nature portfolio

Peer Review File

Block-building with yeast to elucidate an artificial pathway for de novo biosynthesis of glabridin

Corresponding Author: Professor Jingwen Zhou

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 details the engineering of glabridin biosynthesis in baker's yeast, including constructing an artificial pathway, optimizing production, and designing a modular block-based strategy for assembling pathway modules. The authors achieved low mg/L concentrations of glabridin and extend their approach to another natural product using co-culturing of yeast and E. coli. Overall, the work is elegant and comprehensive. The manuscript is clear, rich with data, and broadly applicable.

Major comments:
1) It is not clear to me that an authentic commercial standard was utilized to confirm the identity of glabridin (and perhaps other key compounds such as equol). The authors state that they purchased standards for formononetin, medicarpin, daidzein, and squalene, but not glabridin and equol, which are the most important compounds in the study. The authors show MS/MS fragmentation of yeast-derived glabridin and claim this confirms its identity, but the fragmentation is not compared to an authentic standard. A Google search shows that standards for both glabridin and equol are readily available.
2) Related to structural determination, in Figure 3B the authors screened multiple prenyltransferase orthologs on different substrates (genistein, naringenin, kaempferol, etc.) and show prenylated structures. However, the site of prenylation (6- or 8-) differs depending on the enzyme and/or substrate. How can the authors confidently report these structures and different prenylation sites?
3) The authors cite a key preprint that has since been published in Nature Communications (Zhang, Z. et al. Discover the Maze-like Pathway for Glabridin Biosynthesis.). Since there is significant overlap between these two studies, it is important to differentiate the submitted manuscript. Both studies achieved similar glabridin titers in engineered yeast (0.5 vs 0.24 mg/L), yet neither paper demonstrated improved production in a fed-batch bioreactor. Thus, one way to ostensibly differentiate their study would be to demonstrate improved production in a controlled bioreactor.
4) Related to the above comment, the authors claim that their engineered pathway is advantageous by omitting the methylation + demethylation steps in the native pathway. However, the recent report by Zhang et al. achieved higher titers using the native pathway involving methylation. Thus, it is not clear that circumventing the methylation pathway provides an advantage in production or efficiency. Also, several other studies have reported g/L concentrations of methylated natural products in engineered yeast without modifying SAM production or pathways (e.g., nearly 5 g/L (S)-reticuline using three plant methyltransferases in Narcross et al. bioRxiv 2023 and Pyne et al. Nat. Commun. 2023). Based on these studies, it should be straightforward to engineer yeast to produce mg/L concentrations of glabridin using the methylation route.
5) Overall, the authors should provide more discussion of the related Zhang et al. publication throughout, including highlighting both similarities and differences to their work.

Minor comments:
6) Figure 3 should be modified to avoid the use of a vertical and slanted yeast cell.
7) Figures and figure panels are not cited in order in the main text.
8) Some sentences are missing key references (e.g., lines 229-230 – no citations for key glyceollin biosynthesis studies referred to).

Reviewer #2

(Remarks to the Author)
In this manuscript, the authors establish an innovative "block-building" engineering paradigm that successfully achieves de novo biosynthesis of glabridin and provides a generalizable methodological framework for microbial manufacturing of complex isoflavonoids. This study offers a distinct alternative to traditional transcriptome-guided approaches for uncovering biosynthetic pathways of unknown natural products. The manuscript is nicely designed, comprehensively executed, well written, and accordingly worth publication on Nature Communications. Following please find a few suggestions to further improve this manuscript.
1. The authors should perform systematic comparison with the native pathway. Recently, Zhang et al. reported the natural, maize-like pathways for de novo biosynthesis of glabridin on the same journal. It is suggested to highlight the novelty and specific advantages of the artificial pathway over the natural pathway, including biosynthesis efficiency and by-product accumulation.
2. Although bypassing methylation eliminates the SAM cycle, the introduction of two P450 enzymes (CYP81E12 and GIS1) may lower pathway efficiency and increase NADPH burden. P450 enzymes are known to suffer from low catalytic activities. The authors are suggested to briefly compare the two pathways.
3. What about the generalizability of this modular strategy? Is it possible for the authors to add another example to show the generalizability of this modular strategy?
4. The authors are suggested to better illustrate the specific reasons for abandoning the equol route. Route 1 stopped at the prenylation step. It is suggested to add a brief explanation of whether this was due to the lack of 2'-hydroxyl in equol preventing pentacyclic intermediate formation, or insufficient specificity of PT enzymes toward saturated C-ring substrates. This would help clarify the chemical logic of pathway design.
5. When using LjPTR2 as a probe for BLASTp screening, it is suggested to supplement the e-value threshold, sequence identity cutoff, and specific criteria for selecting 3 candidates from 10 hits (e.g., based on FPKM expression levels or domain integrity).
6. Compared to haploid full integration, it is suggested to demonstrate the genetic stability advantages of yeast diploids during long-term fermentation (e.g., reduced risk of gene loss) to support the rationale for choosing the mating strategy.
7. Figure 1 requires better organization and presentation format.
8. The major difference in the titer of equol and glabridin should be briefly discussed.
9. What is the rationality of using squalene yield as a surrogate indicator for DMAPP supply capacity?
10. Beyond structural similarity, are there any other key considerations for selecting demethylmedicarpin as the core scaffold?
11. In the results regarding the construction of de novo glabridin biosynthesis, some data were not presented in the corresponding figures; it is recommended to display these clearly or provided in SI.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have sufficiently addressed the issues raised by reviewers and improved the overall interpretation and impact of their study

Reviewer #2

(Remarks to the Author)
I'm OK with the revision and have no additional concerns.

Dear Editor and Reviewers,

We are grateful for the opportunity to revise our manuscript and sincerely thank you for the constructive feedback, which has substantially enhanced the quality and clarity of our work. We have carefully considered all comments and revised the text accordingly. Detailed responses to each point are provided below.

Reviewer #1 (Remarks to the Author):

The manuscript details the engineering of glabridin biosynthesis in baker's yeast, including constructing an artificial pathway, optimizing production, and designing a modular block-based strategy for assembling pathway modules. The authors achieved low mg/L concentrations of glabridin and extend their approach to another natural product using co-culturing of yeast and *E. coli*. Overall, the work is elegant and comprehensive. The manuscript is clear, rich with data, and broadly applicable.

Major comments:

Q1: It is not clear to me that an authentic commercial standard was utilized to confirm the identity of glabridin (and perhaps other key compounds such as equol). The authors state that they purchased standards for formononetin, medicarpin, daidzein, and squalene, but not glabridin and equol, which are the most important compounds in the study. The authors show MS/MS fragmentation of yeast-derived glabridin and claim this confirms its identity, but the fragmentation is not compared to an authentic standard. A Google search shows that standards for both glabridin and equol are readily available.

A1: Thank you for raising this point. We confirm that authentic commercial standards for both glabridin and equol were purchased. On the structural characterization

of related compounds, equol has been discussed in our previous article[1], and the HNMR of glabridin was previously submitted along with the manuscript. Meanwhile, we also identified that one additional standard was omitted from the original Materials and Methods; the complete list of standards and suppliers has now been added to the revised manuscript. The related content has been updated as: "Standards of formononetin (CAS: 485-72-3), medicarpin (CAS: 32383-76-9), daidzein (CAS: 486-66-8), glabridin (CAS: 59870-68-7), equol (CAS: 94105-90-5) and squalene (CAS: 7683-64-9) were purchased from Herbest Bio-Technology Co., Ltd. (Shanxi, China). Vestitol (CAS:35878-41-2) were purchased from ChemFaces Biochemical Co., Ltd (Wuhan, China). NaCl, amino acids, glucose, and antibiotics for microorganism culture were all purchased from Sangon (Shanghai, China). Tryptone, peptone, and yeast extract were purchased from Thermo Scientific Oxoid (Basingstoke, UK). The enzymes used for amplifying DNA fragments were purchased from Vazyme Biotech (Nanjing, China). Other chemicals used in the study were purchased from the National Pharmaceutical Group Corporation (Sinopharm, Beijing, China).". Please see Page 22-23, Line 412-420 in the revised manuscript.

Q2: Related to structural determination, in Figure 3B the authors screened multiple prenyltransferase orthologs on different substrates (genistein, naringenin, kaempferol, etc.) and show prenylated structures. However, the site of prenylation (6- or 8-) differs depending on the enzyme and/or substrate. How can the authors confidently report these structures and different prenylation sites?

A2: Thank you for raising this point. In the screening process, the primary experimental readout was LC-based detection of new product peaks that appear only when a prenyltransferase is expressed (and are absent in the corresponding negative controls), *i.e.*, a clear gain-of-function signal in the culture extract chromatograms. We did not intend to imply that "a new peak" by itself is sufficient to *de novo* prove the exact prenylation position for every enzyme–substrate pair. Rather, our regioisomer calls (6- vs 8-) were made mainly because the prenyltransferase orthologs we selected have been previously characterized in the literatures with known regioselectivity on these flavonoid/isoflavonoid scaffolds. Our observed products (retention behavior and LC-MS features) are consistent with those reported products. For example, *La*G6DT (noted as "PT from *Lupinus albus*") has been reported as a genistein 6-prenyltransferase, whereas the earlier *Sf*N8DT-1 is reported to generate 8-prenylated products on relevant flavonoid substrates. In addition, other plant UbiA-family aromatic prenyltransferases used as orthologs in our panel (*e.g.*, lupin isoflavonoid PTs) have published substrate scopes and product regioselectivity that guided our annotations[2-5], which was noted in Result. The related content has been updated as: "Building upon the design, the reported PTs, including G4DT from *G. max*, N8DT from *Sophora flavescens*, F8DT from *Epimedium koreanum*, PT from *Lupinus albus* and *Pc*M4DT from *Psoralea corylifolia*, as well as their signal peptide–truncated forms (Fig. S2), of which the substrate specificity was tested, were introduced into the yeast." A broader review of plant aromatic prenyltransferases also emphasizes that many UbiA PTs exhibit strict product/regioselectivity, which is why prior characterization can be informative for annotation in heterologous systems[6].

We would like to clarify that the primary goal of testing these previously reported prenyltransferases was substrate scope probing-*i.e.*, to determine whether any ortholog was capable of acting on our target scaffolds and generating a new enzyme-dependent LC peak under our yeast-expression conditions. Unfortunately, we did not observe the expected prenylated product peaks in the culture extracts for the relevant targets. Two well-characterized UbiA-type aromatic prenyltransferases that have been reported in the literature-*Pc*M4DT (from *Psoralea corylifolia*) and *Gm*G4DT (from soybean)-also did not yield a positive signal in our system. Based on these negative results across multiple reported enzymes, we speculate that one plausible explanation is insufficient intracellular prenyl-donor availability (DMAPP/IPP-derived pool) under our current conditions, rather than an absence of catalytic potential *per se*, especially because *Pc*M4DT has been reported to show strict donor specificity for DMAPP. This interpretation is consistent with broader metabolic-engineering experience that prenylated product formation can be constrained by prenyl-donor supply and that rewiring/boosting DMAPP availability (*e.g.*, via prenyl donor-supply engineering) can be necessary to unlock prenylation in heterologous hosts.

In addition, we agree that NMR represents the gold standard for unambiguous structural elucidation, including the assignment of prenylation regiochemistry. However, in our study the prenylated products, when detectable, were present only at trace levels in the screening cultures, which made purification to the milligram scale required for full NMR characterization impractical.

Q3: The authors cite a key preprint that has since been published in Nature Communications (Zhang, Z. et al. Discover the Maze-like Pathway for

Glabridin Biosynthesis.). Since there is significant overlap between these two studies, it is important to differentiate the submitted manuscript. Both studies achieved similar glabridin titers in engineered yeast (0.5 vs 0.24 mg/L), yet neither paper demonstrated improved production in a fed-batch bioreactor. Thus, one way to ostensibly differentiate their study would be to demonstrate improved production in a controlled bioreactor.

A3: Thank you for this comment. We have updated our citation of the key preprint to its published Nature Communications version (Zhang, Z. et al. "Discover the maze-like network for glabridin biosynthesis"). We agree that, given the topical overlap, it is essential to clearly differentiate the submitted manuscript. The overlap largely reflects that both studies were initiated and advanced independently over a comparable timeframe to address the same long-standing bottleneck: reconstructing glabridin biosynthesis in yeast. Although both works target *de novo* glabridin production, they differ in pathway architecture and research emphasis. Zhang et al. focus on reconstructing and interpreting a maze-like/branched biosynthetic network with multiple tailoring nodes and demonstrate *de novo* glabridin production in shake-flask fermentation (reported at ~0.5 mg/L)[7]. In contrast, our study emphasizes on: (i) the *de novo* design of an artificial glabridin biosynthetic route that enables glabridin synthesis via a non-methylation strategy, rather than implementing a native-style methylation/demethylation-inclusive logic. (ii) develop and demonstrate a "block-building" modular framework for pathway construction and implementation, organizing pathway development into defined functional blocks/modules and showing how this framework can be used to assemble and execute the artificial route toward glabridin.

Meanwhile, to further differentiate our study from Zhang et al. we performed a 5-L bioreactor fermentation with controlled pH and dissolved oxygen (and feeding strategy, if applicable), optimization of the C:N ratio in the supplemental medium was performed, and achieved a glabridin titer of 1.20 mg/L, improved from our shake-flask titer of 0.24 mg/L. Meanwhile, daidzein (315.34 mg/L) and compound **(27)** (semi-quantitative) were observed to accumulate at relatively high levels, which may provide further reference for subsequent optimization. This provides process-level verification under controlled conditions beyond shake-flask cultivation, and thereby strengthens the distinction between the two studies in terms of validation depth and translational relevance. Future titer improvement efforts should focus on enzyme engineering approaches, as discussed in the revised manuscript. The fermentation in the 5-L bioreactor was carried out using the same method as previously reported[8], which was described in Materials and Methods section.

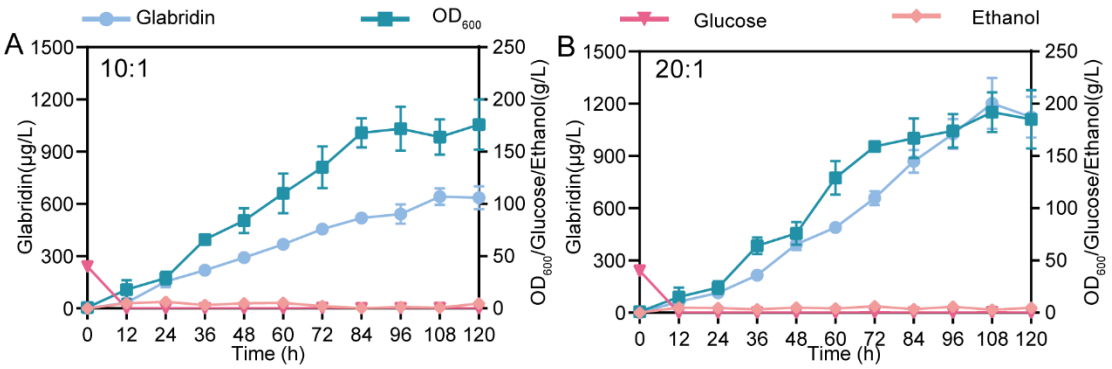

Figure R1 The production of glabridin in 5 L bioreactor

(A) Production of glabridin under a C/N ratio of 10:1; (B) Production of glabridin under a C/N ratio of 20:1, with all other culture conditions kept identical. This comparison investigates how C/N ratio modulation affects the biosynthetic efficiency of glabridin.

Q4: Related to the above comment, the authors claim that their engineered pathway is advantageous by omitting the methylation + demethylation steps in the native pathway. However, the recent report by Zhang et al. achieved higher titers using the native pathway involving methylation. Thus, it is not clear that circumventing the methylation pathway provides an advantage in production or efficiency. Also, several other studies have reported g/L concentrations of methylated natural products in engineered yeast without modifying SAM production or pathways (*e.g.*, nearly 5 g/L (*S*)-reticuline using three plant methyltransferases in Narcross et al. bioRxiv 2023 and Pyne et al. Nat. Commun. 2023). Based on these studies, it should be straightforward to engineer yeast to produce mg/L concentrations of glabridin using the methylation route.

A4: Thank you for raising this point. We agree that high titers of methylated natural products have been achieved in engineered yeast (*e.g.*, gram-per-liter (*S*)-reticuline in an extensively optimized platform)[9,10]. At the same time, we note that production performance in yeast cannot be attributed solely to whether a pathway includes methylation steps, because outcomes are strongly context-dependent—particularly with respect to host background and the specific methyltransferase set used. Both mentioned reticuline studies were built from the BY4741 background (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), whereas our work uses CEN.PK2-1D background (*MATα; ura3-52; trp1-289; leu2-3,112; his3Δ1; MAL2-8C; SUC2*). Reviews of SAM-dependent methyltransferase biotechnology emphasize that methylation flux can be influenced by cofactor availability and SAH inhibition, and often benefits from enzyme/cofactor-cycle engineering[11]. Consistent with this, multiple studies have implemented SAM-

supply engineering in yeast, indicating that methyl-donor status can be relevant depending on the pathway/product context[12,13]. Therefore, we cannot conclude from existing cross-study comparisons whether methyl-donor availability (or strain background differences) explains observed titer differences.

We further note that, for glabridin specifically, the recent pathway mapping/reconstruction study reported *de novo* production at sub-mg/L to low-mg/L levels in shake-flask cultivation (*e.g.*, ~0.5 mg/L) and highlighted a highly branched, "maze-like" tailoring network[7]. This suggests that even when using a methylation-including/native-style architecture, glabridin biosynthesis in yeast remains constrained by overall pathway complexity and other host-/enzyme-dependent factors that are not yet fully resolved.

Crucially, our main motivation for adopting a non-methylation design is selectivity and product-profile control, rather than claiming an inherent titer advantage under all conditions. In preliminary work on isoflavone (daidzein) methylation, we observed off-position methylated regioisomer and over-methylated products, consistent with reported regioselectivity variability and catalytic promiscuity of plant flavonoid/isoflavone *O*-methyltransferases[14]. In line with this, our HPLC chromatograms (Fig. 2C) show multiple side-product peaks when methylation is involved, supporting our rationale to bypass methylation in this study. Accordingly, we revised the manuscript to clarify that the advantage of the non-methylation route is reduced byproduct formation and simplified pathway control, not an a priori guarantee of higher titer.

Finally, our metabolite profiling indicates that the dominant bottleneck in our current strain is downstream of compound **(27)** rather than SAM-dependent methylation capacity: compound **(27)** accumulates substantially in the broth (Fig.

4D), consistent with the PTR-catalyzed five-membered ring-opening conversion of **(27)** to downstream intermediate/product being rate-limiting under our conditions. We added this observation and interpretation to the revised manuscript to clarify the current bottleneck and future optimization direction. The related content has been updated in discussion section as: "Analogous to "the protection and deprotection" strategies in chemical synthesis, the introduction of methylation increases the complexity of the synthetic pathway. Although gram-per-liter titers have been demonstrated for certain methylated natural products such as (*S*)-reticuline in extensively optimized yeast platforms[9,10], broader experience with SAM-dependent methyltransferases suggests that methylation flux can be strongly context-dependent (*e.g.*, sensitivity to SAM/SAH balance) and may benefit from enzyme and/or cofactor-cycle engineering[11,12]. For glabridin, recent pathway mapping highlighted a highly branched "maze-like" tailoring network and reported only sub-mg/L *de novo* titers in shake flasks, supporting the view that pathway architecture, enzyme compatibility, and host background remain major determinants at the current stage[7]. Our rationale for a non-methylation design is primarily improved selectivity and product-profile control: plant flavonoid/isoflavone *O*-methyltransferases can display variable regioselectivity and promiscuity, which can create competing branches and elevate byproduct formation[14]. Consistent with this, we observe multiple side-product peaks when methylation is involved (Fig. 2C). Moreover, *O*-demethylation is commonly proposed to proceed via hydroxylation of the *O*-linked methyl group followed by formaldehyde elimination, potentially adding detoxification burden and another variable impacting product profiles[15] (while alternative THF-dependent *O*-demethylation

routes exist in other biological contexts[16]). The metabolite profiling also shows substantial accumulation of compound **(27)** (Fig. 4D), consistent with a downstream PTR-catalyzed ring-opening step being rate-limiting under our current conditions, thereby providing a focused direction for further optimization independent of methylation capacity." Please see the Page 21-22, Line 377-396 in the revised manuscript.

Q5: Overall, the authors should provide more discussion of the related Zhang et al. publication throughout, including highlighting both similarities and differences to their work.

A5: Thank you for the comment. We therefore revised the manuscript to integrate discussion of Zhang et al. at multiple relevant points (Introduction, Results context, and Discussion), rather than citing it only in passing. In these revisions, we explicitly acknowledge the shared goal of establishing *de novo* glabridin production in yeast and the overlap in key biosynthetic steps, while clearly delineating the distinctions in (i) pathway logic (their maze-like/native-style network versus our artificial, non-methylation route), (ii) engineering emphasis (their network reconstruction/interpretation versus our block-building modular framework and route design rationale), and (iii) experimental validation scope (including the controlled 5-L bioreactor verification added in our revised manuscript). We also added a concise comparison paragraph summarizing these similarities and differences to guide readers and to ensure the novelty and contribution of our work. The related content has been updated in discussion section as: "Analogous to "the protection and deprotection" strategies in chemical synthesis, the introduction of methylation increases the complexity of

the synthetic pathway. Although gram-per-liter titers have been demonstrated for certain methylated natural products such as (*S*)-reticuline in extensively optimized yeast platforms[9,10], broader experience with SAM-dependent methyltransferases suggests that methylation flux can be strongly context-dependent (*e.g.*, sensitivity to SAM/SAH balance) and may benefit from enzyme and/or cofactor-cycle engineering[11,12]. For glabridin, recent pathway mapping highlighted a highly branched "maze-like" tailoring network and reported only sub-mg/L *de novo* titers in shake flasks, supporting the view that pathway architecture, enzyme compatibility, and host background remain major determinants at the current stage[7]. Our rationale for a non-methylation design is primarily improved selectivity and product-profile control: plant flavonoid/isoflavone O-methyltransferases can display variable regioselectivity and promiscuity, which can create competing branches and elevate byproduct formation[14].Consistent with this, we observe multiple side-product peaks when methylation is involved (Fig. 2C). Moreover, oxidative *O*-demethylation is commonly proposed to proceed via hydroxylation of the *O*-linked methyl group followed by formaldehyde elimination, potentially adding detoxification burden and another variable impacting product profiles[15] (while alternative THF-dependent *O*-demethylation routes exist in other biological contexts[16]). The metabolite profiling also shows substantial accumulation of compound **(27)** (Fig. 4D), consistent with a downstream PTR-catalyzed ring-opening step being rate-limiting under our current conditions, thereby providing a focused direction for further optimization independent of methylation capacity." Please see the Page 21-22, Line 377-396 in the revised manuscript.

Minor comments:

Q6: Figure 3 should be modified to avoid the use of a vertical and slanted yeast cell.

A6: Thank you for this suggestion. We have revised Figure 3 to remove the vertical and slanted yeast cell illustration and replaced it with a clearer, conventional schematic.

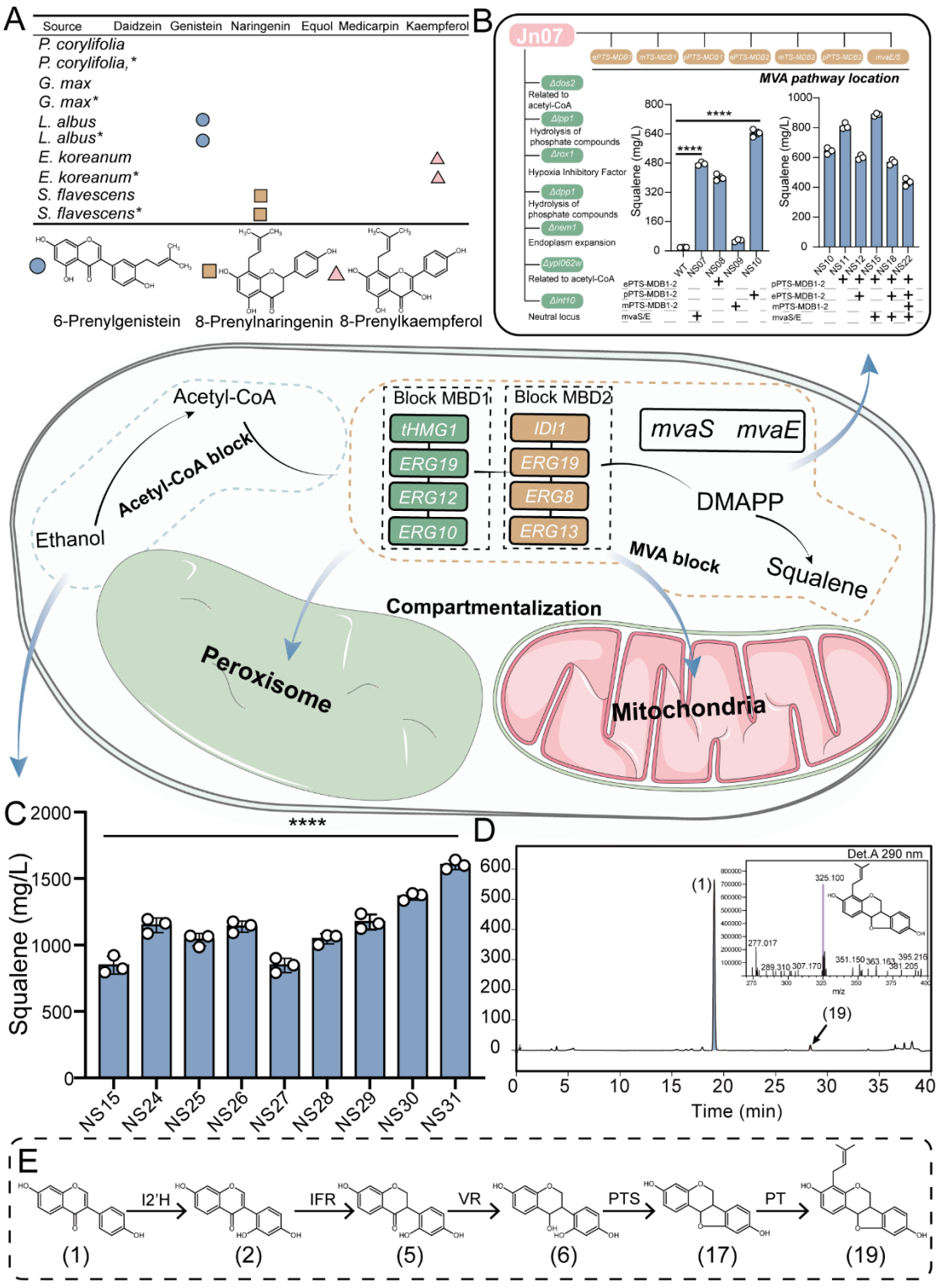

Figure 3. Establishment and optimization of a prenyl-donor platform and pterocarpan prenylation module for producing prenylated isoflavonoids. (A) Substrate-scope screening of prenyltransferase sources against a panel of flavonoid/isoflavonoid acceptors (daidzein, genistein, naringenin, equol,

medicarpin, and kaempferol), identifying products including 6-prenylgenistein, 8-prenylnaringenin, and 8-prenylkaempferol (structures shown). * indicates signal-peptide truncation. (B) Genomic integration strategy and locus selection for assembling MVA-pathway modules in strain Jn07, evaluated using squalene titers to compare pathway location/construct combinations. (C) Squalene production across engineered strains (NS15–NS31) as a proxy for MVA/DMAPP supply capacity. (D) Representative high-performance liquid chromatography trace (with MS inset) confirming the formation of prenylated product (19) from precursor (1). (E) Reconstituted reaction sequence from (1) to (19) via I2′H, IFR, VR, PTS, and PT, with compound numbers matching the intermediate numbering used throughout the study. Please see Page 11-12, Line 192-205 in the revised manuscript.

Q7: Figures and figure panels are not cited in order in the main text.

A7: Thank you for noting this issue. We have carefully checked the entire manuscript and revised the main text to ensure that all figures and figure panels are cited in the correct numerical order (including subpanels).

Q8: Some sentences are missing key references (*e.g.*, lines 229-230 – no citations for key glyceollin biosynthesis studies referred to).

A8: Thank you for noting this. We have revised the manuscript to ensure that all key statements are properly supported by citations and checked the surrounding paragraphs for similar omissions.

Reviewer #2 (Remarks to the Author):

In this manuscript, the authors establish an innovative "block-building" engineering paradigm that successfully achieves *de novo* biosynthesis of glabridin and provides a generalizable methodological framework for microbial manufacturing of complex isoflavonoids. This study offers a distinct alternative to traditional transcriptome-guided approaches for uncovering biosynthetic pathways of unknown natural products. The manuscript is nicely designed, comprehensively executed, well written, and accordingly worth publication on Nature Communications. Following please find a few suggestions to further improve this manuscript.

Q1: The authors should perform systematic comparison with the native pathway. Recently, Zhang et al. reported the natural, maize-like pathways for *de novo* biosynthesis of glabridin on the same journal. It is suggested to highlight the novelty and specific advantages of the artificial pathway over the natural pathway, including biosynthesis efficiency and by-product accumulation.

A1: Thank you for the comment. We agree that, given the recent Nature Communications paper by Zhang et al. describing the native/maze-like glabridin biosynthetic network in yeast, it is important to clearly articulate what is novel in our artificial pathway design and what practical advantages it offers.

In the revised manuscript, we provide a more systematic, side-by-side discussion of the native (maze-like) route logic versus our artificial, non-methylation route, focusing on design objectives that are directly relevant to microbial production. Zhang et al. reconstruct a ladder-like, multi-route tailoring network that includes methylation/demethylation logic and demonstrate de novo glabridin formation in yeast at ~0.5 mg/L under shake-flask conditions. By contrast, our work centers on (i) rational design and construction of an artificial, non-methylation route to

glabridin and (ii) a block-building modular framework for pathway assembly and implementation. We emphasize that our "advantage" claim is not that the artificial route must always outperform the native route in titer at this early stage, but that it can offer pathway economy and controllability: fewer methylation-related branches, reduced risk of methylation-derived product heterogeneity, and a cleaner product profile under our conditions. In particular, we explicitly point to our observed off-target/over-methylation byproducts (Fig. 2C) as a practical motivation for avoiding methylation steps in our engineered context. (This clarification is now included in the Discussion). To address the "systematic comparison," we added a concise comparison in the Discussion section that highlights, for each route, the expected trade-offs in (a) step complexity and branching, (b) cofactor coupling (SAM/SAH dependence vs P450/NADPH dependence), and (c) byproduct risk (methylation-derived regio/over-methylation vs P450-dependent burden), using Zhang et al. as the reference for the native/maze-like pathway logic. This framing is intended to make the novelty and specific engineering advantages of the artificial design transparent without overclaiming absolute efficiency gains absent a head-to-head implementation of the full native route in our chassis. Finally, to strengthen differentiation at the process-validation level (as also suggested elsewhere by the reviewer), we added 5-L bioreactor fermentation verification in the revised manuscript, which extends validation beyond shake-flask cultivation and supports the translational relevance of our modular strategy. The related content has been updated in discussion section as: "Analogous to "the protection and deprotection" strategies in chemical synthesis, the introduction of methylation increases the complexity of the synthetic pathway. Although gram-per-liter titers have been demonstrated

for certain methylated natural products such as (*S*)-reticuline in extensively optimized yeast platforms[9,10], broader experience with SAM-dependent methyltransferases suggests that methylation flux can be strongly context-dependent (*e.g.*, sensitivity to SAM/SAH balance) and may benefit from enzyme and/or cofactor-cycle engineering[11,12]. For glabridin, recent pathway mapping highlighted a highly branched "maze-like" tailoring network and reported only sub-mg/L *de novo* titers in shake flasks, supporting the view that pathway architecture, enzyme compatibility, and host background remain major determinants at the current stage[7]. Our rationale for a non-methylation design is primarily improved selectivity and product-profile control: plant flavonoid/isoflavone *O*-methyltransferases can display variable regioselectivity and promiscuity, which can create competing branches and elevate byproduct formation[14].Consistent with this, we observe multiple side-product peaks when methylation is involved (Fig. 2C). Moreover, oxidative *O*-demethylation is commonly proposed to proceed via hydroxylation of the *O*-linked methyl group followed by formaldehyde elimination, potentially adding detoxification burden and another variable impacting product profiles[15] (while alternative THF-dependent *O*-demethylation routes exist in other biological contexts[16]). The metabolite profiling also shows substantial accumulation of compound **(27)** (Fig. 4D), consistent with a downstream PTR-catalyzed ring-opening step being rate-limiting under our current conditions, thereby providing a focused direction for further optimization independent of methylation capacity." Please see the Page 21-22, Line 377-396 in the revised manuscript.

Q2: Although bypassing methylation eliminates the SAM cycle, the introduction of two P450 enzymes (CYP81E12 and GIS1) may lower pathway efficiency and increase NADPH burden. P450 enzymes are known to suffer from low catalytic activities. The authors are suggested to briefly compare the two pathways.

A2: Thank you for this suggestion. We agree that while bypassing methylation removes SAM-dependent methyltransferase steps, it introduces two cytochrome P450 enzymes (CYP81E12 and GIS1) that require NADPH-supported electron transfer, and thus represents a shift in metabolic/cofactor burden rather than an absolute reduction. We have revised the Discussion to briefly compare these two route concepts: the methylation-inclusive route couples' flux to the SAM/SAH cycle, whereas our non-methylation route removes that coupling but makes P450 performance and NADPH supply key determinants of pathway flux. Plant/microbial P450 steps are widely recognized as frequent bottlenecks in heterologous hosts due to expression and catalytic constraints and their dependence on reducing equivalents. At the same time, we believe the non-methylation design can still provide a net benefit in our context for two reasons. First, SAM synthesis is intrinsically ATP-consuming (ATP is converted to a tripolyphosphate species that is subsequently hydrolyzed), so reducing unnecessary SAM turnover and associated recycling demand can alleviate energy and cofactor coupling. Second, and most importantly for our study, bypassing methylation helps prevent off-target and over-methylation side products (Fig. 2C), which would otherwise divert flux away from the desired product. In addition, we would like to clarify that NADPH supply was already considered during construction of our prenyl-donor module, as prenyl-donor

generation and P450 catalysis are both redox-intensive; accordingly, our platform incorporates redox/cofactor design elements to support NADPH-dependent steps. The related content has been updated in discussion section as: "Analogous to "the protection and deprotection" strategies in chemical synthesis, the introduction of methylation increases the complexity of the synthetic pathway. Although gram-per-liter titers have been demonstrated for certain methylated natural products such as (*S*)-reticuline in extensively optimized yeast platforms[9,10], broader experience with SAM-dependent methyltransferases suggests that methylation flux can be strongly context-dependent (*e.g.*, sensitivity to SAM/SAH balance) and may benefit from enzyme and/or cofactor-cycle engineering[11,12]. For glabridin, recent pathway mapping highlighted a highly branched "maze-like" tailoring network and reported only sub-mg/L *de novo* titers in shake flasks, supporting the view that pathway architecture, enzyme compatibility, and host background remain major determinants at the current stage[7]. Our rationale for a non-methylation design is primarily improved selectivity and product-profile control: plant flavonoid/isoflavone *O*-methyltransferases can display variable regioselectivity and promiscuity, which can create competing branches and elevate byproduct formation[14].Consistent with this, we observe multiple side-product peaks when methylation is involved (Fig. 2C). Moreover, oxidative *O*-demethylation is commonly proposed to proceed via hydroxylation of the *O*-linked methyl group followed by formaldehyde elimination, potentially adding detoxification burden and another variable impacting product profiles[15] (while alternative THF-dependent *O*-demethylation routes exist in other biological contexts[16]). The metabolite profiling also shows substantial accumulation of compound **(27)** (Fig. 4D),

consistent with a downstream PTR-catalyzed ring-opening step being rate-limiting under our current conditions, thereby providing a focused direction for further optimization independent of methylation capacity." Please see the Page 21-22, Line 377-396 in the revised manuscript.

Q3: What about the generalizability of this modular strategy? Is it possible for the authors to add another example to show the generalizability of this modular strategy?

A3: Thank you for this suggestion and we agree that demonstrating generalizability is important. In the manuscript, we already include an independent example-equol biosynthesis-to illustrate that the proposed block-building modular strategy is transferable beyond glabridin. In particular, we implement the equol pathway using the same modular partitioning logic and validate its function at the consortium level, which provides a practical demonstration that the strategy can be applied in a different product context and at a different organizational scale.

To further improve clarity, we revised the text to explicitly position the equol case as a generalizability demonstration of the modular framework, while keeping the central focus of this work on the design and construction of an artificial glabridin biosynthetic route and the establishment of the corresponding modular build strategy that enabled that route.

Q4: The authors are suggested to better illustrate the specific reasons for abandoning the equol route. Route 1 stopped at the prenylation step. It is suggested to add a brief explanation of whether this was due to the lack of

2'-hydroxyl in equol preventing pentacyclic intermediate formation, or insufficient specificity of PT enzymes toward saturated C-ring substrates. This would help clarify the chemical logic of pathway design.

A4: Thank you for this suggestion. We agree that the rationale for discontinuing the equol-based Route 1 should be stated more explicitly. We have revised the manuscript to clarify both the chemical logic we considered and, most importantly, the experimental observations that led us to abandon this route.

In practice, our decision was driven by the LC-based screening results. When we tested Route 1, we did not observe any new product peaks upon introducing the proposed hydroxylation step on the equol scaffold, and we likewise did not detect new prenylated products after introducing the corresponding prenylation step under our yeast-expression conditions. These negative results suggested that, in our system, the relevant enzymes either (i) exhibit insufficient activity toward the equol scaffold and/or (ii) require a different substitution pattern or oxidation state to enable productive conversion.

The related content has been updated as: "We initially considered the equol pathway, but ultimately decided to discontinue it due to several factors. Equol lacks the 2′-hydroxyl group found in the isoflavone intermediates associated with glabridin biosynthesis, which likely affected the downstream chemical logic and the ability to form the expected cyclic intermediate. Additionally, the saturated C-ring of equol may not be as compatible with prenyltransferases, which typically prefer aromatic or unsaturated substrates, potentially limiting prenylation efficiency. Another consideration is the stereochemical difference between equol (which is (*S*)-configured) and glabridin ((*R*)-configured), which

may further hinder productive turnover by downstream tailoring enzymes." Please see Page 20, Line 355-362, in the revised manuscript.

Q5: When using LjPTR2 as a probe for BLASTp screening, it is suggested to supplement the e-value threshold, sequence identity cutoff, and specific criteria for selecting 3 candidates from 10 hits (*e.g.*, based on FPKM expression levels or domain integrity).

A5: Thank you for this suggestion. We agree that the BLASTp screening and candidate-selection criteria should be stated explicitly to improve reproducibility. We have therefore revised the Methods (De novo transcriptome analysis) to include the BLASTp parameters and the downstream filters used to prioritize candidates for functional testing.

Specifically, the related content has been updated as: "using the following thresholds: *E*-value < 1×10^{-5}, sequence identity > 35%, and query coverage > 70%. From the initial 10 hits, we selected three candidates for experimental characterization based on a combination of: (i) predicted protein length of ~300 – 350 aa, consistent with reported PTR family members, and (ii) presence of the characteristic NmrA-like domain (Pfam PF05368), indicating domain integrity.". Please see Page 25, Line479-483 in the revised manuscript.

Q6: Compared to haploid full integration, it is suggested to demonstrate the genetic stability advantages of yeast diploids during long-term fermentation (*e.g.*, reduced risk of gene loss) to support the rationale for choosing the mating strategy.

A6: Thank you for this suggestion. In our work, we integrated different pathway/precursor modules separately into two haploid strains and then mated them to obtain the final diploid production strain for fermentation. Our primary rationale for adopting the mating strategy was practical modular assembly—*i.e.*, using diploid formation as a convenient "connector" to combine independently built modules—rather than claiming that diploidy inherently guarantees genetic stability. This modular build strategy reduces the construction and debugging complexity that would arise if all upstream precursor pathways and downstream modules were assembled in a single haploid strain, and it enables rapid recombination of module combinations through mating (a commonly used and well-understood genetic operation in yeast).

We agree your point is valid that strain robustness/stability is relevant for extended fermentations. Diploid/polyploid backgrounds are widely discussed in the context of industrial yeast performance. Genome duplication has been associated with altered phenotypes and improved robustness in industrially relevant settings, providing a reasonable supporting rationale for choosing a diploid production chassis when performing longer cultivations[17,18].

Q7: Figure 1 requires better organization and presentation format.

A7: Thank you for this suggestion. We have reorganized and reformatted Figure 1 to improve clarity and readability.

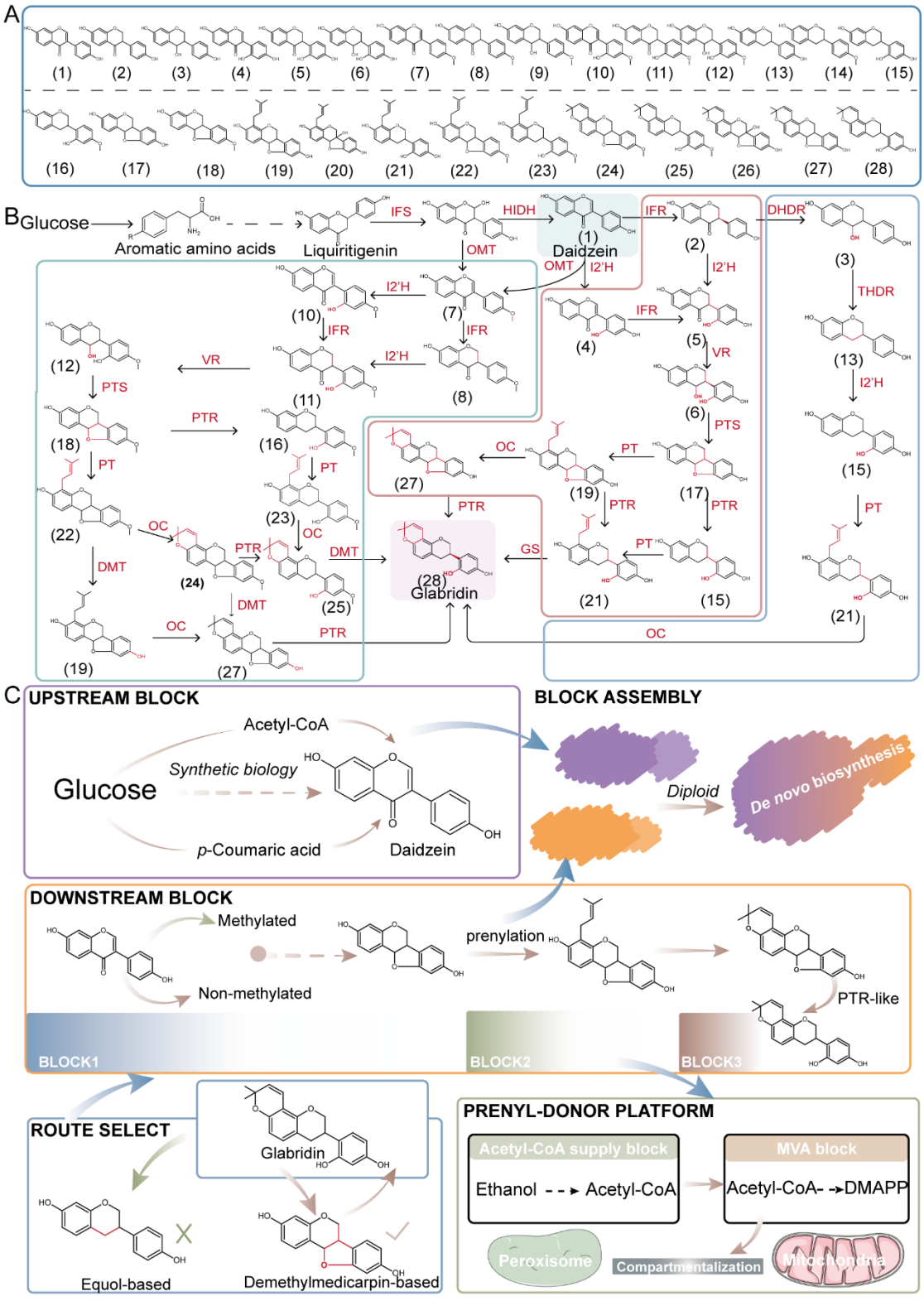

Figure R2. Modular block design for *de novo* biosynthesis of glabridin.

(A) Structural analogs of potential intermediates in the glabridin biosynthetic pathway retrieved from SciFinder (stereochemistry ignored), numbered (1)-(28) for reference. (B) Manually designed schematic of the glabridin biosynthetic pathway. Color-coded

boxes indicate alternative routes: blue, equol-based; orange, demethylmedicarpin-based; green, recently reported native pathway in *G. glabra* (licorice). Enzyme abbreviations: DHDR, dihydrodaidzein reductase; DMT, demethylase; DZNR, daidzein reductase; OC, oxidative cyclase; HIDH, 2-hydroxyisoflavone dehydratase; IFR, isoflavone reductase; IFS, isoflavone synthase; I2′H, isoflavone 2′-hydroxylase; OMT, O-methyltransferase; PT, prenyltransferase; PTR, pterocarpan reductase; PTS, pterocarpan synthase; THDR, tetrahydrodaidzein reductase; VR, vestitone reductase. (C) Block-based assembly strategy linking an upstream precursor-supply block to a downstream tailoring block (prenylation), incorporating a "block-in-block" prenyl-donor platform to enable glabridin biosynthesis with compartmentalization support.

Q8: The major difference in the titer of equol and glabridin should be briefly discussed.

A8: Thank you for your suggestion. We added a brief discussion to explain the large titer gap between equol and glabridin (32.8 mg/L vs 0.24 mg/L) in our system. One contributing factor is likely physicochemical differences: equol is less hydrophobic than glabridin based on ALOGPS-predicted logP values (equol logP = 2.91 vs glabridin logP = 3.81), which can reduce membrane partitioning and product-associated stress and thereby support higher apparent titers.

In addition, the two pathways differ substantially in pathway length/topology and kinetic bottlenecks. Equol biosynthesis in our framework relies mainly on a short set of reductive steps, which has been well-optimized in previous strain, whereas glabridin formation requires a longer sequence of transformations, including regioselective prenylation and more complex downstream scaffold-forming logic. A longer pathway increases the number of potential rate-limiting steps and

opportunities for flux loss to side reactions, which can constrain the achievable titer. The related content has been updated as: "The titer difference between equol and glabridin can be attributed to their different hydrophobicity and the complexity of their biosynthetic pathways. Specifically, equol pathway lacks key features, like prenylation compatibility, that are critical for efficient biosynthesis in our system. The modular approach used for glabridin production allowed us to optimize these complex steps, highlighting the importance of pathway design that is both synthetically feasible and functionally compatible to enhance overall efficiency. This also might support the notion that block-building at the community level can outperform single-strain constructs, which aligns with the foundational goal - divide and conquer." Please see Page 19-20, Line 340-348 in the revised manuscript.

Q9: What is the rationality of using squalene yield as a surrogate indicator for DMAPP supply capacity?

A9: Thank you for the question. Our rationale for using squalene titer as a surrogate readout is that squalene is a major endogenous "sink" for isoprenoid precursors in *S. cerevisiae*. In the mevalonate pathway, DMAPP and IPP are condensed to GPP/FPP, and squalene synthase (Erg9) consumes two molecules of FPP to form squalene. Therefore, when strains are compared under otherwise identical conditions, increases in upstream prenyl-donor supply capacity (IPP/DMAPP to FPP) are expected to increase flux into squalene, making squalene titer a practical indirect indicator of overall isoprenoid precursor supply.

In addition, we selected squalene for practical reasons: (i) squalene is endogenous, so it can be monitored without introducing an additional

heterologous reporter gene that might perturb metabolism, and (ii) squalene extraction and quantification are simple and robust relative to direct measurement of intracellular DMAPP/IPP pools, which are technically challenging and often require specialized quenching and LC–MS workflows. We also clarify in the revised manuscript that this proxy is best interpreted as a relative measure of FPP/IPP/DMAPP-derived flux capacity, rather than a direct measurement of the intracellular DMAPP concentration, and we use it only for within-study comparisons across strains/modules under the same cultivation conditions.

Finally, we note that although we used squalene as a convenient readout here, future strain optimization could also increase prenyl-donor availability by down-tuning squalene formation (*e.g.*, reducing Erg9 flux) to redirect FPP toward the desired prenylation reactions. However, in the current work we did not pursue this strategy because our platform already provided sufficient prenyl-donor supply to support the downstream steps without requiring attenuation of squalene biosynthesis, and we therefore kept the native squalene pathway intact to avoid compromising sterol metabolism and growth.

Q10: Beyond structural similarity, are there any other key considerations for selecting demethylmedicarpin as the core scaffold?

A10: Thank you for the comment. Beyond overall structural similarity, we selected demethylmedicarpin as the core scaffold mainly for engineering feasibility in yeast. First, demethylmedicarpin provides a prenylation-ready pterocarpan framework with an appropriate phenolic substitution pattern, allowing a shorter, more direct route toward glabridin with fewer additional tailoring steps. Second,

using a dimethyl scaffold reduces reliance on SAM-dependent *O*-methylation, which in our system is prone to generating off-target/over-methylated side products and complicates flux control. Third, from an enzyme-compatibility perspective, reported pterocarpan/isoflavonoid enzymes are more likely to accept a demethylmedicarpin-like scaffold, improving the tractability of pathway construction. We have clarified these points in the revised manuscript to make it explicit that the scaffold choice was guided by pathway economy and controllability, not structural similarity alone.

Q11: In the results regarding the construction of *de novo* glabridin biosynthesis, some data were not presented in the corresponding figures; it is recommended to display these clearly or provided in SI.

A11: Thank you for pointing this out. We have now provided the missing data and supporting analyses in the Supplementary Information, and we revised the main text to clearly direct readers to the relevant SI items where these results are presented.

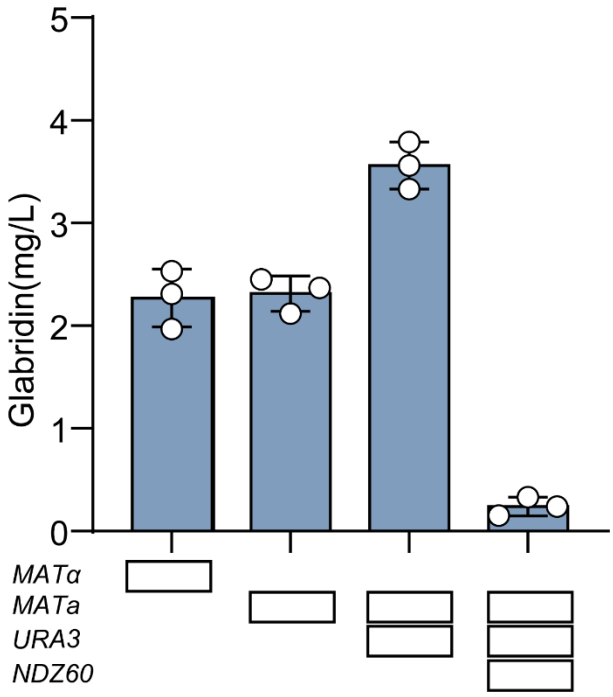

Figure R3 The biosynthesis of glabridin.

The effects of mating-type switching, *URA3* complementation, and diploid strain construction on the synthesis of glabridin in the daidzein-to-glabridin biosynthetic module strain, where the mating-type switching and tag complementation were performed with 250 mg/L of daidzein supplementation.

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Dear Editor and Reviewers,

We sincerely appreciate the opportunity to revise our manuscript and would like to express our gratitude for the thoughtful feedback. We are pleased to hear that you are satisfied with the revisions and agree that no further changes are necessary. Thank you for your time and for helping improve the quality of our work.

Reviewer #1 (Remarks to the Author):

The authors have sufficiently addressed the issues raised by reviewers and improved the overall interpretation and impact of their study

Reviewer #2 (Remarks to the Author):

I'm OK with the revision and have no additional concerns.

Reviewer #2 (Remarks to the Author):

In this manuscript, the authors establish an innovative "block-building" engineering paradigm that successfully achieves *de novo* biosynthesis of glabridin and provides a generalizable methodological framework for microbial manufacturing of complex isoflavonoids. This study offers a distinct alternative to traditional transcriptome-guided approaches for uncovering biosynthetic pathways of unknown natural products. The manuscript is nicely designed, comprehensively executed, well written, and accordingly worth publication on Nature Communications. Following please find a few suggestions to further improve this manuscript.