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Recent advances in synthetic biology for engineering isoprenoid production in yeast

Claudia E Vickers[1,2], Thomas C Williams[3], Bingyin Peng[1] and Joel Cherry[4]

Isoprenoids (terpenes/terpenoids) have many useful industrial applications, but are often not produced at industrially viable level in their natural sources. Synthetic biology approaches have been used extensively to reconstruct metabolic pathways in tractable microbial hosts such as yeast and re-engineer pathways and networks to increase yields. Here we review recent advances in this field, focusing on central carbon metabolism engineering to increase precursor supply, re-directing carbon flux for production of C10, C15, or C20 isoprenoids, and chemical decoration of high value diterpenoids (C20). We also overview other novel synthetic biology strategies that have potential utility in yeast isoprenoid pathway engineering. Finally, we address the question of what is required in the future to move the field forwards.

Addresses
[1] Australian Institute for Bioengineering and Nanotechnology, The University of Queensland, St Lucia, QLD, Australia
[2] Commonwealth Research Organization (CSIRO), Land and Water, Ecosciences Precinct, 41 Boggo Road, Dutton Park 4102, Queensland, Australia
[3] Department of Chemistry and Biomolecular Sciences, Macquarie University, NSW, Australia
[4] Amyris, Emeryville, CA, USA

Corresponding author: Vickers, Claudia E (c.vickers@uq.edu.au)

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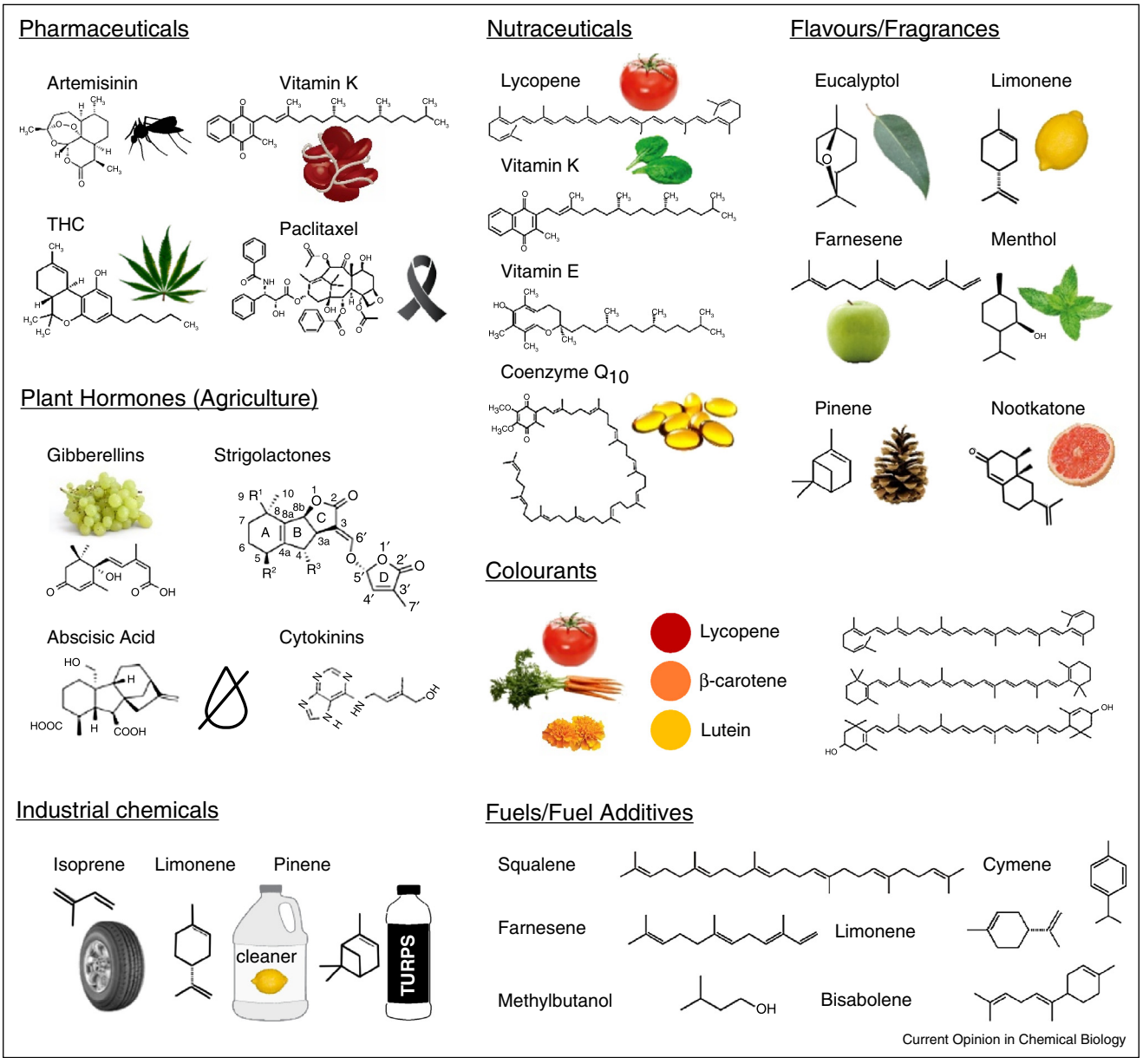
Introduction

The isoprenoid (terpene/terpenoid) family of natural products is home to a large variety of industrially-useful compounds. These range from relatively low-volume, high-value products (e.g. pharmaceuticals) through to high-volume, low value products such as biofuels and bulk industrial chemicals (Figure 1). Regardless of the market size, significant engineering is generally required to achieve sufficient rates, titres, and yields for industrial applications. Isoprenoid biosynthetic pathways have therefore been the subject of extensive research, and have become model systems for development of novel synthetic biology tools aimed at controlling pathway flux for metabolic engineering applications. Of the various production organisms available, yeast has emerged as the most successful due to a wide range of advantages, including: ease of manipulation; depth of genetic and physiological characterisation; availability of engineering tools; high sugar catabolic rate and relatively fast growth rate; relatively high native isoprenoid pathway flux, good capacity to engineer improved isoprenoid pathway flux and to introduce complex functional modifications of products; GRAS status; and amenability to industrial bioprocess conditions.

The rate/titre/yield challenge is obviously most significant for commodity products [1]. Global energy security concerns coupled with environmental concerns around traditional petrochemical products have focussed attention on biofuels in the commodity market. The availability of potential isoprenoid-based drop-in components for a range of fuels from diesel to jet has necessitated substantial innovation in pathway engineering. While we are yet to meet the production efficiencies required for cost-effective production of isoprenoid biofuels, these innovations have provided platform technologies that are now applied to production systems for lower-volume, higher-value products — including mid-range industrial chemicals such as the cosmetic ingredient squalene, liquid farnesene rubber, vitamin precursors, fragrances, and (relatively) high-value pharmaceuticals such as the anti-malarial artemisinin [2,3].

Yeast operates a native mevalonate (MVA) pathway for isoprenoid production (Figure 2). The basic engineering steps for improved pathway flux have been well defined (reviewed in [4–7]). The initial step is usually introduction of a specific product pathway; this serves the dual purpose of providing a metabolic route for production and providing a carbon sink to prevent build-up of prenyl phosphate intermediates, which mediate feedback regulation and may cause toxicity at high intracellular levels [8,9]. MVA pathway flux is then bolstered by expressing native or heterologous MVA pathway genes and further increased by engineering central carbon metabolism. Competing pathways are down-regulated, accumulation of pathway intermediates (which may cause toxicity or simply represent inefficient pathway flux nodes) is minimised by balancing flux throughout the system, and availability of cofactors is balanced. In parallel, the

Figure 1

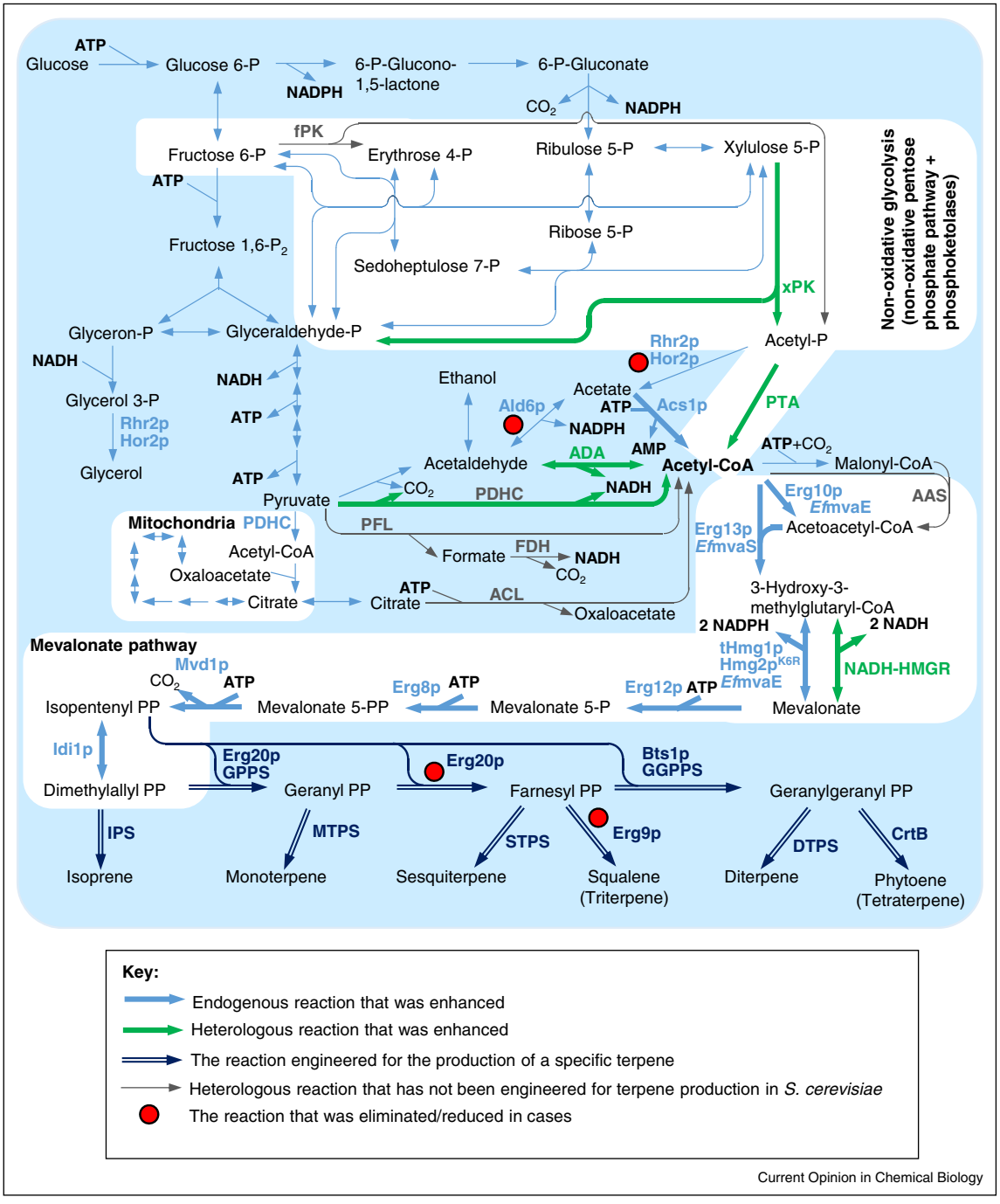


Examples of isoprenoid (terpenoid) products with (real or potential) industrial applications. These include pharmaceuticals, nutraceuticals, flavours, fragrances, colourants, agricultural chemicals, industrial chemicals and chemical feedstocks, and fuels/fuel additives. The authors thank Rebecca Wood for assistance with preparation of this figure. Images used in preparation of this figure were modified from images licensed under creative commons; attributions are provided in the Acknowledgements.

product pathway may require engineering to improve enzyme catalytic properties (rates and/or specificity) for improved titres, specifically for the relevant terpene synthase.

In this review, we will focus on recent advances in synthetic biology tools for engineering isoprenoid production in yeast. In particular, we will examine engineering of central carbon metabolism to increase precursor supply for the MVA pathway, balancing native pathway competition at the farnesyl diphosphate node, and flux redistribution for C10 (monoterpene) and C20 (diterpene) compound production. We will also overview recent advances in engineering for production of high-valued diterpenoid pharmaceuticals and other novel synthetic biology strategies that have potential utility in yeast isoprenoid pathway engineering.

Figure 2



Metabolic network design for terpene production in yeast. (1) Central metabolic pathway: fPK, fructose-6-phosphate phosphoketolase; xPK, xylulose-5-phosphate phosphoketolase; Rhr2p/Hor2p, glycerol-3-phosphate phosphatase; Ald6p, aldehyde dehydrogenase; Acs1p, acetyl-CoA synthase; PTA, phosphate acetyltransferase; ADA, acetaldehyde dehydrogenase (acylating); PDHC, pyruvate dehydrogenase complex; PFL, pyruvate-formate lyase; FDH, formate dehydrogenase; ACL, ATP citrate lyase. (2) Mevalonate pathway: Erg10p, acetoacetyl-CoA thiolase; AAS, acetoacetyl-CoA synthase; Erg13p, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase; EfmvaE, acetoacetyl-CoA thiolase/HMG-CoA reductase; EfmvaS, *Enterococcus faecalis* HMG-CoA synthase; tHmg1p, truncated HMG-CoA reductase 1; Hmg2pK6R, HMG-CoA reductase 2 K6R mutant; NADH-HMGR, NADH-specific HMG-CoA reductase; Erg12p, mevalonate kinase; Erg8p, phosphomevalonate kinase; MVD1p, mevalonate diphosphate decarboxylase; IDI1p, isopentenyl diphosphate:dimethylallyl diphosphate isomerase. (3) Specific terpene synthetic pathway: Erg20p, farnesyl diphosphate synthase; GPPS, geranyl diphosphate synthase; Bts1p/GGPPS, geranylgeranyl diphosphate synthase; IPS, isoprene synthase; MTPS, monoterpene synthase; STPS, sesquiterpene synthase; Erg9p, squalene synthase; DTPS, diterpene synthase; CrtB, phytoene synthase.

Advances in engineering central carbon metabolism to improve acetyl-CoA availability

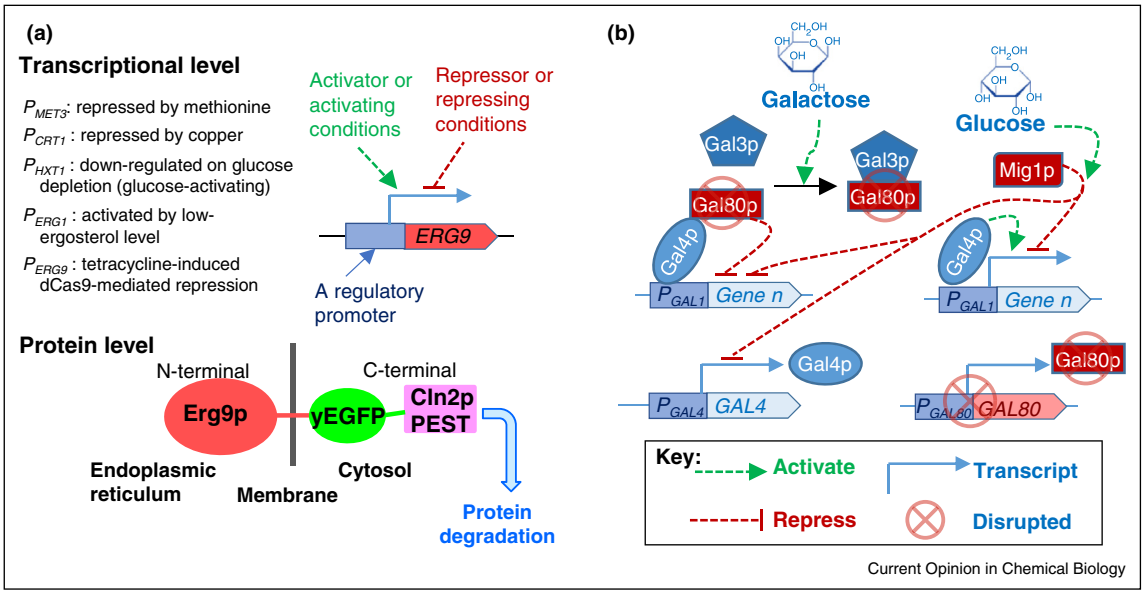
The yeast MVA pathway is connected to central carbon metabolism via acetyl coenzyme A (acetyl-CoA), ATP (energy) and NAD(P)H (reducing power) (Figure 2). Acetyl-CoA is a critical branch-point metabolite in yeast. To initiate isoprenoid biosynthesis, two molecules of acetyl-CoA are condensed to form acetoacetyl-CoA by an acetoacetyl-CoA thiolase (encoded by *ERG10*). Increasing the supply of acetyl-CoA to the MVA pathway, and improving the energetic efficiency of this supply, have therefore been the focus of recent metabolic engineering efforts. However, engineering Acetyl-CoA in yeast is a complex process due to its complex compartmentalised synthesis and involvement in key cellular processes. Cytosolic acetyl-CoA is synthesised from sugar catabolism or during ethanol re-oxidization via acetaldehyde dehydrogenase and acetyl-CoA synthase (ACS), together the pyruvate dehydrogenase (PDH) by-pass; whereas in mitochondria it is produced through the PDH complex (PDHC); there is no direct acetyl-CoA transport between these compartments [10] (Figure 2). Traditional strategies for increasing the supply of acetyl-CoA to the MVA pathway in yeast have involved engineering a PDH bypass by increasing flux from acetaldehyde via overexpressing acetaldehyde dehydrogenase (*ALD6*) and acetyl-CoA synthase (*ACS1* and *ACS2*) enzymes along with native pyruvate decarboxylase expression, resulting in increased amorphadiene production [11]. The PDH bypass was also enhanced by expressing a highly active acetylation resistant acetyl-CoA synthase (L641P) from *Salmonella enterica*, resulting in a further 1.2-fold increase in amorphadiene titre [11]. This approach was recently extended by increasing flux from ethanol to acetaldehyde via alcohol dehydrogenase (*ADH2*) overexpression for isoprene synthesis [12], by eliminating glycerol and ethanol production [13], and by decreasing competition for acetyl-CoA in the glyoxylate cycle with *MLS1* and *CIT2* gene deletions [14]. In another example, pantothanate kinase (*CAB1*) overexpression was combined with established PDH bypass modifications, increasing naringenin titre 24-fold [15].

The ATP requirement of native acetyl-CoA synthases (*ACS1*, *ACS2*) constrains the yields of downstream MVA pathway products, and has therefore become a critical target for improvement of isoprenoid production in yeast. Successful examples include the use of bacterial acetaldehyde dehydrogenase (acetylating) (ADA), pyruvate:formate lyase (PFL), and cytosolic pyruvate dehydrogenases (PDHC) to decouple acetyl-CoA supply from ATP hydrolysis [13,16,17••,18] (Figure 2). A heterologous ATP-citrate lyase has also been used produce acetyl-CoA via TCA cycle derived citrate [19]. Given that this should reduce the overall ATP cost of acetyl-CoA synthesis, it was interesting that isoprenoid yields did not improve. Indeed, choosing the best approach for enhancing acetyl-CoA supply from the many successful examples is difficult at first glance due to different strain performance metrics being used and reported. In an elegant study of pathway stoichiometry, free energy supply, and redox balancing, it was found that the theoretical yield of farnesene from glucose would be highest when a combination of the phosphoketolase/transacetylase reaction and an ATP-independent pyruvate to acetyl-CoA route (as discussed above and depicted in Figure 2) are used [20]. However, achieving the precise distribution of acetyl-CoA synthesis between these two pathways that is required for high farnesene yields (44% and 56% respectively) [20] would necessitate highly advanced synthetic biology tools for balancing relative enzyme expression levels and metabolic fluxes. Rationally engineering this situation would constitute a crowning achievement in the field. As demonstrated by recent achievements, such feats are becoming entirely possible. For example, in a recent *tour de force* of yeast isoprenoid engineering, Meadows *et al.* [21••] combined several strategies to increase the energetic efficiency of the MVA pathway and decrease cellular oxygen demand, resulting in yeast fermentations that generate 15% v/v farnesene. These modifications included the expression of a xylulose-5-phosphate (X5P)-specific phosphoketolase (xPK) along with a phosphotransacetlyase (PTA) to provide a route from X5P to acetyl-CoA (Figure 2) to reduce carbon loss through Embden-Meyerhof-Parnas glycolysis [22]; as well as acetaldehyde dehydrogenase (acylating; ADA) expression to reduce ATP requirements. This non-native pathway increases carbon utilisation (glucose to terpene) by 20% and increases oxygen utilisation by an astounding 280%, facilitating cost-effective terpene production at commercial scale. While these particular modifications reflect those predicted to be optimal from stoichiometric pathway balancing [20], it is likely that fine-tuning the relative supply of acetyl-CoA through the two pathways used would offer further improvements. Central carbon metabolic engineering is emerging as a highly active field, yet there are still many genetic modifications with the potential to increase the MVA pathway fluxes that can be attempted in the future (Figure 2, grey arrows).

Controlling MVA pathway competition at the C15 node

The yeast MVA pathway provides the essential membrane component ergosterol, as well as other important metabolites such as ubiquinone, farnesol, heme A, and dolichol [23]. Sterol synthesis is initiated by the condensation of two C15 farnesyl diphosphate (FPP) molecules by squalene synthase (*ERG9*; Figure 2). Because C15 isoprenoid products such as artemisnin and farnesene also require FPP as a precursor, there is direct competition between target C15 metabolite production and cellular growth and division, as is often the case in metabolic engineering scenarios [24]. This competition has traditionally been controlled by down-regulating squalene

Figure 3



Regulatory control on squalene synthase Erg9p destabilisation **(a)** and engineering galactose-inducible regulatory network to regulate diauxie-inducible transcription from *GAL1* promoter **(b)**: P_{XXXN}, promoter of gene *XXXN*; dCas9, the nuclease-deficient Cas9; yEGFP, yeast enhanced green florescence protein; Cln2pPEST, PEST sequence (rich in Pro, Glu/Asp, Ser, and Thr) from the G1 cyclin Cln2p, which leads protein degradation; Gal3p/Gal80/Gal4p/Mig1p, transcriptional factor.
Figure modified from Refs [27•,31].

synthase expression using promoters that are repressed upon the addition of methionine (P_{MET3} [25]) or copper (P_{CRT3}) [3] to the growth medium, or using the low-glucose repressed *HXT1* promoter [26]. Emerging methods for controlling flux at the FPP node involve the attachment of a protein degradation tag to the C-terminus of squalene synthase (Figure 3a) [27•], and controlling the expression of MVA pathway enzymes using a suite of dynamically regulated promoters [28–30], including modified diauxie-inducible *GAL* expression system (Figure 3b) to couple pathway flux to different growth phases [31]. Native promoters (P_{ERG11}, P_{ERG3}, P_{ERG2}) that are down-regulated by high ergosterol levels have also been used successfully to control *ERG9* expression via an engineered feedback loop, increasing amorphadiene production by 2–5-fold [32]. Deactivated (non-cutting) Cas9 proteins represent versatile tools for regulatory engineering, as they can readily be directed to specific genomic loci by co-expressing homologous guide RNAs. Fine-levels of transcriptional regulation were achieved by targeting a deactivated Cas9 to the promoters of MVA pathway, resulting in simultaneous activation and repression (*ERG9*) of relevant pathway enzymes to control fluxes [33]. RNA based regulators have become highly versatile and easily programmable tools for synthetic biology applications [34]. For example, another recent example in yeast involved the incorporation of an mRNA hairpin cleavage site that is targeted by the yeast Rnt1p RNAse to destabilise the 3′ UTR of *ERG9* mRNA, enabling fine-tuning of MVA pathway flux at the C15 node [35]. General synthetic biology tools that can dynamically regulate gene expression to control competition between growth and production in yeast have also been developed, but not yet applied to isoprenoid synthesis. For example, synthetic quorum sensing [36], RNA interference [37–39], synthetic hybrid promoters [40], synthetic stress-response promoters [41], and synthetic mRNA stem-loop structures and riboswitchs [42,43] all have potential for flux redirection in the MVA pathway.

Engineering prenyl phosphate metabolism for C10 and C20 rather than C15 isoprenoids

The C10 monoterpenes have myriad application as flavours, fragrances, and advanced biofuels, while the C20 diterpenes can form structurally complex molecules such as the anticancer drug precursor taxadiene [44] and the precursor for ambroxan (an ambergris replacement in perfumes), sclareol [45••]. However, engineering the production of C10 and C20 isoprenoids in yeast presents distinct challenges because the native fluxes are 'geared' towards C15 production. There is no dedicated C10 precursor synthase; the native yeast FPP synthase (*ERG20*) condenses the C5 isomers IPP and DMAPP to produce the C10 precursor intermediate geranyl diphosphate (GPP), then adds another IPP to GPP to produce the C15 precursor FPP (Figure 2). *ERG20* does not normally release the C10 precursor intermediate geranyl diphosphate (GPP), so there is not usually a

C10 precursor pool available for monoterpene synthesis. FPP synthase is an essential enzyme, so traditional flux redirection techniques such as gene deletion are not feasible. To overcome this problem, Ignea *et al.* successfully converted the native yeast FPP synthase into a C10 GPP synthase using various protein engineering techniques, enabling a 340-fold increase in the production of sabinene [46•]. Critically, the engineered FPP synthase retained low-level FPP synthesis, enabling ergosterol synthesis and therefore growth. Although yeast has an endogenous geranylgeranyl diphosphate (GGPP) synthase (BTS1) to make the C20 (diterpene) precursor, it does not compete efficiently with Erg9 and Erg20 for the FPP and IPP pools, respectively; this means the native pool of GGPP is relatively small. Avian and bacterial FPP synthase mutants capable of GGPP synthesis are known [47,48]; Ignea *et al.* used structurally homologous regions to and reverse engineer the *ERG20* active site via amino acid substitutions, thereby converting the FFP synthase into a GGPP synthase [45••]. This approach enabled the production of significant amounts of commercially relevant diterpenes [45••].

One classic approach that has been effectively used to engineer fluxes is the use of protein scaffolds to co-localise enzymes and channel substrates [49]. This strategy can be highly effective at redirecting flux at metabolic branch-points such as with the C10, C15, and C20 isoprenoids, as both precursor and product synthase enzymes can be fused. More recently, enzyme co-localisation has been engineered in yeast for the production of the C15 patchoulol with the fusion FPP synthase and patchoulol synthase enzymes [50], for ginsenoside synthesis in *Pichia pastoris* with an *ERG1* and dammarenediol-II synthase scaffold [51], farnesene synthesis with FPP synthase and farnesene synthase co-localised on an affibody scaffold [52], and for sabinene (C10) production via the fusion of GPP synthase and sabinene synthase enzymes [46•]. In all cases, the redirection of fluxes at these metabolic branch-points was demonstrated by increased product yields, emphasising the general effectiveness of this approach as a synthetic biology tool for metabolic engineering.

Cytochromes p450 and diterpene synthesis

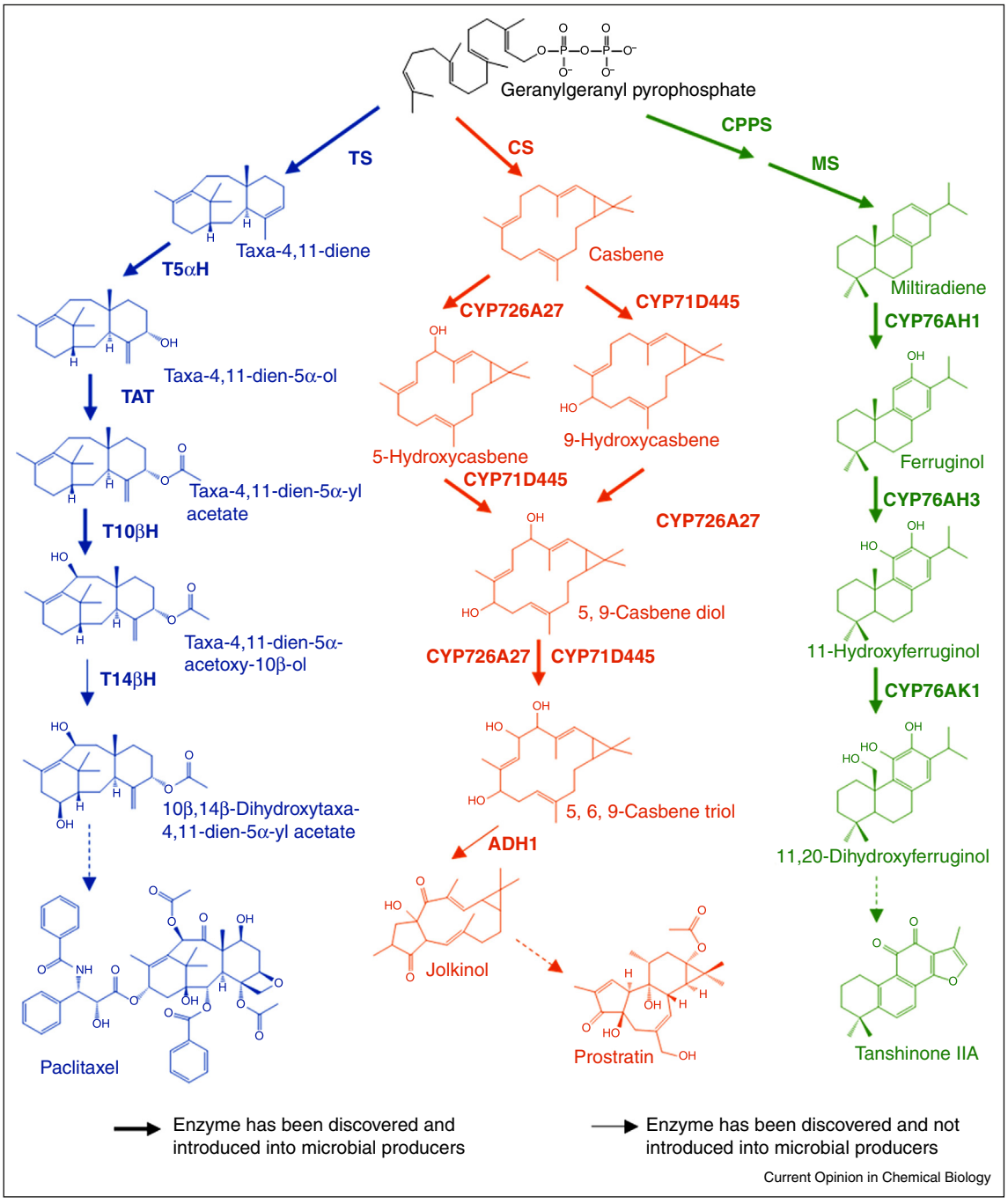
An emerging area of interest in the field of isoprenoid biology has been the production of complex diterpenes (such as the anti-cancer drug paclitaxel, the anti-viral prostratin, and the anti-cancer/anti-inflammatory compound tanshinone) via multiple steps of cytochrome p450-mediated oxidation and modification [53•,54,55] (Figure 4). Utilising yeast as a synthetic biology 'production chassis' for cytochrome p450-derived diterpenes offers the distinct advantage of enabling the functional expression of complex eukaryotic proteins such as cytochrome p450s, with the inherent benefits of large-scale microbial fermentation. However, the biosynthetic pathways have not been fully elucidated, or have only recently been elucidated and have not been implemented in microbial production hosts, for several of these target products (Figure 2). These valuable diterpenes are both difficult to chemically synthesise and impractical to extract from plant material. This situation therefore represents a substantial opportunity for synthetic biology-mediated solutions [56]. Significant challenges confront such projects, as microbial producers such as yeast typically lack compatible redox partners (cytochrome p450 reductases), have low cytochrome p450 expression levels, and can be susceptible to toxicity from foreign metabolites [55,57]. Recently, the cytochrome p450 reductase *ATR1* from *Arabidopsis thaliana* was co-expressed with cytochrome p450s in yeast for the synthesis of tanshinone precursors [53•] (Figure 4). Previous studies have demonstrated that the ratio between cytochrome p450 reductases and cytochrome p450s is important for efficient oxidation of diterpene precursors such as amorphadiene and taxadiene [58,59]. For the production of jolkinol, two cytochrome p450s from the plant *Euphorbia lathyris* were expressed in yeast along with the heterologous alcohol dehydrogenase (*ADH1*) [54] (Figure 4). However, this strain did not produce the cyclic product jolkinol, indicating that further knowledge and/or optimisation is required to enable the synthesis of valuable jolkinol-derived pharmaceuticals such as prostratin. cytochrome p450 mediated diterpene production in synthetic yeast chasses with high MVA pathway flux is a promising but nascent field [56], with significant development required to achieve commercialisation and societal impact.

In addition to the synthesis of diterpenes, cytochrome p450s have already played a significant role in the production of other valuable isoprenoids. For example, the production of artemisinic acid from amorphadiene required the successful expression of an *A. thalina* amorphadiene oxidase (cytochrome p450) and its cognate reductase [3]. Interestingly, this reaction was enhanced by co-expressing a cytochrome b5 that was identified in an A. thaliana complementary DNA library [3]. Executing these steps *in vivo* reduces the costs of downstream processing significantly, and continued development of cytochrome p450 expression systems in yeast will be required to realise a full spectrum of isoprenoid production strains.

Future perspectives for synthetic biology and yeast cell factories

Over the last few decades, genetic engineering has been developing towards large-scaled genetic re-design and reconstruction using synthetic biology methods, with yeast as a key organism. Yeast is set to remain at the forefront of synthetic biology, metabolic engineering, and industrial bioprocessing for the foreseeable future. There are currently dramatic developments occurring in the fields of systems and synthetic biology that will have profound effects on the way that yeast are engineered to produce a

Figure 4



Synthetic pathways of example diterpenoids (paclitaxel, prostratin, and tanshinone). Terpene synthases includes: TS, taxadiene synthase; CS, casbene synthase; CPPS, copalyl diphosphate synthase; MS, miltiradiene synthase. Cytochromes P450 (CYPs) from *Taxus* sp. include: T5αH, taxadiene 5α-hydroxylase; T10βH, taxane 10β-hydroxylase; T14βH, taxoid 14β-hydroxylase. CYPs from *Euphorbia lathyris* L. include CYP726A27 and CYP71D445. CYPs from *Salvia miltiorrhiza* include CYP76AH1, CYP76AH3 and CYP76AK1. TAT is taxadien-5α-ol O-acetyltransferase from *Taxus* sp. ADH1 is alcohol dehydrogenase from *S. miltiorrhiza*.

range of valuable metabolites. The design-build-test (DBT) cycle that is now employed by most synthetic biologists will eventually be replaced with a process more akin to 'Design-Build-Deploy' (DBD) [60] as fields such as synthetic genomics [61], and the practice of automated high-throughput strain construction and screening [62] mature, become cheaper, and gain wider adoption. The synthetic yeast genome project 'Yeast 2.0' [63] will

contribute to the rapid construction of yeast cell factories via the inducible evolution system termed 'SCRaMbLE' (Synthetic Chromosome Recombination and Modification by LoxP mediated Evolution), which enables the creation of millions of yeast genomes with unique genome content and architecture upon the expression of a recombinase enzyme that targets all non-essential genes in the genome [64,65••]. By applying various selection pressures to SCRaMbLEd yeast populations, it will be possible to isolate strains with superior growth characteristics of relevance to industry. High metabolite-production phenotypes can also be selected using high-throughput analytical screening or metabolite-responsive biosensors and fluorescence activated cell sorting [66–69]. An elegant example of biosensor-mediated evolution for diphosphate production was demonstrated when a synthetic transcription factor was used to reduce mutation rates in response to high IPP levels [70]. This system enabled a rapid search of genomic mutation 'solution space', with mutations reducing and the genome stabilising once high levels of IPP had been evolved.

The yawning gap to reach the ultimate goal of synthetic biology is still the lack of knowledge of complicated biochemical and cellular activities. Coupled with the advances in the design, construction, and selection of yeast cell factories, will be the use of systems biology knowledge and techniques to understand fundamental 'design principles' for superior strain performance. Numerous *S. cerevisiae* genomes were recently sequenced and annotated, providing a source of novel alleles and pan-genome functions in the *Saccharomyces* Genome Database [71], genetic 'parts' such as promoters, terminators, and protein degradation tags have been characterised in-depth [72], and a prototrophic yeast deletion collection has been created to enable more accurate analysis of the effects of gene deletions on yeast physiology [73]. Information from systems analysis and/or high-throughput strain construction and screening can then be fed back into future designs as part of a positive feedback loop that ultimately leads to the realisation of the DBD paradigm. To progress this agenda, the community requires an easily-accessible database of engineering processes. Similarly, process automation platforms using computer-aid design [74] and friendly human-computer interfaces will make such large-scaled genetic engineering/screening simpler, cheaper, and faster; leading to a new era in synthetic biology. As isoprenoids represent such a diverse class of compounds with applications as industrial chemicals, biofuels, pharmaceuticals, flavours, and fragrances [75,76], their production in synthetic yeast cell factories will both enable and benefit from the DBD revolution.

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have been highlighted as:

- • of special interest
- •• of outstanding interest

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