Letter

The Aspartic Protease Yps3p and Cell Wall Glucanase Scw10p Are Novel Determinants That Enhance the Secretion of the Antitumor Triterpenoid GA-HLDOA in *Saccharomyces cerevisiae*
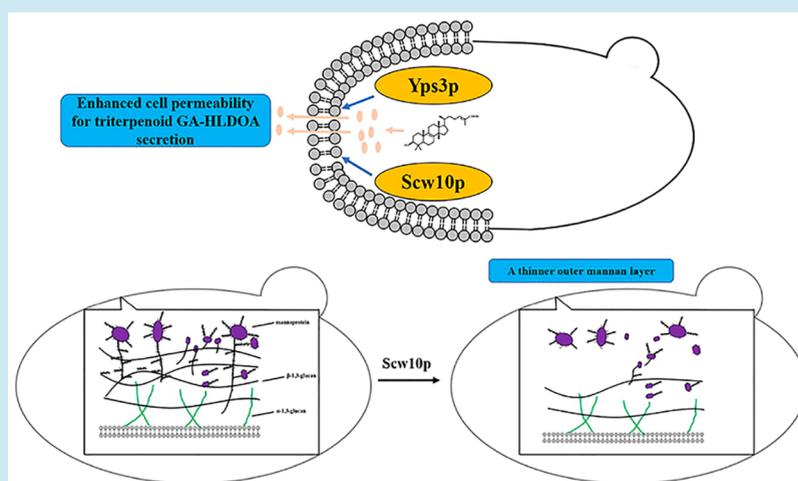
Yubo Fang and Han Xiao*

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ABSTRACT: Efficient bioproduction of triterpenoids is gaining increasing interest because of their significant biological applications; however, the secretion and bioproduction of triterpenoids are hindered by untapped genetic determinants. In our previous study, we observed that different engineered *Saccharomyces cerevisiae* strains exhibit different abilities for secreting the antitumor triterpenoid ganoderic acid 3-hydroxy-lanosta-8,24-dien-26-oic acid (GA-HLDOA). In the present study, we performed comparative proteomics analyses of the engineered strains and identified two genes, encoding an aspartic protease, *YPS3*, and a cell wall glucanase, *SCW10*, as the most effective determinants that enhance the secretion of GA-HLDOA. Compared with this control strain, strain BJ5464-r demonstrated an overexpression of *YPS3* and *SCW10* resulting in 3.9-fold and 4.7-fold higher secretion of GA-HLDOA, respectively, and these increases were accompanied by an increase in cell permeability. Moreover, compared with the *YPS3*-overexpressing strain, the *SCW10*-overexpressing strain had a thinner outer mannan layer. Our findings offer valuable insights into designing microbial cell factories for the efficient secretion of triterpenoids.

KEYWORDS: triterpenoid, Saccharomyces cerevisiae, secretion, GA-HLDOA

■ INTRODUCTION

Owing to their significant biological activities, triterpenoids have considerable practical significance in the pharmaceutical, food, and chemical industries. The increasing demand for triterpenoids has necessitated their efficient bioproduction. Because the intracellular accumulation of triterpenoids often generates feedback inhibition on biosynthetic pathway enzymes, triterpenoid secretion is an effective approach for promoting triterpenoid bioproduction;[1] this approach segregates triterpenoids from the intracellular environment, eliminating the cell lysis step, and thereby facilitating efficient triterpenoid extraction.

The monoterpenoids indole alkaloids and vincamine,[2] the sesquiterpenes arteannuin B and artemisinin,[3] and the diterpenoids labda-8(20),13-diene-15,16-diol, labda-7,13-diene-15,20-diol, and labda-7,13-diene-3,15-diol from *Ceroplastes ceriferus*[4] can be secreted in extracellular environments; in contrast, most triterpenoids naturally accumulate inside the cells.[1] Based on the production and storage processes of triterpenoids by natural hosts, we speculated that vesicles,

Table 1. Comparative Proteomics Analysis Reveals Protein Candidates for Enhanced Secretion of GA-HLDOA by Strain BJ5464-r

| Name	Function	Fold change[1]
Bmh2p	Regulation of vesicle transport	2.2
Gea2p	Involved in vesicle transport between the Golgi and endoplasmic reticulum	*
Gyp7p	Involved in vesicle mediated protein trafficking	*
Sec14p	Phosphatidylinositol transfer protein	3.6
Hxt4p	High-affinity glucose transporter	*
Zrt1p	High-affinity zinc transporter of the plasma membrane	*
Hsp12p	Plasma membrane protein involved in maintaining membrane organization	2.9
Scw4p	Cell wall protein, with similar function as β-1,3-glucanase	9.4
Scw10p	Cell wall protein, with similar function as β-1,3-glucanase	5.2
Pst1p	Cell surface protein involved in cell wall integrity	4.7
Exg1p	Exo-1,3-β-glucanase	3.9
Kre9p	Glycoprotein involved in cell wall β-glucan assembly	3.7
Yps3p	Aspartic protease involved in cell wall growth and maintenance	3.4
Ygp1p	Cell wall-related secretory glycoprotein	3.2
Bgl2p	Endo-β-1,3-glucanase	2.8
Ypk2p	AGC-type S/T protein kinase participating in the cell wall integrity signaling pathway	*
Yps6p	Aspartic protease involved in cell wall growth and maintenance	*

[1]Fold change is the ratio of the protein expression in BJ5464-r and that in INVSc1-r. Star indicates the protein was not identified from the cell supernate samples of INVSc1-r. Vesicle related proteins, transporters, cell membrane related proteins, and cell-wall related proteins were highlighted in gray, blue, yellow, and green, respectively.

transporters, and cell membrane/wall permeability are factors that affect triterpenoid secretion.[1] During the production of the triterpenoid bryonolic acid, swellings of the rough endoplasmic reticulum are released into cytoplasm as vesicles, which eventually establish contact with the cell wall. A previous study reported that bryonolic acid was transported via vesicles.[5] Another previous study reported that decreased transcription of the pleiotropic drug resistance (PDR) transporter *PgPDR3* and decreased production of the triterpenoid ginsenoside in *Panax ginseng* C.A. Meyer were observed in the presence of a PDR transporter inhibitor. Except for *PgPDR3*, no other transporters are involved in triterpenoid secretion.[6] The amphipathic triterpenoid, saponin, was able to spontaneously insert into the artificial membrane, whose hydrophobic moieties were in contact with cholesterol and hydrophilic moieties were in contact with the culture medium.[7] Such interaction leads to membrane bending and pores generation in the membrane, which facilitates the secretion of saponin. However, owing to their strict hydrophobic properties, most triterpenoids do not interact with the cell membrane directly.

Owing to its genetic tractability and the inherent existence of multiple metabolites, *Saccharomyces cerevisiae* is the predominant yeast species used in the synthetic bioproduction of triterpenoids.[8] The metabolites contained in *S. cerevisiae* (e.g., squalene and lanosterol) serve as precursors of triterpenoids. Ganoderic acids (GAs), a group of lanostane-type triterpenoids, are the main bioactive secondary metabolites of the traditional Chinese medicinal mushroom *Ganoderma lucidum*.[9] GAs possess several biological properties, including antitumor,

antioxidative, antidiabetic and anti-inflammatory properties.[10] In our previous study, we reported that the systematic screening of cytochrome P450 from *G. lucidum* enabled the heterologous production of ganoderic acid 3-hydroxy-lanosta-8,24-dien-26-oic acid (GA-HLDOA) in *S. cerevisiae*,[11] most of which was accumulated inside the cell.[12] Although most engineered yeasts cannot secrete various triterpenoids efficiently,[12] there are a few exceptions. Studies have found that *S. cerevisiae* Wat11 and CEN-PK can secrete the pentacyclic triterpenoids betulinic acid and betulin, whereas *S. cerevisiae* INVSc1 and CEN.PK2−1C can secrete the pentacyclic triterpenoid glycyrrhetinic acid.[13,14] However, the mechanism underlying the secretion of all these triterpenoids remains unknown. Moreover, the ABC transporters Pdr5p and Erg6p are two important targets that affect the cell membrane permeability of *S. cerevisiae*; Pdr5p performs cellular detoxification by pumping out toxic substrates,[15] whereas Erg6p decreases cell membrane permeability.[16] However, the findings of our previous study suggest that neither the overexpression of *PDR5* nor disruption of *ERG6* enhanced the secretion of GA-HLDOA in *S. cerevisiae* (data not shown). Cyclodextrins are cyclic oligosaccharides comprising α-D-glucopyranoside units, which can sequester saponins and facilitate their secretion.[17] Moreover, we observed that the addition of methyl-*β*-cyclodextrin or hydroxypropyl-*β*-cyclodextrin at the beginning or during the process of fermentation facilitated the efficient secretion of GA-HLDOA.[12] However, these cyclodextrins (CDs) could not permeate the cell membrane of *S. cerevisiae*; CDs failed to improve the secretion of triterpenoids generated and accumulated in the endoplasmic reticulum or cytoplasm.[12]
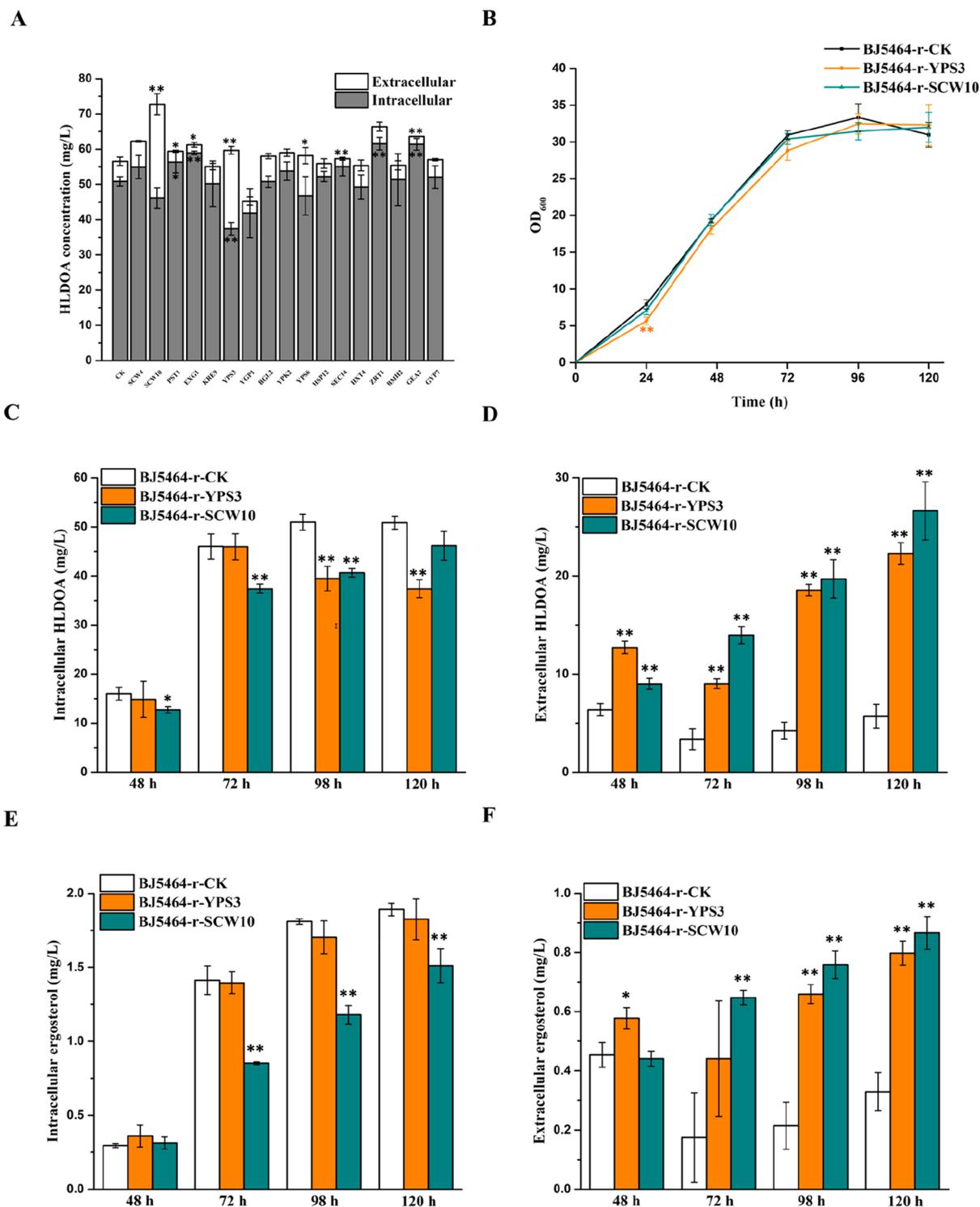
Figure 1. Secretion of GA-HLDOA by the engineered yeast strains. (A) The intracellular and extracellular GA-HLDOA after 120 h fermentation of the engineered yeast strains. Time profile of cell growth (B), intracellular GA-HLDOA (C), extracellular GA-HLDOA (D), intracellular ergosterol (E), and extracellular of ergosterol (F) by engineered yeast strains. CK, SCW4, SCW10, PST1, EXG1, KRE9, YPS3, YGP1, BGL2, YPK2, YPS6, HSP12, SEC14, HXT4, ZRT1, BMH2, GEA2, and GYP7 represented strains BJ5464-r-CK, BJ5464-r-SCW4, BJ5464-r-SCW10, BJ5464-r-PST1, BJ5464-r-EXG1, BJ5464-r-KRE9, BJ5464-r-YPS3, BJ5464-r-YGP1, BJ5464-r-BGL2, BJ5464-r-YPK2, BJ5464-r-YPS6, BJ5464-r-HSP12, BJ5464-r-SEC14, BJ5464-r-HXT4, BJ5464-r-ZRT1, BJ5464-r-BMH2, BJ5464-r-GEA2, and BJ5464-r-GYP7, respectively. The error bars present the standard deviation of three biological replicates. *, $P < 0.05$; **, $P < 0.01$.

In addition, differences in the substrate preference of CDs led to differences in the ability to secrete the triterpenoids β-

amyrin and hydroxylated β-amyrin,[17] indicating that sequestering triterpenoids via CDs is strongly dependent on the
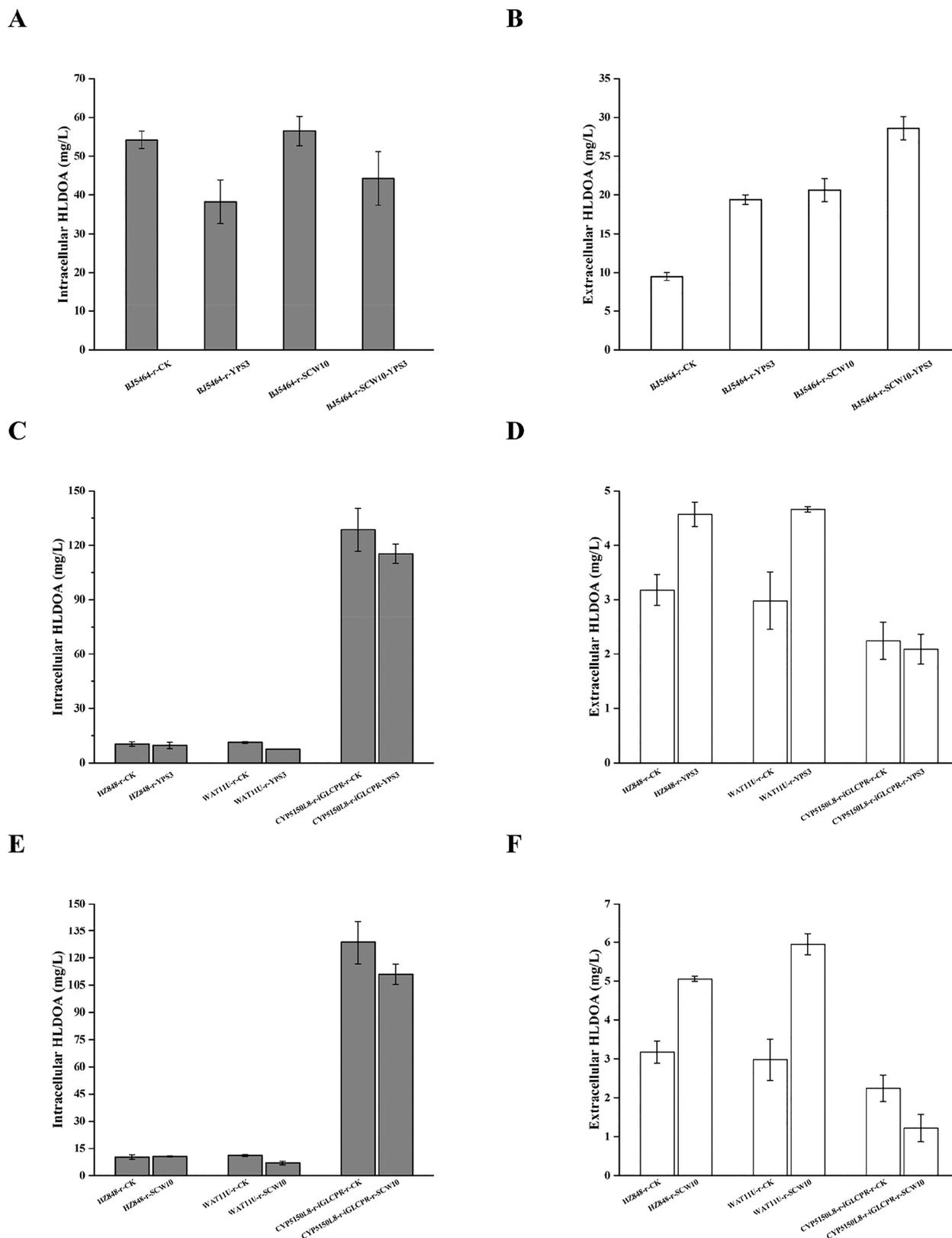
Figure 2. Effect of Yps3p and Scw10p on secretion of GA-HLDOA. (A) The intracellular and (B) extracellular GA-HLDOA after 120 h fermentation of strains BJ5464-r-CK, BJ5464-r-YPS3, BJ5464-r-SCW10, and BJ5464-r-SCW10-YPS3. (C) The intracellular and (D) extracellular GA-HLDOA after 120 h fermentation of strains HZ848-r-CK, HZ848-r-YPS3, WAT11U-r-CK, WAT11U-r-YPS3, CYP5150L8-r-iGLCPR-r-CK, and CYP5150L8-r-iGLCPR-r-YPS3. (E) The intracellular and (F) extracellular GA-HLDOA after 120 h fermentation of strains HZ848-r-CK, HZ848-r-SCW10, WAT11U-r-CK, WAT11U-r-SCW10, CYP5150L8-r-iGLCPR-r-CK, and CYP5150L8-r-iGLCPR-r-SCW10. The error bars present the standard deviation of three biological replicates.

chemical structure of the substrate. Thus, the discovery of novel genetic determinates for promoting the secretion of triterpenoids is urgently required.

In our previous study, we introduced the GA-HLDOA biosynthetic pathway in different *S. cerevisiae* cells. Two engineered yeasts, BJ5464-r and INVSc1-r, produced approximate amounts of GA-HLDOA but with different secretion efficiencies.[12] In the current study, we conducted comparative proteomics analyses of the two engineered strains to discover the determinants that affect the secretion of GA-HLDOA. As a result, we identified several novel targets that enhance the secretion of GA-HLDOA.

■ RESULTS AND DISCUSSION

Comparative Proteomics Analyses Revealed Protein Candidates Related to the Secretion of GA-HLDOA. Before comparing the protein expression of strains BJ5464-r and INVSc1-r, we performed gradient centrifugation of their fermentation broths at 4000*g* for 5 min, 10000*g* for 30 min, and 100 000*g* for 30 min, and detected GA-HLDOA in the resultant precipitates and supernatants. We found that extracellular GA-HLDOA of both strains mainly accumulated in precipitation after centrifugation at 10 000*g* for 30 min. The GA-HLDOA concentration of BJ5464-r, corresponding to the precipitate after centrifugation at 10 000*g* for 30 min, was 3 mg/L after 68 h of fermentation, whereas only 1 mg/L was detected in strain INVSc1-r under the same conditions. All of the precipitates were collected for further comparative proteomics analyses. A total of 157 proteins exhibited significantly higher expression levels in the higher GA-HLDOA secreting-strain BJ5464-r than in strain INVSc1-r, and 153 proteins were only detected in BJ5464-r (Table S3). Our systematic review revealed that vesicle trafficking proteins, transporters, and cell wall- and cell membrane-related proteins are probably involved in the transport of triterpenoids.[1] Of 300 proteins, 3 proteins related to vesicle trafficking, 3 transporters, 10 cell wall-related proteins, and 1 plasma membrane protein (17 proteins in total) were determined as candidates that affect the secretion of GA-HLDOA (Table 1).

Overexpression of *YPS3* and *SCW10* Significantly Enhances the Secretion of GA-HLDOA. To determine whether the 17 aforementioned protein candidates contribute to GA-HLDOA secretion, their corresponding coding genes were individually cloned into the yeast expression plasmid pRS414 under the control of the promoter *TDH3* and the terminator *ADH2*, and subsequently transformed into strain BJ5464-r to yield engineered strains (Table S1). To obtain a clear comparison, the void plasmid pRS414-TDH3p-ADH2t was also transformed into strain BJ5464-r to yield the control strain BJ5464-r-CK. Strains overexpressing the aspartic protease genes *YPS3* and *YPS6* and the cell wall glucanase gene *SCW10* secreted 22.3 mg/L, 11.4 mg/L, and 26.6 mg/L GA-HLDOA after 120 h of fermentation, exhibiting 3.9-fold, 2-fold, and 4.7-fold enhanced secretion compared with the control strain, respectively (Figure 1A). Along with enhanced secretion, intracellular GA-HLDOA concentrations in strains BJ5464-r-YPS3, BJ5464-r-YPS6, and BJ5464-r-SCW10 were reduced to 37.4 mg/L, 46.7 mg/L, and 46.1 mg/L, respectively. In contrast, strain BJ5464-r-CK produced 50.9 mg/L of intracellular GA-HLDOA under similar conditions (Figure 1A). Overall, BJ5464-r-SCW10 produced a total of 72.8 mg/L of GA-HLDOA after 120 h of fermentation, which was significantly higher than that produced by BJ5464-r-CK

(56.6 mg/L). However, no significant difference in the total GA-HLDOA produced was detected among the BJ5464-r-YPS3, BJ5464-r-YPS6, and the control strain (Figure 1A). For the remaining 14 candidates, no enhancement was noted in the secretion of GA-HLDOA by the corresponding strains (Figure 1A). With a 76% similarity in their amino acid sequences, Scw4p and Scw10p were considered paralogs in maintaining cell morphology and releasing cell wall proteins;[18] however, their functions in terms of GA-HLDOA secretion were not similar. Coincidentally, a previous study reported higher expression levels of Scw10p in *S. cerevisiae* cells containing more hydrophobic cell surfaces.[19] Hydrophobic cell surfaces can attract hydrophobic molecules (e.g., the triterpenoid GA-HLDOA) and facilitate the secretion of those molecules.

Having demonstrated that the overexpression of YPS3 and SCW10 considerably increased the secretion of GA-HLDOA, we aimed to explore the fermentation kinetics of strains BJ5464-r-YPS3 and BJ5464-r-SCW10. Although strain BJ5464-r-YPS3 grew a bit slower at 24 h, these two engineered strains exhibited similar cell growth along with the control strain in the following fermentation process (Figure 1B). For BJ5464-r-CK and BJ5464-r-YPS3, the intracellular GA-HLDOA concentrations increased rapidly after 48 h and remained stable after 98 h of fermentation; in contrast, for BJ5464-r-SCW10, the intracellular GA-HLDOA concentrations continued to increase from 48 to 120 h (Figure 1C). Strangely, for BJ5464-r-YPS3, the extracellular GA-HLDOA concentrations decreased after 48 h and increased after 72 h (Figure 1D), the reason for which remains unclear and requires further investigation. For strain BJ5464-r-CK, the extracellular GA-HLDOA concentration decreased from 48 to 72 h and remained stable thereafter. Slightly different, the extracellular GA-HLDOA of strain BJ5464-r-YPS3 first fell after 48 h, and then, increased significantly after 72 h. Moreover, for strain BJ5464-r-SCW10, increased secretion of GA-HLDOA was observed throughout the fermentation process (Figure 1D). Likewise, the secretion of ergosterol in all of these strains exhibited trends similar to the secretion of GA-HLDOA (Figure 1C, D, E, and F). Because both ergosterol and GA-HLDOA possess a tetracyclic skeleton, we speculated that these targets are generally applicable to the secretion of groups of compounds having similar chemical structures.

To determine whether the overexpression of *YPS3* and *SCW10* further increases the secretion of GA-HLDOA, fermentation broths of strain BJ5464-r-SCW10-YPS3 carrying the overexpression cassettes of *YPS3* and *SCW10* were tested. After 120 h of fermentation, the intracellular GA-HLDOA concentrations in strains BJ5464-r-CK, BJ5464-r-YPS3, BJ5464-r-SCW10, and BJ5464-r-SCW10-YPS3 were 54.2 mg/L, 38.3 mg/L, 56.5 mg/L, and 44.3 mg/L, respectively (Figure 2A). Strain BJ5464-r-SCW10-YPS3 secreted 28.6 mg/L GA-HLDOA, which was 1.5-fold, 1.4-fold, and 3-fold higher than the concentrations of GA-HLDOA secreted by BJ5464-r-YPS3, BJ5464-r-SCW10, and BJ5464-r-CK, respectively (Figure 2B). The significant increase of GA-HLDOA secretion was observed when *YPS3* and *SCW10* were coexpressed, indicating that these two genes may act via different pathways.

To investigate whether the enhanced secretion of GA-HLDOA via the overexpression of *YPS3* and *SCW10* is a strain-specific attribute, we overexpressed both genes in three other engineered *S. cerevisiae* strains capable of producing GA-HLDOA: HZ848-r, WAT11U-r, and the hyperproducing GA-HLDOA strain CYP5150L8-r-iGLCPR-r.[11] The secretion of

GA-HLDOA by these strains was tested after 120 h of fermentation. Compared with their respective control strains HZ848-r-CK and WAT11U-r-CK, strains HZ848-r-YPS3 and WAT11U-r-YPS3 secreted 144% and 156% times more GA-HLDOA (Figure 2D). The intracellular GA-HLDOA concentration in strain HZ848-r-YPS3 was similar to that in its control strain HZ848-r-CK, whereas the intracellular GA-HLDOA concentration of strain WAT11U-r-YPS3 was 0.67-fold lower than that in its control strain WAT11U-r-CK (Figure 2C). However, no significant differences in GA-HLDOA concentration were detected either in extracellular or in intracellular in strains CYP5150L8-r-iGLCPR-r-CK and CYP5150L8-r-iGLCPR-r-YPS3 (Figure 2C,D). When *SCW10* was overexpressed in different *S. cerevisiae* strains, similar trends were observed (Figure 2E,F). Strains HZ848-r-SCW10 and WAT11U-r-SCW10 secreted 160% and 199% more GA-HLDOA, whereas CYP5150L8-r-iGLCPR-r-YPS3 did not exhibit enhanced secretion of GA-HLDOA (Figure 2E,F). Taken together, the overexpression of Yps3p and Scw10p enhanced the secretion of GA-HLDOA in strains BJ5464-r, HZ848-r, and WAT11U-r (Figures 1A, D, and 2D,F), indicating that the increased secretion of GA-HLDOA via the overexpression of *YPS3* or *SCW10* is not a strain-specific attribute.

Yps3p is the Most Effective Determinant of Enhanced Secretion of GA-HLDOA among All Glycosylphosphatidylinositol-Anchored Aspartic Proteases. Yps3p, together with Yps1p, Yps2p, Yps6p, and Yps7p, is a yeast yapsin protein, which belongs to the subfamily of aspartic proteases. Yapsins contain the conserved catalytic residues Xaa-Xaa-Asp-Xbb-Gly-Xbb, where Xaa is a hydrophobic residue and Xbb is either Ser or Thr, and cleave proproteins rich in basic amino acids.[20] Increased secretion of GA-HLDOA was observed in both strains BJ5464-r-YPS3 and BJ5464-r-YPS6 (Figure 1A). To investigate whether the enhanced secretion of GA-HLDOA is specific to yapsin, *YPS1*, *YPS2*, and *YPS7*, which encode three other yapsins in *S. cerevisiae*, were individually overexpressed in strain BJ5464-r. Although strains BJ5464-r-YPS3, BJ5464-r-YPS6, and BJ5464-r-YPS7 grew a bit slower at 24 h, all of the *YPS*-overexpressing strains exhibited similar cell growth along with the control strain during fermentation (Figure 3A). After 120 h of fermentation, strains BJ5464-r-YPS1, BJ5464-r-YPS2, BJ5464-r-YPS3, BJ5464-r-YPS6, and BJ5464-r-YPS7 secreted 10.2 mg/L, 9.9 mg/L, 25.6 mg/L, 11.4 mg/L, and 7.5 mg/L of GA-HLDOA, which was 2.1-fold, 2.0-fold, 5.2-fold, 2.3-fold, and 1.5-fold higher than that secreted by the control strain, respectively (Figure 3B). Among all of the tested genes, *YPS3* was found to be the most effective in enhancing GA-HLDOA secretion (Figure 3B). Because four of the five aspartic protease-overexpressing strains exhibited significant improvements in GA-HLDOA secretion (Figure 3B), enzymes such as Yps3p may greatly benefit triterpenoid secretion. However, the currently characterized substrates of yapsins, including β-amyloids, β-endorphins, and Scw4p,[21] may not be involved in the secretion of triterpenoids.

Compared with the overexpression of all other aspartic proteases, that of *YPS3* was the most effective in enhancing the secretion of GA-HLDOA (Figure 3B). We wondered whether the disruption of *YPS3* would reduce the secretion of GA-HLDOA. Hence, we performed CRISPR-Cas9-assisted disruption of *YPS3* in strain BJ5464-r using a previously reported protocol.[22] After 120 h of fermentation, the GA-HLDOA

A

B

C
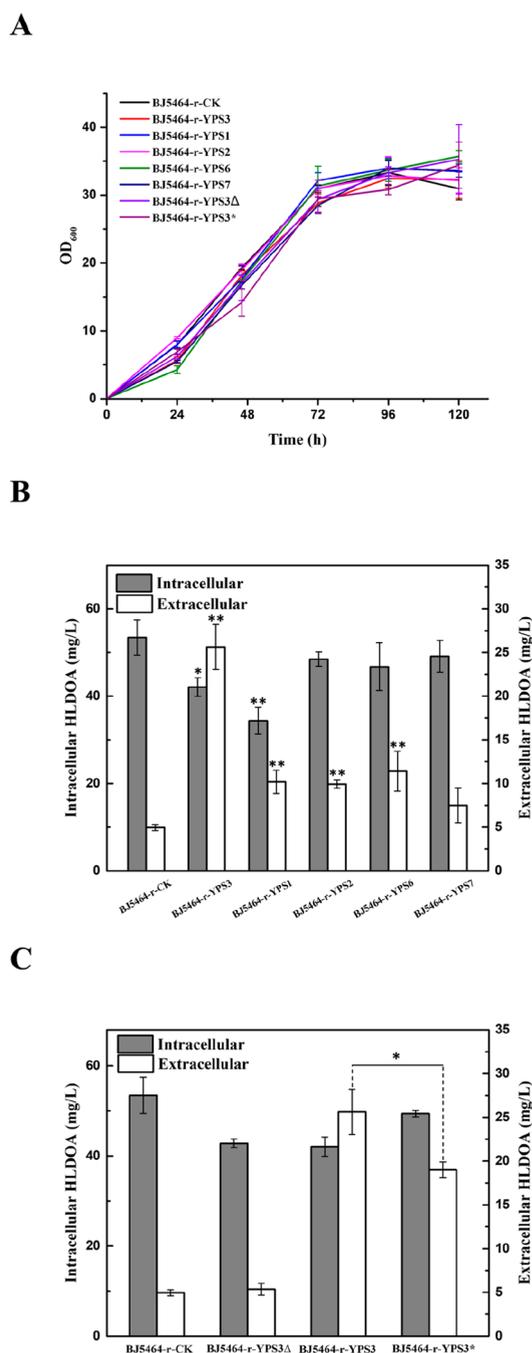
Figure 3. Secretion of GA-HLDOA by strains overexpressing aspartic proteases: (A) cell growth; (B,C) intracellular and extracellular GA-HLDOA concentrations after 120 h of fermentation. Strain BJ5464-r-YPS3* referred to BJ5464-r overexpressing a mutated *YPS3* with D81A and D288A, whereas strain BJ5464-r-CK served as control. The error bars present the standard deviation of three biological replicates. *, $P < 0.05$.

concentration secreted by strain BJ5464-r-YPS3Δ was similar to that secreted by the control strain, suggesting that other enzymes compensate for the functions of Yps3p, thus facilitating the secretion of GA-HLDOA (Figure 3C). Yps3p harbors two conserved residues, Leu-Leu-Asp-Thr-Gly-Ser and Leu-Leu-Asp-Ser-Gly-Thr, with catalytic centers proposed at positions D81 and D288, respectively. To understand whether the catalytic function of the protease contributes to the

enhanced secretion of GA-HLDOA, we introduced a mutated *YPS3* with catalytic centers at D81A and D288A into BJ5464-r to generate strain BJ5464-r-YPS3*. The catalytic function of the mutated Yps3p toward *β*-endorphin, a known substrate for Yps3p,[23] was completely blocked (data not shown). After 120 h of fermentation, strain BJ5464-r-YPS3* secreted 19.0 mg/L GA-HLDOA, which was significantly lower than that secreted by strain BJ5464-r-YPS3 but still higher than that secreted by the strain BJ5464-r-CK (Figure 3C). We speculated that other functions of Yps3p, and not just protease function, contribute to the enhanced secretion of GA-HLDOA.

Enhanced Cell Permeability Was Detected in Strains BJ5464-r-YPS3 and BJ5464-r-SCW10. *Solanum tuberosum* aspartic proteases interact with the surfaces of fungal spores and hyphae of *Fusarium solani* and *Phytophthora infestans* and result in membrane permeabilization,[24] whereas *β*-glucanases facilitate the release of proteins by enhancing cell wall permeability.[25] Based on these findings, we aimed to determine if the enhanced secretion of GA-HLDOA by strains BJ5464-r-YPS3 and BJ5464-r-SCW10 was accompanied by enhanced cell permeability. We performed flow cytometric analysis of these strains using propidium iodide (PI), which is a commonly used method for evaluating cell permeability.[26] PI turns fluorescent upon binding to nucleic acids and cannot permeate intact plasma membranes.[27] A stronger fluorescence signal after PI treatment indicates a greater cell permeability. After 96 h of fermentation, 5.1% of the BJ5464-r-YPS3 cells, and 4.4% of the BJ5464-r-SCW10 cells, and 3.7% of the control strain cells were stained with PI (Figure 4). After 120 h
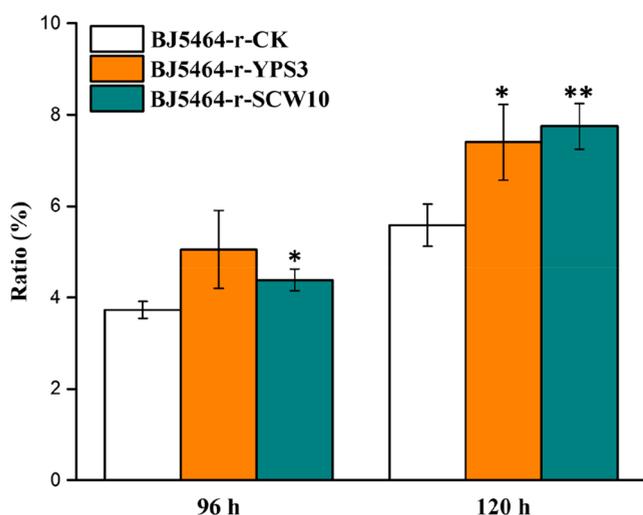
Figure 4. Cell membrane permeability of BJ5464-r-CK, BJ5464-r-YPS3, and BJ5464-r-SCW10. The ratios of the cells stained with propidium iodide after 96 and 120 h fermentation. The corresponding values of strain BJ5464-r-CK were served as control. The error bars represent the standard deviation of three biological replicates. *, $P < 0.05$; **, $P < 0.01$.

of fermentation, 7.4% of the BJ5464-r-YPS3 cells and 7.75% of the BJ5464-r-SCW10 cells were stained with PI, significantly higher than the percentage of the control strain cells stained with PI (5.6%) (Figure 4 and Figure S1). These results suggested that the increased cell permeability of BJ5464-r-YPS3 and BJ5464-r-SCW10 contributes to their enhanced secretion of GA-HLDOA. To eliminate the possibility that the enhanced secretion of GA-HLDOA is caused by increased cell

death rates, we treated the engineered strains with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). Living cells convert MTT into formazan crystals, an indicator of mitochondrial activity, with a maximum absorbance of A570 nm.[28] We did not observe any significant difference in the mitochondrial activities of strains BJ5464-r-YPS3 and BJ5464-r-CK (Figure S2). The mitochondrial activity of BJ5464-r-SCW10 was higher than that of BJ5464-r-CK at 78, 94, and 120 h (Figure S2), suggesting that the secretion of GA-HLDOA is not caused by cell death of the engineered strains. The *SCW10* disruption increased sensitivity to cell wall destabilizing agents, indicating its important role in cell wall assembly and maintenance.[29] We speculated that Scw10p contributed to the increased live ratio of BJ5464-r-SCW10. In accordance with our study, disruption of *SCW10* resulted in a higher cell death rate in a previous study.[30]

A Thinner Mannan Layer and a Thicker Cell Wall Were Observed in Strain BJ5464-r-SCW10. Because the functions of both Yps3p and Scw10p are related to the cell wall, we aimed to observe the cell morphology of BJ5464-r-YPS3 and BJ5464-r-SCW10 using transmission electron microscopy (TEM) (Figure 5). No significant difference was observed in terms of cell shape and size (Figure 5 and Figure S4). Of note, a thinner outer mannan layer with an ill-defined margin was observed for strain BJ5464-r-SCW10 at 96 and 120 h (Figure 5 and Figure S5). Furthermore, the cell wall of BJ5464-r-SCW10 cells was significantly thicker than that of the control (187.8 ± 3.0 nm vs 168.85 ± 1.5 nm), whereas no significant difference in cell wall thickness was noted for strain BJ5464-r-YPS3 (175.4 ± 3.2 nm vs 168.85 ± 1.5 nm) (Figure S4). The thinner outer mannan layer observed in strain BJ5464-r-SCW10 highlighted the reduction of mannoproteins on the cell wall, which enhances cell wall permeability by increasing porosity.[31] With similar functions as those of *β*-1,3-glucanase, Scw10p can cleave *β*-1,3-glucan. The release of *β*-1,3-glucan can cause the release of closely linked mannoproteins from the cell wall.[32] A thicker cell wall was also observed in yeast strains with the cell wall mannoprotein genes *CCW12* and *CCW14* deleted;[33] mannoprotein reduction in these strains led to increased cell wall flexibility and reduced mechanical strength.[33] Reduction of mannoprotein can also increase cell wall porosity.[34] Although a thicker cell wall was observed concomitantly with reduced mannoprotein, no direct connection was reported between a thicker cell wall and increased cell wall flexibility.

In this study, the overexpression of *YPS3* and *SCW10* significantly enhanced the secretion of the triterpenoid GA-HLDOA. Both Yps3p and Scw10p increased the cell permeability without compromising cell growth; this is usually difficult to achieve using traditional methods. In addition to Yps3p, the overexpression of most yapsin proteins significantly enhanced the secretion of GA-HLDOA, indicating the important role of these proteins in triterpenoid secretion. The thin mannan layer observed in strain BJ5464-r-SCW10 suggested that the reduction of mannoproteins on the cell wall facilitates triterpenoid secretion. To our knowledge, these are novel targets for GA-HLDOA secretion. Our findings may provide insights into designing microbial cell factories for the efficient bioproduction and secretion of other triterpenoids.

■ **METHODS**

Yeast Cultivation. *S. cerevisiae* BJ5464-r and INVSc1-r[12] were cultivated in SC-Leu-Ura, whereas the other engineered
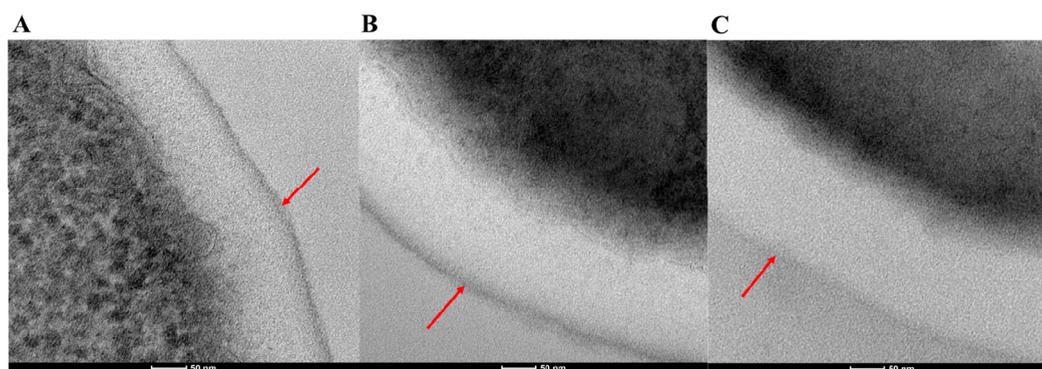
Figure 5. TEM images of BJ5464-r-CK (A), BJ5464-r-YPS3 (B), and BJ5464-r-SCW10 (C) cells after 96 h fermentation. The difference on the outer mannan layer was indicated by the red arrow.

yeast strains were cultivated in SC-Leu-Ura-Trp or yeast extract−peptone−dextrose (YPD) medium, supplemented with 40 g/L glycerol, 500 mg/L G418, and 300 mg/L hygromycin at 30 °C under shaking conditions (220 rpm).

Label-Free Proteomic Analysis. After 68 h of incubation under shaking conditions, the BJ5464-r and INVSc1-r cultures were centrifuged at 4000g for 5 min at 4 °C. The supernatants were then collected and again centrifuged at 10 000g for 5 min at 4 °C to obtain the precipitate. SDT lysis buffer containing 4% sodium dodecyl sulfate, 1 mM dithiothreitol, and 100 mM Tris-HCl (pH7.6) was used for sample lysis and protein extraction. The extracted proteins were sent to Shanghai Applied Protein Technology Co., Ltd. China for sequencing. In brief, the protein concentrations were determined using the BCA Protein Assay Kit (Bio-Rad, USA), according to the manufacturer's instructions. The extracted proteins were digested with trypsin using the filter-aided sample preparation procedure[35] and then desalted. All of the samples were subjected to liquid chromatography with tandem mass spectrometry performed using a Q Exactive Orbitrap mass spectrometer (Thermo Scientific, USA). All protein samples displaying >2-fold changes between strains BJ5464-r and INVSc1-r ($p < 0.05$) were considered differentially expressed proteins.

Construction of Plasmids and Strains. *Escherichia coli* DH5α (Tiangen Biotech, Beijing, China) was used as the cloning host. All primers (Table S2) were ordered from GENEWIZ, Inc. (China). A helper plasmid, pRS414-TDH 3p-ADH2t, was constructed such that it could yield a series of overexpression plasmids. The fragment TDH 3p-PmeI-ADH2t was amplified from the genomic DNA of *S. cerevisiae* YL-T3[36] using the forward and reverse primer pairs THD3p-F and PmeI-R, and PmeI-F and ADH2t-R. The fragment TDH3p-PmeI-ADH2t and linearized pRS414 were ligated to produce pRS414-TDH3p-ADH2t, an overexpression vector, following the procedure described in the Trelief SoSoo Cloning Kit (Tsingke, China). The genes *SCW4*, *SCW10*, *PST1*, *EXG1*, *KRE9*, *YPS3*, *YGP1*, *BGL2*, *YPK2*, *YPS6*, *HSP12*, *SEC14*, *HXT4*, *ZRT1*, *BMH2*, *GEA2*, *GYP7*, *YPS1*, *YPS2*, and *YPS7* were amplified from the genomic DNA of *S. cerevisiae* BJ5464-r and cloned into *Pme*I-linearized pRS414-TDH3p-ADH2t to produce the plasmids pRS414-TDH3p-SCW4-ADH2t, pRS414-TDH3p-SCW10-ADH2t, pRS414-TDH3p-PST1-ADH2t, pRS414-TDH3p-EXG1-ADH2t, pRS414-TDH3p-KRE9-ADH2t, pRS414-TDH3p-YPS3-ADH2t, pRS414-TDH3p-YGP1-ADH2t, pRS414-TDH3p-BGL2-ADH2t, pRS414-TDH3p-YPK2-ADH2t, pRS414-TDH3p-YPS6-

ADH2t, pRS414-TDH3p-HSP12-ADH2t, pRS414-TDH3p-SEC14-ADH2t, pRS414-TDH3p-HXT4-ADH2t, pRS414-TDH3p-ZRT1-ADH2t, pRS414-TDH3p-BMH2-ADH2t, pRS414-TDH3p-GEA2-ADH2t, pRS414-TDH3p-GYP7-ADH2t, pRS414-TDH3p-YPS1-ADH2t, pRS414-TDH3p-YPS2-ADH2t and pRS414-TDH3p-YPS7-ADH2t, respectively. The promoter *PGK1p*, the gene *YPS3*, and the terminator *HXT7t* were amplified from the genomic DNA of *S. cerevisiae* BJ5464 using the primer pairs PGK1p-F and PGK1p-R, YPS3-2-F and YPS3-2-R, and HXT7t-F and HXT7t-R, respectively, and then cloned into *Eco*RI-linearized pRS414-TDH3p-SCW10-ADH2t to produce the plasmid pRS414-TDH3p-SCW10-ADH2t-PGK1p-YPS3-HXT7t. *YPS3* was disrupted using CRISPR-Cas technology; a 20-bp guide sequence targeting 37-bp downstream of the start codon of *YPS3* was selected for *YPS3* disruption. A 100-bp homologous recombinant donor sequence was designed as previously reported.[37] Then, an 8-bp deletion including the PAM sequence was incorporated into the homologous-recombinant donor sequence to introduce a frame-shift mutation 54-bp downstream of the start codon of *YPS3*. The DNA fragment containing the guide sequence and homologous-recombinant donor sequence (Figure S3) was synthesized at GENEWIZ. The plasmid pRS414-TEF1p-Cas9-CYCIt[38] was linearized using *Cla*I and *Bst*EII, and the synthesized DNA fragment was then inserted into the linearized plasmid, according to the procedure described in the Trelief SoSoo cloning kit. The *YPS3* mutation fragment harboring the D81A and D288A mutations was amplified from the plasmid pRS414-TDH3p-YPS3-ADH2t using the primer pair YPS3*-1-F and YPS3*-1-R. Two other fragments were also amplified from the plasmid pRS414-TDH3p-YPS3-ADH2 using the primer pairs of YPS3*-2-F and YPS3*-2-R and YPS3*-3-F and YPS3*-3-R. All three fragments were ligated to produce the plasmid pRS414-THD3p-YPS3*-ADH2t according to the procedure described in the Trelief SoSoo cloning kit. The constructed plasmids were transformed into strain BJ5464-r, HZ848-r, WAT11U-r, and CYP5150L8-r-iGLCPR-r[12] using the standard lithium acetate transformation method. To determine whether *YPS3* was disrupted in the engineered strain, the genomic DNA was amplified using polymerase chain reaction with the primer pair Seq-YPS3Δ-F and Seq-YPS3Δ-R. A 600-bp product was obtained, which was then subjected to Sanger sequencing.

Yeast Fermentation and Analyses of Cell Growth and Metabolites. The yeast strains were cultivated in SC-His-Leu-Ura-Trp medium[41] until an optical density (OD$_{600}$) of four was reached. The cultures were then allowed to ferment in

either round-bottom 15 mL test tubes or 250 mL shaker flasks. The cells were inoculated into 50 mL of YPD medium[41] in a 250 mL shaker flask or into 3 mL of YPD medium in a 15 mL test tube containing 40 g/L of glycerol, 500 mg/L of G418, and 300 mg/L of hygromycin, at an initial OD_{600} of 0.05.

Yeast cell growth was determined as reported previously.[36] For the extraction of ergosterol and GA-HLDOA, 1.5 mL of the fermentation broth was centrifuged at 6000g for 5 min to separate the cells and the supernatant. Then, 1 mL of distilled water and 0.8 mL of ethyl acetate were added to the cell pellet, and 0.8 mL of ethyl acetate was added to the supernatant; the solutions were shaken violently by vortex for 3 min. The organic phase was collected by centrifugation at 12 000g for 10 min. The entire extraction process was repeated twice. After evaporation of the organic phase, the metabolites were redissolved in methanol. The concentrations of the metabolites were measured using the Agilent 1260 Infinity II HPLC system (Agilent, Germany) with an Agilent XDB-C18 column (5 μm; 4.6 mm × 250 mm). Mobile phase A was 100% water, whereas mobile phase B was methanol/acetic acid (100:0.1 v/v). A linear gradient of 90%−100% B in 30 min at 1 mL/min was adopted.

Measurement of Cell Permeability. One OD_{600} of the cells at the late exponential or stationary phase was noted, after which the cells were resuspended in 1 mL of phosphate-buffered saline (PBS). Then, 30 μL of 1 mg/mL PI was added, and the mixture was incubated for 5 min. The samples were then examined using a flow cytometer (Cytoflex, Beckman, USA). The cells treated with 70% ethanol for 10 min were considered the positive control, whereas cells that were not subjected to PI staining were considered the nontreated control.[39]

MTT Assay. After fermentation for 78, 94, and 120 h, 15 OD_{600} values were taken; the cells were then washed with PBS twice by centrifugation (6000g, 5 min) and resuspended in 500 μL of PBS. The suspensions were mixed with 50 μL of 5 mg/mL MTT reagent and incubated at 30 °C under shaking at 220 rpm for 2 h. Then, 500 μL of propan-2-ol containing 0.04 M HCl was added. The mixture was vigorously vortexed to release the MTT-formazan complex from the cells and centrifuged at 12000g for 2 min. The absorbance of the supernatant was measured at 570 nm against the cell-free control treated in an identical manner.[40]

Detection of Cell Morphology. Cell morphology was detected using TEM (Talo L120C G2, USA). Three OD_{600}s of the cells after 96 h of fermentation were taken, and the cells were then fixed in 2% glutaraldehyde for 6 h and postfixed for 1 h in 2% osmium tetroxide. The samples were then dehydrated and embedded in resin, as reported previously,[41] and sectioned with Ultramicrotome EM UC7 (Leica, Germany). For each sample, over 30 cells were randomly selected, and the cell wall thickness was measured at four different positions of each cell. The average cell wall thickness was calculated as the peak height of the fitting curve from the frequency histogram.

ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.2c00005.

Strains and primers used in this study; comparative proteome analysis; PI staining with the engineered yeast

cells; formation of MTT-formazan by the engineered yeast strains; design for *YPS3* disruption; cell wall thickness of the engineered strains; TEM images of the engineered yeast cells (PDF)

AUTHOR INFORMATION

Corresponding Author

Han Xiao − *State Key Laboratory of Microbial Metabolism, Joint International Research Laboratory of Metabolic & Developmental Sciences, and Laboratory of Molecular Biochemical Engineering, School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, China;* ⊙ orcid.org/0000-0003-2895-1847; Email: smallhan@sjtu.edu.cn

Author

Yubo Fang − *State Key Laboratory of Microbial Metabolism, Joint International Research Laboratory of Metabolic & Developmental Sciences, and Laboratory of Molecular Biochemical Engineering, School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acssynbio.2c00005

Author Contributions

H.X. and Y.F. conceived and designed the study. Y.F. and H.X. wrote the manuscript. Y.F. did all the experiments and analyzed the data. H.X. finalized the manuscript. All authors concur with the submission and have seen a draft copy of the manuscript and agree with its publication.

Notes

The authors declare no competing financial interest.

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