Producing aglycons of ginsenosides in bakers’ yeast

Zhubo Dai1,2, Beibei Wang1,2,3, Yi Liu1,2,3, Mingyu Shi1,2,3, Dong Wang1,2,3, Xianan Zhang4,5, Tao Liu1,2, Luqi Huang* & Xueli Zhang1,2

Ginsenosides are the primary bioactive components of ginseng, which is a popular medicinal plant that exhibits diverse pharmacological activities. Protopanaxadiol, protopanaxatriol and oleanolic acid are three basic aglycons of ginsenosides. Producing aglycons of ginsenosides in Saccharomyces cerevisiae was realized in this work and provides an alternative route compared to traditional extraction methods. Synthetic pathways of these three aglycons were constructed in S. cerevisiae by introducing β-amyrin synthase (OAS, a P450 enzyme), which catalyses a three-step, sequential oxidation is the first reaction towards the biosynthesis of oleanane-type ginsenosides.

Insenosides are the primary bioactive components of ginseng, which is a popular medicinal plant. Ginsenosides are a group of triterpenoids that exhibit diverse pharmacological effects on the central nervous, cardiovascular and immune systems. Dammarane-type tetrayclic ginsenosides (Rb1, Rb2, Rg1, Rh2 and Rg3) are the major constituents, while Re, which belongs to oleanane-type pentacyclic ginsenosides, is a minor component of Panax ginseng. These Dammarane-type ginsenosides are divided into two groups according to their aglycon structure: protopanaxadiol (Rb1, Rb2, Re, Rf, Rh2 and Rg3) and protopanaxatriol (Re, Rf, and Rg3).

Currently, ginsenosides are mainly produced through their extraction from ginseng roots. Wild ginseng roots are scarce (i.e., they are endangered species in Asia and North America), but most commercial ginseng roots are collected from farms that cultivate ginseng in fields. However, cultivating ginseng is time-consuming, labour-intensive and is influenced by many conditions such as soil, climate, pathogens and pests.

Bakers’ yeast (Saccharomyces cerevisiae) has been commonly used as a leavening agent in baking bread and bakery products, where it converts fermentable sugars that are present in dough into carbon dioxide and ethanol. In addition, because S. cerevisiae has been genetically and physiologically characterised and multiple genetic engineering tools exist, this microorganism is an ideal host for the heterologous production of valuable natural products and can provide an alternative and attractive route compared to traditional extraction methods. With the development of metabolic engineering and synthetic biology tools, many natural products have been successfully synthesised in S. cerevisiae.

The first committed step in triterpenoid biosynthesis is the cyclisation of 2,3-oxidosqualene. This reaction is catalysed by specific oxidosqualene cyclases (OSCs), e.g., dammarenediol-II synthase (DDS), β-amyrin synthase (βAS), α-amyrin synthase (αAS), lanoster synthase (LAS) and lupeol synthase (LUS). In P. ginseng, cyclisation of 2,3-oxidosqualene to dammarenediol-II by DDS is the first reaction towards the biosynthesis of dammarane-type ginsenosides. Dammarenediol-II is further converted to protopanaxadiol by protopanaxadiol synthase (PPDS), which is a cytochrome P450 enzyme that catalyses the hydroxylation of dammarenediol-II at the C-12 position. Protopanaxadiol is further converted to protopanaxatriol by protopanaxatriol synthase (PPTS). In contrast, cyclisation of 2,3-oxidosqualene to β-amyrin by β-amyrin synthase (βAS) is the first reaction towards the biosynthesis of oleanane-type ginsenosides. β-amyrin is further converted to oleanolic acid by oleanolic acid synthase (OAS, a P450 enzyme), which catalyses a three-step, sequential oxidation
at the C-28 position of β-amyrin (Fig. 1)\textsuperscript{14,15}. These aglycons are further converted to ginsenoside compounds by uridine diphosphate glycosyltransferases (UGTs)\textsuperscript{18,19}.

In this work, \textit{S. cerevisiae} was metabolically engineered for the efficient production of these three aglycons by introducing β-amyrin synthase, oleanolic acid synthase, dammarenediol-II synthase, protopanaxadiol synthase, protopanaxatriol synthase and NADPH-cytochrome P450 reductase from different plants. Genes coding for OAS from \textit{Medicago truncatula} and DDS, PPDS and PPTS from \textit{P. ginseng} have been previously demonstrated to be functional in yeast\textsuperscript{3,16,17}. In addition, precursor supplies were increased to improve aglycon production. The yeast strains engineered in this work can serve as the basis for creating an alternative way for producing ginsenosides in place of extraction from plant sources.

**Results**

**Increasing IPP and DMAPP supplies for terpenoid production in \textit{S. cerevisiae}.** Triterpenoids are derived from two common building blocks, isopentenyl diphasphate (IPP) and dimethylallyl diphasphate (DMAPP), which are synthesised through the mevalonic acid (MVA) pathway in \textit{S. cerevisiae} (Fig. 1). Most IPP and DMAPP precursors enter the ergosterol synthetic pathway\textsuperscript{20–22}. When cultivated in YPD medium with 2% glucose for 7 days, strain BY4742-TRP produced 9.6 mg/L squalene, 2.1 mg/L lanosterol and 10.1 mg/L ergosterol (Fig. 2).

3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase is a key rate-limiting enzyme in the MVA pathway, and overexpression of a truncated HMG-CoA reductase gene (\textit{tHMG1}) has commonly been used to increase the carbon flux through the MVA pathway\textsuperscript{11,25}. This strategy had been used to successfully increase the production of many terpenoids, including sesquiterpenoids\textsuperscript{7,11,26,27}, diterpenoids\textsuperscript{9,10,28}, triterpenoids\textsuperscript{29,30} and carotenoids\textsuperscript{31}.

The \textit{tHMG1} gene (controlled by the \textit{PGK1} promoter) and the \textit{LYS2} gene (controlled by the \textit{TEF1} promoter) were integrated into the chromosome of BY4742-TRP at the DNA site to increase IPP and DMAPP supplies for better terpenoid production. The resulting strain, BY-T1 (Table 1), produced 150.9 mg/L squalene, 5.4 mg/L lanosterol and 42.0 mg/L ergosterol, which were 15.7-, 2.6- and 4.2-fold higher than that of the parent strain (Fig. 2). This result demonstrated that overexpressing \textit{tHMG1} significantly improved terpenoid production, and the engineered strain could be used for further production of aglycons of ginsenosides.

**Construction of the β-amyrin synthetic pathway.** β-amyrin serves as the basic precursor of oleane-type triterpenoids\textsuperscript{48}. β-amyrin is synthesised from 2,3-oxidosqualene in some medicinal plants by 2,3-oxidosqualene cyclases (OSCs)\textsuperscript{13–15,23}. \textit{S. cerevisiae} can produce 2,3-oxidosqualene from IPP and DMAPP through farnesyl diphasphate (encoded by \textit{ERG20}), squalene synthase (encoded by \textit{ERG9}) and squalene epoxidase (encoded by \textit{ERG1}) (Fig. 1).

Squalene synthase and squalene epoxidase are two key enzymes for triterpenoid synthesis. Squalene synthase is the first enzyme dedicated to synthesis of sterols in yeast, while squalene epoxidase catalyses the first oxygenation step in ergosterol biosynthesis and is suggested to represent one of the rate-limiting enzymes in this pathway\textsuperscript{42}. Overexpressing the squalene synthase gene had been used to enhance the production of β-amyrin in yeast\textsuperscript{29,36} and the production
of phytosterols and triterpenoids in plants. Furthermore, over-expressing the squalene epoxidase gene had been used to increase sterol production.

To construct the β-amyrin synthetic pathway and improve its production in *S. cerevisiae*, two different β-amyrin synthase genes of *Glycyrrhiza glabra* and *P. ginseng*, with the *S. cerevisiae* squalene synthase and squalene epoxidase genes, were integrated into the chromosome of strain BY-T1 at rDNA sites, resulting in strains BY-βA-G and BY-βA-P (Table 1), respectively. When cultivated in YPD medium with 2% glucose for 7 days, GC/MS analysis of the cell extractions of both strains confirmed the production of 25.0 mg/L ergosterol (Fig. S1). Strain BY-βA-G and BY-βA-P (Table 1), respectively. When cultivated in YPD medium with 2% glucose for 7 days, GC/MS analysis of the cell extractions of both strains confirmed the production of β-amyrin (Fig. S1). Strain BY-βA-P produced 1.9 mg/L β-amyrin with a yield of 0.2 mg/g (data not shown), while strain BY-βA-G produced 107.0 mg/L β-amyrin with a yield of 9.3 mg/g DCW (Fig. 2). Strain BY-βA-G also produced 183.4 mg/L squalene, 10.0 mg/L lanosterol and 25.0 mg/L ergosterol (Fig. 2).

To verify that increasing 2,3-oxidosqualene supply did improve β-amyrin synthesis, the β-amyrin synthase gene of *G. glabra* (controlled by the *PGK1* promoter) and the *LYS2* gene (controlled by the *TEFI* promoter) were integrated into the chromosome of strain BY4742-TRP at rDNA sites. The resulting strain, BY-βA-G (Table 1), produced 77.7 mg/L β-amyrin, which was 73% of that produced by strain BY-βA-G (Fig. S2). In addition, strain BY-βA-CK produced 34.2 mg/L squalene, 1.4 mg/L lanosterol and 12.1 mg/L ergosterol, which were 19%, 14% and 48% of those produced by strain BY-βA-G (Fig. S2).

**Construction of the oleanolic acid synthetic pathway.** To construct the oleanolic acid synthetic pathway in *S. cerevisiae*, the oleanolic acid synthase gene (OAS) of *Medicago truncatulata* (controlled by *TEFI* promoter), with a cytochrome P450 reductase gene of *Arabidopsis thaliana* (*AtCPR1*) (controlled by *TDH3* promoter) and a new copy of the *G. glabra* β-amyrin synthase gene (controlled by *PGK1* promoter) were integrated into the chromosome of strain BY-βA-G at the Trp1 site, resulting in strain BY-OA (Table 2). After cultivation in YPD medium with 2% glucose for 7 days, LC/MS analysis of the cell extraction of strain BY-OA confirmed the production of oleanolic acid (Fig. S3). Strain BY-OA produced 71.0 mg/L oleanolic acid with a yield of 6.1 mg/g DCW and 88.6 mg/L β-amyrin with a yield of 7.6 mg/g DCW (Fig. 2). This strain also produced 141.2 mg/L squalene, 9.8 mg/L lanosterol and 27.3 mg/L ergosterol (Fig. 2).

**Construction of the synthetic pathways of all three aglycons.** To construct the protopanaxadiol and protopanaxatriol synthetic pathways in strain BY-OA, the *P. ginseng* dammaradiol-II synthase gene (*PgDDS*, controlled by *PGK1* promoter), *P. ginseng* protopanaxadiol synthase gene (*PgPPDS*, controlled by *TEFI* promoter), *P. ginseng* protopanaxatriol synthase gene (*PgPPTS*, controlled by *FBA1* promoter) and *AtCPR1* (controlled by *TDH3* promoter) genes were integrated into the chromosome of strain BY-OA at the *His3* site, resulting in strain GY-1 (Table 1). After cultivation in YPD medium with 2% glucose for 5 days, LC/MS analysis of the cell extractions of strain GY-1 (Table 1) confirmed the production of 27.3 mg/L ergosterol (Fig. 2).

**Table 1 | Strains used in this study**

| Name      | Description                                      | Source            |
|-----------|--------------------------------------------------|-------------------|
| BY4742    | MATα, his3Δ1, leu2Δ10, lys2Δ10, MET15, ura3Δ10 | Brachmann et al.38|
| BY4742-TRP| Deletion of the Trp1 gene of BY4742               | This study        |
| BY-T1     | BY4742-TRP, ΔDNA::PGK1::#HMG1-TADH1-PTEFI-LYS2-TCYC1 | This study        |
| BY-βA-P   | BY-T1, rDNA::PGK1::PgBAS-TADH1-TDEH3-ERG1-TFR1-PTEFI-ERG9-TCYC1 | This study        |
| BY-βA-G   | BY-T1, rDNA::PGK1::GgBAS-TADH1-TDH3-ERG1-TFR1-PTEFI-ERG9-TCYC1 | This study        |
| BY-βA-CK  | BY4742-TRP, rDNA::PGK1::GgBAS-TADH1-PTEFI-LYS2-TCYC1 | This study        |
| BY-OA     | BY-βA-G, Trp1::PGK1::GgBAS-TADH1-TDH3-ERG1-TFR1-PTEFI-ERG9-TCYC1 | This study        |
| GY-1      | BY-OA, His3::PGK1::PgDDS-TADH1-PTEFI-ERG9-PFBA1-SynPgPPDS-TDEH3-AtCPR1-TFR1-PTEFI-ERG9-TCYC1 | This study        |
β-amyrin synthase of *Artemisia annua*, the overexpression of the *tHMG1* gene and down-regulation of the lanosterol synthase gene (encoded by *ERG7*), an engineered *S. cerevisiae* was obtained that produced 6 mg/L β-amyrin 29. By introducing β-amyrin synthase of *Pisum sativum* and overexpressing the phosphomevalonate kinase (encoded by *ERG8*), squalene synthase (encoded by *ERG9*) and acetyl-CoA carboxylase (encoded by *HFA1*) genes, an engineered *S. cerevisiae* was obtained that produced 3.93 mg/L β-amyrin 30. To the best of our knowledge, strain BY-βA-G had the highest β-amyrin titer that was achievable by microbial fermentation. It was suggested that the β-amyrin synthase of *G. glabra* was better at producing β-amyrin than *A. annua*, *P. sativum* and *P. ginseng*. This suggestion was reasonable because *G. glabra* contains a large amount of glycerrhizin (2–8% of the dry weight) 32,33, while the other three plants contain minimal levels of oleanane-type triterpenoids 34.

For strain BY-βA-G, squalene was produced with high productivity (1.60 mg/L-h), while β-amyrin had a relatively lower productivity (0.14 mg/L-h) during the first 72 hours (Fig. 2). From 72 to 144 h, squalene productivity decreased 80% to 0.32 mg/L-h, while β-amyrin productivity increased 9.1-fold to 1.27 mg/L-h. One possibility was that β-amyrin synthase activity was relatively lower for the first 72 h and limited β-amyrin production, while it was high from 72 to 144 h and led to high β-amyrin productivity. Another possibility was that 2,3-oxidosqualene supply was insufficient for the first 72 h, while it was enough from 72 to 144 h and led to high β-amyrin productivity. β-amyrin and dammarenediol-II still accumulated in strain GY-1, suggesting that oleanolic acid synthase, protopanaxadiol synthase and protopanaxatriol synthase might be rate-limiting enzymes for aglycon production in this strain. These synthases are P450 enzymes. Poor coupling between P450 cytochromes and their reductases can result in the release of reactive oxygen species 35, and the CPR levels would thus affect cell health 36. The CPR gene of *A. thaliana* (*AtCPR1*) was used in this study; however, more CPRs from other organisms, such as *M. truncatula* and *G. glabra*, must be further investigated to identify the best enzyme for aglycon production.

The artemisinic acid synthetic pathway had been constructed in yeast by using cytochrome C450 T457AV1, which can catalyse a three-step, sequential oxidation that can convert amorphanedio to artemisinic acid 37. However, the product titer was relatively low, only 100 mg/L. Recently, two plant dehydrogenases and a second cytochrome were discovered that could provide a more efficient biosynthetic route toward artemisinic acid. Cytochrome b5 (CYB5) facilitated the oxidation of amorphanedio to artemisinic alcohol, alcohol dehydrogenase (ADH1) oxidised artemisinic alcohol to artemisinic aldehyde, while artemisinic aldehyde dehydrogenase (ALDH1) oxidised artemisinic aldehyde to artemisinic acid. After reconstituting the entire, heterologous biosynthetic pathway in yeast, the engineered strain produced 25 g/L artemisinic acid 38. Because oleanolic acid synthase also catalyses a three-step sequential oxidation, it was suggested that identification of other cytochromes and dehydrogenases from plants (such as *G. glabra*, *P. ginseng* and *M. truncatula*) and reconstituting them into the engineered yeast would improve oleanolic acid production.

In ginseng, aglycons (protopanaxadiol, protopanaxatriol and oleanolic acid) are further converted to ginsenoside compounds by uridine diphosphate glycosyltransferases (UGTs) 39. Although protopanaxadiol, protopanaxatriol and oleanolic acid have diverse pharmacological effects, ginsenosides (such as Rh2 and Rg3) have more applications in pharmaceutical industries. After functional genomic analysis of *P. ginseng* and other plants, candidate UGTs can be introduced into strain GY-1, which can be used as the host strain for the identification of appropriate UGTs for ginsenoside production.

**Methods**

**Strains and medium.** *S. cerevisiae* BY4742, a derivative of S288C 40, was obtained from EUROSCARF and used as the parent strain for all yeast strains. Engineered yeast strains were grown either in SD medium 41–43 lacking leucine, uracil, tryptophan and.

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**Figure 3** Production of protopanaxadiol, protopanaxatriol and oleanolic acid by engineered *S. cerevisiae* strain GY-1. (A) Titters obtained after fermentation for 7 days; (B) Proportion of protopanaxadiol, protopanaxatriol and oleanolic acid within the total aglycons. Three replicates were performed, and the error bars represented standard deviation.
histidine, where appropriate, or in YPD medium. Histidine was added to the growth medium at 37°C in LB medium with 100 μg/mL ampicillin.

**Plasmid construction.** Panax ginseng cells were induced with 0.1 mg/mL MeJA for 24 h. Total RNA was isolated by the Trizol method, and cDNA was synthesised using the primers 1-M-ADHt-TEF1-F/1-M-ADHt-TEF1-R and 3-M-CYC1t-pEASY-R, followed by selection on SD-URA plates. All strains were verified by PCR analysis, and 12 colonies were screened by GC/MS and HPLC for the selection of the best-producing transformant.

The mixed-solution (600 μL; acetone: methanol=1:1) was added to the tube and crushed by a BeadBeater (BioSpec, USA) 3 times. The samples were then centrifuged at 10,000×g for 1 min, and 1 μL of supernatant was analysed by GC/MS using an Agilent technologies 5975C inert XL MSD with triple-Axis Detector equipped with a HP-5ms (30 m×0.25 mm×0.5 μm) GC column. Compound separation was achieved with an injector temperature of 300°C and a 30 min temperature gradient program for GC-separation starting at 80°C for 1 min followed by heating the column to 300°C at 20°C min⁻¹ and a final constant hold at 300°C for 15 min. Mass detection was achieved with electronic ionisation using SIM-scan mode with diagnostic ions monitored as follows: m/z 69, m/z 218, m/z 363, m/z 411 and m/z 437. A crystallised β-amyrin sample was used as the standard for quantification (purchased from Sigma-Aldrich), and squalene, lanosterol and ergosterol standards (purchased from Sigma-Aldrich) were also used for quantification.

For the determination of oleic acid, acetone and methanol (1:1) extracts (20 μL) were analysed by LC/MS using an Agilent 1200 HPLC system coupled to a Bruker-microOTOF-II with an electrospray ionisation (ESI) interface. Data acquisition and processing were performed with the MicrOTOF control version 3.0/Data Analysis Version 4.0 software. For chromatographic separation, a Waters Symmetry C18® column (25 mm×4.6 mm, 5 μm) was used. The mobile phase consisted of 0.1% ammonium acetate in water (A) and acetonitrile (B), and a program of A: B=15:85 for 30 minutes was used. The solvent flow rate was 1.0 mL/min and the column temperature was set at 30°C. Optimised MS operating conditions were as follows: all spectra were obtained in the negative ion mode over an m/z range of 100–1200; dry gas flow, 6.0 L/min; dry temperature, 180°C; nebuliser pressure, 1 bar; and probe voltage, −4.5 kV. Oleic acid was purchased from Sigma Aldrich and was used as the standard for analysis.

For the determination of protopanaxadiol and protopanaxatriol, acetone and methanol (1:1) extracts (20 μL) were analysed by LC/MS using an Agilent 1200 HPLC system coupled to a Bruker-microOTOF-II with an electrospray ionisation (ESI) interface. Data acquisition and processing were performed with the MicrOTOF control version 3.0/Data Analysis Version 4.0 software. For chromatographic separation, a Waters Symmetry C18® column (25 mm×4.6 mm, 5 μm) was used. The mobile phase consisted of 0.1% formic acid and 10% methanol in water (A) and acetonitrile (B), and a program of A: B=15:85 for 30 minutes was used. The solvent flow rate was 1.0 mL/min, and the column temperature was set at 30°C. Optimised MS operating conditions were as follows: all spectra were obtained in the positive ion mode over an m/z range of 100–1200; dry gas flow, 6.0 L/min; dry temperature, 180°C; nebuliser pressure, 1 bar; and probe voltage +4.5 kV. Protopanaxadiol and protopanaxatriol was purchased from Sigma Aldrich and was used as the standard for analysis.

For quantitative analysis of protopanaxadiol, protopanaxatriol and oleic acid, acetone and methanol (1:1) extracts (20 μL) were injected into an Agilent 1200 HPLC apparatus with UV detection at 203 nm. For chromatographic separation, a Waters Symmetry C18® column (25 mm×4.6 mm, 5 μm) was used. The mobile phase consisted of 0.1% formic acid and 10% methanol in water (A) and acetonitrile (B), and a program of A: B=15:85 for 30 minutes was used. The solvent flow rate was 1.0 mL/min, and the column temperature was set at 30°C. Optimised MS operating conditions were as follows: all spectra were obtained in the positive ion mode over an m/z range of 100–1200; dry gas flow, 6.0 L/min; dry temperature, 180°C; nebuliser pressure, 1 bar; and probe voltage +4.5 kV. Protopanaxadiol and protopanaxatriol and oleic acid was purchased from Sigma Aldrich and was used for quantification.

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Author contributions

Z.D., L.H.T. and X.Z. designed the experiments; Z.D., Y.L., B.W., M.S., D.W. and X.N.Z. performed the experiments; Y.L., Z.D. and X.Z. analysed the data; Z.D. and X.Z. wrote the paper; and all authors reviewed the manuscript.

Additional information

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