



Alleviation of ER stress via targeted genetic engineering enhances recombinant ovalbumin secretion in *Saccharomyces cerevisiae*

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Abstract

Enhancing the secretion of heterologous proteins in *Saccharomyces cerevisiae* is often hindered by endoplasmic reticulum (ER) stress and inefficiencies in intracellular trafficking. To address these limitations, we systematically evaluated 14 genetic modifications associated with the secretory pathway to improve recombinant ovalbumin (OVA) production. Deletion of *PAH1*, a negative regulator of phospholipid biosynthesis, resulted in the highest OVA secretion (5.68 mg/L), representing a 74% improvement over the background strain—likely due to increased ER membrane biogenesis. Similarly, knockout of *GOS1*, a Golgi-to-ER SNARE protein, enhanced secretion, possibly by reducing retrograde trafficking and limiting ER retention of secretory cargo. In contrast, disruption of endosome-to-Golgi transport via *VPS5* deletion resulted in intracellular accumulation of OVA and complete secretion failure. These findings suggest that alleviating ER stress and modulating vesicular trafficking are key strategies for enhancing protein secretion in yeast, offering a functional basis for rational host strain engineering.

Keywords *Saccharomyces cerevisiae* · Ovalbumin · ER stress · Vesicular trafficking

Introduction

Growing concerns over sustainability, food security, and animal welfare have accelerated the search for alternative protein sources (Prajapati et al., 2024). Among these, recombinant production of animal-origin proteins using microbial hosts has emerged as a promising solution (Yang et al., 2025). Ovalbumin (OVA), the major protein component of egg white, constitutes approximately 54% of total egg white protein and is widely utilized in the food industry due to its excellent emulsifying, foaming, and gelling properties (Razi et al., 2023). In addition to its functional roles, OVA has been recognized as a precursor of bioactive peptides with antioxidant, antimicrobial, and immunomodulatory potential, further expanding its applications in the nutraceutical and pharmaceutical sectors (Chang et al., 2018).

Recent advances in synthetic biology and metabolic engineering have enabled the expression of complex eukaryotic proteins in microbial platforms, with *Saccharomyces cerevisiae* being one of the most widely used hosts due to its generally recognized as safe (GRAS) status, well-characterized genetics, and eukaryotic protein folding and secretion machinery (Zhao et al., 2024). However, efficient secretion of heterologous proteins such as OVA remains a major

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bottleneck, often limited by folding inefficiencies, vesicle trafficking constraints, and proteolytic degradation within the secretory pathway (Hou et al., 2012).

To address these challenges, various strategies have been explored, including codon optimization, signal peptide engineering, and co-expression of molecular chaperones (Dong et al., 2024; Jin et al., 2024). Nevertheless, the overall yield and secretion levels of OVA in *S. cerevisiae* remain insufficient for commercial-scale applications. Thus, more comprehensive and systems-level engineering approaches are needed to overcome multiple limitations simultaneously.

In this study, we aimed to enhance recombinant OVA secretion in *S. cerevisiae* by systematically modifying key genetic elements involved in protein production and trafficking. Specifically, 14 genes associated with transcriptional regulation, membrane lipid biosynthesis, endoplasmic reticulum (ER) quality control, vesicle transport, and cell wall remodeling were either knocked out or overexpressed (Fig. 1). This multi-target engineering approach provides new insights into the optimization of yeast as a cell factory

for the animal-free production of functional food proteins such as OVA.

Materials and methods

Strains and plasmids

Escherichia coli TOP10 (Invitrogen, Carlsbad, CA, USA) was used for plasmid construction, and *S. cerevisiae* D452-2 (Hosaka et al., 1992) was used as the expression host for OVA production. The *S. cerevisiae* strains and plasmids used in this study are listed in Table S1.

Genetic manipulation

The primer sets used for amplification of the overexpression targets (*INO2*, *COG5*, and *ERV29*), yeast integrative plasmids (YIPs), and guide RNA (gRNA) plasmids are listed in Table S2. For the gRNA plasmids, primers were designed

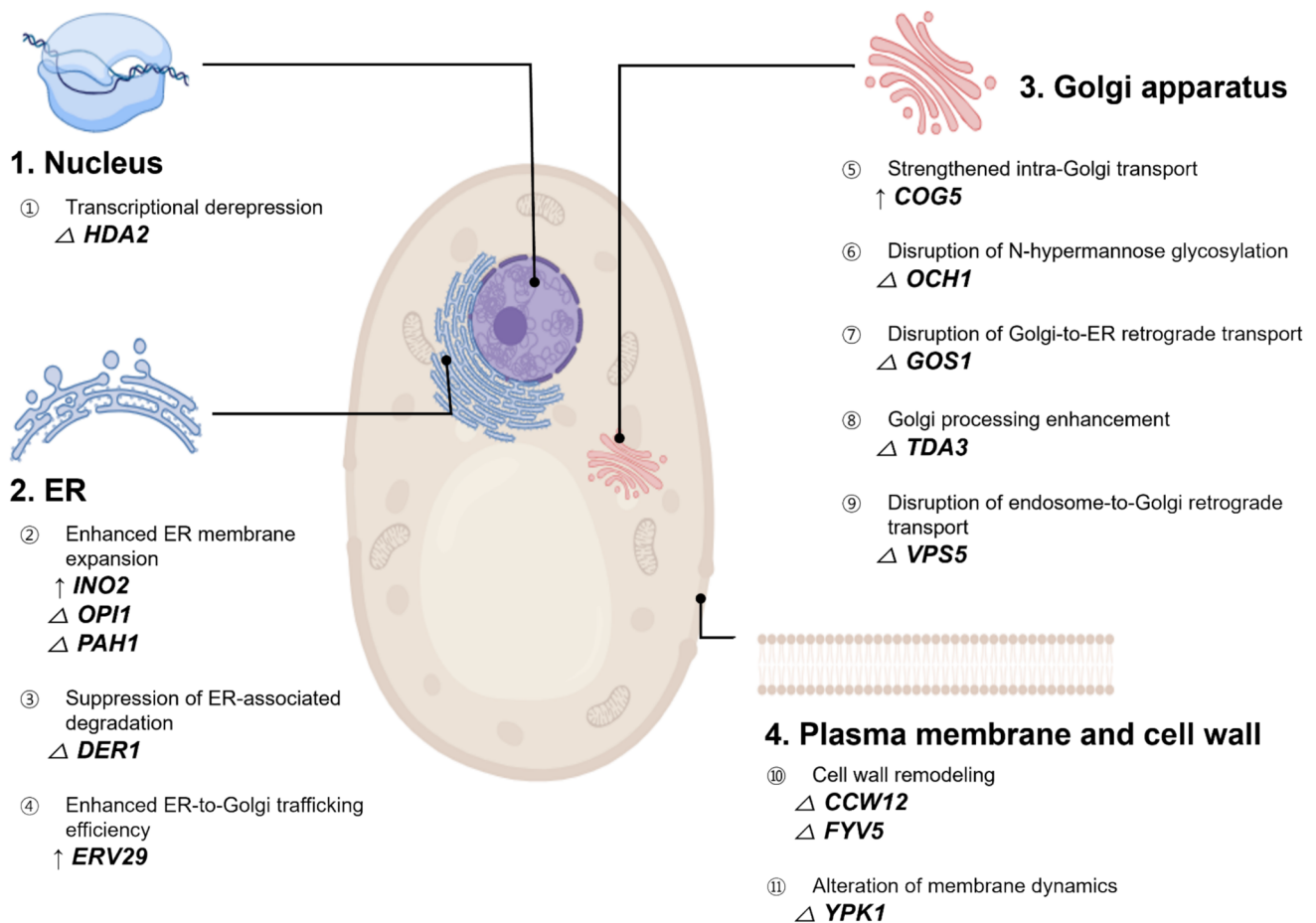


Fig. 1 Schematic diagram of the genetic targets selected in this study to enhance ovalbumin secretion in *Saccharomyces cerevisiae* and their proposed functional effects. Upward arrows (↑) and delta sym-

bols (Δ) indicate genes selected for overexpression and disruption (knockout), respectively

to amplify the pgRNA-*TRP1*-HYB plasmid (Zhang et al., 2014) containing the gRNA expression cassette by replacing only the 20 bp target sequence with that corresponding to the gene intended for knockout. The resulting PCR products were combined using NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions, to construct YIPs for target gene overexpression and gRNA plasmids for clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9)-based target gene knockout.

Target gene knockout in *S. cerevisiae* was performed using a CRISPR/Cas9-based genome editing system, as previously reported (Lee et al., 2017). Briefly, repair DNA fragments were generated by PCR amplification using the primers listed in Table S2. The repair DNA fragments were designed to delete a 56 or 200 bp region within the open reading frame of the target genes, and each fragment contained 50 bp upstream and downstream flanking sequences adjacent to the deleted region. These repair templates, along with the corresponding gRNA plasmids, were co-introduced into *S. cerevisiae* strains harboring pCas9_AUR to achieve targeted gene disruption.

To introduce YIPs containing *INO2*, *COG5*, and *ERV29* into the CS6 locus, *S. cerevisiae* was transformed using a yeast EZ-transformation kit (BIO101, Vista, CA, USA), as specified by the manufacturer. The CS6 locus is an intergenic region between *YNCG0042C* and *YGR191W* in chromosome VII of *S. cerevisiae* (Jeon et al., 2023). Transformants were selected on solid yeast synthetic complete (YSC) medium (6.7 g/L yeast nitrogen base and 20 g/L glucose) containing 15 g/L agar and 200 µg/mL G418 supplemented with appropriate amino acids and nucleotides.

Media and culture conditions

E. coli was cultured in Luria–Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) supplemented with 50 µg/mL ampicillin for plasmid propagation. *S. cerevisiae* strains were precultured at 30 °C and 250 rpm for 48 h in either YSC medium or YP20D medium (10 g/L yeast extract, 20 g/L Bacto™ Peptone, and 20 g/L glucose). After cultivation, the cells were collected and transferred into the main culture medium at an initial optical density at 600 nm (OD_{600}) of 1.0. The main fermentation experiments were performed for 48 h in a baffled flask containing 100 mL of YP50D medium (10 g/L yeast extract, 20 g/L Bacto™ Peptone, and 50 g/L glucose).

Protein preparation and analysis of OVA expression

After 48 h of cultivation in YP50D medium, the culture broth was centrifuged at 15,000 rpm for 10 min to collect the

extracellular protein fraction. A spectrophotometer (OPTI-ZEN POP, Mecasys Co., Ltd., Republic of Korea) was used to measure cell growth at OD_{600} . The cells were harvested at $OD_{600} \times \text{milliliters of culture} = 15$ to collect the intracellular proteins (i.e., if the OD_{600} value was 30, 0.5 mL of culture was harvested). The harvested cells were resuspended in Y-PER™ (Thermo Fisher Scientific, Waltham, MA, USA) and lysed according to the manufacturer's instructions. To visualize the proteins, the protein samples fractionated by 12% (w/v) SDS-PAGE were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). For the immunoblot assays, OVA was detected using an anti-OVA antibody (MA5-15307, Thermo Fisher Scientific), according to the manufacturer's instructions. For the quantification of OVA, standard OVA was purchased from Sigma-Aldrich Co. (A5503, Burlington, MA, USA). Band intensities were quantified using densitometry software (Total Lab 1.01; Nonlinear Dynamics Ltd.). The specific OVA secretion was expressed as the amount of extracellular OVA per dry cell weight (DCW).

Statistical analyses

Statistical analyses were performed using SPSS version 28 (IBM SPSS, Armonk, NY, USA). All data are presented as the means $\pm$ standard deviations. One-way analysis of variance was performed, and statistical significance was assessed using Tukey's HSD tests at a p -value of < 0.05 .

Results and discussion

Functional basis for targeted genetic modifications to improve OVA secretion

In a previous study, we identified a signal peptide derived from EPX1, the most abundant extracellular protein produced by *Pichia pastoris*, as a novel signal peptide that enables efficient secretion of OVA in *S. cerevisiae* (Jin et al., 2024). Furthermore, in the +P1/K2 strain co-expressing the ER-resident molecular chaperones protein disulfide isomerase (PDI1) and the major Hsp70 chaperone (KAR2), OVA secretion efficiency was further enhanced (Jin et al., 2024). To further improve OVA production in the +P1/K2 background, 14 genes previously reported to enhance heterologous protein expression in *S. cerevisiae* were selected in the present study (Table S3). These genes are functionally associated with distinct cellular compartments, including the nucleus, ER, Golgi apparatus, plasma membrane, and cell wall (Fig. 1). Specifically, the nuclear gene *HDA2*, encoding a component of the HDA1 histone deacetylase complex, was disrupted to

relieve transcriptional repression, potentially increasing the expression of genes involved in secretion and protein folding (Huang et al., 2018).

Furthermore, within the ER, several genes were modified to expand the folding capacity and trafficking efficiency. *INO2* encodes a basic helix-loop-helix transcription factor that activates phospholipid biosynthesis genes; its overexpression promotes ER membrane biogenesis (Moon et al., 2023). The genes encoding *OPI1*, a transcriptional repressor of phospholipid metabolism, and *PAH1*, a phosphatidate phosphatase that negatively regulates lipid synthesis, were disrupted to enhance membrane expansion and ER surface area (Besada-Lombana and Da Silva, 2019; Moon et al., 2023). *DER1*, encoding an integral ER membrane protein involved in the retro-translocation of misfolded proteins as part of the ER-associated degradation pathway, was disrupted to reduce proteolytic loss of recombinant proteins (Besada-Lombana and Da Silva, 2019). *ERV29*, encoding a COPII vesicle cargo receptor that mediates ER export of soluble proteins, was overexpressed to facilitate efficient transport of OVA to the Golgi (Besada-Lombana and Da Silva, 2019). In the Golgi apparatus, five genes involved in trafficking and protein processing were targeted. *COG5* encodes a subunit of the conserved oligomeric Golgi (COG) complex responsible for intra-Golgi retrograde vesicle tethering; its overexpression was intended to improve glycoprotein processing (Miller et al., 2013). The genes encoding *GOS1*, a SNARE protein that mediates Golgi membrane fusion, and *VPS5*, a component of the retromer complex involved in endosome-to-Golgi retrograde transport, were disrupted to modify trafficking balance (Huang et al., 2018). *OCH1*, encoding a Golgi α -1,6-mannosyltransferase responsible for initiating outer-chain *N*-glycosylation, was deleted to simplify glycosylation patterns, potentially reducing protein misfolding (Tang et al., 2016). *TDA3*, which encodes a negative regulator of *BTN2*, was disrupted to enhance *BTN2*-dependent endosome-to-Golgi retrieval of membrane proteins (Huang et al., 2018). *BTN2* functions as a sorting factor involved in the retrograde trafficking of proteins such as *KEX2*, a key protease responsible for precursor processing (Huang et al., 2018). The enhanced localization of *KEX2* to the Golgi in response to *TDA3* knockout is expected to improve the maturation efficiency of secretory proteins such as OVA.

Finally, genes associated with the plasma membrane and cell wall were modified to facilitate extracellular release. The genes encoding *CCW12* and *FYV5*, both involved in maintaining cell wall integrity, were disrupted to reduce structural barriers to secretion (Chen et al., 2023; Wentz and Shusta, 2007). *YPK1*, encoding a serine/threonine kinase that regulates sphingolipid biosynthesis and membrane homeostasis, was disrupted to alter membrane dynamics in favor of protein export (Chen et al., 2023).

Optimization of culture conditions for efficient OVA secretion

Before evaluating the effects of the 14 genetic modifications, optimization of the preculture medium and fermentation temperature was performed to establish a baseline for OVA secretion. The +P1/K2 strain was precultured in either YSC as a minimal medium or YP20D as a rich medium, and subsequently inoculated into YP50D medium for the main fermentation. Comparison of the secretion profiles revealed that preculturing in YP20D medium resulted in a 32% increase in maximum DCW and a 63% increase in extracellular OVA concentration compared to YSC medium (Fig. S1A). Accordingly, the specific OVA secretion, defined as the amount of OVA secreted per unit biomass, was also 23% higher in cultures precultured in YP20D medium than in those precultured in YSC medium (Fig. S1B).

Previous studies have reported that culturing *S. cerevisiae* at temperatures lower than the optimal growth temperature of 30 °C can enhance the production of heterologous proteins (Huang and Shusta, 2005; So et al., 2023). To examine whether this observation applies to OVA secretion, the +P1/K2 strain precultured in YP20D medium was cultivated in YP50D at various temperatures (20, 25, and 30 °C). However, no improvement in OVA secretion was observed at the lower temperatures. This result suggests that lowering the cultivation temperature did not enhance OVA secretion, possibly because the reduced expression rate at lower temperatures did not further improve protein folding efficiency under the already optimized conditions of the +P1/K2 strain. Based on these results, all subsequent experiments were conducted using YP20D for preculture and a cultivation temperature of 30 °C (Fig. S2).

Optimization and functional evaluation of genetic factors affecting OVA secretion in *S. cerevisiae*

Among the 14 genetic modifications tested to enhance protein secretion in *S. cerevisiae*, knockout of either *GOS1* or *PAH1* led to a significant increase in OVA secretion (Fig. 2 and S3). Of these, *PAH1* knockout showed the most pronounced effect, resulting in a specific OVA secretion of 0.369 ± 0.007 mg/g cells and a total extracellular OVA concentration of 5.683 ± 0.496 mg/L, representing an improvement of 67% and 74%, respectively, compared to the +P1/K2 background strain. In contrast, *VPS5* knockout in the +P1/K2 background resulted in the intracellular accumulation of OVA with no detectable levels in the extracellular fraction, indicating a defect in secretion rather than protein expression (Fig. 3 and S4).

The enhanced secretion observed in the *PAH1* knockout strain is likely due to increased ER membrane biogenesis. As alluded to above, *PAH1* encodes a phosphatidate

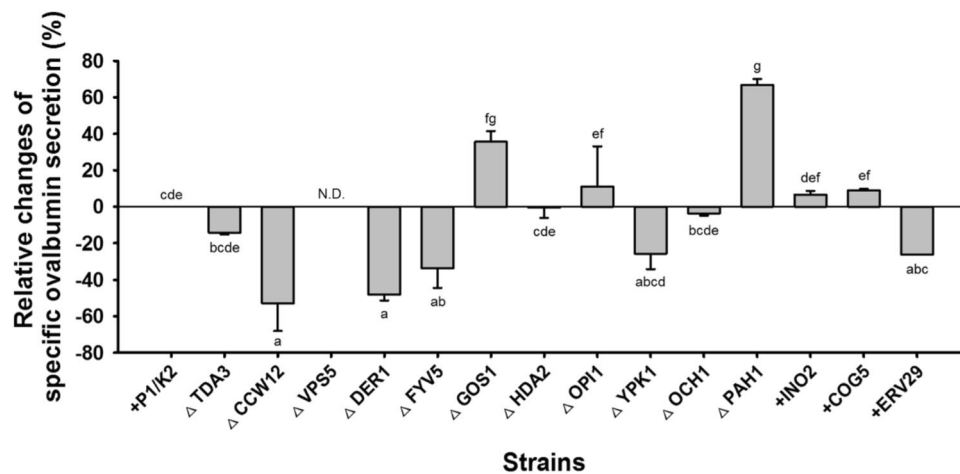


Fig. 2 Effects of various genetic modifications on specific ovalbumin secretion. The relative fold change in specific ovalbumin secretion was calculated for each sample strain, using the value obtained from the +P1/K2 strain as the reference. Results are the means of two experiments, and error bars indicate standard deviation. Different letters represent significantly different means (Tukey's HSD tests, $p < 0.05$). +P1/K2, the *Saccharomyces cerevisiae* D452-2 containing P_{PGK} -EPX1_{SP}-ovalbumin, tHXT7p-PDI1, and tHXT7p-KAR2; ΔTDA3, +P1/K2 with *TDA3* knockout; ΔCCW12, +P1/

K2 with *CCW12* knockout; ΔVPS5, +P1/K2 with *VPS5* knockout; ΔDER1, +P1/K2 with *DER1* knockout; ΔFYV5, +P1/K2 with *FYV5* knockout; ΔGOS1, +P1/K2 with *GOS1* knockout; ΔHDA2, +P1/K2 with *HDA2* knockout; ΔOPI1, +P1/K2 with *OPI1* knockout; ΔYPK1, +P1/K2 with *YPK1* knockout; ΔOCH1, +P1/K2 with *OCH1* knockout; ΔPAH1, +P1/K2 with *PAH1* knockout; +INO2, +P1/K2 containing tHXT7p-INO2; +COG5, +P1/K2 containing tHXT7p-COG5; +ERV29, +P1/K2 containing tHXT7p-ERV29; N.D., not detected

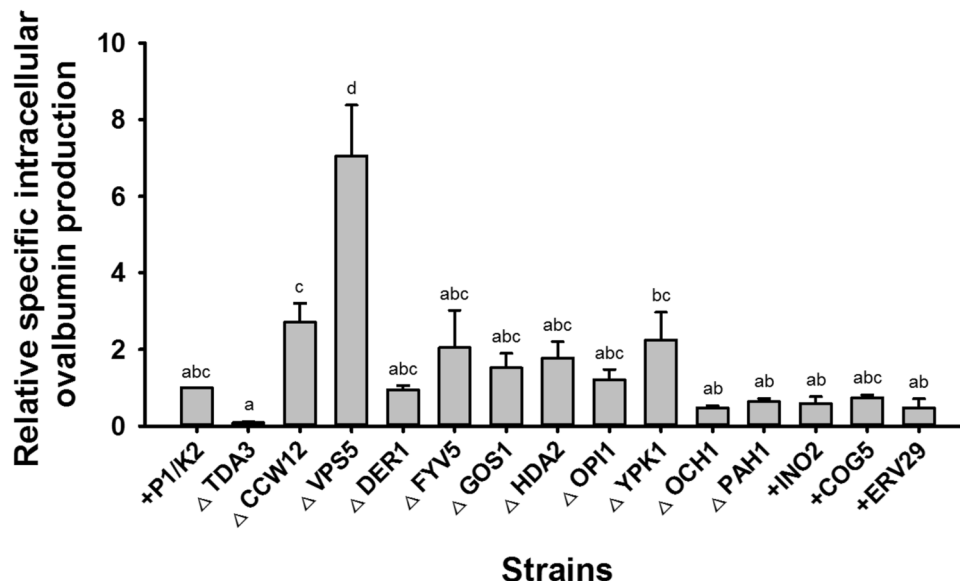


Fig. 3 Effects of various genetic modifications on specific intracellular ovalbumin production. The relative fold change in specific intracellular ovalbumin production was calculated for each sample strain, using the value obtained from the +P1/K2 strain as the reference. Results are the means of two experiments, and error bars indicate standard deviation. Different letters represent significantly different means (Tukey's HSD tests, $p < 0.05$). +P1/K2, the *Saccharomyces cerevisiae* D452-2 containing P_{PGK} -EPX1_{SP}-ovalbumin, tHXT7p-PDI1, and tHXT7p-KAR2; ΔTDA3, +P1/K2 with *TDA3*

knockout; ΔCCW12, +P1/K2 with *CCW12* knockout; ΔVPS5, +P1/K2 with *VPS5* knockout; ΔDER1, +P1/K2 with *DER1* knockout; ΔFYV5, +P1/K2 with *FYV5* knockout; ΔGOS1, +P1/K2 with *GOS1* knockout; ΔHDA2, +P1/K2 with *HDA2* knockout; ΔOPI1, +P1/K2 with *OPI1* knockout; ΔYPK1, +P1/K2 with *YPK1* knockout; ΔOCH1, +P1/K2 with *OCH1* knockout; ΔPAH1, +P1/K2 with *PAH1* knockout; +INO2, +P1/K2 containing tHXT7p-INO2; +COG5, +P1/K2 containing tHXT7p-COG5; +ERV29, +P1/K2 containing tHXT7p-ERV29; N.D., not detected

phosphatase that represses phospholipid biosynthesis and ER expansion (Han and Carman, 2017); its knockout is known to cause ER membrane proliferation, thereby increasing the folding capacity and trafficking surface area required for efficient secretion. Although *INO2* overexpression and *OPII* knockout also target the same phospholipid biosynthetic pathway, these modifications did not enhance OVA secretion in the +P1/K2 background. This discrepancy may be attributed to differences in regulatory control or the degree of ER membrane expansion achieved. Specifically, *PAH1* knockout directly disrupts lipid dephosphorylation and membrane remodeling, while transcriptional regulation in response to *INO2* overexpression and *OPII* deletion may be insufficient to overcome the metabolic and structural bottlenecks present in the already optimized +P1/K2 strain.

Interestingly, *GOS1* and *VPS5* are both involved in retrograde transport but exhibited opposite effects on OVA secretion. *GOS1* encodes a Golgi-localized SNARE protein involved in Golgi-to-ER retrograde transport (McNew et al., 1998). The increased OVA secretion observed upon *GOS1* knockout suggests that retention or accumulation of OVA in the ER negatively affects its secretion efficiency. This finding is consistent with a previous study demonstrating that co-expression of ER-resident chaperones, such as *PDI1* and *KAR2*, can alleviate ER stress and enhance OVA secretion (Jin et al., 2024). These results collectively imply that the ER may represent a bottleneck in OVA secretion, and reducing the retrograde trafficking of secretory cargo from the Golgi to the ER helps prevent its undesirable accumulation in the ER. In contrast, *VPS5* encodes a core component of the retromer complex responsible for endosome-to-Golgi retrieval (Nothwehr et al., 2000). Knockout of *VPS5* likely impairs the retrograde transport of secretory cargo or trafficking machinery from endosomes back to the Golgi, resulting in misrouting of OVA to the endosomal compartment. As the Golgi's processing capacity for OVA appears to be sufficient, diversion of OVA away from this compartment disrupts the secretion pathway. Consequently, OVA accumulates intracellularly and is not detected in the extracellular fraction in the *VPS5* knockout strain, indicating a severe defect in secretion efficiency.

In this study, the +P1/K2 strain, which was used as the background strain, harbors a single copy of the OVA expression cassette integrated into its genome. To investigate whether increasing the gene copy number would enhance OVA secretion, an additional OVA expression cassette was integrated into the genome of the +P1/K2 strain. However, the increase in copy number had no significant effect on OVA secretion levels (Fig. S5). This result suggests that in the +P1/K2 strain carrying only one expression cassette, OVA secretion is not limited by expression level, but rather by unresolved ER stress, which continues to act as a bottleneck. Accordingly, the improvement in OVA secretion

observed upon *GOS1* or *PAH1* knockout is likely due to their direct or indirect roles in alleviating ER stress.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10068-025-02044-1>.

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Declarations

Conflict of interests Sun-Ki Kim, who is the Managing Editor of *Food Science and Biotechnology*, was not involved in the editorial consideration of this manuscript. The authors declare no conflicts of interest.

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