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Rap1 overexpression boosts triterpenoid saponin production in yeast by enhancing precursor supply and heterologous gene expression

Ji-Young Byun[1], Thi Thuy Nguyen[2], Byung-Kwan Cho[3,4], Soo-Hoon Park[2] and Sun-Chang Kim[4,5*]

Abstract

Background Metabolic engineering to increase the supply of precursors, such as 2,3-oxidosqualene (OSQ), and manipulate heterologous biosynthetic pathways through the strategic overexpression of multiple genes is promising for increasing the microbial production of triterpenoid saponins. However, the multiple use of constitutive promoters, typically derived from glycolytic or ribosomal protein promoters, can cause transcription factor competition, reducing the expression of each gene. To avoid this issue, we overexpressed transcriptional factor repressor activator protein 1 (Rap1), known to upregulate glycolytic gene expression and be involved in various metabolic pathways, including pyruvate dehydrogenase (PDH) bypass, the mevalonate (MVA) pathway, and sterol synthesis.

Results Transcriptome analysis of a wild-type yeast strain revealed that Rap1 overexpression significantly upregulated several central carbon metabolism (CCM)-related genes for OSQ production, including glycolytic genes, particularly after the diauxic shift phase. To validate the effect on triterpenoid saponin production, we engineered a *Saccharomyces cerevisiae* strain capable of producing ginsenoside compound K (CK). Notably, compared with the control strain, the CK-producing strain with Rap1 overexpression showed upregulation of heterologous genes controlled by TDH3 promoter, and a continuous supply of precursors to the CK synthesis pathway, resulting in a 4.5-fold increase in CK production.

Conclusion These results highlight Rap1 overexpression as a robust strategy to increase triterpenoid production in yeast cell factories. Additionally, this approach provides a versatile framework for enhancing both precursor supply and heterologous gene expression.

Keywords Triterpenoid saponins, Rap1 overexpression, Metabolic engineering, *Saccharomyces cerevisiae*, Transcription factor (TF), Glycolytic promoter, Ginsenoside Compound K

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Background

Triterpenoid saponins, which are composed of a C30 hydrophobic triterpene aglycone and hydrophilic sugar moieties, have garnered significant attention from the food, cosmetic, and pharmaceutical industries for their diverse biological activities, including insecticidal, antiparasitic, antifungal, antimicrobial, anticancer, antioxidant, and immunostimulatory effects [1–3]. However, their chemical synthesis is limited by structural complexity, and extraction from natural sources presents challenges in isolating them as pure compounds [3]. To overcome these limitations, a sustainable and cost-effective alternative approach utilizing microorganisms, particularly *Saccharomyces cerevisiae*, has been developed. *S. cerevisiae*, renowned for its 'generally regarded as safe' (GRAS) status, is a promising platform for triterpenoid saponin production because of its capacity to express membrane-bound eukaryotic cytochrome P450 oxidases (CYP450s), pivotal catalysts in biosynthetic pathways of plant-derived products such as triterpenoids, and to produce minimal secondary metabolites [4–6]. Furthermore, this microorganism contains an endogenous pathway for 2,3-oxidosqualene (OSQ), a critical precursor for triterpenoid saponin synthesis [7].

Increasing OSQ production in *S. cerevisiae* could increase the production of triterpenoid saponins [8]. OSQ synthesis from glucose involves four sequential pathways: glycolysis, pyruvate dehydrogenase (PDH) bypass, the mevalonate (MVA) pathway, and the sterol synthesis pathway (Fig. 1) [9]. Several studies have successfully enhanced each of these pathways. In glycolysis, regulating a few genes does not affect the entire pathway; however, overexpressing 13 genes with a total of 18 copies enhanced glucose uptake and ethanol production [10]. The other three pathways were respectively enhanced by overexpressing or deleting a few rate-limiting genes. For example, in the PDH bypass pathway, overexpressing the aldehyde dehydrogenase gene (*ALD6*) [11] and acetyl-CoA synthetase (*ACS1*) [12] or deleting alcohol dehydrogenase (*ADH1/4*) [13] increased the level of cytosolic acetyl-CoA. In the MVA pathway, overexpressing *tHMGR1*, a 3-hydroxy-3-methylglutaryl-CoA reductase

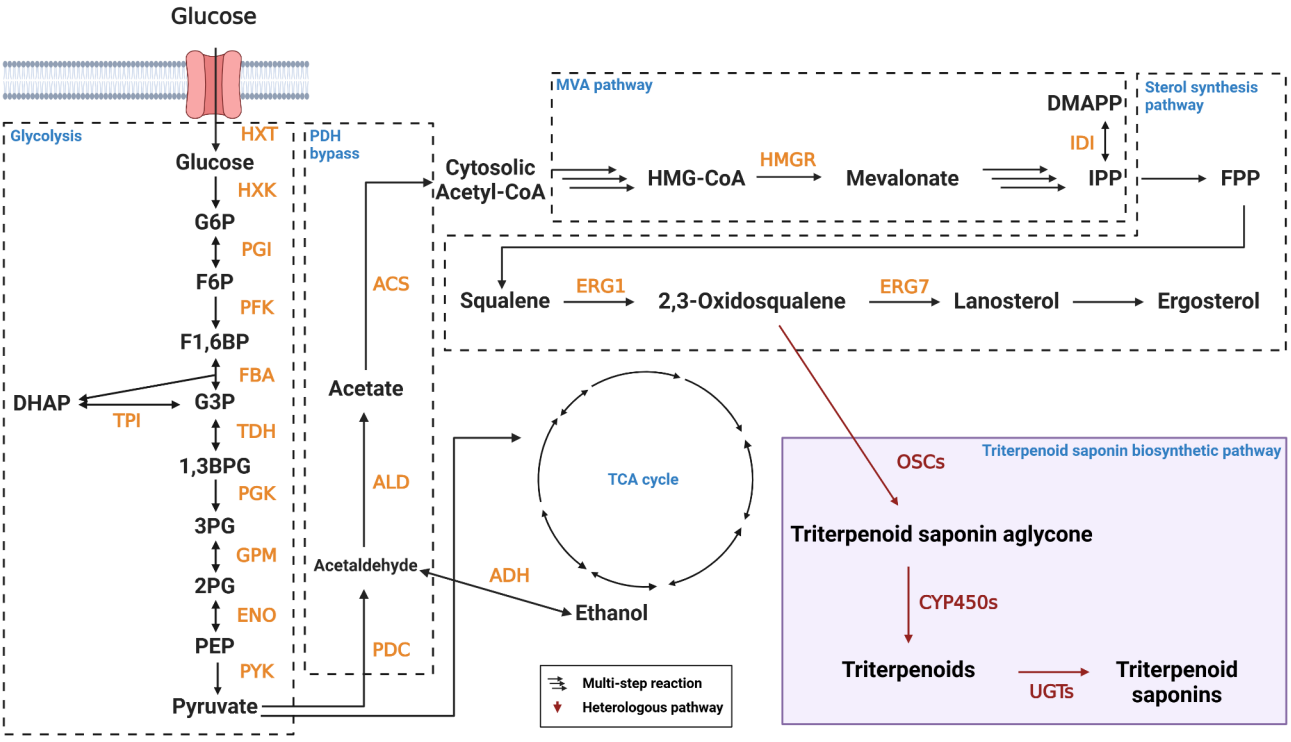

Fig. 1 Pathway for triterpenoid saponin biosynthesis in *S. cerevisiae*. Cytosolic acetyl-CoA, produced via glycolysis and the PDH bypass, is converted to isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) through the MVA pathway. These intermediates then proceed through the sterol biosynthesis pathway, where after the production of 2,3-oxidosqualene (OSQ), they are diverted into the triterpenoid biosynthetic branch, leading to the synthesis of triterpenoid saponins. HXT, Hexose transporter; HXK, Hexokinase; PGI, Phosphoglucose isomerase; PFK, Phosphofructokinase; FBA, Fructose-1,6-bisphosphate aldolase; TPI, Triose phosphate isomerase; TDH, Glyceraldehyde-3-phosphate dehydrogenase; PGK, 3-Phosphoglycerate kinase; GPM, Phosphoglycerate mutase; ENO, Enolase; PYK, Pyruvate kinase; PDC, Pyruvate decarboxylase; ALD, Aldehyde dehydrogenase; ACS, Acetyl-CoA synthetase; HMGR, HMG-CoA reductase; OSCs, 2,3-oxidosqualene cyclases; CYP450s, Cytochrome P450s; UGTs, UDP-glycosyltransferases; G6P, Glucose-6-phosphate; F6P, Fructose-6-phosphate; F1,6BP, Fructose-1,6-bisphosphate; G3P, Glyceraldehyde-3-phosphate; DHAP, Dihydroxyacetone Phosphate; 1,3BPG, 1,3-Bisphosphoglycerate; 3PG, 3-Phosphoglycerate; 2PG, 2-Phosphoglycerate; PEP, Phosphoenolpyruvate; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; FPP, Farnesyl pyrophosphate

1 truncated at the N-terminal region [4], or overexpressing Isopentenyl Diphosphate Isomerase (*IDI1*) [14] increased squalene production. Additionally, in the sterol synthesis pathway, the overexpression of squalene monooxygenase (*ERG1*) [15] and the suppression of lanosterol synthase (*ERG7*) with regulated promoters, such as the methionine-repressible MET3 promoter or the copper-repressible CTR3 promoter, increased OSQ availability for terpenoid saponin production [16, 17].

In our study, we aimed to increase OSQ production by regulating transcription factors (TFs) as opposed to overexpression of individual genes. This strategy was chosen because the excessive use of constitutive promoters, such as TDH3, PGK1 or PYK1—which are primarily derived from glycolytic and ribosomal protein promoters [18, 19] and share a limited number of TFs [20, 21] — might lead to competition among the available TFs, ultimately reducing transcription levels for each gene [22]. Furthermore, since triterpenoid saponin biosynthesis involves multiple enzymatic steps, the use of additional constitutive promoters for these heterologous genes could worsen this issue. Therefore, focusing on TF regulation related to the above pathways can be a more efficient and practical approach to enhance central carbon metabolism (CCM) towards OSQ production, resulting in higher triterpenoid production.

Repressor activator protein 1 (Rap1) and glycolysis regulation 1 (Gcr1) are key transcription factors in *S. cerevisiae* that play crucial roles regulating many genes, particularly for genes involved in glycolysis [20, 23]. However, Gcr1 overexpression has not significantly improved glycolytic flux [24]. Thus, this study focuses on Rap1 overexpression. There have been no studies on Rap1 overexpression, primarily due to its known toxicity when excessive overexpression in yeast cells [25]. Overproduction of Rap1 under its own promoter from YEpFAT4 vector (20–40 copies per cell) did not impair cell growth [26]. However, excessive overexpression of Rap1 driven by the induced GAL1-10 promoter in pRS425 plasmid (20–40 copies per cell) led to very high levels of Rap1 expression, consequently inhibiting cell growth [25]. This inhibition was likely be due to the overstimulation of Rap1 target genes, disrupting essential cellular functions. Nevertheless, studies [27–30], except one by Kalra et al. [31], have demonstrated that down-regulating Rap1 decreases glycolysis gene transcription, suggesting that controlled overexpression of Rap1 could potentially increase the expression of genes under glycolytic promoters. This approach could be efficient when using extra glycolytic promoters to express triterpenoid saponin biosynthetic heterologous genes.

Additionally, Rap1 has been implicated in interactions with many CCM-related genes. DNA-to-cDNA microarray analysis and chromatin immunoprecipitation (ChIP) have confirmed that Rap1 is associated with numerous genes involved in PDH bypass, the MVA pathway, and the OSQ synthesis pathway from IPP and DMAPP, potentially influencing OSQ production [32–34]. Since Rap1 is known to expand its gene targets related to alternative carbon-source utilization after a diauxic shift, its controlled overexpression could affect CCM differently following the transition from fermentation to oxidative metabolism under low-glucose conditions [35]. By controlling Rap1 overexpression, we aimed to upregulate these pathways, thereby increasing the availability of OSQ and subsequently enhancing triterpenoid biosynthesis without causing the detrimental effects associated with high levels of Rap1.

In this study, we overexpressed Rap1 to increase the OSQ supply and heterologous gene expression under the glycolytic promoter in *S. cerevisiae* (Fig. 2). To avoid excessive Rap1 overexpression from inducible promoters,

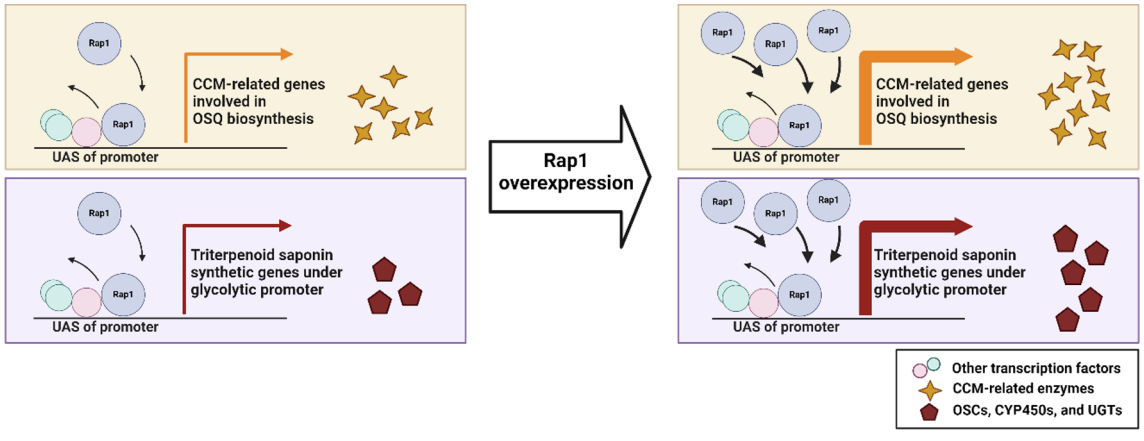

Fig. 2 Strategy for enhancing triterpenoid saponin biosynthesis through Rap1 overexpression in metabolically engineered *S. cerevisiae*. Rap1 overexpression increases the transcriptional activity of central carbon metabolism (CCM)-related genes for OSQ production, as well as triterpenoid saponin synthetic genes under the glycolytic promoters. UAS, upstream activation sequence

which can cause toxicity to yeast cells [25], we utilized constitutive promoters, such as the CCW12 and TDH3 promoters, for Rap1 transcription. We ultimately confirmed the potential benefits of Rap1 overexpression in *S. cerevisiae* on triterpenoid saponin production by focusing on dammarane-type ginsenoside compound K (CK), which is renowned for its various pharmacological activities, including antiallergic, antifatigue, antidiabetic, anti-inflammatory, anticancer, and antiaging properties [36]. To the best of our knowledge, this is the first study demonstrating the application of Rap1 overexpression to enhance triterpenoid production.

Methods

Plasmid and strain construction

The yeast strains used in this study are described in Table 1 and the plasmids and primers are listed in the Supplementary information (Tables S1 and S2). The plasmids used in this study were constructed by the insertion of DNA fragments that were amplified via PCR from yeast genomic DNA or synthesized via codon optimization (BIONEER, Daejeon, South Korea) for expression in *S. cerevisiae* via appropriate primers with p426GPD, which was first digested with the appropriate restriction enzymes. Cassettes controlled under the TDH3 promoter were introduced into pRS423, pRS424, pRS425 or pRS426, which were digested with proper restriction enzymes (Table S3).

For gene integration into the genome, two methods were utilized. Genes in the ginsenoside synthetic pathway were introduced into the genomic DNA through yeast homologous recombination via an auxotrophic complementation-based yeast selection system. The *RAP1* gene was integrated into genomic DNA through CRISPR/Cas9-guided integration. sgRNAs targeting specific sites were designed via an online tool (https://www.atum.bio/eCommerce/cas9/input, accessed 2 July 2018). The integration site used in this study was formerly chosen by our research group [37]. Recombination cassettes of ginsenoside pathway genes or donor DNA cassettes for *RAP1* were amplified via PCR from pRS42x-derived stock plasmids containing a gene with an appropriate promoter and terminator, respectively, via primer sets containing a DNA region homologous to the target recombination site (Table 1, Table S1 and Table S2). For yeast transformation, the standard LiAc/single-stranded carrier DNA/PEG method was used [38]. The CEN.PK-Met-TLH strain was derived from the CEN.PK-Met strain by complementing the auxotrophic mutation (trp1-289, leu2-3, and his3Δ1) under selective pressure to restore prototrophy for tryptophan, leucine, and histidine, while maintaining uracil auxotrophy.

Table 1 List of strains used in this study

Strains	Genotype/description	Source or reference
CEN.PK2-1D	*MATaura3-52trp1-289leu2-3,112his3Δ1MAL2-8^{C}SUC2*	EUROSCARF
CEN.PK-Rap	CEN.PK2-1D P_{CCW12}-*RAP1*-T_{CYC1}	This study
CEN.PK-Met	CEN.PK2-1D P_{MET3}-*ERG7*	[16]
CEN.PK-Met-TLH	CEN.PK-Met *trp1-289::TRP1leu2-3::LEU2his3Δ1::HIS3*	This study
CK01	CEN.PK-Met *trp1-289::*[P_{TDH3}-*tHMGR1*-T_{CYC1}, P_{TDH3}-*PgSE*-T_{CYC1}]-*TRP1 leu2-3::*[P_{TDH3}-*PgDS*-T_{CYC1}, P_{TDH3}-*PgPPDS*-T_{CYC1}, P_{TDH3}-*PgCPR*-T_{CYC1}]-*LEU2 his3Δ1::*[P_{TDH3}-*PgUGT71A27*-T_{CYC1}]-*HIS3*	This study
CK01-CRap	CK01 P_{CCW12}-*RAP1*-T_{RAP1}	This study
CK01-TRap	CK01 P_{TDH3}-*RAP1*-T_{RAP1}	This study

Yeast cultivation in flasks

CEN.PK and CEN.PK-Rap cells were cultured in shake flasks with synthetic defined (SD) medium supplemented with an appropriate amino acid mixture and 2% (w/v) glucose. CEN.PK-Met-TLH, CK01, CK01-CRap, and CK01-TRap were cultured in shake flasks with SD medium supplemented with an appropriate amino acid mixture, 2% (w/v) glucose, and 0.25 g/L methionine. 1mL of glycerol cell stock (25%) was inoculated into a 250 mL baffled flask (TriForest, Irvine, CA, USA) containing 29 mL of medium and incubated at 26 °C with shaking at 180 rpm to a density at an optical density (OD) of 600 nm of up to approximately 1.0 (for CEN.PK2-1D and CEN.PK-Rap) or 2.0 (for CEN.PK-Met-TLH, CK01, CK01-CRap and CK01-TRap) were measured by a GENESYS 20 visible spectrophotometer (Thermo Scientific, Waltham, MA, USA). Subsequently, 1 mL of the preculture was inoculated into a 250 mL baffled flask containing 29 mL of medium and incubated at 26 °C with shaking at 180 rpm for 144 h. All flask fermentation was performed in three independent experiments.

Determination of glucose, ethanol, glycerol, and acetate concentrations

For the quantification of glucose, ethanol, glycerol and acetate, 1 mL of cell culture was harvested, and the supernatants were filtered through a 0.2 μm PVDF syringe filter (Whatman, Germany) after centrifugation at 13,000 × g. The concentrations of glucose, ethanol, glycerol and acetate in the supernatant were measured with a glucose (HK) assay kit, an ethanol assay kit and a glycerol assay kit and an acetate colorimetric assay kit, respectively, from Sigma–Aldrich (Burlington, MA, USA), according to the manufacturer's instructions. Glucose concentrations were determined by measuring the absorbance at 340 nm with a GENESYS 20 visible spectrophotometer. The ethanol and glycerol concentrations were determined by measuring the fluorescence intensity at wavelengths of

535 nm (excitation) and 587 nm (emission) on a microplate reader (Tecan Spark, Tecan, Switzerland), and the acetate concentration was determined by measuring the absorbance at 450 nm with a microplate reader.

Transcriptome analysis

CEN.PK2-1D and CEN.PK-Rap cells at a density corresponding to an OD600 of 20 were harvested from shake flask cultures after cultivation for 9 h, 15 h and 36 h. Total RNA was isolated via an RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions, and contaminant DNA was degraded via RNase-Free DNase Set (Qiagen, Germany) treatment. The library was prepared via the TruSeq Stranded mRNA Library Prep Kit (Illumina, USA) and sequenced via a NovaSeq 6000 (Illumina, USA). The sequencing reads were aligned to the *S. cerevisiae* S288C (GCF_000146045.2_R64) genome via HISAT2 software. The reference genome sequence of *S. cerevisiae* S288C (GCF_000146045.2_R64) and annotation data were downloaded from the NCBI database (https://www.ncbi.nlm.nih.gov/data-hub/genome/GCF_000146045.2/). Gene expression analysis was performed with StringTie (version 2.1.3b), and differentially expressed genes (DEGs) were screened according to the following criteria:|log2-fold change|> 1 and P value < 0.05.

Metabolite extraction and HPLC analysis

Yeast cells at a density corresponding to an OD_{600} of 20 were harvested from shake flask cultures after 72 h and 144 h in 14 mL conical tubes (SPL, South Korea) and centrifuged at 3,000 × g for 5 min. After the supernatant was removed, the collected cells were resuspended in 1 mL of distilled water, transferred to 2 mL safe-lock Eppendorf tubes and centrifuged at 13,000 × g for 5 min. After the supernatant was removed, the collected cells were resuspended in 1 mL of an acetone-methanol mixture (1:1 v/v) and added to a tube containing 400 µL of glass beads (Sigma–Aldrich, Burlington, MA, USA). The cells were then lysed via an MM400 homogenizer (Retsch, Germany) according to the manufacturer's instructions, and the samples were centrifuged at 13,000 × g for 5 min. The supernatant was then injected into an Agilent 1260 Infinity II HPLC system (Agilent, Santa Clara, CA, USA). Chromatography was performed using a Prodigy 5 m ODS-2 LC column (4.6 mm to 150 mm, Phenomenex, Torrance, CA, USA). The solvent flow rate was 1.0 ml/min, the column temperature was 26 °C, and the detection wavelength was 203 nm. The mobile phase was composed of water (A) and acetonitrile (B), and the gradient consisted of 32–65% B at 0–8 min, 65–90% B at 8–12 min, 90% B at 12–20 min, 20–30 min of 90–100% B, and 100% B at 30–62 min. SQ, OSQ, lanosterol and ergosterol standards were purchased from Sigma–Aldrich (Burlington, MA, USA). DD, PPD and CK were purchased from ChemFaces (Wuhan, China).

RNA isolation and real-time PCR analysis

Yeast cells at a density corresponding to an OD_{600} of 10 were harvested from shake flask cultures after 24–48 h. RNA was isolated via a RNeasy Mini Kit (Qiagen, Germany), and contaminant DNA was degraded via an RNase-Free DNase Set (Qiagen, Germany) according to the manufacturer's instructions. Total RNA was transcribed to cDNA by M-MLV Reverse Transcriptase (Thermo Scientific, Waltham, MA, USA). One milligram of total RNA was transcribed to cDNA in a 20 µL reaction mixture containing 200 U of M-MLV Reverse Transcriptase (Thermo Scientific, Waltham, MA, USA), 0.1 mg of oligo(dT) and 1 µL of 10 mM dNTPs and incubated at 65 °C for 50 min. Real-time PCR was performed with a CFX96 thermal cycler (Bio-Rad) using BioFACT™ H-Star Taq DNA Polymerase (Biofact, South Korea), 20X EvaGreen™ (Biofact, South Korea) and gene-specific primers under the following conditions: 95 °C for 15 min, followed by 49 cycles of 95 °C for 20 s, 60 °C for 30 s, 72 °C for 30 s, and 25 °C for 30 s. The expression level of each gene was normalized to that of ACT1, the reference gene. All flask cultures were performed in biological triplicates.

Results

Investigation of Rap1 overexpression in wild-type *S. Cerevisiae* cells

Construction of CEN.PK-Rap strain and its effect on glucose uptake, ethanol production, and OSQ synthesis

In this study, we aimed to overexpress Rap1 without causing toxicity by using constitutive promoters for Rap1 overexpression with a single additional copy of the *RAP1* gene. Even though glycolytic promoters are commonly used as constitutive promoters in yeast, to avoid the autoregulation which can affect its own expression as Rap1 is a known regulator of glycolytic genes, we did not choose a glycolytic promoter for study the overexpression of Rap1 in wildtype strain. We specifically chose the CCW12 promoter, one of the strongest non-glycolytic constitutive promoters [39]. Although CCW12 promoter is stronger than the Rap1 native promoter, its expression from a single additional copy is still not comparable to that from the induced GAL1-10 promoter in the pRS425 plasmid. Therefore, we expected Rap1 overexpression under the control of the CCW12 promoter would not negatively affect cell growth.

To evaluate the impact of Rap1 overexpression in wild-type *S. cerevisiae*, we constructed the strain CEN.PK-Rap by inserting the P_{CCW12}-*RAP1*-T_{RAP1} cassette into the genome of the wild-type strain CEN.PK 2-1D (Table 1). Initially, we compared the growth profiles, glucose

uptake, and ethanol production between CEN.PK2-1D and CEN.PK-Rap cultured in SD medium with an appropriate amino acid mixture and 2% (w/v) glucose, as it was expected that Rap1 overexpression would strongly affect glycolysis. These values were measured every 3 h for up to 15 h to capture the rapid metabolic changes during the exponential phase. After 15 h, glucose depletion induced a diauxic shift, transitioning yeast to aerobic respiration. We also measured these values at 24 h and 36 h to observe the longer-term effects of Rap1 overexpression during the postdiauxic shift. We anticipated that Rap1 overexpression would affect glucose uptake and ethanol production by upregulating glycolysis and CCM-related pathways in yeast cells. However, no significant differences were observed between the two strains in terms of cell growth, glucose uptake and ethanol production for 36 h (Fig. 3A). Interestingly, Rap1 overexpression significantly increased OSQ production compared to the control, particularly after 15 h when glucose was depleted, resulting in a 2-fold increase in OSQ production after 36 h of cultivation (Fig. 3B). These results indicate that even though Rap1 overexpression does not significantly impact glucose utilization, it can alter CCM, which is involved in increasing OSQ production. To comprehensively elucidate the mechanism behind this phenomenon, genome-wide transcriptome analysis at the critical growth phase was further performed.

Genome-wide transcriptome analysis of Rap1-overexpressing yeast cells

According to Buck, M.J. et al. [35], the gene target sets of Rap1 expand despite a decrease in Rap1 protein levels after the diauxic shift. Therefore, we conducted transcriptome analysis via next-generation sequencing (NGS) of the wild-type CEN.PK2-1D and the engineered CEN.PK-Rap strains at three different cell growth phases, the exponential phase (EP) at 9 h, the diauxic shift phase (DSP) at 15 h, and the postdiauxic shift phase (PDSP) at 36 h to explore the effects of Rap1 overexpression on gene expression in yeast (Fig. 3A) [40].

Transcriptome profiling revealed significant differences in gene expression between CEN.PK-Rap and CEN.PK2-1D at the EP, DSP and PDSP, with 416, 916, and 897 DEGs identified, respectively ($|\log_2\text{-fold change}| > 1$ and P value < 0.05) (Tables S5, S6 and S7). These findings indicate that Rap1 overexpression broadly affects the transcription of genes in *S. cerevisiae*, with varying impacts at different growth stages. Functional annotation analysis of the DEGs revealed enrichment in several pathways, ranked by significance at each time point (Fig. 4A and Table S8). In the EP, the top 5 enriched pathways included one carbon pool by folate, the MAPK signaling pathway, and glyoxylate and dicarboxylate metabolism. At the DSP, the top 5 enriched pathways were glycolysis/gluconeogenesis; butanoate; phenylalanine; methane; glyoxylate; and dicarboxylate metabolism. At the PDSP, the top 5 enriched pathways included the pentose phosphate pathway, beta-alanine metabolism, pentose/glucuronate interconversions, ascorbate and aldarate and amino sugar, and nucleotide sugar metabolism. Interestingly, only about 10% of the enriched pathways related to the carbohydrate metabolism at the EP, whereas approximately 50% and 40% of the enriched pathways at the DSP and PDSP, respectively, were classified under carbohydrate metabolism according to KEGG Orthology. This prompted us to focus on the transcript levels of genes involved in CCM related to the biosynthesis of OSQ (Fig. 4B).

As mentioned above, transcriptome analysis of the EP revealed no significant difference in the expression of genes related to carbon metabolism except for the downregulation of hexose transporter genes (*HXT1/2)*, which are involved in glucose uptake, and *PDC5*, which is involved in PDH bypass. HXT genes have different

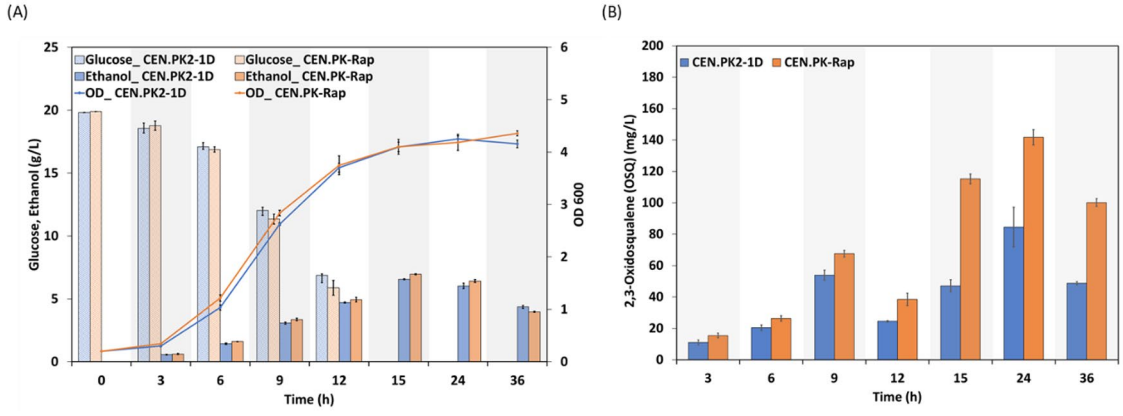

Fig. 3 The growth, glucose uptake, ethanol production, and OSQ synthesis of CEN.PK2-1D and CEN.PK-Rap strains. (**A**) Growth, glucose uptake, and ethanol production; (**B**) OSQ concentration during cultivation for 36 h. Growth was determined by measuring the optical density (OD) of the cells at 600 nm. The mean values of the results of triplicate experiments are shown, with error bars indicating the relative standard deviations

(A)

Gene set enrichment analysis of at EP

# of changed genes (>2-fold) up	# of changed genes (>2-fold) down	MAP name by KEGG	Fold Enrichment
1	2	One carbon pool by folate	16.7
5	8	MAPK signaling pathway - yeast	9.5
2	1	Glyoxylate & dicarboxylate metabolism	8.6
0	5	RNA degradation	6.5
2	4	Cell cycle - yeast	3.9

Gene set enrichment analysis of at DSP

# of changed genes (>2-fold) up	# of changed genes (>2-fold) down	MAP name by KEGG	Fold Enrichment
23	3	Glycolysis / Gluconeogenesis	9.8
4	0	Butanoate metabolism	9.2
0	3	Phenylalanine metabolism	8.9
8	3	Methane metabolism	8.8
4	8	Glyoxylate & dicarboxylate metabolism	8.6

Gene set enrichment analysis of at PDSP

# of changed genes (>2-fold) up	# of changed genes (>2-fold) down	MAP name by KEGG	Fold Enrichment
8	5	Pentose phosphate pathway	8.5
4	2	Beta-Alanine metabolism	8.4
3	2	Pentose & glucuronate interconversions	8.3
3	2	Ascorbate & aldarate metabolism	8.3
7	7	Amino sugar & nucleotide sugar metabolism	7.5

(B)

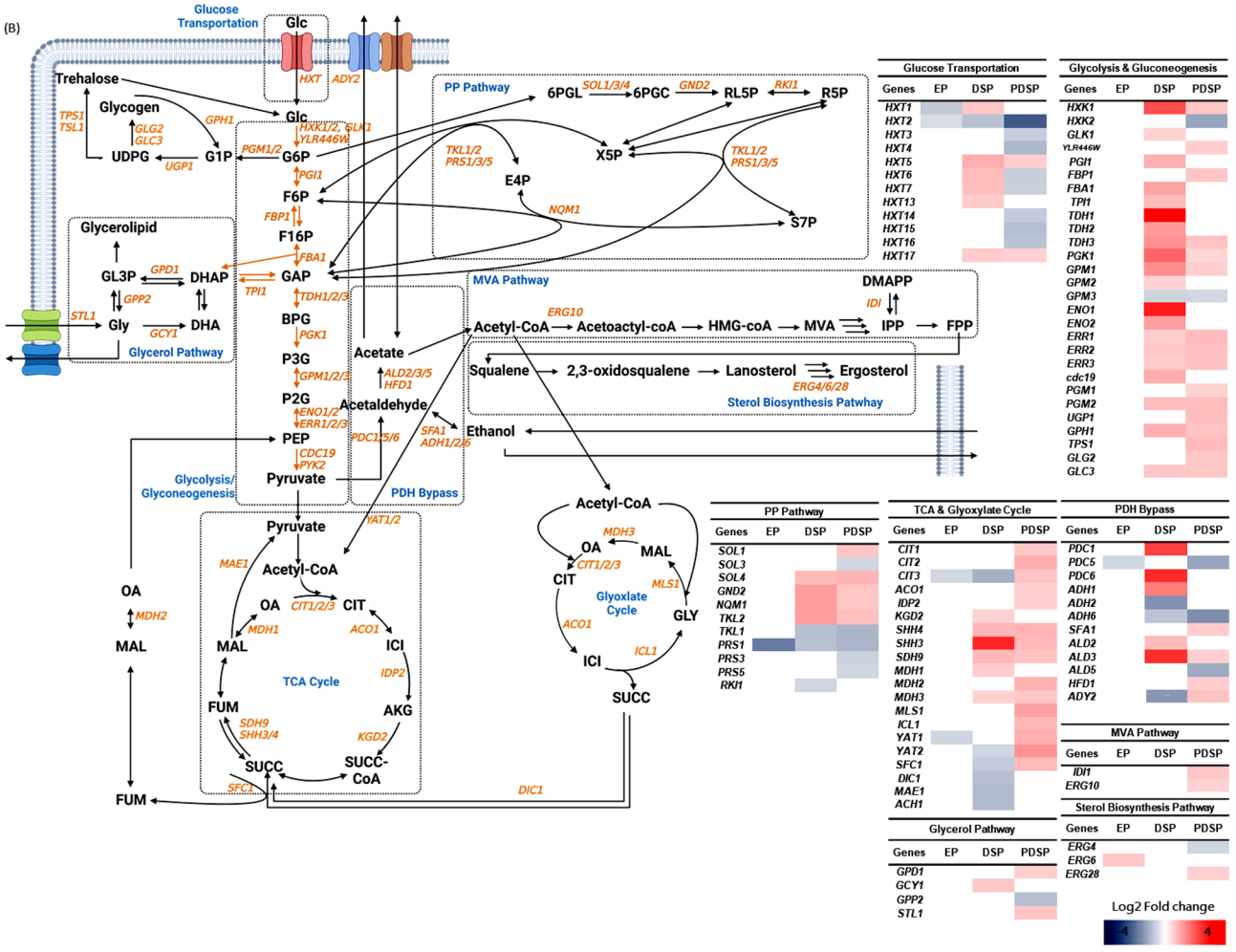

Fig. 4 Transcriptome analysis of CEN.PK-Rap at different growth phases. (**A**) Gene set enrichment analysis of CEN.PK-Rap at exponential phase (EP), diauxic shift phase (DSP) and postdiauxic shift phase (PDSP), shown in that order from top to bottom. The pathways are listed according to their fold-enrichment. The number of up- or downregulated genes in each pathway are indicated ($|\log_2$-fold c hange$|>1$ and P value <0.05). (**B**) Biosynthesis pathway for OSQ production in *S. cerevisiae* and transcriptional changes of genes related to carbohydrate metabolism for OSQ biosynthesis in CEN.PK-Rap compared to the wild-type CEN.PK2-1D. DEGs ($|\log_2$-fold change$|>1$ and P value <0.05) at the EP, DSP or PDSP are highlighted. The abbreviations for enzyme and compound names are provided in Table S4

affinities for glucose, with Hxt1 and Hxt3 being low-affinity transporters, Hxt2 and Hxt4 having moderately low affinity, and Hxt6 and Hxt7 acting as high-affinity transporters [41]. At the EP, when glucose levels dropped to around 1%, our transcriptome analysis showed that the expression of Hxt1/2 was not significant compared to Hxt3/4 (Supplementary Fig. S1). Therefore, even though Hxt1/2 expression was reduced in CEN.PK-Rap strain compared to the control strain, this did not lead to a significant change in glucose uptake. Similarly, Pdc1 and Pdc5, highly similar isozymes (88% identity at the protein level), exhibit functional redundancy. Pdc1 is responsible for most of the pyruvate decarboxylase activity in the wild-type yeast, while Pdc5 activity is only detected in the absence of Pdc1 [42]. Our transcriptome analysis at the EP also revealed that *PDC5* transcription was significantly lower than that of *PDC1*. Therefore, despite the reduced transcription of *PDC5* in CEN.PK-Rap, its activity can be compensated for by *PDC1*, maintaining sufficient PDC activity to support ethanol production. This

explains the lack of marked differences in glucose uptake and ethanol production during the exponential phase, as observed in Fig. 3A.

In contrast, the transcriptome analysis of DSP revealed significant differences between the two strains (Fig. 4B). Genes encoding low-affinity (*HXT1*) and high-affinity (*HXT6/7*) hexose transporters related to glucose uptake, as well as genes (*HXT5/13* and *HXT17*) induced by nonfermentable carbon sources or upregulated in the presence of raffinose and galactose, were upregulated [43, 44]. This finding suggests that substrate utilization capabilities corresponding to diverse environmental conditions increased in the Rap1-overexpressing strain. In addition, the transcription of glycolytic genes (*HXK1*, *GLK1*, *YLR446W*, *PGI1*, *FBA1*, *TPI1*, *TDH1/2/3*, *PGK1*, *GPM1/2/3*, *ENO1/2*, and *ERR1/2/3*), including C*DC19*, a key regulatory gene in glycolysis that can stimulate glycolysis from glucose or glycerol, increased. Furthermore, genes involved in PDH bypass (*PDC1/6* and *ALD2/3)* were also upregulated. These findings suggest that the conversion of pyruvate into cytoplasmic acetyl-CoA may be enhanced, as the fate of pyruvate in the cytosol is determined by PDC activity [45]. In contrast, the PP pathway is not expected to be significantly affected despite the upregulation of some genes (*SOL4*, *GND2*, *NQM1*, and *TKL2*) and the downregulation of others (*TKL1*, *PRS1*, and *RKI1*); this occurred because the upregulation of *GND2* did not greatly increase the PP pathway flux ratio, unlike the upregulation of *SOL3*, which has the strongest positive effect on this pathway [46]. The expression of genes (*KGD2*, *SHH3/4/9*, and *MDH1/3*) involved in the TCA cycle was upregulated, and their transcription is essential for energy production through oxidative phosphorylation via the use of ethanol as a carbon source [47]. *GLC3*, which synthesizes glycogen from UDPG, and *GPH1*, which is required for the formation of glucose-1-phosphate (G1P) from glycogen, were also upregulated.

Moreover, the analysis of the two strains at the PDSP revealed differences, which showed some variations compared with those at the DSP (Fig. 4B). Hexose transporters (*HXT2/3/4/6/7/14/15/16*) were downregulated. However, the expression of genes related to the TCA and glyoxylate cycles (*CIT1/2/3*, *ACO1*, *IDP2*, *ICL1*, and *MLS1*), the gene *SFC1*, a link between the anaplerotic reactions of glyoxylate, and glycolysis and gluconeogenesis-related genes (*TDH3*, *GPM1*, and *ERR1/2/3*), including the rate-limiting gene *FBP1*, was upregulated, indicating that catabolism through nonfermentable carbon sources occurred more actively in the Rap1-overexpressing strains [48]. Additionally, increased transcription of sterol biosynthetic genes (*IDI* and *ERG10*) could increase the supply of IPP and DMAPP [49, 50], and the upregulation of *PGM1* and *UGP1* could increase the supply of UDPG, an important substrate for UGT enzymes for triterpenoid saponin synthesis [51]. On the other hand, the expression of acetyl-carnitine shuttle genes (*YAT1/2*), encoding a shuttle system that transports cytosolic acetyl-CoA to the mitochondrial matrix, was upregulated, but the acetyl-carnitine shuttle was excluded as a possible route due to the absence of carnitine in the medium [11]. In summary, transcriptome analysis revealed that Rap1 broadly influenced the expression of genes related to CCM, particularly after the diauxic shift phase. These results suggest that Rap1 overexpression can alter carbon metabolic pathways, which is likely to increase carbon flux from nonfermentable sources such as ethanol, glycerol and acetate as well as from glucose. Ultimately, increased carbon flux is expected to increase OSQ production, contributing to increased triterpenoid biosynthesis.

Effect of Rap1 overexpression in the CK-producing strain

NGS-based analysis explained the enhanced OSQ production of CEN.PK-Rap compared with the CEN.PK2-1D, suggesting that Rap1 overexpression could be a promising strategy for improving triterpenoid saponin production. Transcriptome analysis revealed the upregulation of glycolytic/gluconeogenic genes in Rap1-overexpressing strains from the diauxic shift phase. Figure 5A shows the $\log_2$-fold change in the transcription levels of *CCW12*, *RAP1* and the top five highly transcribed glycolytic genes in CEN.PK-Rap at the DSP and PDSP (after glucose depletion) compared to those at the EP (before glucose depletion). The transcription levels of *CCW12* and *RAP1* decreased significantly, with $\log_2$-fold changes of -4.62 and − 3.64, respectively, at the PDSP compared to the EP, suggesting that *RAP1* transcription under the CCW12 promoter may decline more notably over time. In contrast, glycolytic genes such as *ENO1*, *TDH1*, *FBA1*, *GPM1*, and *TDH3* increased their expression at the DSP (from least to most), but at the PDSP, their levels returned toward those observed at the EP except *TDH3*, which maintained its elevated expression level at the PDSP. Therefore, the TDH3 promoter was chosen for expressing Rap1 and heterologous genes to prevent a dramatic decrease in Rap1 or CK biosynthetic pathway genes, which might occur if the CCW12 or other glycolytic promoter were used in the CK-producing strain. This selection is expected to effectively mitigate the potential shortage of Rap1 needed to regulate heterologous genes controlled by the glycolytic promoter, thereby upregulating the CK production pathway and improving CK production.

Construction of the CK-producing strain

We constructed a strain capable of producing the triterpenoid saponin CK to explore the potential of Rap1

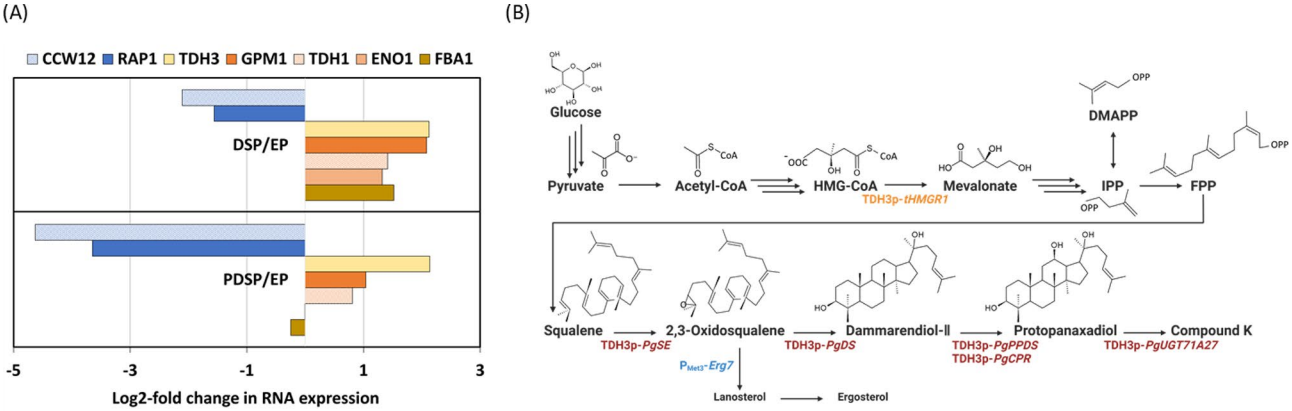

Fig. 5 Construction of a biosynthetic pathway for CK production in *S. cerevisiae*. (**A**) Log_2-fold changes in the transcription levels of *CCW12*, *RAP1*, and the top five highly transcribed glycolytic genes (*TDH3*, *GPM1*, *TDH1*, *ENO1*, and *FBA1*) in CEN.PK-Rap at the DSP and PDSP compared to those at the EP, as observed in the NGS analysis; (**B**) CK biosynthetic pathway in the CK01 strain. Red: heterologous genes from *Panax ginseng*; orange: engineered genes from *S. cerevisiae*. *tHMGR1*, truncated HMG-CoA reductase; *PgSE*, *P. ginseng* squalene epoxidase; *PgDS*, *P. ginseng* dammarenediol-II synthase; *PgPPDS*, *P. ginseng* protopanaxadiol synthase; *PgCPR*, *P. ginseng* cytochrome P450 reductase; *PgUGT71A27*, *P. ginseng* UDP-glycosyltransferases 71A27. All integrated genes are controlled under the TDH3 promoter. Blue: P_{MET3}-*ERG7*: the promoter of yeast lanosterol synthase gene (*ERG7*) was replaced with a methionine-repressible MET3 promoter

overexpression. We used the CEN.PK-Met strain, which was previously constructed from CEN.PK2-1D in our previous study [16]. In CEN.PK-Met, the promoter of the lanosterol synthase gene (*ERG7*), was replaced with a methionine-repressible MET3 promoter to prevent the conversion of OSQ to lanosterol. The construction of the CK-producing strain (CK01) required the introduction of dammarenediol-II synthase (*PgDS*), protopanaxadiol synthase (*PgPPDS*), NADPH-cytochrome P450 reductase (*PgCPR*), and UDP-glycosyltransferase 71A27 (*PgUGT71A27*) derived from *P. ginseng*. Additionally, the overexpression of tHMGR1 derived from *S. cerevisiae* [4, 52] and squalene epoxidase (*PgSE*) from *P. ginseng* [53] is necessary for enhancing the MVA pathway and the supply of OSQ as these native enzymes were strictly regulated and not upregulated by Rap1 overexpression. Thus, we constructed *tHMGR1*, *PgSE*, *PgDS*, *PgPPDS*, *PgCPR* and *PgUGT71A27* expression cassettes that are controlled by the TDH3 promoter and subsequently integrated them into the chromosome of CEN.PK-Met (Fig. 5B; Table 1). We confirmed CK production in CK01 after cultivation for 144 h in shake-flask fermentation (data not shown).

Rap1 overexpression in the CK-producing strain

To confirm the effect of Rap1 overexpression in the CK-producing strain, we generated CK01-TRap by integrating the P_{TDH3}-*RAP1*-T_{RAP1} cassette into the CK01 genome (Table 1). *TDH3* maintains stable expression at DSP and PDSP in CEN.PK-Rap, which is expected to prevent the dramatic decrease in *Rap1* levels in the CK-producing strain that might occur if using the CCW12 promoter. Additionally, we constructed CK01-CRap by integrating the P_{CCW12}-*RAP1*-T_{RAP1} cassette into the CK01 genome (Table 1) to compare the efficiency of TDH3 promoter with CCW12 promoter.

We then examined the growth of three CK-producing strains, CK01, CK01-CRap, and CK01-TRap, and their parent strain, CEN.PK-Met-TLH, over 144 h of cultivation (Fig. 6A). Up to 24 h, the growth of CK-producing strains was similar but not as fast as the parent strain, indicating that these strains might suffer from a metabolic burden caused by the integration of the heterologous pathway. After 24 h, the Rap1-overexpression CK-producing strains (CK01-CRap and CK01-TRap) showed significant growth improvement, and surpassed the parent strain at 72 h. Their OD values were approximately 10% greater than CK01, and these differences persisted until the end of the cultivation. These results suggest that the metabolic burden is not solely due to the heterologous genes but may also be caused by the suppression of the *ERG7* gene. Based on the growth comparison between CEN.PK2-1D and CEN.PK-Rap (data not shown), which indicated that Rap1 overexpression does not affect the growth of the wildtype strain, we conclude that Rap overexpression positively impacts the growth of CK-producing strains. It helps CK-producing strains overcome the metabolic burden caused by the integration of the heterologous pathway and the suppression of the *ERG7* gene, thereby maintaining growth.

We also measured the *RAP1* and *TDH3* mRNA level at 24 h, prior to glucose depletion, and at 48 h, after glucose depletion (Fig. 6B), to compare the Rap1 expression levels and its effect on *TDH3* gene when using CCW12 and TDH3 promoters. Both CK01-CRap and CK01-TRap consistently presented significantly higher Rap1

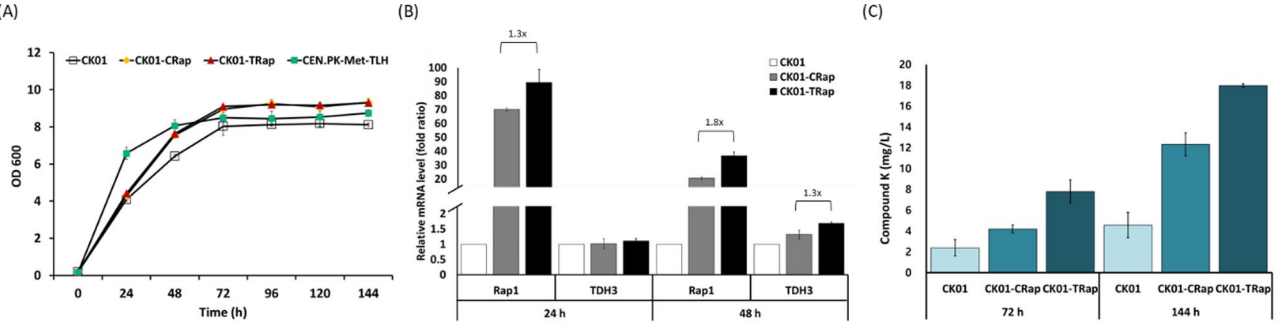

Fig. 6 Confirmation of the effects of Rap1 overexpression in CK01-CRap and CK01-TRap compared with CK01. (**A**) Cell growth of CEN.PK-Met-TLH, CK01, CK01-CRap, and CK01-TRap; (**B**) *RAP1* and *TDH3* transcription levels at 24 h (prior to glucose depletion) and 48 h (after glucose depletion) under different promoters (CCW12 vs. TDH3); (**C**) CK production. Each strain was cultivated for 144 h at 26 °C in 30 mL of SD-His-Leu-Trp medium supplemented with 0.25 g/L methionine and 20 g/L glucose. The samples for the detection of CK and other intermediates were collected after 72 h and 144 h of cultivation. The data are presented as the means ± standard deviations from three independent biological replicates

expression compared to CK01 at both time points, with the TDH3 promoter being 1.3 times stronger than the CCW12 promoter at 24 h and 1.76 times stronger at 48 h. In term of TDH3 expression level, there is no significant differece between three strains at 24 h. However, at 48 h, both Rap1 overexpression strains showed an increased transcriptonal level of *TDH3* compared to CK01, with CK01-TRap exhiniting 1.3 times higher TDH3 expression than CK01-CRap. These data confirmed that the Rap1 overexpression in a glucose-rich environment did not impact TDH3, but after glucose depletion, TDH3 transcription levels were proportional to Rap1 transcription levels, consistent with our observations in the transcriptome analysis in CEN.PK-Rap strain.

After 144 h of cultivation, CK01-TRap produced the highest CK level at 18 mg/L, followed by CK01-CRap at 12 mg/L, both of which were significantly greater than that of the CK01 control strain (4 mg/L) (Fig. 6C). This corresponds to a 4.5-fold increase in CK production in CK01-TRap and a 3-fold increase in CK01-CRap compared with CK01. These findings demonstrate that Rap1 overexpression significantly improves both cell growth and CK production. While both the CCW12 and TDH3 promoters were effective in driving Rap1 expression, the TDH3 promoter provided more stable expression and higher CK production, making it the better choice for maximizing CK production.

Impact of Rap1 overexpression on nonfermentable carbon source utilization and heterologous gene expression in CK production

We verified the effects of Rap1 overexpression on the utilization of nonfermentable carbon sources by the CK01-TRap strain by measuring the consumption of glucose, ethanol, glycerol, and acetate and compared these results with those of the CK01 strain (Fig. 7A). No significant differences were observed in glucose and acetate consumption. However, there were notable differences in the accumulation and consumption rates of glycerol and ethanol between the two strains. CK01-TRap demonstrated more efficient in glycerol and ethanol utilization, particularly at 24 h, 72 h, and 120 h for glycerol and at 72 h, and 120 h for ethanol. These findings confirm that Rap1 overexpression enhances glycerol and ethanol accummulation and consumption during the DSP and PDSP. This effect is likely due to changes in the expression of genes involved in the glycerol pathway (*GPD1*, *GCY1*, *GPP2*, *and STL1*) and the PDH pathway (PDC, ADH, ALD, *SFA1*, *and HFD1*), as suggested by transcriptome analysis of CEN.PK-Rap strain (Fig. 4B). Transcriptome analysis also revealed that the Rap1-overexpressing strain utilizes nonfermentable carbon sources more efficiently, leading to increased OSQ production. We confirmed this by measuring the accumulation of CK intermediates, including squalene (SQ), OSQ, dammarenediol-II (DD), and protopanaxadiol (PPD), in the CK01-TRap strain and compared them with those in the CK01 strain at 72 h and 144 h (Fig. 7B). CK01-TRap accumulated more CK intermediates except for SQ, than did CK01. The total amount of metabolic intermediates was significantly greater in the CK01-TRap strain (Fig. 7B) and remained high even after 144 h, unlike the reduction observed in the CK01 strain. This suggests a continuous and sufficient supply of precursors to the CK synthesis pathway.

Additionally, as heterologous genes for CK production are controlled by the TDH3 promoter, an increase in CK production is also expected through the increased expression of heterologous genes regulated by Rap1 overexpression. We confirmed this improvement by investigating the relative mRNA levels of genes involved in CK synthesis (*tHMGR1*, *PgSE*, *PgDS*, *PgPPDS*, *PgCPR*, *and PgUGT71A27*) in cells harvested after 48 h of cultivation, after which glucose was depleted. The transcription levels of the CK biosynthetic genes under the TDH3 promoter increased up to 2-fold in CK01-TRap compared with those in CK01, except for the *tHMGR1* gene (Fig. 7C),

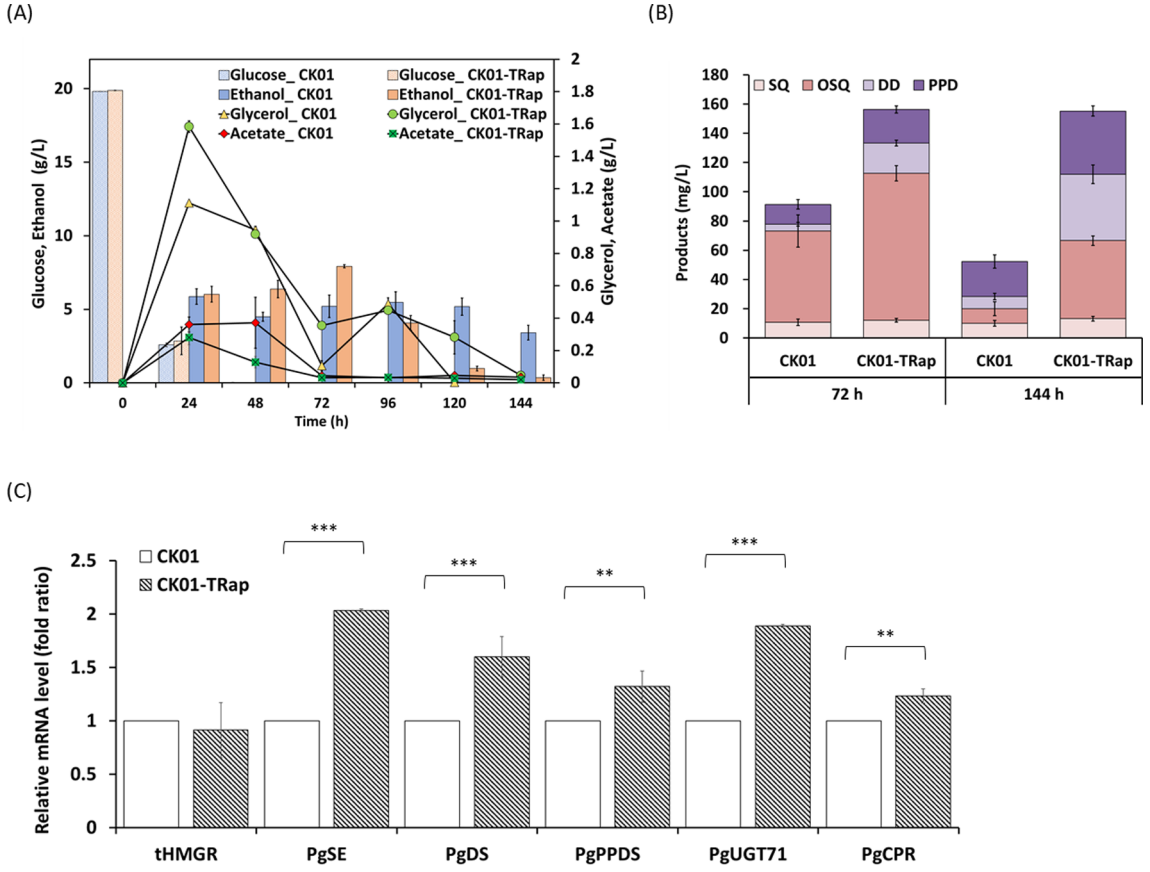

Fig. 7 Effects of Rap1 overexpression in CK01-TRap compared to the CK01 (**A**) Concentrations of glucose, ethanol, acetate and glycerol in the culture medium; (**B**) concentration of SQ, OSQ, DD, and PPD; (**C**) relative mRNA levels of heterologous ginsenoside synthetic genes under the TDH3 promoter. The samples for the detection of CK and other intermediates were extracted after 72 h and 144 h of cultivation. The data are presented as the means ± standard deviations from 3 independent biological experiments. The relative mRNA levels was measured by RT–qPCR after 48 h of cultivation, using *ACT1* as the reference gene. Error bars represent the standard deviations of triplicate sample. Asterisks indicate statistical significance: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$

indicating that Rap1 overexpression upregulated most CK synthesis genes as expected. This increase likely contributed to the enhanced CK production. Therefore, Rap1 overexpression is effective in improving triterpenoid saponin production not only by enhancing the OSQ precursor supply but also by increasing the expression of heterologous genes transcribed by constitutive promoter.

Discussion

In previous studies, the role of Rap1 in regulating glycolytic genes such as *PGK1* or *TDH3* was identified by deleting the Rap1 binding site in the upstream activating sequence of the promoter, as the deletion of Rap1 itself is lethal to cell growth [29, 30, 54]. Functional analysis through Rap1 overexpression had not been pursued due to the results of a study published in 1995 [25], which suggested that overexpression of Rap1 could induce toxicity, although the mechanism underlying this effect was not well established [55]. This led to a focus on studying the downregulation of Rap1 in subsequent research. A recent study [31] revealed that downregulation of Rap1 below average levels positively affected the expression of glycolytic genes during the exponential phase. However, these findings, which suggest that Rap1 can function as a repressor for glycolytic genes during the exponential phase, contradict previous studies and indicate that this aspect remains controversial.

In our study, Rap1 overexpression driven by the CCW12 or TDH3 promoter did not negatively impact cell growth. Furthermore, several interesting findings emerged from the transcriptomic analysis of the Rap1-overexpressing strain, which provides valuable insights into the global effects of Rap1 overexpression and its relation to triterpenoid saponin production.

First, the transcription of glycolytic genes, which are known to be positively regulated by Rap1, remained unaffected by Rap1 overexpression during the exponential phase. This suggests that the natural expression level of Rap1 during the EP is sufficient to direct a transcription program that promotes rapid growth in glucose-rich

environments by increasing protein synthesis and ATP production [56]. In contrast, after a diauxic shift, when Rap1 levels decrease and its transcriptional targets expand, Rap1 overexpression can more strongly upregulate glycolytic genes. This upregulation is significant as it indicates that controlled overexpression of Rap1 can enhance glycolytic activity when the cells transition to utilizing alternative carbon sources, which is crucial for adaptive metabolic responses and compensatory reactions in yeast cells under altered glycolysis or gluconeogenesis gene regulation.

Second, during the DSP or PDSP, Rap1 overexpression is expected to upregulate genes involved in PDH bypass such as PDC, and ADH, the mevalonate (MVA) pathway, and the sterol synthesis pathway. Unexpectedly, it also led to the upregulation of many genes involved in hexose transport, the TCA cycle, and the glyoxylate cycle, which are not typically associated with Rap1 except for *IDP2*, *CIT2/3*, *YAT1*, and *MDH3* [33, 35]. This upregulation suggests that Rap1 may indirectly influence these pathways to increase the supply of cytosolic acetyl-CoA. Acetyl-CoA is a key molecule in triterpenoid production, contributing to ATP and NAD(P)H production, which are expected to be utilized for growth, energy generation, and triterpenoid biosynthesis.

Furthermore, the upregulation of Sip4 (Supplementary Table S7) in Rap1 overexpression during PDSP may regulate genes involved in gluconeogenesis, such as *FBP1* and *PCK1*, as well as *MLS1*, *ACS1*, *MDH2*, *SFC1*, and *IDP2* [57]. The upregulation of these genes may increase the supply of cytosolic acetyl-CoA, which is a key molecule in triterpenoid production, and the production of ATP and NAD(P)H, which are expected to be utilized for growth, energy generation, and triterpenoid production. However, a limitation of this study was the inability to measure ATP and NAD(P)H levels directly.

Finally, Triterpenoid saponins are generally synthesized from OSQ through three steps involving 2,3-oxidosqualene cyclase (OSC), CYP450 and UDP-glycosyltransferase (UGT) (Fig. 1). CYP450 enzymes require NADPH as a cofactor, and UGT enzymes require UDP-glucose as a sugar donor to conjugate sugars to triterpenoids. Previous studies have demonstrated that *ALD3* and *IDP2* regulate NADPH recycling during the postdiauxic shift [58], and *IDP2* has been overexpressed to boost NADPH supply for enhanced triterpenoid saponin production [59]. Similarly, the overexpression of *PGM1* and *UGP1* has been shown to increase UDP-glucose levels, supporting triterpenoid saponin production [51, 60, 61]. As a result, genes like *ALD3*, *IDP2*, *PGM1*, and *UGP*1 are commonly targeted for overexpression to increase triterpenoid saponin production in numerous studies Interestingly, in this study, these key genes were upregulated solely through Rap1 overexpression, highlighting the effectiveness of our approach to enhancing triterpenoid saponin production. These findings align with our goal of strengthening triterpenoid biosynthesis without causing detrimental effects associated with high levels of Rap1.

Conclusion

The strategy of Rap1 overexpression could be generally applicable for the production of other triterpenoid saponins, as it can promote triterpenoid saponin biosynthesis controlled by glycolytic promoters and enhance central carbon metabolism, which increases the uptake of nonfermentable carbon sources from the medium and might promote the circulation of the TCA/glyoxylate cycle for energy generation or cofactor production after the diauxic shift. Furthermore, the benefits of Rap1 overexpression are expected to be more pronounced with longer and more complex heterologous pathways. Overall, this strategy offers a new perspective on the role of Rap1 in cellular metabolism and gene regulation, presenting a viable approach for the production of industrial triterpenoids.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-025-02667-3.

Supplementary Material 1

Supplementary Material 2

Author contributions

J.-Y.B. and S.-C.K. conceived the project. J.-Y.B. performed the experiments. J.-Y.B., T.T.N., B.-K.C., S.-H.P. and S.-C.K. analyzed the data. J.-Y.B. wrote the original draft, and J.-Y.B., T.T.N., and S.-C.K. reviewed and edited the manuscript. All the authors approved the final manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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