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Chaperone overexpression boosts heterologous small molecule production in *Saccharomyces cerevisiae*

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Abstract

Background Chaperones play an important role in maintaining cellular proteostasis by mediating protein folding. As a result, chaperone overexpression has been widely used as a tool for enhancing folding and improving production of heterologous proteins in host organisms such as *Saccharomyces cerevisiae*. In contrast, this strategy has been much less explored for small molecule (SM) production. This is surprising, as SM pathways typically depend on multiple enzymes including large multi-domain synthases or synthetases, which may all benefit from folding assistance to enhance the catalytic power of the pathway.

Results We have established an *S. cerevisiae* strain library of 68 strains overexpressing endogenous cytosolic chaperones and a mating-based method that allows the chaperone library to be combined with a query strain that contains the pathway of a desirable SM. Using the small molecule aspulvinone E from *Aspergillus terreus* as a model compound, we screened the chaperone library for chaperones that improve production of aspulvinone E. Screening of the library identified several chaperones and chaperone combinations that improved aspulvinone E production. Specifically, the combined overexpression of *YDJ1* and *SSA1* was identified as the best hit in our screen. Subsequently, we demonstrated that overexpression of *YDJ1* and *SSA1* improved aspulvinone E production by 84% in 1.5 mL scale batch fermentations. The observed increase is likely due to higher levels of the MelA synthetase responsible for aspulvinone E synthesis, as overexpression of *YDJ1* and *SSA1* increases the amounts of fluorescent MelA-mRFP in cells producing this fusion protein.

Conclusion The endogenous cytosolic chaperone overexpression library and mating based screening method presented in this report constitute a tool allowing for fast and efficient identification of specific chaperones and chaperone combinations that benefit production of a given SM in *S. cerevisiae*-based cell factories.

Keywords *Saccharomyces cerevisiae*, Aspulvinone E, Chaperone overexpression, Mating, Cell factory engineering, Library screening.

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Background

Nature presents a plethora of small molecules (SMs) of biotechnological interest including fragrances, food colors and pharmaceuticals. In many cases, however, the natural producers accumulate the SM in concentrations that are too low for industrial production and/or are incompatible with industrial fermentation [1, 2]. Consequently, there is a significant interest in the establishment of heterologous biosynthesis of SMs in cell factory chassis, which are suitable for industrial production. Baker's yeast, *Saccharomyces cerevisiae*, represents one of the most broadly used chassis for cell factory development due to its many beneficial characteristics including fast and robust growth on a range of carbon sources, well-established fermentation processes, a sophisticated genetic toolbox, and compatibility with high-throughput methods [2, 3]. As such, *S. cerevisiae* has been used as a host organism for the production of a wide range of SMs including non-ribosomal peptides, polyketides and terpenes [2, 4]. Prominent examples include the terpenoid artemisinic acid and the polyketides triacetic acid lactone and 6-methylsalicylic acid, where high-titer production has been achieved via extensive metabolic and process engineering [5–7]. Efficient establishment of a heterologous pathway in a non-native organism is not a trivial task and may require elaborate engineering of host metabolism and physiology to accommodate production [8]. Significantly, the production of non-native proteins in *S. cerevisiae* may suffer from suboptimal folding that eventually reduce yields [9]. This limitation is widely recognized in the design of *S. cerevisiae*-based cell factories engineered for the production of secreted recombinant proteins [10]. Overexpression of genes encoding endogenous chaperones e.g. ER resident chaperones involved in the secretory pathway such as Hsp70 protein Kar2 and the protein disulfide isomerase Pdi1 [11], or cytosolic chaperones such as Ssa1 and Ydj1 aiding in the translocation of proteins from the cytosol into the ER [12], are often used to boost yields. On the contrary, overexpression of chaperone genes to improve heterologous SM production in *S. cerevisiae* is not common and only few examples have been presented in the literature, e.g. to improve terpene biosynthesis [13–15]. Additionally, no systematic tools allowing identification of beneficial chaperone effects are available in *S. cerevisiae*. This is surprising considering that SM production often depends on the proper functionality of many enzymes and inefficient folding of just one of the pathway enzymes is sufficient to reduce yields.

In this report, we present a simple setup enabling identification of cytosolic chaperones that may benefit SM production in *S. cerevisiae*. Specifically, we have constructed a library of 68 strains that overproduce one or two cytosolic *S. cerevisiae* chaperones or co-chaperones, which can be individually combined with a query strain containing an SM pathway by mating via a simple replica-pinning scheme. Beneficial effects on SM production can subsequently be assessed in the resulting diploid strains. As proof of concept, we have used production of the tyrosine derived fungal pigment aspulvinone E as a model compound. Aspulvinone E has previously been produced in *S. cerevisiae* and since it is fluorescent [16, 17], it provides a simple readout to determine product levels in the individual strains of the library. Aspulvinone E is produced by a single non-ribosomal peptide synthetase (NRPS)-like enzyme, MelA, which belongs to a class of multi-domain cytosolic enzymes not produced by *S. cerevisiae*. Like other NRPSs, MelA activity is dependent on posttranslational modification by a phosphopantetheinyl transferase, which converts the synthetase to its holo form. Hence, to produce aspulvinone E, we co-expressed *melA* from *Aspergillus terreus* with the phosphopantetheinyl transferase gene *npgA* from *Aspergillus nidulans*, which is commonly used for PKS and NRPS activation in *S. cerevisiae* [18]. With these enzyme requirements, we speculated that aspulvinone E production in *S. cerevisiae* could potentially benefit from additional chaperone activity that promotes folding of MelA and/or NpgA. In this report, we have identified chaperones and chaperone combinations that enhanced aspulvinone E production on solid medium. Importantly, the effect of the best combination could be reproduced in a liquid batch fermentation where the aspulvinone E yield was improved by 84%.

Results and discussion

A mating-based strategy for identification of chaperones improving heterologous SM production

Our setup to identify chaperones benefitting heterologous SM production requires an arrayed library of haploid strains containing one or more overexpressed chaperone genes and an isogenic haploid SM query strain of the opposite mating type, containing a SM gene expression cassette (GEC) (Fig. 1A). As our library is based on the CEN.PK strain background, query strains can easily be constructed using our well-developed toolbox for gene insertion [19, 20]. Importantly, the library strains and SM query strain contain complementary marker genes, allowing for the selection of diploid cells arising from mating. Hence, in the **first** step of the procedure (Fig. 1B), the arrayed library and query strains are mixed by replica pinning onto solid YPG media, containing galactose as the sole carbon source, to allow for systematic and individual mating of all library stains to the SM query strain. In the **second** step, the arrayed colonies are transferred to a solid medium selecting for heterozygous diploid cells that contain a combination of chaperone gene(s) and the SM pathway gene(s). In the **third** step, the array of diploid strains is transferred to a

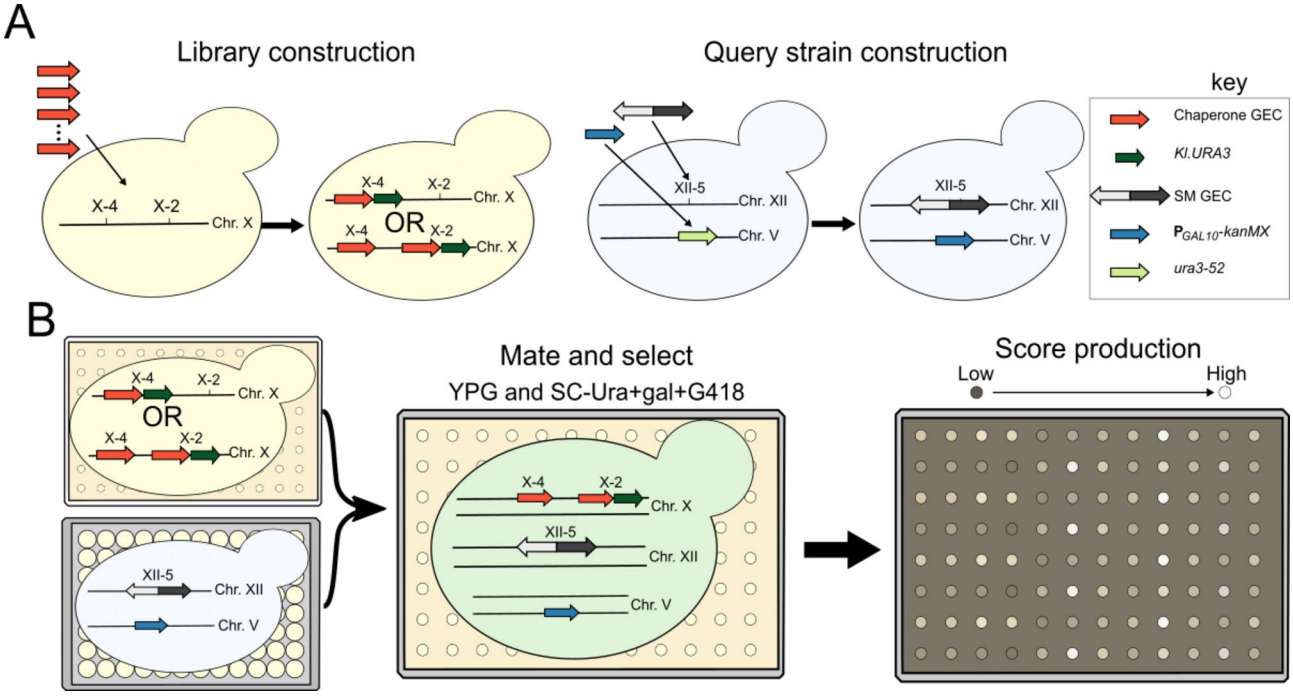

Fig. 1 Mating based setup for identification of beneficial chaperone genes. **(A)** Left: construction of the chaperone strain library by integration of chaperone GECs in integration sites X-2 and X-4. Right: construction of the query strain by integration of an SM GEC in the integration site XII-5 and simultaneous replacement of the *ura3-52* marker gene with a selectable *kanMX* cassette. **(B)** Left: query and chaperone strains are arrayed. Middle: the two strain types are combined on solid YPG medium for mating and transferred to solid SC-Ura + Gal + G418 to select for diploids by replica-pinning. Right: diploids are transferred to solid medium compatible with SM production and quantitative detection

medium of choice to allow the impact of the individual chaperones or chaperone combinations on SM production to be assessed.

Construction of chaperone gene overexpression library

To facilitate folding of heterologous SM pathway enzymes in the cytosol, we have created a library of 68 strains that overproduce one or two cytosolic *S. cerevisiae* chaperones or co-chaperones. The library was designed to include cytosolic chaperone members from the major families: HSP40, HSP70, HSP90, and small heat shock proteins; as well as members that are either categorized as CLIPS (*c*haperones *li*nked to *p*rotein *s*ynthesis), which assist in *de novo* folding of proteins, or HSPs (*h*eat *s*hock *p*roteins), which facilitate refolding of denatured proteins [21, 22], (Table S1). Moreover, chaperones like Ssa1 and Hsc82 belonging to the HSP70 and HSP90 families, respectively, are known to have a high number of chaperone interaction partners [22]. It is therefore possible that Ssa1 and Hsc82, in combination with other chaperone partners, could further benefit production. We therefore included a set of strains expressing *SSA1* or *HSC82* in combination with other chaperone genes in our library. The library was constructed in the CEN.PK113-5D (*MATa*) strain, which contains a *ura3-52* marker (Table S1). Chaperone genes were inserted into the well-characterized genomic insertion sites X-2 and X-4, which support comparable expression levels [19], to produce library strains Chap 001 – Chap 072. Specifically, all strains contain a library gene inserted into the X-4 site and strains harboring two library genes contain the additional gene in the X-2 site, (see Fig. 1A and Table S1). In all cases, the chaperone genes were controlled by the constitutive *TEF1* promoter and the *CYC1* terminator. For library strains Chap 001 – Chap 021 the chaperone gene in the X-4 site was accompanied by the *Kl. URA3* marker while in strains Chap 021 – Chap 072 the *Kl.URA3* marker is present in the X-2 site. In this way all strains in the library are prototrophic.

Construction of an aspulvinone E query strain

To set the stage for aspulvinone E production in yeast, we first constructed a dual GEC harboring *mel*A from *A. terreus*, encoding the MelA synthetase and *npgA* from *A. nidulans* encoding a phosphopantetheinyl transferase. In this aspulvinone E expression cassette *melA* and *npgA* are under the control of the *S. cerevisiae* promoters and terminators P_{TEF1} and T_{CYC1}, and P_{TEF2} and T_{ADH1}, respectively. The aspulvinone E GEC was introduced into the well-characterized expression site XII-5 in CEN. PK113-3B (*MATα*) and the *ura3-52* marker was replaced by *kanMX* under the control of the endogenous P_{GAL10} and T_{TEF1}, as described in (Materials and Methods), resulting in the aspulvinone E query strain AQS002.With

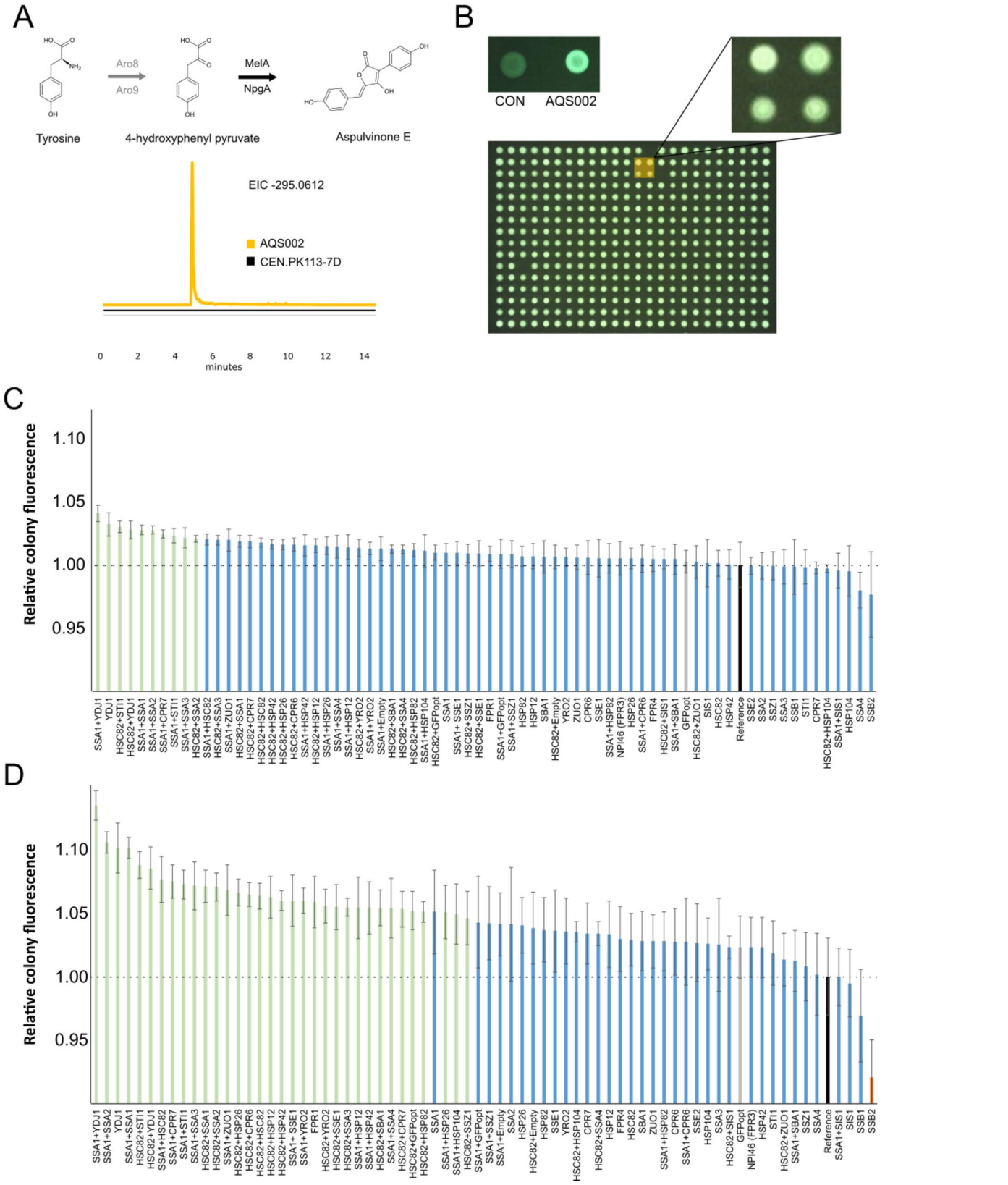

Fig. 2 (See legend on next page.)

(See figure on previous page.)

Fig. 2 The influence of overexpressed chaperone genes on aspulvinone E production. **(A)** Top: Aspulvinone E biosynthetic pathway. Bottom: Extracted ion chromatogram for −295.0612, corresponding to the *m/z* for aspulvinone E $[M\text{-}H]^-$. The aspulvinone E producing strain (AQS002) in orange and non-producing strain CEN.PK113-7D in black. **(B)** Top left: Fluorescent phenotype under UV light of AQS002 and CEN.PK113-7D (CON). Bottom: Fluorescence screen of arrayed diploid strains (chaperone gene library strains mated with AQS002). Zoomed in picture shows the fluorescence of four colonies resulting from mating AQS002 with four different chaperone gene library strains. **(C)** and (**D**) Trial 1 and 2, respectively. For each chaperone gene or gene combination, columns represent average fluorescence intensity measured for 16 independent colonies normalized to the average fluorescence intensity of the reference strain (Chap 001). Strains have been ranked according to the level of fluorescence intensity. The reference and the GFP producing strains are represented by black and gray bars, respectively. Green and red bars represent strains with significantly higher or lower levels of fluorescence than the reference strain ($p < 0.01$) respectively. Blue and gray bars represent strains that do not show significantly different fluorescence levels compared to the reference strain. Error bars represent ± SD, $n = 16$

this genetic setup, it is possible to select diploids resulting from the mating of chaperone library strains and the aspulvinone E query strain on SC-Ura + Gal + G418. As expected, based on previously reported expression of *melA* and *npgA* in *S. cerevisiae* [17], UHPLC-DAD-TOFMS analysis of extracts from YPD cultures of AQS002 and CEN.PK113-7D showed a new peak in AQS002, not observed in CEN.PK113-7D, corresponding to the *m/z* and UV-VIS spectra previously reported for aspulvinone E (Fig. 2A and Fig. S1) [23]. Importantly, for the use of AQS002 in subsequent screening experiments, we observed that AQS002 pinned on solid SC media was fluorescent under UV light while, CEN.PK113-7D was not (Fig. 2B).

A visual screening method for aspulvinone E production in *S. cerevisiae*

Next, we assessed whether aspulvinone E fluorescence could potentially be used to screen for chaperones that influence aspulvinone E production on solid SC medium. To do so, we first pinned the aspulvinone E producing strain APS006, which contains a single copy of the aspulvinone E expression cassette, next to the reference strain CEN.PK113-7D on SC medium and captured pictures under UV-light (see Material and Methods) (Fig. S2). To quantitatively assess colony fluorescence, image analysis was performed using an adaptation of the script described by Cachera et al. [24]. Encouragingly, CEN.PK113-7D and APS006 were easily distinguishable when pictured under UV-light (Fig. S2A). Specifically, APS006 exhibited an approximately 80% hgher signal compared to the non aspulvinone E producing CEN.PK113-7D control ($p < 0.01$). To determine whether we would be able to detect increased production of aspulvinone E in a strain, we introduced an additional copy of the *melA* synthetase into APS006 to generate the strain APS009. Indeed, the APS009 colonies were significantly more fluorescent, 14% ($p < 0.01$), than the APS006 colonies (Fig. S2B). Assuming that the higher *melA* gene dose of APS009, as compared to APS006, is causing the higher aspulvinone E levels, this result indicates that levels of aspulvinone E synthetase limits aspulvinone E production in *S. cerevisiae*. Altogether, our analyses demonstrate that it should be possible to identify overexpressed chaperone genes in the library that benefit, or impair, folding or maturation of the aspulvinone E synthetase via detectable increases or reductions in colony fluorescence levels. Hence, it should be possible to rank chaperone library strains that contain the aspulvinone E expression cassette and use the ranking to identify chaperones that enhance aspulvinone E production.

Screening the chaperone library for candidate genes enhancing aspulvinone E production

To investigate whether overexpression of any of the chaperone genes or gene combinations benefit aspulvinone E production, we combined AQS002 and the chaperone library strains. Given that the differences in fluorescence between colonies might be low we determined average fluorescence levels in screening trials that included 16 independent colonies of each gene combination. This was done to be able to significantly detect even modest differences. Additionally, we performed two independent trials in this manner to evaluate reproducibility of the method. All experiments were performed in a 384-format grid, in a manner ensuring that the individual colonies of a given strain propagated in different environments on the plates (Fig. S3). Mating was initiated by replica-pinning library colonies on top of the AQS002 colonies on solid YPG (Step 1 and Fig. 1B). The plate was incubated for 24 h and the resulting diploids cells formed at this point are exhausted for glucose. Diploid cells will therefore instantly be able to grow when they are transferred to diploid selective medium (Sc-Ura + Gal + G418), due to the presence of the *Kl.URA3* and P_{GAL10}-*kanMX*, as induction of P_{GAL10} should not be impaired by glucose repression. This step was repeated to minimize carry-over of unmated haploid cells. Cells emerging on the second SC-Ura + Gal + G418 plate (Step 2) were transferred to SC medium for three days at 30 °C to allow for aspulvinone E production (Step 3). The mating efficiency of the three-step procedure was 100%, as no strains failed to form colonies on the final SC plate. Aspulvinone E production in individual colonies obtained in trials 1 and 2 was assessed by determining the average level of fluorescence emitted from each strain by analysis of images taken under UV light (see Materials and Methods). Subsequently, strains were ranked according to the intensity

of fluorescence normalized to the reference strain (Chap 001), which does not overexpress any chaperone genes (Fig. 2C-D) (Table S2). This analysis showed that the fluorescence signal only varied moderately from the highest to the lowest producer in trial 1 (6.4%) and trial 2 (21.4%). Encouragingly, plotting the relative colony fluorescence of the two screens showed a positive linear relationship (Fig. S4) and analysis of the data showed a Pearson correlation coefficient of (PCC = 0.91, $p < 0.01$). Moreover, nine of the top-ten hits produced by the two screens were identical. Hence, the ranking of the top-hit effects appears robust, but we note that individual data points obtained in different trials cannot be compared.

Validation of screens observations

It is important to stress that each of the colonies in the screen are not clonal but rather composed of strains resulting from multiple parallel mating events. Moreover, the resulting diploid cells are heterozygous for the aspulvinone E pathway genes and the additional chaperone gene(s). Altogether this could potentially create artificial effects. Hence, to validate the results of the screen, we *de novo* created haploid strains containing the aspulvinone E GEC in combination with selected chaperone genes from the top-ten hit-list. Specifically, we decided to investigate whether the effect of the sole single gene-hit identified in both screens, *YDJ1*, could be reproduced in this setup. Moreover, we also included *YDJ1* in combination with *SSA1* and with *HSC82* in this analysis as these double mutant strains are both in the top-ten hit-lists, the former being the highest scoring strain in both trials. To further examine potential additive effects, we selected gene combinations *HSC82* + *STI1* and *SSA1* + *STI1*, which are also in the top-ten list despite *STI1*, *HSC82*, and *SSA1* alone are scored as genes with little, if any, effect on aspulvinone E production. These six strains were spotted on solid SC medium (Fig. S5), and their levels of fluorescence were compared to the reference strain (APS006), containing the aspulvinone E GEC but no chaperone GEC. The ranking of the selected chaperones was near-identical to that observed in the two screens, except for *HSC82* + *YDJ1*, which in haploid background was ranked higher than *HSC82* + *STI1.* Furthermore, all the selected chaperone overexpression strains demonstrated significantly greater fluorescence relative to that of the reference strain ($p < 0.05$) (Fig. 3).

Characterization of aspulvinone E promoting screen hits in liquid medium

To explore whether the hits identified in the screen could be useful in a metabolic engineering strategy, we next set out to investigate whether the effects obtained on solid SC medium truly represented enhanced aspulvinone E production and could be reproduced in liquid medium in a BioLector. Unfortunately, the first test of the haploid aspulvinone E producing reference strain (APS006) did not produce sufficient amounts of aspulvinone E to allow comparison by UHPLC-DAD-TOFMS, as the peak could barely be distinguished from the baseline produced by the non-producing control strain. This could be due to the presence of both aromatic amino acids and ammonium sulfate in the SC medium. Aromatic amino acids are known to inhibit the shikimate pathway by feedback inhibition of Aro3 and Aro4 [25], while ammonium downregulates transcription of aminotransferases *ARO8* and *ARO9* [26]. We therefore repeated this analysis in synthetic complete drop-out medium pH buffered to 6.0 and using urea as a nitrogen source. In the SD-urea medium a substantial peak representing aspulvinone E was observed (Fig. S6). Hence, we analyzed all the haploid strains equipped with selected chaperone or chaperone combinations, in SD-urea in a BioLector.

Comparison of the observed growth rate of the chaperone overexpression strains and the reference strain did not show any significant differences ($p < 0.05$) (Fig. 4A). When we compared the aspulvinone E titers in the different strains, we observed that the strains overexpressing *HSC82* + *YDJ1*, *SSA1* + *STI1*, and *HSC82* + *STI1* did not produce significantly different amounts of aspulvinone E in comparison to the reference strain, (Fig. 4B). In contrast, the strains overexpressing *YDJ1* and *SSA1* + *YDJ1* produced 29% and 84% more aspulvinone E than the reference strain, respectively, and these differences are both significant ($p < 0.05$). The 43% increase in aspulvinone E production in the double mutant *SSA1* + *YDJ1* relative to the level obtained with the *YDJ1* strain was also significant ($p < 0.05$).

Effect of chaperone overexpression on MelA levels

We hypothesized that increased production of aspulvinone E in the *SSA1* + *YDJ1* overexpression background could simply be explained by increased MelA synthetase levels resulting from improved folding of this enzyme in the presence of the increased chaperone activity. To examine this possibility, we constructed a *npgA* and *melA-mRFP* expression cassette for production of a MelA synthethase, which has been C-terminally tagged by mRFP via a $(GGGGS)_2$ linker. To assess whether the chaperones enhance MelA production and thus increase mRFP signal, this expression cassette was introduced in the *SSA1* + *YDJ1* and empty backgrounds, resulting in APS007 (*SSA1* + *YDJ1*) and APS008 (reference), respectively. Shake-flask cultivation of APS007 and APS008 were conducted in SD-urea and mRFP levels were evaluated over the course of 120 h of the cultivation (Fig. 5A). Importantly, and in agreement with our hypothesis, the mRFP signal from the *SSA1* + *YDJ1* culture was significantly higher ($p < 0.05$) after 48 h in the presence of

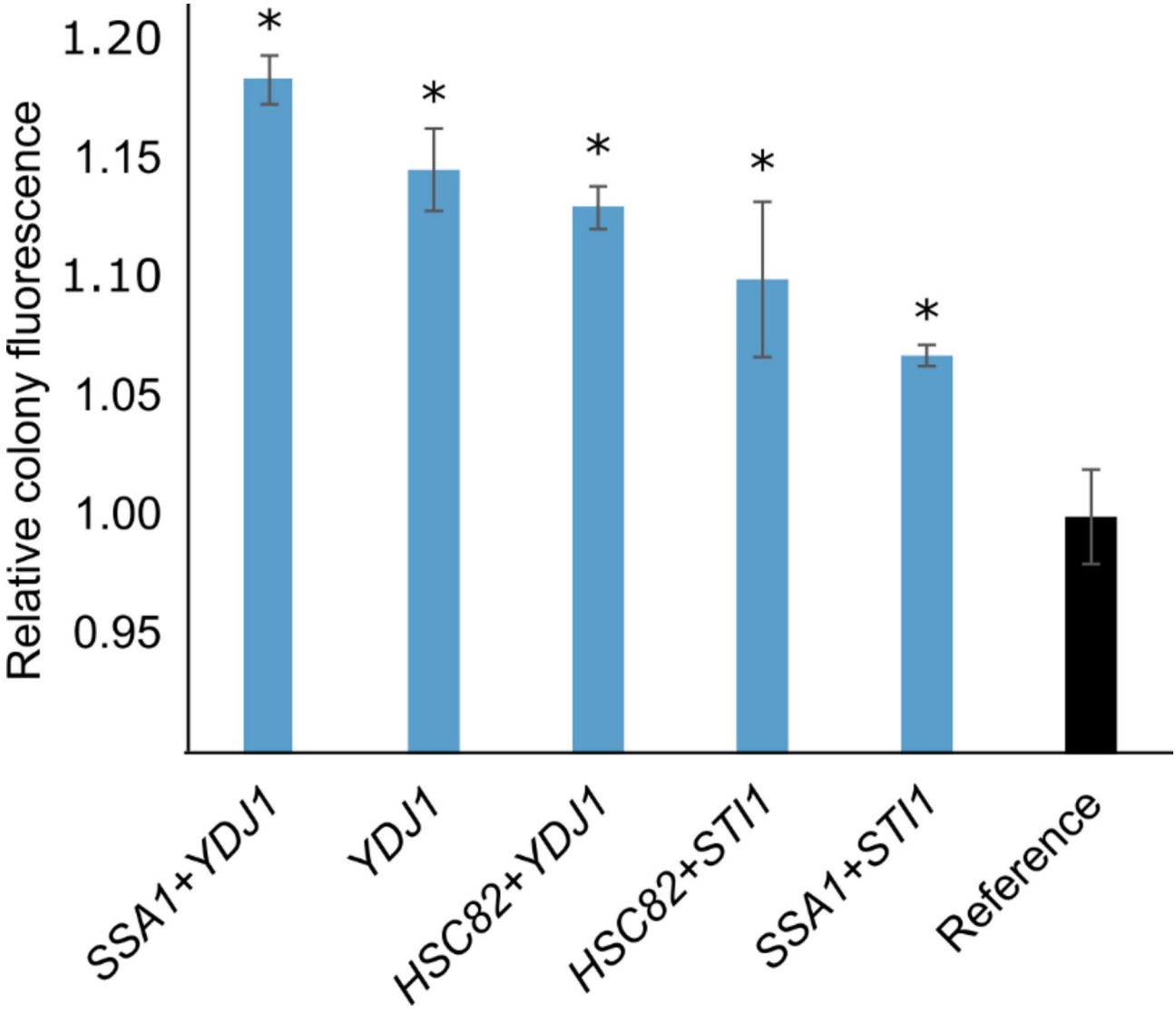

Fig. 3 Relative colony fluorescence. Average colony fluorescence intensity observed for each chaperone GEC and chaperone GEC combination normalized to the reference strain (APS006). Error bars represent ± SD, $n = 24$ (96 for reference). Strains with a significantly higher level of fluorescence than the reference strain ($p < 0.05$) are marked by an asterisk

additional chaperone activity. Comparison of the aspulvinone E titer at the end of the cultivation showed a significant 20% higher titer ($p < 0.05$) in the *SSA1 + YDJ1* strain relative to the reference strain (Fig. 5B). SD-urea cultures of aspulvinone E producing strains APS002 and APS006, neither of which produce the MelA-mRFP fusion protein, showed similar mRFP signal to that observed for CEN. PK113-7D (Fig. S7).

Discussion

In this report, we present a strategy that enables rapid identification of endogenous chaperones that enhance heterologous SM production in *S. cerevisiae*. The core of the strategy is to combine heterologous SM pathway genes with a library of overexpressed chaperones. To explore this strategy, we have developed a simple mating-based method that allows a heterologous pathway to be combined with a library of arrayed strains overexpressing different chaperones or chaperone combinations. This setup allows the effect of all overexpressed chaperones and chaperone combinations on production of an SM of interest to be individually assessed. Aspulvinone E production was chosen to generate proof-of-principle of the strategy, as production levels in colonies could be distinguished by measuring fluorescence. The observed differences in fluorescence levels in our experiment were modest. Nevertheless, several observations support that the ranking order of aspulvinone E production in the experiments are meaningful. Firstly, in trials 1 and 2, we found that 10 and 34 strains, respectively, produced

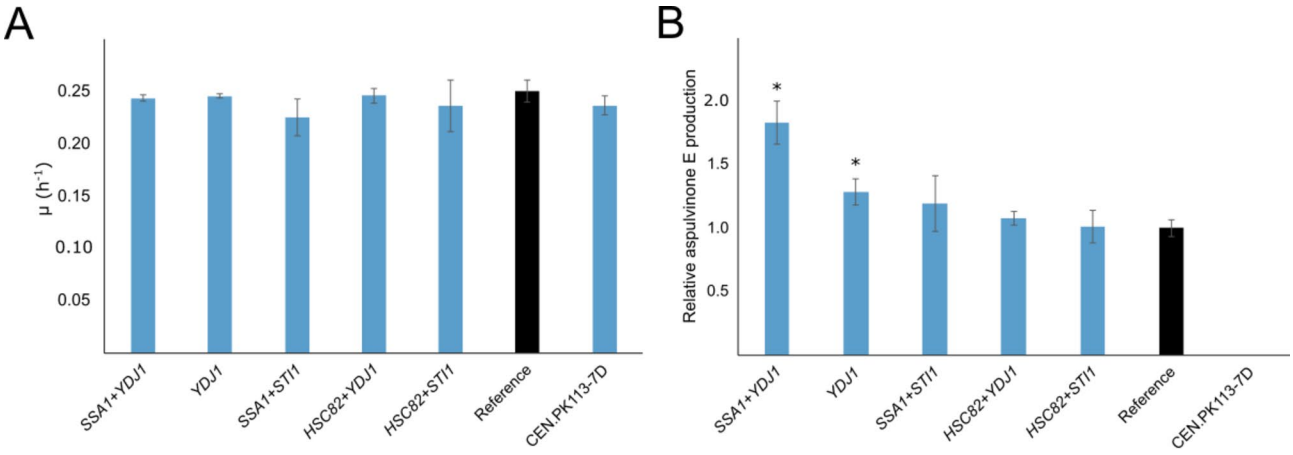

Fig. 4 Effect of chaperone overexpression on aspulvinone E production in Biolector cultivation. **(A)** Growth rate of chaperone overexpression strains, as indicated, and the reference strain shown in black. Growth rate was determined for the exponential growth phase, approximately between 17–22 h, depending on the length of the lag phase. **(B)** Production of aspulvinone E in chaperone overexpression strains after 72 h, relative to the reference (APS006) shown in black. Columns represent average aspulvinone E production as measured by the UV absorption at 368 nm of the peak corresponding to aspulvinone E and normalized to the reference strain. Strains producing significantly more aspulvinone E relative to the reference strain ($p < 0.05$) are indicated by an asterisk. Error bars represent ± SD, $n = 3$

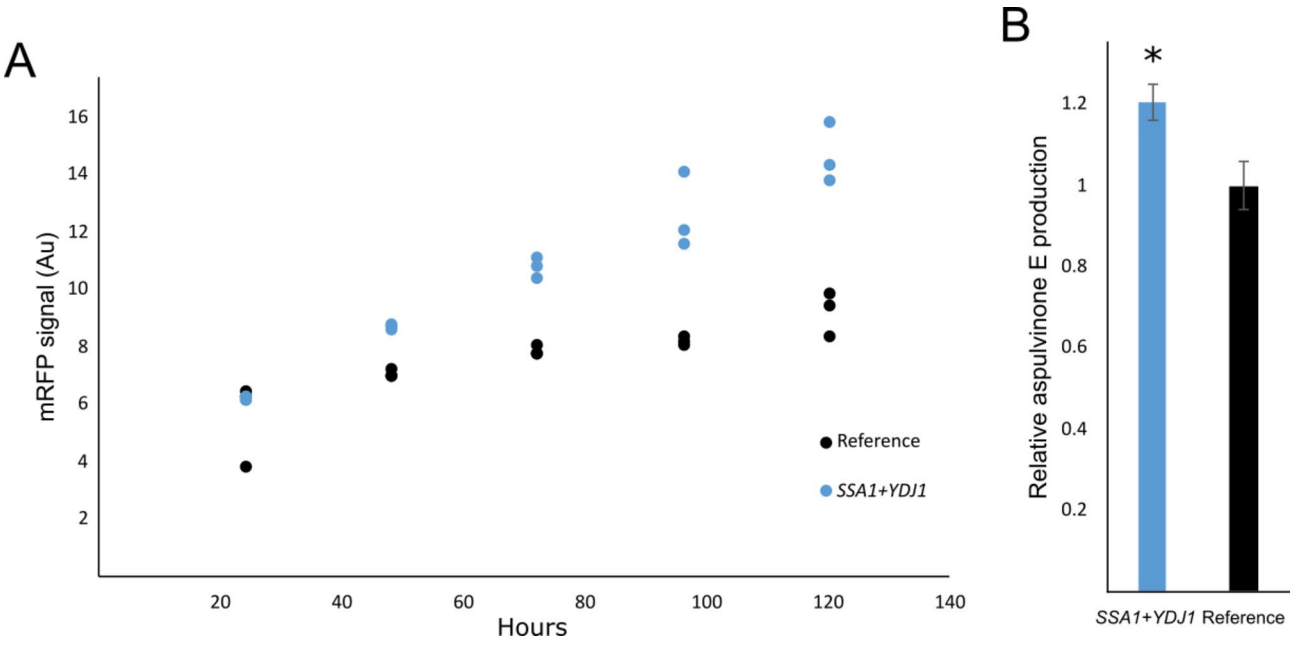

Fig. 5 mRFP signal and aspulvinone E production in strains transformed with MelA C-terminally tagged with mRFP. **(A)** mRFP signal measured for a 100 mL SD-urea shake-flask culture over the course of 120 h. The background signal observed for CEN.PK113-7D was subtracted from the *SSA1* + *YDJ1* strain and the reference strain (APS008) values. **(B)** aspulvinone E production in *SSA1* + *YDJ1* and reference strain harvested at 120 h. Columns represent average aspulvinone E production, as measured by the UV absorption at 368 nm of the peak corresponding to aspulvinone E and normalized to the reference strain. The asterisk indicates that the difference in aspulvinone E production is significantly different from the reference strain ($p < 0.05$). Error bars represent ± SD, $n = 3$

colonies that were significantly ($p < 0.05$) more fluorescent than the reference strain. Secondly, the two trials identified the same strain, *SSA1* + *YDJ1*, as the top hit. Thirdly, a comparison of the top-ten hit-lists of the two trials showed that nine strains are the same. Fourthly, the two strains, *HSC82* + *SSA2* and *SSA1* + *HSC82*, which differ between the two top-ten lists, were both ranked as hit-strain number eleven in the trials where they were not in the top-ten hit-list. Fifth, when we examined the effect of overexpression of a single chaperone in trials 1 and 2, we found only one hit in top-ten, *YDJ1* (ranked second and third in trials 1 and 2, respectively). The remaining top hit strains contain combinations of two different genes and typically they share a gene. For

example, *SSA1* is present in five and seven of the top-ten hit-strains of trials 1 and 2, respectively, including a strain that contains a double dose of *SSA1*. Other genes that appear more than once in each of the top-ten hit-lists are *HSC82*, *SSA2*, *STI1*, and *YDJ1*. Sixth, as a variation of the latter theme, we note that strains containing both *SSA1* and *SSA2* are in the top-ten list of both trials. The two genes are paralogs encoding proteins where the amino acid sequences are 98% identical and have a large overlap in physical protein interactions [27]. Finally, the gene product of the two genes identified as the best hit in our screen, *SSA1* and *YDJ1*, are known to interact. Ydj1 acts as a co-chaperone that stimulates ATPase activity of HSP70 proteins, including Ssa1, to promote maturation of client proteins [28, 29]. In this context, we note that a synergistic effect of simultaneous overexpression of both *SSA1* and *YDJ1* from *S. cerevisiae* on heterologous protein production has previously been reported in *Komagataella phaffii* [30] and our results may well reflect this interaction. When validating the screen observations, all the selected chaperones resulted in increased fluorescence and in the case of the best hit, *SSA1* + *YDJ1*, this translated into an 84% increase in aspulvinone E production in liquid media. Altogether, our results strongly suggest that our screening method is able to identify promising chaperone candidate genes that improve production of aspulvinone E.

It is important to stress that aspulvinone E production depends on only two heterologous pathway enzymes: a simple synthetase containing relatively few functional domains and a PPTase. Production of most other SMs is more demanding and depends on more complex synthases/synthetases with a larger number of functional domains and/or on more pathway enzymes [2]. The fact that a simple pathway like the aspulvinone E biosynthetic pathway benefits from the overexpression of two chaperones suggests that more complex enzymes and biosynthetic pathways may benefit even more from additional chaperone overexpression. Indeed, increasing protein size and number of protein domains, as measured by the number of Pfam annotations, have been found to be positively associated with the number of chaperone interactions in *S. cerevisiae* [22]. It is therefore likely that our approach will also be useful for a large range of other SMs.

The combinatorial effect of chaperone overexpression may not be trivial to predict. For example, in our experiments, we found that individual overexpression of *YDJ1*, but not *SSA1*, results in improved aspulvinone E production. Hence, in the case that no insights into the function of the two genes had been available, it would have been difficult to predict the additive effect of the combination mutant. Simple methods to further combine chaperones in the library are therefore desirable. This can be achieved by performing iterative screens employing new query strains, which contain selected chaperone hit(s) identified in a previous screen.

In the current version of our method, the pathway and chaperone library are combined by simple mating. A potential drawback of this method is that gene doses are diluted in the resulting diploid strains, which are heterozygous for SM genes and chaperone genes. Hence, the possibility exists that chaperone effects are more pronounced in a haploid setup, and it may therefore be advantageous to combine the genes in a haploid background. We envision this can be accomplished by adapting the library for technologies like synthetic genetic array (SGA) or CRI-SPA [24, 31]. Furthermore, the repertoire of libraries could be expanded to include additional genes. For example, it has previously been shown that terpene biosynthesis in *S. cerevisiae* may be improved by co-expression of chaperones originating from the same host plant as the SM pathway of interest [13]. Similarly, genes encoding transporters or genes involved in detoxification could beneficially be included in the library.

Chaperones supporting aspulvinone E production could easily be identified due to the fluorescence of aspulvinone E under UV light. However, many SM products do not allow for simple measurements. For products that are not easy to quantify in a simple assay, strain analysis of production could be achieved by e.g. HPLC-MS analysis of liquid microtiter-plate cultivations. For a library of this size, this is possible but may pose a severe bottleneck. To this end we stress that our premise for optimizing SM pathways by employing chaperones is that they may enhance folding of pathway enzymes. Previous improvement of the terpene protopanaxadiol in *S. cerevisiae* by chaperone gene overexpression has been shown to be linked to increased abundance of pathway enzymes [13]. Indeed, for aspulvinone E production, we demonstrated that enhanced product titers were accompanied by higher levels of a C-terminally mRFP tagged version of the MelA synthetase. For hard to detect products, it may therefore be possible to identify beneficial chaperones by screening the library for chaperones that increase the levels of key proteins in the SM pathway, which have been tagged with fluorescent proteins. With this approach, we envision that our strategy can be used to successfully optimize the production of a wide range of SMs.

Materials and methods

Strains and media

Escherichia coli strain DH5α was used for cloning and plasmid propagation. For selection of DH5α containing plasmids, cells were grown in solid or liquid Luria-Bertani (LB) media supplemented with 100 μg/mL ampicillin. All *S. cerevisiae* strains used in this work were based on CEN.PK113-7D, CEN.PK113-5D and CEN.PK113-3B

kindly provided by Dr. P. Kötter, University of Frankfurt [32]. All strains used in this study are listed in (Table S1). Yeast-extract peptone dextrose (YPD), synthetic complete (SC) media and SC drop out media were prepared as described by Sherman et al. [33]. For preparation of YPG or SC-gal media containing galactose as the sole carbon source, galactose was dissolved in demineralized water and filter sterilized into sterilized YP or SC to a final concentration of 2% (w/v). For *Kl.URA3* counter selection, SC plates were supplemented with 1 g/L 5-fluoroorotic acid (5-FOA) and 30 mg/L uracil. For solid media 20 g/L bacto agar was added to the media prior to autoclavation. For selection on solid medium, plates were supplemented with 200 mg/L geneticin (G418), 100 mg/L nourseothricin (NTC) and 200 mg/L hygromycin (HYG) as indicated. SD-urea contained 1.7 g/L yeast nitrogen base without amino acids and ammonium sulfate, 2.27 g/L urea, 2.44 g/L $Na_2HPO_4.2H_2O$, 11.91 g/L $NaH_2PO_4.H_2O$, 20 g/L glucose and 90 mg/L tyrosine. The pH was adjusted to 6.0 through the addition of KOH.

Plasmid construction and PCR

Generally, DNA assembly was done by USER-fusion using uracil-specific excision reagent (USER™) enzyme from New England Biolabs. PCR fragments were amplified using Phusion U Hot Start DNA Polymerase (Thermo Fisher). Purification of DNA fragments obtained by PCR or from agarose gel bands was done using the illustra GFX PCR DNA and Gel Band Purification Kit (Cytiva). All primers used during the study are listed in (Table S3). All of the plasmids used in this study are listed in (Table S4).

Strain construction

All yeast transformations were carried out using the LiAc/SS carrier DNA / PEG method [34]. When antibiotic resistance genes were used as markers, cells were allowed to recover for 2 h in liquid YPD or YPG, depending on the promoter controlling the resistance gene, prior to plating on solid selective media.

The chaperone gene library members (Chap 001-028) were constructed by transformation of CEN.PK113-5D with NotI (New England Biolabs) linearized plasmids (pX-4 and PL-001-PL-027). The *Kl.URA3* flanked by direct repeats was eliminated in strains Chap 019 and Chap 021 by counterselection on solid SC-5-FOA. The resulting strains were subsequently transformed with NotI linearized plasmids (pX-2 and PL-28-PL-48).

The query strain (AQS002) was constructed by transforming CEN.PK113-3B with NotI linearized PL-49. The *Kl.URA3* flanked by direct repeats was eliminated by counterselection on solid SC-5-FOA resulting in the strain AQS001. Subsequently, 900 bases UP and DOWN from the *ura3-52* locus were amplified and fused with a P_{GAL10}-*KanMX* PCR amplified fragment by treatment with USER enzyme and T4 DNA ligase. The resulting fragment was used as a template for amplification of a gene targeting substrate for replacement of *ura3-52*. The resulting gene targeting substrate was subsequently used to transform AQS001 to replace the endogenous *ura3-52* and selected on solid YPG-G418, resulting in AQS002.

Strains for validating screen observations (APS001-006) were constructed by transforming strains Chap 001, Chap 027, Chap 041, Chap 049, Chap 063 and Chap 071 with NotI digested and gel purified PL-50. Successful transformants were selected on solid YPD-NTC. APS007-APS008 for the evaluation of effect of *SSA1-YDJ1* overexpression on MelA synthetase levels Chap 001 and Chap 049 were transformed with pHO29, pDIV023 and NotI digested and gel purified PL-52. Successful transformants were selected on solid YPD-HYG-G418. To test the effect of an additional copy of the *melA* gene, APS006 was transformed with NotI digested and gel purified PL-51, PL-53 and pDIV023 and successful transformants were selected on solid YPD-HYG-G418 resulting in APS009. All strains were validated by diagnostic colony PCR to ensure correct integration at the intended chromosomal locus.

Mating and screening

Automatic pin replication was carried out using the high-throughput pinning robot ROTOR HDA from Singer Instruments (United Kingdom), along with replica pinning pads (RePads) and rectangular petri dishes (PlusPlates) from the same company. In preparation for screening, the chaperone library organized in six 96 well plates (Table S5) were arrayed on three solid YPD plates in a 384 grid (Fig. S3) and grown at 30 °C O/N. On the same day, AQS002 was inoculated in 10 mL liquid YPD and cultured at 30 °C with 200 rpm shake O/N. The O/N culture of AQS002 was distributed as 150 µL aliquots into a 96 well flat bottom plate (Grenier) serving as a source plate. The AQS002 source plate was pinned in a 384 grid on three solid YPG plates serving as the mating plates. Subsequently the chaperone library was combined with the query strain on the mating plate by pinning from the arrayed chaperone library onto the mating plate and allowed to mate for 8 to 12 h. The mating plates were pinned onto solid SC-Ura + Gal + G418 to select for diploids and incubated at 30 °C for 24 h. To select for diploids the step was repeated by pinning colonies onto fresh SC-Ura + Gal + G418 plates. After 24 h the colonies were pinned onto solid SC plates and incubated at 30 °C for 72 h after which pictures were taken in a Phenobooth with the following parameters: Lighting mode: UV-light, Fluorescence Filter: 527 nm with 20 nm bandwidth (Edmund Optics), Exposure: 400, Gain: 0.1, Hue: 0, Lighting power: 0.3, Saturation: 0.25, Brightness:

0, White Balance Blue: 1, White Balance Red: 1. To assess fluorescence intensity observed for individual colonies, the script previously described by *Cachera et al.* was used [24]. Colony fluorescence intensity was scored using the rgb2gray function from the scikit-image package.

Phenotypic validation on solid media

Strains were inoculated in 3 mL YPD and cultivated O/N at 30 ° C with 200 rpm shake. The following day, aliquots of 200 µL O/N cultures were transferred to 96 well flat bottom Grenier plates and Singer 96 long pins were used to transfer colonies to solid SC Plus plates in a 384 grid. Plates were incubated at 30 °C for 72 h after which images for determining aspulvinone E fluorescence were taken in a Phenobooth.

Biolector cultivation

Cultivations were performed in triplicate. Strains were inoculated in 3 mL YPD and cultivated O/N at 30 °C with 200 rpm shake. The following day the O/N cultures were used to inoculate 1.5 mL SC or SD-urea to a starting OD_{600} = 0.02 and cultivated at 30 °C with 1000 rpm shake and 85% humidity for 72 h, after which a chemical extraction was performed.

Shake-flask cultivation of strains expressing mRFP tagged *melA*

Cultivations were performed in triplicate. Strains were inoculated in 3 mL YPD and cultivated O/N at 30 °C with 200 rpm shake. The following day the O/N cultures were used to inoculate 100 mL SD-urea to a starting OD_{600} = 0.02 and cultivated at 30 °C with 150 rpm shake. Over the course of the cultivation samples were taken every 24 h to measure OD_{600} and mRFP signal. To measure mRFP signal, 200 µL culture broth was transferred to a flat bottom 96 well plate (Grenier) and mRFP signal was measured in CLARIOstar Plus plate reader using excitation of 570 nm ± 15 nm and measuring emission at 620 nm ± 20 nm.

Statistical analysis

Statistical significance was determined by unpaired t-test. For the two screens of the chaperone library, the *p* value was Bonferroni corrected to account for the large number of comparisons.

Chemical extraction and HPLC-MS/MS-DAD analysis

For chemical extraction of aspulvinone E, 1 mL of culture broth was transferred to a 2 mL Eppendorf tube and centrifuged at 16,000 g for 2 min. The supernatant was transferred to a new 2 mL Eppendorf tube and 1 mL of ethyl acetate was added to the cell pellet. The cell pellet was ultrasonicated for 40 min. The supernatant was added to the cell pellet and the mixture was incubated at RT O/N with 150 rpm shake. The extracts were centrifuged at 12,000 g for 2 min and 0.8 mL of the ethyl-acetate phase was transferred to a new 2 mL Eppendorf tube. The extract was dried under a flow of N_2. 150 of µL methanol was added to the dried pellet and the extract was centrifuged at 20,000 g for 10 min. 80 µL cleared extract was transferred to a HPLC vial for analysis.

The chemical analysis of samples from the Biolector cultivations was conducted via ultra-high performance liquid chromatography-diode array detection-time of flight mass spectrometry (UHPLC-DAD-TOFMS) on an Agilent UHPLC-QTOF G6545 mass spectrometer (Agilent Technologies), which used an electrospray ionization source (ESI). The sample separation was carried on an Agilent Infinity 1290 UHPLC with a Poroshell 120 Phenyl Hexyl column (2.1 × 150 mm, 1.9micron) (Agilent Technologies) kept at 40ºC. The mobile phase was a gradient mixture of water (A) and acetonitrile (B). Both A and B contained 20 mM formic acid. The gradient profile was started at 10% B and increased to 100% B within 10 min, then kept at 10% for 2.5 min, dropped at 10% in 10 s and was left to equilibrate in starting conditions until 14 min time. The mass spectra were recorded for a mass range of *m/z* 75-1250 in the negative MS mode, and the UV spectra were collected from 190 to 640 nm.

The chemical analysis of samples from the shake flask cultivations was conducted via ultra-high performance liquid chromatography-diode array detection-time of flight mass spectrometry (UHPLC-DAD-TOFMS) on a Bruker Maxis HD QTOF mass spectrometer, equipped with an electrospray ionization source (ESI) operating in negative mode. The sample separation was carried on an Agilent Infinity 1290 UHPLC with a Poroshell 120 Phenyl Hexyl column (2.1 × 150 mm, 1.9micron) (Agilent Technologies) kept at 40ºC. The mobile phase was a gradient mixture of water (A) and acetonitrile (B). Both A and B contained 20 mM formic acid. The gradient profile was started at 10% B and increased to 100% B within 10 min, then kept at 10% for 2.5 min, dropped at 10% in 10 s and was left to equilibrate in starting conditions until 14 min time. The mass spectra were recorded for a mass range of *m/z* 50-1400 *m/z* in negative MS mode, and the UV spectra were collected from 190 to 640 nm.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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Author contributions
W.N., A.H.M. and U.H.M. designed research. A.H.M. and W.N. performed research. A.H.M., W.N. and P.C. analyzed data. U.H.M. supervised the research. A.H.M., W.N. and U.H.M wrote the paper.All authors revised the manuscript.

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Data availability
Data is provided within the manuscript or supplementary information files.

Declarations

Competing interests
The authors declare no competing interests.

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