MINIREVIEW

Advancing secondary metabolite biosynthesis in yeast with synthetic biology tools

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Abstract

Secondary metabolites are an important source of high-value chemicals, many of which exhibit important pharmacological properties. These valuable natural products are often difficult to synthesize chemically and are commonly isolated through inefficient extractions from natural biological sources. As such, they are increasingly targeted for production by biosynthesis from engineered microorganisms. The budding yeast species *Saccharomyces cerevisiae* has proven to be a powerful microorganism for heterologous expression of biosynthetic pathways. *S. cerevisiae's* usefulness as a host organism is owed in large part to the wealth of knowledge accumulated over more than a century of intense scientific study. Yet many challenges are currently faced in engineering yeast strains for the biosynthesis of complex secondary metabolite production. However, synthetic biology is advancing the development of new tools for constructing, controlling, and optimizing complex metabolic pathways in yeast. Here, we review how the coupling between yeast biology and synthetic biology is advancing the use of *S. cerevisiae* as a microbial host for the construction of secondary metabolic pathways.

Yeast as a host cell for secondary metabolite production

Secondary metabolites are valuable natural products (Williams *et al.*, 1989). Although they do not participate in the central metabolic processes of a living cell, secondary metabolites play important roles in an organism's function. Numerous secondary metabolites are defense compounds that protect against biotic and abiotic stresses such as infection, predation, ultraviolet radiation, and drought (Pichersky & Gang, 2000). Other secondary metabolites act as regulatory molecules, coordinating the activities of cells within a multicellular organism or within a population of single-celled microorganisms (Kell *et al.*, 1995; Davies, 2010). While many organisms synthesize secondary metabolites, the plant kingdom produces the greatest number of these small molecules (Croteau *et al.*, 2000).

Secondary metabolites can exhibit powerful bioactive properties in the human body. Certain plant defense chemicals exhibit similar protective functions in humans, for example, by acting as antioxidants or antimicrobials providing protection against bacterial, viral, fungal, or other parasitic infections. Other plant chemicals are toxic to humans, but at very low doses may cause favorable effects such as analgesia or the death of tumor cells. The human body also synthesizes its own secondary metabolites, which are used to regulate physiological functions such as inflammation and immune responses (Hillier, 2007).

As secondary metabolites have wide-ranging implications for human health, it is essential to ensure a sufficient supply of these molecules. Extraction from natural sources, such as plant biomass or fungal or bacterial cultures, typically suffers from inadequate yields owing to slow biomass accumulation, low quantities of the molecule present in the native host, and the need to purify the desired product from similar chemical species produced by the complex secondary metabolism of the native organism. Total chemical synthesis is similarly difficult because of diminished yield from the multiple transformations required to synthesize complex natural

molecules. As a result, microbial biosynthesis strategies are gaining popularity for providing production methods for secondary metabolites.

Among microorganisms commonly used for biotechnological applications, yeast species are proving to be particularly suited to host diverse secondary metabolite biosynthetic pathways. The budding yeast, *Saccharomyces cerevisiae*, is also a common industrial microorganism used extensively in food and beverage production. *S. cerevisiae* is also a key model organism for fundamental molecular biology research, and it was the first eukaryotic organism to have its genome completely sequenced (Goffeau *et al.*, 1996). This fundamental knowledge base has led to the development of a number of tools for pathway construction in this organism, including methods for controlling functional expression of heterologous genes and ensuring the genetic stability of the introduced genes (Mumberg *et al.*, 1994, 1995). Yeast has the added advantage of exhibiting limited native secondary metabolism, minimizing the interference or competition of any natural pathways with the engineered pathways. In addition, many '–omics' level tools exist for studying the native physiology and genetics of yeast – transcriptomics and metabolomics in particular – that can be used to study the global impact of the introduced enzymes on cellular processes including depletion of substrates and cofactors or responses to toxicity and oxidative stress. Finally, researchers have developed detailed models of metabolic processes in this organism that provide an increased capability to predict the behavior of an altered metabolic network in a host cell (Duarte *et al.*, 2004).

Despite this long list of advantages, several challenges exist in the use of yeast as a host cell for secondary metabolite pathway engineering, especially when contrasted against existing capabilities in bacterial hosts. For example, bacterial hosts can support multigene (polycistronic) transcriptional units, allowing the coordinated expression of many heterologous genes within compact operons, whereas such capabilities do not readily exist in yeast. However, new tools developed through synthetic biology are allowing researchers to take advantage of the unique strengths of a microbial eukaryotic host cell. In particular, yeast have the ability to support the functional expression of heterologous enzymes, such as cytochrome P450s, that exhibit activity within the environment provided by an endomembrane such as the endoplasmic reticulum or mitochondrial inner membrane (Pompon *et al.*, 1996). In addition, yeast carry out post-translational modifications to support the functional expression of enzymes from plants and mammals (Eckart & Bussineau, 1996). Here, we discuss the tools available for the construction and control of secondary metabolic pathways in *S. cerevisiae* as well as the current repertoire of constructed biosynthetic pathways. In reviewing this extensive list of pathways, we will highlight how the coupling between foundational knowledge in yeast biology and modern synthetic biology is advancing the use of *S. cerevisiae* as a microbial chemical factory for the production of secondary metabolites.

Mining and accessing genetic information

Advances in DNA sequencing and synthesis technologies are fueling new paradigms in biosynthetic pathway design, where researchers can gain access to the biological components underlying metabolic pathways of interest without the physical exchange of genetic material (Voigt, 2008). The advancement of sequencing technologies have enabled researchers to sequence the complete genomes of thousands of species and the expressed sequences (mRNAs) of many more, and from this combine computationally derived annotations of genetic elements with those determined experimentally to build vast databases of natural product biosynthetic genes (Caspi *et al.*, 2010; Cochrane & Galperin, 2010; Benson *et al.*, 2011). This immense wealth of sequence data provides the templates from which genes encoding enzymes of known or predicted function can be selected as components of engineered biosynthetic pathways. Assembly of such pathways has been aided by advances in DNA synthesis technologies that enable increasingly rapid and economical gene synthesis. In addition, synthesizing genes de novo has the added advantage of allowing for refinement of the gene sequence to optimize codon usage for a given host cell (Gustafsson *et al.*, 2004). Improved expression by codon optimization has been shown to improve activities of pathway enzymes relative to that of native gene sequences (Redding-Johanson *et al.*, 2011). The predominant method of gene synthesis is to first synthesize short oligonucleotides, which can be carried out with accuracy and purity up to a couple hundred nucleotides (Reese, 2005), that are then assembled into gene-length sequences (~kbs) by polymerase chain reaction (PCR) assembly (Stemmer *et al.*, 1995). With the continuing automation of the design, synthesis, and assembly of oligonucleotides (Hoover & Lubkowski, 2002; Kodumal *et al.*, 2004), many laboratories outsource gene synthesis to commercial suppliers.

The combined advances in sequencing and synthesis are allowing researchers to mine databases for putative enzyme coding sequences, synthesize those sequences directly in desired expression vectors, and then test the activity of candidate enzymes in the desired cellular host. As one recent example, researchers utilized this 'synthetic metagenomics' approach to identify candidate enzymes

for methyl halide production (Bayer *et al.*, 2009). The sequences of known methyl halide transferases (MHTs) were used to search for related sequences across databases accessible through NCBI. Eighty-nine putative enzyme candidates from plants, fungi, bacteria, and archea were synthesized, expressed in yeast, and examined for the desired activity. Approximately 94% of the examined enzymes exhibited activity on at least one of the three halide ions tested, with the greatest activity attributed to a MHT from a halophyte plant. While this work demonstrates the power of the synthetic metagenomics approach, it should be noted that the commercial synthesis of 89 *MHT* genes (averaging *c.* 700 bp each) costs more than $20 000 at standard synthesis rates. Thus, further optimization is required within the 'write' process of DNA synthesis to reduce synthesis costs and realize the opportunity for metabolic engineering provided by the increasing amount of genetic material that is being made available through the 'read' process of DNA sequencing.

Rapid assembly of biosynthetic pathways

While advances in synthesis are enabling broader access to genetic sequences, new techniques for DNA assembly are leading to transformative advances in building biosynthetic pathways in yeast. The ability of *S. cerevisiae* to be transformed with and stably maintain large sequences of DNA has long made it a workhorse for genome sequencing efforts. The central technique for building large heterologous DNA constructs in yeast is transformation-associated recombination (TAR), which leverages yeast's unique capability to efficiently combine many DNA fragments by homologous recombination in a single step (Larionov *et al.*, 1996). Recently, researchers have demonstrated the ability to assemble and clone whole bacterial genomes in yeast (Gibson *et al.*, 2008) using a combination of enzymatic *in vitro* assembly (Gibson, 2011) and TAR-based assembly (Benders *et al.*, 2010) methods. This foundational advance opens up the possibility of rapidly assembling large biosynthetic pathways using a combination of oligonucleotide synthesis, *in vitro* assembly, and yeast-mediated TAR assembly, providing a scalable alternative to traditional recombinant DNA cloning methods.

Initial attempts at applying these techniques to one-step whole-pathway assembly in yeast have yielded promising results. In one example, a random assembly protocol was developed for combinatorial screening of an engineered flavonol pathway (Naesby *et al.*, 2009). Dozens of gene cassettes were designed from mix-and-match promoter and terminator sequences flanking codon-optimized flavonol biosynthesis gene-coding ORFs identified from a variety of species. The cassettes were randomly concatenated by *in vitro* ligation into YAC vectors. A single transformation reaction yielded a library of clones harboring engineered YACs (eYACs) that varied in length from 50 to 500 kb with an estimated average of approximately 50 cassettes (130 kb) per eYAC. Screening of the assembled eYAC libraries identified strains that produced a range of natural flavonol compounds *de novo* as well as strains that converted halogenated precursor molecules to halogenated flavonol compounds. In a second example, an assembly technique based on concatenation of gene cassettes by TAR was applied to simultaneously incorporate a xylose utilization pathway and a carotenoid synthesis pathway into a yeast strain (Shao & Zhao, 2009). Cassettes were designed with flanking overlap homology regions that directed the assembly of the genes in a prescribed order. Additional homology regions were added to the ends of the assembled sequence that either directed assembly into a plasmid vector or integration into a chromosomal delta sequence along with a selectable marker. Assembly efficiency for the gene cassettes was measured with varying lengths of overlapping homology (approximately 50, 125, 270–430 bp) and was generally determined to increase with increasing overlap length. This assembly strategy was later extended to the assembly of polyketide synthesis pathways in yeast (Shao *et al.*, 2011). However, in this later work the yeast-assembled gene sequence was isolated from the yeast strain and transformed into a bacterial production strain.

While these recent examples highlight the power of DNA assembly for constructing large biosynthetic pathways in *S. cerevisiae*, the broader application of these techniques currently faces several challenges. In both examples, a diverse set of appropriate promoter and terminator sequences were meticulously selected prior to cassette assembly. Analogs of the methionine-repressed *MET25* and *MET2* promoters were gathered from four different species and matched with six different terminator sequences to build a combinatorial set of 24 'entry' vectors for the eYAC system. Eight different constitutive promoters and eight separate terminators were matched to each of the different genes in the combined pathway assembly example. The need for variety in promoter-terminator sequences is a result of two important properties: (1) yeast do not readily express polycistronic genes; and (2) yeast harbor highly active homologous recombination machinery, which can lead to deleterious recombination events between two identical or similar sequences. The first property requires that every gene has its own promoter and terminator flanking sequences, whereas the second property requires care in avoiding the reuse of identical or similar regulatory elements when assembling long DNA sequences in yeast. Therefore, researchers

assembling large biosynthetic pathways in yeast would benefit from a large catalog of well-characterized promoter and terminator sequences.

Tools for controlling enzyme expression levels

While advances in DNA sequencing, synthesis, and assembly have eased the identification, procurement, and manipulation of target biosynthetic genes, significant challenges remain in the physical construction and implementation of an engineered biosynthetic pathway. The goal of reaching maximal yield depends on many factors including optimizing pathway flux, reducing toxic intermediates, and balancing stress on the cell host. Therefore, one of the key challenges in pathway engineering is applying appropriate expression and regulation of individual pathway enzymes for optimal activity. A variety of tools for tuning expression of heterologous genes have been described for *S. cerevisiae* that act by modifying gene copy number, transcriptional activity, or post-transcriptional processing (Fig. 1b-d).

Copy number

The first toolset for tuning expression of heterologous genes in yeast is based on modifying gene copy number by selecting a DNA construct to harbor the desired cassette. A number of constructs exist for modifying the copy number of heterologous genes in yeast that are based on expression from plasmids or artificial or native chromosomes. High-copy plasmids rely on the 2 μ origin

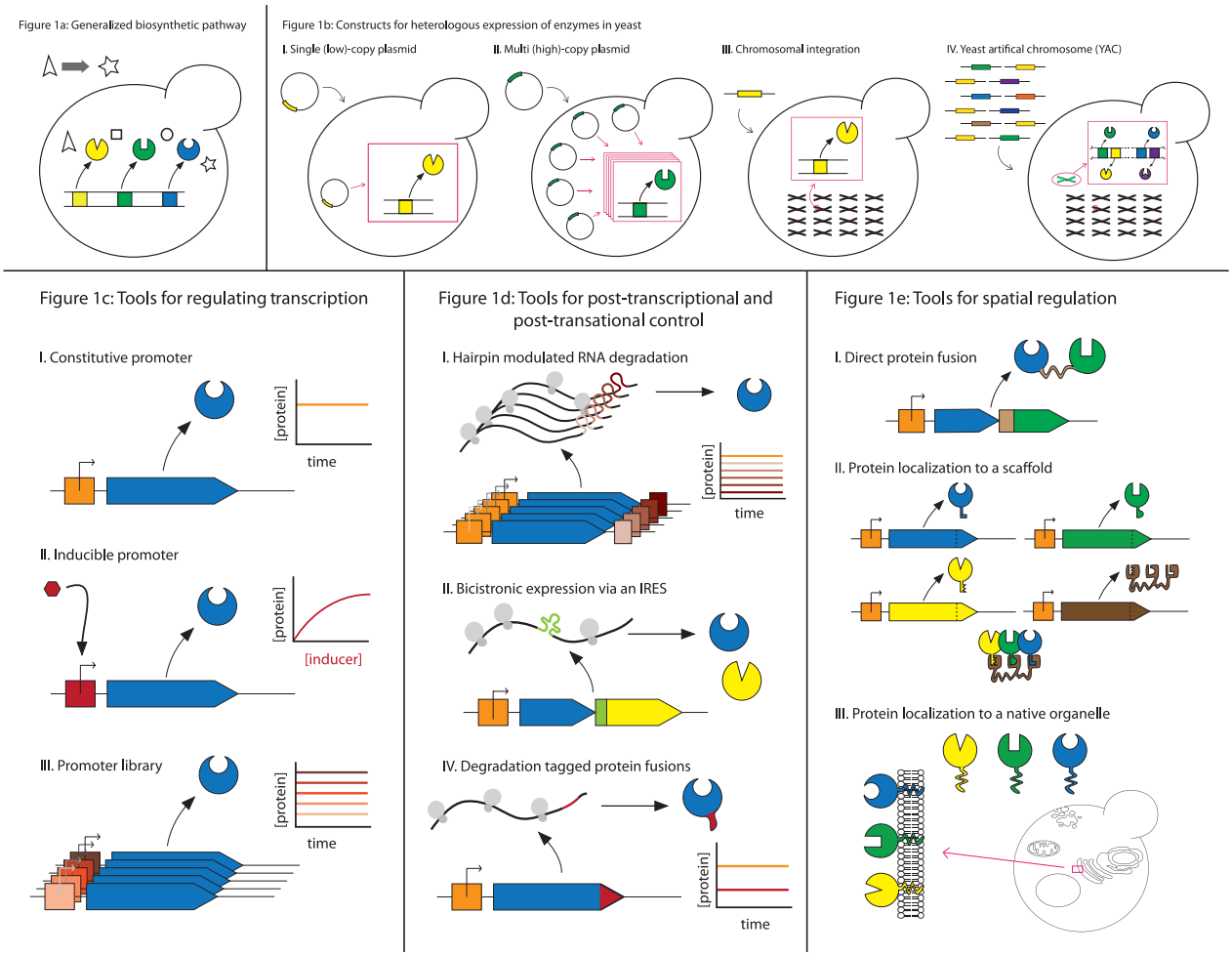


Fig. 1. Tools for controlling enzyme expression in yeast. (a) Metabolic engineering efforts in yeast utilize an array of tools for the expression and regulation of heterologous genes in *Saccharomyces cerevisiae*. Tools enabling heterologous enzyme expression (b), transcriptional regulation (c), post-transcriptional and post-translational regulation (d), and spatial regulation (e) in yeast are illustrated.

of replication for maintenance of a construct at approximately 5–30 copies per cell (Mumberg *et al.*, 1995; Fang *et al.*, 2011). High-copy plasmids allow for strong expression of the encoded genes, which can impart significant burden on the cell and result in instability of the construct. Low-copy plasmids rely on an autonomously replicating sequence paired with a yeast centromere (CEN/ARS) to allow for stable maintenance at very low copy number (approximately 1 copy per cell; Parent *et al.*, 1985). Low-copy plasmids provide a more stable expression platform, but are associated with lower gene expression levels. A large heterologous DNA cassette (up to several Mbps) can also be integrated into an artificial chromosome (YAC; Bruschi *et al.*, 2006; Kouprina & Larionov, 2008). However, selective pressure is required for maintenance of plasmid and YAC constructs in yeast, which requires the use of selective media and can result in incomplete selection of cells harboring the desired constructs.

Researchers can take advantage of the natural ability of *S. cerevisiae* for homologous recombination to insert a DNA cassette into a target locus on the native yeast chromosome. Chromosomal integration provides the most stable expression platform and does not require continuous selective pressure for maintenance of a construct. However, expression levels of genes within integrated cassettes can be significantly impacted by location of the integration locus on the chromosome. Recently, a variety of loci have been characterized based on relative strength of an integrated reporter construct (Flagfeldt *et al.*, 2009; Fang *et al.*, 2011). In addition, targeted integration strategies can be laborious, where building strains with multiple constructs or the integration of multiple copies of a single construct to increase gene copy number typically requires sequential chromosomal integration events. A recent study demonstrated a potential method to build combinatorial libraries by sequential targeted integration for screening enzyme variants within a pathway. This 'Reiterative Recombination' method combined high-efficiency, nuclease-induced homologous recombination with multigene cassettes that harbor biosynthetic pathway genes with alternating, replaceable selection markers. Researchers built a lycopene biosynthetic pathway with this reiterative technique and showed that integration efficiencies were high enough to identify functional biosynthetic genes in a theoretical library of 10^4 variants (Wingler & Cornish, 2011). Even with the utilization of high-efficiency recombination techniques, the multiple rounds of cassette integration, marker selection, and plasmid curing represent a relatively substantial expenditure of labor. As such, automation techniques applied to strain engineering will be an important strategy to scaling these methods for large pathways. In addition, strategies for integration at multiple loci from transformation of a single construct have been developed for strong, heterologous protein expression by targeting repeating ribosomal (Lopes *et al.*, 1989; Fujii *et al.*, 1990; Lopes *et al.*, 1996), delta element (Sakai *et al.*, 1990; Parekh *et al.*, 1996; Lee & Da Silva, 1997a, b; Ekino *et al.*, 2002; Oliveira *et al.*, 2007), and sigma element sequences (Kudla & Nicolas, 1992). The delta element integration sequence has also been used for the construction of biosynthetic pathways. In one study, a two-gene biosynthetic pathway for the production of 1,2-propanediol was constructed using delta element integration cassettes. Strains with three copies of both genes showed the highest production, although a clear correlation between gene number and production was not apparent (Lee & Da Silva, 2006). The single-step pathway assembly strategy described above for carotenoid biosynthesis also used delta element integration cassettes (Shao & Zhao, 2009), but did not confirm a specific copy number of the integration events. As multisite integration strategies continue to be tested for biosynthetic pathway construction in yeast, the technique may provide a useful tool for manipulating activities of specific pathway enzymes.

Current work in pathway engineering tends to rely on systems utilizing either multiple low-copy plasmids (maintained on selective media) or chromosomal integrations at multiple loci. The recent development of methods for rapid pathway assembly relying on many concatenated recombination events (Naesby *et al.*, 2009; Shao & Zhao, 2009) represents an exciting divergence from the traditional approaches to building complex genetic networks in yeast.

Transcriptional-based tools

The second toolset for tuning expression of heterologous genes in yeast is based on modifying transcriptional activity by the choice of promoter controlling the expression of the target genes. A variety of well-characterized promoters exist for the expression of heterologous genes in *S. cerevisiae*. These promoters can be broadly grouped as either providing constitutive or inducible control. A handful of constitutive promoters exhibiting a range of transcriptional activities are commonly used in yeast (Partow *et al.*, 2010; Fang *et al.*, 2011), where the strong promoters *TEF1* and *GPD* (*TDH3*) have been predominately used for overexpression of heterologous genes. Researchers have expanded the selection of constitutive promoters available by generating synthetic promoter libraries through the use of randomized oligonucleotide libraries (Jeppsson *et al.*, 2003) or mutagenesis of promoter regions via error-prone PCR (Alper *et al.*, 2005). As one example, a library of constitutive promoters, generated by mutating the wild-type *TEF1* promoter, exhib-

ited a range in transcriptional activities from 17% to 250% of the original promoter. An alternative approach to expanding the available yeast promoters is to mine promoter sequences from genomes of related species. As one example, a recent study used four promoters homologous to *MET25* or *MET2* from a different, closely related *Saccharomyces* species to regulate enzyme levels within an engineered flavonoid biosynthetic pathway (Naesby *et al.*, 2009). As previously described, the availability of large sets of constitutive promoters exhibiting varying activities and sequences are critical to the design of large biosynthetic pathways.

Inducible promoter systems have the advantage of being able to control enzyme levels in response to the addition of an inducer molecule. A small number of inducible promoter systems have been used in yeast based on either native regulatory networks, such as the *GAL1*, *CUP1*, and *MET25* promoters, or synthetic components, such as the *Tet* promoter adapted from bacteria. These promoters respond to the presence of sugars, copper, methionine, and tetracycline-type antibiotics, respectively. The native promoter systems are easy to implement and provide well-characterized, predictable expression profiles, but have the disadvantage that the inducer molecules for these systems have known pleiotropic effects (Mumberg *et al.*, 1994; Labbe & Thiele, 1999). In addition, the cell consumes inducer molecules that are nutrients, such as galactose and methionine, further complicating the control of gene expression with these systems. The heterologous *Tet* regulatable promoter system can be employed in a modular fashion for *Tet* induction and repression (Belli *et al.*, 1998) and exhibits minimal pleiotropic effects (Wishart *et al.*, 2005), but requires the expression of additional exogenous machinery. However, inducible promoters have limited utility for the design of biosynthetic pathways as the inducer molecules are typically too costly to use for production at scale. Nevertheless, inducible promoter systems can be useful tools for probing the optimal expression level for a particular enzyme in a metabolic network. In one example, an engineered galactose-inducible promoter system, in which the Gal2p galactose-transport protein was deleted to effectively linearize the induction curve (Hawkins & Smolke, 2006), was used to titrate individual enzymes in the main branch of the benzylisoquinoline alkaloid (BIA) biosynthetic pathway in yeast (Hawkins & Smolke, 2008). This titratable promoter system was used to determine the minimum galactose induction (and thus expression level) for each enzyme in the pathway that allowed the highest production of the final metabolite product. This level was matched to the transcriptional activity of a promoter variant from the *TEF* promoter library, and the identified constitutive promoter was then used to express the pathway enzyme at the minimum level at which maximum yield of the end-product BIA was achieved.

Post-transcriptional-based tools

The third toolset for tuning expression of heterologous genes in yeast is associated with modifying the post-transcriptional processing of target transcripts and proteins through a number of different RNA- and protein-based control elements. RNA-based control elements can be applied to regulate the post-transcriptional processing of transcripts encoding pathway enzymes and thus expression levels of those enzymes. Two primary routes by which RNA-based control elements can be used to modulate protein expression in yeast are regulation of translation initiation or mRNA stability (McCarthy, 1998). Libraries of RNA control elements modulating expression through transcript degradation mechanisms have recently been demonstrated. In one example, an RNA-based control module based on Rnt1p-mediated transcript degradation was described based on incorporating engineered Rnt1p hairpin substrates into the 3′ UTR of a target transcript (Babiskin & Smolke, 2011a). A cell-based screen was used to generate a library of Rnt1p hairpin modules that allow for predictable tuning of expression levels in yeast (Babiskin & Smolke, 2011a, b). The RNA hairpins were demonstrated to exhibit predictable expression levels with different promoter, gene, and terminator combinations and span a broad range of expression levels from 8% to 85% of the unmodified protein level. RNA-based control elements have the advantage that they can be combined with any promoter to support titration of regulatory strategies encoded in transcriptional regulators and thus more sophisticated control schemes. To highlight this flexibility, the engineered RNA hairpins were applied to titrate expression of the native yeast *ERG9* gene while maintaining native feedback control at the transcriptional level, resulting in systematic titration of flux through the ergosterol biosynthesis pathway.

RNA control systems have great potential for improving the construction and control of biosynthetic pathways in yeast. For example, a synthetic system supporting polycistronic expression would have a substantial impact on the current workflow associated with building large biosynthetic pathways in yeast, eliminating the requirement for separate promoter and terminator elements for each gene (Hecht *et al.*, 2002). While yeast do not naturally express polycistronic operons, internal ribosome entry sites (IRES) can potentially be used to express multiple genes from a single transcript (Hellen & Sarnow, 2001; Baird *et al.*, 2006). Polycistronic gene expression has been shown in yeast via expression of a viral transcriptional transactivator (Sha *et al.*, 1995), and natural yeast IRES

sequences have been reported (Zhou *et al.*, 2001). A library of short IRES elements was previously developed in yeast (Zhou *et al.*, 2003); however, these synthetic elements result in substantially reduced expression levels relative to normal cap-dependent translation mechanisms, limiting the utility of the resulting library. Thus, the development of a set of IRES elements spanning a broad range in activity and associated polycistronic expression constructs would simplify the design and construction of multi-enzyme complexes and heterologous biosynthesis pathways in yeast.

Protein-based control elements that act through protein degradation have also been used to tune protein levels. As one example, engineered protein degradation tags have been developed in yeast to enhance turnover of heterologously expressed proteins. Specifically, protein degradation systems have been developed using degradation tags taken from native yeast proteins (Mateus & Avery, 2000) and by importing *E. coli* degradation machinery (Grilly *et al.*, 2007). The native degradation tags were taken from the PEST-rich 178 carboxyl-terminal residues of the G1 cyclin Cln2p and were added to the C-terminus of a GFP protein, reducing its half-life from approximately 7 h to 30 min (Mateus & Avery, 2000). The imported *E. coli* degradation system featured tunable rates by LacI repression, demonstrating a range of GFP half-lives between 91 and 22 min (Grilly *et al.*, 2007). Another study constructed a family of CFP reporters with engineered *N*-terminal ubiquitin tags coupled to *N*-degron signals exhibiting half-lives ranging from approximately 5 to 75 min (Hackett *et al.*, 2006). In another recent example, a native amino acid permease, Put4p, was mutated to resist ubiquitination, therefore increasing the half-life of this protein. The mutation resulted in an increase in the effective activity of the amino acid transporter and increased the yeast strain's assimilation of proline by roughly eightfold over a 23-h fermentation (Omura *et al.*, 2005). The control of protein half-lives is essential to maintaining tight dynamic regulation over a biosynthetic pathway. Resistance to native degradation machinery may give a critical boost to the activity of an otherwise underperforming enzyme, while other enzymes, particularly those exposed to highly reactive oxygen species, may exhibit increased overall activity when rapidly cycled between translation and degradation. Thus, libraries of protein tags that modulate rates of protein degradation may prove to be a critical toolset for metabolic engineering.

Strategies for regulating spatial localization of enzymes in yeast

Other efforts to improve pathway flux have been focused on increasing the local concentration of pathway enzymes and intermediates. High local concentrations of biosynthetic enzymes facilitate the movement of intermediates from one active site to the next, preventing the diffusion of intermediates away from the enzyme complex, resulting in increases in pathway flux. In addition, such localization strategies can reduce the loss of intermediates to competing pathways, degradation of unstable intermediates, and the negative effects of toxic intermediates and feedback inhibition. These strategies are inspired by examples from natural metabolic pathways. For example, physical associations between pathway enzymes result in complexes dubbed 'metabolons', and subcellular compartmentalization insulates intermediates from competing pathways and sequesters toxic end-products (Jorgensen *et al.*, 2005; Roze *et al.*, 2011). A number of strategies for manipulating spatial localization of heterologous enzymes have been described for the engineering of secondary metabolite biosynthesis that act through direct protein fusions, localization to scaffolds, and localization to organelles (Fig. 1e).

Direct protein fusions

The most straightforward way to colocalize pathway enzymes is by physically coupling them through direct protein fusion strategies. Protein fusions have been used to redirect flux toward particular pathway branches by fusing key enzymes to reduce intermediate access to competing pathways. In yeast, farnesyl disphosphate (FPP) is a major branch point intermediate of the mevalonate pathway that leads to a variety of valuable compounds in the isoprenoid family. Multiple studies have utilized protein fusion strategies to direct pathway flux from the native pathway downstream of FPP, leading to the production of ergosterol, to an engineered branch (Ohto *et al.*, 2010; Albertsen *et al.*, 2011). In one example, farnesyl diphosphate synthase (Erg20p) was fused to patchoulol synthase (PTS, encoded by *Pogostemon cablin* gene *PatTps177*) to redirect flux to the production of the sesquiterpene patchoulol (Albertsen *et al.*, 2011). A twofold increase was observed in patchoulol production relative to yeast cells expressing each enzyme individually. In another example, Erg20p was fused to geranylgeranyl diphosphate synthase (Bts1p) resulting in an eightfold increase in production of geranylgeranyl diphosphate (GGPP) in yeast relative to that achieved with the enzymes expressed individually (Ohto *et al.*, 2010). In a follow-up study, two protein fusions were used simultaneously to optimize the production of geranylgeraniol (GGOH), an important starting material for the production of vitamins A and E (Tokuhiro *et al.*, 2009). A fusion of Bts1p and diacylglycerol diphosphate phosphatase (DPP1) was engineered to increase production of

GGOH from 0.2 to 75 mg L^{-1}. This protein fusion was subsequently coexpressed with the Erg20–Bts1 fusion protein generated by Ohto *et al.*, resulting in a further increase in GGOH titers to 230 mg L^{-1}. Protein fusion strategies have also been used to improve production of the stilbenoid resveratrol. The enzyme 4-coumaric-CoA ligase (4CL) was fused to stilbene synthase (STS) resulting in an increase in resveratrol production from 0.65 to 5.25 μg mL^{-1} (Zhang *et al.*, 2006). These studies demonstrate that enzyme fusions can be an effective method to redirect flux from a natural pathway to an engineered branch and also to increase flux through consecutive steps to optimize production of an end-product.

The orientation of enzymes in the fusion protein and the linkers used to form the junction between the enzymes can impact the activity of the resulting chimeric protein. For example, an almost fourfold difference was observed in GGPP production between the Erg20–Bts1 and Bts1–Erg20 fusion proteins (Ohto *et al.*, 2010). However, the relative orientation of the Erg20–PTS fusion had no significant effect on patchoulol production (Albertsen *et al.*, 2011). These results demonstrate that the ORF orientation can impact the activity of the fusion protein, but the effect is dependent on the properties of the enzymes. In addition, the length and flexibility of the linker sequence used to couple the enzymes can influence the activity of the resulting fusion protein. To determine the optimal design, linkers of different lengths (3–239 amino acids) and flexibilities (determined by the bulkiness of the residues) were tested with the Erg20–PTS fusions. Patchoulol production was relatively constant across different linker flexibilities; however, the titer decreased with increasing linker length (Albertsen *et al.*, 2011). It is likely that the effect of linker length and ORF orientation may be dependent on the specific properties of the enzymes in the fusion protein and may need to be optimized for each individual case.

While protein fusions have shown promise as a method to increase flux to engineered pathways, this strategy has several limitations. For example, the number of enzymes that can be functionally tethered together is limited by the ability of each protein domain to fold correctly; as more protein sequences are concatenated the likelihood of correct and independent folding is reduced. In addition, a multi-enzyme fusion would be limited to a 1 : 1 ratio of each enzyme, which may not be the optimal ratio for maximizing flux through the pathway if the enzymes exhibit substantially different activities. Finally, not every enzyme is a good candidate to be included in a chimera. For example, an enzyme in which the termini play an important role in catalysis would be more likely to misfold when linked to another protein, and thus lose activity, as the freedom of the termini to achieve the correct fold would be limited.

Localization to scaffolds

An alternative approach to directly linking pathway enzymes is to bring enzymes within close proximity of each other by localizing them to a scaffold. Strategies based on tagging enzymes with short peptide sequences that bind to particular protein or nucleic acid sequences have been used to build enzyme scaffolding systems in bacteria. A protein-based scaffolding system was demonstrated to increase the production of mevalonate by 77-fold over unscaffolded, tagged enzymes in *E. coli* (Dueber *et al.*, 2009). In another example, an RNA-based scaffolding system was applied to increase the production of hydrogen by 48-fold over unscaffolded, tagged enzymes in *E. coli* (Delebecque *et al.*, 2011). While these synthetic scaffolding systems exhibit great potential as a tool for engineering modular protein localization strategies, they have not yet been transferred to yeast.

A strategy based on localizing pathway enzymes to a scaffold can incur its own set of challenges. For example, this strategy requires the expression of additional components to bring together pathway enzymes, in particular the scaffold itself, which can generate an additional metabolic burden on the host cell. In addition, the optimal stoichiometry of pathway enzymes must be determined experimentally to maximize the advantage of colocalizing the enzymes, which is an added optimization step not required by other methods. This strategy also requires tagging the pathway enzymes with protein or peptide domains to direct them to the scaffold, where the addition of the tags can reduce the catalytic activity of the enzymes. Finally, in the case of a protein scaffold, the protein–protein interaction domains must be chosen carefully to prevent crosstalk with endogenous proteins. For example, the system developed by Dueber *et al.* uses metazoan proteins that have homologs in yeast, thus potentially reducing the efficacy of such a strategy in yeast hosts.

Localization to native organelles

A third approach to engineering spatial organization can take advantage of the natural scaffolding provided by the organelles in yeast. Organelles have supplies of important cofactors and can sequester toxic compounds, providing additional advantages to pathway engineering. In natural systems, enzymes are directed to subcellular compartments by sequences that are typically embedded at or near the termini of the protein sequence (von Heijne, 1990). A similar approach can be used by adding tags to

heterologous enzymes to direct them to localize on or within organelles. In one example, a methyl halide transferase was tagged with the N-terminus of carboxypeptidase Y to localize the enzyme to the vacuole in yeast (Bayer *et al.*, 2009). This localization strategy gave the enzyme access to the supply of *S*-adenosyl-methionine (SAM), a required cofactor for the transferase reaction, stored in the vacuole and also sequestered the halogenated products leading to an increase in methyl-iodide production from approximately 130 to 180 mg L^{-1} h.

Targeting enzymes to the mitochondria has been used as a strategy to insulate the production of plant isoprenoids from competing pathways in native yeast metabolism and to take advantage of the pool of FPP, an important branch point intermediate, present in this organelle. In one example, the mitochondrial targeting sequence from the *COX4* gene was fused to the N-terminus of valencene synthase (*TPS1*) and production of valencene was improved by threefold compared to cytosolic TPS1, and the addition of truncated 3-hydroxy-3-methylglutaryl-coenzyme A reductase (tHmg1p) and mitochondrial-targeted Erg20p (mtErg20p) improved valencene titers by an additional twofold. To demonstrate the generalizability of this approach for plant terpenoid synthase, amorpho-4,11-diene synthase (*ADS*) was localized to the mitochondria in the same manner in the presence of tHmg1p and mtErg20p, increasing amorpha-4,11-diene production from approximately 1 to 20 mg L^{-1} (Farhi *et al.*, 2011). These examples demonstrate some key advantages of using yeast as a production host: the availability of precursors and cofactors in subcellular locations, the ability to use native organelles to colocalize and insulate pathways, and the existence of well-characterized subcellular localization tags for the vacuole, mitochondria, endoplasmic reticulum, nucleus, and peroxisome.

Localization of enzymes to native organelles in yeast carries its own set of challenges. As with scaffolding strategies, it is necessary to tag the enzymes to direct their localization and the addition of a tag can negatively affect the activity of the enzyme. While insulation of pathways within an organelle can be advantageous, transport can become an issue as pathway intermediates must be able to reach enzymes within the organelle. Additionally, native regulation of the protein content of each subcellular compartment may lead to the more rapid degradation of the enzymes. Strategies for manipulating spatial localization of pathway enzymes are in the early stages of being applied to metabolic engineering; however, as more advanced techniques are developed, they will prove invaluable to the optimization of engineered biosynthetic pathways.

Secondary metabolite sensing and screening

The optimization of a heterologous secondary metabolite pathway in a yeast host often requires the generation of many enzyme and pathway variants followed by identification of the variants that result in highest productivity and yield by measuring the concentrations of key metabolites in the pathway of interest. However, the slow speed associated with traditional analytical measurement methods limits the efficiency and throughput of the optimization process. Therefore, generalizable approaches that link the concentration of target secondary metabolites to an easily detected output, such as fluorescence or pigment, through a genetically encoded sensor can greatly decrease the time and increase the throughput of the optimization process (Michener *et al.*, 2011).

Metabolite sensors are generally composed of an input domain, which binds the metabolite of interest, and an output domain, which controls the level of a genetic output such as a reporter gene. The binding of the metabolite to the input domain results in a change in the activity of the output domain, thus enabling the state of the pathway to be monitored noninvasively. Genetically encoded sensors can be made from RNA or proteins in yeast. RNA-based sensors, such as RNA switches, typically couple an RNA input binding domain, or aptamer, to an RNA output domain, or RNA regulatory element (Liang *et al.*, 2011). Protein-based sensors, such as transcription factors, typically couple the small molecule-binding domain to an output domain that regulates transcription of a genetic output (Michener *et al.*, 2011). A major limitation in implementing molecular sensing devices in the context of an engineered pathway is the lack of input domains, whether RNA or protein, that recognize secondary metabolites of interest. This limitation can be addressed by advances in synthetic biology research, which are leading to new methods for developing metabolite sensing devices.

RNA-based sensing devices have been demonstrated in yeast that are able to detect different classes of secondary metabolites. In one example, RNA devices responsive to theophylline (an alkaloid) and tetracycline (a polyketide) were demonstrated in yeast that link detection of these secondary metabolites to the levels of a fluorescent reporter protein (Win & Smolke, 2007). One advantage of RNA-based sensors is the ability to generate new sensing modules through the *de novo* generation of aptamers (Ellington & Szostak, 1990; Tuerk & Gold, 1990). RNA aptamers to diverse secondary metabolites, including purine alkaloids, benzylisoquinoline alkaloids, and polyketides, have been generated through *in vitro* selection strategies (Jenison *et al.*, 1994; Berens *et al.*, 2001; Win *et al.*,

2006). In addition, the potential exists to generate sets of aptamers that distinguish between metabolites along a pathway branch, as researchers have demonstrated that aptamers to structurally similar compounds can be developed by screening smaller libraries generated from parent aptamer sequences (Famulok, 1994; Dixon *et al.*, 2010).

Protein-based sensors have been developed that utilize transcription factors to detect cellular metabolites. However, examples to date have focused demonstrations of these sensing systems in *E. coli* and utilized transcription factors that recognize primary metabolites (Sellick & Reece, 2005). While there are likely many transcription factors involved in the regulation of secondary metabolism, few examples currently exist of characterized transcription factors that bind secondary metabolites of interest for production in a microbial host. Therefore, synthetic biology tools can be applied to the identification of transcription factors that bind secondary metabolites from the native host organisms or engineering of these transcription factors to detect specific secondary metabolites of interest.

Secondary metabolite production in yeast

Although many of the described synthetic biology tools have emerged only in recent years, several of these techniques have already been put to use to engineer yeast strains for the production of valuable secondary metabolites. Below, we review progress made toward constructing yeast biosynthetic pathways for four classes of secondary metabolites: phenolics, isoprenoids, alkaloids, and polyketides.

Phenolics

Phenolics are a large group of plant secondary metabolites that are characterized as containing at least one hydroxylated aromatic ring. Among the phenolics produced by the phenylpropanoid pathway in plants, the flavonoids and stilbenes are two classes of metabolites highly valued as nutritional and therapeutic agents (Ververidis *et al.*, 2007). The microbial synthesis of these plant defense compounds is particularly attractive as they are produced in smaller quantities in plants relative to other classes of phenolics, such as the structurally important lignins. Furthermore, many valuable flavonoid and stilbenoid compounds are only found in a limited number of plant species.

Saccharomyces cerevisiae is well suited as a host cell to support the biosynthesis of phenolic compounds. Although yeast do not naturally produce phenylpropanoid phenolics, its central metabolism provides the necessary amino acid precursors for an introduced phenolic biosynthesis pathway. The phenolic-producing yeast strains engineered to date employ basic synthetic biology tools to express a number of genes from the native plant and fungal sources (Table 1). The most optimal of these biosynthesis platforms use the amino acids tyrosine and phenylalanine supplied by the native host cell shikamate pathway as precursors for the heterologous pathway, although several strains have been engineered with partially constructed pathways and thus require specific intermediates provided to the culture.

Flavonoids

Researchers have constructed yeast strains that synthesize a range of flavonoids, starting with the common phenolic intermediates and extending to flavanones, flavones, isoflavones, and flavonols. In the initial steps of the phenylpropanoid pathway, phenylalanine is converted to *p*-coumaric acid, a common phenylpropanoid precursor, in two sequential reactions (Fig. 2). Phenylalanine ammonia lyase (PAL) first catalyzes the deamination of phenylalanine to cinnamic acid, and cytochrome P450 cinnamate-4-hydroxylase (C4H) acting together with its partner reductase (CPR) subsequently catalyzes the hydroxylation of cinnamic acid to *p*-coumaric acid. The construction of a *p*-coumaric acid-producing yeast strain was initially demonstrated by coexpressing Poplar genes for *PAL* and *C4H* (and *CPR*; Ro & Douglas, 2004). A modified version of this strain was later constructed expressing an alternative PAL enzyme from the fungus *Rhodospridium toruloides*, which exhibits an additional tyrosine ammonia lyase (TAL) activity and thus is able to bypass C4H to produce *p*-coumaric acid from tyrosine in addition to the standard route from phenylalanine (Vannelli *et al.*, 2007). This strain produced more than 30 mg L^{-1} *p*-coumaric acid. Both engineered yeast strains could use amino acids supplied by the host cell's primary metabolism, although yield was increased more than twofold when the culture medium was supplemented with approximately 1 mM phenylalanine.

The first class of flavonoid produced from *p*-coumaric acid are the flavanones. The synthesis of naringenin, an important flavanone molecule that acts as a precursor to many downstream flavonoids, by an engineered yeast strain was initially described (Jiang *et al.*, 2005). This system relied on a three-enzyme heterologous pathway, in which *R. toruloides* PAL catalyzes the conversion of tyrosine to *p*-coumaric acid in the absence of C4H activity. In the latter steps, *Arabidopsis thaliana* 4-coumarate:CoA ligase (4CL) catalyzes the formation of an activated

Table 1. Phenolic biosynthetic genes used to build production pathways in yeast

Abbreviation	Gene name	Species	Publications
4CL	4-Coumarate:CoA-ligase	*Petroselinum crispum*, *Nicotiana tabacum*, *Arabidopsis thaliana*, Poplar hybrid (*P. trichocarpa x P. deloides*), *Glycine max*	Becker *et al.* (2003), Jiang *et al.* (2005), Leonard *et al.* (2005), Yan *et al.* (2005), Beekwilder *et al.* (2006), Zhang *et al.* (2006), Yan *et al.* (2007), Trantas *et al.* (2009), Moglia *et al.* (2010), Eudes *et al.* (2011), and Wang *et al.* (2011)
C4H	Cinnamate-4-hydroxylase	Poplar hybrid (*P. trichocarpa x P. deloides*), *Arabidopsis thaliana, Helianthus tuberosus, Glycine max*	Ro & Douglas (2004), Leonard *et al.* (2005), Yan *et al.* (2005), Vannelli *et al.* (2007), and Trantas *et al.* (2009)
CHI	Chalcone isomerase	*Petunia hybrid, Glycine max, Medicago sativa*	Leonard *et al.* (2005), Ralston *et al.* (2005), Yan *et al.* (2005, 2007), and Trantas *et al.* (2009)
CHR	Chalcone reductase	*Medicago sativa*	Yan *et al.* (2007)
CHS	Chalcone synthase	*Petunia hybrid, Medicago sativa, Hypericum androsaemum, Glycine max*	Jiang *et al.* (2005), Leonard *et al.* (2005), Yan *et al.* (2005, 2007), and Trantas *et al.* (2009)
F3H	Flavanone 3β-hydroxylase	*Glycine max*	Ralston *et al.* (2005), and Trantas *et al.* (2009)
F3'H	Flavonoid 3-hydroxylase	*Glycine max*	Trantas *et al.* (2009)
FLS	Flavonol synthase	*Solanum tuberosum*	Trantas *et al.* (2009)
FSI	Flavone synthase I	*Petroselinum crispum*	Leonard *et al.* (2005)
FSII	Flavone synthase II	*Glycine max*, *Antirrhinum majus*	Leonard *et al.* (2005), and Ralston *et al.* (2005)
HCBT	Hydroxycinnamoyl/benzoyl-CoA/ anthranilate *N*–hydroycinnamoyl/ benzoyltransferase	*Dianthus caryophyllus*	Eudes *et al.* (2011)
HCT	Hydroxycinnamoyl-CoA: shikimate/quinate hydroxycinnamoyltransferase	*Cynara cardunculus*	Moglia *et al.* (2010)
IFS	Isoflavone synthase	*Glycine max*	Ralston *et al.* (2005), and Trantas *et al.* (2009)
PAL/TAL	Phenylalanine ammonia lyase/Tyrosine ammonia lyase	Poplar hybrid (*P. trichocarpa x P. deloides*)/*Rhodobacter sphaeroides*, *Rhodosporidium toruloides*	Ro & Douglas (2004)/Jiang *et al.* (2005), Vannelli *et al.* (2007), Trantas *et al.* (2009), and Wang *et al.* (2011)
STS	Stilbene synthase	*Vitis vinifera*	Becker *et al.* (2003), Beekwilder *et al.* (2006), Zhang *et al.* (2006), Trantas *et al.* (2009), and Wang *et al.* (2011)

p-coumaroyl-CoA complex, and *Hypericum androsaeemum* chalcone synthase (CHS) then combines this complex with three malonyl-CoA molecules to make naringenin chalcone. Interestingly, naringenin, and not naringen chalcone, production was observed from the engineered yeast strain, suggesting spontaneous cyclization of the chalcone in the acidic culture medium (Jiang *et al.*, 2005). Other similarly constructed yeast strains have been demonstrated that express a chalcone isomerase (*CHI*) to catalyze the cyclization of chalcones to flavanones (Leonard *et al.*, 2005; Ralston *et al.*, 2005; Yan *et al.*, 2005, 2007; Trantas *et al.*, 2009). While the yeast strain expressing *4CL* and *CHS* produced approximately 7 mg L^{-1} naringenin, similarly constructed strains additionally expressing a *CHI* achieved titers 2–4 times greater (Yan *et al.*, 2005, 2007; Trantas *et al.*, 2009).

Many classes of flavonoids have been synthesized from flavanones in engineered yeast strains. Flavanone-producing yeast strains engineered to express the cytochrome P450 enzymes isoflavone synthase (*IFS*) and flavone synthase II (*FSII*) can synthesize the isoflavone genistein and the flavone apigenin, respectively (Ralston *et al.*, 2005; Trantas *et al.*, 2009). However, greater yields of apigenin were achieved if *FSII* from snapdragon (*Antirrhinum majus* cv. Montego Yellow) was replaced by a parsley (*Petroselinum crispum*) flavone synthase I (*FSI*), a 2-oxoglutarate-dependent dioxygenase that catalyzes the same reaction (Leonard *et al.*, 2005). The biosynthesis of

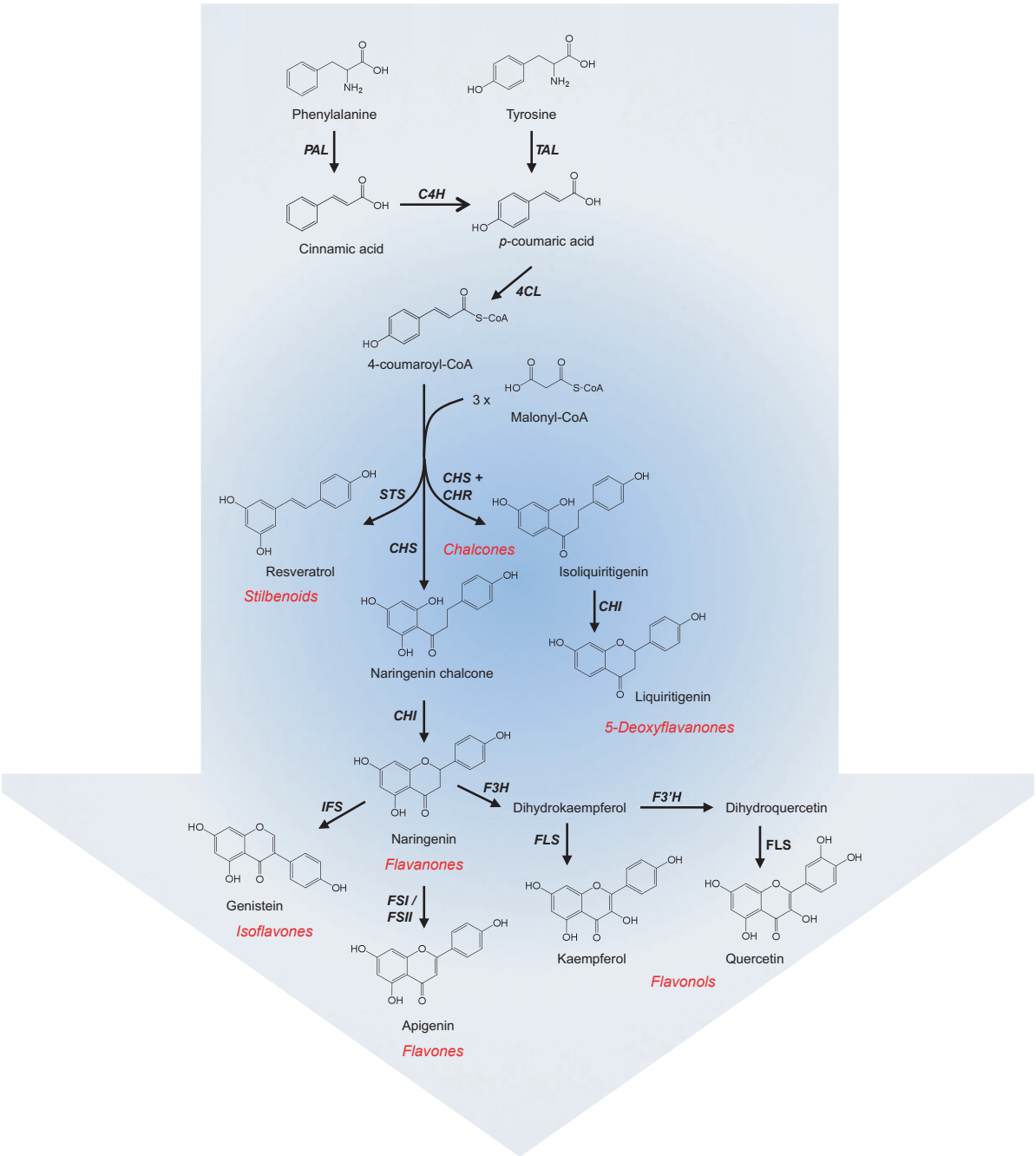


Fig. 2. Pathways constructed for the production of phenolic secondary metabolites in yeast. Pathways have been constructed for the biosynthesis of many types of flavonoid and stilbenoid phenolics in yeast starting from the amino acid precursors phenylalanine and tyrosine. Engineered enzymatic steps are depicted for the production of the stilbenoid resveratrol and flavonoids including flavanones, 5-deoxyflavanones, flavones, isoflavones and favonols. Examples are provided of each compound synthesized from *p*-coumaric acid; however, these same engineered pathways have been demonstrated to produce phenolics from other hydroxycinnamic acid intermediates. Classes of compounds are indicated in red italics; full gene details and references are listed in Table 1. Abbreviations for genes encoding plant and fungal heterologous genes: *4CL*, 4-coumarate:Co1-ligase; *C4H*, cinnamate-4-hydroxylase; *CHI*, chalcone isomerase; *CHR*, chalcone reductase; *CHS*, chalcone synthase; *F3H*, flavanone 3β-hydroxylase; *F3'H*, flavonoid 3-hydroxylase; *FLS*, flavonol synthase; *FSI*, flavone synthase I; *FSII*, flavone synthase II; *IFS*, isoflavone synthase; *PAL*, phenylalanine ammonia lyase; *STS*, stilbene synthase; *TAL*, tyrosine ammonia lyase.

flavonols in flavanone-producing yeast was demonstrated by the coexpression of flavanone 3β-hydroxylase (*F3H*), flavonoid 3-hydroxylase (*F3′H*), and flavonol synthase (*FLS*) to make kaempferol and quercetin (Trantas *et al.*, 2009). Additionally, chalcone reductase (*CHR*) has been expressed in flavanone-producing yeast to act together with CHS to produce both flavanones and the rare 5-deoxyflavanones, a type of phenolic compound unique to leguminous plants (Yan *et al.*, 2007).

Stilbenoids

Among the most well-known stilbenoids is resveratrol, a key health-promoting phenolic component in wine. Resveratrol is extracted from grape skin during fermentation and is therefore present at higher levels in red wine. As wine is a yeast-cultured consumer product, the independent synthesis of resveratrol by yeast strains has been sought, particularly in those strains used for winemaking. STS (or resveratrol synthase, RS) is a type III polyketide synthase closely related to CHS, which uses the same precursors (*p*-coumaroyl-CoA and three molecules of malonyl-CoA) to synthesize resveratrol. Initial attempts to make resveratrol in yeast coexpressed *4CL* and grape *STS* and supplied *p*-coumaric acid in the culture medium (Becker *et al.*, 2003; Beekwilder *et al.*, 2006; Zhang *et al.*, 2006). More recently, resveratrol-producing yeast strains have been engineered to use the amino acids tyrosine and phenylalanine as substrates by expressing the same upstream enzymes used in flavonoid-producing yeast strains (PAL, TAL, C4H, CPR, 4CL; Trantas *et al.*, 2009; Wang *et al.*, 2011). In an important proof-of-concept experiment, researchers used an engineered yeast strain to make white wine with as much resveratrol content as some red wines (0.68 mg L^{-1} in an industrial-scale fermentation; Wang *et al.*, 2011), demonstrating the potential impact of yeast secondary metabolite engineering for the wine industry.

Other phenolics

The examples highlighted above focus on *p*-coumaric acid-derived flavonoids; however, many yeast strains have been constructed that yield flavonoids derived from other hydroxycinnamic acids, including cinnamic acid and caffeic acid (Leonard *et al.*, 2005). With the growing successes of engineered yeast biosynthesis of these and other flavonoids, researchers have recently branched into related compounds such as phenolic esters (Moglia *et al.*, 2010), raspberry ketone (Beekwilder *et al.*, 2007), and cinnamoyl anthranilates including the pharmaceutical tranilast (Eudes *et al.*, 2011).

Yeast as a host cell for the production of phenolics

While yeast has proven a suitable host for phenolic biosynthesis, several challenges associated with target product purity and yield have been encountered. For example, minor nontarget products have been detected and attributed to interactions of the native host cell metabolism with substrates supplied in the culture medium or with phenolic intermediates produced by the introduced pathway (Ro & Douglas, 2004), or conversely, because of substrate promiscuity of the heterologous enzymes that act on native yeast metabolites (Jiang *et al.*, 2005; Moglia *et al.*, 2010). Furthermore, yields obtained to date for individual phenolic compounds from yeast are on the order of 5–20 times lower than those obtained in the well-developed bacterial host *E. coli* (Trantas *et al.*, 2009). In some cases, the reason for this is unclear; there are several examples where the expression of similar or identical heterologous phenolic biosynthetic genes in yeast and bacteria results in lower yields from the yeast host, such as in the production of resveratrol (Beekwilder *et al.*, 2006) or apigenin (Leonard *et al.*, 2005; Miyahisa *et al.*, 2006). In other instances, increased production in the bacterial host can be attributed to the application of protein- and strain-engineering tools. In bacterial cells, the supply of malonyl-CoA is greatly limiting to engineered flavonoid production (Miyahisa *et al.*, 2005); however, researchers have employed methods to increase the levels of this metabolite. In one example, a heterologous acetyl-CoA carboxylase was expressed to engineer malonyl-CoA synthesis from acetyl-CoA (Miyahisa *et al.*, 2005; Leonard *et al.*, 2007). Malonyl-CoA levels have also been enhanced in *E. coli* by introducing heterologous genes for malonate assimilation (Leonard *et al.*, 2008). These strain improvements have increased production of flavonoids, such as naringenin, in *E. coli* to more than four times that which be achieved in yeast (Yan *et al.*, 2005; Leonard *et al.*, 2007, 2008).

Although one key advantage *S. cerevisiae* has over *E. coli* as a host cell is the ability to support the functional expression of many types of plant enzymes, particularly cytochrome P450s, this is another area where the careful implementation of synthetic biology tools and selection of appropriate pathway enzymes have allowed *E. coli* strain engineers to keep yields higher than those obtainable in yeast. For example, the need for functional expression of the cytochrome P450 *C4H* in bacteria has been bypassed by the expression of nonplant *PAL* (Watts *et al.*, 2004; Vannelli *et al.*, 2007) and 4CL (Hwang *et al.*, 2003) enzymes, which allow the engineered phenylpropanoid pathway to proceed down independent routes toward flavonoid biosynthesis using both phenylalanine and tyro-

sine as substrates. In addition, techniques have been demonstrated that allow functional expression of plant cytochrome P450s in bacteria, for example, the construction of a soluble fusion protein of *Catharanthus roseus* flavonoid 3′5′-hydroxylase (F3′5′H) with a P450 reductase partner from the same species (Leonard *et al.*, 2006). However, this bacterial strain produces approximately 0.05 mg L^{-1} quercetin from *p*-coumaric acid compared to 0.26 mg L^{-1} quercetin produced in a yeast strain expressing wild-type potato (*Solanum tuberosum*) *F3′H* cytochrome P450 together with upstream enzymes (Trantas *et al.*, 2009). This suggests that the innate strengths of yeast as a cytochrome P450 host will boost overall phenolic yields for *S. cerevisiae* as synthetic biology tools first developed for *E. coli* strain engineering and the more advanced tools described earlier in this review are implemented in strain and protein optimization in yeast.

Isoprenoids

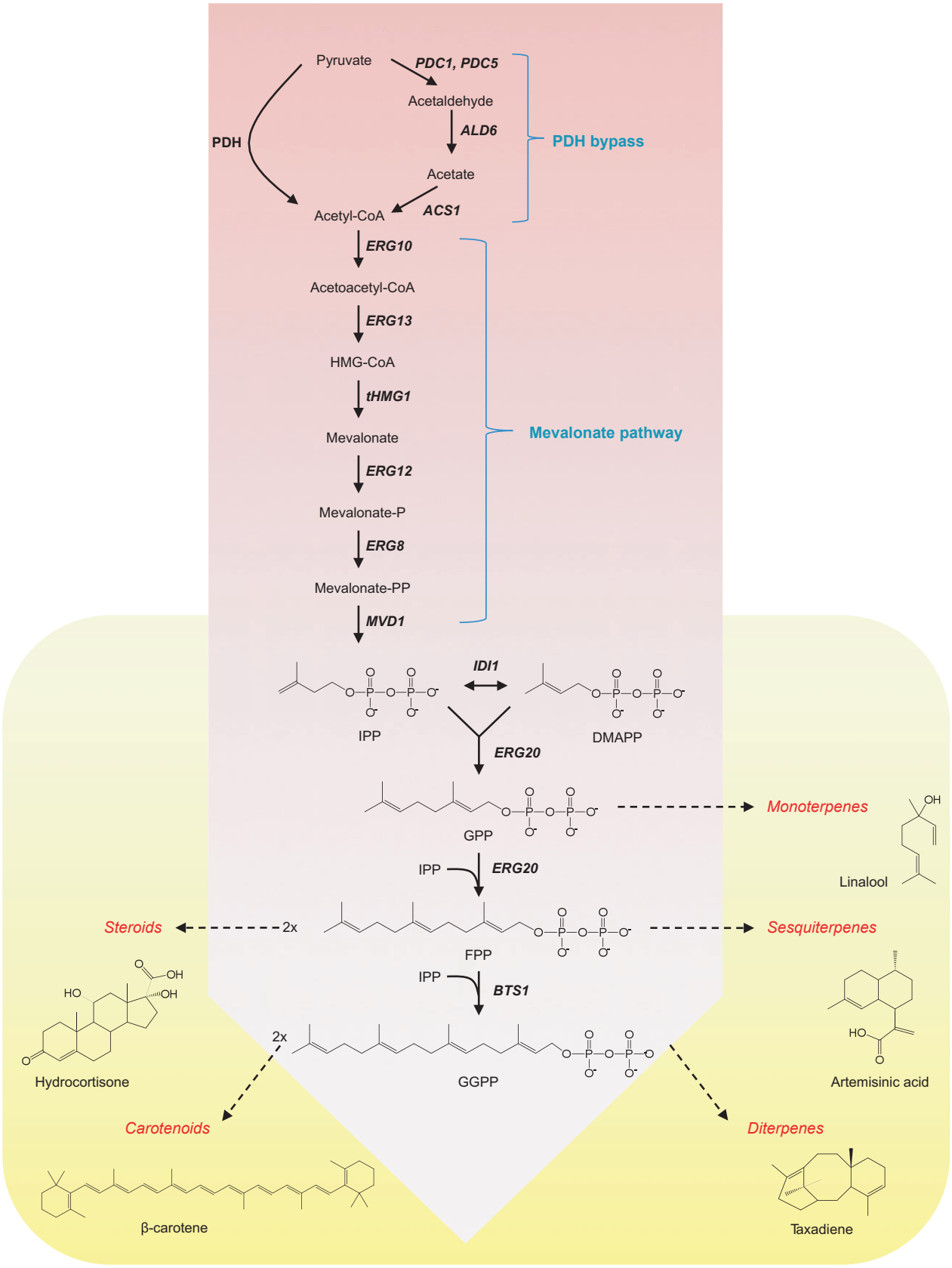
The largest group of plant secondary metabolites is the isoprenoids, which are composed of carbon chains assembled from phosphorylated five-carbon building blocks that are often cyclized and further modified to produce their highly bioactive forms. Some plant isoprenoids, such as taxol and artemisinin, are used as powerful drugs for the treatment of life-threatening disease. Other classes of isoprenoids, including the carotenoids and monoterpenoids, are valued in the food industry for their nutritional, color, and flavor properties.

While plants produce a variety of isoprenoids, many bacteria, fungi, and mammals also make these secondary metabolites. For example, yeast synthesize isoprenoids to control key functions such as membrane fluidity (ergosterol) and mating response (a-factor). Therefore, the use of yeast as a host cell for isoprenoid biosynthesis has benefits and disadvantages. While yeast is able to supply many precursors and intermediates through its native metabolic processes, there is competition between the introduced biosynthetic pathway and the natural cellular processes that rely on these metabolites. Metabolic engineers using yeast as a production host for isoprenoids have thus devised strategies to alter the native upstream pathway for overproduction of key precursors and intermediates and construct downstream heterologous pathways to produce the desired non-native end-products.

Isoprenoid precursors are produced from acetyl-CoA via the mevalonate pathway in yeast (Fig. 3, Table 2). The first precursor is the five-carbon building block molecule, isopentenyl pyrophosphate (IPP), and its isomer dimethylallyl pyrophosphate (DMAPP). IPP and DMAPP are condensed to make the ten-carbon intermediate, geranyl pyrophosphate (GPP), which is combined with an additional IPP molecule to make the 15-carbon intermediate, farnesyl pyrophosphate (FPP), and again with another IPP to make the 20-carbon intermediate, geranylgeranyl pyrophosphate (GGPP). These successive condensations of IPP form the 'core' of the isoprenoid pathway, where each of the intermediates – GPP, FPP, GGPP – are subsequently modified to form functional isoprenoids. Yeast use GPP to make monoterpenoids and FPP to make several essential metabolites, including squalene, from which ergosterol is made, and for the farnesylation of proteins such as a-type mating factor. Yeast also produce a small amount of GGPP for the post-translational modification of proteins by geranylgeranylation. Yeast strains have been engineered to convert GPP, FPP, and GGPP to non-native monoterpenoids, sesquiterpenes, and diterpenes, respectively. In general, heterologous terpene synthases are used to cyclize GPP, FPP, or GGPP, which are then further functionalized by the activity of cytochrome P450s or other enzymes. Alternatively, two of each intermediate (i.e. FPP or GPP) can be combined in the first committed steps to sterol or carotenoid biosynthesis.

Carotenoids

The first class of isoprenoids to be produced in engineered yeast were the carotenoids. Carotenoids are pigment molecules that are structurally comprised of long-chain isoprenoids that are desaturated and terminally cyclized. Engineering efforts sourced carotenoid biosynthetic genes from several microorganisms, in particular the red yeast *Xanthophyllomyces dendrorhous* and the soil bacterium *Erwinia uredovora*, and initially achieved low-level production (tens to one hundred micrograms per dry weight cells) of targeted carotenoids from endogenous precursors (Yamano *et al.*, 1994). The first carotenoid, lycopene, is synthesized from two molecules of GGPP by condensation. Researchers have thus engineered yeast strains with enhanced GGPP availability by either expressing a heterologous GGPP synthase gene termed *crtE* (Yamano *et al.*, 1994; Miura *et al.*, 1998) or overexpressing the native *S. cerevisiae* gene *BTS1* (Verwaal *et al.*, 2007; Ukibe *et al.*, 2009) to increase production of GGPP from FPP. Constructs encoding the expression of combinations of heterologous carotenoid biosynthetic enzymes were introduced into these engineered yeast strains to result in appreciable amounts of lycopene and downstream products, for example up to 5.9 mg g^{-1} (dry weight) β-carotene (Yamano *et al.*, 1994; Miura *et al.*, 1998; Verwaal *et al.*, 2007; Ukibe *et al.*, 2009) and astaxanthin (Miura *et al.*, 1998; Ukibe *et al.*, 2009).



Sesquiterpenes

Sesquiterpenes are produced by the cyclization and further modification of a single FPP molecule. Most notable among yeast strains engineered to produce sesquiterpenes are those which make artemisinic acid, a precursor to the antimalarial artemisinin. In constructing an artemisinic acid-producing strain, researchers used an amorphadiene synthase (ADS) enzyme from *Artemisia annua*, which had previously been shown to catalyze the cyclization of FPP to amorphadiene in microbial systems (Martin *et al.*, 2003). When expression of this heterologous enzyme in yeast was combined with several strain engineering methods to increase intracellular pools of FPP by upregulating flux through the mevalonate pathway and limiting competition for this precursor from the ergosterol biosynthetic pathway, the resulting strain produced 153 mg L^{-1} amorphadiene. However, the enzymes responsible for the following three oxidation steps to convert amorphadiene to artemisinic acid were unknown. Researchers coupled intuition that the likely native enzymes to catalyze these steps were cytochrome P450s with a bioinformatics approach to design degenerate primers to cytochrome P450 cDNAs based on sequences from two species within the Asteraceae family to identify related sequences within a trichome-enriched *A. annua* cDNA library (Ro *et al.*, 2006). An identified cytochrome P450 was tested *in vitro* and when expressed in the amorphadiene yeast production strain was found to catalyze all three reactions, where the resulting artemisinic acid was retained in the cell pellet bound to the cell surface, allowing for a straightforward isolation scheme from the culture. The ongoing optimization of amorphadiene and artemisinic acid production by strain engineering has continued in both yeast and bacteria and is discussed further below.

Diterpenoids

Diterpenoids are produced from one molecule of GGPP by cyclization and further modifications. The most challenging isopreniod for microbial synthesis is taxol, a complex diterpenoid that is currently used as a chemotherapy drug. Taxol can be harvested in small quantities from the bark of tree species in the *Taxus* genus, although it is currently produced commercially by semisynthetic means. Its total biosynthesis involves as many as 19 enzymatic steps, and to date as few as four enzymes from the *Taxus* species have been functionally expressed in yeast. Similar to the construction of carotenoid biosynthesis pathways in yeast, production of diterpene-related taxol benefits from an increased supply of GGPP from FPP. Yeast strains have been engineered for increased production of GGPP for this application by expressing a GGPP synthase (GGPPS) from a *Taxus* species and a GGPPS from *Sulfolobus acidocaldarius,* where the latter utilizes DMAPP to build GGPP and hence does not directly compete with the ergosterol biosynthetic pathway for access to FPP (Engels *et al.*, 2008). The strain coexpressing *S. acidocaldarius* GGPPS with a codon-optimized taxadiene synthase (TS) converted the additional GGPP to taxadiene at a titer of 8.7 mg L^{-1} (strain CEN10), which was 29-fold more than that achieved with the Taxus GGPPS and noncodon-optimized *TS* (strain CEN7; Engels *et al.*, 2008). It has also been reported that a cytochrome P450, THY5α, heterologously expressed in yeast can act on taxadiene to make very low levels of taxadien-5α-ol (Dejong *et al.*, 2006), although another study reported the key product of THY5α heterologous activity in yeast microsomes to be a novel taxane that has not previously been detected in taxol-producing trees (Rontein *et al.*, 2008). Finally, a second *Taxus* cytochrome P450 THY10b, which catalyzes a downstream hydroxylation reaction, has been demonstrated to be functional in yeast microsomal preparations and demonstrates a sevenfold higher activity when coexpressed with a *Taxus* reductase partner (Jennewein *et al.*, 2005). These research efforts represent the initial steps toward constructing a yeast strain for total taxol biosynthesis.

Fig. 3. Pathways constructed for the production of isoprenoid secondary metabolites in yeast. Pathways have been constructed for many isoprenoids in yeast, including monoterpenes, sesquiterpenes, diterpenes, carotenoids and steroids, all synthesized from substrates supplied by the native mevalonate pathway. The pyruvate dehydrogenase (PDH) bypass encoded by genes pyruvate decarboxylase 1 and 5 (*PDC1* and *PDC5*), acetaldehyde dehydrogenase 6 (*ALD6*) and acetyl-CoA synthase 1 (*ACS1*) enhances the flux of acetyl-CoA into the mevalonate pathway, which in turn is encoded by genes *ERG10*, *ERG13*, *ERG12*, and *ERG8* (ergosterol biosynthetic genes 10, 13, 12 and 8, respectively in order of catalysis), truncated HMG-CoA reductase 1 (t*HMG1*), mevalonate pyrophosphate decarboxylase 1 (*MVD1*) and isopentenyl diphosphate:dimethylallyl diphosphate isomerase (*IDI1*) and produces precursors isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). Downstream of the mevalonate pathway, ergosterol biosynthetic gene 20 (*ERG20*) and bet two suppressor (*BTS1*) produce the important intermediates geranyl pyrophosphate (GGP), farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP). Further native and heterologous enzymes produce valuable secondary metabolites; examples from each class are provided. Yeast native metabolism is delineated with a pink background; classes of compounds are indicated in red italics; full gene details and references are listed in Table 2; broken arrows represent many enzymatic steps, which are detailed in the text and Table 2.

Table 2. Isoprenoid biosynthetic genes (both heterologous and native) used to build production pathways in yeast

Type	Name	Species	Publications
Pyrophosphate synthase	*ERG20* (ergosterol biosynthesis 20)	*Saccharomyces cerevisiae*	Ro *et al.* (2006, 2008) and Ohto *et al.* (2010)
	FPPS (farnesyl pyrophosphate synthase)	*Arabidopsis thaliana*	Farhi *et al.* (2011)
	BTS1 (bet two suppressor)	*Saccharomyces cerevisiae*	Verwaal *et al.* (2007), Tokuhiro *et al.* (2009), Ukibe *et al.* (2009), and Ohto *et al.* (2010)
	crtE (carotenoid biosynthetic gene E)	*Xanthophyllomyces dendrorhous, Erwinia uredovora*	Yamano *et al.* (1994), Miura *et al.* (1998), and Verwaal *et al.* (2007)
	GGPPS (geranylgeranyl pyrophosphate synthase)	*Taxus chinensis, Sulfolobus acidocaldarius, Taxus canadensis*	Dejong *et al.* (2006) and Engels *et al.* (2008)
Isoprenoid synthase	*GES* (geraniol synthase)	*Ocimum basilicum*	Oswald *et al.* (2007) and Fischer *et al.* (2011)
	LIS (linalool synthase)	*Clarkia breweri*	Herrero *et al.* (2008), and Rico *et al.* (2010)
	ADS (amorphadiene synthase)	*Artemisia annua*	Ro *et al.* (2006), Paradise *et al.* (2008), Ro *et al.* (2008), and Farhi *et al.* (2011)
	Cubebol synthase	*Citrus* × *paradisi*	Asadollahi *et al.* (2008, 2009, 2010)
	Epicedrol synthase	*Artemisia annua*	Jackson *et al.* (2003)
	Patchoulol synthase	*Pogostemon patchouly*	Asadollahi *et al.* (2008)
	Valencene synthase	*Citrus* × *paradise, Citrus sinensis*	Asadollahi *et al.* (2008) and Farhi *et al.* (2011)
	TS (taxadiene synthase)	*Taxus chiensis, Taxus brevifolia*	Dejong *et al.* (2006) and Engels *et al.* (2008)
Isoprenoid-modifying cytochrome P450*	*CYP71AV1* (amorphadiene oxidase)	*Aretmisia annua*	Ro *et al.* (2006, 2008)
	THY5α (taxadiene 5α hydroxylase)	*Taxus cuspidata*	Dejong *et al.* (2006) and Rontein *et al.* (2008)
	THY10b (taxoid 10β-hydroxylase)	*Taxus cuspidata*	Jennewein *et al.* (2005)
	CYP11A1 (steroid side chain cleavage)	*Bos taurus*	Duport *et al.* (1998, 2003) and Szczebara *et al.* (2003)
	CYP11B1 (11β-steroid hydroxylase)	*Bos taurus, Homo sapiens*	Dumas *et al.* (1996) and Szczebara *et al.* (2003)
	CYP17A1 (17α-steroid hydroxylase)	*Bos taurus*	Szczebara *et al.* (2003)
	CYP21A1 (21-steroid hydroxylase)	*Homo sapiens*	Szczebara *et al.* (2003)
Isoprenoid-modifying other	*DPP1* (diacylglycerol diphosphate phosphatase)	*Saccharomyces cerevisiae*	Tokuhiro *et al.* (2009)
	Δ7-sterol reductase	*Arabidopsis thaliana*	Duport *et al.* (1998, 2003) and Szczebara *et al.* (2003)
	3β-HSD (3β- hydroxyl steroid dehydrogenase/isomerase)	*Bos taurus, Homo sapiens*	Duport *et al.* (1998) and Szczebara *et al.* (2003)
Mevalonate pathway rate-controlling enzyme	*tHMG1* (HMG-CoA reductase truncated), or *HMG1* (wild-type HMG-CoA reductase)	*Saccharomyces cerevisiae*	Ro *et al.* (2006), Engels *et al.* (2008), Ro *et al.* (2008), Tokuhiro *et al.* (2009), Asadollahi *et al.* (2010), Ohto *et al.* (2010), Rico *et al.* (2010), and Farhi *et al.* (2011)
Ergosterol pathway transcription factor	*upc2-1* (uptake control 2)	*Saccharomyces cerevisiae*	Jackson *et al.* (2003), Ro *et al.* (2006), Engels *et al.* (2008), and Ro *et al.* (2008)
Carotenoid biosynthetic genes	*crtY, crtI,crtS, crtR, crtW, crtZ, crtB*	*Xanthophyllomyces dendrorhous, Paracoccus* sp., *Pantoea ananatis, Erwinia uredovora, Agrobacterium aurantiacum*	Yamano *et al.* (1994), Miura *et al.* (1998), Verwaal *et al.* (2007), Ukibe *et al.* (2009), and Verwaal *et al.* (2010)
Pyruvate dehydrogenase bypass enzymes	*ACS1* (acetyl-CoA synthase 1)	*Saccharomyces cerevisiae*	Shiba *et al.* (2007)
	ALD6 (acetaldehyde dehydrogenase 6)	*Saccharomyces cerevisiae*	Shiba *et al.* (2007)

*Isoprenoid-modifying cytochrome P450s were expressed together with heterologous partner redox proteins, namely cytochrome P450 reductase partners (CPR) for sesquiterpenes and diterpenes, adrenodoxin reductase (*ADR*) and adrenodoxin (*ADX*) for steroids.

Steroids

Steroids are molecules with four joined carbon rings, which are produced by the condensation of two FPP molecules to make squalene and then further functionalized by the activity of enzymes including cytochrome P450s. Hydrocortisone is a steroid and an anti-inflammatory drug used widely in formulations for topical ointments and also administered orally or intravenously under prescription. In the human body, native hydrocortisone biosynthesis from cholesterol takes place in the mitochondrial inner membrane and the smooth endoplasmic reticulum membrane of cells within the adrenal gland. Researchers looking to construct a hydrocortisone-producing yeast strain noted the absence of cholesterol in their host cell and instead high-jacked the ergosterol synthesis pathway to supply an unusual sterol precursor. An enzyme from *Arabidopsis thaliana*, *Δ7-sterol reductase*, was expressed in yeast to convert ergosterol and its immediate precursor to two non-native sterols, which could act as precursors for progesterone and hydrocortisone (Duport *et al.*, 1998). The construction of the heterologous hydrocortisone pathway from this point required the functional expression of five key mammalian enzymes, four of which were cytochrome P450s (Szczebara *et al.*, 2003). Two of the cytochrome P450s are localized to the mitochondria in their native cell. Heterologous activity for one of these mitochondrial enzymes, CYP11B1, which catalyzes the final step of hydrocortisone biosynthesis, was established by targeting this enzyme to the yeast mitochondria using a native yeast mitochondrial targeting sequence (Dumas *et al.*, 1996). In contrast, activity for the other mitochondrial cytochrome P450, CYP11A1, which catalyzes the first step in the heterologous pathway, was localized outside of the yeast cell mitochondria in the plasma membrane (Duport *et al.*, 2003). Strains were further engineered to heterologously express mammalian adrenodoxin and adrenodoxin reductase together with a mitochondrial variant of mammalian adrenodoxin and overexpress the native yeast mitochondrial NADPH cytochrome P450 reductase, *ARH1*, to supply reducing equivalents to cytochrome P450s acting in all parts of the heterologous pathway (Szczebara *et al.*, 2003). Titers of more than 10 mg L^{-1} hydrocortisone were reported for this yeast strain without the supply of precursors or intermediates in the culture medium. The completion of this hydrocortisone-producing yeast strain marked one of the first demonstrations of yeast metabolic engineering for secondary metabolite production (Szczebara *et al.*, 2003).

Other isoprenoids

Yeast strains have been engineered to produce many other isoprenoids including the monoterpenoid alcohols geraniol and linalool (Oswald *et al.*, 2007; Herrero *et al.*, 2008; Rico *et al.*, 2010), the sesquiterpene valencene, and the sesquiterpene alcohols epicedrol, cubebol, and patchoulol (Jackson *et al.*, 2003; Asadollahi *et al.*, 2008), and the overproduction of a native yeast diterpenoid alcohol, geranylgeraniol (Tokuhiro *et al.*, 2009; Ohto *et al.*, 2010).

Yeast as a host cell for the production of isoprenoids

In contrast to efforts to engineer the production of most other types of secondary metabolites, pathway construction efforts toward non-native isoprenoids began with the use of host cell-derived precursors and have rarely relied on exogenously fed substrates or intermediates. Strain engineering has therefore focused on optimizing the native isoprenoid biosynthetic pathway.

Efforts to maximize flux through the mevalonate pathway have focused primarily on two proteins: the metabolic enzyme HMG-CoA reductase encoded by *HMG1* and the transcription factor Upc2p. Hmg1p catalyzes the production of mevalonate, is the rate-limiting step in the mevalonate pathway, and is subject to strong regulation. Truncation of the N-terminus of Hmg1p to leave only the catalytic domain results in a deregulated hyperactive HMGR variant, tHmg1p (Donald *et al.*, 1997). Engineered isoprenoid-producing yeast strains that express tHmg1p exhibit increases in yields, for example as high as 5-fold for amorphadiene (Ro *et al.*, 2006) and 1.5-fold for taxadiene (Engels *et al.*, 2008). Upc2p regulates the transcription of ergosterol biosynthetic genes and a mutation of this transcription factor, *upc2-1*, has been shown to enhance its transcription activating function (Davies *et al.*, 2005). Expression of *upc2-1* in engineered yeast strains increases production of non-native isoprenoids, likely by upregulating mevalonate pathway genes in addition to downstream ergosterol biosynthetic genes (Jackson *et al.*, 2003; Ro *et al.*, 2006). The highest isoprenoid titers are usually observed with combined expression of *tHMG1* and *upc2-1* (Jackson *et al.*, 2003).

Increasing flux into the mevalonate pathway has been another approach targeted to enhance yields of isoprenoids in yeast. While the largest supply of acetyl-CoA is produced from pyruvate in the mitochondria by pyruvate dehydrogenase (PDH), this mevalonate precursor is also produced in the cytoplasm by a three-enzyme process termed the PDH-bypass. A yeast strain engineered to overexpress two PDH-bypass genes – a native acetaldehyde dehydrogenase (*ALD6*) and a *Salmonella enterica* acetyl-CoA synthetase ($ACS1^{L641P}$) variant with abolished post-translational regulation – exhibited enhanced amorphadiene production (Shiba *et al.*, 2007), presumably by

supplying greater cytosolic acetyl-CoA to the mevalonate pathway. Mevalonate production is an NADPH-dependent process and increasing the cellular availability of this coenzyme to Hmg1p and other enzymes in the pathway has been shown to increase sesquiterpene production (Asadollahi *et al.*, 2009). Researchers used metabolic modeling to determine that deletion of glutamate dehydrogenase 1 (*GDH1*), an ammonium metabolism enzyme that consumes NADPH, and overexpression of *GDH2* to compensate for the loss of activity with an enzyme that uses NADH would increase intracellular levels of NADPH. This approach resulted in a 1.85-fold increase in production of the sesquiterpene alcohol cubebol (Asadollahi *et al.*, 2009).

As flux through the mevalonate pathway is increased, additional modifications must be made to shuttle increased mevalonate to synthesize the isoprenoid precursors FPP and GGPP. Yeast strains engineered to overexpress the native FPP and GGPP synthases *ERG20* and *BTS1*, respectively, have demonstrated increased levels of these precursors (Verwaal *et al.*, 2007; Tokuhiro *et al.*, 2009; Ukibe *et al.*, 2009; Ohto *et al.*, 2010). For example, overexpression of *BTS1* can provide increases of β-carotene production between 6.5- and 22-fold (Verwaal *et al.*, 2007; Ukibe *et al.*, 2009). In addition, *ERG20* has been overexpressed as a Bts1–Erg20 fusion protein in yeast strains that produce up to 229 mg L^{-1} of the diterpenoid alcohol geranylgeraniol (Tokuhiro *et al.*, 2009; Ohto *et al.*, 2010). Results from early attempts to engineer taxol biosynthesis in yeast suggest that strategies based on the expression of heterologous FPP and GGPP synthases are also useful for this purpose, such as GGPP synthase from *S. acidocaldarius,* as described for taxadiene production above (Engels *et al.*, 2008).

In the yeast host cell, where several native isoprenoid pathways exist, it is particularly important to limit flux to competing pathways. Early indications that native isoprenoid pathways may influence yield through synthetic pathways came from observations of decreased ergosterol content in carotenoid-engineered strains (Yamano *et al.*, 1994) and increased engineered isoprenoid yield in high ergosterol-producing yeast species such as *Candida utilis* (Miura *et al.*, 1998) and also in an a-type mating strain (Jackson *et al.*, 2003). As one strategy to minimize loss of excess FPP to ergosterol, the expression of Erg9p, an enzyme that catalyzes the production of the ergosterol intermediate squalene from FPP, was placed under the control of a methionine-repressible promoter resulting in a twofold increase in the production of non-native isoprenoids such as artemisinic acid (Ro *et al.*, 2006). As an alternative strategy, in cases where the competing enzymes are not required for cell viability, these enzymes can be deleted for strain optimization, such as in the construction of a hydrocortisone producing yeast strain (Szczebara *et al.*, 2003).

Additional strain-engineering efforts have involved analyzing the effect of the heterologous pathway on host cell stress and other cellular processes. For example, transcriptional analysis showed the upregulation of pleiotropic drug-resistant genes encoding ATP-binding cassette (ABC) transporters and also of major facilitator superfamily (MSF) genes in carotenoid- and artemisinic acid-producing strains (Ro *et al.*, 2008; Verwaal *et al.*, 2010). These observations suggest that a cellular stress response occurs as a result of toxicity caused by the synthesis of non-native secondary metabolites. If the upregulation of drug-resistant genes serves to detoxify the cell, it could be expected that blocking these genes might affect yield, either negatively, by enhancing the stress response and limiting growth, or positively, by retaining precursors and intermediates in the cell and thus increasing their effective concentration. However, it has been observed that deletion of several ABC transporter genes has no effect on isoprenoid production levels in a monoterpenoid-producing strain (Oswald *et al.*, 2007), perhaps because of the action of many redundant processes to relieve stress and toxicity in the yeast cell.

Both *S. cerevisiae* and *E. coli* have proven to be suitable hosts for the construction of biosynthetic pathways for a range of isoprenoid end-products. The strategies employed to engineer isoprenoid production in *E. coli* provide a contrast to the work carried out in yeast. In *E. coli*, IPP and DMAPP can be produced from a nonmevolonate pathway; however, to increase the supply of precursors for amorphadiene production researchers have introduced and optimized the yeast mevalonate pathway in *E. coli* (Martin *et al.*, 2003; Kizer *et al.*, 2008; Tsuruta *et al.*, 2009; Morrone *et al.*, 2010). This strategy provides additional FPP from a heterologous pathway free from the endogenous control of the native nonmevalonate pathway. In addition, advances have been made in obtaining cytochrome P450 activities for isoprenoid production in *E. coli* (Chang *et al.*, 2007). However, a similar strategy for increasing non-native isoprenoid titers could potentially be applied in yeast by transferring the nonmevalonate pathway into an engineered yeast strain to further increase IPP and DMAPP levels (Maury *et al.*, 2008). Furthermore, yeast still outperform *E. coli* in the functional expression of cytochrome P450s, the most important enzymes for converting isoprenoid precursors into high-value products. Yeast as a host cell for artemisinic acid production has been successfully guided through scale-up to industrial-scale biofermentation reactors, making the use of yeast for commercial isoprenoid production one of the early success stories for yeast metabolic engineering for secondary metabolite synthesis. Advanced

synthetic biology tools will expand isoprenoid biosynthetic capabilities, increasing yields and reaching valuable therapeutics, such as taxol.

Alkaloids

Alkaloids are a large class of secondary metabolites that include a nitrogen-containing ring as part of their structure. Like phenolic compounds, alkaloids are synthesized from amino acid precursors. Alkaloids can be isolated from many organisms, but those isolated from plants are particularly important because they exhibit a wide range of pharmacological activities including analgesic, antimicrobial, and antiviral properties. Despite the large number of these chemicals found in nature, fewer alkaloid-producing yeast strains have been constructed than have phenolic- and isoprenoid-producing strains. However, knowledge gained from successful strategies for optimizing the biosynthesis of these other secondary metabolites can be leveraged for yeast alkaloid synthesis, particularly for similar classes of enzymes (e.g. cytochrome P450s) and similar precursor molecules (e.g. amino acids).

An early example of engineering yeast to express plant enzymes to synthesize alkaloid molecules demonstrated the production of cathenamine, an indole alkaloid that serves as an intermediate for the biosynthesis of other alkaloids in this class (Geerlings *et al.*, 2001). In this two-enzyme system, a strictosidine synthase (*STR*) from *C. roseus* was expressed in yeast to condense tryptamine and secologanin supplied in the culture medium to 2 g L^{-1} strictosidine (Table 3). Coexpression of a strictosidine β-glucosidase (*SGD*) enzyme from the same plant species resulted in trace amounts of cathenamine detected.

A second heterologous pathway for alkaloid biosynthesis constructed in yeast produced the benzylisoquinoline alkaloid (BIA) reticuline, an intermediate and precursor to many BIAs of medicinal interest including the powerful analgesics codeine and morphine (Hawkins & Smolke, 2008). Yeast strains were engineered to express three methyltransferase enzymes from opium poppy (*Papaver somniferum*) or yellow meadow-rue (*Thalictrum flavum*) to convert a fed substrate norlaudanosoline into both reticuline enantiomers (Table 3). As described earlier, a galactose-titratable expression system was used to select a mutant *TEF* promoter capable of driving expression of the methyltransferases at the lowest level for optimum activity, thus limiting stress to the host cell caused by high-level expression of heterologous genes. To demonstrate the utility of this strain as a BIA-production host cell, opium poppy berberine bridge enzyme (*BBE*) was expressed to make scoulerine, a branch-intermediate for the sanguinarine and berberine pathways, from the available cellular pool of reticuline. Two additional *T. flavum* enzymes were expressed to make canadine, the penultimate metabolite to berberine. The reticuline-producing strain was also used to synthesize salutaridine, an intermediate in the morphine branch, by expression of human cytochrome P450 *CYP2D6* and a reductase partner from *A. thaliana*. While these yeast strains relied on the supply of an intermediate in the culture medium, the production of reticuline from its amino acid precursor, tyrosine, was recently demonstrated in *E. coli*, suggesting that the same will be possible in a yeast host cell (Nakagawa *et al.*, 2011). In addition, co-culture of a reticuline-producing *E. coli* strain with an *S. cerevisiae* strain expressing *Coptis japonica* cytochrome P450 *CYP80G2* was used to produce the alkaloids magnoflorine and corytuberine (Minami *et al.*, 2008), demonstrating how the strengths of these species as production hosts can be combined for alkaloid biosynthesis.

Table 3. Alkaloid biosynthetic genes used to build production pathways in yeast

Abbreviation	Gene name	Species	Publications
4'OMT	3′-hydroxy-*N*-methylcoclaurine 4′-*O*-methyltransferase	*Thalictrum flavum, Papaver somniferum*	Hawkins & Smolke (2008)
6'OMT	Norcoclaurine 6-*O*-methyltransferase	*Thalictrum flavum, Papaver somniferum*	Hawkins & Smolke (2008)
BBE	Berberine bridge enzyme	*Coptis japonica, Papaver somniferum*	Hawkins & Smolke (2008) and Minami *et al.* (2008)
CNMT	Coclaurine *N*-methyltransferase	*Coptis japonica, Thalictrum flavum, Papaver somniferum*	Hawkins & Smolke (2008) and Minami *et al.* (2008)
CYP2D6	Cytochrome P450 2D6	*Homo sapiens*	Hawkins & Smolke (2008)
CYP719A	Canadine synthase	*Thalictrum flavum*	Hawkins & Smolke (2008)
CYP80G2	Corytuberine synthase	*Coptis japonica*	Minami *et al.* (2008)
SGR	Strictosidine β-glucosidase	*Catharanthus roseus*	Geerlings *et al.* (2001)
SMT	(S)-scoulerine 9-*O*-methyltransferase	*Thalictrum flavum*	Hawkins & Smolke (2008)
STR	Strictosidine synthase	*Catharanthus roseus*	Geerlings *et al.* (2001)

Polyketides

Polyketides are polymeric secondary metabolites assembled from acetyl-CoA, malonyl-CoA, or other CoA-activated subunits. Polyketides hold many biological activities, particularly as antibiotics such as erythromycin and tetracycline. The polyketide chain is assembled by the activity of enzymes called polyketide synthases (PKS). Type I PKS are enzymes that carry out repetitive chain assembly reactions either separately at many catalytic modules or iteratively at fewer modules. In type II PKS, the active sites of many enzymes function within a multienzyme complex, whereas type III PKS are composed of simple homodimers. The functional expression of plant type III PKS enzymes in yeast for the production of flavonoids and stilbenes was described in the phenolics section of this review. In this section, we explore preliminary data on the production of bacterial and fungal polyketides by the activity of heterologous type I PKS enzymes in *S. cerevisiae* (Table 4).

The mode of action of PKS enzymes requires several factors. First, an activated CoA-linked starter molecule and further acyl-CoA subunits must be available as substrates for the assembly. In flavonoid and stilbene synthesis, the starter unit is a hydroxycinnamic CoA-linked ester, such as 4-coumaroyl-CoA, which is combined with three malonyl-CoA molecules. Second, some PKS enzyme subunits must be activated by pantetheinylation, which is performed by a phosphopantetheinyl transferase enzyme (PPTase). These two factors – the supply of acyl-CoA precursors and the ability to perform the post-translation modification of PKS – along with the ability to functionally express the PKS subunits comprise the key requirements for a polyketide-synthesizing host cell.

Early success was demonstrated in the expression of a fungal type I iterative PKS in *S. cerevisiae* (Kealey *et al.*, 1998). A gene encoding 6-methylsalicylic acid synthase (6-MSAS) from *Penicillium patulum* required coexpression with *Bacillus subtilis* PPTase to produce the polyketide 6-methylsalicylic acid (6-MSA) at a titer of 1.7 g L^{-1} from endogenous acetyl-CoA and three molecules of malonyl-CoA. A second study tested a PPTase from *Aspergillus nidulans* and reported better performance from this enzyme than a *B. subtilis* PPTase (Wattanachaisaereekul *et al.*, 2007), although overall reported titers were lower owing to factors such as promoter and media selection (approximately 200 mg L^{-1} by batch fermentation). Because the substrates for the heterologous PKS are provided by the host cell metabolism, production of 6-MSA was increased nearly 60% by overexpression of the native *S. cerevisiae* acetyl-CoA carboxylase (*ACC1*) to enhance the conversion of acetyl-CoA to malonyl-CoA (Wattanachaisaereekul *et al.*, 2008). Titers of 6-MSA were further increased to 554 mg L^{-1} by supplying 50 g L^{-1} glucose in the culture medium.

The potential for the functional expression of type I multimodular PKSs in *S. cerevisiae* has also been demonstrated. 6-deoxyerythronolide (6-dEB) is a precursor for erythromycin and is synthesized by the PKS 6-deoxyerythronolide synthase (DEBS) from propionyl-CoA and 6 methylmalonyl-CoA molecules. To build functionality for 6-dEB production in *S. cerevisiae*, researchers expressed propionyl-CoA synthase from *Salmonella typhimurium* and propionyl-CoA carboxylase from *Streptomyces coelicolor* to synthesize these two substrates (propionyl-CoA and methylmalonyl-CoA) from propionate supplied in the culture medium (Mutka *et al.*, 2006). Coexpression of DEBS module 2 to make a triketide lactone demonstrated that these heterologously produced molecules could serve as substrates for DEBS activity. In addition, *S. cerevisiae* has proven useful as an expression host from which other iterative type I fungal PKS enzymes can be purified and reconstituted *in vitro* (Ma *et al.*, 2009), and in several instances researchers have observed PKSs to retain activity in the yeast expression host (Xu *et al.*, 2010; Zhou *et al.*, 2010).

However, the examples of polyketide production in *S. cerevisiae* are overshadowed by the greater successes of polyketide production in *E. coli* to date. *E. coli* strains have been engineered to produce the precursors for DEBS

Table 4. Polyketide biosynthetic genes used to build production pathways in yeast

Abbreviation	Gene name	Species	Publications
6-MSAS	6-methylsalicylic acid synthase	*Penicillium patulum*	Kealey *et al.* (1998), Wattanachaisaereekul *et al.* (2007, 2008)
ACC1	Acetyl-CoA carboxylase	*Saccharomyces cerevisiae*	Wattanachaisaereekul *et al.* (2008)
DEBS	6-deoxyerythronolide synthase (module 2)	*Saccharopolyspora erythraea*	Mutka *et al.* (2006)
PCC	Propionyl-CoA carboxylase	*Streptomyces coelicolor*	Mutka *et al.* (2006)
PPTase	Phosphopantetheinly transferase	*Bacillus subtilis, Aspergillus nidulans*	Kealey *et al.* (1998), Wattanachaisaereekul *et al.* (2007, 2008)
PrpE	Propionyl-CoA synthase	*Salmonella typhimurium*	Mutka *et al.* (2006)

and all active modules of this PKS, and the resulting 6-dEB-producing strain has subsequently been used to synthesize erythromycin (Peiru *et al.*, 2005). *E. coli* strains have been engineered to make additional polyketides including simvastatin and an intermediate of rifamycin (Watanabe *et al.*, 2003). Although *S. cerevisiae* is not as well developed as a production host for polyketides (with the exception of plant type III PKS activities), future work may take advantage of yeast as a host cell for fungal PKSs and the ability of yeast to supply several polyketide precursors from its native metabolism. This will require synthetic biology tools to assemble and tune functional multimodular PKS complexes and re-route native yeast metabolism to supply the needed substrates.

Future perspectives: the application of synthetic biology tools for secondary metabolite pathway and strain engineering

Efforts at engineering secondary metabolite production in yeast to date have generally focused on reconstructing the conversion steps associated with natural biosynthesis pathways, relying predominantly on identification of enzyme activities from the naturally producing organisms with limited knowledge of the regulatory networks that underlie these biosynthetic pathways. However, as synthetic biology tools for yeast metabolic engineering are developed we expect the field to move toward the robust design and construction of pathways that employ sophisticated regulatory networks for optimizing energy and resource usage and increasing product yields.

In the future, natural product-producing yeast strains will increasingly employ synthetic biology tools to engineer new features into heterologous secondary metabolite pathways. As one example, synthetic biology tools will support the embedded regulation of enzyme activities with engineered control systems that dynamically alter levels of pathway enzymes in response to key metabolites. These control systems will require tools such as expanded promoter libraries, RNA control elements, sensing-regulatory devices, and protein degradation tags with well-characterized activities. As another example, engineered pathways will integrate newly discovered enzyme activities from nontraditional source organisms, particularly from microorganisms that are difficult or impossible to culture, with enzymes evolved or designed in the laboratory for novel and optimized functions. The identification of new or enhanced enzyme activities will require more powerful tools for the construction of gene libraries and the screening of enzyme and whole-pathway outputs with genetically encoded metabolite sensors. In addition, these novel and optimized enzyme activities will be incorporated into engineered metabolic complexes constructed on scaffolds or within synthetic organelles to increase flux by increasing the local concentration of precursors and intermediates, limiting interactions with competing pathways, and providing an optimal environment for enzyme activity. These spatial engineering strategies will require tools for scaling the optimization of scaffold- or organelle-targeting strategies to encompass large and complex biosynthetic pathways.

Future efforts in strain engineering may be directed toward developing a catalog of yeast production hosts that are engineered to supply ample precursors for introduced heterologous pathways by the overproduction of central metabolites, especially amino acids and IPP, and of cofactors and cosubstrates such as NADPH and acetyl-CoA. These engineered production strains may also have altered functioning of cell and organelle membrane transport processes to allow for the efficient uptake of substrates, retention and compartmentalization of intermediates, and export of products. Knowledge gained from characterizing the function of native and introduced biosynthetic enzymes in yeast will provide the parameters to enable the development of ever more advanced computational models of metabolic flux, thereby increasing the predictability of pathway engineering. Thus, the tools emerging from synthetic biology have the exciting potential to advance the application of yeast as a production host for the economically feasible and commercial-scale manufacturing of drugs and fine chemicals.

Authors' contribution

M.S.S. and K.T. contributed equally to this work.

References

Albertsen L, Chen Y, Bach LS *et al.* (2011) Diversion of flux toward sesquiterpene production in *Saccharomyces cerevisiae* by fusion of host and heterologous enzymes. *Appl Environ Microbiol* **77**: 1033–1040.

Alper H, Fischer C, Nevoigt E & Stephanopoulos G (2005) Tuning genetic control through promoter engineering. *P Natl Acad Sci USA* **102**: 12678–12683.

Asadollahi MA, Maury J, Moller K, Nielsen KF, Schalk M, Clark A & Nielsen J (2008) Production of plant sesquiterpenes in *Saccharomyces cerevisiae*: effect of ERG9 repression on sesquiterpene biosynthesis. *Biotechnol Bioeng* **99**: 666–677.

Asadollahi MA, Maury J, Patil KR, Schalk M, Clark A & Nielsen J (2009) Enhancing sesquiterpene production in *Saccharomyces cerevisiae* through in silico driven metabolic engineering. *Metab Eng* **11**: 328–334.

Asadollahi MA, Maury J, Schalk M, Clark A & Nielsen J (2010) Enhancement of farnesyl diphosphate pool as direct precursor of sesquiterpenes through metabolic engineering of the mevalonate pathway in *Saccharomyces cerevisiae*. *Biotechnol Bioeng* **106**: 86–96.

Babiskin AH & Smolke CD (2011a) A synthetic library of RNA control modules for predictable tuning of gene expression in yeast. *Mol Syst Biol* **7**: 471.

Babiskin AH & Smolke CD (2011b) Synthetic RNA modules for fine-tuning gene expression levels in yeast by modulating RNase III activity. *Nucleic Acids Res* **39**: 8651–8664.

Baird SD, Turcotte M, Korneluk RG & Holcik M (2006) Searching for IRES. *RNA* **12**: 1755–1785.

Bayer TS, Widmaier DM, Temme K, Mirsky EA, Santi DV & Voigt CA (2009) Synthesis of methyl halides from biomass using engineered microbes. *J Am Chem Soc* **131**: 6508–6515.

Becker JV, Armstrong GO, van der Merwe MJ, Lambrechts MG, Vivier MA & Pretorius IS (2003) Metabolic engineering of *Saccharomyces cerevisiae* for the synthesis of the wine-related antioxidant resveratrol. *FEMS Yeast Res* **4**: 79–85.

Beekwilder J, Wolswinkel R, Jonker H, Hall R, de VosCH & Bovy A (2006) Production of resveratrol in recombinant microorganisms. *Appl Environ Microbiol* **72**: 5670–5672.

Beekwilder J, van der Meer IM, Sibbesen O, Broekgaarden M, Qvist I, Mikkelsen JD & Hall RD (2007) Microbial production of natural raspberry ketone. *Biotechnol J* **2**: 1270–1279.

Belli G, Gari E, Piedrafita L, Aldea M & Herrero E (1998) An activator/repressor dual system allows tight tetracycline-regulated gene expression in budding yeast. *Nucleic Acids Res* **26**: 942–947.

Benders GA, Noskov VN, Denisova EA *et al.* (2010) Cloning whole bacterial genomes in yeast. *Nucleic Acids Res* **38**: 2558–2569.

Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J & Sayers EW (2011) GenBank. *Nucleic Acids Res* **39**: D32–D37.

Berens C, Thain A & Schroeder R (2001) A tetracycline-binding RNA aptamer. *Bioorg Med Chem* **9**: 2549–2556.

Bruschi CV, Gjuracic K & Tosato V (2006) Yeast Artificial Chromosomes. *Encyclopedia of Life Sciences*. John Wiley & Sons Ltd, Chichester.

Caspi R, Altman T, Dale JM *et al.* (2010) The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. *Nucleic Acids Res* **38**: D473–D479.

Chang MC, Eachus RA, Trieu W, Ro DK & Keasling JD (2007) Engineering *Escherichia coli* for production of functionalized terpenoids using plant P450s. *Nat Chem Biol* **3**: 274–277.

Cochrane GR & Galperin MY (2010) The 2010 Nucleic Acids Research database issue and online database collection: a community of data resources. *Nucleic Acids Res* **38**: D1–D4.

Croteau RB, Kutchan TM & Lewis NG (2000) Natural products (secondary metabolites). *Biochemistry and Molecular Biology of Plants* (Buchanan B, Gruissem W & Jones R, eds), pp. 1250–1268. ASPB, Rockville, MD.

Davies PJ (2010) *The Plant Hormones: Their Nature, Occurrence, and Functions Plant Hormones* (Davies PJ, ed.), pp. 1–15. Springer, the Netherlands.

Davies BS, Wang HS & Rine J (2005) Dual activators of the sterol biosynthetic pathway of *Saccharomyces cerevisiae*: similar activation/regulatory domains but different response mechanisms. *Mol Cell Biol* **25**: 7375–7385.

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

Delebecque CJ, Lindner AB, Silver PA & Aldaye FA (2011) Organization of intracellular reactions with rationally designed RNA assemblies. *Science* **333**: 470–474.

Dixon N, Duncan JN, Geerlings T, Dunstan MS, McCarthy JE, Leys D & Micklefield J (2010) Reengineering orthogonally selective riboswitches. *P Natl Acad Sci USA* **107**: 2830–2835.

Donald KA, Hampton RY & Fritz IB (1997) Effects of overproduction of the catalytic domain of 3-hydroxy-3-methylglutaryl coenzyme A reductase on squalene synthesis in *Saccharomyces cerevisiae*. *Appl Environ Microbiol* **63**: 3341–3344.

Duarte NC, Herrgard MJ & Palsson BO (2004) Reconstruction and validation of *Saccharomyces cerevisiae* iND750, a fully compartmentalized genome-scale metabolic model. *Genome Res* **14**: 1298–1309.

Dueber JE, Wu GC, Malmirchegini GR *et al.* (2009) Synthetic protein scaffolds provide modular control over metabolic flux. *Nat Biotechnol* **27**: 753–759.

Dumas B, Cauet G, Lacour T *et al.* (1996) 11 beta-hydroxylase activity in recombinant yeast mitochondria. *In vivo* conversion of 11-deoxycortisol to hydrocortisone. *Eur J Biochem* **238**: 495–504.

Duport C, Spagnoli R, Degryse E & Pompon D (1998) Self-sufficient biosynthesis of pregnenolone and progesterone in engineered yeast. *Nat Biotechnol* **16**: 186–189.

Duport C, Schoepp B, Chatelain E, Spagnoli R, Dumas B & Pompon D (2003) Critical role of the plasma membrane for expression of mammalian mitochondrial side chain cleavage activity in yeast. *Eur J Biochem* **270**: 1502–1514.

Eckart MR & Bussineau CM (1996) Quality and authenticity of heterologous proteins synthesized in yeast. *Curr Opin Biotechnol* **7**: 525–530.

Ekino K, Hayashi H, Moriyama M, Matsuda M, Goto M, Yoshino S & Furukawa K (2002) Engineering of polyploid *Saccharomyces cerevisiae* for secretion of large amounts of fungal glucoamylase. *Appl Environ Microbiol* **68**: 5693–5697.

Ellington AD & Szostak JW (1990) *In vitro* selection of RNA molecules that bind specific ligands. *Nature* **346**: 818–822.

Engels B, Dahm P & Jennewein S (2008) Metabolic engineering of taxadiene biosynthesis in yeast as a first step towards Taxol (Paclitaxel) production. *Metab Eng* **10**: 201–206.

Eudes A, Baidoo EE, Yang F *et al.* (2011) Production of tranilast [N-(3′,4′-dimethoxycinnamoyl)-anthranilic acid] and its analogs in yeast *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* **89**: 989–1000.

Famulok M (1994) Molecular recognition of amino acids by RNA-aptamers: an L-citrulline binding RNA motif and its evolution into an L-arginine binder. *J Am Chem Soc* **116**: 1698–1706.

Fang F, Salmon K, Shen MW *et al.* (2011) A vector set for systematic metabolic engineering in *Saccharomyces cerevisiae*. *Yeast* **28**: 123–136.

Farhi M, Marhevka E, Masci T *et al.* (2011) Harnessing yeast subcellular compartments for the production of plant terpenoids. *Metab Eng* **13**: 474–481.

Fischer MJ, Meyer S, Claudel P, Bergdoll M & Karst F (2011) Metabolic engineering of monoterpene synthesis in yeast. *Biotechnol Bioeng* **108**: 1883–1892.

Flagfeldt DB, Siewers V, Huang L & Nielsen J (2009) Characterization of chromosomal integration sites for heterologous gene expression in *Saccharomyces cerevisiae*. *Yeast* **26**: 545–551.

Fujii T, Kondo K, Shimizu F, Sone H, Tanaka J & Inoue T (1990) Application of a ribosomal DNA integration vector in the construction of a brewer's yeast having alpha-acetolactate decarboxylase activity. *Appl Environ Microbiol* **56**: 997–1003.

Geerlings A, Redondo FJ, Contin A, Memelink J, van derHeijdenR & Verpoorte R (2001) Biotransformation of tryptamine and secologanin into plant terpenoid indole alkaloids by transgenic yeast. *Appl Microbiol Biotechnol* **56**: 420–424.

Gibson DG (2011) Enzymatic assembly of overlapping DNA fragments. *Methods Enzymol* **498**: 349–361.

Gibson DG, Benders GA, Andrews-Pfannkoch C *et al.* (2008) Complete chemical synthesis, assembly, and cloning of a Mycoplasma genitalium genome. *Science* **319**: 1215–1220.

Goffeau A, Barrell BG, Bussey H *et al.* (1996) Life with 6000 genes. *Science* **274**: 546–547.

Grilly C, Stricker J, Pang WL, Bennett MR & Hasty J (2007) A synthetic gene network for tuning protein degradation in *Saccharomyces cerevisiae*. *Mol Syst Biol* **3**: 127.

Gustafsson C, Govindarajan S & Minshull J (2004) Codon bias and heterologous protein expression. *Trends Biotechnol* **22**: 346–353.

Hackett EA, Esch RK, Maleri S & Errede B (2006) A family of destabilized cyan fluorescent proteins as transcriptional reporters in *S. cerevisiae*. *Yeast* **23**: 333–349.

Hawkins KM & Smolke CD (2006) The regulatory roles of the galactose permease and kinase in the induction response of the GAL network in *Saccharomyces cerevisiae*. *J Biol Chem* **281**: 13485–13492.

Hawkins KM & Smolke CD (2008) Production of benzylisoquinoline alkaloids in *Saccharomyces cerevisiae*. *Nat Chem Biol* **4**: 564–573.

Hecht K, Bailey JE & Minas W (2002) Polycistronic gene expression in yeast versus cryptic promoter elements. *FEMS Yeast Res* **2**: 215–224.

von Heijne G (1990) The signal peptide. *J Membr Biol* **115**: 195–201.

Hellen CU & Sarnow P (2001) Internal ribosome entry sites in eukaryotic mRNA molecules. *Genes Dev* **15**: 1593–1612.

Herrero O, Ramon D & Orejas M (2008) Engineering the *Saccharomyces cerevisiae* isoprenoid pathway for de novo production of aromatic monoterpenes in wine. *Metab Eng* **10**: 78–86.

Hillier SG (2007) Diamonds are forever: the cortisone legacy. *J Endocrinol* **195**: 1–6.

Hoover DM & Lubkowski J (2002) DNAWorks: an automated method for designing oligonucleotides for PCR-based gene synthesis. *Nucleic Acids Res* **30**: e43.

Hwang EI, Kaneko M, Ohnishi Y & Horinouchi S (2003) Production of plant-specific flavanones by *Escherichia coli* containing an artificial gene cluster. *Appl Environ Microbiol* **69**: 2699–2706.

Jackson BE, Hart-Wells EA & Matsuda SP (2003) Metabolic engineering to produce sesquiterpenes in yeast. *Org Lett* **5**: 1629–1632.

Jenison RD, Gill SC, Pardi A & Polisky B (1994) High-resolution molecular discrimination by RNA. *Science* **263**: 1425–1429.

Jennewein S, Park H, DeJong JM, Long RM, Bollon AP & Croteau RB (2005) Coexpression in yeast of Taxus cytochrome P450 reductase with cytochrome P450 oxygenases involved in Taxol biosynthesis. *Biotechnol Bioeng* **89**: 588–598.

Jeppsson M, Johansson B, Jensen PR, Hahn-Hagerdal B & Gorwa-Grauslund MF (2003) The level of glucose-6-phosphate dehydrogenase activity strongly influences xylose fermentation and inhibitor sensitivity in recombinant *Saccharomyces cerevisiae* strains. *Yeast* **20**: 1263–1272.

Jiang H, Wood KV & Morgan JA (2005) Metabolic engineering of the phenylpropanoid pathway in *Saccharomyces cerevisiae*. *Appl Environ Microbiol* **71**: 2962–2969.

Jorgensen K, Rasmussen AV, Morant M *et al.* (2005) Metabolon formation and metabolic channeling in the biosynthesis of plant natural products. *Curr Opin Plant Biol* **8**: 280–291.

Kealey JT, Liu L, Santi DV, Betlach MC & Barr PJ (1998) Production of a polyketide natural product in nonpolyketide-producing prokaryotic and eukaryotic hosts. *P Natl Acad Sci USA* **95**: 505–509.

Kell DB, Kaprelyants AS & Grafen A (1995) Pheromones, social behaviour and the functions of secondary metabolism in bacteria. *Trends Ecol Evol* **10**: 126–129.

Kizer L, Pitera DJ, Pfleger BF & Keasling JD (2008) Application of functional genomics to pathway optimization for increased isoprenoid production. *Appl Environ Microbiol* **74**: 3229–3241.

Kodumal SJ, Patel KG, Reid R, Menzella HG, Welch M & Santi DV (2004) Total synthesis of long DNA sequences: synthesis of a contiguous 32-kb polyketide synthase gene cluster. *P Natl Acad Sci USA* **101**: 15573–15578.

Kouprina N & Larionov V (2008) Selective isolation of genomic loci from complex genomes by transformation-associated recombination cloning in the yeast *Saccharomyces cerevisiae*. *Nat Protoc* **3**: 371–377.

Kudla B & Nicolas A (1992) A multisite integrative cassette for the yeast *Saccharomyces cerevisiae*. *Gene* **119**: 49–56.

Labbe S & Thiele DJ (1999) Copper ion inducible and repressible promoter systems in yeast. *Methods Enzymol* **306**: 145–153.

Larionov V, Kouprina N, Graves J, Chen XN, Korenberg JR & Resnick MA (1996) Specific cloning of human DNA as yeast artificial chromosomes by transformation-associated recombination. *P Natl Acad Sci USA* **93**: 491–496.

Lee FW & Da Silva NA (1997a) Sequential delta-integration for the regulated insertion of cloned genes in *Saccharomyces cerevisiae*. *Biotechnol Prog* **13**: 368–373.

Lee FW & Da Silva NA (1997b) Improved efficiency and stability of multiple cloned gene insertions at the delta sequences of *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* **48**: 339–345.

Lee W & Da Silva NA (2006) Application of sequential integration for metabolic engineering of 1,2-propanediol production in yeast. *Metab Eng* **8**: 58–65.

Leonard E, Yan Y, Lim KH & Koffas MA (2005) Investigation of two distinct flavone synthases for plant-specific flavone biosynthesis in *Saccharomyces cerevisiae*. *Appl Environ Microbiol* **71**: 8241–8248.

Leonard E, Yan Y & Koffas MA (2006) Functional expression of a P450 flavonoid hydroxylase for the biosynthesis of plant-specific hydroxylated flavonols in *Escherichia coli*. *Metab Eng* **8**: 172–181.

Leonard E, Lim KH, Saw PN & Koffas MA (2007) Engineering central metabolic pathways for high-level flavonoid production in *Escherichia coli*. *Appl Environ Microbiol* **73**: 3877–3886.

Leonard E, Yan Y, Fowler ZL, Li Z, Lim CG, Lim KH & Koffas MA (2008) Strain improvement of recombinant *Escherichia coli* for efficient production of plant flavonoids. *Mol Pharm* **5**: 257–265.

Liang JC, Bloom RJ & Smolke CD (2011) Engineering biological systems with synthetic RNA molecules. *Mol Cell* **43**: 915–926.

Lopes TS, Klootwijk J, Veenstra AE, van der Aar PC, van Heerikhuizen H, Raue HA & Planta RJ (1989) High-copy-number integration into the ribosomal DNA of *Saccharomyces cerevisiae*: a new vector for high-level expression. *Gene* **79**: 199–206.

Lopes TS, de Wijs IJ, Steenhauer SI, Verbakel J & Planta RJ (1996) Factors affecting the mitotic stability of high-copy-number integration into the ribosomal DNA of *Saccharomyces cerevisiae*. *Yeast* **12**: 467–477.

Ma SM, Li JW, Choi JW *et al.* (2009) Complete reconstitution of a highly reducing iterative polyketide synthase. *Science* **326**: 589–592.

Martin VJ, Pitera DJ, Withers ST, Newman JD & Keasling JD (2003) Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. *Nat Biotechnol* **21**: 796–802.

Mateus C & Avery SV (2000) Destabilized green fluorescent protein for monitoring dynamic changes in yeast gene expression with flow cytometry. *Yeast* **16**: 1313–1323.

Maury J, Asadollahi MA, Moller K, Schalk M, Clark A, Formenti LR & Nielsen J (2008) Reconstruction of a bacterial isoprenoid biosynthetic pathway in *Saccharomyces cerevisiae*. *FEBS Lett* **582**: 4032–4038.

McCarthy JE (1998) Posttranscriptional control of gene expression in yeast. *Microbiol Mol Biol Rev* **62**: 1492–1553.

Michener JK, Thodey K, Liang JC & Smolke CD (2011) Applications of genetically-encoded biosensors for the construction and control of biosynthetic pathways. *Metab Eng*. DOI: 10.1016/j.ymben.2011.09.004.

Minami H, Kim JS, Ikezawa N, Takemura T, Katayama T, Kumagai H & Sato F (2008) Microbial production of plant benzylisoquinoline alkaloids. *P Natl Acad Sci USA* **105**: 7393–7398.

Miura Y, Kondo K, Saito T, Shimada H, Fraser PD & Misawa N (1998) Production of the carotenoids lycopene, beta-carotene, and astaxanthin in the food yeast *Candida utilis*. *Appl Environ Microbiol* **64**: 1226–1229.

Miyahisa I, Kaneko M, Funa N, Kawasaki H, Kojima H, Ohnishi Y & Horinouchi S (2005) Efficient production of (2S)-flavanones by *Escherichia coli* containing an artificial biosynthetic gene cluster. *Appl Microbiol Biotechnol* **68**: 498–504.

Miyahisa I, Funa N, Ohnishi Y, Martens S, Moriguchi T & Horinouchi S (2006) Combinatorial biosynthesis of flavones and flavonols in *Escherichia coli*. *Appl Microbiol Biotechnol* **71**: 53–58.

Moglia A, Comino C, Lanteri S *et al.* (2010) Production of novel antioxidative phenolic amides through heterologous expression of the plant's chlorogenic acid biosynthesis genes in yeast. *Metab Eng* **12**: 223–232.

Morrone D, Lowry L, Determan MK, Hershey DM, Xu M & Peters RJ (2010) Increasing diterpene yield with a modular metabolic engineering system in *E. coli*: comparison of MEV and MEP isoprenoid precursor pathway engineering. *Appl Microbiol Biotechnol* **85**: 1893–1906.

Mumberg D, Muller R & Funk M (1994) Regulatable promoters of *Saccharomyces cerevisiae*: comparison of transcriptional activity and their use for heterologous expression. *Nucleic Acids Res* **22**: 5767–5768.

Mumberg D, Muller R & Funk M (1995) Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene* **156**: 119–122.

Mutka SC, Bondi SM, Carney JR, Da Silva NA & Kealey JT (2006) Metabolic pathway engineering for complex polyketide biosynthesis in *Saccharomyces cerevisiae*. *FEMS Yeast Res* **6**: 40–47.

Naesby M, Nielsen SV, Nielsen CA *et al.* (2009) Yeast artificial chromosomes employed for random assembly of biosynthetic pathways and production of diverse compounds in *Saccharomyces cerevisiae*. *Microb Cell Fact* **8**: 45.

Nakagawa A, Minami H, Kim JS, Koyanagi T, Katayama T, Sato F & Kumagai H (2011) A bacterial platform for

fermentative production of plant alkaloids. *Nat Commun* **2**: 326.

Ohto C, Muramatsu M, Obata S, Sakuradani E & Shimizu S (2010) Production of geranylgeraniol on overexpression of a prenyl diphosphate synthase fusion gene in *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* **87**: 1327–1334.

Oliveira C, Teixeira JA, Lima N, Da Silva NA & Domingues L (2007) Development of stable flocculent *Saccharomyces cerevisiae* strain for continuous Aspergillus niger beta-galactosidase production. *J Biosci Bioeng* **103**: 318–324.

Omura F, Fujita A, Miyajima K & Fukui N (2005) Engineering of yeast Put4 permease and its application to lager yeast for efficient proline assimilation. *Biosci Biotechnol Biochem* **69**: 1162–1171.

Oswald M, Fischer M, Dirninger N & Karst F (2007) Monoterpenoid biosynthesis in *Saccharomyces cerevisiae*. *FEMS Yeast Res* **7**: 413–421.

Paradise EM, Kirby J, Chan R & Keasling JD (2008) Redirection of flux through the FPP branch-point in *Saccharomyces cerevisiae* by down-regulating squalene synthase. *Biotechnol Bioeng* **100**: 371–378.

Parekh RN, Shaw MR & Wittrup KD (1996) An integrating vector for tunable, high copy, stable integration into the dispersed Ty delta sites of *Saccharomyces cerevisiae*. *Biotechnol Prog* **12**: 16–21.

Parent SA, Fenimore CM & Bostian KA (1985) Vector systems for the expression, analysis and cloning of DNA sequences in *S. cerevisiae*. *Yeast* **1**: 83–138.

Partow S, Siewers V, Bjorn S, Nielsen J & Maury J (2010) Characterization of different promoters for designing a new expression vector in *Saccharomyces cerevisiae*. *Yeast* **27**: 955–964.

Peiru S, Menzella HG, Rodriguez E, Carney J & Gramajo H (2005) Production of the potent antibacterial polyketide erythromycin C in *Escherichia coli*. *Appl Environ Microbiol* **71**: 2539–2547.

Pichersky E & Gang DR (2000) Genetics and biochemistry of secondary metabolites in plants: an evolutionary perspective. *Trends Plant Sci* **5**: 439–445.

Pompon D, Louerat B, Bronine A & Urban P (1996) Yeast expression of animal and plant P450s in optimized redox environments. *Methods Enzymol* **272**: 51–64.

Ralston L, Subramanian S, Matsuno M & Yu O (2005) Partial reconstruction of flavonoid and isoflavonoid biosynthesis in yeast using soybean type I and type II chalcone isomerases. *Plant Physiol* **137**: 1375–1388.

Redding-Johanson AM, Batth TS, Chan R *et al.* (2011) Targeted proteomics for metabolic pathway optimization: application to terpene production. *Metab Eng* **13**: 194–203.

Reese CB (2005) Oligo- and poly-nucleotides: 50 years of chemical synthesis. *Org Biomol Chem* **3**: 3851–3868.

Rico J, Pardo E & Orejas M (2010) Enhanced production of a plant monoterpene by overexpression of the 3-hydroxy-3-methylglutaryl coenzyme A reductase catalytic domain in *Saccharomyces cerevisiae*. *Appl Environ Microbiol* **76**: 6449–6454.

Ro DK & Douglas CJ (2004) Reconstitution of the entry point of plant phenylpropanoid metabolism in yeast (*Saccharomyces cerevisiae*): implications for control of metabolic flux into the phenylpropanoid pathway. *J Biol Chem* **279**: 2600–2607.

Ro DK, Paradise EM, Ouellet M *et al.* (2006) Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature* **440**: 940–943.

Ro DK, Ouellet M, Paradise EM *et al.* (2008) Induction of multiple pleiotropic drug resistance genes in yeast engineered to produce an increased level of anti-malarial drug precursor, artemisinic acid. *BMC Biotechnol* **8**: 83.

Rontein D, Onillon S, Herbette G, Lesot A, Werck-Reichhart D, Sallaud C & Tissier A (2008) CYP725A4 from yew catalyzes complex structural rearrangement of taxa-4(5),11(12)-diene into the cyclic ether 5(12)-oxa-3(11)-cyclotaxane. *J Biol Chem* **283**: 6067–6075.

Roze LV, Chanda A & Linz JE (2011) Compartmentalization and molecular traffic in secondary metabolism: a new understanding of established cellular processes. *Fungal Genet Biol* **48**: 35–48.

Sakai A, Shimizu Y & Hishinuma F (1990) Integration of heterologous genes into the chromosome of *Saccharomyces cerevisiae* using a delta sequence of yeast retrotransposon Ty. *Appl Microbiol Biotechnol* **33**: 302–306.

Sellick CA & Reece RJ (2005) Eukaryotic transcription factors as direct nutrient sensors. *Trends Biochem Sci* **30**: 405–412.

Sha Y, Broglio EP, Cannon JF & Schoelz JE (1995) Expression of a plant viral polycistronic mRNA in yeast, *Saccharomyces cerevisiae*, mediated by a plant virus translational transactivator. *P Natl Acad Sci USA* **92**: 8911–8915.

Shao Z & Zhao H (2009) DNA assembler, an *in vivo* genetic method for rapid construction of biochemical pathways. *Nucleic Acids Res* **37**: e16.

Shao Z, Luo Y & Zhao H (2011) Rapid characterization and engineering of natural product biosynthetic pathways via DNA assembler. *Mol Biosyst* **7**: 1056–1059.

Shiba Y, Paradise EM, Kirby J, Ro DK & Keasling JD (2007) Engineering of the pyruvate dehydrogenase bypass in *Saccharomyces cerevisiae* for high-level production of isoprenoids. *Metab Eng* **9**: 160–168.

Stemmer WP, Crameri A, Ha KD, Brennan TM & Heyneker HL (1995) Single-step assembly of a gene and entire plasmid from large numbers of oligodeoxyribonucleotides. *Gene* **164**: 49–53.

Szczebara FM, Chandelier C, Villeret C *et al.* (2003) Total biosynthesis of hydrocortisone from a simple carbon source in yeast. *Nat Biotechnol* **21**: 143–149.

Tokuhiro K, Muramatsu M, Ohto C *et al.* (2009) Overproduction of geranylgeraniol by metabolically engineered *Saccharomyces cerevisiae*. *Appl Environ Microbiol* **75**: 5536–5543.

Trantas E, Panopoulos N & Ververidis F (2009) Metabolic engineering of the complete pathway leading to heterologous biosynthesis of various flavonoids and stilbenoids in *Saccharomyces cerevisiae*. *Metab Eng* **11**: 355–366.

Tsuruta H, Paddon CJ, Eng D *et al.* (2009) High-level production of amorpha-4,11-diene, a precursor of the antimalarial agent artemisinin, in *Escherichia coli*. *PLoS ONE* **4**: e4489.

Tuerk C & Gold L (1990) Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* **249**: 505–510.

Ukibe K, Hashida K, Yoshida N & Takagi H (2009) Metabolic engineering of *Saccharomyces cerevisiae* for astaxanthin production and oxidative stress tolerance. *Appl Environ Microbiol* **75**: 7205–7211.

Vannelli T, Wei QiW, Sweigard J, Gatenby AA & Sariaslani FS (2007) Production of p-hydroxycinnamic acid from glucose in *Saccharomyces cerevisiae* and *Escherichia coli* by expression of heterologous genes from plants and fungi. *Metab Eng* **9**: 142–151.

Ververidis F, Trantas E, Douglas C, Vollmer G, Kretzschmar G & Panopoulos N (2007) Biotechnology of flavonoids and other phenylpropanoid-derived natural products. Part I: chemical diversity, impacts on plant biology and human health. *Biotechnol J* **2**: 1214–1234.

Verwaal R, Wang J, Meijnen JP, Visser H, Sandmann G, van den Berg JA & van Ooyen AJ (2007) High-level production of beta-carotene in *Saccharomyces cerevisiae* by successive transformation with carotenogenic genes from *Xanthophyllomyces dendrorhous*. *Appl Environ Microbiol* **73**: 4342–4350.

Verwaal R, Jiang Y, Wang J, Daran JM, Sandmann G, van den Berg JA & van Ooyen AJ (2010) Heterologous carotenoid production in *Saccharomyces cerevisiae* induces the pleiotropic drug resistance stress response. *Yeast* **27**: 983–998.

Voigt CA (2008) Life from information. *Nat Methods* **5**: 27–28.

Wang Y, Halls C, Zhang J, Matsuno M, Zhang Y & Yu O (2011) Stepwise increase of resveratrol biosynthesis in yeast *Saccharomyces cerevisiae* by metabolic engineering. *Metab Eng* **13**: 455–463.

Watanabe K, Rude MA, Walsh CT & Khosla C (2003) Engineered biosynthesis of an ansamycin polyketide precursor in *Escherichia coli*. *P Natl Acad Sci USA* **100**: 9774–9778.

Wattanachaisaereekul S, Lantz AE, Nielsen ML, Andresson OS & Nielsen J (2007) Optimization of heterologous production of the polyketide 6-MSA in *Saccharomyces cerevisiae*. *Biotechnol Bioeng* **97**: 893–900.

Wattanachaisaereekul S, Lantz AE, Nielsen ML & Nielsen J (2008) Production of the polyketide 6-MSA in yeast engineered for increased malonyl-CoA supply. *Metab Eng* **10**: 246–254.

Watts KT, Lee PC & Schmidt-Dannert C (2004) Exploring recombinant flavonoid biosynthesis in metabolically engineered *Escherichia coli*. *Chembiochem* **5**: 500–507.

Williams DH, Stone MJ, Hauck PR & Rahman SK (1989) Why are secondary metabolites (natural products) biosynthesized? *J Nat Prod* **52**: 1189–1208.

Win MN & Smolke CD (2007) A modular and extensible RNA-based gene-regulatory platform for engineering cellular function. *P Natl Acad Sci USA* **104**: 14283–14288.

Win MN, Klein JS & Smolke CD (2006) Codeine-binding RNA aptamers and rapid determination of their binding constants using a direct coupling surface plasmon resonance assay. *Nucleic Acids Res* **34**: 5670–5682.

Wingler LM & Cornish VW (2011) Reiterative Recombination for the *in vivo* assembly of libraries of multigene pathways. *P Natl Acad Sci USA* **108**: 15135–15140.

Wishart JA, Hayes A, Wardleworth L, Zhang N & Oliver SG (2005) Doxycycline, the drug used to control the tet-regulatable promoter system, has no effect on global gene expression in *Saccharomyces cerevisiae*. *Yeast* **22**: 565–569.

Xu W, Cai X, Jung ME & Tang Y (2010) Analysis of intact and dissected fungal polyketide synthase-nonribosomal peptide synthetase *in vitro* and in *Saccharomyces cerevisiae*. *J Am Chem Soc* **132**: 13604–13607.

Yamano S, Ishii T, Nakagawa M, Ikenaga H & Misawa N (1994) Metabolic engineering for production of beta-carotene and lycopene in *Saccharomyces cerevisiae*. *Biosci Biotechnol Biochem* **58**: 1112–1114.

Yan Y, Kohli A & Koffas MA (2005) Biosynthesis of natural flavanones in *Saccharomyces cerevisiae*. *Appl Environ Microbiol* **71**: 5610–5613.

Yan Y, Huang L & Koffas MA (2007) Biosynthesis of 5-deoxyflavanones in microorganisms. *Biotechnol J* **2**: 1250–1262.

Zhang Y, Li SZ, Li J *et al.* (2006) Using unnatural protein fusions to engineer resveratrol biosynthesis in yeast and Mammalian cells. *J Am Chem Soc* **128**: 13030–13031.

Zhou W, Edelman GM & Mauro VP (2001) Transcript leader regions of two *Saccharomyces cerevisiae* mRNAs contain internal ribosome entry sites that function in living cells. *P Natl Acad Sci USA* **98**: 1531–1536.

Zhou W, Edelman GM & Mauro VP (2003) Isolation and identification of short nucleotide sequences that affect translation initiation in *Saccharomyces cerevisiae*. *P Natl Acad Sci USA* **100**: 4457–4462.

Zhou H, Qiao K, Gao Z, Vederas JC & Tang Y (2010) Insights into radicicol biosynthesis via heterologous synthesis of intermediates and analogs. *J Biol Chem* **285**: 41412–41421.