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Systematic optimization of the yeast cell factory for sustainable and high efficiency production of bioactive ginsenoside compound K

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ABSTRACT

Ginsenoside Compound K (CK) has been recognized as a major functional component that is absorbed into the systemic circulation after oral administration of ginseng. CK demonstrates diverse bioactivities. A phase I clinical study indicated that CK was a potential candidate for arthritis therapy. However, a phase II clinical study was suspended because of the high cost associated with the present CK manufacturing approach, which is based on the traditional planting-extracting-biotransforming process. We previously elucidated the complete CK biosynthetic pathway and realized for the first time *de novo* biosynthesis of CK from glucose by engineered yeast. However, CK production was not sufficient for industrial application. Here, we systematically engineered *Saccharomyces cerevisiae* to achieve high titer production of CK from glucose using a previously constructed protopanaxadiol (PPD)-producing chassis, optimizing UGTPg1 expression, improving UDP-glucose biosynthesis, and tuning down UDP-glucose consumption. Our final engineered yeast strain produced CK with a titer of 5.74 g/L in fed-batch fermentation, which represents the highest CK production in microbes reported to date. Once scaled-up, this high titer *de novo* microbial biosynthesis platform will enable a robust and stable supply of CK, thus facilitating study and medical application of CK.

1. Introduction

Although ginsenoside Compound K (CK) is not naturally present in *Panax* plants, it represents one of the main components detected in mammalian blood and organs following oral administration of *Panax ginseng* or ginseng saponins [1,2]. CK has been widely recognized as the major functional form of protopanaxadiol (PPD)-type ginsenosides that can be absorbed into the systemic circulation. Pharmacological studies have demonstrated that CK confers hepatoprotection [3], anti-diabetes [4,5], and anti-cancer activities [6]. Moreover, multiple studies have demonstrated that CK can suppress the abnormal activation of T-lymphocytes and attenuate inflammatory responses in arthritic mice, thus representing a promising candidate for rheumatoid arthritis therapy [7–10]. Recent phase I clinical trials to evaluate the pharmacokinetics and safety of CK (Trial registration Nos. ChiCTR-TRC-14004824 and ChiCTR-IPR-15006107) have been conducted [11,12]. During the phase I clinical trial, no adverse events were observed following either a single oral administration of 25–800 mg CK in 62 enrolled volunteers, or the repeated oral administration of 100–400 mg/day of CK for 9 consecutive days by 30 enrolled volunteers, indicating that CK was safe and well-tolerated [11]. However, the phase II clinical trial conducted by Zhejiang Hisun Pharmaceutical Co. Ltd. to evaluate the use of CK for the treatment of patients with rheumatoid arthritis (Trial registration No. NCT03755258) was abandoned after two years from the start of the clinical study, because of the poor economic feasibility of current plant-dependent CK production methods.

Currently, the approach for manufacturing CK is dependent on the culture of *Panax* plants, and the extraction and biotransformation of total ginsenosides from the cultured plants. Although much progress has been made in past decades to improve biotransformation of major PPD-type ginsenosides into CK [13], such agriculture-based methods require a long time for the cultivation of *Panax* plants and are susceptible to environmental and climatic changes [14,15]. Moreover, the total saponins in *Panax* plants were found to be very low (~3% of total

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dry-weight), and most of the ginsenosides, including protopanaxatriol-, oleanane-, and ocotillol-type, were not transformed into CK through bio-deglycosylation because of the difference in aglycone [16]. Thus, the current CK extraction–biotransformation manufacturing methods will waste these valuable ginsenoside resources. Total chemical synthesis of CK has not been realized, partially due to the challenges of selective introduction of a glucose moiety to the spatially hindered C–20*S*–OH of PPD.

Development of an alternative microbial fermentation-based CK manufacturing method would be a promising solution, as many attractive natural products, including artemisinic acid, opioids, and tropane alkaloids, have been obtained via microbial fermentation [17–19]. In 2014, we discovered a UDP-glycosyltransferase, UGTPg1, which catalyzes region-specific glycosylation of PPD to produce CK. This discovery led to complete elucidation of the CK biosynthetic pathway and paved the way for production of CK using synthetic biology approaches [20]. By introducing a PPD-producing pathway as well as UGTPg1 into *S. cerevisiae*, we achieved *de novo* CK production from a simple carbon source for the first time. However, the titer produced by this proof-of-principle strain was very low (~1.4 mg/L) [20], making it unsuitable for industrialization. Based on our previous studies, several research groups have taken efforts to increase CK fermentation titers. In 2019, Li et al. reconstructed the CK biosynthetic pathway in *Yarrowia lipolytica* and realized the *de novo* production of CK. Following overexpression of key MVA pathway genes and the fusion of cytochrome P450 PgPPDS with NADPH-P450 reductase ATR1, the titer of CK reached 161.8 mg/L by fed-batch fermentation [21]. More recently, Dong et al. constructed a CK-producing yeast strain by combining the overexpression of genes for the biosynthesis of 2,3-oxidosqualene, the integration of multiple-copy codon-optimized *PgDDS* and *PgPPDS* genes, and the introduction and optimization of a *UGT* gene, achieving a CK titer of 1.17 g/L by fed-batch fermentation [22]. The highest titer production of CK in engineered microbes to date was accomplished by combining pathway optimization and the addition of ethanol and glycerol in fed-batch fermentation, reaching 1.7 g/L [23].

Despite these achievements, current microbial fermentation-based CK titers have been limited to <2 g/L; therefore, CK manufacture by yeast fermentation is not yet economically feasible. Taking into consideration the reported high titer of 11 g/L PPD that was obtained in engineered yeast [24], it is obvious that there is great potential for improving CK production. The problems associated with the low glycosylation efficiency of PPD into CK may involve the activity and expression level of the key biopart (the glycosyltransferase UGTPg1) and the UDP-glucose supply in the yeast cell factory. Previous efforts to increase yeast UDP-glucose supply have been restricted to overexpressing the key genes involved in UDP-glucose biosynthesis, such as *PGM2* and *UGP1* [19,25]. Although the production of glycosylated products could be increased, UDP-glucose supply may be improved using other mechanisms besides overexpression. On one hand, the biosynthesis of UDP-glucose relies on the supply of uridine triphosphate (UTP), which has rarely been considered in previous studies. On the other hand, UDP-glucose is an important intermediate that participates in many metabolic processes in yeast. The generated UDP-glucose could be diverted into these pathways instead of the biosynthesis of target compounds [26].

In the present study, we aimed to develop a high-titer microbial fermentation-based ginsenoside CK production platform. We firstly fulfilled the complete CK pathway in yeast by introducing the key UGT biopart, *UGTPg1*, into a previously constructed PPD-producing chassis. Next, we optimized the cell factories by tuning the expression level of the *UGTPg1* gene, and increased CK titer by more than 4-fold. After boosting UDP-glucose supply by increasing UDP-glucose biosynthesis and throttling down UDP-glucose consumption, we obtained a series of yeast strains with increased CK production. Finally, using fed-batch fermentation in a parallel bioreactor system, the CK titer reached 5.74 g/L, which provided a potential industrial application for CK manufacture at low cost and high efficiency.

2. Materials and methods

2.1. Chemicals

Authentic sample Dammarenediol-II (DM) was purchased from Bio-BioPha Co., Ltd. (Kunming, China). Authentic ginsenoside compound K and protopanaxadiol (PPD) were purchased from Nantong feiyu biological technology Co., Ltd. (Nantong, China). 20S–*O*-β-(D-glucosyl)-dammarenediol-II (DMG) was obtained by purification from the *in vitro* reactions of DM catalyzed by UGTPg1 and its structure was confirmed by NMR in our previous study [20].

2.2. Plasmids and strains

Plasmids pUG66, pAG25 and pROS13 obtained from EUROSCARF were used as the template for the amplification of *ble*, *NatMX*, and *KanMx* selection marker, respectively. High-copy yeast episomal plasmids p2M containing 2μ replicon and *HphMx* gene as hygromycin selection marker was used for the construction of p2M-synUGTPg1. *S. cerevisiae* strain ZW04BY-RS constructed in our previous study was used as the parent strain for all engineering [24]. *E. coli* strain TOP10 was used for the cloning of *galU* gene. Codon-optimized gene *synUGTPg1* was synthesized by Genscript Corporation (Nanjing, China). All the strains used or constructed in this study are listed in Table 1.

2.3. Construction of yeast strains

Strain WPK1 was constructed by transformation ZW04BY-RS with plasmid p2M-synUGTPg1, which was constructed by inserting the synUGTPg1 gene into the *Not* I site of p2M using the ClonExpress II One Step Cloning Kit (Vazyme Biotech Co., Ltd, Nanjing, China). Strains WPK2 to WPK12 were constructed by integrating desired gene expression cassettes into yeast chromosome or by deleting yeast native genes. Generally, the desired genetic modification of each strain was designed *in silico* with each adjacent fragment sharing ~70 bp homologous sequences and the highly efficient yeast homologous recombination based DNA assembly was used to join them together and integrated into target site of chromosome. Two-round of PCR amplification were performed to

Table 1
Strains used in this study.

Strains	Genotype or characteristic	Source
BY4742	MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0	Brachmann et al. (1998)
ZW04BY-RS	BY4742 *HXT7p-tHMG1-ADH1t, TEF2p-synPgCPR1-TDH2t, TPIIp-ERG1-ENO2t, GPM1p-ERG20-CYC1t, PGK1p-ERG9-FBA1t, TDH3p-synDDS-PGT1t, TEF1p-synPPDS-PGK1t, ENO2p-ERG12-CPS1t, TEF2p-ERG13-IDP1t, TPIIp-ERG8-PRM5t, GPM1p-ERG19-HIS5t, PGK1p-IDI-PRM9t, TDH3p-ERG10-SPG5t, TEF1p-tHMG1-ADH1t, TDH3p-synPPDS-CPS1t*	Wang et al. (2019)
WPK1	ZW04BY-RS *p2M-synUGTPg1*	This study
WPK2	ZW04BY-RS *rDNA:: TDH3p-synUGTPg1-PRM9t*	This study
WPK3	WPK2 *TRP1:: TEF1p-synUGTPg1-FBA1t*	This study
WPK4	WPK3 *YPRCt3:: GPD1p-galU-FBA1t*	This study
WPK5	WPK3 *YPRCt3:: GPD1p-UGP1-FBA1t*	This study
WPK6	WPK3 *YPRCt3:: GPD1p-UGP1-FBA1t, PGK1p-PGM2-PRM9t*	This study
WPK7	WPK6 *ΔYND1*	This study
WPK8	WPK6 *YORWδ22:: TEF1p-URA6-ENO2t, TDH3p-YNK1-FBA1t*	This study
WPK9	WPK6 *ΔYND1, YORWδ22:: TEF1p-URA6-ENO2t, TDH3p-YNK1-FBA1t*	This study
WPK10	WPK8 *ΔFKS1*	This study
WPK11	WPK8 *ΔGLC3*	This study
WPK12	WPK8 *ΔALG5*	This study

generate the DNA fragments for yeast transformation: first-round PCR was performed to obtain basic fragments containing each promoter, gene, terminator, selection marker and homologous arm and second-round PCR was performed using the adjacent 2 to 4 basic fragments as template to generated fusion fragments. Finally, the fusion fragments were purified and co-transformed into yeast strain via standard LiAc/ssDNA method [27], the transformants were plated on the YPD agar plate containing corresponding antibiotic for selection and the positive ones were verified by colony PCR. All the primers used for the construction of the plasmids and strains are listed in Supplementary Table 1.

2.4. Yeast cultivation and metabolites extraction

YPD medium (10 g/L Bacto Yeast Extract, 20 g/L Bacto peptone and 20 g/L dextrose) was used for yeast strain construction and shake flask fermentation. For *HphMx*, *KanMx, ble* and *NatMX* marker selection, 300 mg/L hygromycin B (Roche Diagnostics, Basel, Switzerland), 200 mg/L G418 sulfate (Sangon Biotech, Shanghai, China), 20 mg/L phleomycin (InvivoGen, San Diego, CA, USA) and 100 mg/L nourseothricin (Gold Biotechnology, USA) sulfate was added into YPD agar plate, respectively.

For shake flask fermentation of engineered yeast strains, individual clones of the desired stain picked from YPD agar plates were inoculated into the liquid YPD medium and cultivated at 30 °C, 250 rpm overnight. Then the seed cultures were inoculated into 10 mL of liquid YPD medium in 50 mL shake flasks with an initial OD600 of 0.05, fermentation was conducted under 30 °C, 250 rpm for 96 h. The fermentation broth containing the yeast cell and medium was extracted with equal volumes of *n*-butanol and used for the analysis of CK, DMG, DM and PPD.

2.5. Fed-batch fermentation of yeast strain

Fed-batch fermentation of yeast strains were conducted in a 1.3 -L Eppendorf DASGIP Parallel Bioreactor System (Eppendorf, Hamburg, Germany). The synthetic medium for seed, batch fermentation and feed were described previously [24]. Glycerol stock of engineered yeast strains WPK3 and WPK12 were incubated into 50 mL seed medium respectively and cultivated at 30 °C, 250 rpm for 24 h. Then 30 mL seed broth was inoculated into 270 mL batch medium in the 1.3 -L parallel bioreactor to start the fermentation. General parameters for the fermentation was set as following: temperature 30 °C, pH 5.0 (controlled by titration of $NH_3{\cdot}H_2O$), dissolved O_2 >30% (controlled by varying speed of agitation and air flow rate). Feeding rate of fresh medium was programmed controlled to avoid accumulation of ethanol (< 0.5 g/L). Generally, feeding of fresh medium was initiated once the glucose in batch medium was depleted, with gradually increasing feeding rate from 0.5 mL/h to 3 mL/h to maintain ethanol concentration less than 0.5 g/L. Fermentation was finished at 144 h and half the volume of the final fermentation broth of *n*-butanol was added into the fermenters for products extraction.

2.6. Chemical analysis

All the chemicals were analyzed by High Performance Liquid Chromatography (HPLC). A Shimadzu LC20A system equipped with a LC20ADXR pumper, an auto-sampler and a diode array detector (Shimadzu, Kyoto, Japan) was used for HPLC analysis. Chromatographic separation of CK, DMG, DM and PPD was carried out on a Shim-pack XR-ODS column (100 mm × 2.0 mm, 2.2 μm, Shimadzu, Kyoto, Japan) at 35 °C. The mobile phase consisted of water (A) and acetonitrile (B), and separation was conducted using the following gradient procedure: 0–2.5 min (60% B), 2.5–10 min (60%–90% B), 10–11 min (90% B), 11–13 min (60% B) and the flow rate was maintained at 0.45 mL/min. The triterpenoid products were detected at 203 nm. Calibration curves of standard samples based on HPLC integrated peak were generated and used for the quantification of CK, DMG, DM and PPD.

3. Results and discussion

3.1. Optimization of UDP-glycosyltransferase expression for CK biosynthesis

Previously, we characterized the UDP-glycosyltransferase UGTPg1 responsible for C20–*O*-glucosylation of PPD and elucidated the complete biosynthetic pathway of CK. Meanwhile, we constructed proof-of-principle strains, which could produce CK *de novo* from a simple carbon source for the first time; however, the production titer was too low for commercialization [20]. We assumed that the low triterpenoid precursor supply and insufficient glycosylation efficiency were two bottlenecks restricting CK production.

To achieve higher production, we redesigned the complete biosynthetic pathway of CK from the simple carbon source glucose, and divided this pathway into three modules: module I for precursor supply, module II for PPD biosynthesis and module III for glycosylation. Modules I and II enabled *de novo* production of PPD from glucose, and module III was responsible for the conversion of PPD into CK (Fig. 1a). By adopting this strategy, the previously engineered yeast strain ZW04BY-RS harboring modules I and II could produce more than 500 mg/L PPD in shake flasks and 11.0 g/L in fed-batch fermentation [24]. This high titer PPD-producing yeast served as a suitable chassis for CK biosynthesis.

We first introduced the UDP-glycosyltransferase encoding gene *UGTPg*1 into ZW04BY-RS via a 2μ-based high-copy plasmid, with an assumption that the high-copy number of the plasmid might provide a high expression level of *UGTPg*1. However, the production of CK in the resulting strain WPK1 was only 32.7 mg/L, which was much lower than expected. In addition, the total triterpenoid titer, *i.e.*, the sum titers of CK, 20S–*O*-β-(D-glucosyl)-dammarenediol-II (DMG), PPD, and dammarenediol-II (DM), were decreased drastically compared to that of ZW04BY-RS (Fig. 1c and Supplementary Fig. S1). Time course analysis of the cell growth revealed that both the growth and final cell biomass of WPK1 were significantly lower than that of ZW04BY-RS (Supplementary Fig. S2). We thus speculated that the antibiotic dependent high-copy plasmid might be harmful to growth, and thus the production of triterpenoid in the host strain would be affected. Alternatively, we chose to integrate *UGTPg*1 into the chromosome of ZW04BY-RS and constructed strain WPK2. The CK titer of WPK2 increased 4.5-fold to reach 146.9 mg/L. Besides, the total triterpenoid titer of WPK2 was comparable to that of ZW04BY-RS (Fig. 1c and Supplementary Fig. S1). Because a significant amount of PPD accumulated in WPK2, we next tried to improve the conversion of PPD into CK by introducing an additional copy of *UGTPg1* into WPK2. However, the CK titer of the resulting strain WPK3 was 155.6 mg/L, showing no significant increase compared to that of WPK2 (Fig. 1b–c). This result indicated that the expression level of *UGTPg*1 might not be the limiting factor for CK production in WPK2.

3.2. Engineering a UDP-glucose biosynthetic pathway to increase CK production

Increasing the expression level of *UGTPg1* in strain WPK2 did not lead to the expected improvement of CK titer. Therefore, we speculated that the supply of the sugar donor UDP-glucose might not be sufficient for CK biosynthesis. In yeast, UDP-glucose is biosynthesized from glucose via the actions of hexokinase (HXK1, HXK2 or GLK1), phosphoglucomutase (PGM1 or PGM2), and UDP-glucose pyrophosphorylase (UGP1). The phosphoglucomutase catalyzing the formation of glucose-1-phosphate from glucose-6-phosphate (the branch pathway of glycolysis) is the key enzyme to divert metabolic flux to UDP-glucose biosynthesis, and the UDP-glucose pyrophosphorylase directly catalyzes the formation of UDP-glucose from glucose-1-phosphate (Fig. 2a). Thus, to increase UDP-glucose biosynthesis in strain WPK3, we designed and constructed three strains: strain WPK4, which heterologously expresses UDP-glucose pyrophosphorylase gene *galU* from *E. coli*; strain WPK5, which overexpresses the yeast native UDP-glucose

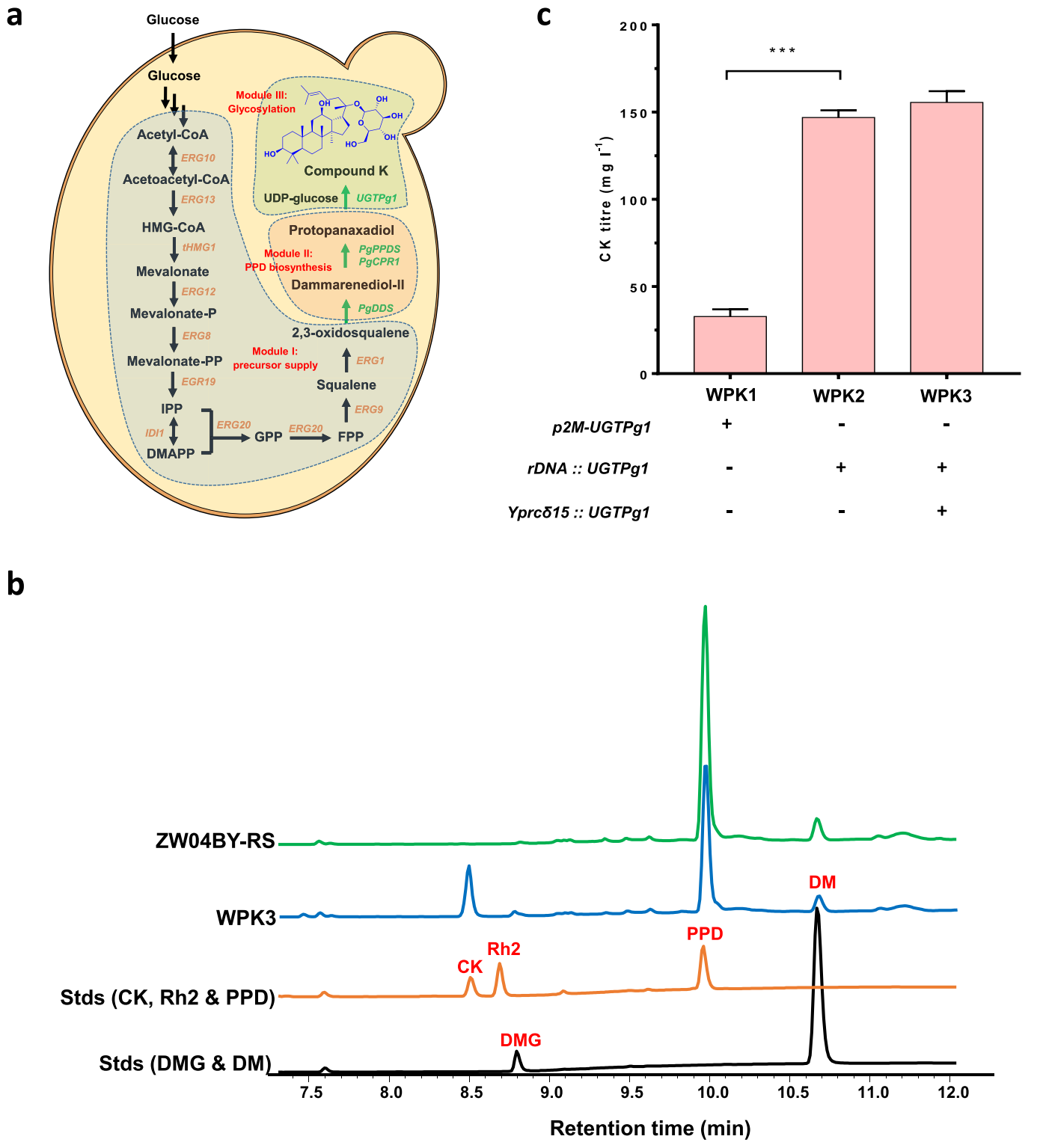

Fig. 1. Optimization of UGTPg1 expression in PPD-producing chassis. (a) Schematic representing the modular construction of the CK biosynthetic pathway in yeast. *Orange*, yeast native genes/enzymes; *green*, *P. ginseng* genes/enzymes; *black arrows*, yeast native pathway; *green arrows*, heterologous pathway. (b) HPLC analysis of the *n*-butanol extracts of the engineered yeast strains. (c) CK production in engineered yeast by expression of *UGTPg1* in ZW04BY-RS via a 2μ based high-copy plasmid (WPK1) or chromosome integration (WPK2). Strain WPK3 was constructed by integration of an additional copy of *UGTPg1* in WPK2. Values are presented as the mean ± S.D. from three biological replicates. Student's two-tailed *t*-test, ***$P < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

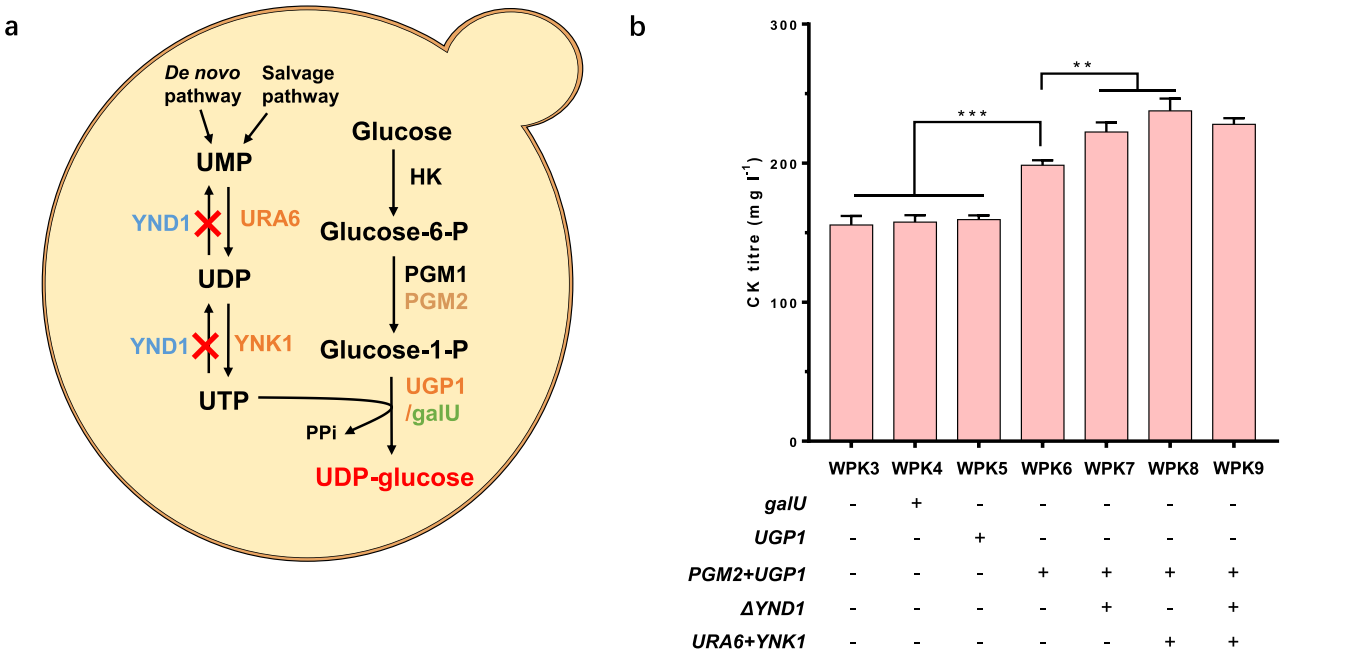

Fig. 2. Boosting CK production by increasing UDP-glucose biosynthesis. (a) Schematic representing the optimization of UDP-glucose biosynthesis in yeast. *Orange*, overexpressed native yeast genes; *blue*, deleted genes; *green*, overexpressed *E. coli* gene; UMP, uridine monophosphate; UDP, uridine diphosphate; UTP, uridine triphosphate; G6P, glucose-6-phosphate; G1P, glucose-1-phosphate. (b) CK production in engineered yeast by overexpressing or deleting genes involved in UDP-glucose biosynthesis. Values are presented as the mean ± S.D. from three biological replicates. Student's two-tailed *t*-test, $^{**}P < 0.01$, $^{***}P < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

pyrophosphorylase gene *UGP1*; and strain WPK6, which overexpresses the yeast native phosphoglucomutase gene (*PGM2*) and UDP-glucose pyrophosphorylase gene (*UGP1*). Shake flask fermentation of the above three strains showed that although overexpression of *galU* or *UGP1* individually did not improve CK production, the combined overexpression of *UGP1* and *PGM2* resulted in a 28% increase in CK production, reaching a titer of 198.5 mg/L (Fig. 2b).

The biosynthesis of UDP-glucose also relied on the supply of UTP, which is biosynthesized from uridine monophosphate (UMP) via the reactions catalyzed by URA6 and YNK1. Besides, UTP could be re-dephosphorylated by YND1 to form UDP and UMP. Hence, we used two strategies to further increase the UTP supply in WPK6 by deletion of the *YND1* gene (strain WPK7) and overexpression of *URA6* and *YNK1* genes (strain WPK8). Subsequent fermentation demonstrated that both WPK7 and WPK8 produced more CK than WPK6, and strain WPK8 gave the highest CK titer of 237.6 mg/L. We then combined the overexpression of *URA6* and *YNK1* and deletion of *YND1* in WPK6; however, no additional increase of CK titer could be observed in the resulting strain WPK9 (Fig. 2b). It seems that one of the two strategies, deletion of the *YND1* gene, or overexpression of *URA6* and *YNK1* genes, was enough to increase the UTP supply in the two strains, WPK7 and WPK8.

At this stage, the efforts to improve UDP-glucose synthesis by the combined overexpression of the genes *PGM2*, *UGP1*, *URA6*, and *YNK1* in WPK3 led to a 53% increase in CK titer, from 155.6 mg/L (WPK3) to 237.6 mg/L (WPK8), and the titer ratio between CK and PPD (CK/PPD) increased from 0.52 to 0.75, which clearly demonstrated that boosting UDP-glucose biosynthesis could promote the glycosylation efficiency and thus CK production in yeast. However, a significant amount of PPD still accumulated in strain WPK8 (Supplementary Fig. S1), indicating that great potential remains for the improvement of CK production.

3.3. Tuning down the UDP-glucose consumption pathways to increase CK production

UDP-glucose is a basic metabolic intermediate in *S. cerevisiae*, which participates in many yeast metabolic processes, including cell wall biosynthesis, glycogen biosynthesis, protein glycosylation, trehalose biosynthesis, and galactose metabolism. Thus the biosynthesized UDP-glucose may be rapidly redirected into the above pathways in addition to the CK biosynthetic pathway. This "leak" of UDP-glucose will inevitably discount the above efforts to improve UDP-glucose biosynthesis (Fig. 3a).

To address this, we tried to throttle down UDP-glucose consumption by manipulating the following three genes: *FKS*1, *GLC*3, and *ALG*5, which are involved in yeast cell wall biosynthesis, glycogen biosynthesis, and protein glycosylation, respectively. FKS1 is the catalytic subunit of yeast 1,3-β-D-glucan synthase, which catalyzes glucan chain elongation using UDP-glucose as a sugar donor during cell wall biosynthesis [28]. GLC3 is the glycogen branching enzyme of yeast, which catalyzes branch formation of glycogen; a *GLC3*-deleted yeast strain does not accumulate glycogen [29]. ALG5 is a glucosyltransferase that adds the first glucose moiety to lipid-linked oligosaccharides using UDP-glucose as a sugar donor during N-linked glycosylation of proteins in yeast [30]. We chose these three gene targets because they use UDP-glucose as a substrate and play a key role in determining cell wall composition, glycogen accumulation, and protein glycosylation. Moreover, deletion of any of the three genes will not cause cell biomass decrease significantly, which is the premise for engineering cell factories. Hence, we constructed the strains WPK10, WPK11, and WPK12 by deleting *FKS1*, *GLC3*, and *ALG5*, respectively, in WPK8. Observations of shake flask fermentation indicated that only strain WPK12 had a 12% increase in CK titer compared to WPK8, while no significant improvement in CK production was observed in the other two strains (Fig. 3b–c). Deletion of *FKS1* or *GLC3* did not improve CK production, nor did it perturb cell growth. It is possible that yeast deletion mutants might have some isozymes, or induce compensatory pathways to rescue cell wall formation and glycogen synthesis to maintain cell growth. For example, studies have demonstrated that the deletion of *FKS1* in yeast induced the chitin biosynthetic process as a compensatory mechanism, which also led to the consumption of UDP-glucose [31]. We believe that further

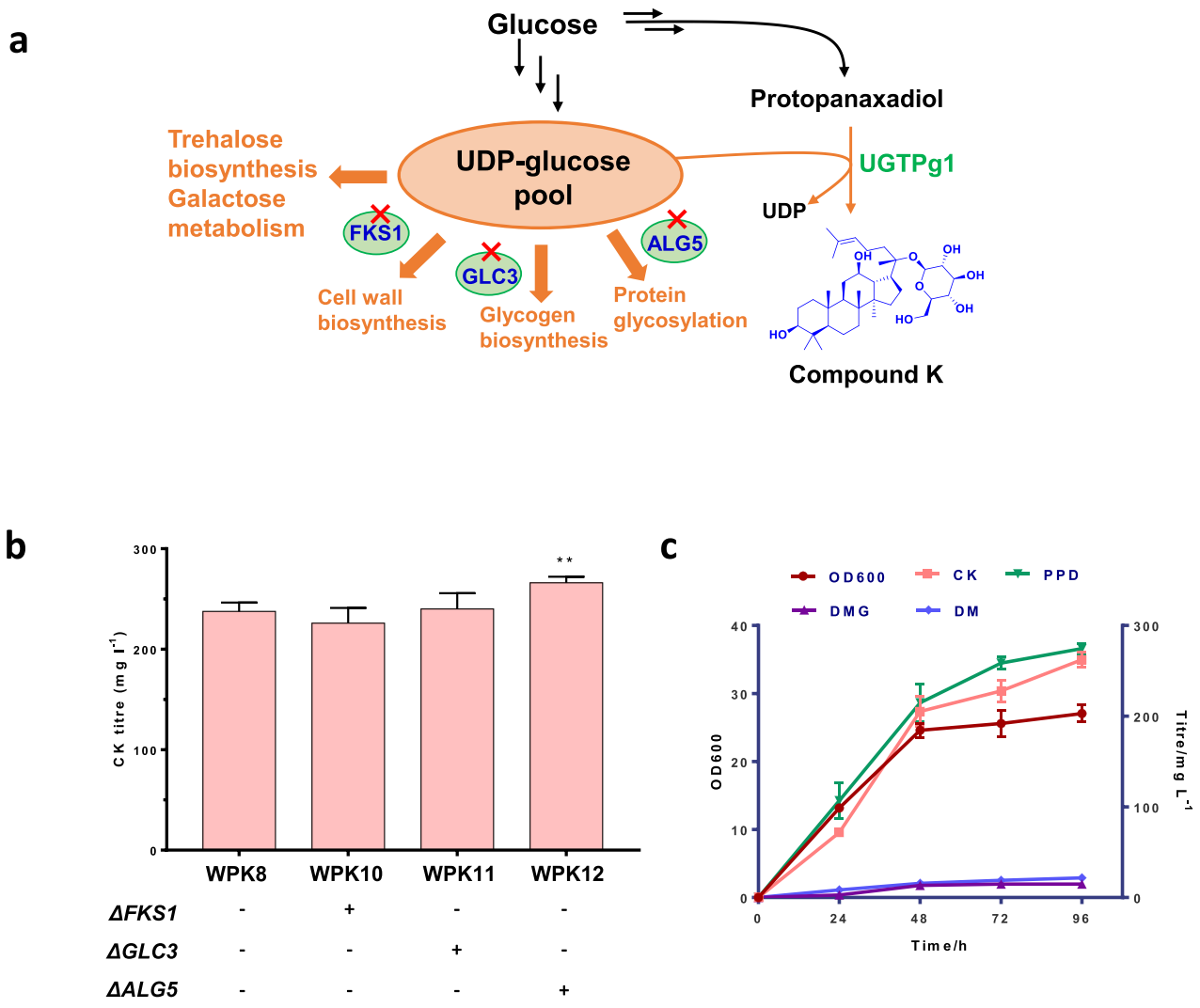

Fig. 3. Increased CK production by deleting UDP-glucose consumption pathway related genes. (a) Schematic representing the major UDP-glucose consumption pathways and the engineering strategies to reduce or block them in yeast; UDP, uridine diphosphate. (b) CK production in engineered yeast by overexpressing or deleting genes involved in UDP-glucose consumption. (c) Time course analysis of cell growth and triterpenoid production by strain WPK12. Values are presented as the mean ± S.D. from three biological replicates. Student's two-tailed *t*-test, $^{**}P < 0.01$.

systematic studies of these UDP-glucose consumption mechanisms will provide additional clues for further CK improvements.

Overall, the CK titer of strain WPK12 increased 8-fold compared to the starting strain WPK1, and more than 190-fold compared to our previously constructed strain AKE [20]. These results clearly demonstrated that the potential of yeast CK biosynthesis could be greatly promoted by addressing the previously mentioned two limiting factors, low triterpenoid precursor supply and insufficient glycosylation efficiency.

3.4. High-titer production of CK by fed-batch fermentation

High cell density fermentation in bioreactors could significantly boost the production of the target natural products, as proven for artemisinic acid [18], salidroside [25], and resveratrol [32] biosynthesis in engineered yeast. To further promote CK production and to compare the performance of engineered strains with or without UDP-glucose supply optimization in a scale-up condition, we performed fed-batch fermentation using the two CK-producing strains, WPK3 and WPK12, in a parallel bioreactor system. The fermentation condition controls for the two strains were the same, *i.e.*, temperature was set to 30 °C, pH controlled at 5.0, and dissolved O_2 maintained at > 30%. Feeding with fresh medium was initiated once the glucose was depleted at approximately 24 h, and the feeding rate was controlled by maintaining ethanol at < 0.5 g/L. The fed-batch fermentation was terminated when cell densities reached a plateau at 144 h. The final biomasses of WPK3 and WPK12 were 149.2 g/L and 152.2 g/L dry cell weight (dcw), respectively. The lack of significant difference in final cell biomass between the two strains suggested that the genetic modifications we performed, including the overexpression and deletion of genes involved in UDP-glucose metabolism, did not affect growth of CK cell factories.

The CK production by WPK3 and WPK12 in fed-batch fermentation was then determined by *n*-butanol extraction and HPLC quantification. The final CK titers for WPK3 and WPK12 were 3.98 g/L and 5.74 g/L, respectively. Compared to WPK3 (strain without UDP-glucose supply optimization), CK production increased by 44.2% in WPK12. Other triterpenoid intermediates were also determined. The DMG, PPD, and DM titers for WPK3 were 1.9 g/L, 6.19 g/L, and 1.75 g/L, respectively; the DMG, PPD, and DM titers for WPK12 were 0.82 g/L, 5.96 g/L, and 0.78 g/L, respectively (Table 2). Although the total triterpenoids of WPK3 (13.8 g/L) and WPK12 (13.2 g/L) showed no significant differences, the CK titer increased by more than 40% for WPK12, indicating that the conversion rate of the triterpenoid intermediates into CK increased significantly. These results demonstrated that boosting UDP-glucose

Table 2
CK and other triterpenoids production by engineered yeast via fed-batch fermentation.

Strains	Cell biomass (g/L, DCW)	CK (g/L)	DMG (g/L)	PPD (g/L)	DM (g/L)	CK dry weight content
WPK3	149.2	3.98	1.9	6.19	1.75	2.67%
WPK12	152.2	5.74	0.82	5.96	0.78	3.77%

supply via increasing UDP-glucose biosynthesis and throttling down UDP-glucose consumption greatly improved the yeast glycosylation efficiency and thus the production of CK. Beyond CK, we believe that our engineered strategies could be leveraged to improve the production of other glycosylated natural products.

CK production by strain WPK12 represented the highest microbial CK biosynthesis reported to date, and is 3.4-fold that of the strain constructed by Nan et al. [23]. The dry weight content of CK in strain WPK12 was 37.7 mg/g dcw (*i.e.*, 3.77% of yeast dry weight), which is comparable to the total saponin content of a 5-year-old ginseng root (~3%) [16]. Except for the need of a much longer cultivation time for ginseng to reach such saponin contents, most of the saponin in ginseng, such as the protopanaxatriol-, oleanane-, and ocotillol-type ginsenosides, could not be converted into CK. Thus, the microbial biosynthesis platform developed in this study will have significant advantages over the traditional plant extraction–biotransformation methods, once scaled up in the future.

4. Conclusion

We developed a high titer microbial fermentation-based ginsenoside CK production platform, which yielded a CK titer of 5.74 g/L in a 1-L bioreactor. Having achieved such a high titer (>5 g/L), the dry weight content of CK in yeast already exceeded the total saponin content in ginseng root, suggesting that the current *Panax* plant farming-based supply of CK could be complemented or replaced by industrial fermentation. Fermentation-based production could have many advantages over agriculture-based approaches, including much shorter manufacturing time, more environmentally friendly, and easier to centralize and scale-up. However, many pilot plant tests in larger fermenters are necessary before initiating commercial production.

Credit authorship contribution statement

Pingping Wang: Conceptualization, Investigation, Writing – original draft. Jiali Wang: Investigation. Guoping Zhao: Conceptualization, Supervision, Xing Yan: Investigation, Writing – review & editing, Supervision. Zhihua Zhou: Conceptualization, Writing – review & editing, Supervision, Project administration.

Declaration of competing interest

The authors have no interests to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2021.03.002.

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