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Producing aglycons of ginsenosides in bakers' yeast

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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, oleanolic acid synthase, dammarenediol-II synthase, protopanaxadiol synthase, protopanaxatriol synthase and NADPH-cytochrome P450 reductase from different plants. In addition, a truncated 3-hydroxy-3-methylglutaryl-CoA reductase, squalene synthase and 2,3-oxidosqualene synthase genes were overexpressed to increase the precursor supply for improving aglycon production. Strain GY-1 was obtained, which produced 17.2 mg/L protopanaxadiol, 15.9 mg/L protopanaxatriol and 21.4 mg/L oleanolic acid. The yeast strains engineered in this work can serve as the basis for creating an alternative way for producing ginsenosides in place of extractions from plant sources.

Ginsenosides 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, endocrine, cardiovascular and immune systems^{1,2}. Dammarane-type tetracyclic ginsenosides (Rb1, Rb2, Rc, Rd, Re, Rf, Rg1, Rh2 and Rg3) are the major constituents, while Ro, which belongs to oleanane-type pentacyclic ginsenosides, is a minor component of *Panax ginseng*³. These Dammarane-type ginsenosides are divided into two groups according to their aglycon structure: protopanaxadiol (Rb1, Rb2, Rc, Rd, Rh2 and Rg3) and protopanaxatriol (Re, Rf, and Rg1)³.

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 fields⁴. However, cultivating ginseng is time-consuming, labour-intensive and is influenced by many conditions such as soil, climate, pathogens and pests⁴.

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 methods⁵⁻⁸. With the development of metabolic engineering and synthetic biology tools, many natural products have been successfully synthesised in *S. cerevisiae*^{7,9-12}.

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 (bAS), α -amyrin synthase (aAS), lanosterol synthase (LAS) and lupeol synthase (LUS)¹³⁻¹⁵. In *P. ginseng*, cyclisation of 2,3-oxidosqualene to dammarenediol-II by DDS is the first reaction towards the biosynthesis of dammarane-type ginsenosides^{16,17}. 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 (Fig. 1)³. Protopanaxadiol is further converted to protopanaxatriol by protopanaxatriol synthase (PPTS) (Fig. 1)¹⁶. In contrast, cyclisation of 2,3-oxidosqualene to β -amyrin by β -amyrin synthase (bAS) 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

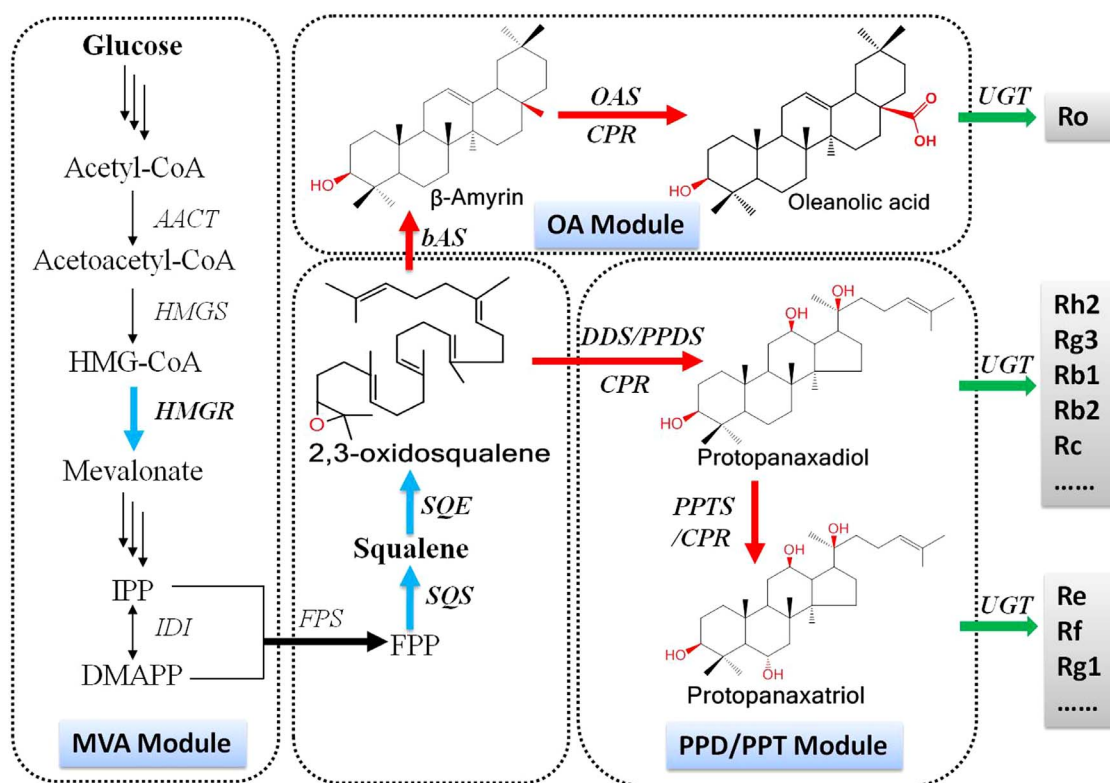


Figure 1 | Biosynthetic pathways for protopanaxadiol, protopanaxatriol and oleanolic acid production in metabolically engineered *S. cerevisiae*. Single arrows represent one-step conversions, while triple arrows represent multiple steps. Bold, blue arrows represent over-expressed yeast endogenous genes. Bold, red arrows represent exogenous plant genes that were introduced into *S. cerevisiae*. OA module includes of *G. glabra* *bAS*, *M. truncatula* *OAS* and *A. thaliana* *CPR* genes, while PPD/PPT module includes *A. thaliana* *CPR* and *DDS*, *PPDS* and *PPTS* of *P. ginseng* genes. Intermediates: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; FPP, farnesyl diphosphate; OA, oleanolic acid; PPD, protopanaxadiol; and PPT, protopanaxatriol. Enzymes: HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; SQS, squalene synthase; SQE, squalene epoxidase; *bAS*, β -amyrin synthase; *OAS*, oleanolic acid synthase; *CPR*, cytochrome P450 reductase; *DDS*, dammarenediol-II synthase; *PPDS*, protopanaxadiol synthase; and *PPTS*, protopanaxatriol synthase.

at the C-28 position of β -amyrin (Fig. 1)^{14,15}. These aglycons are further converted to ginsenoside compounds by uridine diphosphate glycosyltransferases (UGTs)^{18,19}.

In this work, *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 *Medicago truncatula* and *DDS*, *PPDS* and *PPTS* from *P. ginseng* have been previously demonstrated to be functional in yeast^{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 *S. cerevisiae*. Triterpenoids are derived from two common building blocks, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which are synthesised through the mevalonic acid (MVA) pathway in *S. cerevisiae* (Fig. 1). Most IPP and DMAPP precursors enter the ergosterol synthetic pathway^{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 (*tHMG1*) has commonly been

used to increase the carbon flux through the MVA pathway^{11,25}. This strategy had been used to successfully increase the production of many terpenoids, including sesquiterpenoids^{7,11,26,27}, diterpenoids^{9,10,28}, triterpenoids^{29,30} and carotenoids³¹.

The *tHMG1* gene (controlled by the *PGK1* promoter) and the *LYS2* gene (controlled by the *TEF1* promoter) were integrated into the chromosome of BY4742-TRP at the δ 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 *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 oleanane-type triterpenoids¹⁸. β -amyrin is synthesised from 2,3-oxidosqualene in some medicinal plants by 2,3-oxidosqualene cyclases (OSCs)^{13–15,23}. *S. cerevisiae* can produce 2,3-oxidosqualene from IPP and DMAPP through farnesyl diphosphate synthase (encoded by *ERG20*), squalene synthase (encoded by *ERG9*) and squalene epoxidase (encoded by *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 pathway³². Overexpressing the squalene synthase gene had been used to enhance the production of β -amyrin in yeast^{29,30} and the production

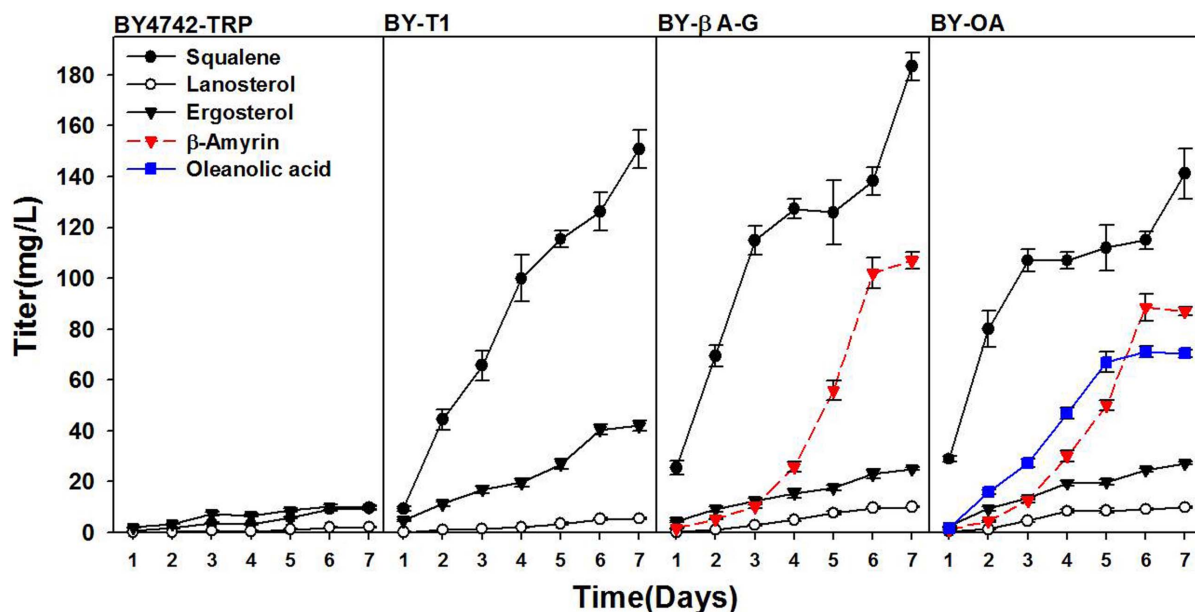


Figure 2 | Production of squalene, lanosterol, ergosterol, β -amyryn and oleanolic acid by engineered *S. cerevisiae* strains BY4742-TRP, BY-T1, BY- β -A-G and BY-OA. All strains were cultivated in YPD medium with 2% glucose for 7 days. Three replicates were performed, and the error bars represented standard deviation. All these five chemicals were measured in the four strains. β -amyryn and oleanolic acid were not detected in strains BY4742-TRP and BY-T1. Oleanolic acid was not detected in strain BY- β -A-G.

of phytosterols and triterpenoids in plants^{33–35}. Furthermore, over-expressing the squalene epoxidase gene had been used to increase sterol production^{21,25}.

To construct the β -amyryn synthetic pathway and improve its production in *S. cerevisiae*, two different β -amyryn 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- β -A-G and BY- β -A-P (Table 1), respectively. When cultivated in YPD medium with 2% glucose for 7 days, GC/MS analysis of cell extractions of both strains confirmed the production of β -amyryn (Fig. S1). Strain BY- β -A-P produced 1.9 mg/L β -amyryn with a yield of 0.2 mg/g (data not shown), while strain BY- β -A-G produced 107.0 mg/L β -amyryn with a yield of 9.3 mg/g DCW (Fig. 2). Strain BY- β -A-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 -amyryn synthesis, the β -amyryn synthase gene of *G. glabra* (controlled by the *PGK1* promoter) and the *LYS2* gene (controlled by the *TEF1* promoter) were integrated into the chromosome of strain BY4742-TRP at *rDNA* sites. The resulting strain, BY- β -A-CK (Table 1), produced 77.7 mg/L β -amyryn, which was 73% of that produced by strain BY- β -A-G (Fig. S2). In addition, strain BY- β -A-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- β -A-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 truncatula*¹³ (controlled by *TEF1* promoter), with a cytochrome P450 reductase gene of *Arabidopsis thaliana* (*AtCPR1*)²⁴(controlled by *TDH3* promoter) and a new copy of the *G. glabra* β -amyryn synthase gene (controlled by *PGK1* promoter) were integrated into the chromosome of strain BY- β -A-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 -amyryn 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* dammarenediol-II synthase gene (*PgDDS*, controlled by *PGK1* promoter), *P. ginseng* protopanaxadiol synthase gene (*PgPPDS*, controlled by *TEF1* 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

Table 1 | Strains used in this study

Name	Description	Source
BY4742	<i>MATα</i> , <i>his3Δ1</i> , <i>leu2Δ10</i> , <i>lys2Δ10</i> , <i>MET15</i> , <i>ura3Δ10</i>	Brachmann <i>et al.</i> ³⁸
BY4742-TRP	Deletion of the <i>Trp1</i> gene of BY4742	This study
BY-T1	BY4742-TRP, δ DNA:: <i>P_{PGK1}-tHMG1-T_{ADH1}-P_{TEF1}-LYS2-T_{CYC1}</i>	This study
BY- β -A-P	BY-T1, rDNA:: <i>P_{PGK1}-PgbAS-T_{ADH1}-P_{TDH3}-ERG1-T_{TRP1}-P_{TEF1}-ERG9-T_{CYC1}</i>	This study
BY- β -A-G	BY-T1, rDNA:: <i>P_{PGK1}-GgbAS-T_{ADH1}-P_{TDH3}-ERG1-T_{TRP1}-P_{TEF1}-ERG9-T_{CYC1}</i>	This study
BY- β -A-CK	BY4742-TRP, rDNA:: <i>P_{PGK1}-GgbAS-T_{ADH1}-P_{TEF1}-LYS2-T_{CYC1}</i>	This study
BY-OA	BY- β -A-G, <i>Trp1</i> :: <i>P_{PGK1}-GgbAS-T_{ADH1}-P_{TDH3}-AtCPR1-T_{TRP1}-P_{TEF1}-MtOAS-T_{CYC1}</i>	This study
GY-1	BY-OA, <i>His3</i> :: <i>P_{PGK1}-PgDDS-T_{ADH1}-P_{FBA1}-SynPgPPTS-T_{TDH2}-P_{TDH3}-AtCPR1-T_{TRP1}-P_{TEF1}-SynPPDS-T_{CYC1}</i>	This study

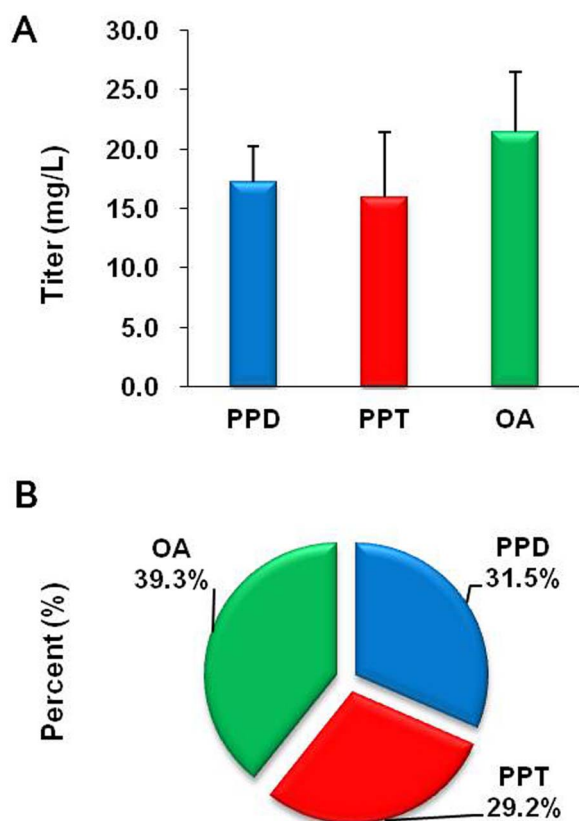


Figure 3 | Production of protopanaxadiol, protopanaxatriol and oleanolic acid by engineered *S. cerevisiae* strain GY-1. (A) Titters obtained after fermentation for 7 days; (B) Proportion of protopanaxadiol, protopanaxatriol and oleanolic acid within the total aglycons. Three replicates were performed, and the error bars represented standard deviation.

extraction of strain GY-1 confirmed the production of protopanaxadiol and protopanaxatriol (Fig. S4 and S5). Strain GY-1 produced 17.2 mg/L protopanaxadiol with a yield of 1.4 mg/g DCW, and 15.9 mg/L protopanaxatriol with a yield of 1.3 mg/g DCW. The oleanolic acid titer decreased from 71.0 to 21.4 mg/L, and its yield decreased from 6.1 to 1.8 mg/g DCW (Fig. 3A). The proportion of protopanaxadiol, protopanaxatriol and oleanolic acid within the total aglycons was 31.5%, 29.2% and 39.3%, respectively (Fig. 3B). In addition, this strain produced 96.0 mg/l squalene, 11.1 mg/l lanosterol, 16.3 mg/l ergosterol, 24 mg/l β -amyryn and 7.9 mg/l dammarenediol-II (data not shown).

Discussion

S. cerevisiae is an ideal host for the heterologous production of valuable natural products. To be economically comparable to the extraction from plant sources, heterologous production of ginsenosides in yeast with high titer and productivity is required. Because 2,3-oxidosqualene is a basic precursor for ginsenoside synthesis, its sufficient supply is very important. In this work, three commonly used engineering strategies for increasing the 2,3-oxidosqualene supply were performed to improve β -amyryn production. After overexpressing the *tHMG1*, *ERG9* and *ERG1* genes, β -amyryn production increased 38% and all squalene-derived terpenoids increased 159% (Fig. S2), demonstrating that these three enzymes were essential for increasing the 2,3-oxidosqualene supply to improve triterpenoid production.

β -amyryn serves as the basic, oleanane-type triterpenoid precursor to variable downstream products¹⁸. Through the introduction of

β -amyryn 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 -amyryn²⁹. By introducing β -amyryn 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 -amyryn³⁰. To the best of our knowledge, strain BY- β A-G had the highest β -amyryn titer that was achievable by microbial fermentation. It was suggested that the β -amyryn synthase of *G. glabra* was better at producing β -amyryn than *A. annua*, *P. sativum* and *P. ginseng*. This suggestion was reasonable because *G. glabra* contains a large amount of glycyrrhizin (2–8% of the dry weight)^{23,36}, while the other three plants contain minimal levels of oleanane-type triterpenoids¹⁶.

For strain BY- β A-G, squalene was produced with high productivity (1.60 mg/L·h), while β -amyryn 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 -amyryn productivity increased 9.1-fold to 1.27 mg/L·h. One possibility was that β -amyryn synthase activity was relatively lower for the first 72 h and limited β -amyryn production, while it was high from 72 to 144 h and led to high β -amyryn 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 -amyryn productivity.

β -amyryn 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 species³⁷, and the CPR levels would thus affect cell health⁷. 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 P450 CYP71AV1, which can catalyse a three-step, sequential oxidation that can convert amorphaadiene to artemisinic acid¹¹. 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 *b*₅ (CYB5) facilitated the oxidation of amorphaadiene 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 acid⁷. 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)^{18,19}. 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 S288C³⁸, was obtained from EUROSCARF and used as the parent strain for all yeast strains. Engineered yeast strains were grown either in SD medium^{9,39} lacking leucine, uracil, tryptophan and



histidine, where appropriate, or in YPD medium³⁹. *Escherichia coli* Trans-10 (TransGen Biotech, Beijing, China) was used for transformation and plasmid DNA extraction. Strains were cultivated at 37°C in LB medium with 100 mg/L ampicillin.

Plasmid construction. *Panax ginseng* cells were induced with 0.1 mg/L MeJA for 24 h. Total RNA was isolated by the Trizol method⁴⁰, and cDNA was synthesised using the PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Dalian, China). The *P. ginseng* β-amyryn synthase (*PgBAS*) were PCR-amplified from the cDNA of *P. ginseng* (using primer sets SexA1-PgPNU/PgPNU-Asc1). The amplified DNA fragments were cloned into pEASY-Blunt, resulting in p-PgBAS.

The *Glycyrrhiza glabra* β-amyryn synthase gene (*GgbAS*) (GenBank: AB037203), *P. ginseng* protopanaxatriol synthase gene (*PgPPTS*) (GenBank: JX036031) and *Medicago truncatula* oleanolic acid synthase gene (*MtOAS*) (GenBank: FN995113), together with the SexA1 and Asc1 restriction sites at their 5′- and 3′- ends, were synthesised by GenScript (Nanjing, China) with codon optimisation for improved expression in *S. cerevisiae*. The synthesised DNA fragments were cloned into pUC57, resulting in p-GgbAS, p-SynPgPPTS and p-MtOAS, respectively.

The plasmids of p-ΔTrp1, pδ-tHMG1, pM13-LYS2, pM3-ERG9, pM11-ERG1, pM11-AtCPR1, pM2-PgBAS, pM2-GgbAS, pM3-MtOAS, pM3-synPgPPTS, pM14-PgDDS, pM8-synPgPPTS, prDNA-LEU, pTrp-HIS and pHis-TRP are described in the supplementary data.

All the plasmids are summarised in Table S1, and the primers used during plasmid construction are summarised in Table S2.

Strain construction. Transformation of *S. cerevisiae* strains was performed by the standard lithium acetate method³⁹. Strain BY4742-TRP was constructed by deleting the *TRP1* gene of strain BY4742, thus leading to tryptophan auxotrophy and was accomplished as follows. The homologous recombination region of the partial *TRP1* gene with the Loxp-HIS3-Loxp cassette was amplified from p-ΔTrp1 using primer sets ZD-TRP1-int-up(400)/ZD-TRP1-int-down(450). The amplified DNA fragment was then transformed into strain BY4742 followed by selection on SD-HIS plates. Strains were verified by PCR analysis, and the correct colony was transformed with plasmid pSH47. The transformant was cultivated at 30°C in SD-HIS-URA medium with 2% galactose as its sole carbon source for 24 h followed by selection on YPD medium with 1 g/L 5-FOA as a selection for the loss of plasmid pSH47, resulting in strain BY4742-TRP. This strain could not grow on SD-HIS and SD-TRP plates.

Strain BY-T1 was constructed by integrating the *tHMG1* and *LYS2* genes into the δ DNA sites of BY4742-TRP using the DNA assembler method as described previously^{41,42}. Three DNA fragments were amplified from pδ-tHMG1 (using primer sets 1-M-HisG-PGK1-F/1-M-ADHt-Tef1-R and 3-M-CYC1t-δ2-F/3-δ2-R) and pM13-LYS2 (using primer set 2-M-ADHt-Tef1-F/2-M-CYC1t-δ2-R), and another DNA fragment was obtained by *Bam*HI and *Xho*I digestion of plasmid pδ-UB. These four DNA fragments were transformed into strain BY4742-TRP followed by selection on SD-URA plates. All strains were verified by PCR analysis, and twelve colonies were screened by GC-MS for the selection of the best squalene-producing transformant.

Strain BY-βA-P was constructed by integrating the *ERG9*, *ERG1* and *PgBAS* genes into the *rDNA* sites of strain BY-T1. Five DNA fragments were amplified from pM2-PgBAS (using primer set 1-M-pEASY-PGK1-F/3G-1-M-ADHt-TDH3-R), pM3-ERG9 (using primer set 3G-2-M-TPI1t-Tef1-F/M-CYC1t-pEASY-R), pM11-ERG1 (using primer set 3G-3-M-ADHt-TDH3-F/3G-3-M-TPI1t-Tef1-R) and prDNA-LEU (using primer sets X1-M-pEASY-r-t-F/X1-r-t-R-rDNA and X2-r-t-F-rDNA/X2-M-pEASY-r-t-R). These fragments were then transformed into strain BY-T1 followed by selection on SD-URA-LEU plates. All strains were verified by PCR analysis, and twelve colonies were screened by GC/MS for the selection of the best-producing transformant.

BY-βA-G was constructed by integrating the *ERG9*, *ERG1* and *GgbAS* genes into the *rDNA* sites of strain BY-T1 to construct strain BY-βA-P.

BY-βA-CK was constructed by integrating the *LYS2* and *GgbAS* genes into the *rDNA* sites of strain BY4742-TRP. Four DNA fragments were amplified from pM2-GgbAS (using primer set 1-M-pEASY-PGK1-F/1-M-ADHt-Tef1-R), pM13-LYS2 (using primer set 2-M-ADHt-Tef1-F/M-CYC1t-pEASY-R) and prDNA-LEU (using primer sets X1-M-pEASY-r-t-F/X1-r-t-R-rDNA and X2-r-t-F-rDNA/X2-M-pEASY-r-t-R) and were then transformed into strain BY4742-TRP followed by selection on SD-LEU plates. All strains were verified by PCR analysis, and 12 colonies were screened by GC/MS for the selection of the best-producing transformant.

Strain BY-OA was constructed by integrating the *GgbAS*, *MtOAS* and *AtCPR1* genes into the *Trp1* site of BY-βA-G. Five DNA fragments were amplified from pM2-GgbAS (using primer set 1-M-pEASY-PGK1-F/3G-1-M-ADHt-TDH3-R), pM3-MtOAS (using primer set 3G-2-M-TPI1t-Tef1-F/M-CYC1t-pEASY-R), pM11-AtCPR1 (using primer set 3G-3-M-ADHt-TDH3-F/3G-3-M-TPI1t-Tef1-R) and pTrp-HIS (using primer sets X1-M-pEASY-r-t-F/ZD-TRP1-interg.-1 and ZD-TRP1-interg.-2/X2-M-pEASY-r-t-R) and were then transformed into strain BY-βA-G followed by selection on SD-URA-LEU-HIS plates. All strains were verified by PCR analysis, and 12 colonies were screened by GC/MS and HPLC for selection of the best-producing transformant.

Strain GY-1 was constructed by integrating the *PgDDS*, *SynPgPPTS*, *SynPPTS* and *AtCPR1* genes into the His3 site of BY-OA. Six DNA fragments were amplified from pM14-PgDDS (using primer set 1-M-pEASY-PGK1-F/4G-1-M-ADHt-FBA1-R), pM8-SynPgPPTS (using primer set 4G-4-M-ADHt-FBA1-F/4G-4-M-TDH2t-TDH3-R), pM11-AtCPR1 (using primer set 4G-3-M-TDH2t-TDH3-F/3G-3-M-TPI1t-Tef1-R), pM3-SynPgPPTS (using primer set 3G-2-M-TPI1t-Tef1-F/M-CYC1t-pEASY-R) and pHis-TRP (using primer sets X1-M-pEASY-r-t-F/ZD-His3

interg.-1 and ZD-His3 interg.-2/X2-M-pEASY-r-t-R) and were transformed into strain BY-OA followed by selection on SD-URA-LEU-HIS-TRP 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.

Primers used in the DNA assembly are summarised in Table S3.

Yeast cultivation. YPD medium was used for cultivating strains BY4742, BY-T1, BY-βA-P, BY-βA-G, BY-βA-CK, BY-OA and GY-1. All strains were first inoculated into 15 ml culture tubes containing 2 ml medium and grown at 30°C and 250 rpm to an OD₆₀₀ of approximately 1.0. Flasks (250 ml) containing 100 ml medium were then inoculated to an OD₆₀₀ 0.05 with the seed cultures. Strains were grown at 30°C and 250 rpm for 7 days, and all optical densities at 600 nm (OD₆₀₀) were measured using a Shimadzu UV-2550 spectrophotometer.

Chemical analysis. Cells were collected from fermentation culture via centrifugation. 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 insert 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 electric ionisation using SIM-scan mode with diagnostic ions monitored as followed: m/z 69, m/z 218, m/z 363, m/z 411 and m/z 437. A crystallised β-amyryn sample was used as the standard for quantification (purchased from BioBioPha, China), and squalene, lanosterol and ergosterol standards (purchased from Sigma Aldrich) were also used for quantification.

For the determination of oleanolic acid, acetone and methanol (1:1) extracts (20 μl) were analysed by LC/MS using an Agilent 1200 HPLC system coupled to a Bruker-microTOF-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 (250 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. Oleanolic 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-microTOF-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 (250 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. The 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 oleanolic 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 (250 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 was held at 30°C during the separation. Protopanaxadiol, Protopanaxatriol and oleanolic acid was purchased from Sigma Aldrich and was used for quantification.

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Author contributions

Z.D., L.H., T.L. 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

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

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