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Strategies for microbial synthesis of high-value phytochemicals

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Phytochemicals are of great pharmaceutical and agricultural importance, but often exhibit low abundance in nature. Recent demonstrations of industrial-scale production of phytochemicals in yeast have shown that microbial production of these high-value chemicals is a promising alternative to sourcing these molecules from native plant hosts. However, a number of challenges remain in the broader application of this approach, including the limited knowledge of plant secondary metabolism and the inefficient reconstitution of plant metabolic pathways in microbial hosts. In this Review, we discuss recent strategies to achieve microbial biosynthesis of complex phytochemicals, including strategies to: (1) reconstruct plant biosynthetic pathways that have not been fully elucidated by mining enzymes from native and non-native hosts or by enzyme engineering; (2) enhance plant enzyme activity, specifically cytochrome P450 activity, by improving efficiency, selectivity, expression or electron transfer; and (3) enhance overall reaction efficiency of multi-enzyme pathways by dynamic control, compartmentalization or optimization with the host's metabolism. We also highlight remaining challenges to — and future opportunities of — this approach.

Phytochemicals exhibit a wide range of bioactivities, and are of great pharmaceutical, ecological and agricultural significance[1]. Plants were recorded for medicinal applications as early as 2600 BC and, so far, phytochemicals comprise approximately 25% of drugs in the United States[2]. One major challenge in developing and utilizing phytochemicals for medicinal and agricultural purposes is their low abundance in nature, resulting in limiting supplies of these molecules that in many cases are unable to meet research and market demand. Thus, alternative approaches for the supply of these valuable molecules have been pursued. Isolating phytochemicals from plant cell or tissue cultures can enable rapid and sustainable phytochemical production[3]. However, the high costs of scaling plant cell culture, challenges in developing plant cell lines, and low phytochemical accumulation has limited the commercial application of this approach to a small number of pharmaceutical phytochemicals such as Taxol. The engineering of recombinant plants, particularly based on model plant hosts, offers the potential of providing phytochemicals more efficiently. However, challenges with plant engineering and low production efficiencies has limited the broad application of this approach[3]. Total chemical synthesis offers an approach to produce phytochemicals without reliance on plant-based production[4], but has faced challenges in providing efficient commercial syntheses for structurally complex phytochemicals (for example, those with numerous chiral centres or polycyclic rings).

Recently, bio-based production of phytochemicals in fast growing, fermentable microorganisms such as yeast and *Escherichia coli* has been pursued as an alternative to the traditional supply chains. Successful semi-synthetic artemisinin[5], rose oil[6] and resveratrol production[7,8] have highlighted the commercial feasibility of this approach. More recently, efforts to engineer microbial biosynthesis of several medicinally important phytochemicals — such as the medicinal opioid alkaloids[9–14] and precursors to vinblastine[15] and Taxol[16,17] — via the reconstruction of complex biosynthetic pathways have lent further support to this alternative production route by harnessing nature's synthesis potential for efficient and rapid phytochemical production.

However, several major challenges remain in advancing microbial bioproduction as a general approach for the supply of valuable phytochemicals, including: (1) the biosynthetic routes for most phytochemicals have not been clearly elucidated; (2) plant enzymes, especially cytochrome P450s, often exhibit poor activity in microbial hosts; and (3) the metabolic flux through complex plant biosynthetic pathways in microbial hosts is typically low. In general, the optimization of enzyme activities and pathway efficiencies has been a key challenge in heterologous pathway reconstitution. A number of synthetic biology tools and metabolic engineering strategies have been developed to enhance the activity and efficiency of heterologous enzymes and pathways and these have been recently reviewed elsewhere[18–20]. In this Review, we focus on recently developed strategies specific to addressing challenges that arise in the reconstitution of plant-specialized metabolic pathways.

Strategies to reconstruct plant biosynthetic pathways

Unlike their microbial counterparts, genes encoding the biosynthetic enzymes in natural product pathways were believed to be primarily distributed throughout the plant genome. Thus, although enzymes responsible for the synthesis of microbial natural products are normally discovered and characterized in batches, those associated with plant natural products are typically discovered individually, which has contributed to the disparity in our understanding of microbial versus plant natural product biosynthesis. The biosynthetic pathways of most phytochemicals are not full elucidated, with enzymes that catalyse certain steps in the proposed pathway remaining unknown. To enable the microbial biosynthesis of target phytochemicals, the cognate enzymes or alternative enzymes from other organisms that can catalyse the unknown biosynthetic steps must be identified or engineered. Currently, enzyme discovery and engineering require significant time and effort. Newer strategies to pathway elucidation and reconstitution are leveraging advances in genomics, bioinformatics and synthetic biology (Fig. 1).

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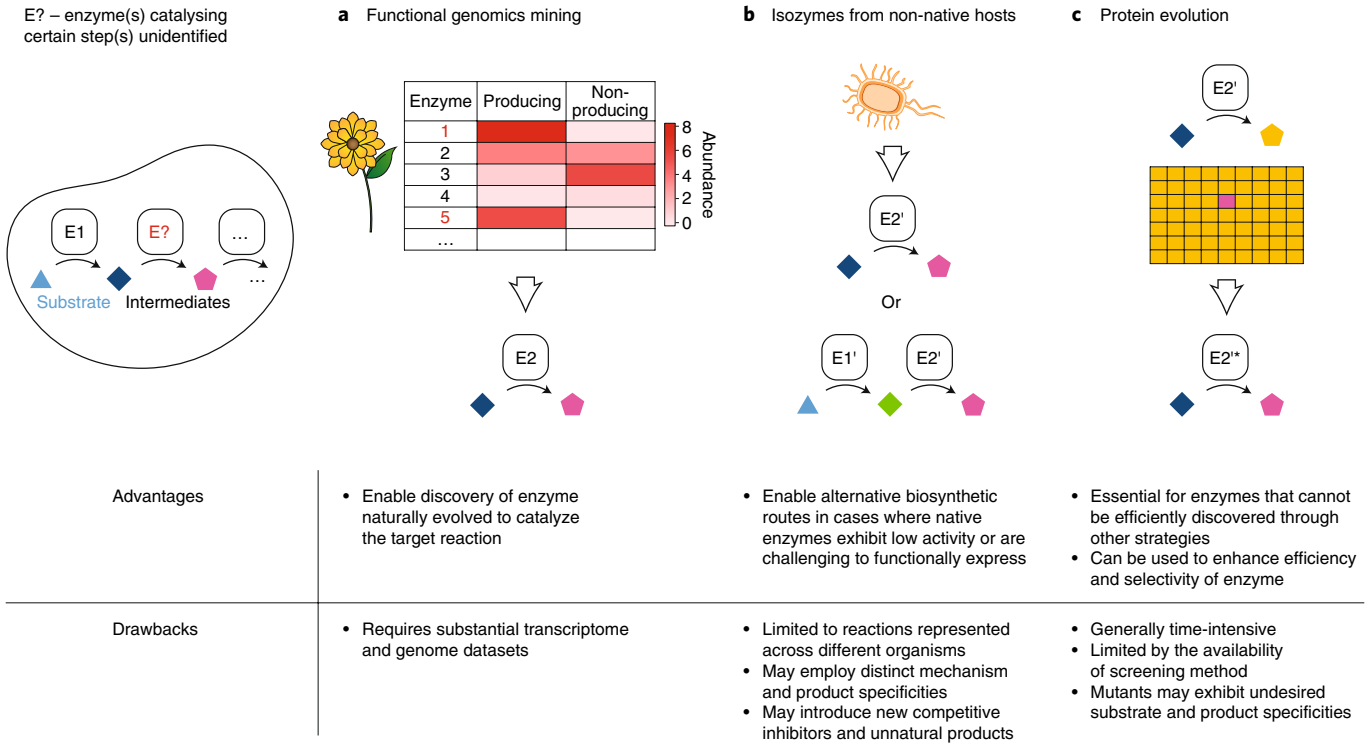

	a Functional genomics mining	b Isozymes from non-native hosts	c Protein evolution
Advantages	• Enable discovery of enzyme naturally evolved to catalyze the target reaction	• Enable alternative biosynthetic routes in cases where native enzymes exhibit low activity or are challenging to functionally express	• Essential for enzymes that cannot be efficiently discovered through other strategies • Can be used to enhance efficiency and selectivity of enzyme
Drawbacks	• Requires substantial transcriptome and genome datasets	• Limited to reactions represented across different organisms • May employ distinct mechanism and product specificities • May introduce new competitive inhibitors and unnatural products	• Generally time-intensive • Limited by the availability of screening method • Mutants may exhibit undesired substrate and product specificities

Fig. 1 | Strategies to reconstruct plant biosynthetic pathways that are not fully elucidated. The biosynthetic pathways underlying most plant natural products are not completely elucidated. Three general approaches have been applied to either discover or engineer the biosynthetic enzyme that can catalyse the target conversion. **a**, Identification of the enzyme from the native plant based on functional genomics analysis. **b**, Identification of an enzyme that can catalyse the desired reaction from organisms other than the native producer. **c**, Engineering an enzyme with the desired activity via protein evolution. E, enzyme.

Identifying biosynthetic enzymes through mining the genomes and transcriptomes of native hosts. Advances in next-generation sequencing technologies have expanded access to plant genomes and transcriptomes, enabling the discovery of enzymes for missing biosynthetic steps via functional genomics strategies. Several recent examples highlight the application of next-generation sequencing, cDNA microarray, and mass spectrometry technologies for advancing elucidation of pathways underlying the biosynthesis of important plant phytochemicals[21]. Many plant biosynthetic enzymes have been identified through functional genomics approaches. A transcriptome comparison between product producing and non-producing plants can be used to identify enzymes that are expressed differentially between them as a starting point. For example, a 10-gene cluster involved in noscapine biosynthesis was discovered through comparative transcriptome profiling of noscapine-producing and non-producing poppy varieties[22]. Additional information regarding the type of target reaction(s) can be used to further narrow the pool of candidate enzyme(s) that might be responsible for the target conversion(s). For example, two enzymes catalysing reactions in the downstream morphine pathway (thebaine 6-O-demethylation, codeine O-demethylation) were identified through a cDNA comparison between morphine-accumulating poppy varieties and a morphine-free/high-thebaine variety[23]. This method enabled the identification of a unique class of enzymes — 2-oxoglutarate/Fe(II)-dependent dioxygenases — catalysing these O-demethylation reactions, which might have otherwise been overlooked if starting from a list of proposed candidate enzymes in the absence of the differential transcriptome data.

Alternatively, transcriptome databases can be searched for specific enzymes based on chemical hypotheses for the missing biosynthetic steps. For example, the isomerase catalysing the conversion from (*S*)-reticuline to (*R*)-reticuline, the gateway intermediate in the biosynthesis of morphine in opium poppy, was recently identified. Based on a prior *in planta* study that observed the accumulation of (*S*)-reticuline upon inactivation of codeinone reductase (COR)[24], researchers queried transcriptome databases and expressed-sequence-tag libraries of poppies to identify a didomain enzyme encoding a fusion between a cytochrome P450 mono-oxygenase and a COR-like aldo-keto reductase, which was subsequently confirmed through bioassay[9,25,26]. In another example, researchers queried the transcriptome of *Cannabis sativa* with sequences from bacterial polyketide cyclases to discover a plant polyketide cyclase required for the biosynthesis of tetrahydrocannabinol[27]. However, the success of this approach is dependent on a strong hypothesis that correctly predicts the type of target enzyme.

Mining biosynthetic enzymes from non-native hosts. In addition to reconstructing a biosynthetic pathway with enzymes from the native producing host, gaps in the pathway can be filled with alternative enzymes from other organisms that catalyse the same reactions and therefore lead to the formation of the same intermediates. This approach leverages the broader biodiversity that is present in the natural world and thus a potentially larger pool of candidate biosynthetic enzymes. However, it requires that similar reactions are represented across different organisms, which may not always be the case for specialized phytochemicals (for example, morphine). Thus, this approach is typically more applicable to reactions involving primary metabolites that are shared across different kingdoms. In addition, a number of enzyme databases have been established that include such information as enzyme nomenclature, catalytic activity and other functional parameters[28–30], facilitating the search process for enzymes that can catalyse reactions of

interest. This approach was recently utilized in reconstructing a biosynthetic pathway to (*S*)-reticuline — a key intermediate in the biosynthesis of benzylisoquinoline alkaloids — which is synthesized via a condensation reaction of dopamine and 4-hydroxyphenylacetaldehyde (4-HPAA), both derived from L-tyrosine. The biosynthetic route by which native producing plants make dopamine has not been fully elucidated, and thus researchers engineered alternative pathways to dopamine from L-tyrosine based on enzymes from non-native producers. Two separate teams of researchers reconstructed a biosynthetic pathway to dopamine using a bacteria tyrosinase and bacteria L-DOPA decarboxylase (DODC) in *E. coli*[31], and through a mammalian tyrosine hydroxylase (TyrH) and bacteria DODC in *Saccharomyces cerevisiae*[13]. One potential challenge with this approach is that enzymes taken from different organisms may catalyse the same reaction through distinct mechanisms, which has to be accounted for in the design of the reconstructed pathway. For example, the activity of mammalian TyrH requires the electron carrier tetrahydrobiopterin, which is non-native to microorganisms. Thus, in reconstructing a biosynthetic route based on TyrH activity, researchers further engineered a tetrahydrobiopterin biosynthesis and recycling pathway based on four additional mammalian enzymes[13]. Another challenge that can arise is that enzymes from other pathways can exhibit substrate and product specificities different from that of the native biosynthetic enzyme[31]. For example, tyrosinases have broad substrate specificities and can catalyse numerous oxidative reactions resulting in the synthesis of undesired products such as DOPA-quinone.

In incorporating enzymes from non-native producing hosts, researchers can also reconstruct alternative biosynthetic routes to desired products that go through different intermediates. This approach further expands the enzymes potentially available to reconstruct the biosynthetic pathway by not being limited to those that follow a specific route. For example, researchers have engineered a reticuline biosynthetic pathway in *E. coli* that proceeds through the non-native intermediate norlaudanosoline via the condensation of dopamine and 3,4-dihydroxyphenylacetaldehyde (3,4-DHPAA), instead of 4-HPAA, by incorporating a monoamine oxidase to convert dopamine to 3,4-DHPAA[31]. Enzymes from non-native producers may also be used in pathway reconstruction when the native enzymes have low activities or are difficult to functionally express in microbial hosts. For example, to achieve efficient synthesis of chalcones in *E. coli*, researchers employed a yeast phenylalanine ammonia lyase (PAL) instead of the plant PAL[32]. The yeast PAL acts on a broader range of substrates to directly achieve the synthesis of both cinnamic acid and 4-coumaric acid, a property utilized by researchers to reconstruct a chalcone biosynthetic pathway that does not require the activity of a plant cytochrome P450, a class of enzymes that is generally challenging to functionally express in bacterial hosts. Similarly, a tyrosine ammonia-lyase (TAL) was used to achieve efficient synthesis of 4-coumaric acid and the downstream product resveratrol from tyrosine in *S. cerevisiae*[33]. Although both strategies for incorporating enzymes from non-native producers can face challenges in differing specificities from native biosynthetic enzymes, strategies based on constructing different biosynthetic routes also change the metabolite profile and pathway intermediates, which can introduce new competitive inhibitors and unnatural products.

Engineering missing biosynthetic enzymes through protein evolution. Another strategy to obtain enzymes for missing steps in reconstructed plant biosynthetic pathways is to engineer the missing catalysts through protein evolution based on structure-inspired site-directed mutagenesis or random mutagenesis and screening. Enzyme engineering is a complementary approach to identifying enzymes from native and non-native hosts as: (1) achieving high activities of some plant enzymes in microbial hosts may be challenging; (2) enhancing the enzyme specificity towards the target molecule can be critical to avoid side reactions toward undesired products; and (3) the enzyme discovery process for some reaction steps can take decades. This approach was utilized to address the low activity that was observed from the *Artemisia annua* cytochrome P450 CYP71AV1 that converts amorphadiene to dihydroartemisinic acid when heterologous expressed in *E. coli*[34]. Researchers developed a ROSETTA-based energy minimization model to identify rational mutations that alter the substrate specificity of the *Bacillus megaterium* cytochrome P450 BM3 to oxidize amorphadiene to artemisinic-11*S*,12-epoxide, which can be readily converted to dihydroartemisinic acid[34,35]. The engineered BM3 was shown to achieve substantially higher oxidation efficiency of amorphadiene than the native CYP71AV1 in *E. coli*. In another example, researchers evolved a plant DOPA oxidase CYP76AD1 to function as a tyrosine hydroxylase in the context of a reconstructed (*S*)-reticuline biosynthetic pathway in *S. cerevisiae*[11]. A fluorescence-based screen was established for CYP76AD1 mutants exhibiting tyrosine hydroxylase activity based on the further conversion of the product (L-DOPA) to betaxanthin by a plant DOPA dioxygenase[11]. However, the CYP76AD1 mutant evolved for increased tyrosine hydroxylase activity also exhibits DOPA oxidase activity, which branches the metabolic flux toward undesired synthesis of L-dopaquinone[11]. In general, enhancing selectivity towards the desired product remains a challenge in strategies based on engineering enzyme activities, which largely relies on the availability of an efficient screening method indicating the presence of the target molecule.

Biosynthetic pathways for phytochemicals can be reconstructed through strategies that leverage genomic information from the native plant hosts, adapting enzymes from other organisms, and engineering new enzymes. In practice, these strategies are often employed in combination to assemble full biosynthetic pathways. Discovery from the native plant host is often the starting point, but requires substantial transcriptome and genome datasets, often from producing and non-producing plants or across different plant tissues. Adapting enzymes from other organisms, requires that similar reactions (and metabolites) be present in other organisms and can face challenges with differences in specificities and mechanisms between the different organisms. Finally, enzyme engineering approaches have been used to enhance activities and engineer entirely new activities, but are generally time-intensive, dependent on the availability of a screen or selection and also suffer from specificity of the resulting engineered enzyme.

Strategies to enhance plant enzyme activity

Microbial hosts exhibit intracellular microenvironments that are different from those present in the native plant hosts, which can lead to incorrect folding, lower expression levels and catalytic efficiencies of plant enzymes in the heterologous context. Among the various classes of biosynthetic enzymes present in plant-specialized metabolism, plant cytochrome P450s occur at a high frequency[36]. Plant cytochrome P450s are membrane-bound oxidoreductase enzymes that are anchored in the endoplasmic reticulum membrane, and the correct folding and localization of plant P450s in microbial hosts has been challenging. In addition, pairing of the endoplasmic-reticulum-bound cytochrome P450 reductase is required for efficient electron transfer from NADPH to the plant P450 enzyme, and increases complexity in achieving the optimal expression ratio of the cytochrome P450s and its reductase partner. Enhancing the heterologous activities of plant P450s has been a major challenge in establishing efficient and high-yielding microbial production platforms for complex phytochemicals (Fig. 2).

Improving the efficiency and selectivity of plant enzymes toward desired products. Increasing the efficiency and selectivity of a rate-limiting pathway enzyme towards the synthesis of the desired product can enhance the metabolic flux to the target phytochemical.

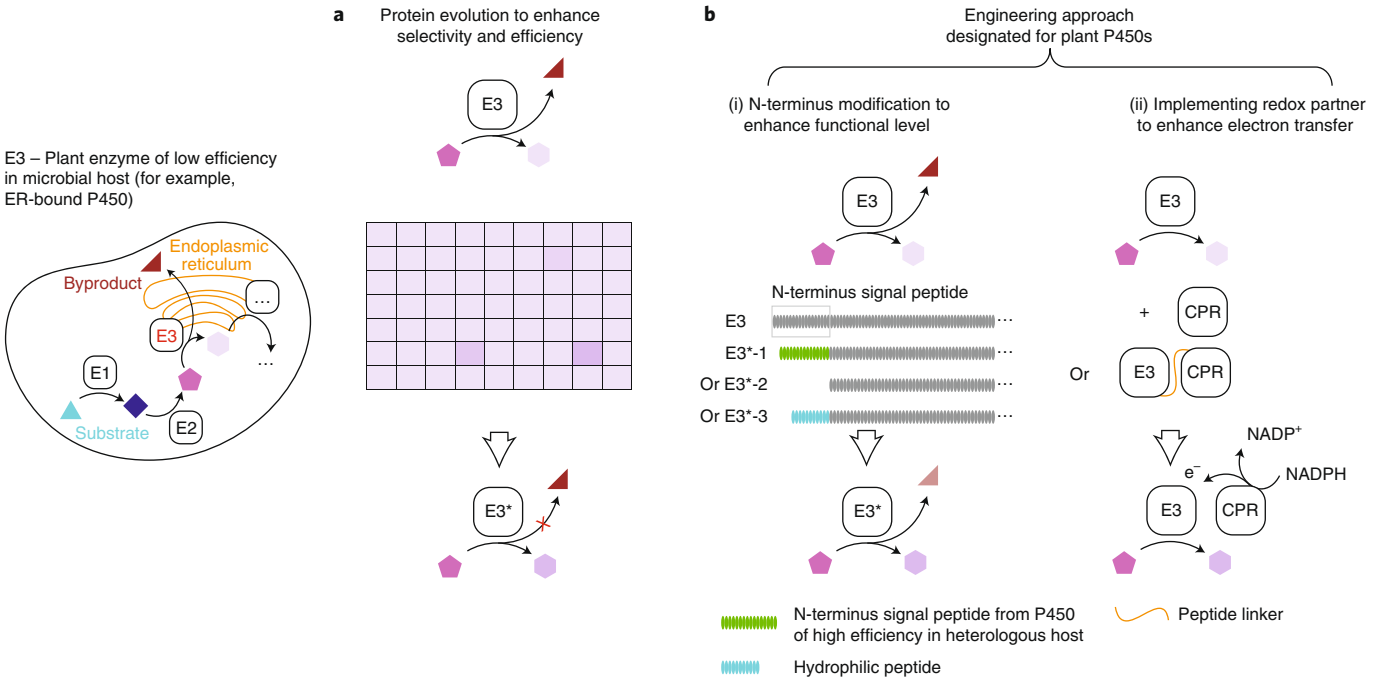

Fig. 2 | Strategies to enhance plant cytochrome P450 activity in microbial hosts. Plant cytochrome P450s are challenging enzymes to functionally express in heterologous microbial hosts. **a**, Protein engineering can be used to enhance the efficiency and selectivity of enzymes, including cytochrome P450s. **b**, The efficiency of plant cytochrome P450s can be improved by: (i) increasing the quantity of functional enzyme through N-terminus engineering, and (ii) improving the electron transfer through optimizing the interaction between the P450 and CPR.

One common approach is to enhance the enzyme expression level through modifications that increase the levels of associated transcripts and proteins[18]. In addition, protein evolution can be applied to improve the folding and kinetic properties, decrease substrate inhibition, and increase selectivity for desired substrates. For example, researchers evolved plant cytochrome P450 CYP76AD1 variants that exhibit increased tyrosine hydroxylase activity based on a combination of higher expression levels and reduced DOPA oxidase activity[11]. Similarly, mutations were rationally incorporated into taxadiene synthase and taxadien-5α-hydroxylase (CYP725A4) in order to achieve enhanced selectivity towards the desired products taxa-4(20)-11(12)-diene and taxadien-5α-ol, respectively, which resulted in improved production of Taxol precursors in *E. coli*[37]. A similar rational mutation approach was applied to steroid 11β-hydroxylase (CYP11B1) to achieve enhanced selectivity towards the desired product 21-deoxycortisol[38]. In another example, protein evolution was applied to enhance the activity of 4-coumarate CoA ligase towards the synthesis of coumaroyl-CoA, with mutant variants exhibiting decreased k_{cat}/K_M for the undesired substrate ferulate and enhanced k_{cat}/K_M for coumarate[39]. The enhancement of catalytic efficiencies through protein engineering has the advantage of not introducing additional expression burden to the microbial host in contrast to expression tuning strategies. Comprehensive analytical methods for pathway performance based on metabolomics and flux analysis[40,41] can be used to determine key limiting steps in a complex pathway on which to focus protein evolution efforts. However, enzyme evolution typically benefits from a high-throughput screen, which is generally not available for most activities of interest. A variety of screening strategies have been applied to evolve biosynthetic enzymes for enhanced isoprenoid production in microbial hosts[42].

Strategies for improving the efficiency of plant cytochrome P450s. *Improving the quantity of functional plant cytochrome P450s.* A common strategy to enhance the activity of plant cytochrome P450s in microbial hosts is to improve the folding or stability through N-terminus engineering. The N-terminus of plant cytochrome P450s is critical in directing the correct anchoring of the nascent polypeptides into the endoplasmic-reticulum membrane. N-terminus engineering has been applied to plant cytochrome P450s for the biosynthesis of complex phytochemicals in both *E. coli* and yeast hosts. Since *E. coli* lacks internal ER membranes, functional heterologous expression of eukaryotic cytochrome P450s and reductase partners in *E. coli* represents a particular challenge. Researchers have addressed this challenge through truncating or replacing the hydrophobic N-terminus of eukaryotic P450 enzymes with a hydrophilic peptide[35,43]. In one example, researchers achieved high level production of 8-hydroxycadinene in *E. coli* (>100 mg l^{-1}), through a combination of codon-optimization and N-terminal transmembrane engineering of the plant P450 8-cadinene hydroxylase (CAH)[35]. However, a recent study examined the effect of different N-terminal modifications on the activity of the CYP725A4 in *E. coli*, and suggested that although hydrophilic tags can enhance the solubility of P450s, the enzyme activity may decrease due to the disrupted enzyme-membrane interaction[44]. Interestingly, the recent N-terminal mutation study on CYP11B1 indicates that mutations within the N-terminus of cytochrome P450s can slightly alter the selectivity[38].

In contrast to *E. coli*, yeast has internal ER membranes and thus the activity of heterologous P450s in yeast can be improved by replacing the native N-terminal sequence with sequences from other P450s or ER-membrane bound proteins to facilitate correct folding and anchoring. For example, to achieve the biosynthesis of opioids in yeast, researchers fused a key pathway cytochrome P450 (salutaridine synthase) with various N-terminal tags from different groups of P450s to achieve a chimeric variant that exhibits a six-fold greater conversion of (*R*)-reticuline to salutaridine[9]. Although a single hydrophilic tag has been demonstrated to enhance the expression of many eukaryotic cytochrome P450s in *E. coli*[44,45], there has been no demonstration of a universally valid N-terminal tag to

enhance plant P450 activity in yeast. Therefore, a set of generalized N-terminal tags for enhancing plant P450 activity are necessary in advancing yeast as a host for complex plant pathways.

Improving the electron transfer to plant cytochrome P450s. The common strategy for pairing plant cytochrome P450s and their reductase partners (CPRs) in *E. coli* is fusing the P450 with the native plant or non-native bacteria CPR on a single peptide[46]. However, excess expression of CPR can be toxic to the cell as it leads to excessive uncoupled electrons and redox activities[47,48]. Further, in constructing biosynthetic pathways harbouring multiple P450s, a fusion chimera would have to be constructed for each P450 enzyme. In one recent study, the activity of CYP725A4 was measured when fused with its native *Taxus cuspidata* CPR and co-expressed with the stand-alone CPR. The results from this study indicate that CYP725A4 exhibits higher activity as a stand-alone enzyme than when fused with the CPR, suggesting that the traditional strategy of fusing the P450 and CPR in *E. coli* may not be necessary[44]. It is unclear how broadly applicable the results from this single study are, but it implies that a multi-P450 pathway can be reconstituted in *E. coli* by introducing one unfused CPR. Yeast encodes an endogenous CPR, which under most cases can facilitate the electron transfer to heterologous plant P450s, but typically with lower efficiency than plant CPRs[14]. Although CPRs native to the target plant pathway have been employed in many examples[9,13,15,47,49,50], the CPR from *Arabidopsis thaliana* (AtATR1) has been shown to serve as an efficient reductase partner to various plant P450s[10,51,52]. In addition, in certain cases cytochrome b_5 has been demonstrated to facilitate the electron transfer. In one example, the production of artemisinic acid was enhanced when the *A. annua* cytochrome b_5 was expressed in the artemisinic-acid-producing yeast[47]. Although there are a number of examples of plant cytochrome P450s being functionally expressed in *E. coli*, in most cases yeast has been shown to be a better host for plant pathways harbouring multiple P450s.

Integrative strategies for improving the heterologous activity of plant cytochrome P450s in microbial hosts must address issues that arise in folding, localization, electron transfer and catalytic activity. Strategies directed to improving functional expression levels and pairing with CPRs are more likely to be generalizable to a group of P450s (for example, N-terminus engineering, CPR fusions). However, the application of protein engineering strategies may often be required to improve catalytic efficiencies or specific folding and coupling issues that may arise in the non-native cellular environment. Enzyme engineering strategies applied to plant cytochrome P450s will face similar challenges in being dependent on the availability of a screen or selection and often suffer from specificity of the resulting engineered enzyme.

Enhancing the efficiency of multi-enzyme pathways

The microbial biosynthesis of complex phytochemicals involves multiple, coordinated reactions within a cell that occur in the context of the host's endogenous metabolic network. Thus, pathway efficiency is influenced by the interactions between metabolites and enzymes within and outside of the target pathway. To enhance efficiencies at a pathway level, researchers have implemented a number of strategies, including: (1) dynamic tuning of the expression levels of the pathway enzymes; (2) compartmentalization of enzymes to direct the interaction of pathway enzymes and metabolites; and (3) globally optimizing the heterologous biosynthetic pathway with the endogenous microbial metabolic network (Fig. 3).

Dynamic control over enzyme expression levels. Dynamic control over enzyme levels and activities can be used to change target metabolite concentrations and pathway flux in response to changing intracellular and extracellular environments. Dynamic flux balancing utilizes regulatory machinery to actively change the levels or activities of pathway enzymes during the growth and production phases. A dynamic control system comprises two elements: a sensing element that is responsive to a target metabolite (for example, a transcription factor); and a regulatory element whose activity is modulated by the sensing element and controls production/consumption of one or more target metabolites in the pathway (for example, a promoter/operator sequence that binds the transcription factor and in turn controls expression of pathway enzymes).

Dynamic control strategies have been used to optimize the production of desired phytochemicals by controlling the production of precursors toxic to the microbial host. In one example, farnesyl pyrophosphate (FPP)-responsive promoters were incorporated into the amorphadiene biosynthetic pathway in *E. coli* to lower the accumulation of a toxic precursor (FPP)[53]. Endogenous *E. coli* promoters responsive to intracellular FPP levels were screened and subsequently used to implement a negative feedback control scheme over the expression of eight enzymes in the upstream FPP-producing pathway, and a positive feedback control scheme over the expression of an amorpha-4,11-diene synthase converting FPP to amorphadiene. This dynamic control strategy resulted in the increase in production of amorphadiene from 700 mg l^{-1} to 1.6 g l^{-1} compared to systems with a static control strategy.

Dynamic control strategies have also been used to control endogenous enzymes catalysing reactions that compete with the heterologous biosynthetic pathway. In one example, researchers used a dynamic control strategy to limit metabolic flux towards ergosterol biosynthesis in *S. cerevisiae* and direct greater flux into a heterologous α-santalene biosynthetic pathway[54]. Researchers replaced the native promoter controlling the expression of squalene synthase with a glucose-induced promoter in order to limit the competitive reaction under glucose-limited cultivation conditions. In this example, an endogenous transcription factor and associated promoter element responsive to glucose levels were utilized as the sensor/regulator pair. The dynamic control strategy in combination with other genetic modifications was shown to increase α-santalene titres by twofold.

The ability to implement dynamic control strategies relies on (and is limited by) the availability of suitable sensor/regulator pairs. So far the majority of effort has focused on the utilization of native transcription factor/promoter pairs in microbial hosts, thereby bypassing challenges associated with constructing new metabolite sensors de novo. To broaden the range of biosynthetic pathways that this strategy can be applied to, the discovery and adaptation of novel sensor/regulator pairs in microbial hosts such as yeast and *E. coli* are critical. Recent progress in engineering biosensors responsive to muconic acid[55], flavonoids (naringenin[55], kaempferol and quercetin[56]) and malonyl-CoA[57,58] will pave the way to implementing dynamic control strategies in complex biosynthetic pathways for phytochemical production in yeast and *E. coli*, providing powerful tools for pathway optimization. In addition, the development of RNA-based switches that respond to phytochemicals is an emerging research area that will contribute additional sensor/regulator pairs. Although current research has focused on using phytochemical-responsive RNA switches to monitor target concentrations or strain screening[59,60], these RNA-based sensors may play an important role advancing dynamic control strategies in future.

Subcellular compartmentalization of heterologous enzymes. Subcellular compartmentalization of heterologous enzymes has been used to increase local concentrations of essential substrates and co-factors, and to provide intracellular environments that support optimal enzyme kinetics. In some cases, pathways that occur across multiple compartments may achieve increased efficiencies by being relocalized to one compartment. For example, the biosynthetic pathway for L-ornithine, which is a precursor for plant tropane alkaloids, involves upstream reactions that synthesize

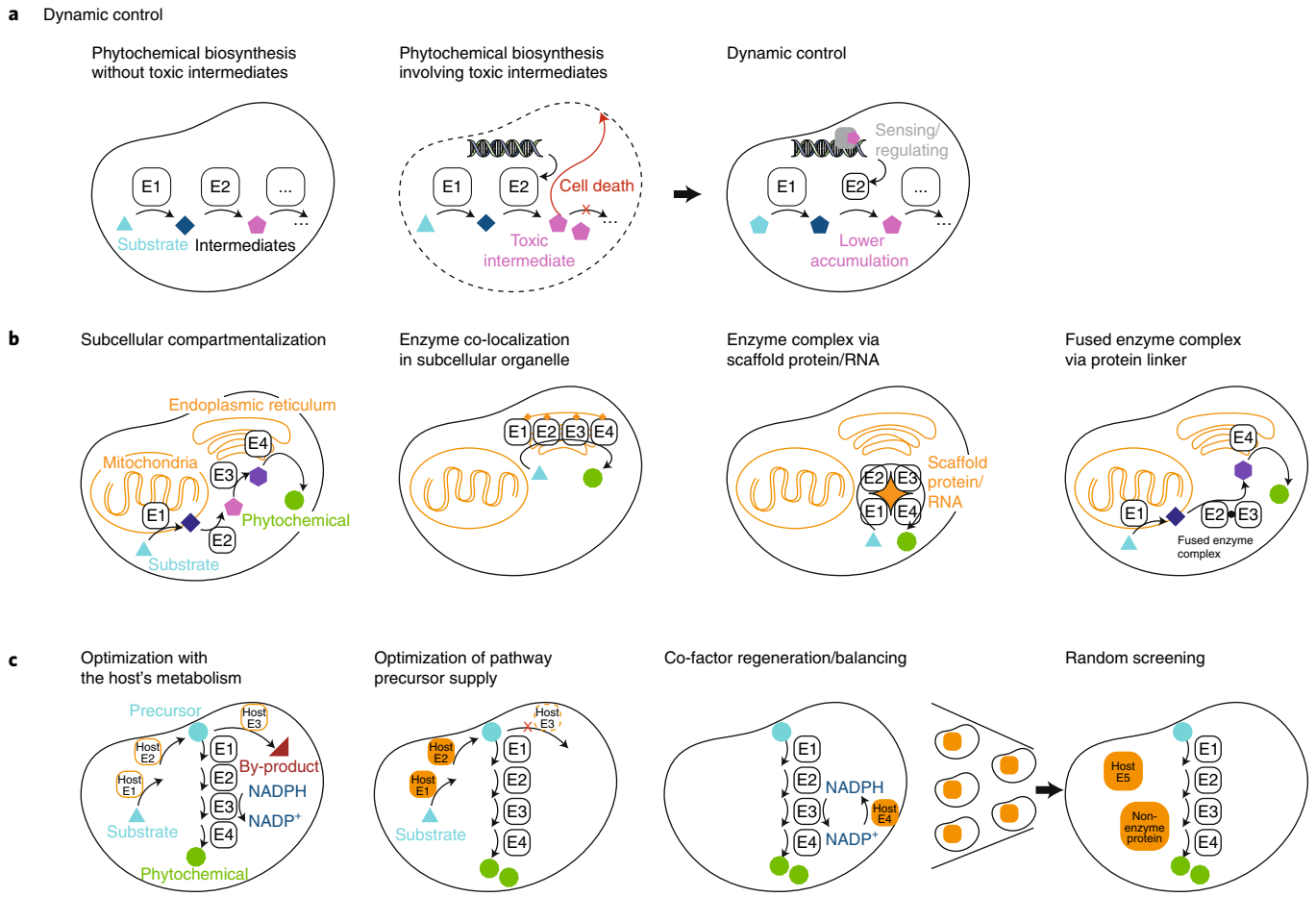

Fig. 3 | Strategies to enhance the overall efficiency of multi-enzyme pathways within a microbial cell. a, Dynamic control: a multi-enzyme pathway including multiple enzymes and their corresponding products (intermediates) is depicted. The accumulation of a toxic intermediate triggers the sensing-regulating system, leading to the repression of E2 that produces the toxic intermediate, thus lowering the cell toxicity and enabling the production of the downstream phytochemical. **b**, Subcellular compartmentalization: a multi-enzyme pathway including four enzymes (E1 localized in mitochondria, E2 and E3 in cytoplasm, and E4 in endoplasmic reticulum) is depicted. E1–E4 can be co-localized into the endoplasmic reticulum (left), assembled into a synthetic protein/RNA scaffold (middle), or E2 and E3 can be fused via a protein linker to make a fused protein complex in cytoplasm. **c**, Optimization with the host's metabolism: a multi-heterologous enzyme pathway (E1–E4) and endogenous enzymes (hosts E1–E3) are depicted. Overexpression of hosts E1 and E2, which produce the precursor, along with the deletion of host E3, which converts the precursor to a by-product, can increase the precursor supply (left); overexpression of a host enzyme E4 that enhances NADPH regeneration can optimize the phytochemical production by co-factor regeneration (middle); and random screening from the genes in the host may find other enzymes (that is, host E5) and non-enzyme proteins to enhance phytochemical production (right).

L-glutamate in the cytoplasm, and downstream reactions converting L-glutamate to L-ornithine in the mitochondria. In one study, researchers examined various localization strategies in yeast to enhance L-ornithine production by: (1) enhancing internal trafficking between the mitochondria and cytoplasm and internal trafficking within mitochondria without interrupting the native enzyme compartmentalization strategy; (2) localizing upstream glutamate biosynthetic enzymes to the mitochondria; or (3) localizing the mitochondrial L-ornithine biosynthetic enzymes to the cytoplasm[61]. Of the strategies examined, the localization of the enzymes involved in L-ornithine biosynthesis from the mitochondria to the cytoplasm resulted in the largest improvement in L-ornithine production.

Enzyme compartmentalization can also be used to delocalize enzymes as a way to direct specificity toward certain reactions. In reconstructing the biosynthetic pathway to morphine in yeast, researchers observed two branched pathways producing either the target molecule morphine or an undesired by-product, neomorphine. The branched pathway was a result of a slow spontaneous reaction step between the reactions catalysed by thebaine 6-O-demethylase and a plant aldo-keto reductase enzyme: COR, where COR was observed to exhibit activity on both the substrate and product of the spontaneous reaction to make the by-product and the target molecule. Researchers targeted the pathway enzymes to different subcellular compartments to spatially delocalize the two enzymes to provide more time for the spontaneous reaction to occur and thus lower production of the undesired by-product, thereby enhancing both the reaction efficiency and specificity[62].

Synthetic scaffolds have also been applied to achieve increased production of phytochemicals through the engineering of enzyme–enzyme interactions and enhanced substrate channelling. For example, synthetic protein scaffolds were used in *E. coli* to co-localize three enzymes converting fed naringenin or eriodictyol to a plant secondary metabolite, catechin, with enhanced titre[63]. In other examples, DNA and RNA scaffolds have been used in *E. coli* to enhance L-threonine[53], pentadecan and succinate production[64] by co-localizing up to four pathway enzymes. Compared to the co-localization in native organelles such as mitochondria or endoplasmic reticulum membranes, synthetic scaffolds may enable greater

flexibility in organizing enzymes in a rational or multidimensional manner for restricted substrate diffusion and enzyme interactions.

Direct protein fusion is another co-localization approach that can be used to enhance enzyme-enzyme interactions by increasing intermediate accessibility. Fused enzymes may exhibit higher activities than those from the two separate cytoplasmic enzymes. In one example, the fusion of two enzymes, farnesyl diphosphate synthase (ERG20) and *Catharanthus roseus* geraniol synthase (CrGES), in *S. cerevisiae* resulted in 15% higher production of the acyclic monoterpene alcohol geraniol than the unfused enzymes[65]. Enzymes are typically covalently fused through protein linkers; however, other noncovalent interactions, such as electrostatic forces, may also affect the distance between the component enzymes. Charge distribution on the surfaces of enzymes may vary, thus the electrostatic force resulting from the different orientations of two component enzymes in a fused enzyme complex may affect the enzyme–enzyme interactions[66,67]. For example, ERG20 is uniformly negatively charged, whereas CrGES has a positively charged region on the surface of N-terminus and negatively charged region on the surface of C-terminus, which may further affect the activity of fused enzymes[65]. Therefore, surface electrostatic distribution analysis will be a promising method for improving the design of fused proteins.

Optimization of heterologous plant pathways in the context of the microbial host's metabolism. Systematic optimization of the heterologous pathway with endogenous metabolic pathways in the microbial host plays a key role in enhancing overall pathway efficiencies. Heterologous pathways may interact with the host's metabolic network in a number of ways. For example, precursors, co-factors and co-substrates are provisioned from and shared with the host's metabolism. In addition, metabolites produced in the heterologous pathway may be further acted on by enzymes native to the microbial host; in effect competing away key metabolites from the desired pathway.

Optimizing the production of pathway precursors provisioned by the host cell is an effective strategy to enhance phytochemical production. Precursor availability can generally be enhanced by increasing precursor production and/or reducing precursor consumption from competing pathways. In one example, the availability of the isoprenoid precursor FPP to a heterologous artemisinic acid biosynthetic pathway was increased by limiting a competing FPP-consuming reaction by using a copper-regulated promoter to repress the transcription of the *ERG9* gene[47]. In a separate example of yeast engineered to produce α-santalene, FPP consumption was lowered by replacing the native promoter of *ERG9* with a glucose-repressed promoter, and by the deletion of two lipid phosphate phosphatases that convert FPP to undesired farnesol[68]. In another study in which yeast was engineered to produce strictosidine, the levels of the precursor geranyl pyrophosphate (GPP) were enhanced by reducing feedback inhibition in its upstream mevalonate pathway, increasing expression of the enzyme catalysing upstream substrate formation, and lowering the consumption of GPP precursors by deleting the competing FPP synthase[15].

Aromatic amino acids, chorismic acid, and their derivatives are precursors to diverse phytochemicals including the benzylisoquinoline alkaloids and flavonoids, and thus have been the target of numerous strategies to enhance their availability in microbial hosts. L-tyrosine levels were enhanced in yeast by the expression of enzyme mutants that are insensitive to feedback inhibition (that is, DHAP synthase, chorismate mutase)[69]. Enhanced supply of L-tyrosine also contributed to increased production of reticuline, a major intermediate in BIA pathway, which was achieved by the expression of feedback insensitive DHAP synthase mutants, deletion of the glucose-6-phosphate dehydrogenase ZWF1, and overexpression of the transketolase TKL1 in the pentose phosphate pathway[9,13]. In another example, the overproduction of the flavonoid precursor *p*-coumaric acid was achieved in yeast by deleting enzymes in competing pathways (for example, ARO10, PDC5) and introducing feedback insensitive enzymes (DHAP synthase, chorismate mutase) in the biosynthetic pathway to *p*-coumaric acid[70]. The engineered strain produced 1.89 g l^{-1} *p*-coumaric acid and can serve as a platform for flavonoid and other phenylpropanoid biosynthesis. In a separate example, the supply of the flavonoid precursor malonyl-CoA was increased by using CRISPRi technology to repress the expression of enzymes associated with competing pathways in *E. coli* central metabolism, such as fatty acid biosynthesis. By increasing the supply of malonyl-CoA in an *E. coli* strain engineered with a heterologous plant flavonoid pathway, the titre of naringenin was increased by sevenfold[71].

Many enzymes in plant specialized metabolism require common co-factors, and thus the incorporation of co-factor regeneration and balancing strategies can have significant impact on overall reaction efficiencies. In one example, a stoichiometric-based model was used to predict gene deletion targets for enhancing NADPH availability in *E. coli*. Implementation of the model predictions resulted in enhanced plant flavonoid biosynthesis due to improved activities of NADPH-dependent enzymes[72]. In a second example, replacing *E. coli* endogenous NAD-dependent glyceraldehyde 3-phosphate dehydrogenase with a NADP-dependent enzyme from *Clostridium acetobutylicum* was shown to improve NADPH regeneration and corresponding biosynthesis of the plant carotenoid lycopene[73]. In another example, the availability of NADPH was enhanced by replacing the NADP-dependent glutamate dehydrogenase GDP1 with a NAD-dependent GDP2, thereby resulting in increased production of α-santalene[68].

Although rational design approaches have been used to enhance precursor and co-factor supply by targeting known metabolic genes, large-scale random screening strategies have also been implemented to yield both known and unidentified genetic targets. In one study, *E. coli* strains producing high titres of lycopene were obtained from gene deletion libraries generated by rational design and random transposon-based mutation. Subsequent gene identification revealed the deletions enhanced lycopene production by increasing precursor supply or altering regulation[74]. Similarly, preliminary screening of gene deletion strains in a carotenoid-producing yeast identified multiple genes targets. The gene targets were then deleted in a sesquiterpene bisabolene biosynthetic strain for enhanced production. The deletion of one transcription factor (ROX1) and two uncharacterized proteins (YJL064W and YPL062W) in combination with other gene modifications enhanced bisabolene production by 20-fold[75]. These gene targets have subsequently been applied to improve the biosynthesis of other isoprenoids in yeast; for example, deletion of ROX1 in combination with another gene modification resulted in enhanced lycopene production[76]. In another example, a transcription factor-based sensor was used to couple naringenin concentration to cell fitness in *E. coli*. After four rounds of genome mutagenesis and selection, a strain was achieved that exhibited 36-fold higher naringenin production. Sequencing of the resultant high-producing strains identified multiple gene modifications enhancing the supply of precursors such as malonyl-CoA and tyrosine[77].

Generally, a rational combination of numerous strategies is important to enhance the overall reaction efficiency of complex biosynthetic pathways. Dynamic control can be applied to heterologous and endogenous enzymes to tune the reaction activity at a suitable level for phytochemical biosynthesis. The genetic targets most suitable for this approach are typically enzymes that are essential for the phytochemical biosynthesis, yet overexpression results in cell toxicity or substrate competition. However, effective dynamic control relies on the availability of suitable sensing and regulatory elements, which are often limiting. The development of hybrid transcription factors or synthetic RNA switches may help to address this limitation. Compartmentalization approaches

are applicable to heterologous enzymes in the microbial host for enhanced reaction activity and better intermediate trafficking. Modular protein localization tags have been developed to apply these strategies more generally. Recently, microbial co-culture strategies have highlighted the potential of enhancing the efficiency of phytochemical biosynthesis via a compartmentalization strategy that occurs across different cells. Co-cultivations involving engineered *E. coli* (or *E. coli* and *S. cerevisiae*) strains have been described for the production of taxadiene-5-ol[17], catechin[78] or anthocyanins[79], highlighting a cell-based compartmentalization strategy for enhancing phytochemical production. Optimization of the heterologous pathway with the host's metabolism focuses on engineering the host's endogenous enzymes to increase the supply of compounds essential to the phytochemical biosynthesis. Numerous genetic engineering and genome editing methods are available to alter the activity or specificity of endogenous enzymes. Recent genome-wide screens have indicated the important roles of non-enzyme proteins in phytochemical production[75,76]. Recent advances in engineering microbial chromosomes may be used to remove unnecessary metabolic processes and increase genome stability while maintaining cell fitness, thus addressing the challenges in enhancing phytochemical production[80–82].

Conclusion and future outlook

A number of approaches for producing high-value phytochemicals are being pursued as alternatives to extraction from the native plant producer. Each of these approaches has different strengths and weaknesses, and the best approach may depend on the particular phytochemical of interest. For example, chemical synthesis presents a number of advantages such as reliability and control of the process. However, for phytochemicals of complex structure, chemical synthesis will not be a commercial feasible route as evidenced by the number of phytochemicals that are still extracted from native producers. Bio-based production in microorganisms presents similar advantages to chemical synthesis, with the additional benefits of providing a commercially feasible route for more complex phytochemicals by leveraging enzymatic catalysis. However, microbial biosynthesis requires knowledge of the biosynthesis route and underlying enzymes, which for many phytochemicals will not be known and require significant research and discovery. In these situations, plant extraction or plant cell culture may provide the only feasible routes as, although often inefficient and expensive, these approaches can be applied without knowledge of the biosynthesis process.

Over the past ten years, a number of technological advances have made the microbial synthesis of valuable and complex phytochemicals increasingly feasible. Numerous examples now exist of phytochemical production in microbial platforms that can achieve high titres and support complex pathways of dozens of heterologous enzymes. However, scalable measurement and discovery remain as two major challenges to the field. Measurement technologies are key to providing information on the sophisticated biological activities within the cellular context, which is essential for understanding and engineering the phytochemical biosynthesis process. Yet, there is a tradeoff among data quality, scale, and speed in measurement technologies. For example, analytical methods such as mass spectrometry generate high quality, quantitative data; however, the low-throughput limits its application in collecting large-scale, real-time, non-invasive data. In contrast, synthetic biosensors that convert intracellular chemical concentrations to measurable signals (for example, fluorescence or colorimetric readouts, cell growth rate) enable scalable, rapid assays; however, these indirect measurements are likely coupled with information loss. The availability and specificity of biosensors also limit their target chemicals to a small group of phytochemicals, reaction intermediates, or by-products, which results in further loss of information and data quality. Thus, the development of tools and technologies that enable scalable and information-rich measurements will provide an opportunity to accelerate the pace of complex phytochemical production.

The intersection of advances in genomics, bioinformatics, and synthetic biology offers the potential to transform the discovery of plant phytochemicals and their underlying biosynthetic pathways. Despite the unprecedented increase in availability of datasets (for example, plant genomics, functional genomics) and rapid development of data analytics, transitions from raw data to useful knowledge demand the systematic interface between computational study and experimental science. In the future, advances in the interpretation of plant genomics information, the analysis of plant secondary metabolism–transcriptome correlation, and the reconstruction of plant biosynthetic pathways in microorganisms may be integrated into a systematic pipeline to transform theoretical chemical hypotheses to scalable microbial biosynthesis platforms, and thus experimental platforms for rapidly confirming these hypotheses. New technologies and approaches developed in addressing these challenges will drive the engineering of microorganisms that synthesize complex phytochemicals and advance our understanding of the sophisticated natural product biosynthesis schemes evolved in plants.

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Author contributions

S. L., Y. L. and C. D. S. contributed to discussions and wrote the manuscript.
S. L. and Y. L. contributed equally.

Competing interests

The authors declare no competing interests.

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