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PERSPECTIVE

Opportunities in metabolic engineering to enable scalable alkaloid production

Effendi Leonard, Weerawat Runguphan, Sarah O'Connor & Kristala Jones Prather

Numerous drugs and drug precursors in the current pharmacopoeia originate from plant sources. The limited yield of bioactive compounds in plant tissues, however, presents a significant challenge for large-scale drug development reliant on whole plant extracts. Metabolic engineering has facilitated the development of plant cell and tissue systems for the overproduction of high-value plant pharmaceuticals that can be conveniently scaled up in a controlled environment. Nevertheless, effective metabolic engineering approaches and the predictability of genetic transformations are often obscured owing to the myriad complexities of cellular biology. Progress in systems biology has aided the understanding of the genome-wide interconnectivity in plant-based systems. In parallel, the bottom-up assembly of plant biosynthetic pathways in microorganisms demonstrated the possibilities of a new means of production.[AU: OK?] In this Perspective, we discuss the advances and challenges of metabolic engineering implementation in various scalable production platforms for the bio-based synthesis of natural and unnatural plant alkaloids.

Bioactive compounds with "privileged structures" are highly sought paradigms in drug development. Functionally, a privileged structure is a molecular scaffold that can accommodate various pharmacophores arranged to promote interaction with biological targets[1–4]. Though many have been synthetically designed, nature remains the largest source of highly sophisticated biologically active privileged compounds because presumably they play a key role in increasing the survival fitness of organisms[5–7]. In fact, about one third of the ~980 new pharmaceuticals in the past two and a half decades originated from or were inspired by natural products[8].

With over 10,000 structurally characterized members, plant alk loids are important privileged compounds of many pharmacological activities[9–12].[**AU: "with many pharmacological activities"?**] The endogeneous[**AU: endogenous?**] role of alkaloids in plants has not been fully elucidated. However, current evidence suggests that alkaloids are generally involved in plant defense against pathogens, insects and herbivores owing to their potent toxicity[13]. For example, the indolizidine, indolizine and β-carbolines paradigmatic alkaloid backbone structures can exert over 25 biological activities, such as dopamine reuptake inhibitor, glucosidase inhibitor, sodium channel blocker and 5HT1D agonist[9]. In fact, alkaloid-containing plants have been recognized and exploited since ancient human civilization, from the utilization of *Conium maculatum* (hemlock) extract containing neurotoxin alkaloids to poison Socrates, to the use of coffee and tea as mild stimulants[11]. Today, numerous alkaloids are pharmacologically well characterized and are used as clinical drugs, ranging from cancer chemotherapeutics to analgesic agents (**Table 1**).

Despite their importance, the inefficiency of extracting some alkaloids remains a significant barrier toward inexpensive bioprospecting for drug development. The process of separating, purifying and structurally characterizing compounds of interest from a myriad of other metabolites is time-consuming and expensive. Bioactive alkaloids are also usually present in small quantities (**Table 1**). Furthermore, the yield consistency cannot always be guaranteed because it depends heavily on the source organisms and the geographical and climate conditions. The scarcity of some alkaloids in plants is exemplified by vincristine (**1**), the cancer chemotherapeutic compound in *Catharanthus roseus*, which exists at concentrations that only reach 0.0003% by dry weight[14].

The field of organic synthesis has advanced tremendously in the past decades in creating various methodologies suitable for constructing bisindole alkaloids with multiple functionalities and stereocenters[14–17]. Nevertheless, total or semisynthesis of many other alkaloids remains a daunting challenge that is far from being practical at the industrial level. Plant tissue and cell cultures can serve as alternative production platforms in which the biosynthesis of alkaloids has been improved through various elicitation and culture manipulation strategies. In addition, metabolic engineering, which is supported by the availability of systems biology datasets, now has the potential to more effectively maximize the capacity for alkaloid biosynthesis in cellular systems.[**AU: Sentence correct as edited?**] For the most part, major advancements in alkaloid metabolic engineering occurred within the last decade. In this Perspective, we first focus on the milestones and challenges in engineering plant tissue and cell lines for improving natural alkaloid production, and for facilitating the synthesis of unnatural alkaloids. Recently, there has been an increasing interest in the engineering of microorganisms for the synthesis of high-value metabolites. To this end, we highlight the recent construction of artificial alkaloid biosynthetic pathways in *Escherichia coli* and *Saccharomyces cerevisiae* and discuss the potential for the use of microbes as novel alkaloid production platforms.

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Table 1 Examples of medicinally important alkaloids

Alkaloid	Type	Approximate yield* (organ)	Source plant	Pharmaceutical applications
Ajmaline (**46**)	MIA	1 (root)[83]	Rauwolfia sellowii	Anti-arrhythmic
Berberine (**43**)	BIA	1 (root)[84]	Berberis vulgaris	Antimicrobial
Caffeine (**19**)	Purine	1 (bean)[85]	Coffea arabica	Stimulant
Camptothecin (**47**)	MIA	0.05 (bark)[86]	Camptotheca acuminata	Chemotherapeutics
Cocaine (**48**)	Tropane	0.8 (leaves)[87]	Erythroxylon coca	Stimulant
Codeine (**24**)	BIA	0.5 (seed capsule)[88]	Papaver somniferum	Antitussive, analgesic
Hyoscyamine (**22**)	Tropane	0.4 (leaves)[89]	Hyoscyamus muticus	Anticholinergic, antimuscarinic
Irinotecan (**49**)	MIA (ss)	–	–	Chemotherapeutics
Morphine (**23**)	BIA	10 (seed capsule)[88]	Papaver somniverum	Analgesic
Nicotine (**17**)	Tropane	3 (leaves)[90]	Nicotiana tabacum	Stimulant
Noscapine (**50**)	BIA	2 (seed capsule)[91]	Papaver somniverum	Analgesic, antitussive
Oxycodone (**51**)	BIA (ss)	–	–	Analgesic
Oxymorphone (**52**)	BIA (ss)	–	–	Analgesic
Papaverine (**53**)	BIA	1 (seed capsule)[91]	Papaver somniverum	Vasodilator
Quinidine (**54**)	MIA	0.2 (bark)[92]	Cinchona ledgeriana	Antiarrhythmic
Quinine (**55**)	MIA	6 (bark)[92]	Cinchona ledgeriana	Antimalarial, analgesic
Reserpine (**56**)	MIA	0.03 (root)[93]	Rauwolfia nitida	Antihypertensive
Sanguinarine (**57**)	BIA	0.001 (root)[93]	Sanguinaria canadiensis	Antibacterial (in dental products)
Scopolamine (**21**)	Tropane	0.1 (leaves)[89]	Hyoscyamus muticus	Anticholinergic, antimuscarinic
Strychnine (**58**)	MIA	2 (root)[94]	Strychnos nux-vomica	Stimulant, pesticide
Topotecan (**59**)	MIA (ss)	–	–	Chemotherapeutics
Vinblastine (**35**)	MIA	0.0001 (whole plant)[95]	Catharanthus roseus	Chemotherapeutics
Vincristine (**1**)	MIA	0.0003 (whole plant)[14]	Catharanthus roseus	Chemotherapeutics
Vindesine (**60**)	MIA (ss)	–	–	Chemotherapeutics
Vinflunine (**34**)	MIA (ss)	–	–	Chemotherapeutics
Vinorelbine (**61**)	MIA (ss)	–	–	Chemotherapeutics
Yohimbine (**62**)	MIA	1 (bark)[96]	Pausinystalia yohimbe	Erectile dysfunction treatment

*Yield represents percentage dry weight. ss indicates semisynthetic derivative.

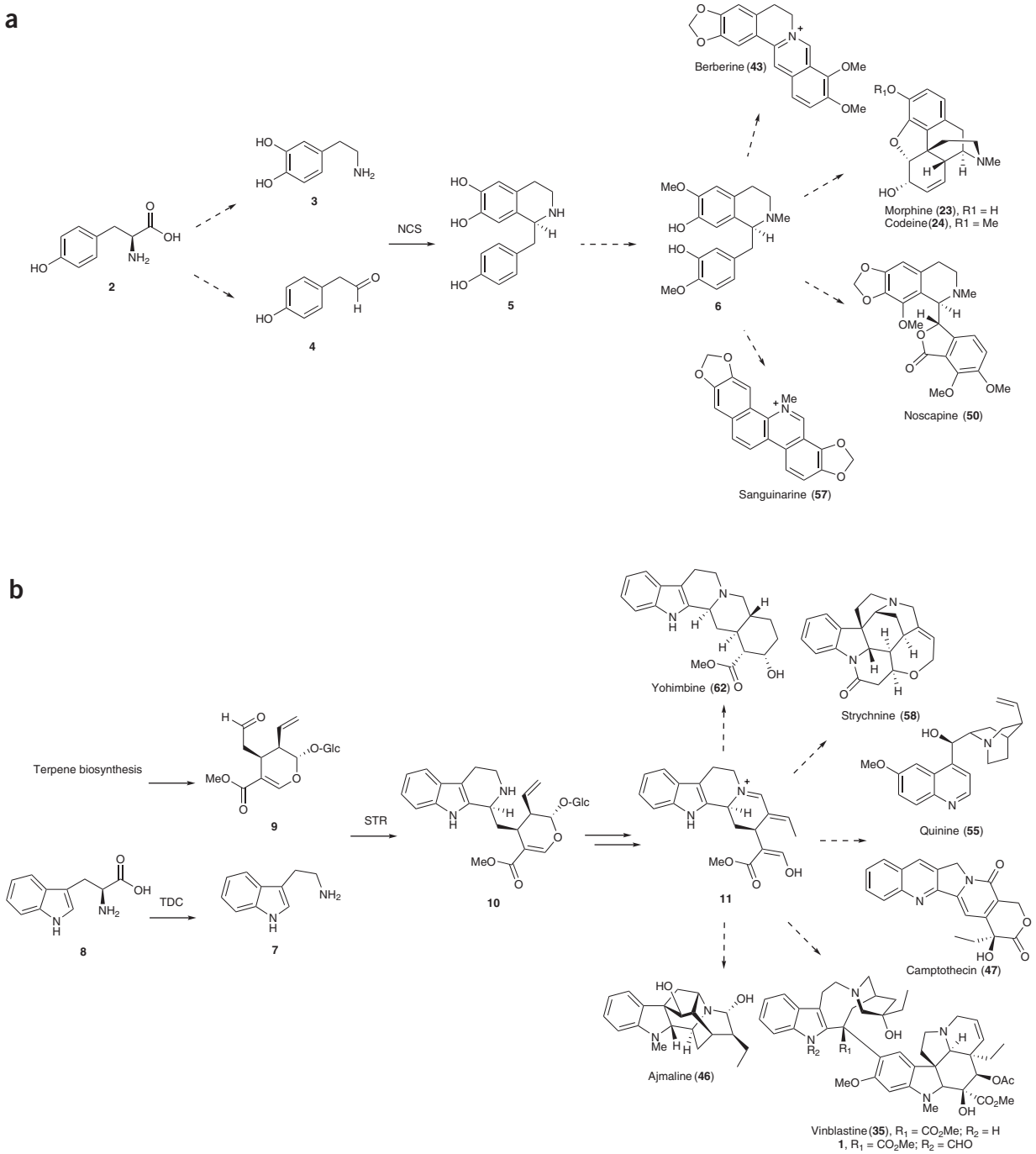
Alkaloid biosynthesis and manipulations in plants

The significance of alkaloids has motivated the characterization of their biosynthetic pathways. Mechanistic elucidation of enzymatic steps typically begins by tracking isotopically labeled metabolites in differentiated plants or plant cell cultures. Further steps commonly involve reverse genetics, in which (following plant enzyme isolation and purification) partial sequence data of the purified protein are used to obtain the corresponding gene from a complementary DNA library. This allows the identification of the starting substrates, and enables the proposal of a series of logical biosynthetic transformations. Recently, genomic and transcriptomic technologies have been used to rapidly identify biosynthetic steps. There are currently over 40,000 expressed enzyme tags (ESTs) generated from alkaloid-producing plants that have been used to isolate genes involved in the alkaloid pathway[10]. Nevertheless, the availability of genome sequences of alkaloid producer plants is urgently needed to further speed the elucidation of their biosynthesis. So far, the biosynthetic routes of four alkaloid subclasses have been partially characterized: the benzylisoquinoline, monoterpenoid indole, purine and tropane alkaloids. Benzylisoquinoline alkaloids (BIAs) are derived from tyrosine (**2**) and are comprised of ~2,500 defined structures found mainly in the Papaveraceae, Ranunculaceae, Berberidaceae and Menispermaceae[18]. The first committed step of BIA biosynthesis begins with the stereoselective Pictet-Spengler condensation of dopamine (**3**) and 4-hydroxyphenylacetaldehyde (**4**) to form (*S*)-norcoclaurine (**5**). Through a series of methylations and hydroxylations, **5** is converted into (*S*)-reticuline (**6**), the pivotal intermediate of many pharmaceutically important BIAs in the downstream pathways (**Scheme 1a**). The second subclass, the monoterpenoid indole alkaloids (MIAs), are derived from tryptophan metabolism. MIAs are some of the most structurally diverse natural products. With over 2,000 structures, they are mainly found in the Apocynaceae, Loganiaceae and Rubiaceae[19]. Much like BIA biosynthesis, the committed step of MIA biosynthesis begins with the condensation of tryptamine (**7**) (derived from tryptophan (**8**)) and secologanin (**9**) (derived from terpene biosynthesis) to form strictosidine (**10**)[20]. Following the deglucosylation of **10**, equilibrium of the unstable aglycon intermediates leads to the formation of 4,21-dehydrogeissoschizine (**11**), the branchpoint precursor of MIAs (**Scheme 1b**). Tropane alkaloids are the third subclass whose biosynthetic pathways have been investigated. They are found primarily in the Solanaceae[21]. The first committed step of tropane biosynthesis is the N-methylation of putrescine (**12**) (derived from L-ornithine (**13**)) to form *N*-methylputrescine (**14**). Following the conversion to 1-methyl-Δ^1 pyrrolinium cation (**15**), its condensation with nicotinic acid (**16**) leads to nicotine (**17**) synthesis while other chemical conversions lead to the formation of tropinone (**18**), the branchpoint intermediate of many tropane alkaloids (**Scheme 2a**). The fourth alkaloid subclass is derived from purine nucleotides instead of amino acids. A well-known example of a purine alkaloid is caffeine (**19**), whose biosynthetic route has been largely studied in *Camellia*, *Coffea*, *Theobroma* and *Ilex*[22]. Its upstream pathway involves four enzymatic steps that consist of three SAM-dependent methyl transfers and one nucleotide removal reaction from xanthosine (**20**) (**Scheme 2b**).

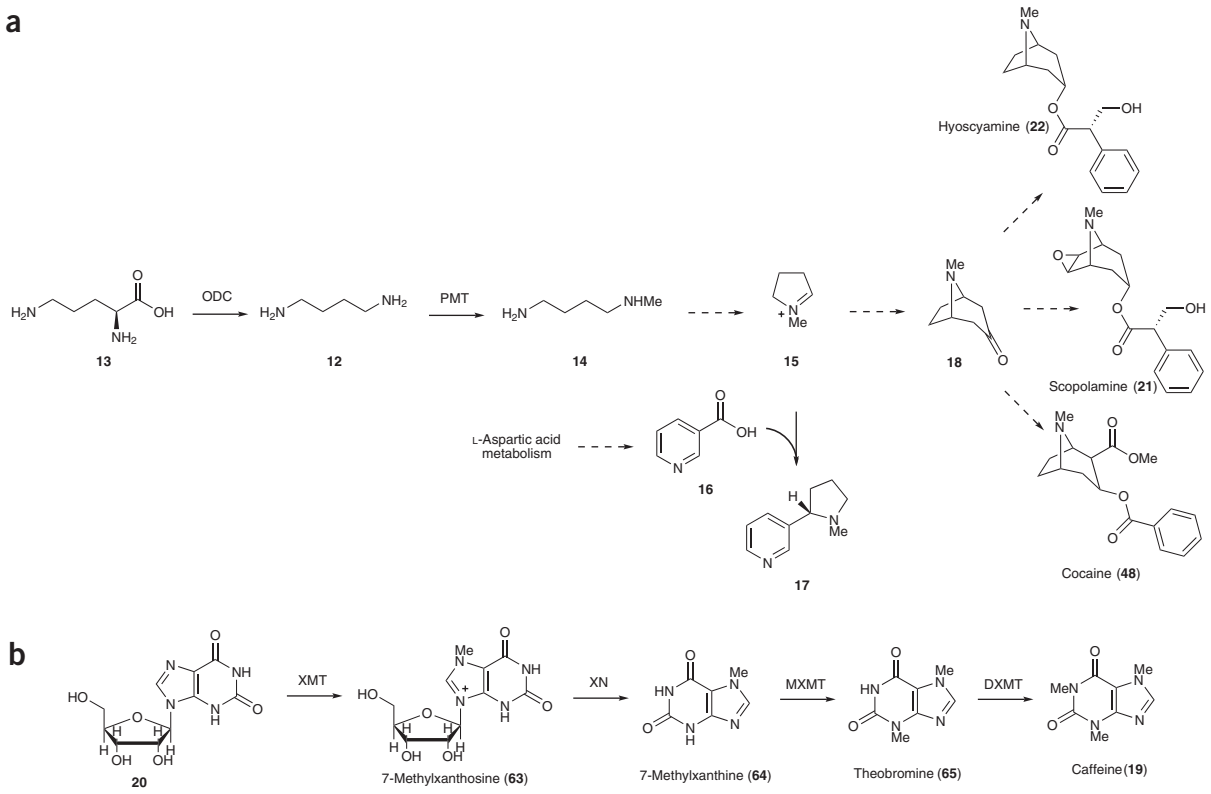
While efforts to complete elucidation of alkaloid metabolism are progressing, known enzymatic steps have been used as a basis for plant metabolic engineering strategies to increase the biosynthesis of alkaloids of interest or to eliminate undesired metabolites. For example, a transgenic coffee cultivar with 70% reduction of caffeine content was created by the introduction of RNA interference constructs in order to downregulate threobromine synthase (MXMT)[23]. A more pest resistant tobacco cultivar has also been engineered by expressing three N-methyltransferases from coffee to divert flux from **20** to synthesize **19**[24]. Furthermore, up to 1.2% dry weight of the pharmaceutically valuable scopolamine (**21**) has been made available in *Atropa belladonna*, a plant that normally accumulates hyoscyamine (**22**), by expressing *Hyoscyamus niger* hyoscyamine 6β-hydroxylase (H6H), the enzyme that converts **22** into **21**[25].

Although there are many examples of successful attempts to achieve a desired alkaloid production phenotype, the outcome of plant metabolic engineering strategies is often unpredictable. For instance, consider *COR1*,[**AU: Please ensure that only official (Entrez Gene) gene symbols for the appropriate organism are used.**] which encodes for codeinone

reductase, the final enzyme in morphine biosynthesis. By overexpressing this single gene, morphine (**23**) and codeine (**24**) content in transgenic opium poppy were moderately increased by ~22% and ~58%, respectively[26]. However, thebaine (**25**), an upstream metabolite in the **23** branch pathway, was also unexpectedly and significantly amplified[26]. The outcome of the downregulation of codeinone reductase with RNAi was also puzzling. It was expected that this strategy would lead to suppression of **23** formation and the accumulation of codeinone (**26**) and morphinone (**27**), the immediate precursors of codeinone reductase. Although the amount of the morphinan alkaloids was decreased, biosynthesis of **6**, an early upstream metabolite in the pathway, was increased instead of **26** or **27**[27]. On the other hand, the overexpression of another enzyme in the pathway, the cytochrome P450 monooxygenase (*S*)-N-methylcoclaurine 3′-hydrolase (CYP80B3), resulted in an up to 450% increase of total morphinan alkaloids without altering the product distribution[28]. This result suggests that although there are multiple control points in the BIA pathway, CYP80B3 is an important target toward improving morphine biosynthesis. The suppression of this gene by an antisense construct, which led to a reduced total alkaloid content in the transgenic opium poppy, supported this hypothesis[28].



Scheme 1 The general biosynthetic schemes of BIA and MIA alkaloid subclasses. Some important alkaloid products are represented. (**a**) BIA (NCS, norcoclaurine synthase). (**b**) MIA (TDC, tryptophan decarboxylase; STR, strictosidine synthase).



Scheme 2 The general biosynthetic schemes for tropane and purine alkaloids. (**a**) Tropane alkaloid (ODC, ornithine decarboxylase; PMT, putrescine N-methyltransferase). (**b**) Purine alkaloid (XMT, xanthosine N-methyltransferase/7-methylxanthosine synthase; XN, 7-methylxanthosine nucleotidase; MXMT, 7-methylxanthine N-methyltransferase/theobromine synthase; DXMT, dimethylxanthine N-methyltransferase/caffeine synthase).

Alkaloid production in plant tissue and cell lines

The demand for highly abundant plant alkaloids (**Table 1**), such as the morphinan opiates, can be met through plant extraction[29]. However, for some scarcely available alkaloids, alternative production platforms are desirable. It was discovered as early as the 1950s that undifferentiated plant cells have the capacity to produce many of the same secondary metabolites as whole plants[30]. Today, several plant cell lines have been developed to synthesize some important pharmaceuticals at industrial levels. For example, concentrations of paclitaxel as high as 0.5% of dry weight have been achieved in plant cell culture with methyl jasmonate elicitation[31]. This is a stark yield improvement over the concentration of paclitaxel in Pacific yew, which makes up as little as 0.01% of the dry weight[32].[**AU: Sentence correct as edited?**] Shikonin, a naphthoquinone pigment used in cosmetics, has also been successfully derived from *Lithospermum erythrorhizon* cell suspension cultures[33].

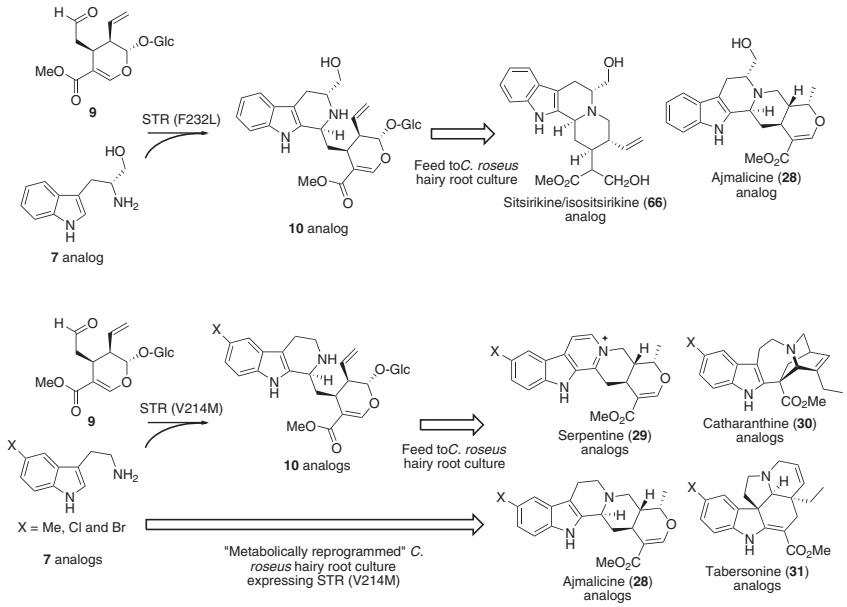
Extensive efforts have focused on optimizing plant cell cultures for improving the yield, controllability and reproducibility of several pharmaceutically important alkaloids[34]. However, because the activation of many alkaloid biosynthetic pathways is tissue specific and a function of developmental stage[35], plant tissue cultures (rather than cell lines) are often used as production platforms[36].[**AU: Correct as edited?**] For both tissue and cell culture systems, the elicitation of alkaloid synthesis often involves the utilization of certain small molecules[11] and light[37,38]. Aside from developing optimal culture conditions, various metabolic engineering manipulations have also been explored in plant tissue and cell lines to obtain alkaloid overproduction phenotypes. An example of a successful metabolic engineering strategy is the simultaneous overexpression of putrescine N-methytransferase and H6H, which resulted in the synthesis of ~400 mg l^{-1} of **21** in transgenic *H. niger* hairy root culture (~ninefold increase over the wild-type line)[39]. As in whole plants, however, the outcomes of chosen metabolic engineering strategies in plant tissue and cell lines are often difficult to predict or control. In one instance, while the overexpression of STR[**AU: Spell out.**] (the key enzyme in the MIA pathway; **Scheme 1b**) in *C. roseus* cell lines improved the levels of ajmalicine (**28**), serpentine (**29**), catharanthine (**30**) and tabersonine (**31**), the highly productive lines were deemed to be unstable[40]. In another case, it was previously known that the biosynthesis of **8** is feedback inhibited; hence its availability might be a limiting factor in MIA biosynthesis. However, the introduction of *Arabidopsis thaliana* feedback-resistant anthranilate synthase (*At*AS) and induction of tryptophan decarboxylase (TDC) in *C. roseus* hairy roots did not significantly improve downstream MIAs even though the levels of early MIA precursors **7** and **8** were increased[41–43]. Again, this result suggested that the availability of the early amino acid precursor is not limiting for MIA biosynthesis, which confirms the finding that the availability of **9** was the important rate-limiting step in MIA biosynthesis. Improvements in the precursor branch pathway of **9** successfully increased MIA synthesis in suspension cells and hairy roots[44–46].[**AU: Correct as edited?**] When the activity of the terminal step of vindoline (**32**) biosynthesis was amplified in *C. roseus* hairy roots by the overexpression of deacetylvindoline-4-O-acetyltransferase (DAT), the accumulation of horhammericine (**33**), an alkaloid not in the **32** pathway, was elevated by fourfold[47]. Further experiments revealed the existence of cross-talk by DAT overexpression because this enzyme inhibited the activity of minovincinine-19-O-acetyltransferase (MAT), the enzyme that turns over **33**.

As with whole-plant systems, the intricate relationships among metabolic pathways and regulatory schemes in plant cells and tissues are among the many factors that limit the robustness of a metabolic engineering

design to achieve a specific overproduction target. Various systems biology approaches have been developed to portray the complex metabolic interplay in plant tissue and cell systems. In one instance, flux analysis using isotopomer[48,49] and 'bond-isomer'[50,51] labeling and balancing has been used to profile flux distribution in the central metabolism of *C. roseus* hairy roots. Significant progress has also been made toward elucidating the genome-wide interconnectivity among biological functions, resulting in datasets that contain gene-to-gene and gene-to-metabolite networks that reveal regulatory differences in key alkaloid pathways in *C. roseus* cells[52]. Alkaloid metabolic regulatory machineries have also been probed by using transcriptome analysis, leading to the identification of several transcription factors in MIA biosynthesis[53,54]. All together, this information can potentially be useful in determining metabolic engineering targets that can effectively deliver a desired improvement in a specific alkaloid branch pathway. For instance, a metabolic engineering strategy to increase MIA production was devised to exploit the utility of the ORCA3 transcription factor to upregulate the expression of many MIA biosynthetic genes simultaneously[53]. However, initial ORCA3 overexpression in *C. roseus* cell cultures did not significantly improve MIA synthesis. It was discovered that even though ORCA3 positively regulates the expression of many genes that lead to the synthesis of **10**, it does not upregulate the expression of geraniol 10-hydroxylase (G10H), the enzyme in the terpenoid pathway that leads to the synthesis of **9**. Upon supplemental feeding of loganin, the precursor of **9**, the overexpression of ORCA3 resulted in ~0.6% of dry weight of MIAs (~threefold increase). Another transcriptome study revealed the complexity of MIA biosynthetic control by a variety of transcriptional regulators. In this case, it was discovered that although the MIA biosynthetic enzymes that were upregulated upon ORCA3 overexpression in *C. roseus* hairy root lines are similar to those upregulated upon ORCA3 overexpression in cell lines, the transcriptional repressors ZCT1 and ZCT2 were also upregulated in the hairy root lines[55].[**AU: Sentence correct as edited?**] This finding provided an explanation for the insignificant improvement of MIA synthesis in *C. roseus* hairy roots upon ORCA3 overexpression.

Mutasynthesis of novel alkaloid analogs

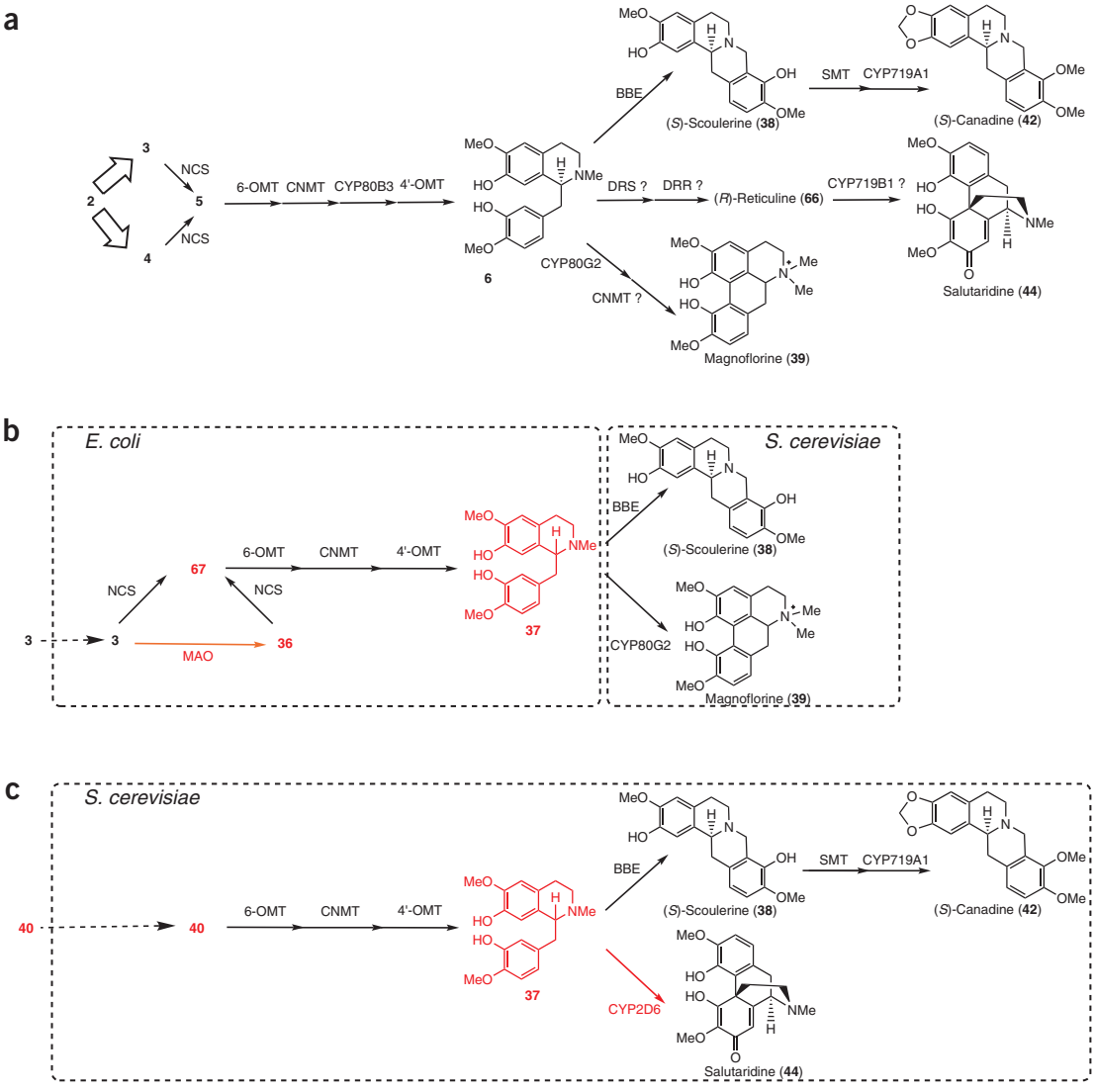
Functional group substitution of natural alkaloids can lead to the generation of compounds with improved pharmacological properties. For example, vinflunine (**34**) (4′-deoxy-20′,20′-difluoro-C′-norvincaleukoblastine), a new compound that is currently in clinical trials, was created by the introduction of two fluoro groups into vinblastine (**35**)[56]. The current availability of novel alkaloids, however, remains limited because they are still semisynthetically derived from naturally isolated precursors. Precursor-directed biosynthesis, or a "mutasynthetic" approach, is a powerful strategy for increasing the availability of alkaloid derivatives. The technology that harnessed whole-cell biocatalysts for mutasynthetic purposes arose from the discovery that several fluorinated tropane alkaloids could be produced by simply feeding fluorinated phenyllactic acid analogs to *Datura stramonium* root cultures[57]. Similarly, a wide variety of **7** and **9** analogs could be introduced into *C. roseus* root cultures and seedlings in order to synthesize unnatural MIAs[58,59]. The apparent flexibility of downstream alkaloid pathways opened the possibility of generating enzyme variants with increased selectivity toward unnatural substrate analogs, thereby improving the efficiency of precursor-directed biosynthesis and increasing the number of unnatural alkaloids. Several STR variants with altered substrate specificity have been successfully engineered. In one instance, the structural elucidation of *Rauvolfia serpentina* STR[60] led to the identification of several amino acid residues that form the binding pocket of *C. roseus* STR[61]. Using *in vitro* assays that incorporated **9** derivatives, an enzyme variant containing a D177A mutation that exhibited increased selectivity toward an analog of **9** (with a pentynyl group) was identified. This strategy clearly demonstrated the benefit of re-engineering STR plasticity for mutasynthetic purposes. However, the ability to explore a widely diverse mutational space was still limited owing to the lack of a facile screening assay. In approaching this challenge, a medium-throughput colorimetric assay was developed in order to identify functional STR mutants that can accept **7** analogs[62]. The medium-throughput assay took advantage of the formation of products downstream of STR that can be visualized when metabolized by strictosidine glucosidase. By applying a saturation mutagenesis strategy on several residues that form the binding pocket, two STR mutants (V214M and F232L) that turned over unnatural **7** compounds to synthesize β-carboline analogs were identified using the *in vitro* assay[61]. When the newly synthesized **10** analogs were fed to *C. roseus* hairy root cultures, a number of novel MIA analogs were obtained (**Scheme 3**). This finding set the stage for rational metabolic engineering of unnatural products within the plant cell environment, or "plant metabolic reprogramming"[63]. Indeed, upon feeding with the tryptamine analogs that the reengineered enzyme was designed to accept, transgenic *C. roseus* hairy roots expressing the V214M mutant enzyme produced a variety of unnatural alkaloid compounds (**28**, **29**, **30** and **31**). These results show the power of biocatalysis to facilitate the synthesis of unnatural alkaloids—an approach that has now been demonstrated to be applicable to plant cell culture.



Scheme 3 Mutasynthetic strategy for generating unnatural alkaloids. STR mutants (identified from the development of colorimetric medium-throughput assay) that can efficiently turn over substrate analogs were used to synthesize unnatural strictosidine (**10**) from unnatural tryptamine (**7**) and secologanin (**9**). Unnatural complex alkaloids can be generated from feeding the unnatural **10** into *C. roseus* hairy root culture. Analogs of **7** can also be directly converted into complex alkaloids by metabolically reprogramming *C. roseus* hairy root.

Engineering alkaloid biosynthesis in microorganisms

Owing to the smaller genome size, the degree of complexity in microorganisms is significantly lower than that of plants. Moreover, microorganisms have fewer intracellular organelles compared to plant cells; hence metabolite transport between enzymatic steps can be negligible. Indeed, the relative simplicity and tractability of microorganisms has sparked significant interest in their engineering for the synthesis of high-value plant metabolites. To this end, the bacterium *E. coli* and yeast *S. cerevisiae* were recently explored as production hosts of plant alkaloids. In both cases, the metabolic engineering efforts in microorganisms entailed the reconstruction of the plant biosynthetic pathways (**Scheme 4a**). In plants, **6**, the direct precursor of many BIAs, is derived from the condensation of **3** and **4** by norcoclaurine synthase (NCS) to form **5**. Subsequently, **5** is methylated by norcoclaurine 6-O-methyltransferase (6-OMT) and coclaurine-N-methyltransferase (CNMT), hydroxylated by the cytochrome P450 CYP80B3 and further methylated by 3′-hydroxy-N-methylcoclaurine-4′-O-methyltransferase (4′-OMT) (**Scheme 3**). To assemble an artificial pathway to achieve **6** biosynthesis in *E. coli*, *Micrococcus luteus* monoamine oxidase (MAO) was introduced together with *Coptis japonica* NCS, 6-OMT, CNMT and 4′-OMT in plasmid-based expression systems[64] (**Scheme 4b**). In this strategy, the utilization of the microbial MAO allowed the incorporation of the hydroxyl group early in the reticuline pathway through the synthesis of 3,4-dihydroxyphenylacetaldehyde (**36**) from **3**, hence obviating the need to express the plant P450 CYP80B3 in the bacterium, which is often problematic. Upon induction of enzyme expression, and supplementation with ~780 mg l^{-1} **3**, ~11 mg l^{-1} (*R*,*S*)-reticuline (**37**) could be detected in the culture medium of the recombinant *E. coli*. Because plant NCS exclusively synthesizes *S* enantiomers, the generation of the racemic products by the artificial pathway was confounding. Further investigation concluded that when **36** and **3** were sufficiently available, a spontaneous chemical conversion that resulted in the synthesis of **37** occurred. Nevertheless,



Scheme 4 Reconstruction of BIA pathway in microorganisms. (**a**) Native plant pathway. (**b**) Microbial biocatalysts using both *E. coli* and *S. cerevisiae*. (**c**) A microbial biocatalyst using only *S. cerevisiae*. Enzymatic and metabolite modifications are indicated in red. NCS, norcoclaurine synthase; 6-OMT, norcoclaurine 6-O-methyltransferase; CNMT, coclaurine-N-methyltransferase; 4′-OMT, 3′-hydroxy-N-methylcoclaurine-4′-O-methyltransferase; DRS, 1,2-dehydroreticuline synthase; DRR, 1,2-dehydroreticulene reductase; BBE, berberine bridge enzyme; SMT, scoulerine 9-O-methyltransferase; MAO, bacterial monoamine oxidase; CYP2D6, human cytochrome P450 enzyme; CYP80G2, CYP719B1 and CYP719A1, plant cytochrome P450 enzymes.**[AU: Correct as edited? If not, please define CYP80G2 and CYP719B1.]**

the synthesis of **37** allowed the biosynthesis of downstream BIAs. By coculturing the reticuline-producing *E. coli* with *S. cerevisiae* expressing the *C. japonica* berberine bridge enzyme (BBE) or CYP80G2 in the presence of **3**, (*S*)-scoulerine (**38**) and magnoflorine (**39**) could be detected at ~8 mg l^{-1} and ~7 mg l^{-1}, respectively, after 48 to 72 h of incubation.[**AU: Sentence correct as edited?**]

The use of two microbial systems for pathway construction reduced the efficiency of alkaloid synthesis owing to necessary metabolite transport between cells. In another study, *S. cerevisiae* was used as a sole host organism for the assembly of artificial BIA pathways[65] (**Scheme 4c**). In this work, the biosynthesis of **37** from (*R*,*S*)-norlaudanosoline (**40**) was enabled by expressing 6-OMT, CNMT and 4′-OMT derived from either *Thalictrum flavum* or *Papaver somniferum*. After stable insertion into the yeast genome under a reduced-strength promoter variant (TEF7)[66], the heterologous gene expression resulted in the creation of an artificial plant pathway with reduced transcriptional activities, while maintaining high catalytic activities. Furthermore, the plasmid-based expression of *P. somniferum* BBE together with *T. flavum* (*S*)-scoulerine 9-O-methyltransferase (SMT) in (*R*,*S*)-reticuline–producing yeasts resulted in the synthesis of ~60 mg l^{-1} (*S*)-tetrahydrocolumbamine (**41**) from ~1 g l^{-1} of **40** in 48 h. Additional plasmid-based expression of the *C. japonica* P450 enzyme CYP719A1 and the integration of the *A. thaliana* P450 redox partner protein ATR1 in the genome gave rise to the accumulation of an estimated ~30 mg l^{-1} (*S*)-canadine (**42**), the direct precursor of the pharmaceutically important berberine (**43**). The synthesis of **37** by the engineered yeast strain also enabled the synthesis of salutaridine (**44**), an intermediate in the branch pathway of **23**, through a shorter route. In plants, the synthesis of **44** from **6** includes[**AU: Correct?**] multiple enzymatic steps, many of which are not characterized. However, by expressing a human cytochrome P450 involved in morphine metabolism (CYP2D6) together with human CPR1 reductase in the reticuline-producing yeasts, ~20 mg l^{-1} **44** could be synthesized from **40**. Yeast have also been engineered to accommodate the biosynthesis of high-value MIAs from feeding **7** and **9**[67]. Transgenic yeast were created by expressing *C. roseus* STR and strictosidine β-glucoside (SGD) using a plasmid-based expression system. Upon supplemental feeding of the STR substrates, ~2 g l^{-1} of **10** was detected in the medium where the heterologously expressed STR was exported. Permeabilization of yeast cells to allow the diffusion of strictosidine into the cells was necessary for its metabolism by SGD to result in the generation of cathenamine (**45**), also at the yield of ~2 g l^{-1}.

The bottom-up assembly of artificial biosynthetic pathways in *E. coli* and yeast enabled the biosynthesis of plant alkaloids in a short period of time (48–72 h). One advantageous feature of yeast is the ability to support the functionality of plant membrane-bound cytochrome P450 enzymes that are rendered difficult in *E. coli* due to the absence of the endoplasmic reticulum, which is required for anchorage. However, protein engineering strategies to allow the functional expression of plant P450s in *E. coli* have been reported recently[68,69]. In general, the current technology of supplying alkaloids from engineered microorganisms is not economical because it still relies on the supplementation of expensive intermediate metabolites. However, the complete elucidation of alkaloid biosynthetic pathways from the early amino acid precursors could lead to the generation of inexpensive microbial production platforms. In fact, the feasibility of high-level plant metabolite synthesis from inexpensive precursors in both *E. coli* and *S. cerevisiae* has been demonstrated. For example, high-level synthesis of plant flavonoid at ~400 mg l^{-1} from engineered *E. coli*[70] could be facilitated by redirecting various metabolic fluxes from glucose toward malonyl-CoA (a flavonoid building block). This titer was further improved up to ~700 mg l^{-1} by partially repressing fatty acid metabolism in the *E. coli* hosts[71]. In the case of the production of plant natural product in *S. cerevisiae*, the synthesis of ~100 mg l^{-1} artemisinic acid from glucose could be achieved by the upregulation of the mevalonate pathway and the downregulation of a competing pathway (sterol biosynthesis)[72].

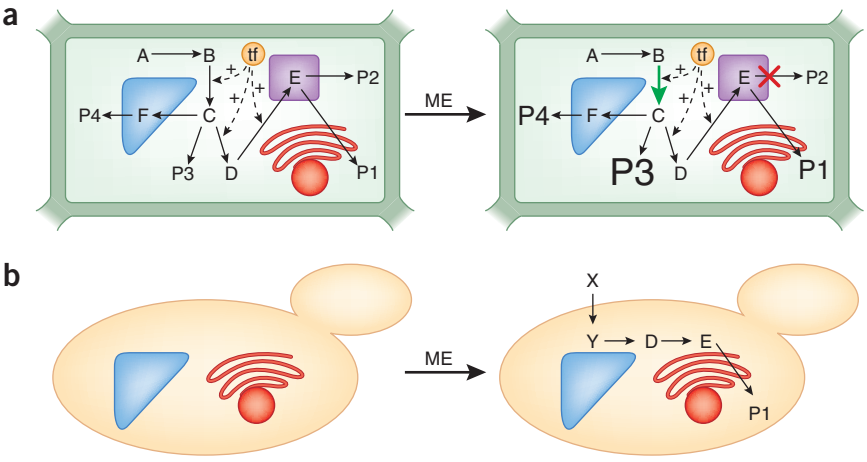


Figure 1 **[AU: Please provide a brief overall title sentence that does not refer to specific parts or panels.]**(**a**,**b**) Metabolic engineering (ME) of a plant system (**a**) and a microorganism (**b**). Multiple branch pathways exist (A–F) in plant cells that lead to the formation of diverse alkaloid products (P1, P1, P3). These pathways are also fragmented in different intracellular compartments such as the vacuole (blue triangle), plastid (purple square) and endoplasmic reticulum (red curve). Moreover, alkaloid biosynthesis in plant cells is also regulated by transcription factors (tf). Microorganisms, on the other hand, have fewer (or no) intracellular organelles, and are devoid of preexisting alkaloid pathways and transcription factors. Rational metabolic engineering strategies (overexpression, green arrow; deletion, red cross) to increase a particular alkaloid product (for example, P1) often lead to unexpected outcomes (for example, the significant amplification of P3 and P4) due to the inherent complexities of plant cellular biology and the lack of understanding of alkaloid biosynthetic networks. Microorganisms can facilitate the biosynthesis of a single alkaloid product (for example, P1) by the construction of an artificial biosynthetic pathway.**[AU: Correct as edited?]** However, synthetic intermediates (X) have to be provided.**[AU: Please check that color descriptions match the figure.]**

Perspective

Plant tissue and cell cultures are prospective scalable alkaloid production platforms. The utility of these systems is exemplified by the industrial-scale production of **21** and **43** from cell culture by Sumitomo Chemical Industries and Mitsui Petrochemical Industries[73,74].[**AU: respectively?**] One major drawback of plant tissues and cell lines, however, is the inability to produce certain alkaloids owing to the lack of specialized cell types[75]. For instance, **1** and **35** are not significantly produced in *C. roseus* cell suspension and hairy root cultures because the precursor pathway of **32** is only fully activated in aerial plant parts[76,77]. Metabolic reconstruction will therefore be required for increasing the utility of plant cell lines and tissues for commercial production systems, and this necessitates the enrichment and development of robust genetic tools for plant transformation. [**AU: Sentence correct as edited?**] However, as in whole plant systems, pathway compartmentalization and the existence of multiple alkaloid

biosynthetic pathways and regulatory control mechanisms are among the factors that significantly increase the degree of unpredictability of metabolic engineering efforts in plant cell lines and tissues (**Fig. 1a**).[**AU: Sentence correct as edited?**] The lack of complete understanding of the complex alkaloid biosynthetic networks also hinders the determination of an effective metabolic engineering strategy to achieve a specific production phenotype. The development of mathematical models of plant metabolism[78] together with systems biology analyses[79] can eventually be used to aid in determining effective metabolic engineering strategies. Additionally, because the inherent complexity of plant cellular systems often causes single gene manipulations to be ineffective for altering a biosynthetic phenotype, methods that are capable of effecting simultaneous changes in multiple metabolic points, such as the use of transcription factors, are promising[80].

Microbes are even more scalable than plant tissue and cell cultures, with a long and successful history as chemical factories for the large-scale production of both bulk and specialized chemical products. The degree of complexity in microorganisms is significantly lower than that of plant systems (**Fig. 1b**), such that the lack of preexisting branch alkaloid pathways and transcription factors in microbes should also simplify the choice of metabolic engineering targets and approaches. The recent demonstrations of engineering alkaloid pathways in microbes are promising, but are limited by the need to provide expensive intermediate precursors exogenously. Thus the complete elucidation of alkaloid biosynthesis is needed to enable the synthesis of complex downstream alkaloids from simple precursors. However, this strategy will likely entail the implantation of numerous biosynthetic steps, which is not trivial. For example, the reconstruction of the plant biosynthetic pathway of **23** from **2** in microbes will involve the functional expression of more than 17 enzymes. There are several other challenges that must be met before microorganisms can be used as an industrial alkaloid production platform. For example, because many steps in alkaloid biosynthesis require methylation, high-level production in microbial systems will likely be limited by the intracellular availability of *S*-adenosyl-L-methionine (SAM). Therefore, this bottleneck motivates further metabolic engineering efforts to increase the SAM pool in the microbial host. The cytotoxicity of alkaloids in yeast has also been implicated[67], and is presumably a factor in other microbes as well. Therefore, practical and effective strategies need to be devised to mitigate toxicity in order to generate alkaloid-overproducing microbes. A transcriptomic approach has been recently used to diagnose the effect of metabolite toxicity, and resulted in a strategy to dampen the negative impact of the toxicity on growth[81].[**AU: Sentence correct as edited?**] Moreover, a new strategy to increase microbial tolerance toward toxic metabolites by engineering transcription factors[82] can also potentially be applied in alkaloid-producing microbes.

In conclusion, although both plant cell/tissue and microbial systems offer tremendous advantages as scalable alkaloid production platforms, many opportunities still lie in the cellular and metabolic engineering sectors to create the multifaceted phenotypic traits (for example, high productivity, product tolerance and stability) required for use in industrial bioprocesses. Moreover, because the characteristics and metabolic capacities of plant cell/tissue and microbial systems are inherently different, they can serve as complimentary unit operations in order to solve the long-standing problem of robust alkaloid production.[**AU: Sentence correct as edited?**]

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