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Secretory helpers for enhanced production of heterologous proteins in *Yarrowia lipolytica*

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ABSTRACT

Depending on the suboptimal factor, the target protein secretion can be over 1000-fold below the theoretical maximum. The bottlenecks may be alleviated by co-overexpression of "secretory helpers" (*SHs*). Here we proposed twelve *SHs,* functionally spanning the whole transcription-translation-translocation-folding-maturation-excretion pipeline. The genes were co-transformed with an easy-to-track reporter, and tested less than two temperatures. Our results indicated a clear distinction in the effects triggered by *SHs* involved in either synthesis or trafficking of the heterologous polypeptides. For superior operation of synthesis-related *SHs*, namely *RPL3, SSA5* and *SSA8*, the secretory pathway's capacity must be released by applying decreased temperature (25 °C). The other *SHs* considered (e.g. *SSO1, CWP11*) did not give such spectacular results in the amounts of the target heterologous polypeptide, but allowed to maintain secretory capacity under unfavorable thermal conditions. This study provides generalizable guidelines for cloning/culturing strategies aiming at enhancement of heterologous protein secretion in *Y. lipolytica*.

Abbreviations

(sp_)exYFP – (specific) extracellular fluorescence of YFP expressed in (FU $OD600nm^{-1}$) FU
(sp_)inYFP – (specific) intracellular fluorescence of YFP expressed in (FU $OD600nm^{-1}$) FU
SCYFP/SH(s) – genes encoding the target proteins: secretory YFP/secretory helper (s) (capitalized and italicized)
scYFP/SH (s) – proteins: secretory YFP/secretory helper (s) (non-capitalized and non-italicized)
FL – Fluorescence

1. Introduction

While yeast cells offer a multitude of benefits when used as heterologous protein production platforms (*i.a.* ease of genetic engineering and cultivation, eukaryotic post-translational modifications and secretion, large quantities of the product in grams per liter, if optimized), they are also subjected to numerous limitations. It has been estimated, that, depending on the suboptimal factor, the target protein secretion titers are 100- to 1000-fold lower than the theoretical maximum [1]. To account for this, plenty of inventive engineering strategies have been adopted to reach the desired product gain, including process and genetics optimization [2–9]. One of the possible approaches is to co-overexpress a gene involved in the process of protein synthesis, folding, maturation or trafficking, as it has been shown that the stresses imposed by heterologous protein synthesis and secretion may be diminished by overexpression of genes encoding products involved in the secretory pathway. The so-called secretory helpers (SHs), secretion enhancers or secretion helper factors have been adopted for assisting the process of heterologous proteins synthesis in several yeast species, including *Komagataella phaffii* (traditional name *Pichia pastoris* will be used hereafter) [10,11], *Saccharomyces cerevisiae* [12–16], *Kluyveromyces lactis* [17], or *Hansenula polymorpha* [18] (for comprehensive reviews on the subject see [2,3,8,15]. The biggest challenge in such approaches is to first accurately identify the bottleneck from amongst a multitude of possibilities found across the translational-secretory machinery, and then–to identify an operable and efficient *SH* that would alleviate the limitation.

When it comes to identification of the rate-limiting step within the transcription-translation-translocation-folding-maturation and secretion pipeline, what is already known for sure, is that it depends on both–the host cell and the biochemical characteristics of the overproduced polypeptide [12,19–22]. All these studies, clearly

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demonstrated that the host cell reacts differently to overproduction of biochemically-different polypeptides, and that different *SHs* are thus efficient in enhancing the target protein synthesis. Nevertheless, several *SHs* with high potential of generalizability have been discovered and described for *P. pastoris* and *S. cerevisiae*.

As previously demonstrated, transcriptomics, functional genomics and metabolic models are valuable and reliable tools for fishing out and selection of genetic engineering targets for enhancing synthesis of heterologous secretory proteins. Microarrays-based transcriptomics enabled identification of completely new, efficient *SHs* operating as chaperones, ATPase, or exocytosis-associated kinase [10]. Whole genome metabolic model with incorporated synthesis of heterologous protein was proved to be functional for prediction of targets (*i.a.* dehydrogenases, decarboxylases, transferases) for genetic engineering enhancing synthesis of heterologous proteins [11]. High-throughput genomic library screens allowed to identify unexpected secretion enhancers within the genes involved in cell wall biogenesis, but also expectedly–ribosomal elements and foldases [12]. Microfluidic screening combined with high-throughput whole-genome sequencing resulted in many insightful observations and allowed to identify new engineering targets within genes involved in genome maintenance, trafficking of polypeptides, respiration, stress response *etc*. [23]. In our previous study we conducted comparative profiling of global transcriptomes in chemostat-maintained *Yarrowia lipolytica* cells overexpressing several different heterologous proteins [24]. The following careful analysis of the expression profiles, revealed genes that were differentially regulated upon overexpression of specific, or any heterologous secretory protein. These genes were considered as natural targets for further modifications with the aim to improve secretory capacity of this yeast species, which has not been attempted, to date.

Therefore, in the present study we used literature premises and our own experimental indications for selection of twelve potential *SHs*, and co-cloned them in *Y. lipolytica* with an easy-to-track reporter. The strains were tested in batch cultivations for their secretory capacity, synthetized proteins retention, and the target gene expression. Cultivations were conducted under two temperatures, based on the previous literature data. The research yielded some interesting conclusions in the interplay between function of the co-overexpressed *SH* and the temperature. The most beneficial cloning-cultivation engineering strategy for enhancing the heterologous secretory protein production in *Y. lipolytica* was indicated.

2. Materials and methods

2.1. Strains, growth and culture conditions

All strains used in this study are listed in Tab.S1. *Y. lipolytica* and *E. coli* strains were maintained as described in [25,26]. *E. coli* JM109 strain, used for sub-cloning, and its derivatives were grown at 37 °C with 250 rpm shaking in LB medium ((g L^{-1}): yeast extract (BTL, Lodz, Poland), 5; bactopeptone (BTL), 10; NaCl (POCh, Gliwice, Poland), 5), supplemented with kanamycin (Sigma Aldrich, Merck KGaA, St. Louis, USA, 40 (μg mL^{-1})) and agar (Biomaxima, Lublin, Poland; 15 (g L^{-1})), when required. *Y. lipolytica* Po1f strain (*ura- leu-*, ATCC MYA2613) was used as a parental strain for co-transformations. *Y. lipolytica* Po1h strain (*ura-, leu+)* transformed with a URA3 marker cassette was used as a negative control strain for assessment of background fluorescence. Yeast strains were routinely maintained in a minimal yeast nitrogen base medium (YNB; (g L^{-1}): YNB (Sigma-Aldrich, Merck KGaA), 1.7; $(NH_4)_2SO_4$ (PoCh), 5; glucose (PoCh), 20), or in a rich yeast extract-peptone-dextrose medium (YPD; (g L^{-1}): yeast extract, 10; bactopeptone, 20; glucose, 20), solidified with agar (15 (g L^{-1})) when required, at 30 °C and with 250 rpm shaking, for liquid cultures.

2.2. Molecular biology techniques and reagents

Transformations of *E. coli* (heat-shock) and *Y. lipolytica* (lithium acetate heat-shock) strains were performed as described in [25,26]. Restriction digestion of DNA fragments was done using *Bam*HI, *Avr*II, *Not*I enzymes (New England Biolabs, Ipswich, USA; Thermo Fisher Scientific, Waltham, USA), as indicated. Ligation of DNA fragments into pCR Blunt II TOPO vector (Thermo Scientific, Waltham, USA) or JMP62 vectors was conducted using 200 U T4 DNA ligase (NEB) and T4 DNA ligase buffer (NEB). PCR was performed using Phire DNA polymerase (Thermo Fisher Scientific, Waltham, MA USA) or RUN DNA polymerase (A&A Biotechnology, Gdynia, Poland). DNA plasmids isolation, DNA fragment extraction from agarose gel, and purification of DNA fragments were all conducted using appropriate kits from A&A Biotechnology (Gdynia, Poland). All the reactions and protocols were used in accordance with the manufacturer's recommendations.

2.3. Cloning strategy

A DNA sequence encoding YFP (26 kDa) was transcriptionally fused with a signal sequence SP1, native for exo-1,3-beta-glucanase (*YALI0B03564g*) to assure its efficient secretion [27]. The *SH* genes were amplified directly on *Y. lipolytica* genomic DNA using Phire DNA polymerase with specific oligonucleotides listed in Tab.S2. *CNE1* gene DNA sequence was purchased from SYNGEN (Wrocław, Poland) as a completely synthetic DNA element, due to the presence of numerous *Bam*HI recognition sites, that were later required as unique for cloning. The oligonucleotides contained restriction sites *Bam*HI and *Avr*II, which enabled cloning in JMP62 plasmids [28]. JMP62-URA3ex was used as a vector for the model reporter protein (scYFP), and JMP62-LEU2ex was used as a vector for the *SH* genes Fig.S1. All the genes were cloned under the control of a constitutive pTEF promoter. The presence of expression cassettes in recombinant *E. coli* strains was verified by colony PCR. The expression cassettes were sequenced (Genomed, Warsaw, Poland) to verify correctness of the DNA constructions. Positive bacterial strains were deposited as glycerol stocks at–80 °C.

Prior to the transformation into *Y. lipolytica* Po1f strains, the JMP62-based constructions were digested with *Not*I endonuclease to remove bacterial elements. The JMP62-LEU2ex empty expression vector and the JMP62-URA3ex expression vector containing the *SCYFP* were co-transformed into *Y. lipolytica* Po1f strain, that was used as a reference for fluorescence evaluation and external calibrator for relative quantification of the expression level. Recombinant fluorescent strains were obtained by co-transformation with the vector JMP62-URA3ex-*SCYFP* and JMP62-LEU2ex-*SHs* into *Y. lipolytica* Po1f parental strain (Fig.S1). All transformants were selected on YNB medium at 30 °C for 48 h, and then, replica-plated on fresh YNB and YPD agar plates. The fluorescence phenotype (scYFP) was verified via fluorescence microscopy observations (ZEISS AxioVert, AxioCam 350 color; filterset: 09) and quantitatively assessed in microcultures using an automatic plate reader/fluorimeter (Tecan Infinite M200; Tecan Group Ltd., Männedorf, Switzerland). The presence of genes encoding *SHs* in the recombinant strains was verified by colony PCR using specific primers. Positive yeast strains were deposited as glycerol stocks at–80 °C.

2.4. Screening for representative sub-clones

Four sub-clones of each variant were selected from amongst all *Y. lipolytica* positive transformants and screened to select a representative strain for further studies. The screening was conducted in 50 mL shake flasks containing 5 mL YNB medium at 30 °C and 220 rpm shaking for 72 h. Samples were periodically withdrawn from the cultures and centrifuged. The biomass was washed twice in a sterile saline solution (0.85% NaCl), resuspended in equal volume of the same solution and appropriately diluted, to assure readout within the method linearity range. The supernatant and the washed biomass were subjected to

fluorimetric measurements. The biomass was also analyzed via spectrophotometric measurements for biomass growth determination. All the cultures were performed in biological duplicate.

2.5. Batch cultivations

Four milliliters of YPD medium were inoculated with a single colony of the representative strain (YPD agar plates) and grown at 30 °C with 250 rpm shaking. After 22 h, three milliliters (10% of working volume) of these pre-cultures were transferred into 30 mL of the YPD medium in 250 mL shake flasks and incubated at 30 °C for 24 h with 220 rpm shaking. For the cultures conducted at 25 °C, the temperature downshift was executed at 24 h time-point and continued until the end of culturing. All the cultures were conducted for 96 h in batch culture mode. Samples (1 mL) were periodically collected, centrifuged and analyzed as described in 2.4 and 2.6. All the cultures were conducted in at least two biological replicates.

2.6. Analytical methods

2.6.1. Optical density determination (OD600)

The growth of *Y. lipolytica* strains was monitored by measuring optical density at 600 nm (OD 600 nm). The yeast biomass was suspended and diluted in a sterile saline solution (0.85% NaCl) and measured in flat-bottomed MTP plates (Corning; Sigma-Aldrich) in Tecan Infinite M200 automatic plate reader in 200 µL. All the measurements were conducted in technical duplicate out of each biological replicate.

2.6.2. Fluorimetry (in / exYFP)

The fluorescence (FL) measurements of biomass and supernatant samples were performed in flat-bottomed MTP plates (Corning; Sigma-Aldrich) in Tecan Infinite M200 automatic plate reader at the wavelengths (excitation/emission) 495/527 nm. The extracellular FL was measured in 200 µl in supernatant samples. The intracellular FL was measured in 200 µl pre-washed culture pellet samples, resuspended and diluted in the saline solution (0.85% NaCl). Each fluorimetric measurement was normalized vs background fluorescence, either biomass of the Po1h_Ura3 negative control strain or fresh YNB/YPD media for inYFP and exYFP readouts, respectively. FL results were expressed as: i) inYFP–raw FL readout of the washed biomass in [FU], ii) sp_inYFP–-specific fluorescence of the washed biomass in [FU OD600nm^{-1}], iii) exYFP–raw FL readout of the supernatant in [FU], iv) sp_exYFP–specific fluorescence of the supernatant in [FU OD600 nm^{-1}]. All the measurements were conducted in technical duplicate out of each biological duplicate. Additionally, the fluorimetry results were randomly verified through observations under fluorescence microscope.

2.6.3. Gene expression analysis (RTqPCR)

Determination of the *SH*- and *YFP*-encoding genes expression level in the recombinant *Y. lipolytica* strains was conducted by RTqPCR. The samples were collected after 24 h of growth in batch cultures under 30 °C or 25 °C. Biomass from 1 mL of the culture was used for isolation of total RNA using Bead-Beat Total RNA Mini Kit (A&A Biotechnology). After qualitative (agarose gel electrophoresis) and quantitative (spectrophotometry) verification of total RNA preparations, the material was reverse transcribed to cDNA using SuperScript III Reverse Transcriptase and oligo(dT) primer, according to the manufacturer's instructions (Thermo Fisher Scientific). cDNA preparations were used as templates in RTqPCR, carried out in an Applied Biosystems 7500 device (Applied Biosystems, Foster City, USA). The reactions were set up using RT HSPCR Mix SYBR® B (A&A Biotechnology) according to the manufacturer's specifications. LoROX dye was used as a passive reference. Actin-encoding gene (*ACT1*) was used as the internal calibrator for normalization of the expression level. The gene-specific primer pairs are listed in Tab.S2. The following thermal profile was used: 95 °C 3 min, (95 °C 15 s, 60 °C 30 s, 72 °C 30 s) × 40, 72 °C 1 min, Melt Curve 94 °C 15 s, 60 °C 60 s, 95 °C 30 s, 60 °C 15 s. The target genes' expression was quantified using the $2^{-\Delta\Delta Ct}$ method [29]. cDNA preparations of the control strain (expressing *scYFP*, without *SH*'s overexpression) was used as the external calibrator, to which 1.0 expression level was assigned. All the samples were analyzed in technical duplicates.

2.6.4. Data analysis

For convenience of comparison, some data were additionally expressed as fold change (FC) values calculated by making a fraction of two specific values expressed in the same units. Such FC values prepared using sp_exYFP were fed into Morpheus (https://software.broadinstitute.org/morpheus/) online tool to draw illustrative heatmaps. Kinetic graphics representing growth and evolution of (sp_)in/exYFP in batch cultures were prepared using Microsoft Excel. Likewise, bar charts illustrating gene expression data were prepared using Excel graphic tools. Statistical analysis was performed with R (version 4.1.0 https://www.R-project.org/). Tukey HSD (p-value of 0.05) was performed using the agricolae package (https://cran.r-project.org/package=agricolae). The rstatix package was used to perform t-tests (https://CRAN.R-project.org/package=rstatix). Boxplots were prepared using ggplot2 (https://ggplot2.tidyverse.org) and ggpubr (https://CRAN.R-project.org/package=ggpubr) packages.

3. Results

3.1. Cloning of homologous secretory helpers in Y. lipolytica

A list of *SHs* studied here is given in Table 1. The selection was based on our previous indications inferred from comparative global transcriptome profiling in *Y. lipolytica* [24] and literature data demonstrating benefits of overexpression of a given *SH* in *S. cerevisiae, K. phaffii, H. polymorpha, K. lactis* or *Aspergillus oryzae*, e.g. [2,10,12]. Cloning strategy for co-transformation of the reporter protein (*scYFP*) and the *SHs* is shown in Fig.S1. For each *SCYFP-SH* combination, four *Y. lipolytica* sub-clones were initially screened in micro-cultures for biomass growth, as well as intra- and extra-cellular fluorescence, to select a single representative strain. As can be observed in Fig.S2.x.1 (where x is a letter assigned to cloned *SH*), the inter-clonal variation was reasonably low and overexpression of *SHs* did not impaired growth of the transformants, when compared to the control strain ($p < 0.05$). On the other hand, depending on the co-transformed *SH*, the strains exhibited variability in sp_inYFP/exYFP parameters Fig.S2.x.1 and S2.x.2. Based on that initial screening, we selected one sub-clone from each co-transformation type, that demonstrated the most similar growth curve to the reference strain, and was the most representative for the group of recombinants in terms of sp_inYFP/exYFP parameters (avoiding outliers). Enhanced expression of the *SH*-encoding genes in these selected double-transformants over the *YFP*-expressing control was confirmed by RTqPCR (not shown). Twelve representative strains were subjected to further, more detailed analyses.

3.2. Batch cultivations of Y. lipolytica strains with elevated dosage of SHs

3.2.1. Kinetics of growth, heterologous protein synthesis and secretion

The twelve representative *Y. lipolytica* strains, overexpressing both the secretory reporter (scYFP) and one of the *SHs*, were subjected to a series of batch cultivations (scaled up vs the preliminary screens) in parallel with the control strain (expressing solely *SCYFP*). The cultures were continued over 96 h under two thermal conditions–25 °C and 30 °C. Kinetics of biomass growth, as well as inYFP and exYFP evolution is presented in Fig. 1.x.3, 0.1.x.1 and 0.1.x.2, respectively. As inferred from the preliminary micro-culture screens, overexpression of *SHs* did not significantly affect the growth of either the recombinant and control strain ($p < 0.05$). In contrast, the decrease in the temperature by 5 °C (to 25 °C) significantly impacted biomass accumulation ($p < 0.05$), in favor of the lower (Fig. 1.x.3). The course of growth curves clearly indicates

Table 1
Secretory helpers analyzed in this study with short description of their function.

Y. lipolytica	Component	Cellular localization	Description
YALI0B12716g	*HAC1*	Nucleus	Transcription factor that regulates the unfolded protein response, via UPRE binding, and membrane biogenesis. Hac1p mediates activation of hundreds of molecular events, including increased provision of chaperones and membranes, to concertedly relieve burdened secretory pathway
YALI0C21560g	*RPL3*	Ribosome	Protein component of the large (60S) ribosomal subunit
YALI0F25289g *YALI0D22352g*	*SSA5* *SSA8*	Cytosolic	Cytoplasmic members of the HSP70 family; play a key role in folding, targeting and post-translational translocation. The protection of the released polypeptide in extended conformation (translocation competent state) is secured by the action of cytosolic chaperones.
YALI0E03036g	*PDI*	ER lumen	Protein disulfide isomerase essential for disulfide bond formation, which relies on stochastic oxidation–reduction of cysteine side chains; consumes considerable amounts of oxidating and reducing agents (O2 and GSH, respectively) during the folding of secretory proteins
YALI0E32703g	*SLS1*	ER lumen	Sls1, a nucleotide exchange factor for Kar2/BiP, has important functions in regulating ER stress and the interaction of Kar2/BiP and Ire1. Sls1 regulates this interaction, by stimulating the conversion of BiP from the ADP-bound to the ATP-bound state, which favors its interaction with Ire1. Sls1p acts in the protein translocation process, interacting directly with translocating polypeptides to facilitate their transfer and/or help their folding in the ER.
YALI0B13156g	*CNE1*	ER membrane	Calnexin involved in glycosylation and monitoring the folding state of the nascent glycosylated polypeptides. Key component of the quality control mechanism in the ER
YALI0E26026g	*YET3*	ER membrane / ER-Golgi transport	Yet3 involved in transport between the ER membrane and Golgi (homologous to the mammalian Bap31); its traffic is dependent on vesicular transport and is associated with new synthesized membrane polypeptide
YALI0D23947g	*USO1*	ER-Golgi transport	Essential protein involved in vesicle-mediated ER to Golgi transport; binds membranes and functions during vesicle docking to the Golgi; required for assembly of the ER-to-Golgi SNARE complex
YALI0E22044g	*SEC1*	Golgi-Plasma membrane	Sm-like protein involved in docking and fusion of exocytic vesicles; binds to assembled SNARE complexes at the membrane and stimulates membrane fusion. SEC1 is required for transport of polypeptides from Golgi to plasma membrane
YALI0E23243g	*SSO1*	Plasma membrane	Plasma membrane t-SNARE functioning at the targeting/ fusion of the Golgi-derived secretory vesicles to the plasma membrane
YALI0E22286g	*CWP11*	Cell wall	GPI-anchored cell wall protein. Essential protein involved in the cell wall remodeling during production of the secretory protein

that the differentiation in growth rate was initiated once the temperature downshift was applied. This general observation holds valid for all the twelve strains, irrespective of the *SH* cloned. Only at some specific time-points and for specific gene combinations, minor temporary differences in the biomass accumulation between the *SH* recombinants and the control strain could be observed (e.g. strain *SCYFP-SSO1* at 48 h, 25 °C; or *SCYFP-YET3* at 48 h, 30 °C; $p < 0.05$).

In contrast, overexpression of different *SHs* triggered significant and highly variable changes in inYFP and exYFP evolution over the culturing time (Fig. 1.x.1 and Fig. 1.x.2). Primarily, a clear impact of increased dosage of *RPL3, SSA5* and *SSA8* on enhanced synthesis and retention of YFP was observed, irrespectively of the adopted temperature (Fig. 1.B/C/D.1). For all these strains, the inYFP accumulation level reached the highest values from amongst all the strains (compare Y axis ranges in Fig. 1.x.1). Interestingly, in the late stationary phase of growth, particularly high inYFP accumulation was observed when the *RPL3*-, *SSA5*- and *SSA8*-overexpressing strains were cultured under 25 °C. Temperature-dependence was also observed in the case of *SSO1*-overexpressing strain, where significantly increased inYFP accumulation was recorded under 30 °C, while no difference in inYFP was found between the *SH*-overproducing strain and control cultured under 25 °C (Fig. 1.K.1). For the remaining cases studied here, no clear difference in the inYFP parameter were observed between the modified and the control strains, except for *YET3*-overexpressing strain, for which the *SH's* dose increase caused a decrease in intracellular YFP under both temperatures applied (Fig. 1.H.1).

When it comes to exYFP absolute values (Fig. 1.x.2; Table 2), we observed three spectacular improvements caused by overexpression of *RPL3, SSA5, SSA8* in *Y. lipolytica* strains cultured at 25 °C (Fig. 1.B/C/D.2). The effect of these genetic modifications was not that remarkable, when these strains were grown at 30 °C. Some minor improvements were also observed due to increased dosage of *SSO1* (Fig. 1.K.2), and to a lesser extent of–*HAC1, PDI1, SLS1, CNE1, USO1,* SEC*1, CWP11* (Table 2). In majority of those cases, combined action of the lowered temperature and the genetic modification was required for the enhancement in exYFP level ($p < 0.05$). SEC*1, YET3, SLS1, PDI1*, and *USO1* overexpression did not exert positive effect on exYFP levels when the strains were cultured under 30 °C. In fact, overexpression of *USO1* combined with cultivation at 30 °C contributed to lower exYFP levels ($p < 0.05$); such a tendency was also observed for *PDI1* and *SLS1*.

Careful analysis of the inYFP and exYFP evolution, as well as biomass growth curves (Fig. 1) showed that all the processes reached their plateau at ~ 48 h (biomass growth) to ~ 72 h (in/exYFP). At that stage, the effects of the temperature and the genetic modification could be observed macroscopically, by reading absorbance or fluorimetry. In addition, to evaluate the actual impact of the two given factors (*SH* overexpression and temperature) on the cell's secretory capacity and not

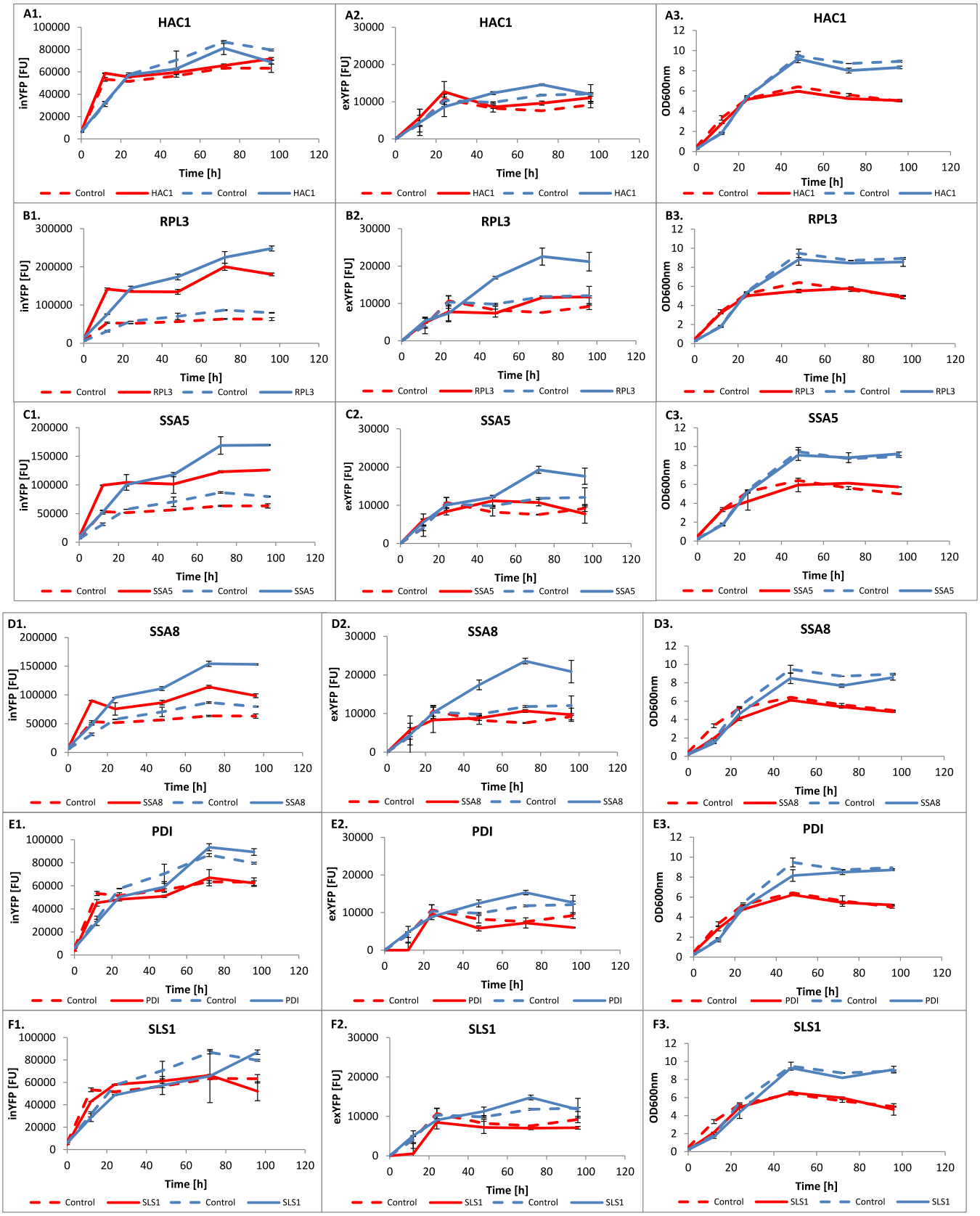

Fig. 1. Kinetics of inYFP (0.1 [FU]) exYFP (0.2 [FU]) and biomass growth (0.3 [OD600 nm]) in batch cultures of *Y. lipolytica* co-transformants (solid lines) and the reference strain (dashed lines) under 25 °C (blue lines) and 30 °C (red lines). X axis: culturing time [h]; Y axis: inYFP (0.1 [FU]), exYFP (0.2 [FU]) and biomass growth (0.3 [OD600 nm]). In the case of sub-figures 0.1, please mind Y axis range. Error bars indicate mean values ± SD from biological duplicate, each measured in technical duplicate.

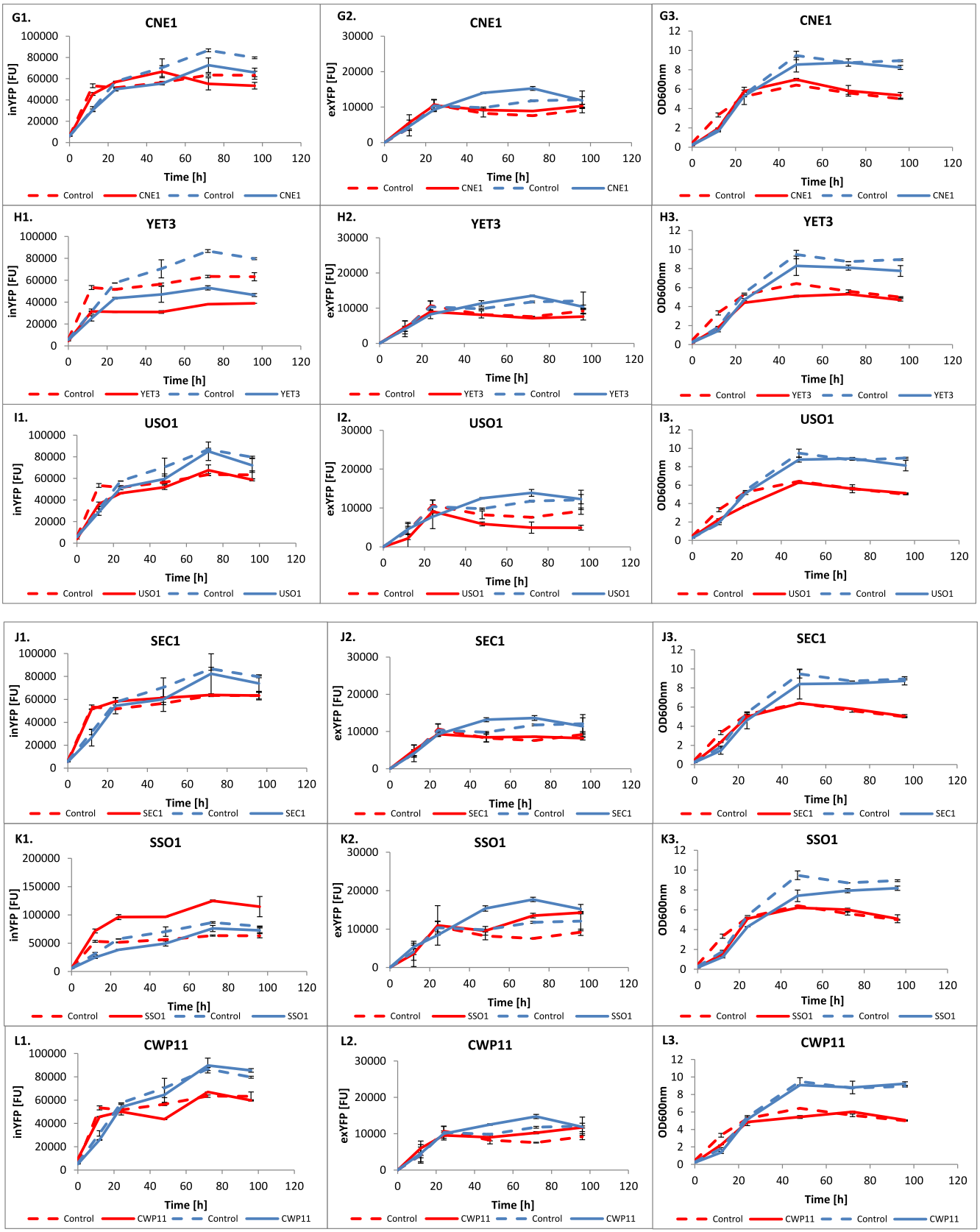

Fig. 1. (*continued*).

its growth, in our further analyses we use specific fluorescence, understood as specific secretion (sp_exYFP in [FU OD600 nm^{-1}]) parameter as the one enabling most accurate description of the studied biological process. Since the kinetics of growth was corresponding in all of the processes, we could compare different cultures using sp_exYFP [FU OD600 nm^{-1}] at a specified time point from the indicated plateau range.

Table 2
Numerical data for growth (OD 600 nm), extracellular fluorescence (exYFP [FU]) and extracellular fluorescence normalized per biomass accumulation (sp_exYFP [FU OD600nm^{-1}]) of *Y. lipolytica* co-transformants at stationary phase of growth (72 h) in batch cultures under 25 °C or 30 °C. Fold change values were calculated in reference to a control strain overexpressing solely *YFP*. Numbers are given in the indicated units ± SD from biological duplicate, each measured in technical duplicate.

Helper gene	72h									
	25 °C					30 °C				
	OD 600nm	exYFP [FU]	Fold change [exYFP]	sp_exYFP [FU OD600 nm^{-1}]	Fold change [sp_exYFP]	OD 600nm	exYFP [FU]	Fold change [exYFP]	sp_exYFP [FU OD600 nm^{-1}]	Fold change [sp_exYFP]
Control_scYFP	8.72 ± 0.00	11,780.50 ± 252.44	1.00	1352.53 ± 26.06	1.00	5.62 ± 0.15	7572.50 ± 103.94	1.00	1348.60 ± 16.81	1.00
scYFP_HAC1	8.02 ± 0.24	14,569.75 ± 264.81	1.24	1818.34 ± 20.21	1.34	5.24 ± 0.02	9610.50 ± 552.25	1.27	1834.90 ± 97.52	1.36
scYFP_RPL3	8.44 ± 0.04	22,542.50 ± 2281.83	1.91	2673.97 ± 284.69	1.98	5.79 ± 0.16	11,557.00 ± 141.42	1.43	1995.94 ± 80.20	1.48
scYFP_SSA5	8.83 ± 0.52	19,294.25 ± 930.20	1.64	2193.26 ± 237.35	1.62	6.13 ± 0.03	10,712.00 ± 869.74	1.42	1747.53 ± 132.04	1.30
scYFP_SSA8	7.69 ± 0.19	23,576.25 ± 720.19	2.00	3067.31 ± 20.51	2.27	5.38 ± 0.14	10,647.00 ± 379.01	1.41	1980.28 ± 123.52	1.47
scYFP_PDI	8.49 ± 0.24	15,305.25 ± 579.474	1.30	1804.33 ± 15.72	1.33	5.45 ± 0.53	7235.00 ± 1350.57	0.96	1322.81 ± 119.70	0.98
scYFP_SLS1	8.19 ± 0.04	14,784.50 ± 591.85	1.26	1804.26 ± 63.05	1.33	5.96 ± 0.10	7012.00 ± 397.39	0.93	1176.21 ± 46.17	0.87
scYFP_CNE1	8.75 ± 0.38	15,253.50 ± 551.54	1.30	1745.04 ± 13.90	1.29	5.84 ± 0.55	8891.00 ± 12.73	1.17	1530.60 ± 147.40	1.14
scYFP_YET3	8.09 ± 0.26	13,537.75 ± 172.18	1.15	1675.45 ± 32.31	1.24	5.31 ± 0.03	7154.50 ± 17.99	0.95	1346.05 ± 16.56	1.00
scYFP_USO1	8.89 ± 0.12	13,901.00 ± 869.03	1.18	1564.42 ± 118.57	1.16	5.61 ± 0.43	4949.50 ± 1441.79	0.65	875.56 ± 189.38	0.65
scYFP_SEC1	8.48 ± 0.01	13,626.75 ± 658.67	1.16	1607.63 ± 75.05	1.19	5.81 ± 0.03	8625.00 ± 90.51	1.14	1483.34 ± 8.89	1.10
scYFP_SSO1	7.92 ± 0.21	17,724.50 ± 560.74	1.51	2243.24 ± 128.82	1.66	6.00 ± 0.11	13,511.00 ± 593.97	1.78	2317.25 ± 239.95	1.67
scYFP_CWP11	8.80 ± 0.71	14,741.75 ± 590.79	1.25	1681.11 ± 69.87	1.24	6.02 ± 0.06	10,269.50 ± 301.94	1.36	1705.45 ± 34.12	1.27

3.2.2. *Effect of the SHs elevated dosage on secretory capacity*

Fig. 2 illustrates the results of Tukey HSD test classifying the double-transformants in terms of their normalized secretory capacity (sp_exYFP; [FU OD600 nm^{-1}]) under two thermal conditions at 72 h of the culturing. Irrespective of the culturing temperature, increased dose of *SSA8, SSA5, RPL3, SSO1* and *HAC1* turned the strains into most efficient secretors of the model protein (Fig. 2.A.B.; Fig. 3.A.B.; numerical data can be found in Table 2). Yet, the absolute numbers for sp_exYFP were in general higher for the strains grown under 25 °C (scYFP_SSA8 1.5-fold, scYFP_RPL3 1.34-fold, scYFP_SSA5 1.25-fold), with some minor exceptions. Under 30 °C, SEC*1, CNE1* and *CWP11* overexpression positively impacted sp_exYFP levels, but the level of improvement (10–27%) was not statistically significant ($p > 0.05$). *PDI1* and *YET3* overexpression had no observable effect on the normalized secretory capacity of the strain when cultured at 30 °C. On the other hand, *USO1* overexpression led to nearly 40% decrease in both exYFP and sp_exYFP measures when the strain was grown under 30 °C. It was also the only case, where the genetic modification triggered inferior outcome compared to the control strain ($p < 0.05$) and to majority of the strains bearing the other *SH*-overexpression (Fig. 3.A).

Culturing the modified strains under decreased temperature (25 °C) caused significant changes in the ranking of *SHs* (Fig. 2.B). In this case, any *SH* overexpression had some positive effect on the normalized secretory capacity of *Y. lipolytica* ($p < 0.05$; a tendency for *USO1* and SEC*1*; see also Fig. 3.B). Applying the lower temperature triggered a kind of polarization of the results, observed as a more clear distinction of the homogeneous groups, clearly defining the most beneficial genetic modifications (Fig. 2.B). Under these conditions, the unambiguous prevalence of the *SSA8*-overexpression strain over the control (~2.3-fold improvement in sp_exYFP; Table 2), and all the remaining strains, could be seen (Fig. 3.B). The leader strain was directly followed by the "*RPL3* overexpressor" (~100% improvement vs the control; Table 2), making a homogeneous group of its own. The *SSO1* and *SSA5*-overexpressing strains were categorized together (Fig. 2.B), demonstrating significant superiority over the control strain (~60% improvement; Table 2) and the remaining eight *SH*-overexpressing strains ($p < 0.05$; Fig. 2.B and Fig. 3.B). Further specific details on the fold change in the sp_exYFP measures by all the strains at two time points (48 h and 72 h) and the two temperatures (25 °C and 30 °C) are given in Fig.S3.

3.2.3. *Effect of the temperature on SHs action in Y. lipolytica*

The results presented in Fig. 1.B.2, C.1, C.2, D.1, D.2 K.1 indicate a clear modulating effect of the temperature on the YFP accumulation and/or secretion. This indication led us to the hypothesis that maybe the action of the specific SHs is somehow temperature-dependent. To test this, we again selected a specific time point (72 h) and sp_exYFP parameter, describing synthesis and secretion potential without growth-related phenomena, and run *t*-test statistical comparison. Results of this direct comparison are shown in Fig. 4. Indeed, for half of the SH studied here, the decreased temperature had a significant positive impact in terms of the resultant secretory potential ($p < 0.05$). Notably, it was actually the interaction between *SH* overexpression and the temperature that contributed to elevated sp_exYFP, as such effect could not be seen for the control strains ($p = 0.931$) and several other *SH*-overexpressing strains cultured under 25 °C. That beneficial interaction could be seen for all the cytoplasmic helpers–RPL3, SSA5, SSA8, but also ER-resident SLS1, and two SHs operating within vesicular transportation–YET3 and SEC1. Some insignificant tendency of this beneficial interaction occurrence was also observed for PDI1 and USO1 ($p < 0.1$), which was significant at the earlier time point for USO1 (Fig.S4).

3.2.4. *Expression level of the heterologous gene upon SHs overexpression*

To investigate whether the macroscopically observed changes in synthesis and secretion of YFP were underlain by the *YFP* gene expression level, we conducted comparative gene expression analysis for all the representative strains cultured under 25 °C and 30 °C (Fig. 5.A.B; Fig. 6). Biological sense of this experiment was to see whether the elevated dose of the *SHs* impacted transcription of the *YFP*, as previous

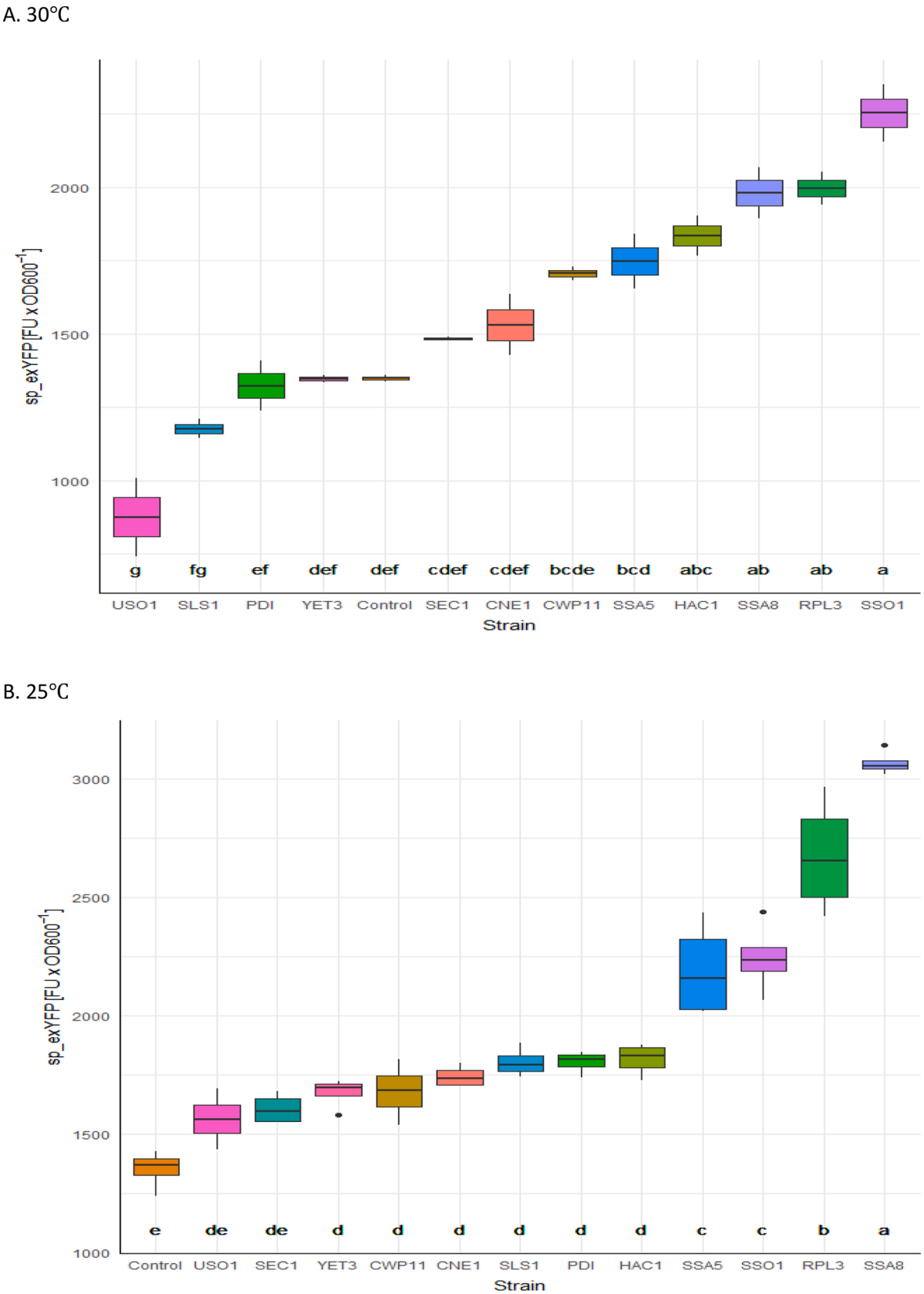

Fig. 2. Specific secretion (fluorescence) of the heterologous reporter protein (scYFP) by *Y. lipolytica* co-transformants and the control strain in stationary phase of growth (72 h) in batch cultures under 30 °C (A) and 25 °C (B). X axis: co-overexpressed *SH*; letters indicate homogenous groups calculated by Tukey HSD test. Y axis: specific secretion (sp_exYFP) of YFP in [FU OD600 nm^{-1}]. The boxes cover the 50% of input data points and a median indicated as solid line.

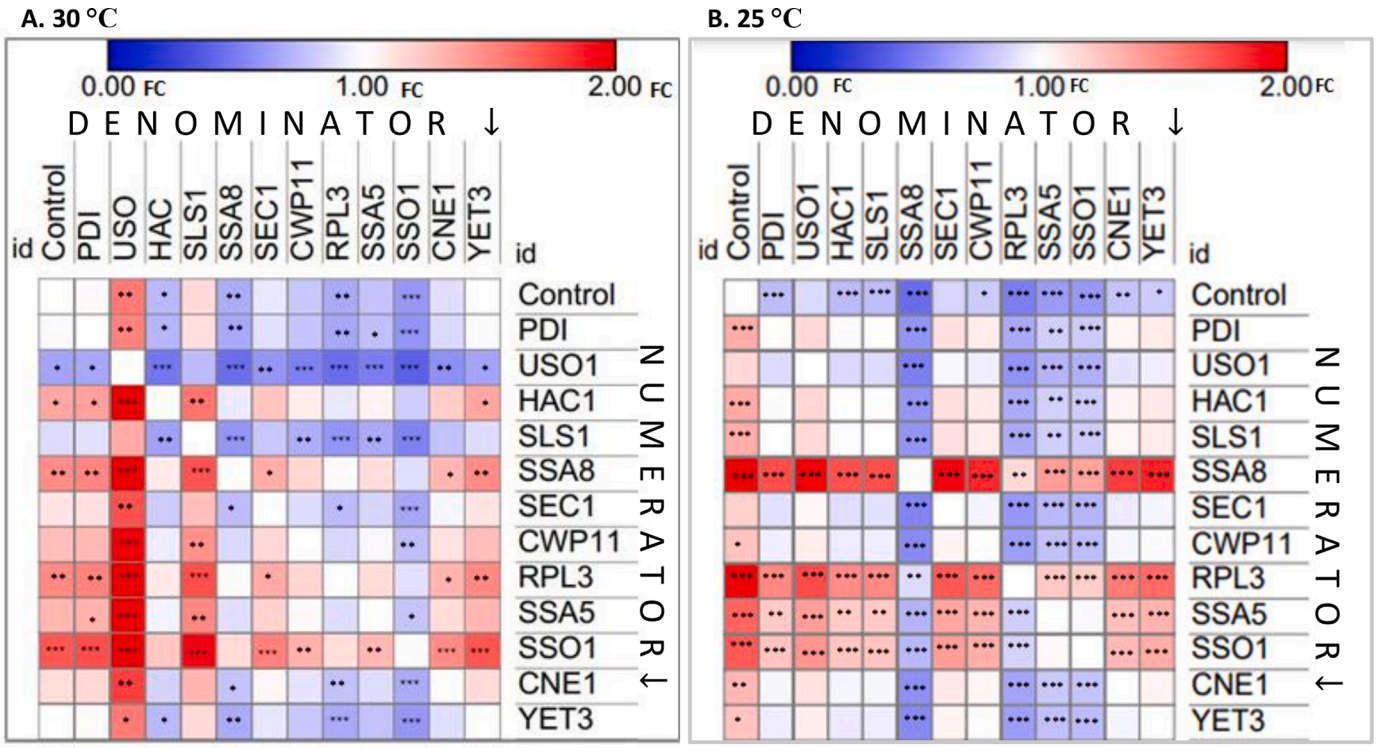

Fig. 3. Multiple comparisons of *Y. lipolytica* co-transformants and the control strain presented as fold change of specific secretion measures (sp_exYFP; $\frac{NUMERATOR\_FU\_OD600nm-1}{DENOMINATOR\_FU\_OD600nm-1}$) between the strains, cultured under 30 °C (A) and 25 °C (B). To use the heatmap correctly, first identify the strain on the left and use it as a NUMERATOR in fold change calculation, than, select a strain from the top of the heatmap, to be used as DENOMINATOR in the fold change calculation. Red squares represent prevalence of the numerator in the adopted measure (1.01–2.00 FC) and blue squares represent prevalence of the denominator in the adopted measure (0 - 0.99 FC). Statistical significance of the comparisons was determined by a Tukey HSD test. *** $P \leq 0.001$ ** $P \leq 0.01$ * $P \leq 0.05$.

research demonstrated that, in specific cases, transcription of heterologous genes can be reversibly modulated by the downstream processes like translation and folding events [24]. If no specific mechanism of reverse regulation occurs, the rate of transcription initiation events should be decreased under decreased temperature, according to the classic Arrhenius model. While in majority of cases the common model held valid, for several specific cases we observed a slight tendency for increased *YFP* expression level at elevated temperature (not significant at $p < 0.05$; Fig. 6). Primarily, the increased dosage of *RPL3* had a tremendous effect on the gene of interest overexpression ($p < 0.05$), which was, in addition, doubled when the strain was grown at 25 °C (significant over the control at both temperatures, $p < 0.05$; Fig. 5.A.B; but not significant between the temperatures, $p = 0.166$; Fig. 6). Likewise, doubling the *YFP* RQ value under 25 °C was observed for the strains overexpressing *YET3* and SEC*1* (significant over the control at $p < 0.05$; Fig. 5.B., but not significant between the temperatures, p~0.15; Fig. 6), but those genes overexpression under 30 °C had no significant effect on RQ (not significant over the control at $p < 0.05$; Fig. 5.A). The other genes with the highest impact on inYFP/exYFP synthesis (as illustrated in Fig. 1, Fig. 2, Fig. 3, Fig. 4), *SSA5* and *SSA8*, contributed to significant upregulation of the *YFP* gene under both temperatures (Fig. 5.A.B). On the other hand, that was not the case for the other "efficient *SH*", namely *SSO1*, as its overexpression had no impact on *YFP* gene transcription (Fig. 5.A.B). Intriguingly, overexpression of *HAC1, SLS1, PDI1* triggered a significant increase in the heterologous gene expression when the strains were grown under 30 °C (Fig. 5.B; a tendency observed for *CNE1*, but $p > 0.05$). In fact, all these genes exerted their impact on *YFP* gene expression level in a temperature-dependent manner (Fig. 6). Such a mode and direction of the temperature-dependent action was also observed for a one more gene, namely *USO1* ($p < 0.05$). An opposite trend was observed for the strains co-overexpressing *RPL3*, SEC*1, YET3*, for which coordinately higher expression (not significant) and secretion (significant at $p < 0.05$) were seen under 25 °C.

To get a global insight into the relationship between: i) the temperature, ii) the heterologous gene expression level and iii) synthesis of scYFP polypeptide, we conducted Pearson correlation analysis between total amount of produced YFP (sum of FU for inYFP+exYFP) and RQ values for *YFP*-encoding gene. Strikingly, we noted, that such correlation was also temperature-dependent, as the r factor for the values read in 30 °C-maintained strains was 0.397, while for those cultured under 25 °C – $r = 0.855$, showing high linearity between transcription and translation under the lowered temperature.

Finally, to get an insight into the relationship between Hac1p transcription factor and its known downstream "interaction partners", we analyzed gene expression level of *HAC1, PDI1, KAR2*, as well as sc*YFP,* in the *HAC1*-overexpressing strain (Fig. 7). It was expected that upon elevated provision of *HAC1* transcript, its downstream targets will be expressed at higher rates as well. According to a known model, an Ire1p-spliced *HAC1* mRNA is translated into a functional transcription factor and binds to the UPRE motives in the promoter regions of UPR–regulated genes, activating expression of a multitude of ER-resident chaperones and foldases (e.g. *KAR2, PDI1*) [30,31]. From the obtained data, we inferred that the increased dose of native *HAC1* transcript had no effect on its typical regulon elements, as neither *KAR2* nor *PDI1* expression was enhanced. Elevated *HAC1* transcript level triggered enhanced expression of *YFP*, but only under 30 °C, according to its already discussed temperature-dependent expression pattern.

4. Discussion

In the present study, the choice of the *SHs* (the engineering targets) was based on our previous transcriptomics study conducted with *Y. lipolytica* [24], as well as the literature data [2,10,12]. The targets

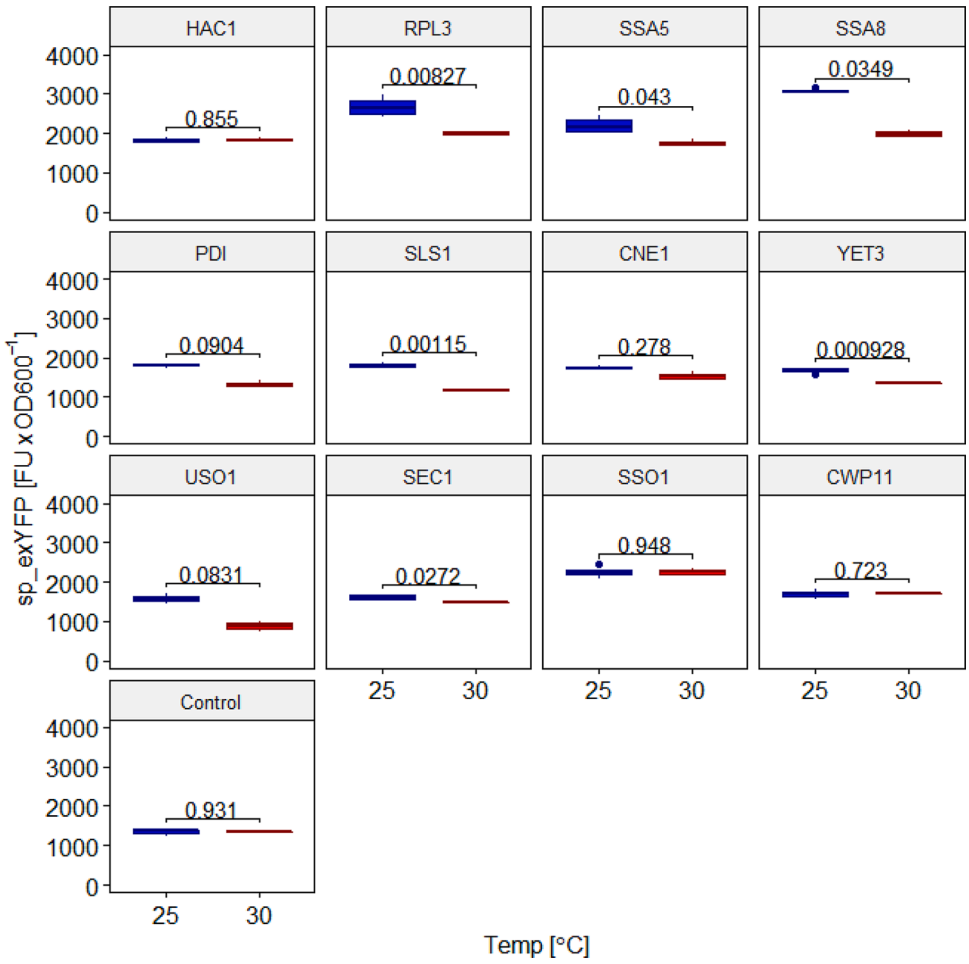

Fig. 4. Direct comparison of specific secretion values [FU OD600 nm^{-1}] reached at 72 h with an indicated co-transformants (top of each panel) under two cultivation temperatures 25 °C (blue box) and 30 °C (red box). Numbers indicate p value of t-Student's test. X axis: cultivation temperature 25–25 °C, 30–30 °C. Y axis: specific secretion (sp_exYFP) of YFP in [FU OD600 nm^{-1}]. The boxes cover the 50% of input data points and a median indicated as solid line.

were previously identified as differently regulated genes (DEGs; either up- or downregulated) in response to overexpression of one of three different heterologous reporter proteins targeted for secretion – a small fluorescent protein (scYFP), an alpha-amylase rich in cysteines (SoA), and a glucoamylase rich in glycosylation sites (TlG), as well as one intracellular reporter (inYFP). Hence, according to a rationale and strategy adopted previously by Gasser et al. (2007), different polypeptides were used across the study (from identification of the targets to studying of the effects of their overexpression), which precludes consideration of protein-specific effects, and leads to generalizable conclusions. The set of *SHs* studied here (Table 1) covers those operating throughout a transcription-translation-folding-maturation-secretion pipeline, including a major transcription factor governing UPR activation (HAC1), a ribosome element (RPL3), cytosolic chaperones (SSA5 and SSA8), ER-residents involved in folding and stress signaling (PDI1, SLS1, CNE1), as well as components of proximal (YET3, USO1) and distal (SEC1, SSO1, CWP11) vesicular transportation of the cargo protein outside the cell. As shown by the transcriptomics analysis [24], the genes included in the set, responded differently to the imposed metabolic burden, e.g. *CWP11* was highly upregulated when SoA and scYFP reporters were intensively synthesized and secreted, which also applies to *SSAs* and *USO1*; on the other hand, SEC*1* and *SSO1* were significantly downregulated under these conditions. Yet, in the present study, we attempted solely overexpression (and not deletion) of the selected *SHs*, irrespective of the previously observed regulation direction. The approach was based on former literature reports on beneficial outcomes of the *SHs* overexpression in different fungal production platforms (specific references are given hereafter).

In addition, we decided to implement two different temperatures for testing the *SHs* operation. The rationale driving this approach were the findings by [32] and [12] on significant temperature-dependency of multiple secretory enhancers co-overexpressed in *P. pastoris* and *S. cerevisiae*, respectively, studied in a range from 20 °C to 37 °C. In the former study, 40% improvement in the target protein production was achieved by the reduction of the temperature from 25 °C to 20 °C. On the other hand, the latter research demonstrated that, for example, *ERO1* was the only *SH* from amongst tested, that exhibited beneficial impact on synthesis and secretion of a specific reporter at 20 °C. In contrast, most significant effects of several other *SHs* overexpression (including cell wall genes *CCW12/CWP2* and ribosomal *RPP0*) towards secretion of specific reporter proteins, were elicited under elevated temperatures 30–37 °C [12]. That study indirectly indicated that under regular/-elevated temperatures, the secretory pathway is burdened due to excessive transcripts provision, and hence, only in those conditions the effect of several *SHs* overexpression was apparent. Still, that previous research founded a solid rationale behind testing the double transformants constructed here under different temperatures. In the present study, we adopted the induction temperature of 25 °C, as used previously for testing different *SHs* in *P. pastoris* [10]. In addition, since the former transcriptomics study, that provided data for the *SHs* selection [24], was conducted under 30 °C, which is a typical temperature for *Y. lipolytica* cultivations, that thermal condition was used as a reference.

Kinetic studies (Fig. 1.x.3) showed that while overexpression of *SHs* did not have any impact on growth rate of the recombinant strains, the

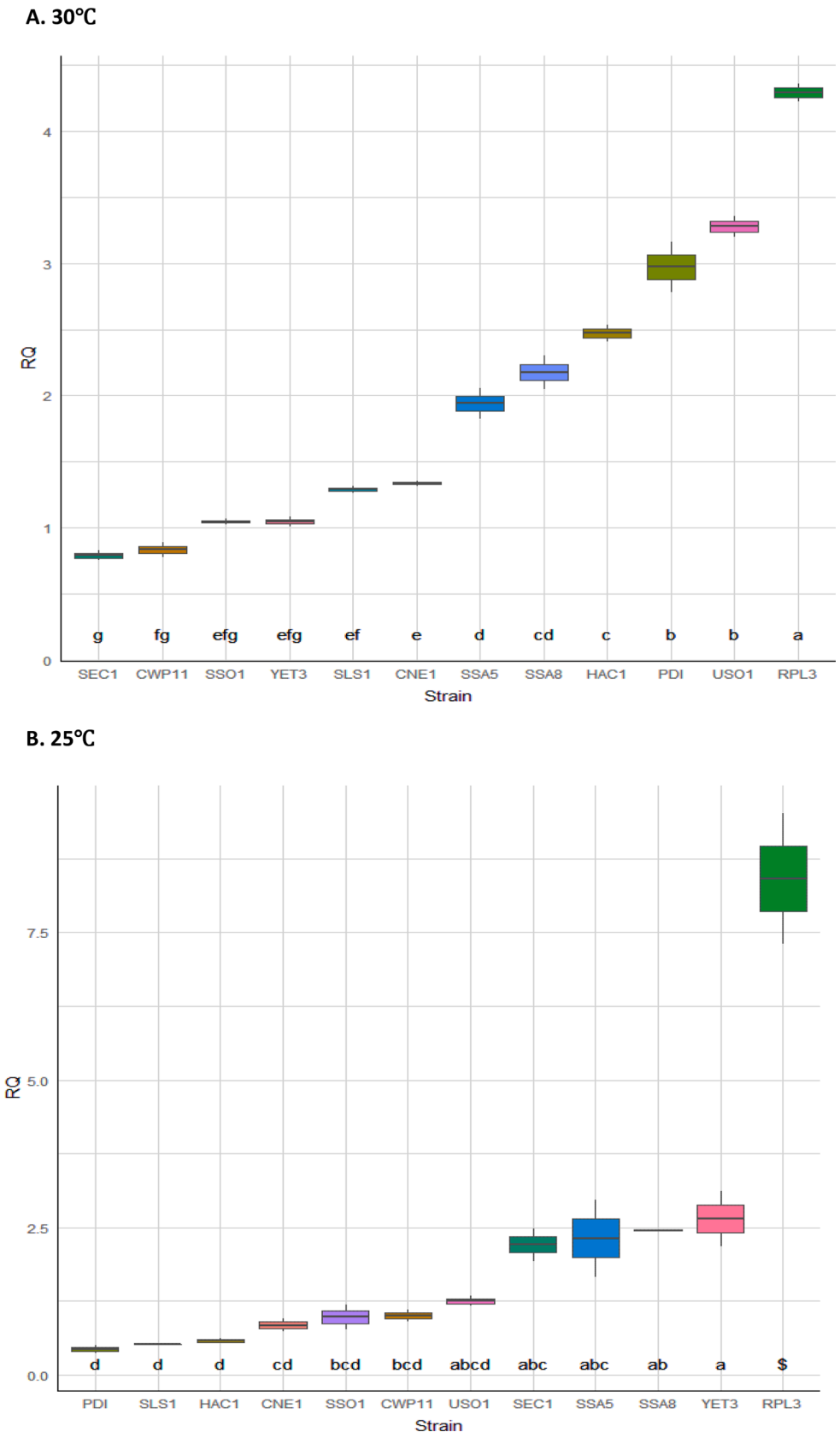

Fig. 5. Expression level of the heterologous gene encoding YFP in the *Y. lipolytica* co-transformants under two cultivation temperatures 30 °C (A) and 25 °C (B). X axis: co-overexpressed *SH*; letters indicate homogenous groups calculated by Tukey HSD test. Y axis: relative quantitation value calculated according to ddCt model. The boxes cover the 50% of input data points and a median indicated as solid line. Value 1.0 indicates lack of change in the YFP expression level between the control strains and the indicated co-transformants.

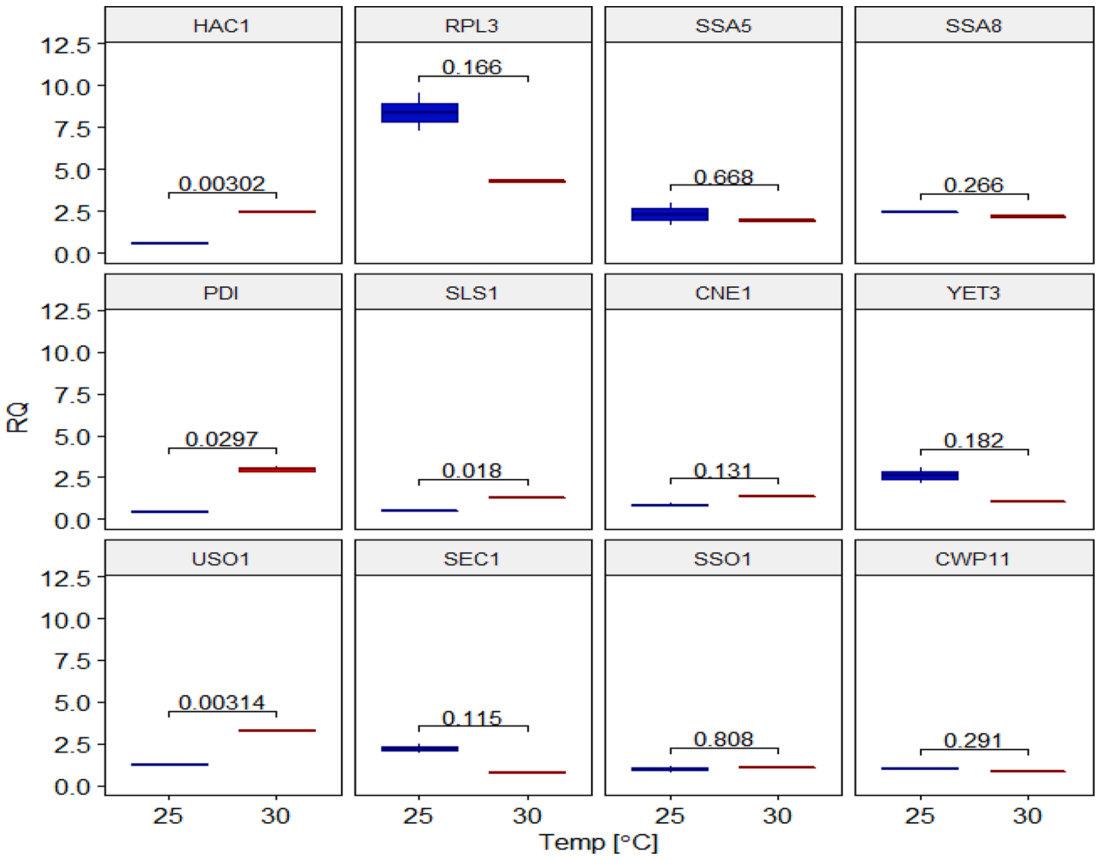

Fig. 6. Direct comparison of the expression level of the heterologous gene (YFP) in the indicated co-transformants (top of each panel) under two cultivation temperatures 25 °C (blue box) and 30 °C (red box). Numbers indicate p value of t-Student's test. X axis: cultivation temperature 25–25 °C, 30–30 °C. Y axis: relative quantitation value calculated according to ddCt model. The boxes cover the 50% of input data points and a median indicated as solid line. Value 1.0 indicates lack of change in the YFP expression level between the control strains and the indicated co-transformants.

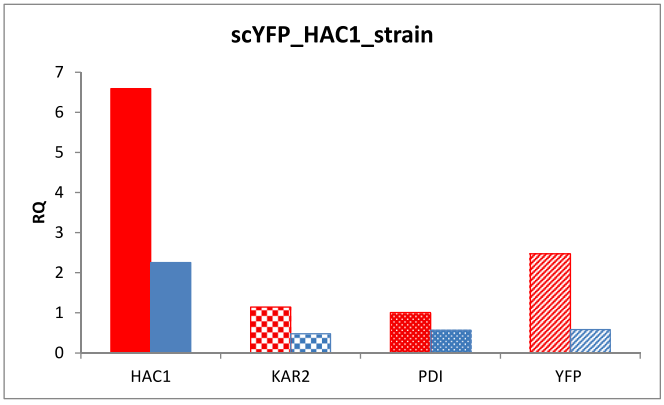

Fig. 7. Expression level of *HAC1* gene, its two direct downstream targets – *KAR2* and *PDI1*, and the heterologous gene encoding YFP in the *Y. lipolytica* strain overexpressing *YFP* and *HAC1* under two cultivation temperatures 30 °C (red bars) and 25 °C (blue bars). X axis: RTqPCR-targeted gene. Y axis: relative quantitation value calculated according to ddCt model. Value 1.0 indicates lack of change in the YFP expression level between the control strains and the indicated co-transformants.

decreased temperature uniformly promoted higher biomass accumulation. These observations stay in agreement with the findings by [10] on lack of detrimental impact of *SHs* overexpression on *P. pastoris'* growth; but also, with our recent results showing that decreased temperature in fact promotes biomass growth (and heterologous protein synthesis and secretion) in *Y. lipolytica* [33]. Analysis of kinetic data presented in Fig. 1.x.1 and 1.x.2, allowed to withdraw several interesting conclusions. Based on Fig. 1.B/C/D.1 we inferred that overexpression of *SHs* involved in translation (*RPL3*) and chaperoning activity executed in cytoplasm, directly after translation, (*SSA5* and *SSA8*) contribute to significantly increased intracellular accumulation of the reporter (inYFP), irrespectively of the adopted induction temperature. In contrast, their beneficial impact was elicited for the extracellular fraction of YFP (exYFP) solely under decreased temperature (Fig. 1. B/C/D.2). Hence, we postulate, that overexpression of *SHs: RPL3, SSA5* and *SSA8* enhanced the synthesis of the reporter protein in temperature-independent manner (as the inYFP fold change over the control at 25 °C and 30 °C was similar), but the decreased temperature was permissive for the release of the secretory pathway's capacity. Therefore, it is the combined effect of both the *SHs* overexpression and permissive temperature (25 °C) that contributes to the increases in exYFP visualized in Fig. 1.B/C/D.2 (numerical values 1.98-, 1.62- and 2.27-fold over the control [sp_exYFP] in Table 2 and Fig. 2.B). Hence, the observation visualized in Fig. 4, on statistical significance of the temperature on sp_exYFP parameter, relates to such a combined effect of genetic and environmental factors, rather than direct operation of the *SHs* in a temperature-dependent manner. Our data on the target gene expression levels corroborate this statement, as *YFP* expression was significantly enhanced due to overexpression of all these three *SHs* under both adopted temperatures (even slightly higher at 30 °C for *RPL3*), (Fig. 5.A.B), and for all three *SHs* the temperature was a not-significant factor affecting *YFP* expression (Fig. 6). However, the macroscopic outcomes, seen as significantly elevated exYFP, were observed solely under 25 °C. On top of that, since the effect in inYFP (Fig. 1.B/C/D.1) was seen under both temperatures, it is a direct

evidence on relived secretory pathway under 25 °C. In the literature, overexpression of *SSA8*'s homologue (*SSA4*) brought 40% improvement in heterologous protein synthesis and secretion in *P. pastoris* [10]. *S. cerevisiae*'s *SSA1* overexpression in *P. pastoris* resulted in 4-fold enhancement in a recombinant protein synthesis [34]. In that latter study, it was postulated and experimentally evidenced that combined overexpression of chaperones and folding helpers, such as *KAR2* or *PDI1*, can further improve the secretion levels. Overexpression of a ribosomal protein *RPP0* (as *RPL3* in this study) in *S. cerevisiae* enhanced final yields of secretory reporters by over 2.5-fold [12]. Based on our and the literature data, it can be concluded that the genes involved in translation and/or having chaperoning activity are useful as *SHs*, enabling substantial enhancement of the heterologous secretory protein synthesis. Combination of the *SHs* co-overexpression and the temperature downshift, allows to reach even better results in terms of extracellular yields of the target protein.

Significant temperature-dependency was also observed in intracellular retention of YFP upon overexpression of syntaxin *SSO1* (Fig. 1.K.1). In this case, accumulation (inYFP) of the reporter was significantly higher at 30 °C, but the protein's secretion was more efficient under 25 °C (Fig. 1.K.2). Careful analysis of the data presented in Fig. 1.K.1/2, Fig. 2.A.B and Table 2, combined with argumentation presented by [12] on burdened secretory pathway under elevated temperatures, imply that 30 °C was the permissive environmental condition that allowed demonstration of the *SSO1*-overexpression phenotype. In fact, the combined action of the *SSO1* overexpression and 30 °C resulted in the highest sp_exYFP readout under this temperature, from those studied here. It can be thus further inferred that the targeting/fusion of the Golgi-derived secretory vesicle to the cell membrane, at which SSO's operates, is the bottleneck of the secretory pathway under regular temperature of *Y. lipolytica* cultivation, which can be alleviated by either *SSO1* overexpression OR decreased temperature (exYFP: 13,511 ± 593.97 vs 11,780.5 ± 252.44 [FU]±SD, for the 25 °C and *SSO1* overexpression, respectively). As reported previously, overexpression of homologous syntaxins *SSO1* and *SSO2* in *S. cerevisiae* resulted in 4–6-fold increase in secretion of heterologous bacterial enzyme [35], which well aligns with our observations and founds the rationale behind the manipulations within late-Golgi to cell membrane traffic. Overexpression of *S. cerevisiae's SSO2* in *P. pastoris* triggered 20% increase in the secretory reporter synthesis when induced under 25 °C [10]. Another inter-species modification including *K. lactis*' *SSO1* homologue's overexpression in *S. cerevisiae* led to enhanced production of secreted proteins in the host cell [13], confirming its universally important role in the process of polypeptides secretion.

It was very surprising to see the negative effect of *USO1* co-overexpression on exYFP levels under 30 °C (reduced by nearly 40%; Fig. 1.I.2, Fig. 2.A, Table 2) and lack of impact of increased *USO1* dose on exYFP under 25 °C (Fig. 1.I.1/2, Fig. 2.B, Table 2). *USO1* is involved in vesicle mediated ER to Golgi transport, which is most frequently pointed as a key bottleneck in the secretory pathway [2,36]. Previously, USO1-encoding gene was identified as one of the most up-regulated DEGs upon high synthesis and secretion of two secretory reporters (SoA and scYFP; [24]), which well aligned with the claimed, key limitation at this stage of the secretory pathway. Interestingly, *USO1* was also identified as a promising genetic engineering target, based on microfluidic screening and whole genome sequencing in *S. cerevisiae* [23]. Our current data suggest that *USO1* co-overexpression results in no significant change (25 °C, Fig. 3.B) or inferior levels of sp_exYFP (30 °C, Fig. 3.A) in *Y. lipolytica*, when compared to the control strain. Results shown in (Fig. 5.A, Fig. 6) indicate that *USO1* had no regulatory role in promoting *YFP*'s expression (which was significantly higher at 30 °C, as expected), but that elevated expression was accompanied by significantly reduced (sp_)exYFP levels (Fig. 1.I.2, Fig. 2.A). It is thus suggested, that excessive provision of *YFP* and *USO1* transcripts triggered substantial stress for ER-localized foldases / translocon elements, which awaken UPR leading to the YFP degradation. Under 25 °C, its impact on *YFP* expression, accumulation and secretion was negligible (Fig. 5.B, Fig.1.1.1/2, Fig. 2.B, Table 2). In previous studies, several targets localized to Golgi compartment were tested for their efficiency as *SHs* [10], including *COG6, COY1, IMH1*, and SEC*31*. Their overexpression showed no significant improvement in the target protein secretion, which well corresponds with our current observations. It also leaves an open question on both–mode of these genes regulation / operation, which contribute to such unexpected outcomes; but also–on correctness of assigning the key limiting role of this specific stage of the secretory pathway (as commonly postulated). In contrast, moderate overexpression of SEC*16* (involved in protein translocation from ER to Golgi) increased the secretion of a heterologous enzymatic reporter, and two other proteins [37].

The set of *SHs* studied in the present research, also included also the most straightforward targets, frequently adopted as secretion enhancers in fungi, namely transcription factor *HAC1*, and ER resident chaperones *PDI1, SLS1*, and *CNE1*. Overexpression of these genes is considered one of most useful approaches in the yeast secretion engineering, as ER-localized protein folding is claimed, by many authors, the most rate-limiting bottleneck in heterologous protein secretion [2]. Overexpression of *PDI1* and *HAC1* had a significant positive impact on secretion of a target protein in *P. pastoris*, yielding 1.5–2.3-fold improvement, depending on the culturing mode and measure [10]. Co-overexpression of calnexin *CNE1* with any of four reporters (three glycosylated and one glycosylation-free) triggered uniformly positive impact on the reporters secretion in *H. polymorpha* [18]. On the other hand, the effect of *PDI1* co-overexpression is known to be strongly dependent on biochemical characteristics of the target polypeptide (for excellent reviews see: [2,3,8]. For example, in *K. lactis* the effect was variable, depending on presence of disulfide bonds in the target proteins [17]. In that study, the beneficial effect was observed solely, when the target protein was "disulfidebonded", while secretion of disulfide-free interleukin remained unaffected. It could, at least to some extent, explain our observations on lack of any substantial improvement in (sp_)exYFP levels upon *PDI1* co-overexpression under 30 °C (Fig. 1.F. 2, Fig. 2.A, Fig. 3.A, Table 2). In contrast, significant but moderate improvement (~30%) in (sp_)exYFP due to *PDI1* overexpression could be seen after the temperature downshift (Fig. 1.F. 2, Fig. 2.B, Fig. 3.B, Table 2). The mechanism, by which this temperature dependency was executed, seems similar to what was postulated above for *USO1*, especially considering the expression profile of the target gene (Fig. 5.A.B). Exactly the same relationship between the gene of interest expression level, secretion of the reporter and the temperature, was observed for the remaining *SHs* from this group: *HAC1, SLS1, CNE1* (Fig. 1. A/E/F/G.2, Fig. 2.A.B, Fig. 3.AB, Fig. 4, Fig. 5.A.B, Fig. 6, Table 2). For all these genes, any improvement in expression level of the *YFP* gene over the control was seen under 30 °C (Fig. 5.A), but it was associated with no improvements in its synthesis and secretion (Fig. 2.A). Decreased expression of the reporter under 25 °C (Fig. 5.B) was accompanied by significantly improved secretion of the heterologous protein (all grouped in a distinct group "d"; Fig. 2.B). The same observation was reported previously for *P. pastoris*, for which, the change in the cultivation temperature from 25 °C to 20 °C led to a 1.4-fold increase of specific product secretion rate, although the transcriptional levels of the product genes (Fab light and heavy chain) were significantly reduced [32]. Therefore, based on our current and the literature data, it is postulated that without any specific reverse regulation mechanism, the transcription of the heterologous genes is decreased under the decreased temperature, which gives sufficient capacity to the folding machinery to correctly process the nascent polypeptides. Under these conditions more correctly folded proteins could be synthesized and secreted.

Due to its regulatory role, exploitation of *HAC1* as the secretion enhancer seems to be a particularly interesting approach. HAC1 mediates activation of hundreds of molecular events, including increased provision of chaperones and membranes, to concertedly relieve

burdened secretory pathway [38,39]. The list of successful strategies comprising exploitation of *HAC1* as *SH* is very long, including strategies executed in *P. pastoris* [10] and *S. cerevisiae* [16,20,32,40]. In our study, co-overexpression of *HAC1* triggered significant, ~30% improvement in (sp_)exYFP levels over the control strain, under both temperatures (Fig. 2.A.B, Fig. 3.A.B, Table 2). Comparable level of improvement, reaching 50%, was previously observed in *P. pastoris* [10]. Interestingly, in the present study, the% improvements were similar under both temperatures, but the level of exYFP was > 50% higher under 25 °C. Expression of YFP-encoding gene was elevated due to *HAC1* co-overexpression solely under increased temperature, but the final sp_exYFP values remained strikingly similar under both temperatures (1818.34 ± 20.21 vs 1834.9 ± 97.52 [FU OD600 nm^{-1}] for 25 °C and 30 °C, respectively; Table 2). By overtaking the argumentation by [12], it could be stated that both temperatures adopted here allowed to visualize the effects of *HAC1* co-overexpression. Mechanistically, none of implemented temperatures specifically relieved / burdened biological processes in which HAC1 is involved, or rather–HAC1's scope of activity is so broad that it exerts its positive effect when either transcription (under 25 °C) or secretion (under 30 °C) are limited. Considering the key role of HAC1 in managing the proteins synthesis and secretion process, and our previous, unexpected findings on lack or very slight upregulation of *HAC1* upon overproduction heterologous proteins [24], we paid particular attention to the strain co-overexpressing this specific *SH* and conducted gene expression analysis of the two genes *PDI1* and *KAR2*, known to be the direct downstream targets of HAC1 (Fig. 7). It was again very surprising to see, that neither of the genes was upregulated due to upregulation of *HAC1*. While it well corroborates our previous transcriptomics data, earlier studies conducted with *P. pastoris* [41], and *S. cerevisiae* [22] reported that upregulation of *HAC1* is accompanied by enhanced expression of its downstream targets. It could be speculated that since we co-overexpressed native *HAC1*, and not its spliced variant, the level of transcript was significantly increased (Fig. 7), but the level of operable protein was not that substantially improved to induce massive UPR (marked by *PDI1* and *KAR2* upregulation). For being translated, *HAC1* requires unconventional splicing by dimerized IRE1. Under balanced conditions, IRE1 is bounded with KAR2, mediated by SLS1, and is not available for the *HAC1* transcript processing [42–44]. Elevated dose of SLS1 strengthens the interaction of KAR2-IRE1, disallowing *HAC1* processing, while *Δsls1* genotype displays a constitutive UPR at a "maximal" level [43]. The previously postulated key role of *SLS1* in regulation of *HAC1* translation in *Y. lipolytica* [42,44], was recently corroborated by transcriptomics data showing its uniform downregulation upon overproduction of any heterologous secretory protein [24], which probably promoted *HAC1* splicing and fine-tuning of secretion. Yet, SLS1 is a multifunctional protein, which, in addition to mediating IRE1-KAR2 interaction, promotes the SEC63-mediated activation of KAR2′s ATPase activity [44,45]. It is plausible that this specific molecular function of SLS1 underlies the observed ~30% enhancement in (sp_)exYFP under *SLS1* co-overexpression (Table 2). It also well aligns with a previous observation that *Δsls* genotype decreased the level of secretory proteins production [42,44]. Interestingly, the beneficial effect of *SLS1* co-overexpression required implementation of permissive, lowered temperature, releasing the secretory pathway from burden. Definitely, more insightful studies merging approaches addressing gene expression, protein abundance and the reporter protein secretion assessment is required to understand this mechanism in *Y. lipolytica*.

5. Conclusions

In summary, the present study allowed to withdraw several interesting conclusions on the impact of selected *SHs* overexpression and the modulating role of temperature in synthesis and secretion of heterologous proteins in *Y. lipolytica*. Here we demonstrated that under decreased temperature, the biological processes transcription and translation are balanced, as the amount of transcript and protein products are then highly correlated. We also observed a clear distinction in the effects of adopting as the *SHs,* the genes involved in either SYNTHESIS or TRANSPORTATION of the heterologous polypeptides. The former (*RPL3, SSA5, SSA8*), significantly enhance synthesis of the protein irrespective of culturing temperature, however, for efficient secretion of the protein accumulated in large amounts, the secretory pathway's capacity must be released by applying decreased temperature (25 °C). Exploitation of the genes involved in the protein trafficking as the *SHs*, does not give such spectacular results in terms of the amounts of synthesized target polypeptide, however, their overexpression allows to assist the secretory pathway in maintaining the capacity under not favorable thermal conditions (*SSO1, CWP11*). What underlies that relationship between the secretory pathway's capacity and the temperature, is, either increased or decreased, rate of transcription, which, if excessive - induces stress in the secretory pathway and causes loss in the final product, and if balanced - gives sufficient capacity to the folding machinery to correctly process the nascent polypeptides. This study provides generalizable guidelines for application in cloning/culturing strategies aiming at enhancement of heterologous protein secretion in *Y. lipolytica*.

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Data availability

All data generated within the study are available directly in the article or in the supplementary online materials.

Supplementary Materials

Fig.S1. Schematic representation of cloning strategy followed in this study. Two vectors from JMP62 series were co-transformed into *Y. lipolytica* recipient strain. Elements of the vectors were indicated in colors: zeta region (*orange*), LoxP and LoxR (*dark blue*), constitutive p*TEF* promoter (*green*), genes of interest: scYFP (A. *yellow*) or *SH* (B. *blue*) equipped in the *Bam*HI-*Avr*II fragments, the URA3ex (A. *red*) or LEU2ex (B. *light blue*) excisable auxotrophic markers. Bacterial *ori* of replication (*pink*) and kanamycin resistance gene *KanR* (*purple*), contained in the "bacterial" part of the vectors, were discarded before the yeast cell transformation by *Not*I restriction digestion.

Fig.S2. Kinetics of growth (0.1) and specific fluorescence of intracellular (sp_inYFP; 0.1) and extracellular (exYFP; 0.2) fractions of the heterologous protein YFP in randomly selected four sub-clones of each co-transformation type. The strains were cultures in shake flask cultures. Sub-charts "x.1″: X axis: Time [h]. Y axis: biomass accumulation (continuous lines) [OD 600 nm]; Y axis auxiliary: Specific intracellular fluorescence of YFP (sp_inYFP; dashed lines) in [FU OD600nm^{-1}]. Sub-charts "x.2″: X axis: sub-clones and time of culturing [h]; Y axis: extracellular fluorescence of YFP (exYFP) in [FU]. Given values are means ±SD from at least biological duplicate.

Fig.S3. Multiple comparisons of *Y. lipolytica* co-transformants and the control strain presented as fold change of specific secretion measures (sp_exYFP; $\frac{NUMERATOR\_FU\ OD600nm-1}{DENOMINATOR\ \_FU\ OD600nm-1}$) between the strains, cultured under 30 °C (A, C) and 25 °C (B, D) for 48 h (A, B) and 72 h (C, D). To use the heatmap correctly, first identify the strain on the left and use it as a NUMERATOR in fold change calculation, than, select a strain from the top of the heatmap, to be used as DENOMINATOR in the fold change calculation. Red squares represent prevalence of the numerator in the adopted measure (1.01–2.00 FC) and blue squares represent prevalence of the denominator in the adopted measure (0–0.99 FC). Statistical

significance of the comparisons was determined by a Tukey HSD test. Numbers within the comparison squares indicate the exact value of FC.

Tab.S1. *E. coli* and *Y. lipolytica* strains used in this study.

Tab.S2. Oligonucleotides used in this study. Bold sequences correspond to the part of targeted gene. Underlined sequences correspond to introduced restriction sites (*Bam*HI/*Avr*II). Oligonucleotides used for RTqPCR are preceded by "r-t" suffix.

CRediT authorship contribution statement

Paulina Korpys-Woźniak: Investigation, Methodology, Visualization, Data curation, Writing – original draft, Writing – review & editing. **Piotr Kubiak:** Formal analysis, Software, Visualization. **Ewelina Celińska:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing.

Declarations 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.

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Supplementary materials

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References

[1] M. Schröder, The cellular response to protein unfolding stress, Exploit. Fungi Symp. Br. Mycol. Soc. Held Univ. Manchester Sept. 2005 (2008) 117–139, https://doi.org/10.1017/CBO9780511902451.009.

[2] A. Idiris, H. Tohda, H. Kumagai, Engineering of protein secretion in yeast : strategies and impact on protein production, (2010) 403–417. 10.1007/s00253-010-2447-0.

[3] R.J. Zahrl, B. Gasser, D. Mattanovich, P. Ferrer, Detection and elimination of cellular bottlenecks in protein-producing yeasts, Methods Mol. Biol. 1923 (2019) 75–95, https://doi.org/10.1007/978-1-4939-9024-5_2.

[4] R.J. Zahrl, D.A. Peña, D. Mattanovich, B. Gasser, Systems biotechnology for protein production in *Pichia pastoris*, (n.d.) 1–31. 10.1093/femsyr/fox068. 2019.

[5] E. Celińska, J.-.M. Nicaud, Filamentous fungi-like secretory pathway strayed in a yeast system: peculiarities of *Yarrowia lipolytica* secretory pathway underlying its extraordinary performance, Appl. Microbiol. Biotechnol. 103 (2019) 39–52, https://doi.org/10.1007/s00253-018-9450-2.

[6] C. Madzak, *Yarrowia lipolytica* strains and their biotechnological applications: how natural biodiversity and metabolic engineering could contribute to cell factories improvement, J. Fungi. 7 (2021) 548, https://doi.org/10.3390/jof7070548.

[7] C. Madzak, Engineering *Yarrowia lipolytica* for use in biotechnological applications: a review of major achievements and recent innovations, Mol. Biotechnol. 60 (2018) 621–635, https://doi.org/10.1007/s12033-018-0093-4.

[8] V. Puxbaum, D. Mattanovich, B. Gasser, Quo vadis? the challenges of recombinant protein folding and secretion in *Pichia pastoris*, Appl. Microbiol. Biotechnol. 99 (2015) 2925–2938, https://doi.org/10.1007/s00253-015-6470-z.

[9] E. Celińska, J.-.M. Nicaud, W. Białas, Hydrolytic secretome engineering in *Yarrowia lipolytica* for consolidated bioprocessing on polysaccharide resources: review on starch, cellulose, xylan, and inulin, Appl. Microbiol. Biotechnol. 105 (2021) 975–989, https://doi.org/10.1007/s00253-021-11097-1.

[10] B. Gasser, M. Sauer, M. Maurer, G. Stadlmayr, D. Mattanovich, Transcriptomics-based identification of novel factors enhancing heterologous protein secretion in yeasts, Appl. Environ. Microbiol. 73 (2007) 6499–6507, https://doi.org/10.1128/AEM.01196-07.

[11] J. Nocon, M.G. Steiger, M. Pfeffer, S.B. Sohn, T.Y. Kim, M. Maurer, H. Rußmayer, S. Pflügl, M. Ask, C. Haberhauer-Troyer, K. Ortmayr, S. Hann, G. Koellensperger, B. Gasser, S.Y. Lee, D. Mattanovich, Model based engineering of *Pichia pastoris* central metabolism enhances recombinant protein production, Metab. Eng. 24 (2014) 129–138, https://doi.org/10.1016/j.ymben.2014.05.011.

[12] A.E. Wentz, E.V. Shusta, A novel high-throughput screen reveals yeast genes that increase secretion of heterologous proteins, Appl. Environ. Microbiol. 73 (2007) 1189–1198, https://doi.org/10.1128/AEM.02427-06.

[13] J.H. Toikkanen, L. Sundqvist, S. Ker, *Kluyveromyces lactis* SSO1 and SEB1 genes are functional in *Saccharomyces cerevisiae* and enhance production of secreted proteins when overexpressed, Yeast 2 (2004) 1045–1055, https://doi.org/10.1002/yea.1151.

[14] J. Hou, T. Österlund, Z. Liu, D. Petranovic, J. Nielsen, T. Osterlund, Z. Liu, D. Petranovic, J. Nielsen, Heat shock response improves heterologous protein secretion in *Saccharomyces cerevisiae*, Appl. Microbiol. Biotechnol. 97 (2013) 3559–3568, https://doi.org/10.1007/s00253-012-4596-9.

[15] J. Hou, K.E.J. Tyo, Z. Liu, D. Petranovic, J. Nielsen, Metabolic engineering of recombinant protein secretion by *Saccharomyces cerevisiae*, FEMS Yeast Res 12 (2012) 491–510, https://doi.org/10.1111/j.1567-1364.2012.00810.x.

[16] J. Hou, H. Tang, Z. Liu, T. Österlund, J. Nielsen, D. Petranovic, Management of the endoplasmic reticulum stress by activation of the heat shock response in yeast, FEMS Yeast Res 14 (2014) 481–494, https://doi.org/10.1111/1567-1364.12125.

[17] T. Lodi, B. Neglia, C. Donnini, Secretion of human serum albumin by *Kluyveromyces lactis*, Overexpressing KlPDI1 and KlERO1, Appl. Environ. Microbiol. 71 (2005) 4359–4363, https://doi.org/10.1128/AEM.71.8.4359.

[18] J. Klabunde, G. Kunze, G. Gellissen, C.P. Hollenberg, Integration of heterologous genes in several yeast species using vectors containing a *Hansenula polymorpha*-derived rDNA-targeting element, FEMS Yeast Res 4 (2003) 185–193, https://doi.org/10.1016/S1567-1356(03)00148-X.

[19] P. Korpys-Woźniak, P. Kubiak, W. Białas, E. Celińska, Impact of overproduced heterologous protein characteristics on physiological response in *Yarrowia lipolytica* steady-state-maintained continuous cultures, Appl. Microbiol. Biotechnol. 104 (2020) 9785–9800, https://doi.org/10.1007/s00253-020-10937-w.

[20] K.E.J. Tyo, Z. Liu, D. Petranovic, J. Nielsen, Imbalance of heterologous protein folding and disulfide bond formation rates yields runaway oxidative stress, BMC Biol 10 (2012) 16, https://doi.org/10.1186/1741-7007-10-16.

[21] J.L. Martínez, E. Meza, D. Petranovic, J. Nielsen, The impact of respiration and oxidative stress response on recombinant α-amylase production by *Saccharomyces cerevisiae*, Metab. Eng. Commun. 3 (2016) 205–210, https://doi.org/10.1016/j.meteno.2016.06.003.

[22] Z. Liu, J. Hou, J.L. Martínez, D. Petranovic, J. Nielsen, Correlation of cell growth and heterologous protein production by *Saccharomyces cerevisiae*, Appl. Microbiol. Biotechnol. 97 (2013) 8955–8962, https://doi.org/10.1007/s00253-013-4715-2.

[23] M. Huang, Y. Bai, S.L. Sjostrom, B.M. Hallström, Z. Liu, D. Petranovic, M. Uhlén, H. N. Joensson, H. Andersson-Svahn, J. Nielsen, Microfluidic screening and whole-genome sequencing identifies mutations associated with improved protein secretion by yeast, Proc. Natl. Acad. Sci. U. S. A. 112 (2015) E4689–E4696, https://doi.org/10.1073/pnas.1506460112.

[24] P. Korpys-Woźniak, E. Celińska, Global transcriptome profiling reveals genes responding to overproduction of a small secretory, a high cysteine- and a high glycosylation-bearing protein in *Yarrowia lipolytica*, Biotechnol. Reports. 31 (2021) e00646, https://doi.org/10.1016/j.btre.2021.e00646.

[25] G. Barth, C. Gaillardin, *Yarrowia lipolytica*, in: K. Wolf (Ed.), Nonconv. Yeasts Biotechnol. A Handb, Springer Berlin Heidelberg, 1996, pp. 313–388, https://doi.org/10.1007/978-3-642-79856-6.

[26] J. Sambrook, D. Russell, Molecular Cloning: A Laboratory Manual, 3rd ed., Cold Spring Harbor Laboratory Press, New York, 2001.

[27] E. Celińska, M. Borkowska, W. Białas, P. Korpys, J.M. Nicaud, Robust signal peptides for protein secretion in *Yarrowia lipolytica*: identification and characterization of novel secretory tags, Appl. Microbiol. Biotechnol. 102 (2018) 5221–5233, https://doi.org/10.1007/s00253-018-8966-9.

[28] J.M. Nicaud, C. Madzak, P. Van Den Broek, C. Gysler, P. Duboc, P. Niederberger, C. Gaillardin, Protein expression and secretion in the yeast *Yarrowia lipolytica*, FEMS Yeast Res 2 (2002) 371–379, https://doi.org/Pii S1567-1356(02)00082-X.

[29] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method, Methods 25 (2001) 402–408, https://doi.org/10.1006/meth.2001.1262.

[30] J.S. Cox, P. Walter, A novel mechanism for regulating activity of a transcription factor that controls the unfolded protein response, Cell 87 (1996) 391–404, https://doi.org/10.1016/S0092-8674(00)81360-4.

[31] K. Mori, T. Kawahara, H. Yoshida, H. Yanagi, T. Yura, Signalling from endoplasmic reticulum to nucleus : transcription factor with a basic-leucine zipper motif is required for the unfolded protein-response pathway, Genes cells (1996) 803–817. 10.1046/j.1365-2443.1996.d01-274.x.

[32] B. Gasser, M. Maurer, J. Rautio, M. Sauer, A. Bhattacharyya, M. Saloheimo, M. Penttilä, D. Mattanovich, Monitoring of transcriptional regulation in *Pichia pastoris* under protein production conditions, BMC Genomics 8 (2007) 1–18, https://doi.org/10.1186/1471-2164-8-179.

[33] M. Kubiak, W. Białas, E. Celińska, Thermal treatment improves a process of crude glycerol valorization for the production of a heterologous enzyme by Yarrowia lipolytica, Biotechnol. Reports. 31 (2021) e00648, https://doi.org/10.1016/j.btre.2021.e00648.

[34] W. Zhang, H.L. Zhao, C. Xue, X.H. Xiong, X.Q. Yao, X.Y. Li, H.P. Chen, Z.M. Liu, Enhanced secretion of heterologous proteins in Pichia pastoris following overexpression of Saccharomyces cerevisiae chaperone proteins, Biotechnol. Prog. 22 (2006) 1090–1095, https://doi.org/10.1021/bp060019r.

[35] L. Ruohonen, J. Toikkanen, V. Tieaho, M. Outola, H. Soderlund, S. Keranen, Enhancement of protein secretion in saccharomyces cerevisiae by overproduction of Sso protein, a late-acting component of the secretory machinery, Yeast 13 (1997) 337–351, 10.1002/(sici)1097-0061(19970330)13:4<337::aid-yea98>3.3.co;2-b.

[36] J.R. Shuster, Gene expression in yeast: protein secretion, Curr. Opin. Biotechnol. 2 (1991) 685–690, https://doi.org/10.1016/0958-1669(91)90035-4.

[37] J. Bao, M. Huang, D. Petranovic, J. Nielsen, Moderate expression of SEC16 increases protein secretion by Saccharomyces cerevisiae, Appl. Environ. Microbiol. 83 (2017), https://doi.org/10.1128/AEM.03400-16.

[38] K.J. Kauffman, E.M. Pridgen, F.J. Doyle, P.S. Dhurjati, A.S. Robinson, Decreased protein expression and intermittent recoveries in BiP levels result from cellular stress during heterologous protein expression in Saccharomyces cerevisiae, Biotechnol. Prog. 18 (2002) 942–950, https://doi.org/10.1021/bp025518g.

[39] M. Guerfal, S. Ryckaert, P.P. Jacobs, P. Ameloot, K. Van Craenenbroeck, R. Derycke, N. Callewaert, The HAC1 gene from Pichia pastoris: characterization and effect of its overexpression on the production of secreted, surface displayed and membrane proteins, Microb. Cell Fact. 9 (2010) 1–12, https://doi.org/10.1186/1475-2859-9-49.

[40] J. Liu, Q. Han, Q. Cheng, Y. Chen, R. Wang, X. Li, Y. Liu, D. Yan, Efficient expression of human lysozyme through the increased gene dosage and Co-expression of transcription factor hac1p in Pichia pastoris, Curr. Microbiol. 77 (2020) 846–854, https://doi.org/10.1007/s00284-019-01872-9.

[41] A.R. Hesketh, J.I. Castrillo, T. Sawyer, D.B. Archer, S.G. Oliver, Investigating the physiological response of Pichia (Komagataella) pastoris GS115 to the heterologous expression of misfolded proteins using chemostat cultures, Appl. Microbiol. Biotechnol. 97 (2013) 9747–9762, https://doi.org/10.1007/s00253-013-5186-1.

[42] A. Boisramé, J.M. Beckerich, C. Gaillardin, Sls1p, an endoplasmic reticulum component, is involved in the protein translocation process in the yeast Yarrowia lipolytica, J. Biol. Chem. 271 (1996) 11668–11675, https://doi.org/10.1074/jbc.271.20.11668.

[43] A. Babour, M. Kabani, A. Boisramé, J. Beckerich, Characterization of Ire1 in the yeast Yarrowia lipolytica reveals an important role for the Sls1 nucleotide exchange factor in unfolded protein response regulation, (2008) 337–346. 10.1007/s00294-008-0190-1.

[44] A. Boisramé, M. Kabani, J.M. Beckerich, E. Hartmann, C. Gaillardin, Interaction of Kar2p and Sls1p is required for efficient co- translational translocation of secreted proteins in the yeast Yarrowia lipolytica, J. Biol. Chem. 273 (1998) 30903–30908, https://doi.org/10.1074/jbc.273.47.30903.

[45] M. Kabani, J.-.M. Beckerich, C. Gaillardin, Sls1p stimulates Sec63p-mediated activation of Kar2p in a conformation-dependent manner in the yeast endoplasmic reticulum, Mol. Cell. Biol. 20 (2000) 6923–6934, https://doi.org/10.1128/mcb.20.18.6923-6934.2000.