Engineering isoprenoid quinone production in yeast

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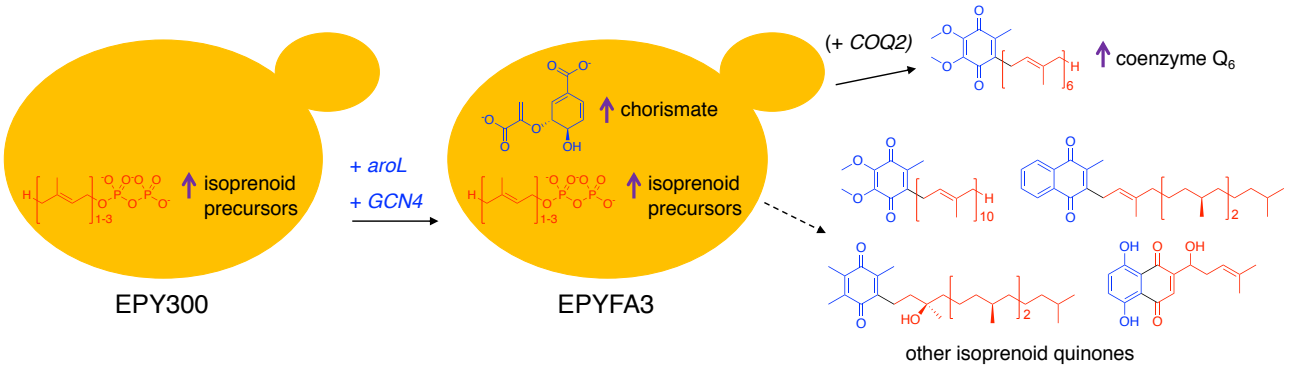


Supporting Information Placeholder

ABSTRACT: Isoprenoid quinones are bioactive molecules that include an isoprenoid chain and a quinone head. They are traditionally found to be involved in primary metabolism, where they act as electron transporters, but specialized isoprenoid quinones are also produced by all domains of life. Here, we report the engineering of a baker's yeast strain, *Saccharomyces cerevisiae* EPYFA3, for the production of isoprenoid quinones. Our yeast strain was developed through overexpression of the shikimate pathway in a well-established recipient strain (*S. cerevisiae* EPY300) where the mevalonate pathway is overexpressed. As a proof of concept, our new host strain was used to overproduce the endogenous isoprenoid quinone coenzyme Q_6, resulting in a nearly three-fold production increase. EPYFA3 represents a valuable platform for the heterologous production of high value isoprenoid quinones. EPYFA3 will also facilitate the elucidation of isoprenoid quinone biosynthetic pathways.

KEYWORDS: *isoprenoid quinones, yeast, coenzyme Q_6, shikimate pathway, specialized metabolites*

Isoprenoid quinones (IQs) are biologically active metabolites that are made of a hydrophobic isoprenoid tail and a polar quinone head.[1] Traditionally, IQs have been found to act as electron transporters in respiratory and photosynthetic electron transport chains.[2] In this context, the isoprenoid moiety allows IQs to be anchored to phospholipid bilayer membranes, whereas the polar head enables their interaction with proteins. Naphthoquinones (menaquinone and phylloquinone) contain a naphthalene-related quinone head, whereas benzoquinones (ubiquinone and plastoquinone) include a benzene-related quinone ring. IQs have mixed biosynthetic origins. Their isoprenoid chain comes from isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP).[3] The quinone head can come from different sources, in particular from the *O*-succinylbenzoate pathway *via* chorismate or from the shikimate pathway *via* chorismate, tyrosine, or phenylalanine.[2,4] Specialized IQs often include a polyketide head group, as commonly seen for fungal meroterpenoids.[5] Ubiquinone-10 (coenzyme Q_{10}),[1] phylloquinone (vitamin K1)[6] and α-tocopherol quinone (vitamin E quinone)[7] are examples of widespread IQs (Figure 1). Species-specific IQs, which are generally regarded as specialized metabolites, include the antitumor furaquinocin A,[8] the antiviral 4-hydroxypleurogrisein,[9] the antioxidant naphterpins[10] and the antitumor antibiotics BE-40644[11] and shikonin (Figure 1).[12,13]

The biosynthetic pathways to ubiquitous IQs have been investigated extensively, although a complete picture is still lacking for compounds such as coenzyme Q.[14] The biosynthesis of specialized IQs is often directed by cryptic metabolic pathways. Their elucidation requires the use of interdisciplinary approaches, such as total synthesis and reconstitution of *in vitro* reactions with recombinant biosynthetic enzymes, as shown for instance by Murray *et al.*[10] for the biosynthesis of naphterpins and marinones. To increase IQs production and to facilitate the elucidation of their cryptic biosynthetic routes, a suitable biological platform providing ample precursor supply is needed.

Here we report the engineering of a *Saccharomyces cerevisiae* (baker's yeast) strain that overproduces the building blocks required for IQ biosynthesis.

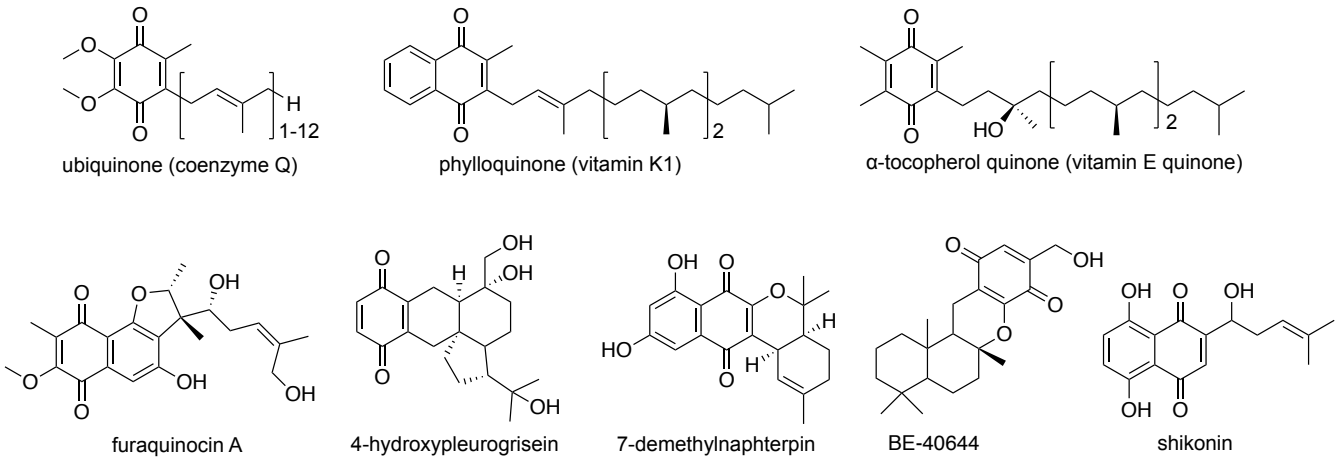


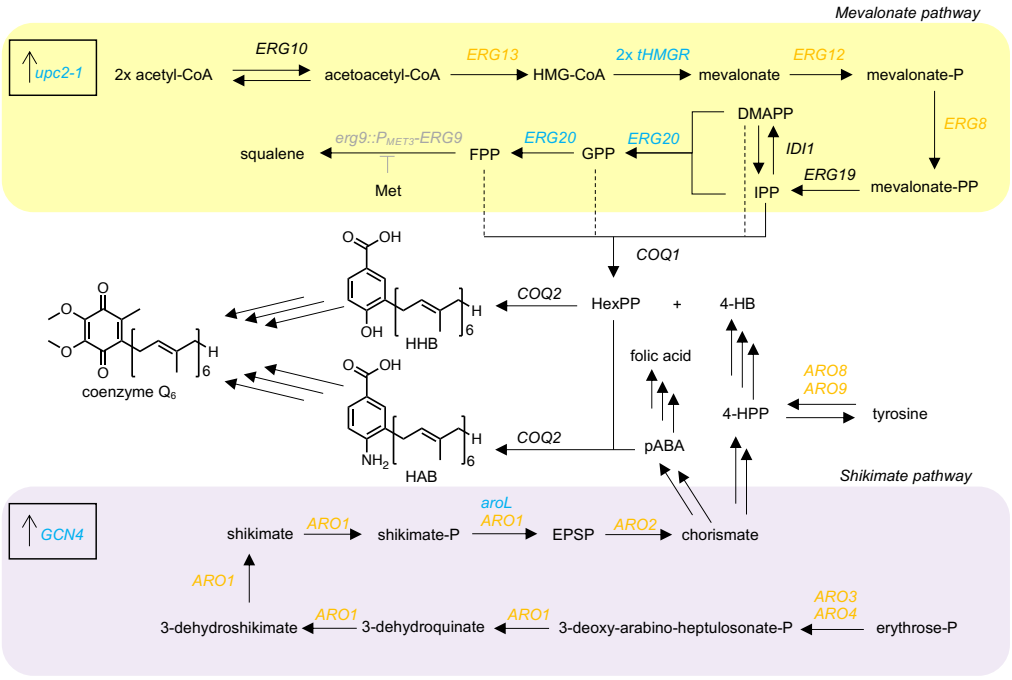
Figure 1. Examples of isoprenoid quinones.

As a proof of concept, we used our strain to improve the production of coenzyme Q_6. *S. cerevisiae* has been metabolically engineered in the past to overproduce isoprenoids.[15] Notably, the Keasling lab has engineered the mevalonate pathway in yeast, in order to provide increased amounts of IPP and DMAPP for the assembly of various downstream products, such as the antimalarial drug precursor artemisinic acid,[16] the biofuel precursor bisabolene,[17] as well as natural and unnatural cannabinoids.[18] Strain *S. cerevisiae* EPY300 (Supporting Table S1) has been used to produce terpenoids, such as artemisinic acid[16] and costunolide,[19] in high amounts.

EPY300 includes two copies of a truncated, soluble variant of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (*tHMGR*), as well as an additional copy of the farnesyl-pyrophosphate (FPP) synthase gene *ERG20*, a copy of the semi-dominant allele *upc2-1* for upregulation of genes involved in the biosynthesis of sterols, and a methionine inducible downregulation mechanism for the control of *ERG9*, which codes for squalene synthase (Scheme 1). Paradise *et al.*[20] showed that in the presence of 1 mM methionine the vast majority of the isoprenoid flux in *S. cerevisiae* EPY213 was diverted away from squalene and ergosterol production to yield amorphadiene. In EPY300 (which compared to EPY213 lacks the amorphadiene synthase-containing plasmid pRS425ADS and contains an additional chromosomal copy of *tHMGR* and *ERG20* respectively) the majority of the isoprenoid flux would similarly be expected to be available to assemble the isoprenoid tail of coenzyme Q_6 upon induction with methionine in a medium that contains either pure galactose or a mixed carbon source glucose/galactose.

In the present work, we acquired *S. cerevisiae* EPY300 from the Keasling lab and engineered it further to overexpress the shikimate pathway. The gene *aroL* codes for the shikimate kinase II in *Escherichia coli*,[21] and its overexpression in *S. cerevisiae* has been shown to improve production of the shikimate pathway-derived *p*-coumaric acid.[22] *GCN4* codes for a leucine zipper transcriptional activator (Gcn4p) in yeast, which is induced under amino acid starvation, and in turn triggers the upregulation of biosynthetic genes involved in the production of most amino acids.[23] The targets of Gcn4p include genes *ARO1*, *ARO2*, *ARO3* and *ARO4*, which in yeast code for the enzymes that catalyze the seven steps of the shikimate pathway leading to chorismate. Chorismate can then be used for downstream processes, such as the biosynthesis of aromatic amino acids and that of the head group of IQs *via* 4-hydroxyphenylpyruvate (4-HPP). Genes *ARO8* and *ARO9*, encoding aromatic aminotransferase I and II, respectively, are also among the targets of Gcn4p. Aro8p and Aro9p catalyze the conversion of 4-HPP into tyrosine and the reverse deamination reaction.[24] Their upregulation can therefore provide additional 4-HPP, starting from the tyrosine present in the culturing medium, that can be used to make the quinone head of IQs. Gcn4p also targets vitamin-cofactor biosynthetic genes, such as those that code for the enzymes responsible for the biosynthesis of NAD+, FAD and coenzyme-A, as well as for the biosynthesis of tryptophan, a precursor of nicotinamide, which in turn is a component of NAD+ and NADP+.[25] Therefore, overexpression of *GCN4* can also lead to increased amounts of the cofactors that are required by secondary metabolite biosynthetic enzymes, including those oxidoreductases that catalyze the assembly of IQs. Eukaryotic cytochrome P450 enzymes, such as CYP76B74 involved in the biosynthesis of the IQ shikonin,[26] require the cofactor NADPH as a source of electrons, and some dehydrogenases use NAD+ or NADP+ as electron acceptors.

In order to produce a yeast strain that would accumulate precursors to IQs in high amounts, we set out to overexpress the shikimate pathway in EPY300. To introduce the *E. coli aroL* and overexpress the endogenous *GCN4*, we assembled the multigene vector pFA011 using the MoClo Yeast Toolkit[27] (see Experimental Methods in the Supporting Information for details on plasmid assembly). The sequence of the shikimate kinase II gene *aroL* (NCBI Gene ID: 945031) was codon-optimized for expression in *S. cerevisiae* (Supporting Table S2), whereas *GCN4* (NCBI Gene ID: 856709) was amplified from the genomic DNA of *S. cerevisiae* EPY300. The strong constitutive promoters *pTEF1* and *pTEF2* were placed upstream of *aroL* and *GCN4*, respectively, as they are known to drive steady gene expression in media with different carbon sources.[28] Upon linearization with NotI, pFA011 was introduced into *S. cerevisiae* EPY300 *via* the LiAc/single-stranded carrier DNA/PEG method,[29] producing strain EPYFA3 (Scheme 1).



Scheme 1. Schematic representation of the engineered metabolism of *S. cerevisiae* EPYFA3. Genes from the mevalonate pathway are grouped in the yellow panel (adapted from Ro *et al.*[16]); genes from the shikimate pathway are grouped in the purple panel. Overexpressed genes are highlighted in light blue; genes indirectly upregulated (from *upc2-1* for the mevalonate pathway and from *GCN4* for the shikimate pathway) are highlighted in orange. Repression of *ERG9* through the methionine-repressible promoter P_{MET3} is represented in grey. Usage of DMAPP, GPP or FPP by *COQ1* is shown through dashed lines as it is unclear which of these allylic pyrophosphates is used *in vivo* to make HexPP. The first reaction in the biosynthetic pathway to coenzyme Q_6, the condensation of HexPP and 4-HB catalyzed by *COQ2*, is also shown. HMG-CoA: 3-hydroxy-3-methyl-glutaryl-coenzyme A; IPP: isopentenyl pyrophosphate; DMAPP: dimethylallyl pyrophosphate; GPP: geranyl pyrophosphate; FPP farnesyl pyrophosphate; HexPP: hexaprenyl pyrophosphate; 4-HB: 4-hydroxybenzoic acid; HHB: 3-hexaprenyl-4-hydroxybenzoic acid; HAB: 3-hexaprenyl-4-aminobenzoic acid; 4-HPP: 4-hydroxyphenylpyruvate; pABA: 4-aminobenzoic acid; EPSP: 5-enolpyruvylshikimate-3-phosphate.

Correct integration of linearized pFA011 in the *LEU2* locus was verified through PCR amplification of the *LEU2* 5' region (Supporting Figure S3a), as well as of the *LEU2* 3' region (Supporting Figure S3b).

In order to confirm that the target genes of interest were upregulated by the transcriptional activator Gcn4p in EPYFA3, we performed RT-qPCR analysis, targeting the aromatic amino acid biosynthetic genes *ARO1*, *ARO2*, *ARO3*, *ARO4*, *ARO8* and *ARO9*. The expression of *GCN4* itself was also evaluated in EPYFA3 and compared to that of the *GCN4* single copy-containing strain EPY300. The expression of *GCN4* was confirmed to be higher in EPYFA3 than in EPY300 by almost a five-fold (Figure 2), due to the introduction of a second copy of *GCN4* under control of the strong constitutive *pTEF2* promoter in EPYFA3. Upregulation of *ARO1*, *ARO2*, *ARO3*, *ARO4*, *ARO8* and *ARO9* was confirmed upon overexpression of *GCN4*, in accordance with what described by Natarajan *et al.*,[23] with values ranging from a three- to a 22-fold increase in EPYFA3 compared to EPY300 (Figure 2).

Next, we tested the ability of *S. cerevisiae* EPYFA3 to be used as a host for the increased production of IQs. As a proof of concept, we decided to test for the overproduction of ubiquinone-6 (coenzyme Q_6), since this is the yeast endogenous benzoquinone and is assembled by condensing building blocks deriving from the mevalonate and the shikimate pathways (see the review of Stefely and Pagliarini[14] for an extensive overview of coenzyme Q biosynthesis). The quinone head of coenzyme Q_6 in yeast comes from either 4-hydroxybenzoic acid (4-HB) or 4-aminobenzoic acid (pABA).[30] In turn, 4-HB comes from 4-HPP, which can either be made *de novo* through the shikimate pathway (involving Aro1p, Aro2p, Aro3p and Aro4p) or derive from the deamination of tyrosine (involving Aro8p and Aro9p), which is an ingredient of synthetic defined (SD) medium at 76 mg/L. Conversely, the isoprenoid tail of coenzyme Q_6 in yeast comes from the mevalonate pathway, presumably as a result of the condensation of IPP with the allylic pyrophosphates DMAPP, geranyl-pyrophosphate (GPP) or FPP.[3]

To assess the ability to redirect building blocks towards a specific metabolite of interest in EPYFA3, we overexpressed the gene *COQ2*, which codes for the 4-HB polyprenyltransferase, a UbiA family prenyltransferase that catalyzes the first committed reaction in the biosynthesis of coenzyme Q_6.[31] The sequence of gene *COQ2* (NCBI Gene ID: 855778) was optimized to remove a BsmBI site through the introduction of a synonymous substitution (G885A).

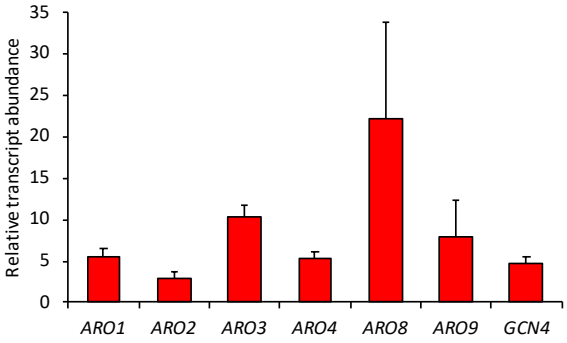


Figure 2. RT-qPCR expression analysis. Relative transcript abundance of genes *ARO1*, *ARO2*, *ARO3*, *ARO4*, *ARO8*, *ARO9* and *GCN4* in *S. cerevisiae* EPYFA3. Actin (*ACT1*) was used as the reference gene, and the average Ct value of each target gene in *S. cerevisiae* EPY300 was used as the calibrator (the expression of each gene in *S. cerevisiae* EPYFA3 is shown relative to the expression of the same gene in *S. cerevisiae* EPY300). Values and error bars reflect mean and standard deviation of three biological replicates.

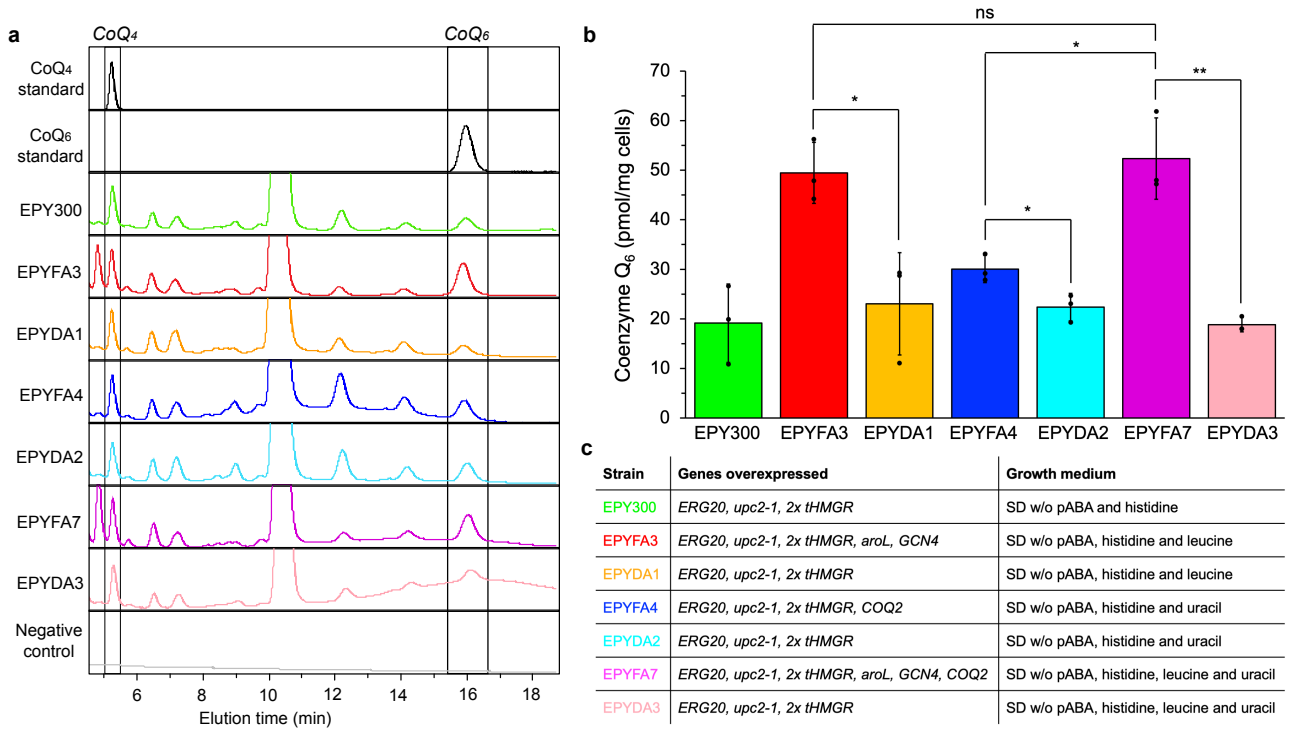
We assembled an expression vector, pFA004, that contained a copy of *COQ2* under control of the strong constitutive promoter pCCW12 (see Experimental Methods in the Supporting Information for details on plasmid assembly). Upon linearization with NotI, pFA004 was introduced into both *S. cerevisiae* EPY300 and EPYFA3 *via* the LiAc/single-stranded carrier DNA/PEG method,[29] producing strains EPYFA4 and EPYFA7 respectively. Correct integration of linearized pFA004 in the *URA3* locus was verified through PCR amplification of the *URA3* 5' region (Supporting Figure S3c), as well as of the *URA3* 3' region (Supporting Figure S3d). Control strains with matching auxotrophy requirements to EPYFA3, EPYFA4 and EPYFA7 were also generated through the integration in EPY300 of empty vectors pFA010 (giving strain EPYDA1), pYTK096[27] (giving strain EPYDA2) and both pFA010 and pYTK096 (giving strain EPYDA3). Correct integration of these vectors in the *URA3* and *LEU2* loci was verified through PCR amplification (Supporting Figure S4a-d).

We determined the amount of coenzyme Q_6 accumulated in strains EPY300, EPYFA3, EPYFA4 and EPYFA7, as well as in control strains EPYDA1, EPYDA2 and EPYDA3. Most of the coenzyme Q_6 produced by *S. cerevisiae* in synthetic medium is known to originate from the pABA that is either acquired from the medium or synthesized endogenously.[30] To obtain a clear insight into the effect of the genetic modifications object of our study, we performed coenzyme Q_6 measurements from yeast cells grown in SD media lacking pABA. Folic acid, which is assembled using pABA and may therefore be depleted in its absence, was added to all cultures at a concentration of 20 µM. Lipids were extracted from pelleted cells using a modified protocol from Marbois *et al.*[32] (see Experimental Methods in the Supporting Information). Coenzyme Q_4 (Sigma-Aldrich) was used as an internal standard and added to all samples in a known amount (expected final concentration 5 pmol μL^{-1}). Samples were analyzed through reversed-phase HPLC diode-array base-peak chromatogram obtained at 274 nm. Quantification was performed using a coenzyme Q_6 (Sigma-Aldrich) external standard and a calibration curve, through the integration of the area underneath the UV peak corresponding to coenzyme Q_6; the recovery of the internal standard coenzyme Q_4 from the lipid extracts was determined through a calibration curve and used to adjust the measured amounts of coenzyme Q_6 for the extraction efficiency. The identity of coenzyme Q_6 in the yeast extracts was confirmed through mass spectrometry (Supporting Figure S5); other metabolites observed in the UV chromatograms could not be unambiguously assigned based on their m/z values. An increase in coenzyme Q_6 production could be observed upon overexpression of the shikimate pathway in strain EPYFA3 (Figure 3), which accumulated 49.4 (± 6.2) pmol mg^{-1} wet cell weight (WCW), compared to EPYDA1, which produced 23.0 (± 10.3) pmol mg^{-1} WCW. Similarly, increased production was detected when *COQ2* was being overexpressed in EPYFA7, corresponding to 52.4 (± 8.2) pmol mg^{-1} WCW, compared to strain EPYFA4 where *COQ2* was also overexpressed but the shikimate pathway was not, which accumulated 30.1 (± 2.7) pmol mg^{-1} WCW. Interestingly, no significant difference could be observed between strains EPYFA3 and EPYFA7, suggesting that overexpression of the shikimate pathway was the main player in the increased production of coenzyme Q_6 in this strain as opposed to overexpression of *COQ2*.

It should be noted that methionine is used to downregulate the expression of the squalene synthase gene *ERG9* but it is also consumed during coenzyme Q_6 biosynthesis in *S. cerevisiae*, as *S*-adenosylmethionine (SAM) is employed as a cofactor by the *O*-methyltransferase Coq3p in the catalysis of two coenzyme Q_6 biosynthetic steps.[33] However, the amount of coenzyme Q_6 produced by EPYFA7 corresponds to 70.71 (± 6.44) nM. Therefore, only 141.42 (± 12.88) nM equivalent of methionine is used for the production of coenzyme Q_6 by the highest producing strain, meaning that ample methionine is available to direct downregulation of *ERG9* through the methionine-repressible promoter P_{MET3} (Scheme 1).

The growth of the four yeast strains was monitored in the same conditions used to extract lipids, measuring the OD_{600} at various timepoints (Supporting Figure S6). A significant difference in growth was observed only between EPYFA7 and EPYDA3, with EPYFA7 showing a 15% ($p<0.001$; unpaired two-tailed t test) higher OD_{600} at stationary phase (7-day timepoint) than EPYDA3, putatively due to an improved fitness upon accumulation of additional coenzyme Q_6.

Noteworthily, since *S. cerevisiae* EPYFA3 is a derivative of BY4742, it harbors unused auxotrophic markers that can be employed to introduce other expression vectors. Loci *LYS2* (mutated to *lys2Δ0*), *URA3* (mutated to *ura3Δ0*) and *HO* can be used for the chromosomal integration of up to three plasmids. *LYS2*, *URA3*, as well as expression cassettes for resistance to antifungals, such as HygromycinR and KanMXR, can be used as selectable markers for the maintenance of integrative and/or replicative plasmids. This will allow EPYFA3 to be used to reconstitute the biosynthesis of IQs, as well as that of any other compounds derived from the mevalonate and the shikimate metabolic pathways.



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Strain	Genes overexpressed	Growth medium
EPY300	*ERG20, upc2-1, 2x tHMGR*	SD w/o pABA and histidine
EPYFA3	*ERG20, upc2-1, 2x tHMGR, aroL, GCN4*	SD w/o pABA, histidine and leucine
EPYDA1	*ERG20, upc2-1, 2x tHMGR*	SD w/o pABA, histidine and leucine
EPYFA4	*ERG20, upc2-1, 2x tHMGR, COQ2*	SD w/o pABA, histidine and uracil
EPYDA2	*ERG20, upc2-1, 2x tHMGR*	SD w/o pABA, histidine and uracil
EPYFA7	*ERG20, upc2-1, 2x tHMGR, aroL, GCN4, COQ2*	SD w/o pABA, histidine, leucine and uracil
EPYDA3	*ERG20, upc2-1, 2x tHMGR*	SD w/o pABA, histidine, leucine and uracil

Figure 3. Quantification of coenzyme Q_6 production. (a) HPLC diode-array base-peak chromatograms (274 nm) of lipid extracts for detection of coenzyme Q_6 (CoQ_6) and coenzyme Q_4 (CoQ_4). Representative chromatograms from one of three biological replicates are shown. (b) Quantification of coenzyme Q_6 from the *S. cerevisiae* strains with HPLC-diode-array detection. Values and error bars reflect mean and standard deviation of three biological replicates; ns, no significant difference; * $p < 0.05$, ** $p < 0.01$; unpaired two-tailed t test. (c) List of *S. cerevisiae* strains with corresponding overexpressed genes and growth medium.

In conclusion, we have engineered a yeast strain that overexpresses both the mevalonate and the shikimate pathways and can therefore be used to produce IQs in which the quinone ring derives from the shikimate pathway. Our approach involved the overexpression of the *E. coli* shikimate kinase II gene *aroL* and that of the transcriptional activator *GCN4*, which in turn led to the upregulation of shikimate pathway genes *ARO1*, *ARO2*, *ARO3* and *ARO4*, as well as of *ARO8* and *ARO9*. Our strategy was similar to the one adopted by Ro *et al.*[16], who achieved upregulation of mevalonate pathway genes through the overexpression of the transcriptional activator *upc2-1*. Further improvement of our strain EPYFA3 may be achieved through a targeted overexpression of each shikimate pathway gene, similarly to what done by Westfall *et al.*[34] for the mevalonate pathway. Multiple copies of *aroL* could be introduced, as the encoded enzyme has a high metabolic flux control.[22] Galactose-inducible promoters may be used to drive the overexpression of shikimate pathway genes (and *COQ2*), in order to coordinate their expression with that of mevalonate pathway genes. Additionally, it should be noted that coenzyme Q_6 measurements in this work were carried out in SD medium lacking pABA to appreciate the effects of the genetic modifications that targeted the *ARO* genes. Inclusion in the medium of pABA, which is the main precursor of the head group of coenzyme Q_6 in *S. cerevisiae*,[30] is expected to lead to higher amounts of coenzyme Q_6.

We envisage that our strain will be particularly useful to express prenyltransferase enzymes and other IQ biosynthetic enzymes, which have recently gained attention as potential biocatalysts.[35] It will also enable to support accumulation of metabolites in amounts sufficient for purification and structure determination. Therefore, it will be a valuable platform to refactor and characterize the cryptic biosynthesis of specialized IQs for which a fast-growing heterologous host is needed.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Experimental Methods; Supporting Figures S1-S6: (i) Assembly of plasmid pFA011, (ii) Assembly of plasmid pFA004, (iii) PCR screening for chromosomal integration in yeast strains EPYFA3, EPYFA4 and EPYFA7, (iv) PCR screening for chromosomal integration in yeast strains EPYDA1, EPYDA2 and EPYDA3, (v) Mass spectrometry detection of coenzyme Q_6 (CoQ_6), (vi) Growth of yeast strains shake-flask; Supporting Tables S1-S2: (i) Strains and plasmids used in this study, (ii) List of oligonucleotides used in this work; Supporting References.

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Author Contributions

# D.K. and D.A. contributed equally to this work. D.K., D.A. and F.A. performed experiments and data analysis. F.A. and C.C provided equipment and materials. F.A., with the assistance of C.C, conceived and designed the study and wrote the manuscript. All authors have given approval to the final version of the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

ACKNOWLEDGMENT

The authors would like to acknowledge E.M. Wellington for providing equipment. E. Silvano and L. Song are thanked for guidance with lipid extraction and analysis. J. Keasling (UC Berkeley) is thanked for providing strain *S. cerevisiae* EPY300. J.E. Dueber (UC Berkeley) is thanked for providing the MoClo Yeast Toolkit. The authors would like to acknowledge funding from the Leverhulme Trust (ECF-2018-691) and the University of Warwick, through an Early Career Fellowship to F.A.; EPSRC and BBSRC (EP/L016494/1) for a scholarship to D.K. through the Centre for Doctoral Training in Synthetic Biology (SynBioCDT); CARA and the University of Warwick for a scholarship to D.A. The Warwick Integrative Synthetic Biology Centre (WISB), a UK Synthetic Biology Research Centre grant from EPSRC and BBSRC (BB/M017982/1), is also acknowledged.

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