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Peer Review File

Design of the biosensor-dependent coupling system stabilizes the high-synthesis phenotype of cell factory

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript describes a strategy for constructing a metabolite-based biosensor responsive to GA and applying it to improve product formation through evolution engineering. Specifically, the study begins by identifying GA-responsive gene using comparative transcriptomic analysis. Following biosensor optimization, GA production was coupled to cell growth by placing the essential gene HIS3 under the control of a GA-responsive promoter (PDR5). Through serial transfer cultivation, a single colony was identified that exhibited significantly improved production of GA and licorice triterpenoids. Furthermore, single-cell transcriptomic analysis revealed that a substantial portion of the evolved population remained high biosynthetic efficiency. While many trategies utilized have been previously reported, the study presents some new insights into PDR5 regulation and heterogeneity issue in cell factories. However, several critical issues concerning the experiment design and data interpretation require further clarification.
Major Concerns:
1, The GA (or upstream intermediates)-responsive expression of PDR5 appears to rely on transcriptional regulation. The manuscript suggests that transcription factors Pdr1/3 and/or Stb5 may be involved. However, additional data are required to confirm whether these transcriptional factors directly respond to GA or its intermediates. Clarifying this would significantly enhance our understanding of the regulatory mechanisms involved.
2, If the improved dynamic range of the biosensor corresponds to increased PDR5 expression, this may reduce the selective pressure when HIS3 is placed under its control. Since the aim of coupling biosensor activity to essential gene expression is to apply selective pressure that stabilizes genotype during evolution, the rationale for increasing dynamic range needs to be clearly explained.
Additionally, if 3AT is introduced to strengthen selection via the biosensor system, how stable is the genotype of the evolved strains under no selective pressure? For instance, what is the performance of the evolved strain A8 after being transferred for 6 or 18 days without 3AT?
Other Issues:
L117, it stated " a series of genes were selected". How many genes were initially selected and tested? If only 12 genes were evaluated (as shown in Fig1c), what was the basis for selecting these 12 out of 215 upregulated genes? Moreover, why did only PDR5 show enhanced fluorescence while the others showed decreased signal (Fig1c), especially if they were all selected from the same upregulated gene set?
Fig1c, the figure seemed to imply GA directly interacts with the promoter regions of the tested genes. Is this correct, or is the interaction mediated through transcription factor(s)?
Fig 1d, e, and f showed intracellular GA production in relation to the fluorescence response. Was GA accumulation exclusively intracellular under these conditions, or was there also extracellular GA detected?
Fig1g, when GA was added into the culture, how much was taken up by the cells (i.e., what were the intracellular concentrations)?
Fig2, it was not clear whether the compounds shown were produced endogenously by the yeast cell factories or externally supplemented. Fig2a, in particular, needs clearer explanation, i.e., does the indicated concentration refer to the maximum fluorescence achieved or the maximum concentration that the engineered yeast cell factories were able to produce?
L240, what regulatory features did PE25 and PE31 promoters contain?
L245, "single colonies were then randomly picked" was mentioned. Could the authors clarify the source of these colonies? Were they picked at the final stage of the evolution experiment?
Fig4b shows normalized GA production per biomass. Could the authors provide the actual biomass concentrations used under these different conditions?

Reviewer #2

(Remarks to the Author)
Reviewer Summary
The authors discovered a responsive element (PDR5 gene) to the presence of GA; by fusing its translation to fluorescent protein production it formed a biosensor for GA. Firstly its responsiveness to GA concentration, specificity and its potential interference by cell stress conditions was tested, then an attempt at dynamic range enhancement by promoter engineering which was initially modest (5-fold) but greatly enhanced by knocking out two genes that interact with PDR5: Ssz1 and Rdr1. Using a 5-fold enhanced dynamic range PDR5 promoter, coupled to HIS3 gene expression, histidine production (hence cell growth) was regulated by PDR promoter activity. In addition, an azole histidine synthesis inhibitor was co-incubated to further impede histidine synthesis (as the coupled receptor was not impactful enough). Ultimately an engineered form of the PDR promoter with lower dynamic range, and in the absence of azole, was most effective at enhancing GA production by the cell.
Bulk cell transcriptomic analysis was conducted comparing the highest producing isolate (A8) and GA184, which was a control but unspecified. The basis for the GA production differences between the strains was confirmed by higher expression of HIS3 and major GA pathway genes, upregulated INO1 indicating phospholipid synthesis and downregulation of ergosterol synthesis genes.
Finally, single cell transcriptomic analysis was conducted on approx. 14000 cells divided between A8 and the control. This sorted the transcriptomes into patterns of upregulation/downregulation and into 9 clusters of similar gene expression patterns among the strains. This analysis showed clear delineation of gene expression between A8 and the control and confirmed the bulk cell transcriptomic analysis and higher HIS3 and GA pathway gene expression in A8. The analysis also identified several potential gene targets in the control which could influence GA production. Two strains that had deletion of 1 of 2 target genes were deleted gave higher GA yields; other knockouts identified from the single cell transcriptomic analysis resulted in lower GA yields.

Review
The manuscript does not universalise the concept that was promised in this work. It was claimed that the manuscript “developed a universal paradigm for the construction of biosensors and their subsequent application in stabilizing and evolving the genotype of cell factories” but the work is restricted to the example, GA biosynthesis, and no attempt to broaden the concept in either the Results or Discussion was made.

Indeed, some key aspects are missing (e.g. how 12 upregulated genes in GA exposure were selected as potential biosensors, what role does biosensor dynamic range have in potentially increasing yield, what is considered a ‘control’ in this kind of research). This knowledge is essential for extending the applicability of this concept. Some of these queries are detailed further below.
The single cell transcriptomics analysis produced some interesting results as any opportunity to look deeply into a yeast culture and infer its performance via gene expression can do. In this case, the analysis seemed an add-on and not central to the point of the paper. The bulk cell transcriptome analysis already showed the key gene expression differences between GA184 and A8.

The concept of using biosensors to indicate positive directions of genetic/metabolic engineering and high throughput screening or adaptive laboratory evolution is not new. The selection of PDR was based on earlier work and others that have coupled biosensor response to growth. A selection of relevant research and review articles is given below.

Li et al (2019) quoted by the authors identified the action of PDR and used this biosensor approach (fluorescent biosensor based on PDR) in their study. That is, they state “here we developed a small molecule-responsive biosensor that couples transcriptional induction of PDR genes to growth rate in the yeast Saccharomyces cerevisiae “ (Li J, Kolberg K, Schlecht U, St Onge RP, Aparicio AM, Horecka J, Davis RW, Hillenmeyer ME, Harvey CJB. A biosensor-based approach reveals links between efflux pump expression and cell cycle regulation in pleiotropic drug resistance of yeast. J Biol Chem. 2019 Jan 25;294(4):1257-1266. doi: 10.1074/jbc.RA118.003904)
Krüger, A., Göddecke, J., Osthege, M. et al. Biosensor-based growth-coupling as an evolutionary strategy to improve heme export in Corynebacterium glutamicum. Microb Cell Fact 23, 276 (2024). https://doi.org/10.1186/s12934-024-02556-1
Dacquay LC, McMillen DR. Improving the design of an oxidative stress sensing biosensor in yeast. FEMS Yeast Res. 2021 May 1;21(4):foab025. doi: 10.1093/femsyr/foab025. PMID: 33864457; PMCID: PMC8088429.
Yongkun Lv, Shuai Qian, Guocheng Du, Jian Chen, Jingwen Zhou, Peng Xu, Coupling feedback genetic circuits with growth phenotype for dynamic population control and intelligent bioproduction, Metabolic Engineering,54,2019,109-116, https://doi.org/10.1016/j.ymben.2019.03.009.
Lv Y, Gu Y, Xu J, Zhou J, Xu P. Coupling metabolic addiction with negative autoregulation to improve strain stability and pathway yield. Metab Eng. 2020 Sep;61:79-88. doi: 10.1016/j.ymben.2020.05.005.
Many approaches to growth coupled chemical bioproduction have been reviewed in the following (and others)
Gazi Sakir Hossain, Mukesh Saini, Ryoma Miyake, Hua Ling, Matthew Wook Chang, Genetic Biosensor Design for Natural Product Biosynthesis in Microorganisms, Trends in Biotechnology,38, 2020, 797-810, https://doi.org/10.1016/j.tibtech.2020.03.013.
Yoshihiro Toya, Hiroshi Shimizu, Coupling and uncoupling growth and product formation for producing chemicals, Current Opinion in Biotechnology, 87, 2024, 03133, https://doi.org/10.1016/j.copbio.2024.103133.
Alter TB, Ebert BE. Determination of growth-coupling strategies and their underlying principles. BMC Bioinformatics. 2019 Aug 28;20(1):447. doi: 10.1186/s12859-019-2946-7

Further comments regarding suggestions on improving the manuscript, the lack of or unclear experimental details, language, and results interpretation are provided.

Abstract

It is difficult to get any sense of the novelty of the study from this abstract. There is some strange phrasing used (see General comments) which adds to the difficulty in getting value from it. There are no measures of impact in the whole abstract – no indication of the extent of enhancement for example is given; no gene names, no promoter name or how the biosensor works, the overall impact etc.

Introduction

It may be a personal dislike but I don't find the idea that a research area is a "hot research topic" a very compelling reason to do research. Also, proponents of ALE are unlikely to consider the approach 'irrational'.

The introduction would benefit from being rewritten to improve clarity and to do justice to the literature that is touched on. Given the complexity (lines 71-75) of developing a biosensor and using it to evolve strains, taking this approach needs stronger justification. What are the specific drivers of this research? What is the novelty? Why is this a "universal paradigm" or "universal workflow" when only GA synthesis is described?

The sentences (lines 76-88) relating to within culture differentiation are a sharp direction change from the rest of the introduction and look like they have been parked there to justify undertaking single cell transcriptomics. Line 84 suggests within isogenic cultures experiencing nutritional variability there is always a differentiation of labour among cells but then it is confusingly concluded that "We surprisingly found that the high-production yeast population exhibits division of labor, using single-cell transcriptome sequencing." .

Results

Line 115

Fig 1b shows 1109 genes were upregulated in the GA109 strain compared to control and the text reads "215 with significantly upregulated expression and 936 genes with significantly downregulated expression"

Ultimately only 12 genes are listed in Table S1 and ultimately tested. How were these selected? What was considered "significant"? Table S1 does not show fold upregulation for the selected genes and the criteria for selection of these 12 genes are not clear.

Line 188

"and fluorescent proteins were utilized to characterize the promoter response" Suggest restating this to more accurately reflect what you have shown in Fig 1c.

PDR response in GA180 is enhanced compared with the control strain because the former is producing GA

In Fig 1f and 1g,

was GA added to control yeast strains to test the responsiveness of PDR to GA? This would seem like the logical experiment to show that this is an accurate biosensor.

Glycyrrhetinic acid is considered water insoluble. How was it delivered to yeast cultures?

In Fig 2,

the potential impact of a range of stressors and environmental agents were tested against non-GA producing yeast expressing the PDR biosensor. In the absence of stressor (Fig 1a) control yeast produced up to 250 FU at 120 h. If all conditions were the same for Fig 2a and b, why are the FU so different? That is, control is 100 FU and GA elicits a response of 205 FU at 120 h.

What is meant by "(b) The response of the GA biosensor to other natural products in the corresponding engineered strains." What are the corresponding engineered strains? Werent molecules 1 – 10 added to the flasks at different concentrations?

Line 159 onwards

Extensive empirical promoter engineering was undertaken to attempt to increase the dynamic range of its response with little impact. Was this result surprising?

Fig 3 data.

Do all these attempted promoter engineering results need to be in the main document? Most do not make an important difference.

Line 228 :

"Consequently, the strains evolved in YPD media exhibited a dramatic reduction in triterpenoid production indicating that the GA-producing trait is not steadily inherited as the production process of GA may exerting a burden on cell growth"

This looks to be a classic case of loss of plasmid pRS415 from the cells in rich media. The selection pressure to keep the plasmid was removed as histidine is drawn from the media. As cells divide there is no benefit to retaining the plasmid. Also the azole is not effective.

Line 231

"The heterologous genes were almost lost as the strain continuous cultivation without a selective pressure in YPD media (Fig. 4e and Supplementary Fig. 2)."

This is very surprising as these heterologous genes (2CYPs and CPR) are integrated into the yeast genome. The absence of the genome-integrated pathway genes at 18 d in YPD suggests that either the gene insertions are unstable and prone to excision or that the culture was overgrown by wild type yeast. Could you please comment.

Line 233

I'm struggling to understand these two seemingly contradictory points:

"In contrast, the evolution in SD-His media made the producing phenotype and genotype more stable, due to the coupling of growth with biosynthesis. Furthermore, the addition of 3-AT led to an increased correlation between growth and GA production"

The more the yeast needs to produce histidine via the PDR-PE23 promoter (with 5-fold dynamic range) plus the effect of azole, the more GA is produced. That makes sense.

"As anticipated, the strains employing PE25 and PE31 exhibited substantial increases in GA production. The strains with

PE31 evolved for 6 days achieved the highest production level, in which the production of GA increased by 17.5% and the production of total triterpenoids increased by 11.4% (Fig. 4d)."
PDR-PE25 and -PE31 have lower dynamic range (~2-3.5-fold) and no azole was added to enhance the endogenous histidine requirement. Why was it anticipated that this would result in a substantial increase in GA production?
Line 265
"strain A8 and the control strain (cultured in YPD medium for 6 days) were analyzed"
What is the "control" strain for these experiments and why was this strain selected? In Fig 5 is shown as GA184 but what does that signify?
Line 281
The downregulation of ergosterol synthesis pathway genes in the A8 in comparison to GA184 was interesting and no doubt beneficial for obtaining a higher yield of GA in that strain. It is stated that "It is evident that, through biosensor-dependent evolution, this balance can be rapidly achieved."
But this was not made clear in the manuscript. Were there any negative phenotypical consequences for the ergosterol synthesis downregulation evident in the A8 strain? (these effects have been extensively reported e.g. Johnston EJ, Moses T, Rosser SJ. The wide-ranging phenotypes of ergosterol biosynthesis mutants, and implications for microbial cell factories. Yeast. 2020 Jan;37(1):27-44. doi: 10.1002/yea.3452). There may have to be a choice between poorly growing strains/high GA and normal growth/moderate GA.
Line 381
PIR1 is a described as a "newborn marker gene", "cluster 0 are in a state of neogenesis"
PIR1 is a structural constituent of the cell wall involved in intracellular protein transport and cell wall organization; also localizes to the bud scar and nuclear periphery. According to ref 41 PIR1 is a marker for early G1 phase but does not distinguish between mother or daughter cells (they suggest use of DSE2 which is only expressed in daughter cells).
The genes upregulated PIR1, EXG1, HSP150, CRH1, NCW2, PST1, and CWP2 are all involved in cell wall synthesis and remodelling. Together their upregulation could indicate budding or cell wall remodelling for other reasons.
It is not clear from the clustering description (line 381 onwards) what the authors are suggesting. Doesn't the clustering represent major patterns of gene expression identified in individual cells. Most of the cells have patterns of gene expression (up-, down and no change) that locate to one of nine patterns. These are a different way of grouping the cells compared with the UMAP graphs.
Line 329:
"The low RNA feature of cluster 7 indicates that this cluster may be undergoing the process of aging"
what does the low RNA feature mean? Cluster 7 has a lower number of "features" compared to some of the clusters as does cluster 5. The gene expression pattern for cells in cluster 7 shows upregulation of stress response genes like HSP12, DDR2 and down regulation of GA pathway genes. Interestingly, it is mainly A8 cells this cluster.
Line 343
"In cluster 6, gene ADH2, ADY2, ATO3, and CTA1 were significantly upregulated. These genes have been shown to regulate the treatment with ethanol, acetic acid, ammonia, and reactive oxygen species, respectively."
These are either metabolism genes or transporters. I don't think it is accurate to say they regulate the treatment with those substances.
Line 412:
"For the control strain, subculture results in a loss of the strain capacity for synthesizing licorice triterpenoids."
Again, what strain is meant by 'control' is never clear. Loss of GA production seemed to occur during subculture in rich media (YPD) where the plasmid coding for HIS3 was lost during replication.
Line 420:
"It can therefore be inferred that their role may be to maintain the sustainability of high-production cells (cell regeneration and protein renaturation) and the stability of growth environments"
This inference is likely projecting well beyond the data. The different gene clustering within the high performance A8 strain could represent cells in a distinct life stage to cluster 0, or the gene expression pattern may result from the gradient of nutrient and oxygen availability across the culture.
Line 425:
"Whereas, within the control strain, there was a progressive decline in the capacity for product synthesis, attributable to the absence of biosensor-dependent synthesis and growth coupling."
There was no time based analysis in this single cell transcriptomic analysis so what is meant by a progressive decline? The single cell transcriptomes show that A8 and Control have different gene expressions which are consistent with the differences in GA production and lower HIS3 expression.
"Consequently, the cells gradually degenerated into metabolic characteristics dominated by growth"
This is poor phrasing. Cell undertaking normal metabolism for growth shouldn't be termed degenerated.

General:
At many points, the manuscript lacks scholarly treatment, ie sentences lack key words and details which results in them sounding very general or facile. I recommend a complete and thorough edit and a rewrite of the Introduction.

Some phrasing needs to be reconsidered such as:
Line 219 and onward "growth-production coupling strategy"? Perhaps coupled growth-production is better
"evolutionary strains" should be evolved strains

These terms and phrases need to be reconsidered:
"improving the efficiency of synthetic products"
high-effective screening of cell factory products
strategy of coupling synthesis and growth prevents the degeneration of cell synthesis ability.

the biosensor-dependent evolution of synthetic chemicals in cell factories
difficulties of enabling strains to evolve in a synthesis-oriented manner.
lead to strain degeneration
an absence of methodologies capable of impeding the descent of cells towards a state of diminished synthesis ability.
can potentially trigger exclusionary responses in the organism

Reviewer #3

(Remarks to the Author)
This study focuses on designing a biosensor-dependent coupling system to stabilize the high-synthesis phenotype of yeast cell factories for licorice triterpenoid production, It constructs a biosensor targeting glycyrrhetinic acid (GA), a licorice triterpenoid, optimizes its dynamic range through promoter engineering, and designs genetic circuits coupling product synthesis with cell growth. Via adaptive evolution, it significantly enhances licorice triterpenoid yields. Single-cell transcriptomics reveals a population division of labor in evolved strains, with "highly efficient synthetic cells" and "viability-maintaining cells," offering new strategies for regulating microbial cell factory stability and improving synthesis efficiency. However, the study has limitations in experimental design rigor, such as insufficient specificity verification of the biosensor, and lack of reproducibility in single-cell sequencing, which somewhat undermine the persuasiveness of conclusions and the universal promotion of the method.

Detail comments below:
For experimental design details, regarding the biosensor, although the responsiveness of the PDR5 promoter to GA is confirmed, the dynamic concentrations of compounds like naringin chalcone, which it weakly responds to, in the GA synthesis system are not detected. Additionally, the molecular mechanism (e.g., interaction with Pdr1) by which Ssz1 knockout improves the dynamic range lacks verification. In the growth-synthesis coupling system, the non-specific metabolic effects of 3-AT are not excluded, the independent impact of nutrient differences in different media (YPD, SD-His, SD-His+3AT) on evolutionary results is not distinguished, and the passage stability of the high-producing strain A8 is not tested. In transcriptome and single-cell analysis, single-cell sequencing lacks biological replicates, the ADY2 and CTA1 knockout experiments lack complementary verification, and the function of population division of labor (e.g., sorting cells from different clusters for separate culture to verify synthesis and growth capabilities) is not confirmed through experiments.

For article structure, the "unclear mechanism of microbial population division of labor" proposed in the introduction only describes phenomena without delving into mechanisms in the single-cell analysis of results. Bulk transcriptome (Fig. 5) and single-cell data (Fig. 6) lack correlation analysis, such as the enrichment of upregulated genes in specific cell clusters. The discussion overly emphasizes the universality of the "universal paradigm" and insufficiently addresses limitations such as PPDR5's dependence on hydrophobic compounds and the off-target risk of the coupling system in complex networks. Moreover, it fails to compare with similar studies in Cell Rep (2021) to clarify incremental contributions. Key information is missing in chart annotations (e.g., the correlation between the abscissa range and saturation points in Fig. 1g, the clusters corresponding to marker genes in Fig. 6k). Supplementary materials, such as the retention rate of heterologous genes in Supplementary Fig. 2, are not quantified, and the selection criteria for candidate genes in Supplementary Table S1 are not explained, resulting in insufficient support.

Judging from the title, there may be a lack of more examples to verify the author's concept of "biosensor-dependent coupling system stabilizes the high-synthesis phenotype of cell factory.

Lines 117-118 state, "To identify GA-responsive elements, a series of genes were selected from those with significantly upregulated expression". What is the basis for selecting these genes?

Line 119 mentions, "As a result, the PDR5 promoter (PPDR5) displays a marked fluorescent response to GA (Fig. 1c)". From Fig. 1c, it appears that other promoters (such as REE) also show responses to the target compound. What is the rationale for selecting only PPDR5?

Lines 146-148 state, "To examine the spectrum of PPDR5, its capacity to respond to other natural products (triterpenoids: compound 1-6, flavonoids: compound 7-10)". The article focuses on the response to GA throughout, so why test these seemingly unrelated substances here? This seems inconsistent with the main theme of the article, and it is suggested that the authors should instead consider substances related to the GA synthesis pathway.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have added additional information that addresses my concerns.

Reviewer #2

(Remarks to the Author)
Thank you for the comprehensive response and corresponding updates you have made to the manuscript and supplementary information. This revised manuscript has been a substantial undertaking and it has been done with care and attention to detail. My queries have been satisfactorily addressed.
One minor point is your use of the terms "strain degradation" and "degradation in cell factories". I think you could be more specific as to your meaning as they could be confused with cell death.

Reviewer #3

(Remarks to the Author)
All comments have been well replied. I suggest to accept the manuscript.

Reviewer #1 (Remarks to the Author):

The manuscript describes a strategy for constructing a metabolite-based biosensor responsive to GA and applying it to improve product formation through evolution engineering. Specifically, the study begins by identifying GA-responsive gene using comparative transcriptomic analysis. Following biosensor optimization, GA production was coupled to cell growth by placing the essential gene HIS3 under the control of a GA-responsive promoter (PDR5). Through serial transfer cultivation, a single colony was identified that exhibited significantly improved production of GA and licorice triterpenoids. Furthermore, single-cell transcriptomic analysis revealed that a substantial portion of the evolved population remained high biosynthetic efficiency. While many strategies utilized have been previously reported, the study presents some new insights into PDR5 regulation and heterogeneity issue in cell factories. However, several critical issues concerning the experiment design and data interpretation require further clarification.

Major Concerns:

1, The GA (or upstream intermediates)-responsive expression of PDR5 appears to rely on transcriptional regulation. The manuscript suggests that transcription factors Pdr1/3 and/or Stb5 may be involved. However, additional data are required to confirm whether these transcriptional factors directly respond to GA or its intermediates. Clarifying this would significantly enhance our understanding of the regulatory mechanisms involved.

Response:

We thank Reviewer for the positive evaluation of our work and their constructive suggestions, which significantly improved the manuscript. Below, we address each comment in detail.

PDR5 responds to GA, but not its intermediates (such as β-amyrin, 11-O-β-amyrin, glycerrhetol and glycerrhizin). This has been tested and shown in Fig.S2, and more explanation has been added in manuscript (Line 171-175).

Furthermore, Pdr1 was reported as a nuclear receptor that directly interacts with several drugs to stimulate expression of PDR5. To investigate the role of these TFs (Pdr1/Pdr3/Stb5) on signal transduction, these TFs was knocked out (Fig. 2d). Results showed that both pdr1, pdr3 and stb5 are necessary to correspond to GA. Docking result also shows that Pdr1 can directly bind to GA (Fig.S3).

2, If the improved dynamic range of the biosensor corresponds to increased PDR5 expression, this may reduce the selective pressure when HIS3 is placed under its control. Since the aim of coupling biosensor activity to essential gene expression is to apply selective pressure that stabilizes genotype during evolution, the rationale for increasing dynamic range needs to be clearly explained.

Response:

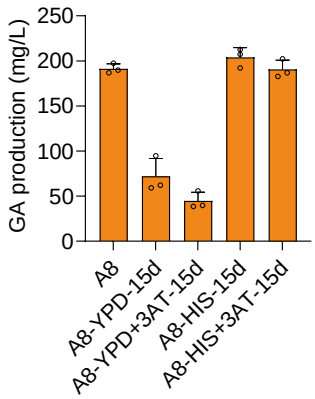
Biosensors need to increase or decrease their dynamic range depending on the application requirements. Since the aim of coupling biosensor activity to essential gene expression is to apply selective pressure, the dynamic range needs to be decreased (Fig. 3c). Subsequent experiments confirmed this (Fig. 3e). Here we revised the statement as “To enhance the applicability of GA biosensor for strain screening and evolution, it is imperative to be able to adjust its dynamic range” (Line 183).

Additionally, if 3AT is introduced to strengthen selection via the biosensor system, how stable is the genotype of the evolved strains under no selective pressure? For instance, what is the performance

of the evolved strain A8 after being transferred for 6 or 18 days without 3AT?

Response:

The selective pressure in this study mainly comes from the defects of His. And 3AT only plays a role in regulating the selective pressure. Our supplementary experimental results indicate that the phenotype (synthetic ability) of A8 remains stable after passage in a medium with selective pressure for 15 days (A8-HIS-15d) (see the figure below). While its synthetic ability still decreases after passage without selective pressure (A8-YPD-15d). The decrease in A8 production is not as significant as that of the starting strain GA184. This result is consistent with the design principles of our biosensors and reflects the important role of biosensors in constraining product synthesis.



Other Issues:

L117, it stated " a series of genes were selected". How many genes were initially selected and tested? If only 12 genes were evaluated (as shown in Fig1c), what was the basis for selecting these 12 out of 215 upregulated genes? Moreover, why did only PDR5 show enhanced fluorescence while the others showed decreased signal (Fig1c), especially if they were all selected from the same upregulated gene set?

Response:

Because GA is a lipophilic exogenous component, it may cause the expression of stress-related yeast drug transporters and other similar proteins. Therefore, the basis for the selection was the genes encoding transporters or hypothetical proteins with upregulated expression. Based on this, 12 genes were selected, and we listed the protein functions and fold changes in Table S1. The related statements in manuscript were revised.

The reason why some genes showed reduced induced expression in the experimental validation, except for *PDR5*, is probably due to the differences in sampling time, or the context effect of genes. More detailed information was supplied in Fig.1 Caption.

Fig1c, the figure seemed to imply GA directly interacts with the promoter regions of the tested genes. Is this correct, or is the interaction mediated through transcription factor(s)?

Response:

According to the experiments and the literature (Fig.2 and Fig.S4), GA plays direct binding role on Pdr1. Pdr1 was a nuclear receptor that directly interacts with GA to stimulate expression of PDR5.

Fig 1d, e, and f showed intracellular GA production in relation to the fluorescence response. Was

GA accumulation exclusively intracellular under these conditions, or was there also extracellular GA detected?

Response:

GA was detected primarily within cells. Following endogenous GA synthesis and exogenous GA addition, the intracellular and extracellular GA concentrations were quantified, as shown in Fig.S1.

Fig1g, when GA was added into the culture, how much was taken up by the cells (i.e., what were the intracellular concentrations)?

Response:

The intracellular and extracellular GA concentrations were quantified, as shown in Fig.S1.

In addition, we tested the GA solubility in different solvents. The solubility of GA in pure water is reported as 6.62 μg/L. Because GA solubility in complex growth media and fermentation broths has not been systematically reported, we directly measured it under conditions relevant to our study. A 4 g/L GA stock solution was prepared using ethyl acetate as solvent. To test the solubility of GA in the cell culture, the GA stock solution was added to the culture to achieve a final concentration of up to 200 mg/L. The soluble fraction of GA in YPD medium without yeast was 13 mg/L, whereas the cell-free fermentation broth collected after 5 days showed a higher apparent solubility of 34 mg/L. The increase observed during fermentation may be explained by medium changes that promote GA dispersion. For example, shifts in pH, accumulation of metabolic by-products, secretion of amphiphilic proteins or surfactants, and/or formation of colloidal or micellar phases that enhance apparent solubility. Crucially, when the yeast was added into the YPD medium (OD=2.71), the intracellular GA (measured after cell harvest, washing and organic extraction) reached 160 mg per liter fermentation broth, equivalent to a GA loading of 59 mg/L per OD cell culture. This indicates strong partitioning and accumulation within yeast and demonstrating that the GA levels are sufficient to support the function of the biosensors used in this study. GA is a membrane-affinity component, so it readily binds to organelles with membrane structures. Therefore, a GA concentration below 200 mg/L ensures complete absorption by the yeast.

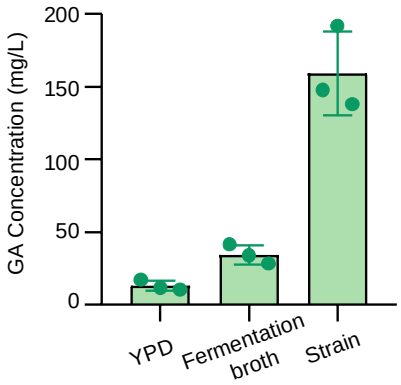


Fig2, it was not clear whether the compounds shown were produced endogenously by the yeast cell factories or externally supplemented. Fig2a, in particular, needs clearer explanation, i.e., does the indicated concentration refer to the maximum fluorescence achieved or the maximum concentration that the engineered yeast cell factories were able to produce?

Response:

In Fig.2a (current Fig.S2a), the compounds were exogenously added to the culture media in a

reasonable amount. The concentrations were designed based on the concentrations of cell metabolites under normal conditions.

In fig.2b (current Fig.S2b), the compounds were endogenously synthesized by the corresponding engineered strains. The detailed explanation has been added in manuscript and the Fig. S1 Caption and Table S2.

L240, what regulatory features did PE25 and PE31 promoters contain?

Response:

The regulatory features can be found in Fig.2e. And the explanation was added in the context: "To further reinforce the correlation between production and growth, we selected other engineered biosensors with reduced dynamic ranges (PE25 and PE31, which regulatory features are shown in Fig. 2e) to reduce the synthesis amount of histidine (Fig. 3c)." (Line 271-274)

L245, "single colonies were then randomly picked" was mentioned. Could the authors clarify the source of these colonies? Were they picked at the final stage of the evolution experiment?

Response:

The colonies were isolated after the evolution with PE31 for 6 days. Subsequently, yeast culture was dispersed onto SD-His medium plate. After the colony growth, 58 single colonies were randomly selected and inoculated into GM1 medium. Fermentation was conducted for 5 days, after which the yield was measured. This explanation was added in the context (Line 278) and Fig.3 Caption.

Fig4b shows normalized GA production per biomass. Could the authors provide the actual biomass concentrations used under these different conditions?

Response:

The actual biomass concentrations have been supplemented in Fig.3b.

Reviewer #2 (Remarks to the Author):

Reviewer Summary

The authors discovered a responsive element (PDR5 gene) to the presence of GA; by fusing its translation to fluorescent protein production it formed a biosensor for GA. Firstly its responsiveness to GA concentration, specificity and its potential interference by cell stress conditions was tested, then an attempt at dynamic range enhancement by promoter engineering which was initially modest (5-fold) but greatly enhanced by knocking out two genes that interact with PDR5: Ssz1 and Rdr1.

Using a 5-fold enhanced dynamic range PDR5 promoter, coupled to HIS3 gene expression, histidine production (hence cell growth) was regulated by PDR promoter activity. In addition, an azole histidine synthesis inhibitor was co-incubated to further impede histidine synthesis (as the coupled receptor was not impactful enough). Ultimately an engineered form of the PDR promoter with lower dynamic range, and in the absence of azole, was most effective at enhancing GA production by the cell.

Bulk cell transcriptomic analysis was conducted comparing the highest producing isolate (A8) and GA184, which was a control but unspecified. The basis for the GA production differences between

the strains was confirmed by higher expression of HIS3 and major GA pathway genes, upregulated INO1 indicating phospholipid synthesis and downregulation of ergosterol synthesis genes.
Finally, single cell transcriptomic analysis was conducted on approx. 14000 cells divided between A8 and the control. This sorted the transcriptomes into patterns of upregulation/downregulation and into 9 clusters of similar gene expression patterns among the strains. This analysis showed clear delineation of gene expression between A8 and the control and confirmed the bulk cell transcriptomic analysis and higher HIS3 and GA pathway gene expression in A8. The analysis also identified several potential gene targets in the control which could influence GA production. Two strains that had deletion of 1 of 2 target genes were deleted gave higher GA yields; other knockouts identified from the single cell transcriptomic analysis resulted in lower GA yields.

Review
The manuscript does not universalise the concept that was promised in this work. It was claimed that the manuscript "developed a universal paradigm for the construction of biosensors and their subsequent application in stabilizing and evolving the genotype of cell factories" but the work is restricted to the example, GA biosynthesis, and no attempt to broaden the concept in either the Results or Discussion was made.
Response:
We thank Reviewer for the positive evaluation of our work and their constructive suggestions, which significantly improved the manuscript. Below, we address each comment in detail.
We supplemented another experiment to demonstrate the generality and scalability of the concept—medicarpin (Fig.1i-k). Through supplementary experiments, the universal workflow for the in-situ discovery of target-responsive biosensors in chassis cells has been demonstrated to be reliable. And more discussion was added (Line 431-436).

Indeed, some key aspects are missing (e.g. how 12 upregulated genes in GA exposure were selected as potential biosensors, what role does biosensor dynamic range have in potentially increasing yield, what is considered a 'control' in this kind of research). This knowledge is essential for extending the applicability of this concept. Some of these queries are detailed further below.
Response:
The basis for the selection was the genes encoding transporters or hypothetical proteins with upregulated expression. Based on this, 12 genes were selected, and we listed the protein functions and fold changes in Table S1. The related statements in manuscript were revised (Line 131).
The effect of dynamic range on the production was added and discussed in manuscript (Line 246), and a figure (Fig. 3c) was added to illustrate this impact. Theoretically, both increasing 3-AT concentration and decreasing dynamic range can enhance the constraint of biosensor on cell growth.
All the 'control' was clarified and explained in manuscript (Line 137, 147, 289, 309, 419).

The single cell transcriptomics analysis produced some interesting results as any opportunity to look deeply into a yeast culture and infer its performance via gene expression can do. In this case, the analysis seemed an add-on and not central to the point of the paper. The bulk cell transcriptome analysis already showed the key gene expression differences between GA184 and A8.
Response:
We added the comparison between the bulk and single-cell RNA-seq. The two methods

demonstrate overall consistency (Fig. S7). However, single-cell sequencing has demonstrated irreplaceability. The significant differentially expressed genes discovered through it in subgroups do not show any differences in bulk transcriptome, such as ADY2 and CTA1 (Fig. S10). These DEGs in subgroups have also been shown to be key factors of product synthesis. Moreover, the conclusion of cell division in this article is entirely based on single-cell sequencing. These conclusions cannot be obtained using bulk transcriptome. More discussion was added (Line 486-493).

The concept of using biosensors to indicate positive directions of genetic/metabolic engineering and high throughput screening or adaptive laboratory evolution is not new. The selection of PDR was based on earlier work and others that have coupled biosensor response to growth. A selection of relevant research and review articles is given below.

Li et al (2019) quoted by the authors identified the action of PDR and used this biosensor approach (fluorescent biosensor based on PDR) in their study. That is, they state “here we developed a small molecule-responsive biosensor that couples transcriptional induction of PDR genes to growth rate in the yeast Saccharomyces cerevisiae “ (Li J, Kolberg K, Schlecht U, St Onge RP, Aparicio AM, Horecka J, Davis RW, Hillenmeyer ME, Harvey CJB. A biosensor-based approach reveals links between efflux pump expression and cell cycle regulation in pleiotropic drug resistance of yeast. J Biol Chem. 2019 Jan 25;294(4):1257-1266. doi: 10.1074/jbc.RA118.003904)

Krüger, A., Göddecke, J., Osthege, M. et al. Biosensor-based growth-coupling as an evolutionary strategy to improve heme export in Corynebacterium glutamicum. Microb Cell Fact 23, 276 (2024). https://doi.org/10.1186/s12934-024-02556-1

Dacquay LC, McMillen DR. Improving the design of an oxidative stress sensing biosensor in yeast. FEMS Yeast Res. 2021 May 1;21(4):foab025. doi: 10.1093/femsyr/foab025. PMID: 33864457; PMCID: PMC8088429.

Yongkun Lv, Shuai Qian, Guocheng Du, Jian Chen, Jingwen Zhou, Peng Xu, Coupling feedback genetic circuits with growth phenotype for dynamic population control and intelligent bioproduction, Metabolic Engineering,54,2019,109-116, https://doi.org/10.1016/j.ymben.2019.03.009.

Lv Y, Gu Y, Xu J, Zhou J, Xu P. Coupling metabolic addiction with negative autoregulation to improve strain stability and pathway yield. Metab Eng. 2020 Sep;61:79-88. doi: 10.1016/j.ymben.2020.05.005.

Many approaches to growth coupled chemical bioproduction have been reviewed in the following (and others)

Gazi Sakir Hossain, Mukesh Saini, Ryoma Miyake, Hua Ling, Matthew Wook Chang, Genetic Biosensor Design for Natural Product Biosynthesis in Microorganisms, Trends in Biotechnology,38, 2020, 797-810, https://doi.org/10.1016/j.tibtech.2020.03.013.

Yoshihiro Toya, Hiroshi Shimizu, Coupling and uncoupling growth and product formation for producing chemicals, Current Opinion in Biotechnology, 87, 2024, 03133, https://doi.org/10.1016/j.copbio.2024.103133.

Alter TB, Ebert BE. Determination of growth-coupling strategies and their underlying principles. BMC Bioinformatics. 2019 Aug 28;20(1):447. doi: 10.1186/s12859-019-2946-7

Response:

Yes, coupling is not a novel approach, and the references reviewer mentioned have been included or added in the introduction (Line 47-63). We clearly highlight the strengths and challenges of this

work in both the introduction and discussion sections: the acquisition of biosensors (Line 64-78) and the analytical methods for identifying differences (Line 79-98).

Further comments regarding suggestions on improving the manuscript, the lack of or unclear experimental details, language, and results interpretation are provided.

Abstract

It is difficult to get any sense of the novelty of the study from this abstract. There is some strange phrasing used (see General comments) which adds to the difficulty in getting value from it. There are no measures of impact in the whole abstract – no indication of the extent of enhancement for example is given; no gene names, no promoter name or how the biosensor works, the overall impact etc.

Response:

Abstract was rephrased by adding the whole impact and quantitative information such as the extent of enhancement, promoter name, the biosensor works, etc.

Introduction

It may be a personal dislike but I don't find the idea that a research area is a "hot research topic" a very compelling reason to do research. Also, proponents of ALE are unlikely to consider the approach 'irrational'.

Response:

The introduction was rewritten. These issues have been revised to scientific expressions.

The introduction would benefit from being rewritten to improve clarity and to do justice to the literature that is touched on. Given the complexity (lines 71-75) of developing a biosensor and using it to evolve strains, taking this approach needs stronger justification. What are the specific drivers of this research? What is the novelty? Why is this a "universal paradigm" or "universal workflow" when only GA synthesis is described?

Response:

The introduction was rewritten. First, the coupling strategy in the literature is elaborated more comprehensively, thereby highlighting the importance and necessity of developing biosensors and identifying the bottlenecks in their development (Line 47-63). Subsequently, when explaining the reasons for yield enhancement through coupling, it is noted that evolutionary differential analysis still lacks high-resolution methods (Line 79-98). Finally, addressing the above two issues, the innovation of this study lies in developing a relatively rapid acquisition process for biosensors and revealing differences in cellular division of labor within coupled strains (Line 99-117). Moreover, we added experiments with two additional biosensors to demonstrate their versatility.

The sentences (lines 76-88) relating to within culture differentiation are a sharp direction change from the rest of the introduction and look like they have been parked there to justify undertaking single cell transcriptomics. Line 84 suggests within isogenic cultures experiencing nutritional variability there is always a differentiation of labour among cells but then it is confusingly concluded that "We surprisingly found that the high-production yeast population exhibits division of labor, using single-cell transcriptome sequencing." .

Response:

The introduction was rewritten. The description for this literature has been deleted. The semantics of this paragraph have been modified to be more coherent. By analyzing the coupling mechanism, the issue of single-cell sequencing required for cell division of labor is raised, and it is ultimately proposed that the division of labor mechanism in cells under the condition of synthetic products in cell factories is still unclear (Line 79-98).

Results

Line 115

Fig 1b shows 1109 genes were upregulated in the GA109 strain compared to control and the text reads "215 with significantly upregulated expression and 936 genes with significantly downregulated expression"

Response:

There was an error in the figure, which has been corrected. "215 with significantly upregulated expression and 936 genes with significantly downregulated expression" was the correct data. The data used in the figure can be found in the supplementary materials.

Ultimately only 12 genes are listed in Table S1 and ultimately tested. How were these selected? What was considered "significant"? Table S1 does not show fold upregulation for the selected genes and the criteria for selection of these 12 genes are not clear.

Response:

Because GA is a lipophilic exogenous component, it may cause the expression of stress-related yeast drug transporters and other similar proteins. Therefore, the basis for the selection was the genes encoding transporters or hypothetical proteins with upregulated expression. Based on this, 12 genes were selected, and we listed the protein functions and fold changes in Table S1. The related statements in manuscript were revised (Line 128).

Line 118

"and fluorescent proteins were utilized to characterize the promoter response" Suggest restating this to more accurately reflect what you have shown in Fig 1c.

Response:

This has been restated: "The inducibility of these gene promoters is characterized using red fluorescent protein mcherry by constructing expression cassettes into GA180 and SynV strains, respectively" (Line 131-133).

PDR response in GA180 is enhanced compared with the control strain because the former is producing GA

Response:

Yes, I agree with your point of view. As shown in Fig.1.

In Fig 1f and 1g,

was GA added to control yeast strains to test the responsiveness of PDR to GA? This would seem like the logical experiment to show that this is an accurate biosensor.

Response:

Yes. Both the addition experiment (Fig. 1g and h) and endogenous experiment (Fig. 1d-f) were

conducted. The statement has been revised to be clearer in manuscript and Figure Caption: “To validate the capability and dynamic range of P_{PDR5} in response to GA, GA was added to control strain SynV with varying concentrations”

Glycyrrhetinic acid is considered water insoluble. How was it delivered to yeast cultures?

Response:

As you correctly point out, GA is essentially water-insoluble (reported solubility in pure water is extremely low, 6.62 μg/L). Because GA solubility in complex growth media and fermentation broths has not been systematically reported, we directly measured it under conditions relevant to our study. A 4 g/L GA stock solution was prepared using ethyl acetate as solvent. To test the solubility of GA, the GA stock solution was added to the culture to achieve a final concentration of up to 200 mg/L. The soluble fraction of GA in YPD medium without yeast was 13 $mg \cdot L^{-1}$, whereas the cell-free fermentation broth collected after 5 days showed a higher apparent solubility of 34 mg/L. The increase observed during fermentation may be explained by medium changes that promote GA dispersion. For example, shifts in pH, accumulation of metabolic by-products, secretion of amphiphilic proteins or surfactants, and/or formation of colloidal or micellar phases that enhance apparent solubility. Crucially, when the yeast was added into the YPD medium (OD=2.71), the intracellular GA (measured after cell harvest, washing and organic extraction) reached 160 mg per liter fermentation broth, equivalent to a GA loading of 59 mg/L per OD cell culture. This indicates strong partitioning and accumulation within yeast and demonstrating that the GA levels are sufficient to support the function of the biosensors used in this study. GA is a membrane-affinity component, so it readily binds to organelles with membrane structures.

In Fig 2,
the potential impact of a range of stressors and environmental agents were tested against non-GA producing yeast expressing the PDR biosensor. In the absence of stressor (Fig 1a) control yeast produced up to 250 FU at 120 h. If all conditions were the same for Fig 2a and b, why are the FU so different? That is, control is 100 FU and GA elicits a response of 205 FU at 120 h.

Response:

The fluorescence value of microplate reader is an "arbitrary unit (a.u.)" because it is a relative measurement value highly dependent on specific instrument hardware and software settings (such as Gain value). For this set of data, the difference in control comes from the different Gain set. Its value lies not in its absolute value, but in its ability to make relative comparisons within an experiment. A method to make it comparable between groups is to normalize the data, but this is not necessary.

What is meant by “(b) The response of the GA biosensor to other natural products in the corresponding engineered strains.” What are the corresponding engineered strains? Werent molecules 1 – 10 added to the flasks at different concentrations?

Response:

Molecules 1-10 were endogenously synthesis in the corresponding engineered strains. The additional information of these strains was supplemented in Fig.S2 Caption and Table S2.

Line 159 onwards

Extensive empirical promoter engineering was undertaken to attempt to increase the dynamic range of its response with little impact. Was this result surprising?

Response:

The results are as expected; they align with our expectations. Previous literatures demonstrating the feasibility of modulating dynamic range through promoter engineering confirmed that this approach is viable [28-30]. Similarly, we have successfully achieved modifications in response capacity using this methodology.

Fig 3 data.

Do all these attempted promoter engineering results need to be in the main document? Most do not make an important difference.

Response:

The original Fig. 3d,e,f have been moved to the Supplementary Figure S4.

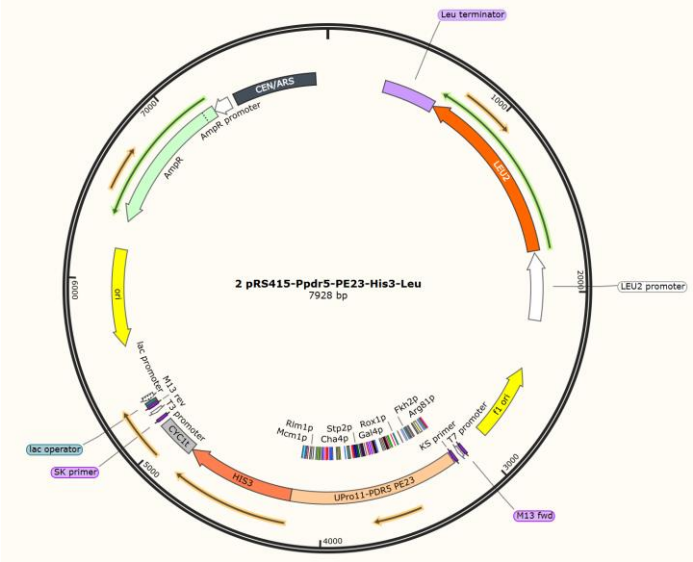
Line 228 :

"Consequently, the strains evolved in YPD media exhibited a dramatic reduction in triterpenoid production indicating that the GA-producing trait is not steadily inherited as the production process of GA may exerting a burden on cell growth"

This looks to be a classic case of loss of plasmid pRS415 from the cells in rich media. The selection pressure to keep the plasmid was removed as histidine is drawn from the media. As cells divide there is no benefit to retaining the plasmid. Also the azole is not effective.

Response:

The genes involving GA synthesis were integrated in the genome as shown in Table S2. The reduced production is not related to the loss of plasmid. pRS415 only contains the biosensor. Ultimately, the decrease in yeast production in YPD is due to the lack of pressure to screen synthetic products. In SD-His media, the plasmid must exist to maintain cell growth. The related statement has been revised as: "The heterologous genes were almost lost as the strain continuous cultivation without the production-dependent selective pressure in YPD media (Fig. 3f and Supplementary Fig. 6), which may be due to unstable gene integration or overgrowth of the wild-type yeast. This phenomenon of strain degradation also occurs in industrial production. In contrast, the evolution in SD-His media made the producing phenotype and genotype stable, due to the coupling of growth with biosynthesis." (Line 262-268)



Line 231

“The heterologous genes were almost lost as the strain continuous cultivation without a selective pressure in YPD media (Fig. 4e and Supplementary Fig. 2).”

This is very surprising as these heterologous genes (2CYPs and CPR) are integrated into the yeast genome. The absence of the genome-integrated pathway genes at 18 d in YPD suggests that either the gene insertions are unstable and prone to excision or that the culture was overgrown by wild type yeast. Could you please comment.

Response:

Yes, I agree with your point of view that both these factors are possible. The discussion has been added in manuscript: “The heterologous genes were almost lost as the strain continuous cultivation without the production-dependent selective pressure in YPD media (Fig. 4f and Supplementary Fig. 2), which may be due to unstable gene integration or overgrowth of the wild-type yeast. This phenomenon of strain degradation also occurs in industrial production.” (Line 262-268)

Line 233

I’m struggling to understand these two seemingly contradictory points:

“In contrast, the evolution in SD-His media made the producing phenotype and genotype more stable, due to the coupling of growth with biosynthesis. Furthermore, the addition of 3-AT led to an increased correlation between growth and GA production”

The more the yeast needs to produce histidine via the PDR-PE23 promoter (with 5-fold dynamic range) plus the effect of azole, the more GA is produced. That makes sense.

“As anticipated, the strains employing PE25 and PE31 exhibited substantial increases in GA production. The strains with PE31 evolved for 6 days achieved the highest production level, in which the production of GA increased by 17.5% and the production of total triterpenoids increased by 11.4% (Fig. 4d).”

PDR-PE25 and -PE31 have lower dynamic range (~2-3.5-fold) and no azole was added to enhance the endogenous histidine requirement. Why was it anticipated that this would result in a substantial increase in GA production?

Response:

This reason can be explained by Fig. 4c. Both increasing 3-AT concentration and decreasing

dynamic range can enhance the constraint of biosensor on cell growth. There is an optimal catalytic validity of His3 that maximizes GA production. The explanation has been added in manuscript (Line 244-249) and Fig. 3 Caption.

Line 265

"strain A8 and the control strain (cultured in YPD medium for 6 days) were analyzed"

What is the "control" strain for these experiments and why was this strain selected? In Fig 5 is shown as GA184 but what does that signify?

Response:

Here the "control strain" has been revised to "the control strain GA184Y". The control strain, named GA184Y, was obtained from the strain GA184 evolved in YPD medium at 6th day. The evolved strain A8 and the control strain GA184Y were cultivated in GM1 media for a period of 5 days and subjected to RNA-seq. The evolved strain A8 and the control strain GA184Y represent the biosensor-dependent evolved high-production strain and the normal passaged strain, respectively, utilized to explore the gene expression differences of high-production strain.

Line 281

The downregulation of ergosterol synthesis pathway genes in the A8 in comparison to GA184 was interesting and no doubt beneficial for obtaining a higher yield of GA in that strain. It is stated that "It is evident that, through biosensor-dependent evolution, this balance can be rapidly achieved."

But this was not made clear in the manuscript. Were there any negative phenotypical consequences for the ergosterol synthesis downregulation evident in the A8 strain? (these effects have been extensively reported e.g. Johnston EJ, Moses T, Rosser SJ. The wide-ranging phenotypes of ergosterol biosynthesis mutants, and implications for microbial cell factories. Yeast. 2020 Jan;37(1):27-44. doi: 10.1002/yea.3452). There may have to be a choice between poorly growing strains/high GA and normal growth/moderate GA.

Response:

We cited this reference and added more discussion about the balance between cell growth and terpene production. We also provided yeast biomass data (Fig. 3i) to support the result. The high production strain A8 indeed exhibits lower biomass, which is consistent with the metabolic changes of ergosterol synthesis pathway (Line 333-336).

Line 318

PIR1 is a described as a "newborn marker gene", "cluster 0 are in a state of neogenesis"

PIR1 is a structural constituent of the cell wall involved in intracellular protein transport and cell wall organization; also localizes to the bud scar and nuclear periphery. According to ref 41 PIR1 is a marker for early G1 phase but does not distinguish between mother or daughter cells (they suggest use of DSE2 which is only expressed in daughter cells).

The genes upregulated PIR1, EXG1, HSP150, CRH1, NCW2, PST1, and CWP2 are all involved in cell wall synthesis and remodelling. Together their upregulation could indicate budding or cell wall remodelling for other reasons.

It is not clear from the clustering description (line 318 onwards) what the authors are suggesting. Doesn't the clustering represent major patterns of gene expression identified in individual cells. Most of the cells have patterns of gene expression (up-, down and no change) that locate to one of

nine patterns. These are a different way of grouping the cells compared with the UMAP graphs.

Response:

The inaccurate description “newborn” was revised to: “budding”. The marker genes of cluster 0 are indeed related to budding.

The paragraph structure in this section posed comprehension challenges, so we revised it. First, this study employed a single clustering method—UMAP—to partition cells into nine clusters (Line 348). These nine clusters remain consistent throughout the text. This clustering grouped cells with similar gene expression patterns together. This section (line 358) primarily describes the marker genes and inferred characteristics of each cluster within the evolved strain, specifically clusters 0, 1, and 7, elucidating the community structure of the evolved strain.

Line 329:

“The low RNA feature of cluster 7 indicates that this cluster may be undergoing the process of aging” what does the low RNA feature mean? Cluster 7 has a lower number of “features” compared to some of the clusters as does cluster 5. The gene expression pattern for cells in cluster 7 shows upregulation of stress response genes like HSP12, DDR2 and down regulation of GA pathway genes. Interestingly, it is mainly A8 cells this cluster.

Response:

'RNA feature' refers to the types of RNA that can be detected. Low RNA feature indicates that cells are in a physiological resting state (e.g. the G0 phase), experiencing environmental stress, or in a senescent state.

Cluster 7 belongs to A8 cells. In the final paragraph of this section, we emphasize the division of labor mechanism in the evolved strain: “The evolved strain exhibits high expression of synthetic pathway genes, yet it also demonstrates a pronounced division of labor mechanism: not all cells are highly productive. Instead, one group exhibits stronger cell regeneration characteristics (cluster 0), while another group displays distinct quiescent traits (cluster 7).” (Line 400-405)

Line 343

“In cluster 6, gene ADH2, ADY2, ATO3, and CTA1 were significantly upregulated. These genes have been shown to regulate the treatment with ethanol, acetic acid, ammonia, and reactive oxygen species, respectively.”

These are either metabolism genes or transporters. I don’t think it is accurate to say they regulate the treatment with those substances.

Response:

ADH2 catalyzes the conversion of ethanol to acetaldehyde, which is further transformed into acetic acid, marking the initial step in acetic acid metabolism.

ADY2, as an acetate transporter, is involved in acetate uptake and ammonia export, playing a role in acetate utilization and resistance mechanisms.

ATO3 is responsible for ammonia export, aiding in the maintenance of nitrogen balance and pH stability, and is involved in ammonia processing during acetate metabolism.

CTA1 decomposes hydrogen peroxide to protect cells from oxidative stress, which is particularly important during acetate metabolism (such as β-oxidation in peroxisomes).

As a conclusion, these genes respond to the metabolic stress and oxidative stress induced by acetate to ensure cellular homeostasis. Therefore, this sentence was revised to: “These genes have

been shown to regulate the adaption to metabolic stress such as ethanol, acetic acid, ammonia, and reactive oxygen species." (Line 386)

Line 412:
"For the control strain, subculture results in a loss of the strain capacity for synthesizing licorice triterpenoids."
Again, what strain is meant by 'control' is never clear. Loss of GA production seemed to occur during subculture in rich media (YPD) where the plasmid coding for HIS3 was lost during replication.
Response:

The control was specified as "For the control strain GA184Y, subculture without selective pressure in rich media results in a loss of synthesis genes".

In reality, the decrease in yield of the control strain GA184Y is due to the loss or the silence of synthesis genes during replication without selective pressure (Fig. 3f). Although *HIS3* gene may also be lost, it is not the direct cause of the decrease in yield.

Line 420:
"It can therefore be inferred that their role may be to maintain the sustainability of high-production cells (cell regeneration and protein renaturation) and the stability of growth environments"
This inference is likely projecting well beyond the data. The different gene clustering within the high performance A8 strain could represent cells in a distinct life stage to cluster 0, or the gene expression pattern may result from the gradient of nutrient and oxygen availability across the culture.
Response:

Yes, Discussion was rewritten. The sentences beyond the data were deleted. The revised discussion focuses more on discussing the new findings of this study and comparing them with the results of other literature (Line 472-493).

Line 425:
"Whereas, within the control strain, there was a progressive decline in the capacity for product synthesis, attributable to the absence of biosensor-dependent synthesis and growth coupling."
There was no time based analysis in this single cell transcriptomic analysis so what is meant by a progressive decline? The single cell transcriptomes show that A8 and Control have different gene expressions which are consistent with the differences in GA production and lower HIS3 expression.
"Consequently, the cells gradually degenerated into metabolic characteristics dominated by growth"
This is poor phrasing. Cell undertaking normal metabolism for growth shouldn't be termed degenerated.
Response:

Discussion was rewritten. The poor phrasings were deleted.

The "progressive decline" is mainly reflected in the passage process of Fig.3e.

General:
At many points, the manuscript lacks scholarly treatment, ie sentences lack key words and details which results in them sounding very general or facile. I recommend a complete and thorough edit and a rewrite of the Introduction.

Response:

The introduction was rewritten.

Some phrasing needs to be reconsidered such as:
Line 219 and onward “growth-production coupling strategy”? Perhaps coupled growth-production is better
“evolutionary strains” should be evolved strains

Response:

Thanks again for your attentive and constructive suggestions!

This statement has been revised throughout the entire text.

These terms and phrases need to be reconsidered:
“improving the efficiency of synthetic products”
high-effective screening of cell factory products
strategy of coupling synthesis and growth prevents the degeneration of cell synthesis ability.
the biosensor-dependent evolution of synthetic chemicals in cell factories
difficulties of enabling strains to evolve in a synthesis-oriented manner.
lead to strain degeneration
an absence of methodologies capable of impeding the descent of cells towards a state of diminished synthesis ability.
can potentially trigger exclusionary responses in the organism

Response:

These expressions have been revised as follows:

“enhancing product synthesis efficiency”

“high-throughput screening of cell factories for efficient synthesis of products”

“Coupling synthesis and growth can prevent the degradation of cellular synthetic capacity.”

“the evolution of cell factories dependent on biosensors”

“the complexity of evolving strains in a synthesis-directed manner”

“potentially exacerbating cellular differentiation and reducing synthesis efficiency”

“However, there is currently no effective method to prevent the decline in synthetic capacity caused by cellular differentiation.”

“can potentially trigger a stress response within the cell”

Reviewer #3 (Remarks to the Author):

This study focuses on designing a biosensor-dependent coupling system to stabilize the high-synthesis phenotype of yeast cell factories for licorice triterpenoid production, It constructs a biosensor targeting glycyrrhetinic acid (GA), a licorice triterpenoid, optimizes its dynamic range through promoter engineering, and designs genetic circuits coupling product synthesis with cell growth. Via adaptive evolution, it significantly enhances licorice triterpenoid yields. Single-cell transcriptomics reveals a population division of labor in evolved strains, with "highly efficient synthetic cells" and "viability-maintaining cells," offering new strategies for regulating microbial cell factory stability and improving synthesis efficiency. However, the study has limitations in experimental design rigor, such as insufficient specificity verification of the biosensor, and lack of

reproducibility in single-cell sequencing, which somewhat undermine the persuasiveness of conclusions and the universal promotion of the method.

Detail comments below:

For experimental design details, regarding the biosensor, although the responsiveness of the PDR5 promoter to GA is confirmed, the dynamic concentrations of compounds like naringin chalcone, which it weakly responds to, in the GA synthesis system are not detected.

Response:

We thank Reviewer for the positive evaluation of our work and their constructive suggestions, which significantly improved the manuscript. Below, we address each comment in detail.

We showed the responsiveness of the PDR5 promoter to some common natural products. These molecules were endogenously synthesis in the corresponding engineered strains with the PDR5 biosensor system, and the response at the end of fermentation was detected. The additional information of these strains was supplemented in Fig.S2 Caption and Table S2. The purpose of this experiment is to detect and avoid the influence of GA precursors on the response. It is not necessary to detect the dynamic concentration of naringin chalcone (which does not exist in the GA synthesis system). Additionally, for smoother logic, we only retained the response of PDR5 to GA analogs (Fig.S2). And we also supplemented the exploration of other natural product-medicarpin biosensors in specific cell factory (Fig. 1i-k). The additional discussion has been added in manuscript (Line 171-181).

Additionally, the molecular mechanism (e.g., interaction with Pdr1) by which Ssz1 knockout improves the dynamic range lacks verification.

Response:

We supplemented the reference [30,31,34] and the experiments (Fig. 2d and Fig.S3) (Line 189-203) to illustrate the molecular mechanism. Through the literature [34] and the molecular simulation (Fig.S3a), it is speculated GA directly binds to transcriptional factor Pdr1. The knockout of Pdr1 results in Pdr5 becoming completely unresponsive to GA (Fig. 2d). And a pull-down assay was conducted, which showed Ssz1 interacts with Pdr1 (Fig.S3b). It was verified that Ssz1 knockout affects the response of PDR5.

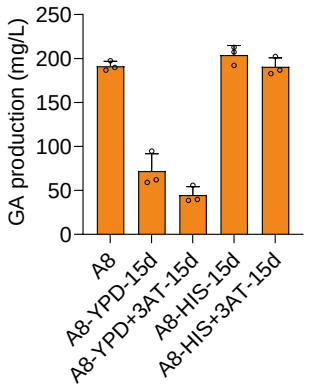
In the growth-synthesis coupling system, the non-specific metabolic effects of 3-AT are not excluded, the independent impact of nutrient differences in different media (YPD, SD-His, SD-His+3AT) on evolutionary results is not distinguished, and the passage stability of the high-producing strain A8 is not tested.

Response:

In the concept validation (Fig. 3b), we added the influence of only adding 3-AT in YPD. It has a weak inhibitory effect on growth and synthesis. In principle, 3-AT plays a role in inhibiting His3 activity, so in YPD medium with sufficient histidine, 3-AT will not have a significant impact on cells. The experimental results confirmed this principle. Based on this, strain evolution under YPD+3AT is not considered (in Fig. 3e).

We also supplemented the validation experiment of A8 passage stability (see the figure below). Results indicate that the phenotype (synthetic ability) of A8 remains stable after passage in a medium with selective pressure for 15 days (A8-HIS-15d). While its synthetic ability still decreases

after passage without selective pressure (A8-YPD-15d). The decrease in A8 production is not as significant as that of the starting strain GA184. This result is consistent with the design principles of our biosensors and reflects the important role of biosensors in constraining product synthesis.



Supplementary Figure: Validation of A8 passage stability

In transcriptome and single-cell analysis, single-cell sequencing lacks biological replicates,

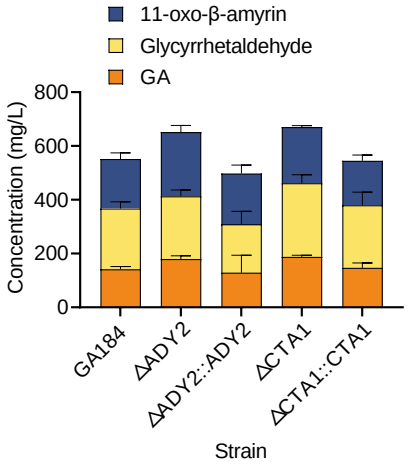
Response:

Single-cell sequencing data have included biological replicates. Samples of yeast cells from two biological replicates were pooled into a single trial when we analyzed the data. This detail is supplemented in Method (Line 603 and 616).

the ADY2 and CTA1 knockout experiments lack complementary verification,

Response:

The complementary verification was supplemented as figure below. Gene complementation brought yields back to the levels of the original strain.



and the function of population division of labor (e.g., sorting cells from different clusters for separate culture to verify synthesis and growth capabilities) is not confirmed through experiments.

Response:

The experiment of the function of population division of labor was supplemented (Fig. S9). Cells were sorted from different clusters for separate culture to verify synthesis and growth capabilities.

The result confirmed that cluster 0 and 1 contribute the product synthesis, as well as cluster 3. And cluster 7 is not a high productivity cluster. The related discussion was added (Line 406-414, 472-485).

For article structure, the "unclear mechanism of microbial population division of labor" proposed in the introduction only describes phenomena without delving into mechanisms in the single-cell analysis of results.

Response:

The introduction was rewritten. We have re-analyzed the coupled production mechanism and cellular division of labor mechanism reported in the article, as well as the issues that have not yet been explained. About the mechanism behind enhanced synthetic capacity in growth-synthesis coupled strains, conventional wisdom posits that this strategy confers a growth advantage to high-yielding cells by suppressing the proliferation of deceptive cells, thereby reducing metabolic phenotype heterogeneity within the population and enabling autonomous enrichment of high-yielding strains [3]. However, this theory remains lacking in robust evidence at the microscopic level. So single-cell transcriptomics sequencing has emerged as an effective tool for resolving gene expression heterogeneity among individual microbial cells and dynamically revealing cellular functional differentiation. Previous studies indicate that when facing environmental stress or resource limitations, microbial populations tend to differentiate into functionally specialized subpopulations: one accelerates division and proliferation to maintain population size, while the other reduces division and extends lifespan to produce specific bioactive molecules [22]. However, no studies have yet demonstrated whether similar division-of-labor patterns exist during microbial heterogenous product synthesis, nor how such functions are regulated and optimized. (Line 79-98)

Bulk transcriptome (Fig. 5) and single-cell data (Fig. 6) lack correlation analysis, such as the enrichment of upregulated genes in specific cell clusters.

Response:

The global correlation analysis between bulk transcriptome and single-cell data has been added in Fig. S7c, and the illustration has been added in manuscript. The marker genes found in each cluster has been enriched in the bulk transcriptome analysis (Fig. S10), and the significance of single-cell sequencing has been discussed (Line 486-493).

The discussion overly emphasizes the universality of the "universal paradigm" and insufficiently addresses limitations such as PPDR5's dependence on hydrophobic compounds and the off-target risk of the coupling system in complex networks.

Response:

The limitations were added in discussion to analyze the possible regulation function and the limitations on the application (Line 439-455).

Moreover, it fails to compare with similar studies in Cell Rep (2021) to clarify incremental contributions.

Response:

The discussion was added to clarify incremental contributions.

In contrast to the previous studies on growth-synthesis coupling research [4,7,10,11], this study

elucidates the mechanism by which growth-synthesis coupling stabilizes and enhances product synthesis efficiency from the perspective of non-genetic cellular phenotypic differentiation. Contrary to previous understanding [3], community differentiation persists in biosensor-dependent high production strain, indicating that growth-synthesis coupling does not entirely eliminate "deceptive cells". Growth-synthesis coupling induces high-production metabolic profiles in the majority of cells, yet a minority remains allocated to maintaining cellular activity or repairing damage. This indicates that these few cells are necessary for coping with environmental changes or cellular aging [42-44]. (Line 472-487)

Key information is missing in chart annotations (e.g., the correlation between the abscissa range and saturation points in Fig. 1g, the clusters corresponding to marker genes in Fig. 6k).

Response:

GA concentrations below 200 mg/L ensure complete absorption by the yeast. And the saturation points was varied with different conditions. The solubility of GA in pure water is reported as 6.62 μg/L. Because GA solubility in complex growth media and fermentation broths has not been systematically reported, we directly measured it under conditions relevant to our study. A 4 g/L GA stock solution was prepared using ethyl acetate as solvent. To test the solubility of GA in the cell culture, the GA stock solution was added to the culture to achieve a final concentration of up to 200 mg/L. The soluble fraction of GA in YPD medium without yeast was 13 $mg \cdot L^{-1}$, whereas the cell-free fermentation broth collected after 5 days showed a higher apparent solubility of 34 mg/L. The increase observed during fermentation may be explained by medium changes that promote GA dispersion. For example, shifts in pH, accumulation of metabolic by-products, secretion of amphiphilic proteins or surfactants, and/or formation of colloidal or micellar phases that enhance apparent solubility. Crucially, when the yeast was added into the YPD medium (OD=2.71), the intracellular GA (measured after cell harvest, washing and organic extraction) reached 160 mg per liter fermentation broth, equivalent to a GA loading of 59 mg/L per OD cell culture. This indicates strong partitioning and accumulation within yeast and demonstrating that the GA levels are sufficient to support the function of the biosensors used in this study. GA is a membrane-affinity component, so it readily binds to organelles with membrane structures. Therefore, a GA concentration below 200 mg/L ensures complete absorption by the yeast.

The clusters corresponding to marker genes in Fig. 6k have been added.

Supplementary materials, such as the retention rate of heterologous genes in Supplementary Fig. 2, are not quantified,

Response:

The quantified result of Supplementary Fig. 2 (current Fig.S6) was shown in Fig.3f. The explanation has been added in Fig.S6 Caption.

and the selection criteria for candidate genes in Supplementary Table S1 are not explained, resulting in insufficient support.

Response:

The basis for the selection was the genes encoding transporters or hypothetical proteins with upregulated expression. Because GA is a lipophilic exogenous component, it may cause the expression of stress-related yeast drug transporters and other similar proteins. Based on this, 12

genes were selected, and we listed the protein functions and fold changes in Table S1. The related statements in manuscript were revised. (Line 128-131)

Judging from the title, there may be a lack of more examples to verify the author's concept of "biosensor-dependent coupling system stabilizes the high-synthesis phenotype of cell factory.

Response:

We supplemented another experiment to demonstrate the generality and scalability of the concept—medicarpin (Fig.1i-k). Through supplementary experiments, the universal workflow for the in-situ discovery of target-responsive biosensors in chassis cells has been demonstrated to be reliable. And more discussion was added (Line 431-436). Therefore, our two examples and multiple examples in the literature [4,7,10,11] can support the concept proposed in this article.

Lines 117-118 state, "To identify GA-responsive elements, a series of genes were selected from those with significantly upregulated expression". What is the basis for selecting these genes?

Response:

The basis for the selection was the genes encoding transporters or hypothetical proteins with upregulated expression. Based on this, 12 genes were selected, and we listed the protein functions and fold changes in Table S1. The related statements in manuscript were revised. (Line 128-131)

Line 119 mentions, "As a result, the PDR5 promoter (PPDR5) displays a marked fluorescent response to GA (Fig. 1c)". From Fig. 1c, it appears that other promoters (such as REE) also show responses to the target compound. What is the rationale for selecting only PPDR5?

Response:

Promoters with improved response were annotated with their p-values. The PDR5 promoter displays the most significant response enhancement to GA, compared to REE1, etc. The related statements in manuscript were revised: “As a result, the PDR5 promoter (PPDR5) displays the most significant response enhancement to GA”. (Line 133)

Lines 146-148 state, "To examine the spectrum of PPDR5, its capacity to respond to other natural products (triterpenoids: compound 1-6, flavonoids: compound 7-10)". The article focuses on the response to GA throughout, so why test these seemingly unrelated substances here? This seems inconsistent with the main theme of the article, and it is suggested that the authors should instead consider substances related to the GA synthesis pathway.

Response:

Compounds 1-6 have relation with GA synthesis. As shown in Fig. S2b, compounds 2, 3, and 4 are the main precursors of GA, compound 1 is an analogue to compound 2, and compound 6 is the glycation product of GA. Compounds 7-10 have no relationship with GA, which was deleted. The detailed explanation has been added in manuscript. (Line 171-175)

Reviewer 2: Thank you for the comprehensive response and corresponding updates you have made to the manuscript and supplementary information. This revised manuscript has been a substantial undertaking and it has been done with care and attention to detail. My queries have been satisfactorily addressed.
One minor point is your use of the terms "strain degradation" and "degradation in cell factories". I think you could be more specific as to your meaning as they could be confused with cell death.

Response:

We thank Reviewer for the positive evaluation of our work and the constructive suggestions. We have made modifications to the word 'degradation' based on your suggestion:

P51 'strain degradation' was revised to 'degeneration'.

P105 'the degradation of high-yield traits during passage' was revised to ' the instability of high-yield traits during passage '.

P266 'strain degradation' was revised to 'strain deterioration'.

P429 'the issue of degradation in cell factories' was revised to 'the issue of degeneration in cell factories'.