ARTICLE

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Optimization of a Cytochrome P450 Oxidation System for Enhancing Protopanaxadiol Production in *Saccharomyces cerevisiae*

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ABSTRACT: Ginsenosides, the major bioactive components of *Panax ginseng*, are regarded as promising high-value pharmaceutical compounds. In ginseng, ginsenosides are produced from their precursor protopanaxadiol. Recently, an artificial biosynthetic pathway of protopanaxadiol was built in *Saccharomyces cerevisiae* by introducing a *P. ginseng dammarenediol-II synthase*, a *P. ginseng cytochrome P450-type protopanaxadiol synthase* (PPDS), and a *Arabidopsis thaliana NADPH-cytochrome P450 reductase* (ATR1). In this engineered yeast strain, however, the low metabolic flux through PPDS resulted in a low productivity of protopanaxadiol. Moreover, health of the yeast cells was significantly affected by reactive oxygen species released by the pool coupling between PPDS and ATR1. To overcome the obstacles in protopanaxadiol production, PPDS was modified through transmembrane domain truncation and self-sufficient PPDS–ATR1 fusion construction in this study. The fusion enzymes conferred approximately 4.5-fold increase in catalytic activity, and 71.1% increase in protopanaxadiol production compared with PPDS and ATR1 co-expression. Our in vivo experiment indicated that the engineered yeast carrying fusion protein effectively converted 96.8% of dammarenediol-II into protopanaxadiol. Protopanaxadiol production in a 5 L bioreactor in fed-batch fermentation reached 1436.6 mg/L. Our study not only improved protopanaxadiol production in yeast, but also provided a generic method to improve activities of plant cytochrome P450 monooxygenases. This method is promising to be applied to other P450 systems in yeast.

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KEYWORDS: dammarenediol-II; protopanaxadiol; *Saccharomyces cerevisiae*; protopanaxadiol synthase; self-sufficient P450s; synthetic biology

Introduction

Ginsenosides are a special group of triterpenoid saponins in *Panax ginseng*, which exhibit potential anticarcinogenic, immunomodulatory, anti-fatigue, and antiallergic activities (Christensen, 2009). They are usually used in pharmaceutical and health food preparations (Lee et al., 2010). Ginsenosides are extracted from ginseng or its hairy root, which is time consuming and labor-intensive. Thus, mass production of ginsenosides in this traditional way seems to be a big challenge for reduction of their price and therefore widely application (Murthy et al., 2014).

Protopanaxadiol is a dammarane-type aglycon precursor of ginsenosides. In ginseng, protopanaxadiol is converted to ginsenosides by various glycosyl transferases (GTs) (Sun et al., 2010). Biosynthesis of protopanaxadiol starts from cyclization of 2, 3-oxidosqualene by dammarenediol-II synthase (DS) (Tansakul et al., 2006). Then, dammarenediol-II is hydroxylated at the C-12′ position through a cytochrome P450-type protopanaxadiol synthase (PPDS, also known as CYP716A47) (Han et al., 2011).

In order to improve the production of ginsenosides, researchers have engineered yeast strains to produce ginsenosides (Dai et al., 2013; Wang et al., 2014, 2015). For instance, an artificial biosynthetic pathway of ginsenosides was built in *S. cerevisiae* by introducing *DS*, *PPDS*, and *Arabidopsis thaliana NADPH-cytochrome P450 reductase* (*ATR1*). This engineered yeast strain, however, accumulated large amount of an intermediate precursor dammarenediol-II, which was due to the low enzymatic activity of PPDS (Dai et al., 2013). Increasing copy numbers of PPDS and its reductase partner NADPH-cytochrome P450 reductase (CPR) could not increase protopanaxadiol production (Dai et al., 2013). PPDS turns out to be a rate-limiting enzyme for high

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efficient production of ginsenosides in yeast. Similarly, cytochrome P450s often present low catalytic activities in heterologous hosts, which indicates that the bottleneck of cytochrome P450-dependent bioconversion is a common challenge in transgenic microorganisms (DeJong et al., 2006; Gavira et al., 2013; Jung et al., 2011).

Another obstacle for the utilization of plant cytochrome P450 in microorganism is the reactive oxygen species (ROS) released by the low coupling between cytochrome P450 and CPR. Over expression of CPRs often makes oxidative stress to cells or even causes severe cytotoxicity (Zangar et al., 2004). Normally, cytochrome P450s are found in large excess to CPRs in plant or animal (Renault et al., 2014). However, metabolic engineering in microbial host generally needs to maximize the metabolic flux of a target pathway, which is usually achieved by enhancing expression of related enzymes. Therefore, cytochrome P450 oxidation system should be optimized for engineering applications. To bypass these limitations, one potential solution is to construct self-sufficient P450 reduction enzyme by fusing P450 to the reductase domain (Sadeghi and Gilardi, 2013). This strategy is inspired by a natural self-sufficient fatty acid hydroxylase, CYP102A1 from *Bacillus megaterium*, which exists as a fusion enzyme combining a cytochrome P450 oxidase domain with a reductase domain. CYP102A1 possesses the highest turnover rates ever measured for cytochrome P450s, with a k_{cat} value of 17,000/min (Warman et al., 2005).

To address these two challenges facing the PPDS heterologous expression in *S. cerevisiae* and to obtain a yeast strain with high-yield of protopanaxadiol, PPDS was modified through transmembrane domain truncation and self-sufficient P450 construction in this study. The results indicated that this strategy resulted in high conversion rate from dammarenediol-II to protopanaxadiol (96.8%) in *S. cerevisiae*. The strain carrying the PPDS–ATR1 fusion protein could produce 1436.6 mg/L protopanaxadiol in well controlled fed-batch fermentation in a 5 L bioreactor. This artificial self-sufficient PPDS construction approach will be valuable for efficient utilization of other plant cytochrome P450s.

Materials and Methods

Strains, Vectors, and Media

S. cerevisiae W303-1a was used as a parent strain for all engineered strains. Engineered yeast strains were grown in SD medium (Adams et al., 1998) lacking leucine, uracil, adenine, and histidine where appropriate. *Escherichia coli* DH-5α was used for gene transformation and plasmid DNA extraction. All strains were listed in Table I. Plasmid pRS403, pXP218, and pXP320 were obtained from ATCC. Genes including *DS* (GenBank: AB265170.1), *PPDS* (GenBank: JN604537.1), and *ATR1* (GenBank: BT008426.1) were synthesized by GENEWIZ (Suzhou, China) with codon optimization. The synthesized genes were cloned into pUC57 vector to generate p-DS, p-PPDS, and p-ATR1 plasmids, respectively (Table SII).

Engineering *S. cerevisiae* for Protopanaxadiol Production

Primers used for plasmids construction were summarized in Table SI. Yeast promoters, genes, and terminators were amplified from the genomic DNA of *S. cerevisiae* W303-1a. *DS*, *PPDS*, and *ATR1* were amplified from plasmids p-DS, p-PPDS, and p-ATR1, respectively. *PGK1p-ERG20-CYC1t*, *TPI1p-ERG9-ADH2t*, *TDH3p-tHMG1-ADH3t*, *TEF1p-ERG1-ADH1t*, *PGK1p-DS-CYC1t*, *PGK1p-PPDS-CYC1t*, and *TDH3p-ATR1-ADH3t* expression cassettes were constructed by fusion PCR, and were cloned into pEASY-Blunt (TransGen Biotech, Beijing, China), resulting in pM-tHMG1, pM-ERG20, pM-ERG9, pM-ERG1, pM-DS, pM-PPDS, and pM-ATR1, respectively (Table SII). *S. cerevisiae* transformations were performed using DNA assembler (Shao et al., 2009). Overlaps of 50 bp were designed between adjacent fragments. The expression cassettes overlap and integrated order for multi-copy integration were illustrated in Figure S1.

Transmembrane Domain Truncation and Construction of Artificial Self-Sufficient PPDS–ATR1 Fusion Proteins

Transmembrane domain of PPDS was predicted using the TMHMM program (Chen et al., 2003). Subsequently, 17, 26, 31, and 43 amino acid residues were truncated from the N-terminal sequence, respectively (Fig. S2). Primers used for transmembrane domain truncation of PPDS were listed in Table SIII. For constructing fusion proteins, 46 amino acid residues of ATR1 were truncated from its N-terminal (Schuckel et al., 2012).

PPDS heme domain and *46tATR1* reductase domain were fused by overlap PCR. Three polypeptide linkers, GSTSSGSSG (linker1), GSTSSG (linker2), and GGG (linker3), were used to investigate the influence of different linkers between two domains on fusion protein activities. A fusion protein without linker was also constructed. These four fusion proteins were named as PPDS-linker1-46tATR1, PPDS-linker2-46tATR1, PPDS-linker3-46tATR1, and PPDS-nolinker-46tATR1, respectively. In addition, 31tPPDS heme domain and 46tATR1 reductase domain were linked by polypeptide GSTSSGSG. All fusion cassettes were integrated into δ DNA site of strain W2 using

Table I. Strains used in this study.

Name	Description
W303-1a	*MATa {leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15}*
W1	*DS*, *ERG1*, and marker *HIS3* gene expression cassettes were integrated into *rDNA* site of W303-1a
W2	*tHMG1*, *ERG20*, *ERG9*, and marker *LEU2* expression cassettes were integrated into *rDNA* site of W1
W3	*PPDS*, *ATR1*, and marker *URA3* expression cassettes were integrated into δ site of W2
W3 plus	*PPDS* and *ATR1* expression cassettes were integrated into *LEU2* site of W3 using *ADE2 as* marker
W3t	*31tPPDS*, *ATR1*, and marker *URA3* expression cassettes were integrated into δ site of W2
W3a	*PPDS*-linker1-*46tATR1* and marker *URA3* expression cassettes were integrated into δ site of W2
W3at	*31tPPDS*-linker1-*46tATR1* and marker *URA3* expression cassettes were integrated into δ site of W2
W3b	*PPDS*-linker2-*46tATR1* and marker *URA3* expression cassettes were integrated into δ site of W2
W3c	*PPDS*-linker3-*46tATR1* and marker *URA3* expression cassettes were integrated into δ site of W2
W3d	*PPDS*-nolinker-*46tATR1* and marker *URA3* expression cassettes were integrated into δ site of W2

URA3 as the selection marker (Fig. S1D). Sequences of oligonucleotide primers used for fusion proteins construction were listed in Table SIV..

Quantification of PPDS and ATR1 Copy Numbers in *S. cerevisiae* by qPCR

Gene copy numbers was quantified by qPCR with the absolute quantification method (Abad et al., 2010). *ARG4* gene was used as a reference gene. Open reading frames of *ARG4*, *PPDS*, and *ATR1* were amplified and quantified to generate standard curves, respectively. Genome DNAs of different colonies were extracted using a TIANamp Yeast DNA kit (Tiangen, Beijing, China). TaqMan probe-based determinations were performed as described by Zhang (Zhang et al., 2014). Primers used for qPCR were listed in Table SV.

Intracellular ROS Level Determination

Yeast cells were collect at 15 and 30 h by centrifugation. ROS levels were detected with 2′, 7′-dichlorofluorescein diacetate as described previously (Liu et al., 2013).

CO Difference Spectra Analysis

PPDS level was estimated by analyzing the CO difference spectra (Omura and Sato, 1964). *S. cerevisiae* microsomes were prepared as described previously (Olsen et al., 2010). CO difference spectra analysis was performed on a TU-1810 UV-Vis spectrophotometer (PERSEE, China) to record the development of difference spectra at 450 nm. The cytochrome P450s content was determined using an extinction coefficient of 91.9 $mM^{-1}\,cm^{-1}$ at 450 nm.

Determination of Catalytic Activity and Coupling Efficiency of PPDS and ATR1

To determinate the catalytic activity and coupling efficiency of co-expressed and fusion-expressed PPDS and ATR1, 1 mg of microsomal fraction and 300 μM of dammarenediol-II were mixed in 100 mM potassium phosphate buffer (pH 7.4) and the final volume was adjusted to 500 μL. The reaction initiated by adding NADPH (final concentration 1 mM). The reaction mixture was incubated at 30°C. Consumption of NADPH was measured by NADPH depletion at 340 nm ($\varepsilon = 6.22\ mM^{-1}\,cm^{-1}$) (Kille et al., 2011). The reaction was monitored until the absorption value for NADPH reached constant. The product was extracted with hexane and analyzed by HPLC. The percentage coupling efficiency was calculated as protopanaxadiol produced/NADPH oxidized × 100% (Degregorio et al., 2011).

Yeast Cultivation and Fermentation Process

YPD medium (Adams et al., 1998) was used for cultivating yeast strains. All strains were inoculated into flask containing 30 mL YPD medium, which were cultured at 30°C and 220 rpm for 6 days. All the flask fermentations were performed in triplicate.

The protopanaxadiol-producing strains were used for fed-batch fermentation in 250 mL shaking flasks. Seed was prepared by inoculating single colony into 30 mL YPD medium, where was incubated at 30°C and 220 rpm for 24 h. Three milliliters of the seed culture was then transferred to a new 250 mL shaking flask containing 30 mL YPD medium. Fermentation was carried out at 30°C and 220 rpm with an initial pH of 5.5. At 48 h, 5 mL fed solution (pH 5.5) containing 400 g/L glucose, 9 g/L KH_2PO_4, 5.12 g/L $MgSO_4 \cdot 7H_2O$, 3.5 g/L K_2SO_4, 0.28 g/L Na_2SO_4, 0.5 g/ L adenine, 0.6 g/L uracil, and 1.2 g/L lysine, were fed to the flask. For two phase extractive fermentation, 3 mL dodecane was added into 30 mL YPD medium at 24 h after inoculation.

Strains W3 and W3a were subsequently cultured in 2.5 L YPD medium in a 5 L bioreactor (ShangHai BaoXing Bio-Engineering Equipment Co. Ltd., China) with an initial OD600 of 0.5. Fermentation was carried out at 30°C with an agitation speed of 600 rpm and air flow rate of 2 L/min. pH was automatically controlled at 5.5 with ammonia hydroxide (5 M). Totally 500 mL fed solution as mentioned above was fed into medium from 36 h after inoculation by controlling glucose concentration less than 1 g/L.

Extraction and Analysis of Metabolites

The cellpellets were harvested and disrupted. The intracellular metabolites were extracted with ethyl acetate. For two phase fermentation, 1 mL dodecane was collected and diluted in 9 mL ethyl acetate. Protopanaxadiol, squalene and 2, 3-oxidosqualene standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dammarenediol-II standard was purchased from BioBioPha (Kunming, China).

HPLC analysis was carried on an Elite P230II high-pressure pump system coupled with UV detection at 203 nm. Chromatographic separation was realized on Grace Apollo C18 column ($4.6 \times 250\ mm^2$, 5 μm).

LC/APCI/MS analysis was performed on a surveyor LC system (Thermo Finnigan, San Jose) equipped with an Agilent Zorbax SB-Aq($2.1 \times 100\ mm^2$, 3 μm). An APCI /MS system was used for detection. All system control, data acquisition, and processing were performed by Xcalibur 2.0.5 (Thermo Scientific, Bremen, Germany).

Results

Protopanaxadiol Production in *S. cerevisiae*

To construct a yeast strain for protopanaxadiol production, *DS*, *PPDS*, and *ATR1* cassettes were integrated into chromosome of *S. cerevisiae* W303-1a. A truncated *3-hydroxy-3-methylglutaryl coenzyme A (tHMG1)*, *farnesyl diphosphate synthase* (*ERG20*), *squalene synthase* (*ERG9*), and *Squalene epoxidase* (*ERG1*) genes were also overexpressed to increase squalene supply (Fig. 1D), and the top-performing strain W3 for protopanaxadiol production was selected. Strain W3 produced 155.3 mg/L of protopanaxadiol as well as 96.5 mg/L dammarenediol-II (Table II), suggesting insufficient conversion of dammarenediol-II by PPDS. Then, the copy numbers of *PPDS* and *ATR1* were further increased in strain W3 plus to enhance conversion ratio of dammarenediol-II. In strains W3 and W3 plus, copy numbers of ATR1 and PPDS were estimated as 3, 3

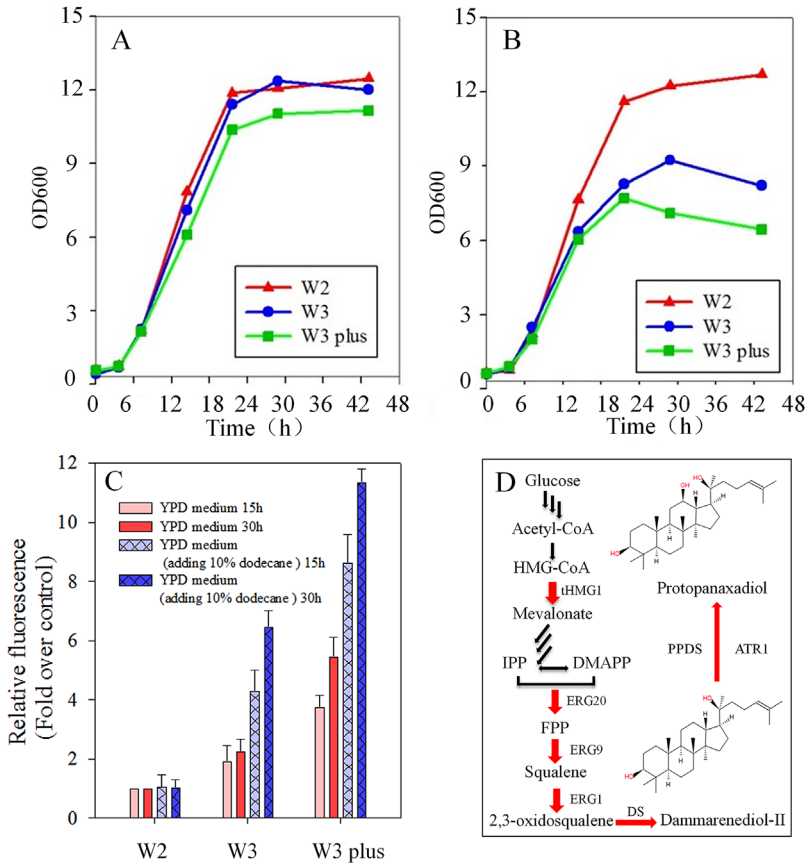

Figure 1. Growth status and intracellular ROS level of strains W2, W3, and W3 plus. Growth curves of engineered strains in YPD medium (**A**) and YPD medium with addition of 10% dodecane (**B**). (**C**) ROS level of strains when cultured in YPD medium and in YPD medium with addition of 10% dodecane. Values of the bars indicated the fold changes of ROS levels over control sample W2. (**D**) Construction and optimizing of biosynthetic pathway for protopanaxadiol production in *S. cerevisiae*. *tHMG1*, *ERG20*, *ERG9*, and *ERG1* were overexpressed in *S. cerevisiae* to enhance precursor supply (red arrows represent over-expressed genes and introduced heterologous genes in yeast). tHMG1, truncated 3-hydroxy-3-methylglutaryl coenzyme A reductase; ERG20, farnesyl diphosphate synthetase; ERG9, squalene synthetase; ERG1, squalene monooxygenase; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; FPP, farnesyl diphosphate.

and 5, 5, respectively (Fig. 4). However, no obvious improvement was observed in protopanaxadiol production in W3 plus (Table II). These results were consistent with previous study when dammarenediol-II and protopanaxadiol were produced in *S. cerevisiae* BY4742 (Dai et al., 2013).

However, when the expression of PPDS and ATR1 was enhanced, the OD600 of W3 plus decreased (Fig. 1A). It was reported that high levels of heterologous P450 and CPR expression would cause poor coupling between cytochrome P450s and their reductases, and thus resulted in the genetation of ROS (Zangar et al., 2004). In another study, the viability of the *S. cerevisiae* culture also decreased remarkably after CYP71AV1 and CPR1 high-expression (Paddon et al., 2013). Thus, we deduced that the engineered strains W3 plus may be affected by high-level ROS released from poor coupling of

Table II. Metabolites analysis of engineered strains W2, W3, and W3 plus during fermentations. The values refer to total production of dammarenediol-II and protopanaxadiol. Intracellular squalene and 2, 3-oxidosqualene were measured.

Strain	Dammarenediol-II (mg/L)	Protopanaxadiol (mg/L)	Squalene (mg/L)	2, 3-oxidosqualene (mg/L)
W2[a]	210.3 ± 8.3	—	38.9 ± 6.5	12.5 ± 4.6
W3[a]	96.5 ± 6.6	155.3 ± 6.2	42.3 ± 3.6	13.4 ± 3.6
W3 plus[a]	98.8 ± 6.9	152.9 ± 8.1	45.2 ± 4.3	12.6 ± 3.5
W2[b]	223.5 ± 3.9	—	48.1 ± 6.4	14.7 ± 6.4
W3[b]	46.8 ± 4.5	78.7 ± 5.6	41.2 ± 3.5	12.5 ± 4.7
W3 plus[b]	38.5 ± 6.3	67.5 ± 4.5	46.5 ± 4.3	11.6 ± 6.3

[a]Fermentation in YPD medium.
[b]Two phase fermentation with addition of 10% dodecane in YPD medium.

PPDS and ATR1. Moreover, during two phase fermentation, the concentrations of target compounds were conspicuous less than that produced in YPD medium without dodecane (Table II). In addition, OD600 of W3 and W3 plus decreased remarkably in two phase fermentation (Fig. 1B). Since biomass and dammarenediol-II production of W2 did not change, heterologous co-expression of *PPDS* and *ATR1* in W3 and W3 plus should be responsible for the decrease of biomass. P450 and CPR coupling efficiency are also associated with the level of substrate content (Zangar et al., 2004). In the absence of substrates, efficient coupling in cytochrome P450s catalysts are almost disrupted (Whitehouse et al., 2010). In two phase fermentation, dammarenediol-II is easily extracted into dodecane. Low concentration of intracellular substrate may result in low coupling efficiency of PPDS and thus high level of intracellular ROS was detected in two phase fermentation. When ROS accumulates to a high dose, yeast cells begin to lose viability and apoptosis is initiated (Madeo et al., 1999).

To evaluate the oxidative stress induced by co-expression of *PPDS* and *ATR1*, ROS levels of strains W2, W3 and W3 plus were determined at 15 h (log phase) and 30 h (stationary phase), respectively, with and without adding dodecane. Strain W2 was selected as a control sample. In YPD medium, ROS level of W3 was about two- to threefolds higher than W2 (Fig. 1C), whereas, OD600 of W3 was not affected (Fig. 1A). This result suggested that *S. cerevisiae* W303-1a could endure two- to threefolds higher oxidative stress. W3 plus generated about four- to fivefolds of more ROS comparing to W2, which induced a decline in biomass. When strain W2, W3, and W3 plus were cultured in two phase fermentation, ROS levels of W3 and W3 plus increased remarkably (Fig. 1C), which were accompanied with decrease of biomasses (Fig. 1B) and triterpenoid production (Table II).

Modifying PPDS Through Transmembrane Domain Truncation and Self-Sufficient P450 Construction

Most natural plant cytochrome P450s and their redox proteins are membrane-bound enzymes (Bolwell et al., 1994), while the soluble artificial P450s can work in prokaryotic microorganism. In this study, we firstly studied whether locating in cytoplasm or membrane is suitable for efficient catalysis using self-sufficient PPDS in eukaryotic yeast. The N-terminal transmembrane domain of PPDS was truncated at different sites and the modified enzymes were tested for their activity (Fig. S2). Only 31tPPDS has PPDS activity, though it is lower than natural PPDS (Fig. 4B). Other truncated PPDS failed to convert dammarenediol-II to protopanaxadiol in yeast cells (Fig. S2B).

Subsequently, PPDS and 31tPPDS heme domains were linked with truncated ATR1 reductase domain by a GSTSSGSG polypeptide, individually, to generate *PPDS-linker1-46tATR1* and *31tPPDS-linker1-46tATR1* cassettes. The cassettes were integrated into chromosome of yeast strain W2 to generate transgenic strains W3a and W3at, respectively (Fig. 2C and D). Yeast strain W3a cultured in YPD medium produced 265.7 mg/L protopanaxadiol and 8.7 mg/L dammarenediol-II (Fig. 2D). That is, the conversion ratio of dammarenediol-II to protopanaxadiol is resulted in 96.8%. On the contrary, no protopanaxadiol was detected in W3at (Fig. 2D). These results indicated that transmembrane domain was crucial for efficient catalysis of fusion PPDS in yeast.

Effects of the Linkers' Length on PPDS-ATR1 Fusion Proteins

To further investigate the effects of the linkers' length on PPDS-ATR1 fusion proteins, four fusion proteins, PPDS-linker1 (GSTSSGSSG)-46tATR1, PPDS-linker2(GSTSSG)-46tATR1, PPDS-linker3(GGG)-46tATR1, and PPDS-nolinker-46tATR1 were constructed and transformed into chromosome of strain W2 at δloci. As a result, strains W3a, W3b, W3c, and W3d were obtained, respectively. When being cultured in shaking flasks, all strains produced similar amount of protopanaxadiol (about 260 mg/L).

Multi-copy integration strategy was used for strain constructs in this study. In order to identify the difference between PPDS-ATR1 fusion proteins and co-expressed proteins, copy numbers of PPDS and ATR1 from different colonies were measured during screening of strains W3, W3a, W3b, W3c, and W3d (Fig. 3). The results showed that the conversion of dammarenediol II increased with increasing copy numbers of PPDS and ATR1 in W3. However, the production of protopanaxadiol was no longer increasing after the copy number arrived at 3. Three copies of the fusion protein gene, no matter what linker sequence was used, could convert 96% of darmmarenediol II, which indicated the improvement of catalytic activity. The results also reflected that the influence of linkers' length on the activities of PPDS-ATR1 fusion enzymes was not critical.

Catalytic Activity and Coupling Efficiency of PPDS and ATR1

To estimate the content of cytochrome P450s, carbon monoxide difference spectra method was used. The content of PPDS in each strain was estimated and listed in Table III. Activities of fusion proteins in microsomes were also measured in a reconstituted redox system with dammarenediol-II as the substrate. Catalyst activities of PPDS-ATR1 fusion enzymes were compared with that of the co-expressed PPDS and ATR1. As shown in Figure 4A, the reaction rate of the fusion protein was about 4.5-fold higher than separately existing PPDS and ATR1 within 20 min, indicating that self-sufficient PPDS-ATR1 enzymes were more efficient in dammarenediol-II conversion.

Although with a slight increase, coupling values of these fusion enzymes are still less than 30%, indicating that uncoupling still occur to a large extend. ROS levels of the strains W3a, W3b, W3c, and W3d were two- to threefolds higher than control strain W2 (Fig. 4B). Under these conditions, biomasses of W3a, W3b, W3c, and W3d were not affected by oxidative stress (Fig. S4).

Production of Protopanaxadiol Through Fed-Batch Fermentation

In order to assess the effect of the four PPDS–ATR1 fusion proteins on protopanaxadiol production, fed-batch fermentations were firstly performed in shaking flasks. All strains containing PPDS–ATR1 fusion proteins produced about 1400 mg/L protopanaxadiol in

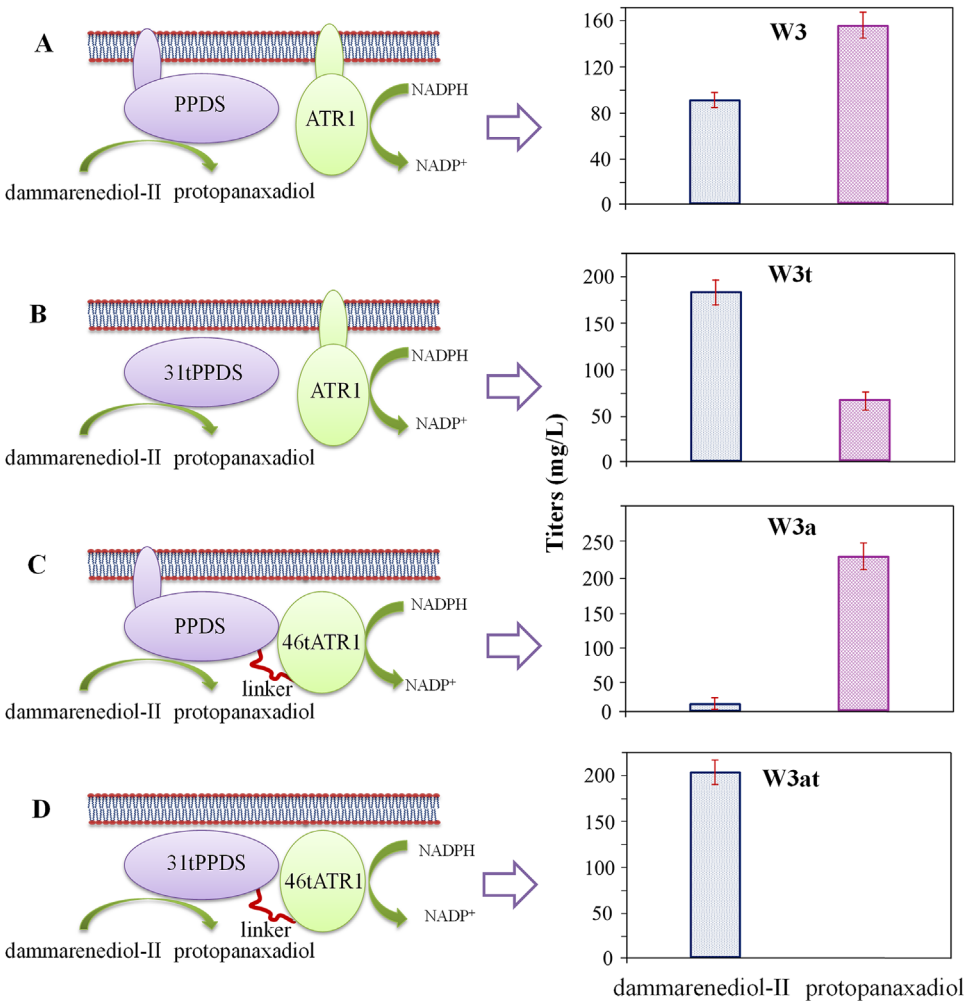

Figure 2. Transmembrane domain truncation and self-sufficient PPDS constructions. (**A**) Natural existing state of PPDS and ATR1 in endoplasmic reticulum; (**B**) transmembrane domain of PPDS was truncated; (**C**) PPDS-46tATR1 fusion protein in yeast cells; (**D**) 31tPPDS-46tATR1 fusion protein in yeast cells. Strains were cultured in YPD medium for 6 days. Experiments were performed in triplicate. Histograms demonstrated the productions of corresponding strains.

fermentation processes, in which strain W3a had the highest protopanaxadiol production of 1412.5 mg/L (Table IV). In addition, part of target products were secreted out of yeast cells (Table SVI). Protopanaxadiol and dammarenediol-II can pass the membrane of yeast cells when they accumulated in large amount (Dai et al., 2013). Addition of dodecane in medium accelerated secretion process, preventing conversion of dammarenediol-II through PPDS. Thus, two phase fermentation process was excluded in our study.

To further explore the performance of the PPDS–ATR1 fusion protein in protopanaxadiol production, yeast strain W3a was subsequently cultured in 5 L fermentor with W3 as the control. W3a and W3 had a similar biomass accumulation but different production of protopanaxadiol and dammarenediol-II (Fig. 5). With the addition of fed solution, biomass continued to increase up to 120 h. The maximum OD600 of W3 and W3a were 42.6 and 43.3, respectively. From 36 to 84 h, dammarenediol-II in W3a accumulated gradually (Fig. 5B), which illustrated that DS generated more dammarenediol-II than self-sufficient PPDS could consume during that period. After 84 h, dammarenediol-II level decreased from 156.3 to 19.7 mg/L within 36 h. While protopanaxadiol production of W3a kept increasing up to 1436.6 mg/L in 144 h. Finally, almost all dammarenediol-II (98.8%) was converted

Table III. Concentration of PPDS heme domain and coupling efficiency analysis of PPDS-ATR1 fusion proteins.

Strain	W3	W3a	W3b	W3c	W3d
Concentration (μM/g microsome)	0.98 ± 0.08	0.93 ± 0.06	0.10 ± 0.08	0.95 ± 0.08	0.88 ± 0.07
Coupling efficiency	22.3%	29.6%	26.2%	28.5%	27.1%

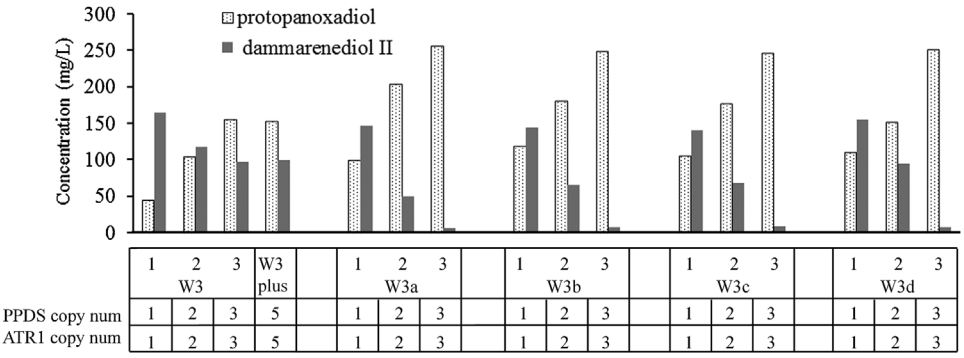

Figure 3. Copy numbers of PPDS and ATR1 in different colonies during screening of strains W3, W3a, W3b, W3c and W3d.

to protopanaxadiol in W3a. Comparing with the curves of protopanaxadiol and dammarenediol-II accumulation in W3, PPDS-ATR1 fusion protein significantly increased protopanaxadiol production in W3a.

Discussion

Plants cytochromes P450s are usually used in combinatorial biosynthesis of natural compounds due to their versatile function. However, the application of these enzymes is affected by their notoriously low activity in heterologous hosts (Urlacher and Girhard, 2012). Here we described the development of a generic method for increasing cytochrome P450 activity. The construction of self-sufficient protein successfully overcame the challenge caused by the negative effect of low conversion rate of cytochrome P450.

Normally, cytochromes P450s require interaction with redox partners to exert their oxidative activities. The redox partners transfer electrons originating from NADP (H) to the cytochrome P450s heme center. In catalytic cycles, inefficient electron transfer efficiency obviously affects enzyme activity (Nazor et al., 2008). One remarkable exception is CYP102A1, which exists as a fusion between a heme domain and a reductase domain (Warman et al., 2005). Thus, artificial self-sufficient P450s have been explored as a strategy to engineer plant cytochrome P450s with enhanced turnover and efficiency, for example, CYP725A4 for oxidation of taxadiene (Ajikumar et al., 2010) and CYP105D7 for hydroxylation of daidzein (Choi et al., 2012). However, all of those plant fusion P450s were expressed in prokaryotic microorganism such as *E. coli* with N-terminal transmembrane truncation (Ajikumar et al., 2010; Choi et al., 2014; Schuckel et al., 2012) or substitution (Leonard and Koffas, 2007). *S. cerevisiae* has not been used as a platform for plant fusion P450s expression. In this study, removing transmembrane domain of PPDS reduced PPDS activity or even inactivated the fusion protein (Fig. 2B and D), indicating that transmembrane domain is important for catalytic activity of P450 in compartmental yeast cells.

It was reported that closing the spatial distance between two active domains through a polypeptide would enhance protein-protein interaction (Zhou et al., 2012). However, a too short linker may cause steric hindrance and thus influence free motion of activate domains. The activities of P450s fusion enzymes according to changes in electron transfer efficiencies are significantly affected by the length of linker peptides (Haga et al., 2013). In this study, three flexible polypeptides (GGG, GSTSSG, and GSTSSGSG) were inserted between PPDS and ATR1 activated domains, respectively. Enzymatic determination revealed that the catalyst activities were not affected by length of linkers, and the fusion enzyme without linker also functions efficiently.

Table IV. Results of fed-batch fermentation. Values represent the sum of intra- and extracellular concentration.

Strain	OD600	Dammarenediol-II (mg/L)	Protopanaxadiol (mg/L)	Total triterpenoids (mg/L)	Ratio[a]
W3[b]	39.1 ± 1.5	424.8 ± 12.7	768.5 ± 16.3	1193.3	1.81:1
W3a[b]	38.3 ± 1.3	16.8 ± 3.2	1412.5 ± 22.6	1429.3	84.07:1
W3b[b]	38.5 ± 1.2	18.4 ± 5.2	1396.8 ± 19.3	1415.2	75.86:1
W3c[b]	39.2 ± 1.2	19.2 ± 4.2	1402.1 ± 18.4	1421.3	73.02:1
W3d[b]	39.7 ± 1.6	21.3 ± 3.6	1367.5 ± 16.8	1388.8	64.20:1
W3[c]	27.6 ± 1.4	456.3 ± 15.6	255.8 ± 14.5	712.1	0.56:1
W3a[c]	36.6 ± 1.7	523.7 ± 18.7	312.0 ± 41.6	835.7	0.59:1

[a]Indicate the ratio of protopanaxadiol to dammarenediol-II.
[b]Engineered strains were inoculated to shake flasks by fed-batch fermentation.
[c]Engineered strains were inoculated to shake flasks by two phase fed-batch fermentation.

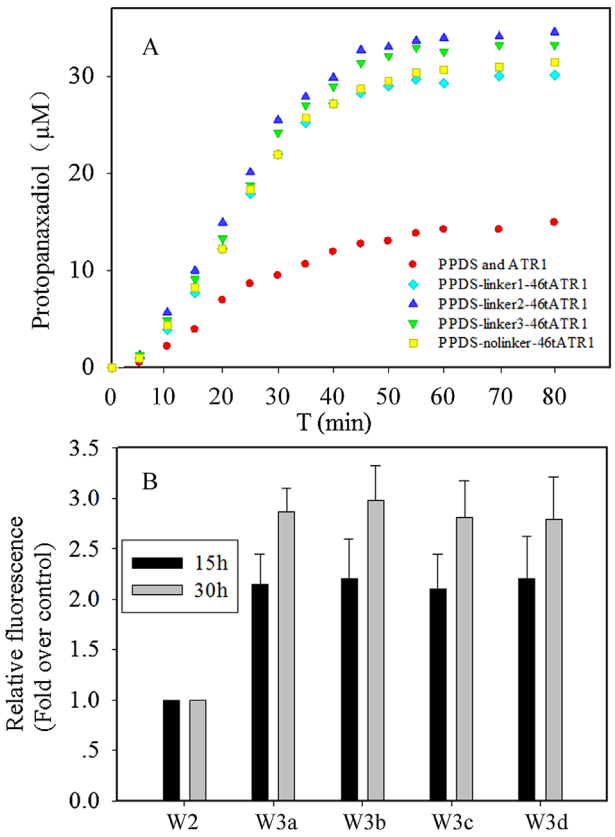

Figure 4. Comparison of catalytic activities of different PPDS (**A**), and ROS level changes of engineered strains during cultured in YPD medium (**B**).

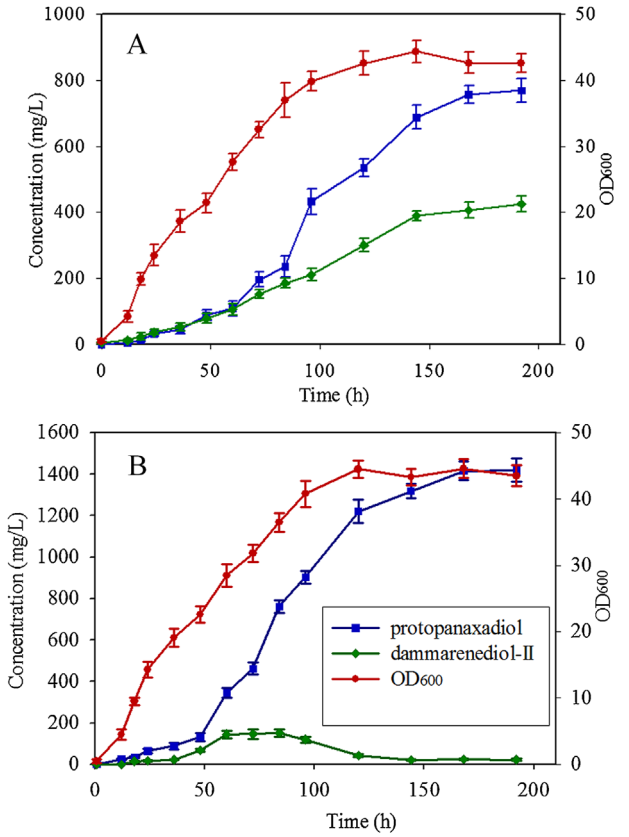

Figure 5. Productions of protopanaxadiol and dammarenediol-II by strain W3 (**A**) and W3a (**B**) through fed-batch fermentation in 5 L bioreactor.

Mechanisms of coupling efficiency are associated with the level of substrate and CPR content (Zangar et al., 2004). In two phase fermentation, dammarenediol-II is easily extracted into dodecane, low concentration of intracellular substrate resulted in low coupling efficiency of PPDS, and thus high level of intracellular ROS was detected (Fig. 1C). Expression of CPR in excess reduced production of sesquiterpene and decreased yeast viability (Paddon et al., 2013). Herein, enhancing expression of *PPDS* and *ATR1* also increased ROS level. In order to relieve oxidative stress, the copy numbers of these genes were well controlled in engineered yeast. Our results indicated that 3 copies of PPDS-ATR1 fusion protein were enough for efficient conversion of dammarenediol-II to protopanaxadiol. In W3a carrying fusion PPDS, 96.8% of dammarenediol-II could be converted to protopanaxadiol, and the growth of cells was not affected by ROS. Collectively, a high-yield strain was constructed for protopanaxadiol production by generating a self-sufficient PPDS-ATR1 fusion protein and regulating its expression. This work also provided a common method to use plant self-sufficient P450s construction in industrial microbiology and biotechnology.

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References

Abad S, Kitz K, Hrmann A, Schreiner U, Hartner FS, Glieder A. 2010. Real-time PC R-based determination of gene copy numbers in *Pichia pastoris*. Biotechnol J 5:413–420.

Adams A, Gottschling D, Kaiser C, Stearns T. 1998. Methods in yeast genetics: A laboratory course manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Ajikumar PK, Xiao WH, Tyo KE, WangY, Simeon F, Leonard E, Mucha O, Phon TH, Pfeifer B, Stephanopoulos G. 2010. Isoprenoid pathway optimization for Taxol precursor overproduction in *Escherichia coli*. Science 330:70–74.

Bolwell GP, Bozak K, Zimmerlin A. 1994. Plant cytochrome P450. Phytochemistry 37:1491–1506.

Chen YJ, Yu P, Luo JC, Jiang Y. 2003. Secreted protein prediction system combining CJ-SPHMM, TMHMM, and PSORT. Mamm Genome 14:859–865.

Choi KY, Jung E, Jung DH, An BR, Pandey B, Yun H, Sung C, Park HY, Kim BG. 2012. Engineering of daidzein 3′-hydroxylase P450 enzyme into catalytically self-sufficient cytochrome P450. Microb Cell Fact 11:81.

Choi KY, Jung E, Yun H, Yang YH, Kim BG. 2014. Engineering class I cytochrome P450 by gene fusion with NADPH-dependent reductase and *S. avermitilis* host development for daidzein biotransformation. Appl Microbiol Biotechnol 98:8191–8200.

Christensen LP. 2009. Ginsenosides chemistry, biosynthesis, analysis, and potential health effects. Adv Food Nutr Res 55:1–99.

Dai ZB, Liu Y, Zhang XN, Shi MY, Wang BB, Wang D, Huang LQ, Zhang XL. 2013. Metabolic engineering of *Saccharomyces cerevisiae* for production of ginsenosides. Metab Eng 20:146–156.

Degregorio D, Sadeghi SJ, Di Nardo G, Gilardi G, Solinas SP. 2011. Understanding uncoupling in the multiredox centre P450 3A4-BMR model system. J Biol Inorg Chem 16:109–116.

DeJong JM, Liu Y, Bollon AP, Long RM, Jennewein S, Williams D, Croteau RB. 2006. Genetic engineering of taxol biosynthetic genes in *Saccharomyces cerevisiae*. Biotechnol Bioeng 93:212–224.

Gavira C, Höfer R, Lesot A, Lambert F, Zucca J, Werck-Reichhart D. 2013. Challenges and pitfalls of P450-dependent (+)-valencene bioconversion by *Saccharomyces cerevisiae*. Metab Eng 18:25–35.

Haga T, Hirakawa H, Nagamune T. 2013. Fine tuning of spatial arrangement of enzymes in a PCNA-mediated multienzyme complex using a rigid poly-L-proline linker. PLoS ONE 8:e75114.

Han JY, Kim HJ, Kwon YS, Choi YE. 2011. The Cyt P450 enzyme CYP716A47 catalyzes the formation of protopanaxadiol from dammarenediol-II during ginsenoside biosynthesis in *Panax ginseng*. Plant Cell Physiol 52:2062–2073.

Jung ST, Lauchli R, Arnold FH. 2011. Cytochrome P450: Taming a wild type enzyme. Curr Opin Biotech 22:809–817.

Kille S, Zilly FE, Acevedo JP, Reetz MT. 2011. Regio- and stereoselectivity of P450-catalysed hydroxylation of steroids controlled by laboratory evolution. Nat Chem 3:738–743.

Lee HS, Lee HJ, Cho HJ, Park SS, Kim JM, Suh HJ. 2010. Cosmetic potential of enzymatic treated ginseng leaf. J Gins Res 34:227–236.

Leonard E, Koffas MAG. 2007. Engineering of Artificial Plant Cytochrome P450 Enzymes for Synthesis of Isoflavones by *Escherichia coli*. Appl Environ Microbiol 73:7246–7251.

Liu JD, Zhu YB, Du GC, Zhou JW, Jian C. 2013. Response of *Saccharomyces cerevisiae* to D-limonene-induced oxidative stress. Appl Microbiol Biotechnol 97:6467–6475.

Madeo F, Frohlich E, Ligr M, Grey M, Sigrist SJ, Wolf DH, Frohlich KU. 1999. Oxygen stress: A regulator of apoptosis in yeast. J Cell Biol 145:757–767.

Murthy HN, Georgiev MI, Kim YS, Jeong CS, Kim SJ, Park SY, Paek KY. 2014. Ginsenosides: Prospective for sustainable biotechnological production. Appl Microbiol Biotechnol 98:6243–6254.

Nazor J, Dannenmann S, Adjei RO, Fordjour YB, Ghampson IT, Blanusa M, Roccatano D, Schwaneberg U. 2008. Laboratory evolution of P450 BM3 for mediated electron transfer yielding an activity-improved and reductase-independent variant. Protein Eng Des Sel 21:29–35.

Olsen KM, Hehn A, Jugde H, Slimestad R, Larbat R, Bourgaud F, Lillo C. 2010. Identification and characterisation of CYP75A31, a new flavonoid 3′5′-hydroxylase, isolated from *Solanum lycopersicum*. BMC Plant Biol 10:21.

Omura T, Sato R. 1964. The carbon monoxide-binding pigmet of liver microsomes. I. Evidence for its hemoprotein nature. J Biol Chem 239:2370–2378.

Paddon CJ, Westfall PJ, Pitera DJ, Benjamin K, Fisher K, McPhee D, Leavell MD, Tai A, Main A, Eng D, Polichuk DR, Teoh KH, Reed DW, Treynor T, Lenihan J, Jiang H, Fleck M, Bajad S, Dang G, Dengrove D, Diola D, Dorin G, Ellens KW, Fickes S, Galazzo J, Gaucher SP, Geistlinger T, Henry R, Hepp M, Horning T, Iqbal T, Kizer L, Lieu B, Melis D, Moss N, Regentin R, Secrest S, Tsuruta H, Vazquez R, Westblade LF, Xu L, Yu M, Zhang Y, Zhao L, Lievense J, Covello PS, Keasling JD, Reiling KK, Renninger NS, Newman JD. 2013. High-level semi-synthetic production of the potent antimalarial artemisinin. Nature 496:528–532.

Renault H, Bassard JE, Hamberger B, Werck-Reichhart D. 2014. Cytochrome P450 mediated metabolic engineering: Current progress and future challenges. Curr Opin Plant Biol 19:27–34.

Sadeghi SJ, Gilardi G. 2013. Chimeric P450 enzymes: Activity of artificial redox fusions driven by different reductases for biotechnological applications. Biotechnol Appl Bioc 60:102–110.

Schuckel J, Rylott EL, Grogan G, Bruce NC. 2012. A gene-fusion approach to enabling plant cytochromes p450 for biocatalysis. Chembiochem 13:2758–2763.

Shao ZY, Zhao H, Zhao HM. 2009. DNA assembler, an in vivo genetic method for rapid construction of biochemical pathways. Nucleic Acids Res 37:e16.

Sun C, Li Y, Wu Q, Luo HM, Sun YZ, Song JY, Lui EM, Chen SL. 2010. De novo sequencing and analysis of the *American ginseng* root transcriptome using a GS FLX Titanium platform to discover putative genes involved in ginsenoside biosynthesis. BMC Genomics 11:262.

Tansakul P, Shibuya M, Kushiro T, Ebizuka Y. 2006. Dammarenediol-II synthase, the first dedicated enzyme for ginsenoside biosynthesis, in *Panax ginseng*. FEBS Lett 580:5143–5149.

Urlacher VB, Girhard M. 2012. Cytochrome P450 monooxygenases: An update on perspectives for synthetic application. Trends Biotechnol 30:26–36.

Wang PP, Wei YJ, Fan Y, Liu QF, Wei W, Yang CS, Zhang L, Zhao GP, Yue JM, Yan X. 2015. Production of bioactive ginsenosides Rh2 and Rg3 by metabolically engineered yeasts. Metab Eng 29:97–105.

Wang L, Zhao SJ, Cao HJ, Sun Y. 2014. The isolation and characterization of dammarenediol synthase gene from *Panax quinquefolius* and its heterologous co-expression with cytochrome P450 gene *PqD12H* in yeast. Funct Integr Genomics 14:545–557.

Warman AJ, Roitel O, Neeli R, Girvan HM, Seward HE, Murray SA, McLean KJ, Joyce MG, Toogood H, Holt RA, Leys D, Scrutton NS, Munro AW. 2005. Flavocytochrome P450 BM3: An update on structure and mechanism of a biotechnologically important enzyme. Biochem Soc T 33:747–753.

Whitehouse CJ, Yang W, Yorke JA, Rowlatt BC, Strong AJ, Blanford CF, Bell SG, Bartlam M, Wong LL, Rao Z. 2010. Structural basis for the properties of two single-site proline mutants of CYP102A1 (P450BM3). Chembiochem. 11:2549–2556.

Zangar RC, Davydov DR, Verma S. 2004. Mechanisms that regulate production of reactive oxygen species by cytochrome P450. Toxicol Appl Pharm 199: 316–331.

Zhang XM, Xue CY, Zhao FL, Li DS, Yin J, Zhang CB, Caiyin Q, Lu WY. 2014. Suitable extracellular oxidoreduction potential inhibit rex regulation and effect central carbon and energy metabolism in *Saccharopolyspora spinosa*. Microb Cell Fact 13:98.

Zhou YJ, Gao W, Rong Q, Jin G, Chu H, Liu W, Yang W, Zhu Z, Li G, Zhu G, Huang L, Zhao ZK. 2012. Modular pathway engineering of diterpenoid synthases and the mevalonic acid pathway for miltiradiene production. J Am Chem Soc 134:3234–3241.

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