

Heterogenous biosynthesis of medicarpin using engineered *Saccharomyces cerevisiae*

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Research Article

Keywords: Medicarpin, Glycyrrhiza glabra L., *Saccharomyces cerevisiae*, Medicarpin biosynthesis pathway, Transcriptome sequencing, Pterocarpan

Posted Date: April 13th, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-2645193/v1>

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Abstract

Background

Medicarpin is one important bioactive compound with multiple medicinal activities, including anti-tumor, anti-osteoporosis, anti-bacterial effects. Medicarpin is assigned to pterocarpan derived from medicinal plants, such as *Sophora japonica*, *Glycyrrhiza uralensis* Fisch. and *Glycyrrhiza glabra* L. However, these medicinal plants only contain low amounts of medicarpin. Moreover, the planting area for medicarpin-producing plants is limited, thus, current medicarpin supply cannot satisfy the great demands of medicinal markets.

Results

In this study, eight key genes involved in medicarpin biosynthesis were identified by comparative transcriptome analysis and bioinformatic analyses. *In vitro* and *in vivo* enzymatic activities assays confirmed the catalytic functions of candidate enzymes were responsible for the biosynthesis of medicarpin and medicarpin intermediates. Further engineering of these genes in *Saccharomyces cerevisiae* achieved the heterogenous biosynthesis of medicarpin using liquiritigenin as the substrate, and the final medicarpin titer was 0.82 ± 0.18 mg/L. By increasing the gene copy number of *VR* and *PTS*, the final titer of the medicarpin increased to 2.05 ± 0.72 mg/L.

Conclusion

This study provides a solid foundation for the economical and sustainable production of medicarpin by synthetic biology strategy.

Background

Pterocarpan belongs to the group of dihydroisoflavonoids with a benzopyran-benzofuran four ring structure skeleton. Pterocarpan is widely distributed in various medicinal plants, such as *Pueraria lobata*, *Hedysari Radix*. Pterocarpan has many bioactivities, including anti-tumor, anti-osteoporosis, anti-bacterial, anti-inflammation, antioxidant, anti-estrogenic, inhibition of acetylcholinesterase and neuraminidase, and immunosuppressive activities[1]. Thus, pterocarpan is the potential drug for the treatment of several different diseases.

Medicarpin, one pterocarpan compound, is widely distributed in medicinal plants, including *Sophora japonica*, *Zollernia paraensis*, *Platymiscium yucatamun*, *Machaerium aristulatum*, *Platymiscium floribundum*, *Brazilian red propolis*, *Glycyrrhiza uralensis* Fisch. and *Glycyrrhiza glabra* L.[2]. Medicarpin has anti-osteoporotic[3], bone protection activities[4, 5], and shows cytotoxic activities in human breast cancer cells and human epidermoid carcinoma cells with low IC_{50} [6]. Besides, it can lead to apoptosis of multi-drug-resistant P388 leukemia cells[7], and display antifungal[8], antibacterial properties[9, 10]. Medicarpin is mainly extracted from *Glycyrrhiza* plants, and its composition in the *G. glabra* L. is 0.09%,

and its composition in *G. uralensis* Fisch. is 0.00012%[11, 12]. The *Glycyrrhiza* plants mainly grow in arid and semiarid areas. Previous overharvest of *Glycyrrhiza* plants led to the desertification and disappearance of desertification in nature. Planting *Glycyrrhiza* plants occupies large amounts of field, and it takes 2–3 years obtain high-level medicarpin in *Glycyrrhiza* plants. Therefore, other new alternative sources to obtain sufficient amount of medicarpin is of great interest.

The chemical synthesis process of medicarpin is complex, and the reaction conditions are severe. Moreover, the yield of chemical synthesis for medicarpin is low. Plant cell culture can be used to collect biomass and extract medicarpin, but the conditions for plant cell cultivation are relatively harsh, and plant cell grow slowly, leading to the unstable yields of medicarpin. Recently, synthetic biology have been applied to produce artemisinic acid[13, 14], ginsenoside[15–17], and other complex plant natural products. Especially, some flavonoid biosynthetic pathways in plants, such as quercetin, resveratrol, kaempferol, baicalein and scutellarein, have been identified[18], and efficient production of these flavonoids in yeasts have been realized using synthetic biology and metabolic engineering strategy[19–21].

As a valuably medicinal plant, the genome and transcriptome of *G. uralensis* have been sequenced, and the biosynthesis pathways of the glycyrrhizin[22] and liquiritin[23] have been revealed. The liquiritin biosynthesis pathway was reconstructed in yeast, and the heterologous synthesis of the main flavonoid skeleton of *G. uralensis* was realized[23]. Currently, the daidzein biosynthesis was rebuilt in yeast and optimized, and the final titer of daidzein was 85.4 mg/L[24]. Further introducing of plant glycotransferases in yeast led to the production of 72.8 mg/L puerarin and 73.2 mg/L daidzin. The conversion of vestitone to medicarpin in *Medicago sativa* L. was catalyzed by vestitone reductase and 7,2'-dihydroxy-4'-methoxyisoflavanol dehydratase[25]. These provide the possibility to produce *G. uralensis* flavonoids at economically and sustainably way via synthetic biology and other cutting-edge biotechnologies. Thus, identifying the full medicarpin biosynthetic pathway in *Glycyrrhiza* and engineering yeasts to produce medicarpin are of great interest.

In this study, comparative analysis of *Glycyrrhiza uralensis* Fisch. and *Glycyrrhiza glabra* L. transcriptome identified some potential keystone genes involving in the biosynthesis of medicarpin from liquiritin. The functions of these genes were further verified *in vitro*. Then, the genes used for medicarpin biosynthesis were engineered in *Saccharomyces cerevisiae*, and the natural product metabolites of the engineered yeast strains were characterized. This study provides a new strategy for the sustainable production of complex bioactive natural products derived from traditional Chinese medicines using microbial cell factories.

Results And Discussion

Transcriptomic analysis of *G. glabra* L. and *G. uralensis* Fisch. root

The medicarpin was detected in the roots of the *G. glabra* L. and *G. uralensis* Fisch (**Fig. S1**). Though the medicarpin compositions in these two different *Glycyrrhiza* plants are different, their contents are low and

can't be obtained. In order to reveal the possible key enzymes for medicarpin biosynthesis in *Glycyrrhiza*, deep sequencing of the *G. glabra* L. and *G. uralensis* Fisch. root transcriptome was performed (**Tables S5-6 and Fig. S2**)[26, 27], and *Glycyrrhiza* genes were comprehensively annotated.

For the transcriptomic data, 35953 of the 36159 unigenes from *G. glabra* L. and *G. uralensis* Fisch. have high identities with genes in Nr database, whereas 27077 unigenes have high identities with sequences in Swiss-Prot database. All unigenes > 1000 bp has blast matches, and they are similar with homologies among several different plant species (**Fig. S3**). Among them, 7854 contigs were matched to *Cicer arietinum*, and 4899 and 4768 contigs were matched to *Glycine max* and *Cajanus cajan*, respectively. GO analysis suggested that the biological process was the most abundant (57453, 38.29%), followed by cellular component (46775, 31.17%) and molecular function (45802, 30.52%) (**Fig. S4**). For biological process, cellular process and metabolic process were dominant, suggesting that some important cellular processes and metabolic activities occurred in *Glycyrrhiza* roots. All annotated unigenes were assigned to 25 COG categories (**Fig. S5-6**). Amino acid transport and metabolism category is the largest group, followed by cell wall/membrane/envelop biogenesis and cytoskeleton.

To identify the active biological pathways and understand the biological gene functions, all unigenes were further analyzed by the KEGG pathway database. In summary, 8528 unigenes were assigned to six KEGG categories[28]. The metabolism category included ten sub-categories, and the four main sub-categories were carbohydrate metabolism, amino acid metabolism, lipid metabolism and energy metabolism (**Fig. S7**). A total of 464 unigenes were assigned to the biosynthesis of other secondary metabolites sub-category, and most of them were annotated to be the enzymes accounting for the synthesis of flavonoids, flavone and flavonol. These hinted that *Glycyrrhiza* roots harbored diverse natural product biosynthetic genes and might synthesize a variety of metabolites.

Prediction of medicarpin biosynthetic genes

Differentially expressed transcript analysis identified more than 2-fold changes in different gene expression and false discovery rate (FDR) < 0.05, and 1241, 1882, and 3123 genes were up-regulated, down-regulated, and differentially expressed, respectively in the roots of *G. glabra* L. and *G. uralensis* Fisch. (**Fig. S8**). Based on the degree of KEGG enrichment analysis, selenocompound metabolism, and other natural product biosynthetic pathway were differentially expressed in these two *Glycyrrhiza* roots (**Fig. S9-10**). The differentially expressed gene analysis based on GO, COG and KOG annotation identified some genes associated with biosynthesis, transport, and catabolism of secondary metabolites, amino acid transport and metabolism, and defense mechanisms (**Fig. S11-12**). Besides, 2431 unigenes were predicted to be transcription factors (**Fig. S13**)[29].

Medicarpin were predicted to the precursor of glybridin[30], and glybridin was mainly extracted from *G. glabra* L.[31–35]. Therefore, the high-level expression genes in the natural product biosynthetic pathway of *G. glabra* L.. may be responsible for the biosynthesis of medicarpin or its intermediates. A total of 176 genes were annotated to encode the key enzymes for medicarpin biosynthesis in the transcriptome data

(Fig. 1, S2 and Table S6). Expression analysis of these candidate genes revealed that genes annotated as CHS, CHR, CHI, 2-hydroxyisoflavanone synthase (2-HIS), cytochrome P450 reductase (CPR), isoflavone 4'-O-methyltransferase (I4'OMT), 2-hydroxyisoflavanone dehydratase (HID), isoflavone 2'-hydroxylase (I2'H), isoflavone reductase (IFR), vestitone reductase (VR), and pterocarpan synthase (PTS) had relatively higher expression levels in *G. glabra* L. than that in *G. uralensis* Fisch. (Fig. 1, and Table S6).

Functional characterization of predicted medicarpin biosynthetic enzymes

The candidate genes 2-HIS, CPR, I4'OMT, HID, I2'H, IFR, VR, and PTS, were expressed in *E. coli* or yeast. The expressed proteins were purified for *in vitro* enzymatic activity assays. Incubation of liquiritigenin with 2-HIS and CPR yielded 2,7,4'-trihydroxyisoflavanone (Fig. 2A). The extraction of yeast harboring 2-HIS, CPR and I4'OMT can generate 2,7-dihydroxy-4'-methoxyisoflavanone using liquiritigenin as substrate (Fig. 2A). For the yeast strain DWY1 harboring 2-HIS, CPR and HID, the yeast extraction can catalyze liquiritigenin to daidzein[24]. When yeast DWY1 crude enzyme solution and I4'OMT protein were added into the reaction system together, can catalyze liquiritigenin to formononetin (Fig. 2B)[36, 37]. Besides, some by-product was generated in the yeast fermentation metabolites (Fig. 2B). Further analysis suggested that the by-product was generated by I4'OMT with liquiritigenin as substrate, and generated by-products were identified as liquiritigenin 4'-methyl ether and liquiritigenin 7-methyl ether by LC-MS and NMR (Fig. 2B and Fig. S14-16)[38, 39]. Thus, I4'OMT could catalyze flavonoids to novel products. The enzyme mixture of I2'H, CPR, IFR, VR and PTS could catalyze formononetin to 2'-hydroxyformononetin, vestitone, and medicarpin (Fig. 2C and Fig. S17)[40, 41]. 2-HIS, CPR, I4'OMT, HID, I2'H, IFR, VR and PTS can catalyze liquiritigenin to medicarpin *in vitro*, showing the biosynthetic pathway from liquiritigenin to medicarpin was recovered. Thus, introduction of the pathway in yeasts might produce medicarpin from liquiritigenin directly.

Heterologous production of medicarpin using engineered yeast

In order to verify the medicarpin synthesis in yeasts, we introduced the eight identified genes into wide-type yeasts, and three engineered yeast strains, DW08, DW09, and DW10, were constructed (Fig. 3A and Table S3). DW09 harboring 2-HIS, CPR, I4'OMT and HID genes can produce formononetin using liquiritigenin as substrate (Fig. 3B and 3C), while DW08 harboring I2'H, CPR, IFR, VR, and PTS genes can produce medicarpin using formononetin as substrate. Further shake flask analysis showed the final medicarpin title of DW08 using formononetin as substrate was 9.21 ± 1.97 mg/L (Fig. 3B and 3D). DW10 contained eight key enzyme genes of 2-HIS, CPR, I4'OMT, HID, I2'H, IFR, VR and PTS, and it can produce medicarpin directly using liquiritigenin as substrate (Fig. 3B and 3E). However, the medicarpin titer of DW10 using liquiritigenin as substrate was 0.82 ± 0.18 mg/L, which was lower than that of the DW08 using formononetin as substrate, suggesting limited formononetin was provided during the transfer from liquiritigenin to formononetin. Besides the targeted product of medicarpin, the intermediates daidzein,

formononetin, and vestitone were detected in DW10 biomass. As daidzein was the completing byproduct for formononetin, *I4'OMT* enzyme activity should be enhanced to switch the metabolic flux to formononetin synthesis, which might increase the final product medicarpin synthesis.

The titer of vestitone produced by strain DW10 was high and the titer of medicarpin was low, suggesting the enzyme activities of *VR* and *PTS* should be increased to convert vestitone to medicarpin. Therefore, strain DW11 was further built by increasing the gene copy number of *VR* and *PTS* in DW10. After feeding liquiritigenin as substrate, the final medicarpin titer of DW11 was 2.05 ± 0.72 mg/L and it was 2.5 times of DW10 (Fig. 3B and 3F).

In the *in vitro* enzyme activity assay of *I4'OMT*, no daidzein was detected, and formononetin and LM were detected (Fig. 2B). During DW09, DW10 and DW11 fermentation with liquiritigenin as substrate, large amounts of by-product daidzein and small amounts of formononetin were detected. In the meanwhile, no LM was detected. Extra *I4'OMT* was available *in vitro* reaction system, and the *I4'OMT* activity might be low *in vivo*, suggesting *I4'OMT* may be the rate-limiting enzyme in medicarpin biosynthesis. Therefore, high-level expression of *I4'OMT* genes might increase medicarpin titer of DW11. *I4'OMT* was reported to be an isoflavone 4'-O-methyltransferase. In this study, *I4'OMT* was verified to catalyze C-4' and C-7 hydroxyl groups of flavonoid liquiritigenin *in vitro*, showing that *I4'OMT* may have versatile bioactivity and play essential roles in the biosynthesis of several different flavonoids. Structure biology would give insights into the catalytic mechanism of *I4'OMT* when using liquiritigenin as its substrate, which may help to increase/strengthen *I4'OMT* specificity and activities by protein engineering strategy.

Metabolite accumulation in the fermenter varies with the amount of cells

The starting substrates and fermentation time might affect the medicarpin production of DW11 (Fig. 4). During 120 h flask fermentation, the medicarpin titer of DW11 using formononetin as substrate was higher than that of DW11 using liquiritigenin as starting substrate. The final medicarpin titer is 4.27 ± 0.08 mg/L using formononetin as substrate (Fig. 4B), while the final medicarpin titer is 3.77 ± 0.25 mg/L using liquiritigenin as starting substrate (Fig. 4A). As the metabolic pathway from formononetin to medicarpin is shorter than that from liquiritigenin to medicarpin, more medicarpin might be produced and accumulated when using formononetin as substrate. In this study, further engineering the flavonoid biosynthetic precursors, such as *p*-coumaroyl-CoA and Malonyl-CoA would generate efficient yeast strains for medicarpin production.

The synthesis of liquiritigenin *de novo* in yeast has been realized in *S. cerevisiae* and *Yarrowia lipolytica*[42, 43]. The final liquiritigenin titer of one *Y. lipolytica* strain was 62.4 mg/L[42]. Though *de novo* production of medicarpin was not tried in this study, introducing several known plant genes and increasing liquiritigenin precursor supply in DW11 can synthesize medicarpin using glucose as sole carbon source in the future. The medicarpin was predicted to be the precursor of glabridin[30], and the medicarpin can be detected in *G. glabra* L. and *G. uralensis* Fisch.. Glabridin is only available in *G. glabra* L.[31–35], thus, whether medicarpin are the precursor of glabridin should be further investigated.

Conclusion

In this study, the comparative transcriptome analysis of *G. glabra* L. and *G. uralensis* Fisch. identified 176 genes which may function in the medicarpin synthesis. Further enzymatic assays confirmed eight key enzymes in the pathway from liquiritigenin to medicarpin. Introduction of these eight genes in *S. cerevisiae* led to the final medicarpin titer of 0.82 ± 0.18 mg/L; the final medicarpin titer of was further increased to 2.05 ± 0.72 mg/L in another optimized yeast strain, which lay the foundation to produce the valuable product of medicarpin using engineered yeasts. This study demonstrates that the integration of omics technology and synthetic biology strategies could recover complex plant natural product biosynthetic pathway.

Methods

Plant materials and Chemicals

The root samples of *G. glabra* L. and *G. uralensis* Fisch. were collected from Urumqi, Xinjiang province, China during the middle stage of root formation. The middle stage of root formation was two years. The root samples of *G. glabra* L. and *G. uralensis* Fisch. from the middle stage were set with three biological replicates (marked as G1, G2, G3 and W1, W2, W3, respectively). All the root samples were frozen at -80°C for RNA extraction. The purity of all the chemical compounds, including liquiritigenin (CAS: 578-86-9), daidzein (CAS: 486-66-8), formononetin (CAS: 485-72-3), medicarpin (CAS: 32383-76-9), NADH (CAS: 606-68-8), and NADPH (CAS: 2646-71-1), are $> 98\%$. All the chemical compounds were commercially available (Yuanye, Shanghai, China).

Deep Illumina sequencing and transcriptome analysis

Total RNAs were extracted from the root samples of *G. uralensis* Fisch. and *G. glabra* L. using the Column Plant RNAout2.0 (Tiandz Inc., Beijing, China). Approximately 100 mg of each sample was used to extract RNA. The RNA was evaluated using agarose gel electrophoresis, Nanodrop One (Nanodrop Technologies Inc., DE, USA), and Agilent 2100 (Agilent Technologies Inc., CA, USA) to confirm the purity, concentration, and integrity, respectively. The 260/280 nm ratios and 260/230 nm ratios of 1.8–2.2 and 1.4–1.8, respectively, from the Nanodrop were regarded as pure (**Table S4**). Next, the RNA library was constructed, and sequencing was performed by Genepioneer technologies corporation (Nanjing, China)[44]. Novaseq 6000 platform (Illumina Inc.) was used for high-throughput sequencing[45, 46] with pair-end 150 bp. The reference genome of *G. uralensis* (<http://ngs-data-archive.psc.riken.jp/Gur-genome/download.pl>) was used for transcriptome analysis, and gene functions were comprehensively annotated based on the following databases of Nr (NCBI nonredundant protein sequences), Pfam (protein families), KOG/COG (clusters of orthologous groups of proteins), SwissProt (a manually annotated and reviewed protein sequence database), KEGG (Kyoto encyclopedia of genes and genomes database), GO (gene ontology), and KO (KEGG Orthology). Fragments per kilobase per million mapped reads (FPKM) value was used to

estimate the expression level of the genes. DEGs between libraries were identified by DESeq2 (<http://www.bioconductor.org/packages/release/bioc/html/DESeq.html>). Fold change represents the ratio of expression quantity between two samples, and the Benjamini-Hochberg approach was used to adjust the *P* values for controlling the FDR. Unigenes with FDR < 0.05 and an absolute value $\log_2$ (Fold change) ≥ 1 were seen as differentially expressed[47]. The full-length cDNAs of the targeted genes were amplified by PCR using High-Fidelity PCR Master Mix (Tolo Biotech, shanghai, China), and the primers were designed based on the transcriptomic data (**Table S1**). The transcriptomic data used in this study were submitted to the Sequence Read Archive (SRA) of NCBI database with the accession numbers as SRR22859373, SRR22859372, SRR22859371 for three biological replicates from *G. glabra* L. and SRR22859370, SRR22859369, SRR22859368 for three biological replicates from *G. uralensis* Fisch., respectively.

Based on the annotation results, seventeen candidate genes involved in the medicarpin biosynthesis pathway, including the sequences which encoding PAL (phenylalanine ammonialyase), C4H (cinnamate 4-hydroxylase), 4CL (4-coumaroyl CoA ligase), TAL (tyrosine ammonialyase), PD (pyruvate dehydrogenase), ACC (acetyl-CoA carboxylase), CHS (chalcone synthase), CHR (chalcone reductase), CHI (chalcone isomerase), 2-HIS (2-hydroxyisoflavanone synthase), CPR (cytochrome P450 reductase), I4'OMT (isoflavone 4'-O-methyltransferase), HID (2-hydroxyisoflavanone dehydratase), I2'H (isoflavone 2'-hydroxylase), IFR (isoflavone reductase), VR (vestitone reductase), PTS (pterocarpan synthase) were highly expressed in the root of *G. glabra* L. Since the enzyme activities of CPR and PTS from *G. glabra* L. were weak, CPR of soybean[24] and PTS of *G. pallidiflora* Maxim[48]. were selected and used for the heterogenous biosynthesis of medicarpin.

Gene expression

The genes *HID*, *I4'OMT*, *IFR* and *PTS* were amplified from the cDNAs from the roots of *G. glabra* L., and *G. pallidiflora* Maxim, respectively, and then cloned into the pET-28a vector, and the gene *VR* was amplified from the cDNA from the root of *G. glabra* L. and cloned into the pGEX-4T-1 vector using the EasyGeno Assembly Cloning kit (Tolobio, Shanghai, China). The plasmids were transferred into *Escherichia coli* BL21 (DE3). All primers used in vector construction are listed (**Table S1**). Transformants were screened on solid culture medium (containing 100 µg/mL kanamycin sulfate or ampicillin sodium), and single colonies were picked for sequencing verification.

Recombinant cells were cultured in 1 L Luria-Bertani (LB) medium (37 °C, 165 rpm) containing 100 µg/mL kanamycin sulfate or ampicillin sodium. When OD₆₀₀ value of the reaches 0.6–0.8, isopropyl β-D-thiogalactoside (IPTG) with a final concentration of 0.5 mmol/L was added. The cultivation was further carried out at 18 °C for 12 hours. 1 L of bacterial suspension was centrifuged to obtain expression cells (5000 rpm, 15 min), and the precipitant was resuspended in 20 mL buffer C solution (10 mmol/L Tris-HCl, 5% glycerol, 0.2 mol/L NaCl). The cells were disrupted with a high-pressure cell crusher (ATS Engineering Limited, Suzhou, China), then centrifuged at 4 °C and 12000 rpm. The recombinant protein was purified

using the His-Tagged Protein Purification Kit or the GST-Tagged Protein Purification Kit (Smart-Lifesciences, Changzhou, China), and the protein concentration was determined by ultraviolet spectrophotometer (Allsheng, Hangzhou, China).

Cytochrome P450 2-HIS and I2'H were first cloned from the cDNA from the roots of *G. glabra* L, and expressed in *S. cerevisiae* IMX581 using pESC-LEU vector. Cytochrome P450 reductase (CPR) was amplified from the cDNA of soybean, and expressed in IMX581 using the vector pESC-URA. The recombinant plasmid was transferred into IMX581 by electroporation, and transformants were grown on corresponding SC-Leu or -Ura media with 2% glucose at 30 °C for 3 days. The positive colonies were picked and shaken at 30 °C and 200 rpm until OD₆₀₀ value reaches about 0.8. The 2% glucose medium was replaced with an induction medium (containing 2% galactose), and cultivated at 30 °C and 200 rpm for 72 h.

Characterization of enzymatic activity in vitro and in vivo

The enzymatic reaction systems using liquiritigenin as substrate contained 0.1 mol/L dipotassium hydrogen phosphate, 0.5 mol/L sucrose, 0.5 mmol/L glutathione, 1 mmol/L NADPH, 1 mmol/L NADH, 5 mmol/L magnesium chloride, 100 µmol/L substrate liquiritigenin and 0.5 mmol/L S-adenosyl-L-methionine (SAM), and 500 µL crude yeast extraction supernatant solution of strain DWY1, and 30 µL I4'OMT protein derived from *E. coli* expression. The enzymatic reaction systems[49] using formononetin as substrate contained 0.1 mol/L potassium phosphate (pH = 8.0), 0.4 mol/L sucrose, 0.5 mmol/L glutathione, 2 mmol/L NADPH, 2 mmol/L NADH, 5 mmol/L magnesium chloride, 50 µmol/L substrate formononetin, and 250 µL crude yeast extraction supernatant solution of strain DW08.

In vitro enzymatic activity measurement mixture was incubated at 30 °C for 12 h with gentle shaking, and the reaction mixture was extracted with an equal volume of ethyl acetate for three times. The extracted metabolites were dried and re-dissolved in methanol. The metabolites were further purified by passing through a 0.22 µm polytetrafluoroethylene (PTFE) filter and used for UPLC-ESI-Q-TOF-MS/MS analysis.

For *in vivo* analysis, the yeast strains were cultivated in medium containing 20 mg liquiritigenin or formononetin at 30 °C and 210 rpm for 24 hours; Then 20 mg of liquiritigenin or formononetin was added to the medium, and continued to cultivate for another 72 hours. The fermentation broth was extracted with an equal volume of ethyl acetate for three times, and the final products were redissolved in methanol and used for UPLC-ESI-Q-TOF-MS/MS analysis.

UPLC–ESI-Q-TOF-MS/MS analysis of catalytic and final products

An Acquity UPLC system coupled with a Synapt mass spectrometer (Waters Corp., Milford, MA, USA) and equipped with an electrospray ionization (ESI) device were used to analyze the catalytic products. The separation was carried out on a Waters C18 column (100 mm × 2.1 mm, 1.7 µm). The column was eluted

with a gradient mobile phase which consisted of 0.1% aqueous formic acid (solvent system A) and acetonitrile (B). The mobile phases during operation were: 0–1 min, 5% B; 1–20 min, 5%-100% B; 20–30 min, 100% B; 30–31 min, 100%-5% B; 31–33 min, 5% B. The injection volume was 2 μ L, and the flow rate was 0.4 mL/min.

Extraction of medicarpin and other metabolites from *G. uralensis* Fisch. and *G. glabra* L.

The dried roots of *G. uralensis* Fisch. or *G. glabra* L., were pulverized into powder, and 4 g of the powder was dissolved in 400 mL of 70% methanol. The power in the methanol was treated with ultrasonic (KQ-500DE numerical control ultrasonic cleaner, working frequency: 40 kHz, power: 500 W, Kunshan Ultrasonic Instrument Co., Ltd.) at 30 °C for 60 min to extract metabolites in the roots. After centrifugation, the supernatant was filtered through a 0.22 μ m PTFE filter and used for qualitative analysis.

Reconstitution of the medicarpin biosynthetic pathway in yeast

The chassis strain used in this experiment was *S. cerevisiae* IMX581 (MATa ura3-52 can1::cas9-natNT2 TRP1 LEU2 HIS3)[50]. Heterologous genes were integrated into the targeted genomic loci via the CRISPR/Cas9 system[23]. Plasmid pMEL10 was used as gRNA vector. Phanta Max Super-Fidelity DNA Polymerase (Vazyme Biotech Co. Ltd) was used for DNA fragment amplifications, and PrimeStar DNA polymerase (TaKaRa Bio) was used for *in vitro* fusion PCR. The ClonExpress MultiS One Step Cloning Kit (Vazyme Biotech Co.,Ltd) was used for *in vitro* fragment recombinant. FastPure Plasmid Mini Kit (Vazyme Biotech Co.,Ltd) was used for plasmid extraction. FastPure Gel DNA Extraction Mini Kit (Vazyme Biotech Co.,Ltd) was used for DNA extraction. Super Yeast Transformation Kit (Coolaber) was used for transformation. All primers, the plasmids, and the strains used in this study were listed (**Table S1 -3**). The genes used in this study were submitted to Genbank database with the accession numbers of OQ067102(CHS), OQ067258(CHI), OQ067259(CHR), OQ102530(2-HIS), OQ102529(HID), OQ067262(I2'H), OQ067263(IFR), OQ067264(VR), OQ067265(I4'OMT), OQ067266(C4H), OQ067267(4CL).

Fermentation and product analysis

S. cerevisiae strain DW11 was used for medicarpin biosynthesis in shake flask. DW11 was grown at 30 °C and 200 rpm for 12 h, and then transferred into 1 L of fresh medium containing 0.2 g/L liquiritigenin or formononetin. DW11 was further cultivated at 30 °C and 200 rpm for 5 days, and yeast sample was fetched every 24 h during cultivation. The collected yeast samples were disrupted with a high-pressure cell crusher, and then centrifuged at 10000 g for 20 min to collect the supernatant. The metabolites in the supernatant were extracted with the same volume of ethyl acetate (v/v = 1:1) for three times. The extraction was dried and redissolved with methanol for UPLC analysis.

Quantitative UPLC analysis

The yeast product was detected using a Waters C18 column (100 mm × 2.1 mm, 1.7 μm) on an Acquity UPLC system (Waters Corp., Milford, MA, USA) by gradient elution with a mobile phase comprising 0.1% (v/v) formic acid aqueous solution (A) and acetonitrile (B) at a flow rate of 0.4 mL/min. The mobile phases were programmed as: 0–1 min, 5% B; 1–10 min, 5%-100% B; 10–11 min, 100% B for 1 min; 11–12 min, 100%-5% B; 12–13 min, 5% B. The injection volume was 2 μL. The column temperature during operation was 25 °C. For the product vestitone with no available standard compound, the semi-preparative RP-HPLC (Shimadzu, Kyoto, Japan) was used to collect and concentrate, which was further dried to obtain solid powder by rotary evaporator (Buchi, Switzerland). The verstitone could be used as standard for quantification analysis.

Declarations

Supplementary Information

The online version contains supplementary material available at

Acknowledgements

We thank the staff at the experiment center for science and technology of Nanjing University of Chinese Medicine for experimental supports. We appreciate Zhaozhu Lin, Mingming Xu, Xinyi Wei, Fulin Wang, and Jing Yang from Nanjing University of Chinese Medicine for technical assistance and discussions.

Author contributions

M.Z., YJ.W., and W.L. designed experiments. H.F., JZ. Z, R.D. and CJ.L. performed the sample collection and performed the bulk of the experiments. CJ.L. contributed to gene identification and cloning, protein expression, purification, and crystallization. CJ.L. contributed to enzymatic assay experiments. M.Z., YJ.W., and W.L. analyzed the data and wrote the manuscript. M.Z., YJ.W., and W.L. conceived the project.

Funding

This work was financially supported by the National Natural Science Foundation of China (81903526, 81991523, 82072240, and 32270192), the Open Project of Chinese Materia Medica First-Class Discipline of Nanjing University of Chinese Medicine (No. 2020YLXK008 to W.L.), the Open Project of State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences (No. SIMM2205KF to W.L.), the Open Project of State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences (No. SKLMR-20220704 to W.L.), the Open Funding Project of the State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology to W.L., the Fok Ying Tung Education Foundation, and Jiangsu Specially-Appointed Professor Talent Program to W.L.

Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing of interests.

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Figures

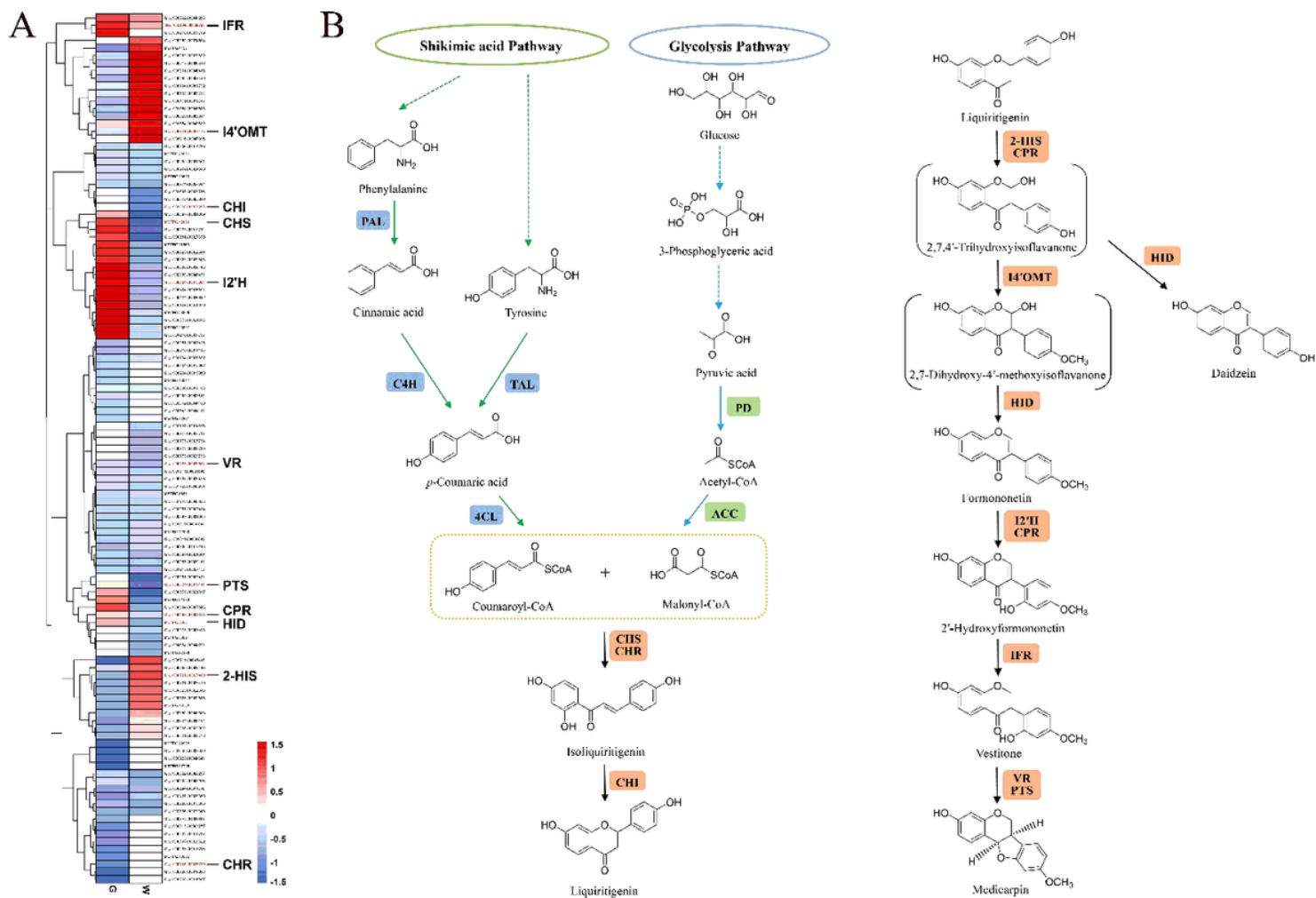


Figure 1

Schematics of the putative medicarpin biosynthesis pathway, and the differential expression of the unigenes involved in *Glycyrrhiza glabra* L. and *Glycyrrhiza uralensis* Fisch.

(A) The heatmap of the differentially expressed unigenes across the medicarpin biosynthesis pathway. G, *Glycyrrhiza glabra* L.; W, *Glycyrrhiza uralensis* Fisch. The heatmap was drawn using Heml 1.0 with log₂ values of FPKM (Fragments Per Kilobase of exon per Million mapped fragments) of the candidate genes, and the sequences with log₂FPKM < -1 were assigned as low expression in the samples which were excluded from the statistical data. Depths of color in the red and green rectangles indicate higher and lower Z-scores (log₂) of the corresponding RNA expression levels. The red font indicates the sequences used for medicarpin pathway reconstruction in yeast. **(B)** Proposed pathway of medicarpin biosynthesis. Left panel, the biosynthesis route of Liquiritigenin from Shikimic acid pathway and Glycolysis pathway; Right panel, the biosynthesis route of medicarpin starting from Liquiritigenin. Abbreviations: PAL (phenylalanine ammonialyase), C4H (cinnamate 4-hydroxylase), 4CL (4-coumaroyl CoA ligase), TAL (tyrosine ammonialyase), PD (pyruvate dehydrogenase), ACC (acetyl-CoA carboxylase), CHS (chalcone synthase), CHR (chalcone reductase), CHI (chalcone isomerase), 2-HIS (2-hydroxyisoflavanone synthase), CPR (cytochrome P450 reductase), I4'OMT (isoflavone 4'-O-methyltransferase), HID (2-

hydroxyisoflavanone dehydratase), I2'H (isoflavone 2'-hydroxylase), IFR (isoflavone reductase), VR (vestitone reductase), PTS (pterocarpan synthase).

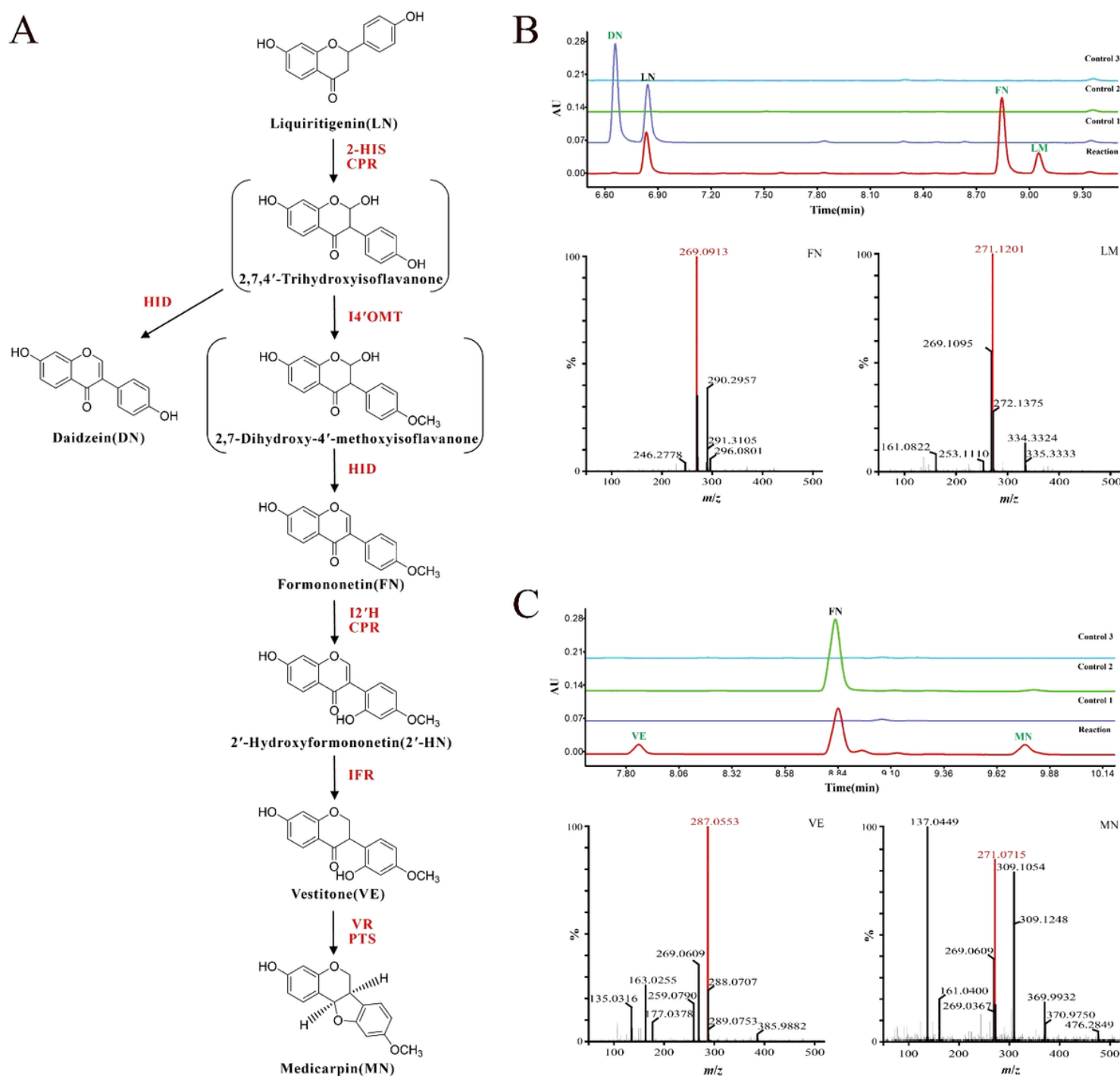


Figure 2

Analysis of catalytic products of the enzymes encoded by the candidate genes.

(A) The medicarpin biosynthesis pathway of *Glycyrrhiza glabra* L.; **(B, C)** UPLC-ESI-Q-TOF-MS/MS profiles of the *in vitro* enzymatic products of recombinant 2-HIS, CPR, I4'OMT, HID (B, liquiritigenin as substrate) expressed in *E. coli* or yeast using pET-28a or pESC-LEU/URA as expression vector; UPLC-ESI-Q-TOF-

MS/MS profiles of the fermentation products of yeast harboring the recombinant vector pESC-LEU-I2'H, pESC-URA-CPR, IFR, VR, PTS (C, formononetin as substrate) with external precursor addition. The peaks and mass spectrum of the product indicated by the green font marks, the substrate indicated by the black font marks, are shown (electron ionization in positive-ion mode, $[M+H]^+$). Extracted-ion chromatogram (EIC) of the analyte, as indicated, at m/z 269, 271 **(B)**, m/z 287, 271 **(C)**. All the reactions were performed with a gene-free strain or a strain without substrate as control. LN, liquiritigenin; DN, daidzein; FN, formononetin; LM, liquiritigenin 4'-methyl ether and liquiritigenin 7-methyl ether; VE, vestitone; MN, medicarpin.

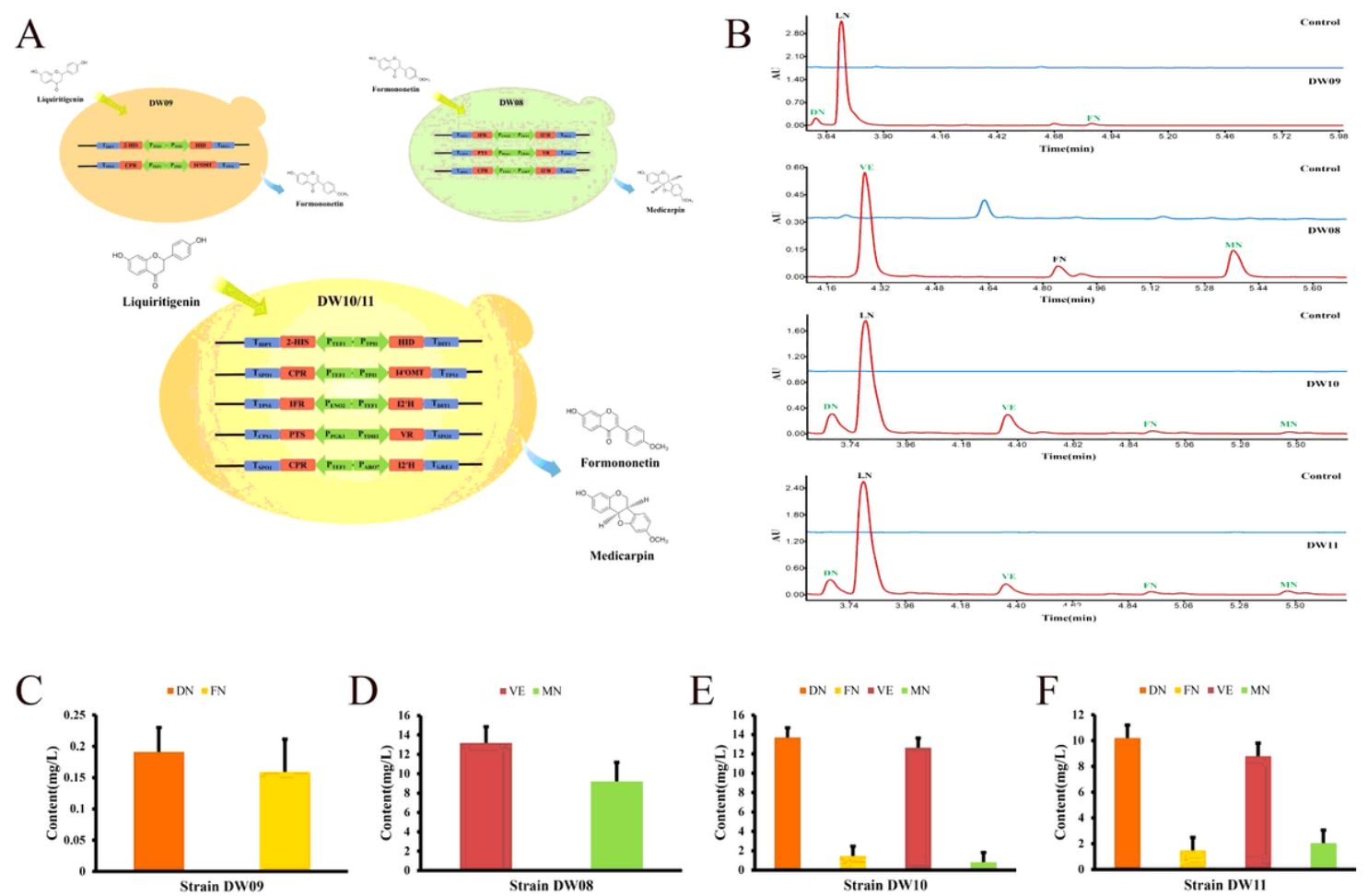


Figure 3

Reconstitution of the biosynthesis pathway of medicarpin in yeast.

(A) Schematic of the recombinant yeast strain DW08, DW09 and DW10. **(B)** The products of DW09 (feeding liquiritigenin), DW08 (feeding formononetin), DW10 (feeding liquiritigenin) and DW11 (feeding liquiritigenin) were detected by UPLC; **(C-F)** Production of daidzein and formononetin by yeast stains DW09 (harboring 2-HIS, CPR, I4'OMT and HID) after cultured for 72 h; the production of vestitone and medicarpin by yeast stains DW08 (harboring I2'H, CPR, IFR, VR and PTS) after cultured for 72 h; production of daidzein, formononetin, vestitone and medicarpin by yeast stains DW10 (harboring 2-HIS, CPR, I4'OMT, HID, I2'H, IFR, VR and PTS) after cultured for 72 h; The titer of daidzein, formononetin,

vestitone and medicarpin by yeast stains DW11 (increasing the gene copy number of *VR* and *PTS* in DW10) after cultured for 72 h was shown.

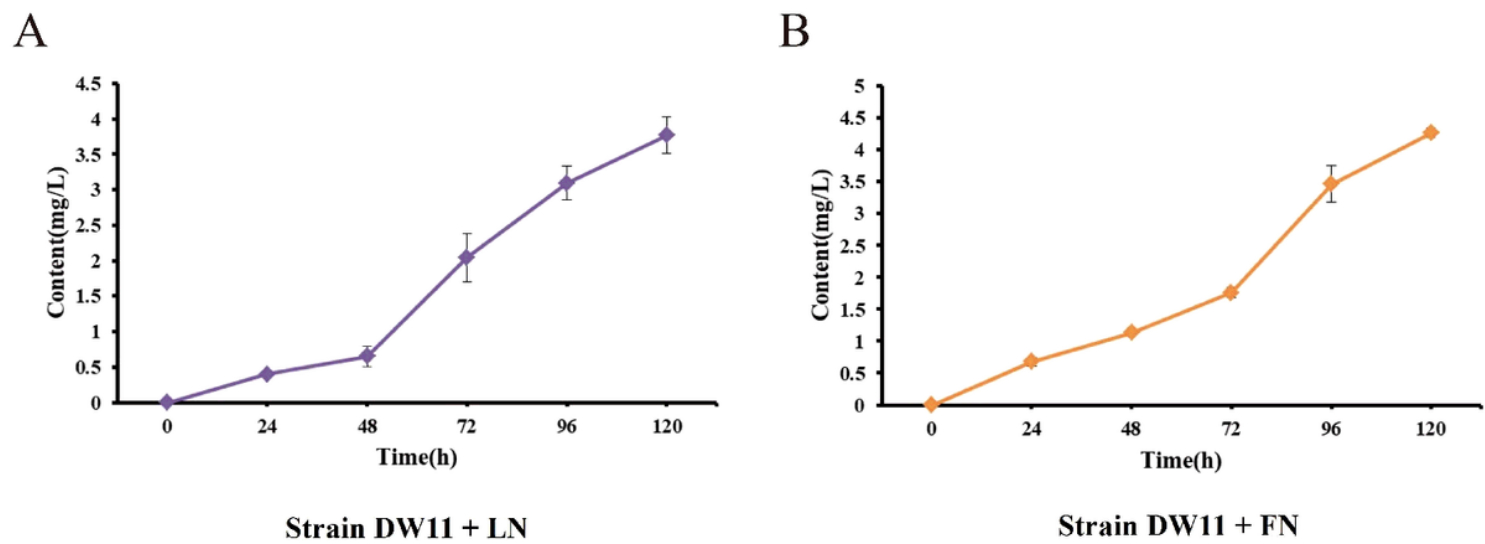


Figure 4

Fermentation product of strain DW11 in fed-batch fermentation.

(A) Strain DW11 was fed with liquiritigenin as substrate. (B) Strain DW11 was fed with formononetin as substrate. Three biological replicates were performed for each analysis and the error bars represented the standard deviation (SD).

Supplementary Files

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