



Engineering protein translocation pathway to improve recombinant proteins in *Pichia pastoris*



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ABSTRACT

Pichia pastoris is one of the most commonly used hosts for producing heterologous proteins, whereas production levels vary depending on the protein of interest and are also regulated by regulatory factors. We conducted RNA-seq by expressing the reporter EGFP and observed significant upregulation of certain subunits (Sec61p, Sbh1p, Sss1p, Sec66p and Sec72p) of the Sec complex in the high-expression recombinant GS115 stains. The overexpression of these genes may increase the expression levels of heterogeneous proteins. In this study, the endogenous promoters of the Sec complex subunits Sbh1p, Sss1p, Sec66p and Sec72p were isolated and verified their activity using the Lac-Z reporter gene. *Sss1*, *Sbh1*, *Sec66* and *Sec72* were overexpressed under the control of their own promoters in *Pichia pastoris*, respectively. The overexpression of *Sss1*, *Sbh1*, *Sec66* and *Sec72* in cells was confirmed by fluorescent microscope and Western blot analysis. The α-amylase was employed to evaluate the effect of overexpression of the Sec subunits on the heterologous protein expression. The results demonstrated that the α-amylase activity increased by 16%, 58%, 16% and 17% in the strains overexpressing *Sss1*, *Sbh1*, *Sec66* and *Sec72*, respectively. Engineering the protein translocation pathway can be an alternative to enhance heterogeneous proteins in *Pichia pastoris* expression system.

1. Introduction

Pichia pastoris (reclassified as *Komagataella phaffii*) is a methylo-trophic yeast widely used to produce heterologous proteins and bio-products via various genetic engineering or metabolic engineering (Duman-Ozdamar & Binay, 2021; Ergun et al., 2022; Yang & Zhang, 2018). *Pichia pastoris* has a secretory pathway like that of higher eukaryotes in having stacked Golgi cisternae, which ensures proper protein folding, processing and post-translational modification of secreted proteins (Bevis et al., 2002; Rossanese et al., 1999). The secretory pathway of *Pichia pastoris* also supports efficient secretion for heterologous protein production. The expression levels of certain proteins in *Pichia pastoris* can exceed 10 g/L in the large-scale industrial production (Clare et al., 1991; Hasslacher et al., 1997; Zhu et al., 2021). *Pichia pastoris* secretes only low levels of endogenous protein, which simplifies further down-stream procedures, based on these features, most of heterologous proteins produced in *Pichia pastoris* are generally secreted into the culture medium.

Despite these advantages, specific problems associated with the high-yield production and secretion of recombinant proteins or enzymes in *Pichia pastoris* remain a challenge. Significant progress has been made in enhancing recombinant protein expression through vector design, development of promoters and signal peptides, co-expression of chaperones, optimization of fermentation processes, and metabolic engineering (Tulek et al., 2021; Vogl et al., 2018; Yang & Zhang, 2018; Zhang et al., 2022; Zou et al., 2022). However, some proteins still fail to meet industrial application requirements in terms of expression levels. For secreted proteins, increasing the rate of entry and exit from the secretion pathway can effectively enhance protein yield.

For expressing secreted proteins, a signal peptide must be employed. The α-mating factor (MF) secretion signal from *Saccharomyces cerevisiae* is the most common and widely used signal sequence for heterologous protein secretion in *Pichia pastoris* (Cereghino & Cregg, 2000). Using α-MF as secretion signal, heterologous proteins were synthesized completely in the cytoplasm. Synthesized proteins were then recognized by heat shock protein 70 (Hsp70) and heat shock protein 40 (Hsp40) and

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pulled to the endoplasmic reticulum (ER) (Barrero et al., 2021; Ngosuwan et al., 2003; Tripathi et al., 2017). After reaching the surface of the ER, proteins were transferred to the interior of the endoplasmic reticulum. The process is known as post-translational translocation. Post-translational translocation of heterologous proteins into the ER were mediated by the Sec61 complex, consists of three subunits Sec61 α , Sbh1 β , and Sss1 γ in yeast (Sec61 α , Sec61 β , and Sec61 γ in mammals), and the Sec62/Sec63 complex, consists of four subunits Sec62p, Sec63p, Sec71(66)p and Sec72p (Harada et al., 2011; Jung et al., 2019; Junne et al., 2006; Zhang et al., 2013). Sec71 is identical to Sec66 (Fang & Green, 1994).

Sec61p is a key element in the protein transport mechanism and is an internally hydrophobic protein transport channel (Rapoport, 2007). It contains ten transmembrane helices (TM) in the shape of an hourglass, through which all peptides are transported to the ER (Itskanov et al., 2021). In the initial phase of post-translational translocation, after the N-terminal of the peptide enters the Sec61p channel, the N-terminal signal sequence is cleaved and separated by signal peptidase. In the non-translocating state, the translocation channel is blocked by a valve plug domain, which moves away when the protein starts translocating, thus opening the channel. The channel also contains a lateral gate that releases the peptide into the lipid phase and from which the signal sequence is released after cleavage (Li et al., 2016; Voorhees & Hegde, 2016). The opening of the lateral gate is required for the recognition of hydrophobic signal sequences of soluble secretory proteins and the integration of transmembrane proteins (Itskanov et al., 2021). Sss1p and Sbh1p are small in size, attach around Sec61p, and promote protein translocation. During protein translocation, lipid penetration inhibits peptide entry into the translocation channel and thus affects the efficiency of protein translocation, while Sec62p can form a barrier to prevent lipid penetration into the interior of the translocation channel (Itskanov et al., 2021). It has been shown that the silencing of Sec62p leads to a decrease in the efficiency of small secreted proteins entering the ER through post-translational translocation (Lang et al., 2012), and the detailed function of Sec62p in protein translocation is still not fully determined, but it has been found that polypeptides of less than 100 amino acids rely more on Sec62p for efficient translocation (Tyedmers et al., 2000). Sec63p consists of three transmembrane structural domains, including a J domain in the lumen of the ER (Bhadra et al., 2021), which mediates the interaction between translocated proteins and molecular chaperones in the endoplasmic reticulum. It has been demonstrated that the interaction of Sec63 with chaperone BiP can effectively facilitate the entry of some translocated proteins into the Sec61 channel (Matlack et al., 1999; Misselwitz et al., 1999), but not for all proteins, so the specificity of Sec63 in the transport mechanism remains to be investigated. Sec71 and Sec72 often appear as a complex, and the integration of Sec71 into the Sec complex requires the presence of Sec72, which in turn is essential for the stability of Sec72 *in vivo* (Feldheim & Schekman, 1994). In particular, the C-terminal tetratricopeptide repeat domain of Sec72 interacts with the molecular chaperones Ssa1 and Ssb1 in the cytoplasm to recruit post-translational translocated proteins and co-translational translocated proteins to the Sec71-Sec72 complex, respectively (Tripathi et al., 2017).

Several studies indicated that restriction of secretory pathway can lead to reduced production of target proteins in *Pichia pastoris* (Damasceno et al., 2007; Inan et al., 2006). As the first biogenesis step for secreted protein into the ER, the regulation of translocation should play a key role for extracellular secretion efficiency. Upregulated expression of *Sbh1*, *Sss1*, *Sec66* and *Sec72*, encoding the subunits of Sec61 and Sec62/63 complex, was observed by RNA-seq in *Pichia pastoris* strains overexpressing the reporter protein EGFP. In this study, Increasing the gene dosage of subunits of Sec61 and Sec62/Sec63 complex to affect the expression levels of the reporter α -amylase was evaluated.

2. Materials and methods

2.1. Strains and plasmids

Pichia pastoris GS115 was used as the expression strain, and all recombinant strains in this study were derived from this strain. *Escherichia coli* TOP10 was used as the host for plasmid construction. The expression vector pPICZA was used to express the reporter α -amylase. The pPIC9K was employed as a backbone to construct the vectors for expressing the subunits of Sec complex. These vectors were designed to have the subunits placed under the control of their individual promoters. This arrangement allowed for the regulated expression of the subunits in the Sec complex.

2.2. Cloning and activity analysis of promoters

The promoter regions of the *Sss1*, *Sbh1*, *Sec66* and *Sec72* were amplified by genomic PCR using specific primer pairs. The information of primer sequences was listed in supplementary file (Table S1). The *LacZ* was employed as the reporter gene to analyze promoter activity. The *LacZ* was amplified from plasmid pAd/CMV/V5-GW/LacZ by PCR and cloned into pPIC9K between *Sna*B I and *Eco*R I sites to construct pPIC9K-LacZ plasmid. The promoter regions of the *Sss1*, *Sbh1*, *Sec66* and *Sec72* were cloned into the pPIC9K-LacZ plasmid between *Sac* I and *Sna*B I sites to replace the *AOX1* promoter and α -mating factor signal sequence (Fig. S1). Activity analysis of promoters were performed as described previously (Zhang et al., 2022).

2.3. Construction of expression vectors

To construct the expression vectors of fusion proteins between Sss1p, Sbh1p, Sec66p or Sec72p and red fluorescent protein DsRed, the *DsRed* gene was amplified from plasmid pDsred-N1 by PCR specific gene primers and cloned into pPIC9K between *Sna*B I and *Eco*R I sites to construct pPIC9K-DsRed plasmid. The DNA sequences including the promoter regions and coding sequences without stop codon of the *Sss1*, *Sbh1*, *Sec66* and *Sec72* were amplified by genomic PCR using specific primer pairs (Table S2) and then cloned into the pPIC9K-DsRed plasmid between *Sac* I and *Sna*B I sites to replace the *AOX1* promoter and α -mating factor signal sequence (Fig. S2A).

To construct the overexpression vectors of *Sss1*, *Sbh1*, *Sec66* and *Sec72* under the control of their individual promoters, The DNA sequences including the promoter regions and coding sequences were amplified by genomic PCR using specific primer pairs (Table S3). Targeted sequences were cloned into pPIC9K between *Sac* I and *Eco*R I sites (Fig. S2B).

To construct the expression vectors of the reporter α -amylase, the coding sequence of *Aspergillus oryzae* α -amylase (GenBank ID: ACP34134) was synthesized by a gene company (Wuhan GeneCreate Biological Engineering Co., Ltd.) and cloned into pPICZ α A between *Eco*R I and *Not* I sites.

2.4. Expression of the reporter

A single colony of *Pichia pastoris* strains on YPD plates (1 % yeast extract, 2 % peptone, 2 % dextrose, and 1.5 % agar) was picked into a 50 ml flask containing 5 ml of BMGY medium (1 % yeast extract, 2 % peptone, 1 % YNB, 1 % glycerol, 0.4 % biotin, 100 mM potassium phosphate, pH 6.0) and incubated at 29 °C with shaking at 220 rpm for 24 h. The OD₆₀₀ value was measured at the end of incubation. The culture was transferred into a 50 ml centrifuge tube and centrifuged at 5000 × g for 5 min. The supernatant was discarded, and the pellet was diluted to OD₆₀₀ = 1 with BMMY medium (1 % yeast extract, 2 % peptone, 1 % YNB, 1 % methanol, 0.4 % biotin, 100 mM potassium phosphate, pH 6.0), and then 5 ml of the broth was transferred to a 50 ml flask and fermented continuously at 29 °C and 220 rpm for 48 h, with 1

% methanol added every 24 h. After the fermentation, the culture was centrifuged at 4 °C at 12000 × g for 5 min, and the supernatant was used to analyze the expression levels of the reporter α-amylase.

2.5. RNA-seq analysis

GS115 and the recombinant strains expressing reporter protein were incubated in BMGY to induce the expression of the reporter protein at 29°C and 220 rpm for 48 h. Three biological replicates of GS115 and the recombinant strains were performed for RNA-seq to ensure data reliability. The RNA was extracted using HiPure Yeast RNA Kit (Guangzhou Magen Biotechnology Co., Ltd.) through the operation manual. The cDNA generation, library amplification, and sequencing were provided by Igenebook, Wuhan, China (<https://www.igenebook.com>).

2.6. Real-time PCR

The transcription levels of genes were confirmed by real-time PCR. Total RNAs of samples were extracted according to HiPure Yeast RNA Kit (Guangzhou Magen Biotechnology Co., Ltd.). The cDNAs were synthesized by using All-in-One First-Strand Synthesis Master Mix Kit (baimengtech.com) with 1 µg of the total RNA as a template based on the manufacturer's operation manual. The real-time PCR was performed in the ABI StepOne Plus Real-Time PCR System (ABI, Germany) with three biological replicates. The Primers used in real-time PCR are shown in Table S4.

2.7. Detection of DsRed fusion protein expression

A single colony of strains expressing Sbh1p, Sss1p, Sec66p or Sec72p-DsRed fusion protein under the control of their respective promoters was picked and incubated in 5 ml of BMGY medium at 29 °C with 220 rpm for 36 h. The cells were then harvested by centrifugation at 5000 × g for 5 min at 4 °C, washed with phosphate buffered saline (PBS) and fixed in 4 % paraformaldehyde solution for 15 min. After fixation, the cells were further washed with PBS. The expression of Sbh1p, Sss1p, Sec66p or Sec72p-DsRed fusion protein was determined using a confocal microscope.

2.8. Analysis of α-amylase activity

The α-amylase activity was assayed following the method described (Trabelsi et al., 2019). In brief, 100 µl of fermentation broth, 0.5 ml of 1 % starch, and 0.4 ml of a 0.1 M acetate solution (pH 5.6) were mixed and incubated in a water bath at 60 °C for 30 min, the reducing sugar content was determined using 3,5-dinitrosalicylic acid method. One unit (U) of α-amylase was defined as the amount of enzyme that liberates 1 µmol glucose per minute under the specified assay conditions. The experiments were performed in triplicate, and the mean values were reported.

3. Results

3.1. Increase in transcript levels of Sss1, Sbh1, Sec66, and Sec72 in recombinant GS115 strains

Sec61 complex in *Pichia pastoris* is a heterotrimeric protein that consists of Sec61p, Sss1p and Sbh1p subunits. The Sec62/Sec63 complex is composed of Sec62p, Sec63p, Sec66p, Sec72p subunits (Table 1). Both the heterotrimeric Sec63 complex and the dimeric Sec62/Sec63 complex are located in the membrane of the ER and play a central role in the secretion of precursor polypeptides via alpha-mating factor secretion signal into the ER lumen. In order to investigate the involvement of subunits of Sec61 and Sec62/Sec63 complexes in regulating the secretion of heterologous proteins, we constructed recombinant strains expressing the reporter protein and performed RNA-seq analysis to identify differentially expressed mRNAs between GS115 with an empty

Table 1

The list of subunits of Sec complexes in *Pichia pastoris*.

Complex	Protein Name	Locus_tag	Length
Sec61 complex	Sec61p	PAS_chr1-3_0202	480aa
	Sss1p	PAS_chr1-1_0023	80aa
	Sbh1p	PAS_chr2-2_0210	82aa
Sec62/Sec63 complex	Sec62p	PAS_chr3_1014	274aa
	Sec63p	PAS_chr4_0395	663aa
	Sec66p	PAS_chr2-1_0433	206aa
	Sec72p	PAS_chr2-1_0448	193aa

vector and the recombinant strains. The results showed an increase in the mRNA expression levels of *Sss1*, *Sbh1*, *Sec66*, and *Sec72* in the recombinant strains compared to GS115 with an empty vector (Fig. 1A.). This increase was further confirmed by real-time PCR (Fig. 1B). These findings suggest a potential role for the subunits of the Sec61 and Sec62/Sec63 complexes in the regulation of heterologous protein secretion.”

3.2. Isolation and identification of active promoters of *Sss1*, *Sbh1*, *Sec66* and *Sec72*

Based on the RNA-seq data, we observed that the genes *Sss1*, *Sbh1*, *Sec66*, and *Sec72* responded to the expression of heterologous proteins by upregulating their expression, thereby facilitating the translocation of nascent polypeptides into the ER lumen. In *Pichia pastoris*, most of the developed promoters for heterologous protein expression are strong, constitutive, or inducible promoters that are not specifically regulated by the expression of heterologous proteins. To mimic the physiological states of yeast cells, we aimed to isolate active promoters for *Sss1*, *Sbh1*, *Sec66*, and *Sec72*. We then used the isolated active promoter to achieve overexpression of the respective genes in the recombinant strain. Through Lac Z reporter assays, we confirmed that the approximately 1000 bp upstream regions of *Sss1*, *Sec66*, and *Sec72*, and the approximately 1500 bp upstream region of *Sbh1* possessed promoter activities (Fig. 2). These isolated promoters were utilized to drive the expression of *Sss1p*, *Sbh1p*, *Sec66p*, and *Sec72p*. To visualize the expression driven by their own promoters, we fused the DsRed protein to the C-terminus of *Sss1p*, *Sbh1p*, *Sec66p*, and *Sec72p*, respectively. The results indicated successful expression of *Sss1p*, *Sbh1p*, *Sec66p*, and *Sec72p* using their respective promoters (Fig. 3).

3.3. Overexpression of *Sss1*, *Sbh1*, *Sec66* and *Sec72* improve the expression levels of the reporter

To assess the impact of *Sss1p*, *Sbh1p*, *Sec66p*, and *Sec72p* on the expression levels of a heterologous protein, the α-amylase was employed as a reporter. The α-amylase gene was transcribed under the regulation of the *AOX1* promoter. Each of the genes for *Sss1*, *Sbh1*, *Sec66*, and *Sec72* were separately expressed under their respective promoters. To achieve their integration into the GS115 genome, electroporation was employed, and successful overexpression of *Sss1*, *Sbh1*, *Sec66*, and *Sec72* was confirmed through real-time PCR analysis (Fig. 4). We then examined the effects of *Sss1p*, *Sbh1p*, *Sec66p*, and *Sec72p* overexpression on the expression of α-amylase. Our findings demonstrated a significant enhancement in α-amylase expression levels within the culture supernatant for all strains overexpressing *Sss1p*, *Sbh1p*, *Sec66p*, or *Sec72p*. The overexpression strains displayed a 1.2 ~ 1.6-fold increase in α-amylase expression compared to the control strain (Fig. 5).

4. Discussion

The α-mating factor secretion signal mediates the translocation of secretory proteins into the ER through a post-translational translocation mechanism (Rothblatt & Meyer, 1986; Rothblatt et al., 1987). Post-translational translocation allows proteins to cross the Sec61 channel and target the ER, similar to co-translational translocation. However, the

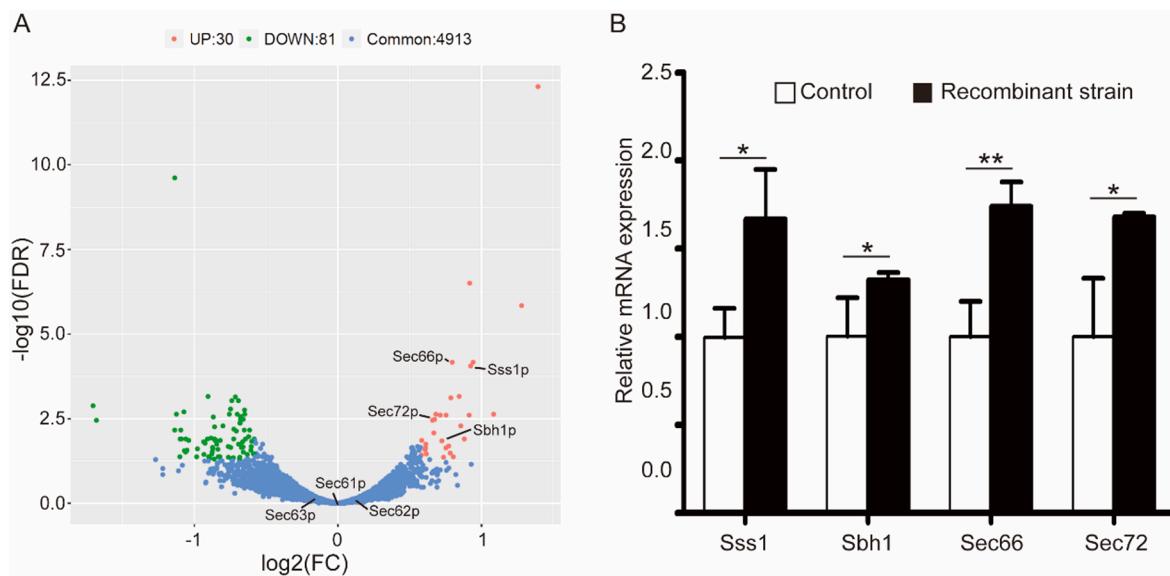


Fig. 1. Transcript levels of the subunits of Sec61 and Sec62/Sec63 complex. (A) The volcano plots of the RNA-seq analysis depict the expression changes in the recombinant strains expressing the reporter protein compared to the control, GS115 with an empty vector. (B) Real-time PCR was conducted to analyze the expression of the subunits of the Sec61 and Sec62/Sec63 complexes. Each experiment was conducted with three replicates. * $P < 0.05$, ** $P < 0.01$.

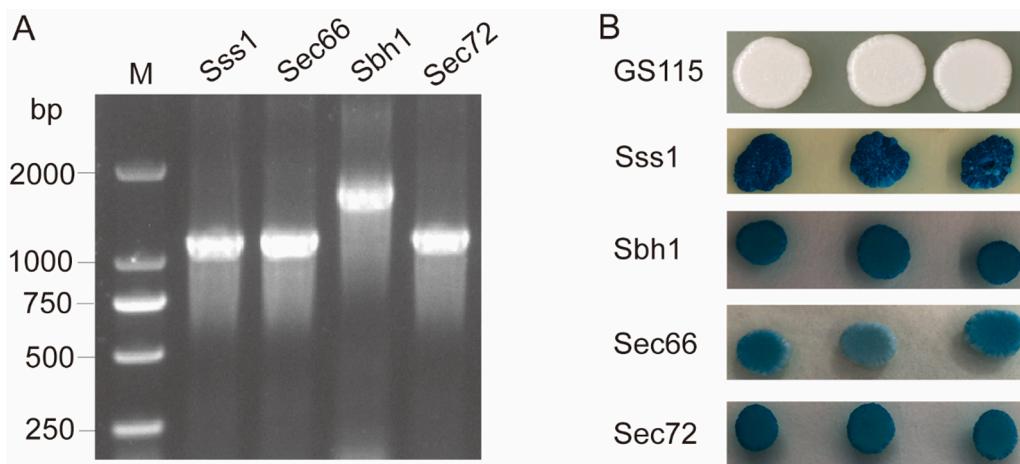


Fig. 2. Cloning and detection of promoter activity of Sss1, Sbh1, Sec66 and Sec72. (A) Promoter cloning of Sss1, Sbh1, Sec66, and Sec72 was performed, and the resulting cloned promoter regions were analyzed using agarose gel electrophoresis. (B) To assess promoter activity, *LacZ* reporter assays were conducted for Sss1, Sbh1, Sec66, and Sec72. The strains expressing *LacZ* under the control of the cloned promoter regions were grown on YPD plates supplemented with the X-Gal substrate. Three independent colonies of each strain were tested, and GS115 was used as a negative control.

key difference is that post-translational translocation not only involves the Sec61 complex but also requires the participation of the Sec62/Sec63 complex (Weng et al., 2021; Wu et al., 2019). Regardless of whether it is co-translational or post-translational translocation, secretory proteins enter the ER through the protein-conducting channel formed by the Sec61p protein.

In high-expression recombinant strains, cells synthesize a large amount of proteins destined for secretion. This necessitates the cellular machinery responsible for protein secretion to react accordingly, facilitating rapid entry of proteins into the ER and preventing the accumulation of heterologous proteins in the cytoplasm. Cells can increase the number of Sec61 channels or enhance the speed of protein translocation through the Sec61 channel to increase the quantity of secretory proteins entering the ER. RNA-seq results indicated that in the high-secreting recombinant strains, there is no enhancement in the transcription of *Sec61* compared to the control strain, suggesting that the number of Sec61 channels on the ER membrane remains relatively stable. Sbh1p

promotes ER translocation of substrates by a conformational change induced by N-terminal phosphorylation (Barbieri et al., 2023). Mutations in *Sec71(66)* and *Sec72* partially block translocation (Fang & Green, 1994). Sss1p regulates translocation efficiency by a direct interaction with a subunit of oligosaccharyl transferase (OST), Wbp1p. *In vitro* testing, Translocation of α -mating factor precursor into Sss1 - mutant microsomes was significantly slower, and maximal import was reduced by ~20 % compared with wild type (Schepet et al., 2003). These studies demonstrated that Sss1p, Sbh1p, Sec66p, and Sec72p play significant roles in regulating the efficiency of secretory protein translocation into the ER. These proteins exert their influence over the translocation process via a variety of mechanisms which collectively ensure the proficient passage of proteins through the Sec61 channel, which is the primary gateway into the ER for secretory proteins. In our RNA-seq analysis, significant upregulation of Sss1, Sbh1, Sec66, and Sec72 was observed in recombinant strains compared to the control strain (Fig. 1). This transcriptional response points to a cellular

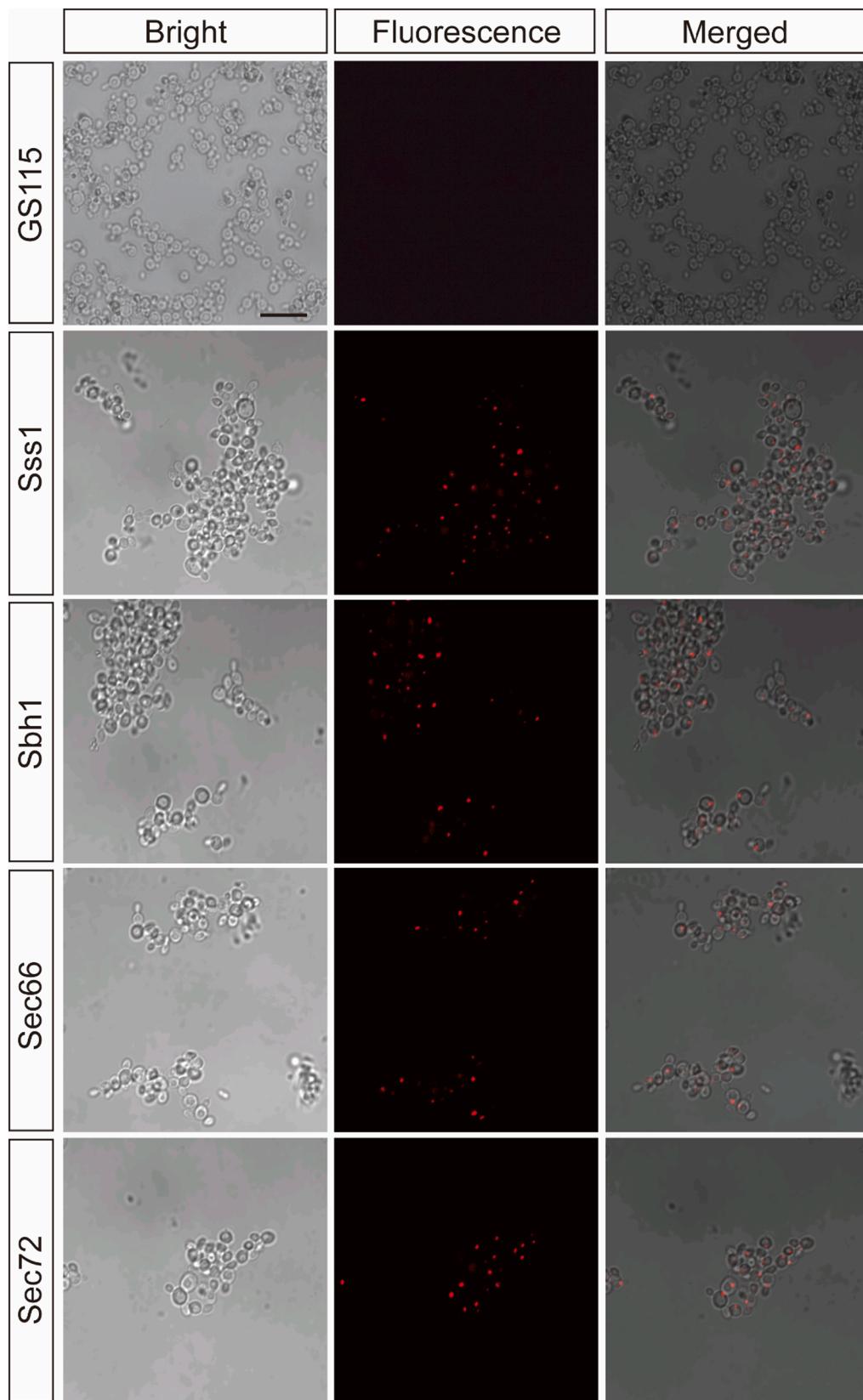


Fig. 3. Expression analysis of Sss1p, Sbh1p, Sec66p, and Sec72p. After 48 h cultivation in YPD medium, red fluorescence was observed in the strains expressing Sss1p, Sbh1p, Sec66p, Sec72p, and DsRED fusion proteins, as well as in the control strain GS115 integrated an empty vector, using fluorescence microscopy. Scale bars = 40 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

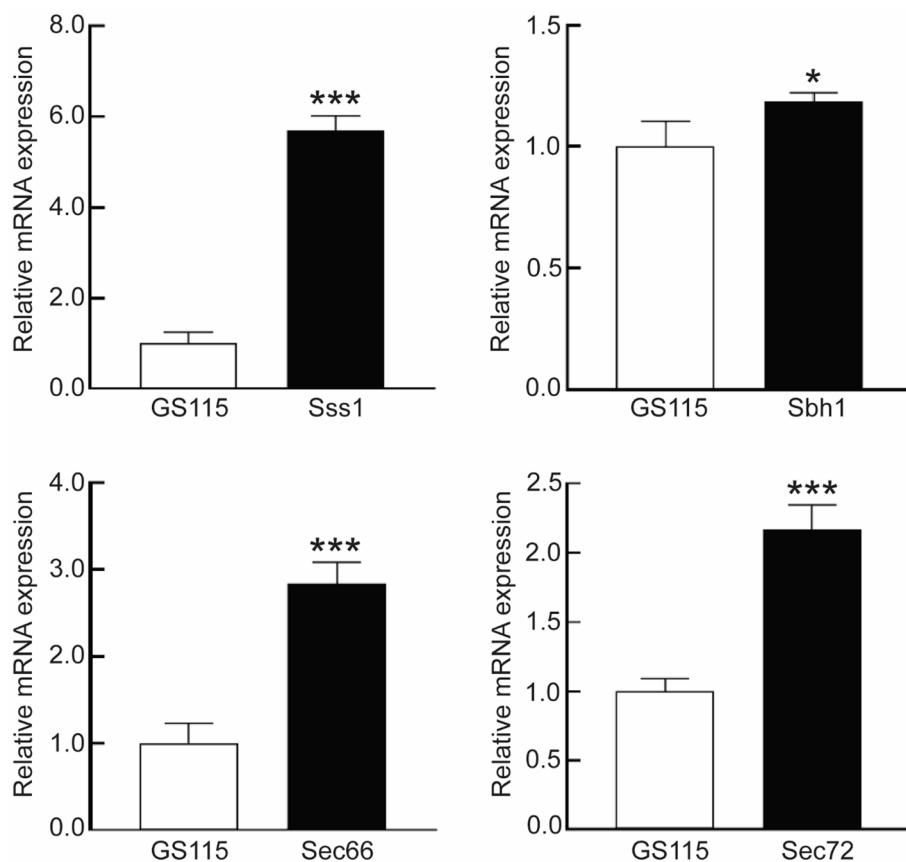


Fig. 4. Transcriptional analysis of *Sss1*, *Sbh1*, *Sec66* and *Sec72*. Total RNA was extracted from both the overexpression and control strains. Transcriptional levels of *Sss1*, *Sbh1*, *Sec66* and *Sec72* were assessed and analyzed through real-time PCR. Each experiment was conducted with three replicates. * $P < 0.05$, *** $P < 0.001$.

adaptation strategy wherein the upregulation of these key components is potentially correlated with an increased capacity for translocating secretory proteins into the ER. Such a mechanism may involve the enhanced performance of the Sec61 channel, perhaps through the stabilization of its structure or the facilitation of its protein-conducting functions, resulting in a higher throughput of proteins entering the ER lumen. Crucially, this adaptive response may be integral to optimizing protein production in recombinant strains, and further studies to dissect the exact contributions of each protein to the secretory capacity will be essential. Investigating the interplay among these proteins and the potential for their cooperative action could provide valuable strategies for enhancing protein production in biotechnological applications.

Sec61 and Sec62/63 complexes are integral components of the Sec complex, which is responsible for regulating the entry of newly synthesized proteins into the ER lumen. The secretion of heterologous proteins is a preferred method to avoid the formation of intracellular inclusion bodies and *Pichia pastoris* is often employed for this purpose. Proper folding of the secreted proteins is crucial when using *Pichia pastoris* as an expression system for heterologous proteins. However, the secretory pathway is not well-adapted to handle high levels of secretory protein, presenting challenges to efficient protein secretion.

One approach to enhance the secretion of heterologous proteins is to engineer the Sec complex in the host strain. Overexpression of the *Sbh1* gene from *Saccharomyces cerevisiae* or *Kluyveromyces lactis* has been shown to increase the production of secreted proteins in *Saccharomyces cerevisiae* (Toikkanen et al., 2004). Overexpression of *Sbh1p* in *Pichia pastoris* resulted in a significant increase in the secretion of the recombinant protein HyHEL-Fab (Marsalek et al., 2019). Overexpression of Sec63p in *Pichia pastoris* led to a 1.4-fold increase in the expression level of *Candida antarctica* lipase B (CalB) compared to the control strain (Samuel et al., 2013). Our study also demonstrated a 1.2 ~ 1.6-fold

increase in α -amylase expression in recombinant strains overexpressing *Sss1p*, *Sbh1p*, *Sec66p*, or *Sec72p*, compared to the control strain. The studies suggest that targeting the engineering of the Sec complex within the strain is a promising approach to improve the secretion of heterologous proteins.

The intricate coordination among the subunits of the Sec complex, including Sec61 and Sec62/63 complexes, is crucial for the efficient function of the Sec61 protein-conducting channel. It has been suggested that overexpression of individual subunits of the Sec complex can enhance the expression level of heterologous proteins to some extent. Future research may explore not only the overexpression of individual Sec components but also a more comprehensive amplification of the entire Sec complex to determine if this can lead to even higher yields of secretory protein production. Exploring this avenue could potentially address the current limitations in secretion capacity and pave the way for more efficient production of recombinant proteins in *Pichia pastoris*.

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Human and animal rights and informed consent

This paper does not contain any studies with human participants or animals performed by any of the authors.

CRedit authorship contribution statement

Shengyan Wang: Conceptualization, Writing – original draft. **Huijia Dai:** Resources, Methodology, Writing – original draft. **Qingling Tang:** Methodology, Writing – original draft. **Yujing Yu:** Data curation, Methodology, Writing – original draft. **Yaying Xie:** Data curation, Formal analysis. **Tao Wang:** Conceptualization, Data curation, Formal

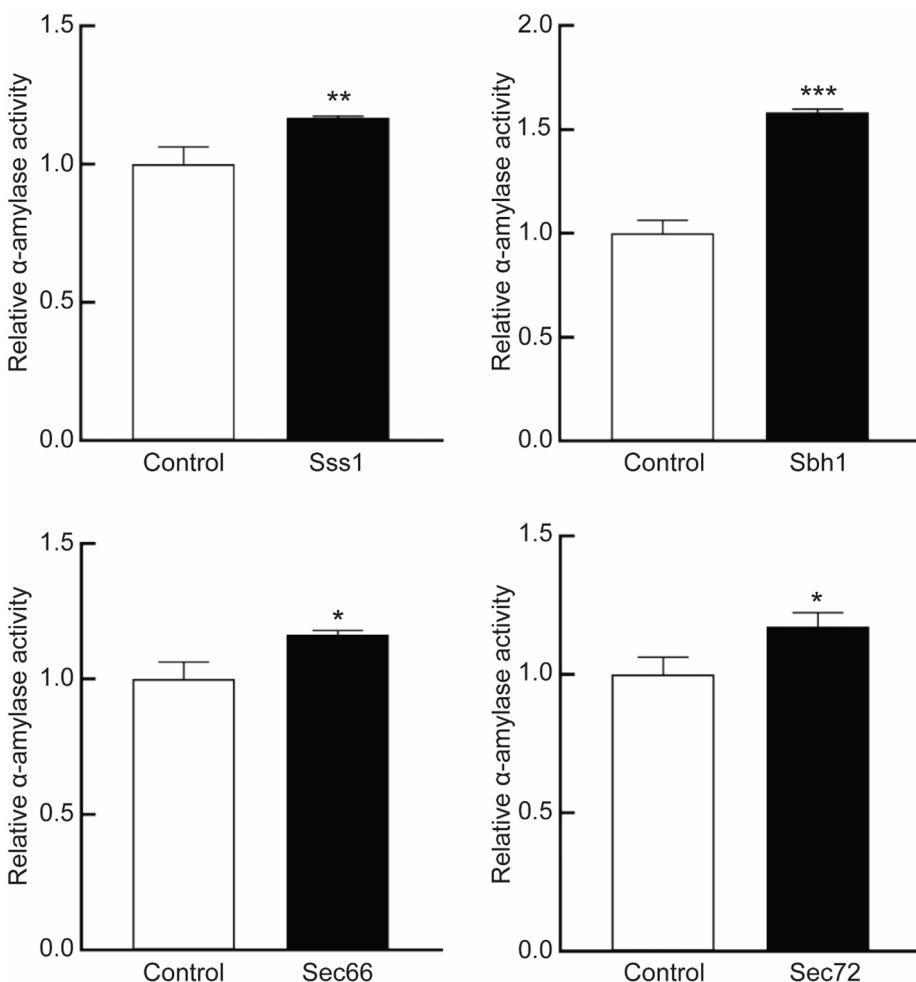


Fig. 5. Overexpression of *Sss1*, *Sbh1*, *Sec66* and *Sec72* on the effect of the α -amylase expression. After 48 h of methanol induction, the relative activity of α -amylase in the fermentation supernatant was compared between the overexpression strains and the control strain. Each experiment was conducted with three replicates. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.crbiot.2024.100182>.

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