 to target the transcription factor $Cg$Yap6, followed by its translocation from the cytoplasm to the nucleus. Inside the nucleus, phosphorylated $Cg$Yap6 recruits $Cg$Med2 to induce the expression of genes involved in the glycerophospholipid pathway, resulting in membrane integrity enhancement.

Previous studies have indicated that Med2 regulates cell growth under stress conditions. In *Candida albicans*, Med2 affects the ability to form adherent biofilms and invasive hyphae to resist various environmental and nutrient stresses (30, 32). In *Candida dubliniensis*, Tlo1 (a Med2 ortholog) is required for the response to carbon source and oxidative stress (33). In this study, *C. glabrata* lacking Med2 exhibited a severe growth defect at pH 2.0, whereas overexpression of $Cg$Med2 enhanced cell growth and survival rates compared to those of the wild-type strain. These results suggest that Med2 may play different roles under various conditions of environmental stress. Furthermore, a previous study on *C. glabrata* reported that $Cg$Med3 plays an important role in the response to acid pH stress, by regulating the expression of genes involved in lipid metabolism, ribosome biosynthesis, and carbohydrate metabolism (20). Our transcriptome analysis demonstrated that in the $Cg$Med2 deletion strain, genes involved in the translation process, posttranslational modification, lipid metabolism, and carbohydrate metabolism were downregulated at pH 2.0, suggesting that $Cg$Med2 and $Cg$Med3 function redundantly at acid pH but that deletion of individual tail subunits causes different effects on gene expression.

The protein kinase Yak1 regulates a variety of cellular functions, such as cell growth and stress response. Previous studies reported that Yak1 is involved in resistance to oxidative stress (34) and organic acid stress (35). In addition, inactivation of Yak1 causes abnormal polarized apical growth in *Trichoderma reesei*, as well as sensitivity to various stresses (36). In this report, we highlighted a new physiological function of Yak1 in *C. glabrata*. Specifically, $Cg$Yak1 phosphorylates the transcription factor Yap6 in response to acid pH stress. In *S. cerevisiae*, Yak1 substrates include transcription factors, metabolic enzymes, and other protein kinases (31). Therefore, transcription factors can be phosphorylated by Yak1 under stress conditions (37, 38). For example, in *S. cerevisiae*, the conserved protein kinase Yak1 was activated under heat stress, and its target transcription factors (i.e., Msn2 and Msn4) were phosphorylated, thereby affecting DNA-binding activity. Perturbation of this pathway considerably affected cell survival upon heat stress (38). These findings are consistent with our results that $Cg$Yak1 targeted $Cg$Yap6 to induce its translocation from the cytoplasm to the nucleus under pH 2.0 stress. It has been reported that nutrient starvation acts as a cellular signal to also activate Yak1 (39). This led us to speculate that the absorption of nutrients by *C. glabrata* is hampered under pH 2.0 stress, and that $Cg$Yak1 then targets the transcription factor $Cg$Yap6 to promote its translocation to the cell nucleus, where it performs its functions.

Here, we demonstrated that $Cg$Yap6 can recruit $Cg$Med2 to regulate the expression of glycerophospholipid genes at pH 2.0. The subunits of the tail module regulate the expression of genes involved in diverse metabolic pathways by either directly or indirectly interacting with their respective transcriptional activators (25). For example, the Med15 subunit of the tail module has been shown to regulate the expression of genes involved in fatty acid metabolism by directly interacting with the activator Oaf1 in *S. cerevisiae* (40). Similar results have also been obtained in human cells and *Caenorhabditis elegans* (41, 42). This study provides a new link between the tail module of the $Cg$Med2 subunit and the transcription factor $Cg$Yap6 at pH 2.0. Our results confirm that the tail module functions mainly as a target for transcription factors to active gene expression. Several studies have proposed that some transcription factors can recruit Med2 to resist environmental stress (43, 44). In *C. glabrata*, the increased susceptibility of $Cg$Med2 mutants to azole antifungal drugs is due to the inability to activate the zinc-finger transcription factor $Cg$Pdr1 to transcribe multidrug efflux pumps.
CgYak1 (43). In addition, several pieces of evidence suggest that some transcription factors require posttranslational modifications to be activated in order to recruit Med2 (45, 46). For example, under arsenate stress, the cysteine sulfhydryl group of Yap8 is modified into its activated form. This active form interacts with the tail subunit Med2, which facilitates the Acr2/Acr3 promoter recruitment of the Mediator complex, leading to upregulation of Acr2 and cell tolerance to the stressor (45). This previous result recalls our data, according to which CgYak1-mediated phosphorylation of CgYap6 facilitates the recruitment of the mediator CgMed2 to regulate downstream glycerophospholipid gene expression and glycerophospholipid composition in response to pH 2.0 stress (see Fig. S5 in the supplemental material). In industrial microorganisms, adverse environmental pressure often causes damage to the cell membrane (47, 48). Enhancement of the phospholipid content has been proven to be effective in increasing cell membrane integrity and stress tolerance. For example, in Escherichia coli, overexpression of phosphatidylserine synthetase (PssA) increased the content of PE in the cell membrane, thereby enhancing the bacterium’s tolerance to octanoic acid and increasing yield (49). In S. cerevisiae, modularized engineering was used to change the composition of the phospholipid head, thereby increasing the integrity of the cell membrane and improving salt tolerance (50). These results indicate that alteration of the phospholipid content in the cell membrane is a potential strategy to improve the robustness of industrial microorganisms.

In conclusion, when C. glabrata is subjected to acid pH stress, the protein kinase CgYak1 integrates the stress signals and transmits them to the transcription factor CgYap6. Specifically, CgYak1 induces the translocation of CgYap6 from the cytoplasm to the nucleus, where it binds to the promoter region of glycerophospholipid-related genes and then recruits CgMed2 to regulate the glycerophospholipid composition of the cell membrane and its integrity. Therefore, this study may provide a potential strategy for enhancing the resistance of C. glabrata to acid pH stress during organic acid fermentation.

MATERIALS AND METHODS

Strains, media, and culture conditions. The yeast strains and plasmids used in this study are listed in Table 3. Escherichia coli JM109 was used for cloning and plasmid propagation. All yeast strains used in this study were derived from a C. glabrata CgHTUΔ (his3Δ trp1Δ ura3Δ) strain. The deletion strains were constructed by homologous recombination of CgHI53, CgTRP1, and CgURA3 markers in the CgMed2, CgYap6, and CgYak1 loci. The marker genes were amplified from the C. glabrata strain ATCC 2001 genome and fused between the upstream and downstream regions of the CgMed2, CgYap6, and CgYak1 genes open reading frames by fusion PCR. PCR products were transformed in the C. glabrata strain CgHTUΔ as described previously, and the deletion strains were confirmed by genomic PCR and DNA sequencing.

C. glabrata JM109 cells were grown in LB medium (2% tryptone, 2% NaCl, 1% yeast extract) and incubated at 37°C with shaking at 200 rpm. Ampicillin (100 mg/liter) was added for the selection of cells carrying the relevant plasmid. Unless otherwise stated, yeast strains were grown in yeast nitrogen base (YNB) (0.67% yeast nitrogen base, 2% glucose) medium supplemented with essential nutrients and incubated at 30°C with shaking at 200 rpm.

Plasmids. The CgMed2 gene was amplified from C. glabrata genomic DNA using the primers listed in Table 4. For establishment of the overexpression strains, we used the GPD promoter of plasmid pY26 (copy number of 2 μm plasmid); the target genes were amplified from the genome of C. glabrata using primers containing BamHI and HindIII restriction sites and cloned into pY26 to generate CgMed2/CgMed2, CgYap6/CgYap6, CgYak1/CgYak1, CgHap5/CgHAP5, CgCom/2/CgCOM2, CgSuc1/CgSU1, CgHap1/CgHAP1, and CgYap6/CgYAP6 strains. Clones were confirmed by DNA sequencing.

The CgYap6-His and CgMed2-Myc constructs were amplified from C. glabrata genomic DNA using the primers listed in Table 4. CgMed2-Myc was cloned into the vector pY26 using the NotI and BglII restriction sites, and the construct was named pY26-CgMed2-Myc. CgYap6-His was cloned into the plasmid pY26-CgYap6-Myc using the HindIII restriction site and a ClonExpress II one-step cloning kit (C112-01; Vazyme), and the construct was named pY26-CgMed2-Myc-CgYap6-His. Similarly, clones were confirmed by DNA sequencing.

The CgYap6 and CgMed2 genes were amplified from C. glabrata genomic DNA using the primers listed in Table 4. The CgMed2 gene was cloned into the vector pGBKT7 using the ClonExpress II one-step cloning kit (C112-01; Vazyme), and the construct was named pGBKT7-CgMed2. The CgYap6 gene was cloned into the vector pGADT7 using the ClonExpress II one-step cloning kit (C112-01; Vazyme), and the construct was named pGADT7-CgYap6.

Spot assay. Yeast cells were cultivated in logarithmic phase and diluted to an absorbance at 660 nm (A660) of 1.0 in phosphate-buffered saline (PBS). Aliquots of 10-fold serial dilutions were spotted onto YNB
Quality controlled by SeqPrep (https://github.com/jstjohn/SeqPrep) and Sickle (https://github.com/)

After quantified by TBS380, the paired-end RNA-seq library was sequenced with the Illumina HiSeq construction protocol. Libraries were size selected for DNA target fragment of 300 bp on 2% Low Range cDNA synthesis kit (Invitrogen, CA) with random hexamer primers (Illumina). The synthesized cDNA was isolated according to the poly(A) selection method with oligo(dT) beads and then fragmented by.

To analyze cell survival, yeast cells were cultivated in logarithmic phase and then treated with

g/liter. To analyze cell survival, yeast cells were cultivated in logarithmic phase and then treated with
taking at regular time intervals, and the dry weight of cells (DCW) on the basis of a standard curve where an
660 was calibrated against the

Growth and survival analysis. To analyze the growth of C. glabrata strains at pH 2.0, logarithmic-phase cells were diluted into fresh YNB medium at pH 5.5 or 2.0 at an initial A660 of 0.1. Cultures were taken at regular time intervals, and the A660 values were recorded. The A660 was calibrated against the dry weight of cells (DCW) on the basis of a standard curve where an A660 of 1 is equal to a DCW of 0.23 g/liter. To analyze cell survival, yeast cells were cultivated in logarithmic phase and then treated with various concentrations of HCl for 1 h at 30°C with shaking at 200 rpm. Cells were then centrifuged and washed with sterile water three times. After dilution, cells were plated on YNB medium plates with the

RNA extraction. C. glabrata cells were cultured to logarithmic phase and then reincubated into fresh YNB medium at an initial A660 of 0.1. After incubation for 6 h, cells were harvested and released to YNB medium at pH 5.5 and pH 2.0 for 2 h. The experiments were performed in biological triplicate. Cells were then recollected and washed twice with phosphate-buffered saline by resuspension and centrifugation at 3,500 × g for 10 min at 4°C. Total RNA was isolated by using a MiniBEST universal RNA extraction kit (TaKaRa Bio, Shiga, Japan). The concentration and quality of total RNA were determined by microspectrophotometry using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

RNA-seq analysis. A transcriptome sequencing (RNA-seq) transcriptome library was prepared with a TruSeq RNA sample preparation kit from Illumina (San Diego, CA) using 1 μg of total RNA. Briefly, mRNA was isolated according to the poly(A) selection method with oligo(dT) beads and then fragmented by fragmentation buffer. Next, double-stranded cDNA was synthesized using a SuperScript double-stranded cDNA synthesis kit (Invitrogen, CA) with random hexamer primers (Illumina). The synthesized cDNA that was subjected to end repair, phosphorylation, and “A” base addition according to Illumina’s library construction protocol. Libraries were size selected for cDNA target fragments of 300 bp on 2% Low Range Ultra agarose, followed by PCR amplification using Phusion DNA polymerase (NEB) for 15 PCR cycles. After quantified by TBS380, the paired-end RNA-seq library was sequenced with the Illumina HiSeq xten/NovaSeq 6000 sequencer (2 × 150-bp read length). The raw paired-end reads were trimmed and quality controlled by SeqPrep (https://github.com/jstjohn/SeqPrep) and Sickle (https://github.com/najoshi/sickle) with default parameters. Clean reads (see Table S2 in the supplemental material) then were aligned to the reference genome of Candida glabrata CBS 138 (https://www.ncbi.nlm.nih.gov/ genome/192?genome_assembly_id=28426). The differential expression analysis was performed using the DESeq software.

| Table 3 Strains and plasmids used in this study |
|------------------------------------------------|
| Strain or plasmid | Relevant characteristics | Reference |
|-------------------|--------------------------|-----------|
| C. glabrata ATCC 2001 | his3Δ trp1Δ ura3Δ | 51 |
| C. glabrata HTUΔ | his3Δ trp1Δ ura3Δ CgMED2::CgHIS3 | 51 |
| CgMedΔΔ | his3Δ trp1Δ ura3Δ CgMED2::CgHIS3 | This study |
| CgMedΔ2/CgMed2 | his3Δ trp1Δ ura3Δ med2Δ CgMED2::CgHIS3 pY26-PGPD/CgMed2 | This study |
| CgYap6Δ | his3Δ trp1Δ ura3Δ CgYap6::CgTRP1 | This study |
| CgHap5Δ | his3Δ trp1Δ ura3Δ CgHAP5::CgHIS3 | This study |
| CgCom2Δ | his3Δ trp1Δ ura3Δ CgCOM2::CgHIS3 | This study |
| CgAR1Δ | hisΔ trp1Δ ura3Δ CgAR1::CgHIS3 | This study |
| CgSur1Δ | hisΔ trp1Δ ura3Δ CgSur1::CgHIS3 | This study |
| CgYap5ΔΔ | his3Δ trp1Δ ura3Δ CgYap5::CgHIS3 | This study |
| CgYak1Δ | his3Δ trp1Δ ura3Δ CgYak1::CgHIS3 | This study |
| CgMedΔ2/CgYap6Δ | his3Δ trp1Δ ura3Δ CgYap6::CgTRP1 CgMED2::CgHIS3 | This study |
| CgYap6Δ/CgYap6Δ | his3Δ trp1Δ ura3Δ CgYap6::CgTRP1 pY26-PGPD/CgYap6 | This study |
| CgHap5Δ/CgHAP5 | his3Δ trp1Δ ura3Δ CgHAP5::CgHIS3 pY26-PGPD/CgHAP5 | This study |
| CgCom2Δ/CgCOM2 | his3Δ trp1Δ ura3Δ CgCOM2::CgHIS3 pY26-PGPD/CgCOM2 | This study |
| CgAR1Δ/CgAF1T | his3Δ trp1Δ ura3Δ CgAF1T::CgHIS3 pY26-PGPD/CgAF1T | This study |
| CgSur1Δ/CgSur1Δ | his3Δ trp1Δ ura3Δ CgSur1::CgHIS3 pY26-PGPD/CgSur1 | This study |
| CgYap5ΔΔ/CgYap5Δ | his3Δ trp1Δ ura3Δ CgYap5::CgHIS3 pY26-PGPD/CgYap5 | This study |
| CgSur1Δ/CgSur1Δ | his3Δ trp1Δ ura3Δ CgSur1::CgHIS3 pY26-PGPD/CgSur1 | This study |
| AH109 | his3Δ trp1Δ ura3Δ CgSur1::CgHIS3 pY26-PGPD/CgSur1 | This study |
| CgMedΔ2/CgMed2-Myc | his3Δ trp1Δ ura3Δ CgMED2::CgHIS3 pY26-PGPD/CgMed2-Myc | This study |
| CgMedΔ2/CgYap6Δ/CgMed2-Myc/ | his3Δ trp1Δ ura3Δ CgYap6::CgTRP1 CgMED2::CgHIS3 pY26-PGPD/CgMed2-Myc | This study |
| CgYap6-His | his3Δ trp1Δ ura3Δ CgYap6::CgTRP1 CgMED2::CgHIS3 pY26-PGPD/CgMed2-Myc | This study |
| Plasmids | | |
| pY26 | 2μm URA3 P_ura3 P_1REF | This study |
| pGBK7 | Kan TRP1 GAL4 DNA-Binding domain fusion | This study |
| pGAD7 | Amp LEU2 GAL4 DNA-binding domain fusion | This study |
| Category and name | Sequence (5′→3′)* |
|-------------------|------------------|
| **Deletion**      |                  |
| L-CgMED2-F1       | TACAAATTAGCTATTATACCAA |
| L-CgMED2-F2       | CTTAACAGCCCGCACTTCAGACGACCTGAT |
| CgHS3(CgMed2)-F1  | GCCCGTCTGAAAGTAATGGCGTTTGTTAAGAGGGT |
| CgHS3(CgMed2)-F2  | AAAGGGGGGATGCAGAGATCTTGAAGAGAGGGT |
| R-CgMED2-F1       | GGTGTCCTAGCATAGCTTGGTCTTCCTATTGTTAACTATT |
| R-CgMED2-F2       | GGATAAATTTTTTGATAGTTTAGTA |
| L-CgYAP6-F1       | AGTACACAGGATACGATATATATTGCTTGCTGTTGCTGCTG |
| L-CgYAP6-F2       | ACTATATTTCTTGGGAATAGGACACCTTGAT |
| CgTRP1(CgYap6)-F1| AGTGACATGGGATCTGATTCATTGTTTCTTTTTGATTCATGCTAG |
| CgTRP1(CgYap6)-F2| AAAATGCAAAGAAACAATGACTCTGAACTCCGAATGCAATT |
| R-CgYAP6-F1       | TTTGATAGTTTAGTA |
| R-CgYAP6-F2       | AGTACACAGGATACGATATATATTGCTTGCTGTTGCTGCTG |
| L-CgHAP5-F1       | TTTCCTTAAATGCTTAAGTGCT |
| L-CgHAP5-F2       | ACCCTCTTAAACCGCATCTTGAGTTTAAGAGAGGGT |
| CgHS3(CgHap5)-F1  | GATGGTGGGGAAGAAGAGAGGGT |
| CgHS3(CgHap5)-F2  | CTTTGGGATTATCCGATAGGACACCTTGAT |
| R-CgHAP5-F1       | TTTGATAGTTTAGTA |
| R-CgHAP5-F2       | AGTACACAGGATACGATATATATTGCTTGCTGTTGCTGCTG |
| L-CgCOM2-F1       | TTTCCTTAAATGCTTAAGTGCT |
| L-CgCOM2-F2       | ACCCTCTTAAACCGCATCTTGAGTTTAAGAGAGGGT |
| CgHS3(CgCom2)-F1  | GATGGTGGGGAAGAAGAGAGGGT |
| CgHS3(CgCom2)-F2  | CTTTGGGATTATCCGATAGGACACCTTGAT |
| R-CgCOM2-F1       | TTTGATAGTTTAGTA |
| R-CgCOM2-F2       | ACCCTCTTAAATGCTTAAGTGCT |
| L-CgAF1T-F1       | TATAAGGAAGGAAAGAGAGAGCT |
| L-CgAF1T-F2       | ACCCTCTTAAACCGCATCTTGAGTTTAAGAGAGGGT |
| CgHS3(CgAf1t)-F1  | TATGGTGGGGAAGAAGAGAGGGT |
| CgHS3(CgAf1t)-F2  | CTTTGGGATTATCCGATAGGACACCTTGAT |
| R-CgAF1T-F1       | TTTGATAGTTTAGTA |
| R-CgAF1T-F2       | ACCCTCTTAAATGCTTAAGTGCT |
| L-CgHI-F1         | TTTCCTTAAATGCTTAAGTGCT |
| L-CgHI-F2         | ACCCTCTTAAACCGCATCTTGAGTTTAAGAGAGGGT |
| CgHS3(CgHi)-F1    | GATGGTGGGGAAGAAGAGAGGGT |
| CgHS3(CgHi)-F2    | CTTTGGGATTATCCGATAGGACACCTTGAT |
| R-CgHI-F1         | TTTGATAGTTTAGTA |
| R-CgHI-F2         | ACCCTCTTAAATGCTTAAGTGCT |
| L-CgYAP5-F1       | TTTCCTTAAATGCTTAAGTGCT |
| L-CgYAP5-F2       | ACCCTCTTAAACCGCATCTTGAGTTTAAGAGAGGGT |
| CgHS3(CgYap5)-F1  | GATGGTGGGGAAGAAGAGAGGGT |
| CgHS3(CgYap5)-F2  | CTTTGGGATTATCCGATAGGACACCTTGAT |
| R-CgYAP5-F1       | TTTGATAGTTTAGTA |
| R-CgYAP5-F2       | ACCCTCTTAAATGCTTAAGTGCT |
| L-CgYAK1-F1       | TTTCCTTAAATGCTTAAGTGCT |
| L-CgYAK1-F2       | ACCCTCTTAAACCGCATCTTGAGTTTAAGAGAGGGT |
| CgHS3(CgYak1)-F1  | GATGGTGGGGAAGAAGAGAGGGT |
| CgHS3(CgYak1)-F2  | CTTTGGGATTATCCGATAGGACACCTTGAT |
| R-CgYAK1-F1       | TTTGATAGTTTAGTA |
| R-CgYAK1-F2       | ACCCTCTTAAATGCTTAAGTGCT |

| Overexpression    |                  |
|-------------------|------------------|
| CgMED2-F1(pY26)   | GATCTCAAAATAGCTGGATGGTGTTATGGGAGAGAGGGCAGTTACCC |
| CgMED2-F2(pY26)   | GATCTCAAAATAGCTGGATGGTGTTATGGGAGAGAGGGCAGTTACCC |
| CgYAP6-F1(pY26)   | GATCTCAAAATAGCTGGATGGTGTTATGGGAGAGAGGGCAGTTACCC |
| CgYAP6-F2(pY26)   | GATCTCAAAATAGCTGGATGGTGTTATGGGAGAGAGGGCAGTTACCC |
| CgHAP5-F1(pY26)   | GATCTCAAAATAGCTGGATGGTGTTATGGGAGAGAGGGCAGTTACCC |
| CgHAP5-F2(pY26)   | GATCTCAAAATAGCTGGATGGTGTTATGGGAGAGAGGGCAGTTACCC |
| CgCOM2-F1(pY26)   | GATCTCAAAATAGCTGGATGGTGTTATGGGAGAGAGGGCAGTTACCC |
| CgCOM2-F2(pY26)   | GATCTCAAAATAGCTGGATGGTGTTATGGGAGAGAGGGCAGTTACCC |
| CgAF1T-F1(pY26)   | GATCTCAAAATAGCTGGATGGTGTTATGGGAGAGAGGGCAGTTACCC |
| CgAF1T-F2(pY26)   | GATCTCAAAATAGCTGGATGGTGTTATGGGAGAGAGGGCAGTTACCC |
| CgHI-F1(pY26)     | GATCTCAAAATAGCTGGATGGTGTTATGGGAGAGAGGGCAGTTACCC |
| CgHI-F2(pY26)     | GATCTCAAAATAGCTGGATGGTGTTATGGGAGAGAGGGCAGTTACCC |
| CgYAP5-F1(pY26)   | GATCTCAAAATAGCTGGATGGTGTTATGGGAGAGAGGGCAGTTACCC |
| CgYAP5-F2(pY26)   | GATCTCAAAATAGCTGGATGGTGTTATGGGAGAGAGGGCAGTTACCC |
| CgYAK1-F1         | GATCTCAAAATAGCTGGATGGTGTTATGGGAGAGAGGGCAGTTACCC |
| CgYAK1-F2         | GATCTCAAAATAGCTGGATGGTGTTATGGGAGAGAGGGCAGTTACCC |

(Continued on next page)
TABLE 4 (Continued)

| Category and name | Sequence (5’→3’)a |
|--------------------|-------------------|
| Yeast two-hybrid assay |                 |
| BD-CgMED2-F1        | TTTGACTTGTATGCCCGGAATTCATGAGTTACAAAGAAACGGCTTACGG |
| BD-CgMED2-F2        | CTATAGGGCTCTAGAGTCGACTTAGATATTAAAGCCATTTAGGTCTAGG |
| AD-CgYAP6-F1        | AAGAGATGCAATTAGGATCCTGAGCGCACAGTTAACATGCG |
| AD-CgYAP6-F2        | AAGCATTAGAATTGAAATTCTAGGACTTCTGGTGGCAGCAGGA |
| Coimmunoprecipitation |             |
| pY26/PGPD-CgMED2-F1 | ATTTCTAAGACTTGAATGGATCTGTGAGTTACAAAGAAACCTTACGG |
| pY26/PGPD-CgMED2-F1 | TCGACGGTATCTGAAACTTTCATTTACAGATCTCCTGAGATGATCTTCTG |
| pY26/PTEF-CgYAP6-F2 | GAAATGTTAAATAAAAAGCTCTGACGATGAGTTTCTG |
| pY26/PTEF-CgYAP6-F2 | CGGCCAGCAATTG |
| RT-PCR              |                   |
| YAP5-F1             | GGAGGATCCCAATGCTA |
| YAP5-F2             | GTTCGCTAAGAAAGCTG |
| HAP5-F1             | ACAGAGATAGCTTCTCC |
| HAP5-F2             | GCAGAATATTATAGGCCC |
| COM2-F1             | GAGGAACACTCTCAATACATA |
| COM2-F2             | CGTCATTAAGAAGTCTTACCA |
| YAP6-F1             | GACATCAAGACCACCTAG |
| YAP6-F2             | GACCATTTTGGAAGAAGA |
| AFT1-F1             | GCCAGATTCGACTATAAAC |
| AFT1-F2             | TGTTGACATGTATATGGAC |
| SUT1-F1             | CTGTTGAGTATGTTACAA |
| SUT1-F2             | AGCCCTCTCAGCTCTTAG |
| SCT1-F1             | AGTGGCATTGGATTTTC |
| SCT1-F2             | TGAGGATGGAAGATTTACCA |
| CDS1-F1             | ACCTGACCTGTGATTTG |
| CDS1-F2             | TGGCTAACAGTGATTTA |
| PAH1-F1             | CTCTGATGGCTTATGAAAAAG |
| PAH1-F2             | CACAGTAGTAAAGACAG |
| ALE1-F1             | GCTGGAAGAAGTTGCTCA |
| ALE1-F2             | AGTCGCGATAGTGATAG |
| PSD1-F1             | CGGAAGAAGACTAACTATA |
| PSD1-F2             | CCAATTTGATGGTAAGAAG |
| CH01-F1             | AAGGCGAAGTCTTACAGT |
| CH01-F2             | GCAAGAAGAAGTGAAGATG |
| OPI3-F1             | GCTCTCATTCTCTTCTG |
| OPI3-F2             | CAGCAATCTTGGTGGAG |

aUnderlining indicates sequences of regions flanking a target gene or a restriction site.

qRT-PCR analysis. C. glabrata cells were cultured as described for transcriptome sequencing analysis. Total RNA was extracted using a MiniBEST universal RNA extraction kit (TaKaRa Bio, Shiga, Japan), and 1 μg was used to synthesize cDNA with a PrimeScript II first-strand cDNA synthesis kit (TaKaRa Bio, Shiga, Japan). The cDNA mixture was diluted to approximately 100 ng/μl and used for quantitative real-time PCR (qRT-PCR) with TB Green Premix Ex Taq (TaKaRa Bio, Shiga, Japan) on an iQ5 continuous fluorescence detector system (Bio-Rad, Hercules, CA). Data were normalized to values for ACT1 mRNA.

Coimmunoprecipitation. Cells expressing CgMed2-Myc and CgYap6-His were grown to logarithmic phase in YNB medium. The cells were then cultured at pH 5.5 and pH 2.0 for 1 h and harvested at 4°C. Pellets were resuspended in lysis buffer (45 mM HEPES-KOH [pH 7.5], 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 2 mM diethiothreitol [DTT]) with protease and phosphatase inhibitors, followed by the addition of glass beads and sonication at 4°C. Protein extracts were clarified by centrifugation at 6,000 × g for 10 min at 4°C. Proteins were incubated with primary antibody at 4°C overnight and then incubated with protein A-agarose beads (Sangon Biotech) at 4°C for 6 to 8 h. Beads were washed six times with lysis buffer and one time with PBS and then boiled in SDS loading buffer for 10 min. The binding proteins were resolved by 10% SDS-PAGE and detected by Western blotting.

Two-hybrid analysis. The yeast two-hybrid assays were performed using a Matchmaker library construction and screening kit (Clontech), and two-hybrid analysis was carried out by using pGADT7 (Gal4AD) as the activation domain (AD) plasmid and pGBK7 (Gal4BD) as the DNA-binding domain (BD) plasmid. The plasmid pGADT7-CgYap6 was cotransformed with pGBK7-CgMed2 using the AH109 reporter strain. Positive clones were selected and further tested as follows. The transformed yeast strains were grown until mid-log phase in YNB medium, diluted on synthetic dextrose (SD)-Leu-Trp plates and

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SD-Leu-Trp-His selective plates with the histidine biosynthesis inhibitor 1,2,4-aminotriazole (3-AT), and incubated for 2 to 4 days at 30°C.

**Alkaline phosphatase treatment.** Samples used for the alkaline phosphatase treatment were processed as described for Western blotting, except that after the lysis buffer washes, PBS was added to the immunoprecipitated proteins bound to the beads. Samples were treated with alkaline phosphatase (D7027; Beyotime) in the presence or absence of alkaline phosphatase inhibitor (P1081; Beyotime) and incubated for 1 h at 30°C with occasional shaking. Untreated samples were used as controls. The immunoprecipitated proteins were then washed twice with the wash buffer without protease inhibitors and released from the beads by boiling in SDS loading buffer.

**ChIP assay.** Cells were grown to the logarithmic phase and then cultured at pH 5.5 and pH 2.0 for 1 h. The cells were cross-linked with 1% formaldehyde for 20 min at room temperature. Glycine was added to a final concentration of 330 mM, and the incubation was continued for 15 min. The cells were collected, washed four times with cold Tris-buffered saline (TBS) (20 mM Tris-HCl [pH 7.5], 150 mM NaCl), and maintained at −20°C for further processing. Cell pellets were resuspended in 0.3 ml of cold lysis buffer (50 mM HEPS-KOH [pH 7.5], 150 mM NaCl, 1 mM EDTA, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride [PMSF]) supplemented with 1% Triton X-100 and lysed with a bead beater. The cross-linked chromatin was sonicated to yield an average DNA fragment size of 350 bp (range, 100 to 850 bp). Finally, the sample was clarified by centrifugation at 12,000 g for 5 min at 4°C. An aliquot of chromatin solution was used for immunoprecipitation (IP), input (IN), and control immunoprecipitation (CIP), and the remaining samples were stored at −20°C. The IP and CIP samples were incubated with anti-His monoclonal antibody and anti-IgG monoclonal antibody, respectively, which were precoated to magnetic beads (9006; Cell Signaling Technology). After shaking for 2 h at 4°C on a rotator, the beads were washed twice with lysis buffer, twice again with lysis buffer plus 500 mM NaCl, twice with washing buffer (10 mM Tris-HCl [pH 8.0], 0.25 M LiCl, 1 mM EDTA, 0.5% N-P40, 0.5% sodium deoxycholate), and once with TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). The chromatin was eluted, and cross-linking was reversed by incubation at 65°C overnight. After extraction with phenol-chloroform-isooamyl alcohol (25:24:1, vol/vol/vol), DNA was ethanol precipitated for 4 h at −20°C and resuspended in TE buffer. qRT-PCR was used to analyze the DNA with TB Green Premix Ex Taq (TaKaRa Bio) on an IQ5 continuous fluorescence detector system (Bio-Rad, Hercules, CA, USA). Relative fold enrichment was calculated using the following formula: fold change = (IP intensity – CIP intensity)/IN intensity. The primers used in this study are listed in Table 4.

**Fluorescence microscopy analysis.** Yeast strains carried the plasmids pY26-CgMed2-eGFP and pY26-CgYap6-eGFP were cultivated in the logarithmic phase and then incubated at pH 2.0 for 1 h. Cells were then harvested and released into fresh YNB medium at pH 5.5 or 2.0 for 4 h. Cells were harvested, washed twice with PBS, and freeze-dried. Fifty milligrams of dried cells was resuspended in a solution of ammonium acetate. The A/B ratios were 95:5, 95:5, 70:30, 60:40, and 95:5 at run times of 0, 2, 15, 17, and 20 min, respectively. Data analysis was based on the following commercial standards at a concentration of 0.3 ml -1. The mobile-phase gradient was formed by buffer A (acetonitrile) and buffer B (11 mM ammonium acetate). The A/B ratios were 95.5, 95.5, 70:30, 60:40, and 95.5 at run times of 0, 2, 15, 17, and 17.10 min, respectively. The capillary voltage was set at +3.5 kV or −3.5 kV for the positive or negative mode, respectively. Data analysis was based on the following commercial standards at a concentration of 1 mg · liter−1: 160 PA (830855; Avanti Polar Lipids), 160 PC (850355; Avanti Polar Lipids), 160 PS (840037; Avanti Polar Lipids), 160 PG (840455; Avanti Polar Lipids), Avanti Polar Lipids). The mass amounts of glycerophospholipid were calculated by the following equation: content of glycerophospholipid = a × c/v × m, where a0 is the peak area of the sample, c0 is the peak area of the standard, v is the total volume of the sample, and m is the mass of freeze-dried cells.

**Cell membrane integrity analysis.** Logarithmic-phase C. glabrata cells were harvested and released into fresh YNB medium at pH 5.5 or 2.0 for 4 h. Samples were centrifuged, washed twice with PBS, and diluted to an A660 of 0.5. The diluted sample (500 μl) was incubated with 5 μl of propidium iodide (Sangon Bio, Shanghai City, China) for 5 min at room temperature in the dark and then harvested, washed twice with PBS, and resuspended in the same volume of PBS. The cell number and fluorescence intensity (excitation, 536 nm; emission, 617 nm) of cell suspensions were measured by flow cytometry analysis using a FACSCalibur apparatus (BD Biosciences, Shanghai City, China). More than 20,000 events were analyzed for each sample and at a rate of 600 to 1,000 events/s. The data were acquired and analyzed using CellQuest software.

Cell membrane integrity was analyzed by confocal fluorescence microscopy. Logarithmic-phase C. glabrata cells were harvested and released onto fresh YNB medium at pH 5.5 or 2.0 for 4 h. The samples were centrifuged and washed twice with PBS (pH 7.4). The samples were then subjected to SYTOX green...
and FM4-64 uptake for 20 min and placed on a microscope slide covered with a coverslip. Images were acquired using a Nikon ECLIPSE 80i microscope equipped with a Nikon DS-R1i camera.

**Statistical analysis.** Experimental data are shown as the means ± standard deviations. All quantitative data were analyzed using Student’s t test or one-way analysis of variance (ANOVA). Each experiment was repeated at least three times.

**Data availability.** The RNA-seq raw reads were submitted to NCBI under BioProject number PRJNA630869, and the Sequence Read Archive (SRA) entries are SRR11723916, SRR11723917, SRR11723918, and SRR11723919. The data that support the plots within this paper and other findings of this study are available from the corresponding author on reasonable request.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**
PDF file, 0.9 MB.

**SUPPLEMENTAL FILE 2**
XLSX file, 0.1 MB.

**SUPPLEMENTAL FILE 3**
XLSX file, 0.1 MB.

**SUPPLEMENTAL FILE 4**
XLSX file, 0.1 MB.

**SUPPLEMENTAL FILE 5**
XLSX file, 0.04 MB.

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P.Z. and L.L. designed the research. P.Z. and X.Y. performed the research. P.Z., H.L., Y.Q., and X.C. analyzed the research. P.Z. and L.L. wrote the paper.

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