Opportunities in metabolic engineering to enable scalable alkaloid production

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Submitted for publication in: Nature Chemical Biology

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Abstract

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. Because of their sophisticated molecular architecture, efficient chemical syntheses for many plant-derived drugs are also seldom available. 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 due to the myriad complexity 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 production means. 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{Costantino, 2006 #1; Evans, 1988 #74; Horton, 2003 #3; Maclean, 2000 #2}. While 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 an organism{Koehn, 2005 #75; Maplestone, 1992 #76; Williams, 1989 #77}. 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{Newman, 2007 #5}.

With over 10,000 structurally characterized members, plant alkaloids are important privileged compounds from which many key clinical medicines are derived (Table 1){Beghyn, 2008 #4; Facchini, 2008 #13; Kutchan, 1995 #6; Verpoorte, 2000 #11}. The endogeneous 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 due to their potent toxicity{Hartmann, 2004 #12}. 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{Beghyn, 2008 #4}. The bioactivities of alkaloids have been recognized and exploited since ancient human civilization, from the utilization of Conium maculatum (hemlock) extract containing the
neurotoxin alkaloid coniine to poison Socrates, to the use of caffeine in coffee and tea as a mild stimulant{Kutchan, 1995 #6}. Today, numerous alkaloids are pharmacologically well-characterized and 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 towards 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 can not always be guaranteed because it depends heavily on the source organisms as well as geographical and climate conditions. The scarcity of some alkaloids in plants is exemplified by the cancer chemotherapeutic compound vincristine, which is found in Catharanthus roseus at concentrations that only reach 0.0003% by dry weight{Kuboyama, 2004 #9}.

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{Kuboyama, 2004 #9; Miyazaki, 2007 #7; Uchida, 2006 #8; Yokoshima, 2002 #10}. Nevertheless, total or semi-synthesis 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.
Additionally, supported by the availability of systems biology datasets, metabolic engineering now has the potential to more effectively maximize the capacity for alkaloid biosynthesis in cellular systems. 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.

### Alkaloid biosynthetic pathway elucidation and manipulation 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 where, following plant enzyme isolation and purification, partial sequence data of the purified protein is used to obtain the corresponding gene from a cDNA 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 [Facchini, 2008 #13]. Nevertheless, the availability of genome sequences of alkaloid producer plants is urgently needed to further speed the elucidation of their biosynthesis. To date, the biosynthetic routes of four alkaloid sub-classes have been partially characterized, namely the benzylisoquinoline, monoterpenoid indole, purine, and tropane alkaloids. Benzylisoquinoline alkaloids (BIAs) are derived from tyrosine and are comprised of ~2500 defined structures found mainly in the *Papaveraceae*, *Ranunculaceae*, *Berberidaceae*, and *Menispermaceae* [Liscombe, 2008 #16]. The first committed step of BIA biosynthesis begins with the stereoselective Pictet-Spengler condensation of dopamine and 4-hydroxyphenylacetaldehyde (4HPAA) to form norcoclaurine. Through a series of methylations and hydroxylations, (S)-norcoclaurine is converted into (S)-reticuline, 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 2000 structures, they are mainly found in the *Apocynaceae*, *Loganiaceae* and *Rubiaceae* [O'Connor, 2006 #15]. Similar to BIA, the committed step of MIA biosynthesis begins with the condensation of tryptamine and secologanin (derived from terpene biosynthesis) to form strictosidine [Maresh, 2008 #14]. Following the deglucosylation of strictosidine, equilibrium of the
unstable aglycon intermediates leads to the formation of 4, 21-dehydrogeissoschizine, the branch-point precursor of MIAs (Scheme 1b). Tropane alkaloids are the third sub-class whose biosynthetic pathways have been investigated. They are found primarily in Solanaceae (Sato, 2001 #17). The first committed step of tropane biosynthesis is the N-methylation of putrescine to form N-methylputrescine. Following the conversion to 1-methyl-Δ¹ pyrroline cation, its condensation with nicotinic acid leads to nicotine synthesis while other chemical conversions lead to the formation of tropinone, the branch-point intermediate of many tropane alkaloids (Scheme 1c). The fourth alkaloid sub-class is derived from purine nucleotides instead of amino acids. A well-known example of a purine alkaloid is caffeine, whose biosynthetic route has been largely studied in Camellia, Coffea, Theobroma, and Ilex (Ashihara, 2008 #18). Its upstream pathway involves four enzymatic steps which consist of three SAM-dependent methyl transfers, and one nucleotide removal reaction to form xanthosine, the first committed precursor of purine alkaloids (Scheme 1d).

While efforts to complete elucidation of alkaloid metabolism is 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 RNAi constructs in order to down-regulate theobromine synthase (MXMT) (Ogita, 2003 #19). A more pest resistant tobacco cultivar has also been engineered by expressing three N-methyltransferases from coffee to divert flux from xanthosine
to synthesize caffeine (Uefuji, 2005 #20). Furthermore, the pharmaceutically valuable scopolamine has been made available in *Atropa belladonna*, a plant that normally accumulates hyoscyamine, by expressing *Hyoscyamus niger* hyoscyamine 6β-hydroxylase (H6H), the enzyme which converts hyoscyamine into scopolamine (Yun, 1992 #21).

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* which encodes for codeinone reductase, the final enzyme in morphine biosynthesis. By only overexpressing this single gene, morphine and codeine content in transgenic opium poppy was moderately increased by ~22% and ~58%, respectively (Larkin, 2007 #22). However, thebaine, an upstream metabolite in the morphine branch pathway was also unexpectedly and significantly amplified (Larkin, 2007 #22). The outcome of the down-regulation of codeinone reductase with RNAi was also puzzling. It was expected that this strategy would lead to suppression of morphine formation and the accumulation of codeinone and morphinone, the immediate precursors of codeinone reductase. Although the amount of the morphinan alkaloids was decreased, biosynthesis of (S)-reticuline, an early up-stream metabolite in the pathway was increased instead of codeinone or morphinone (Allen, 2004 #24). 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\cite{Frick, 2007 #23}. This result suggests that although there are multiple control points in the BIA pathway, CYP80B3 is an important target towards 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\cite{Frick, 2007 #23}.

**Engineering alkaloid production in plant tissue and cell lines**

The demand for highly abundant plant alkaloids, such as the morphinan opiates, can be met through plant extraction\cite{Ye, 1998 #39}. 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\cite{Angela M. Stafford, 1998 #63}. Today, several plant cell lines have been developed to synthesize some important pharmaceuticals at industrial levels. For example, concentrations of taxol as high as 0.5% of dry weight have been achieved in plant cell culture with methyl jasmonate elicitation\cite{Yukimune, 1996 #25}. This is a stark yield improvement over taxol concentrations in Pacific yew, which account for as little as 0.01% of the dry weight\cite{Witherup, 1990 #79}. Shikonin, a naphthoquinone pigment used in cosmetics, has also been successfully derived from *Lithospermum erythrorhizon* cell suspension cultures\cite{Touno, 2005 #26}.
Extensive efforts have focused on optimizing plant cell cultures for improving the yield, controllability and reproducibility of several pharmaceutically important alkaloids (Gamborg, 2002 #80). However, because the activation of many alkaloid biosynthetic pathways is tissue specific and a function of developmental stage (Filner, 1969 #40), plant tissue cultures are often utilized as production platforms instead of cell lines (Shanks, 1999 #62). For both tissue and cell culture systems, the elicitation of alkaloid synthesis often involves the utilization of certain small molecules (Kutchan, 1995 #6) and light (De Luca, 2000 #42; Vazquez-Flota, 2000 #41). 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. Similar to 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, the key enzyme in the MIA pathway (Scheme 1b), in C. roseus cell lines improved the levels of ajmalicine, serpentine, catharanthine and tabersonine, the highly productive lines were deemed to be unstable (Canel, 1998 #27). In another case, it was previously known that tryptophan biosynthesis is feedback inhibited, hence tryptophan availability might be a limiting factor in MIA biosynthesis. However, the introduction of Arabidopsis thaliana feedback-resistant anthranilate synthase (AtAS) 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 tryptophan and tryptamine were increased (Hughes, 2004 #43;
This result suggested that the availability of tryptophan and tryptamine are not limiting for MIA biosynthesis, confirming the finding that secologanin availability was the important rate-limiting step in MIA biosynthesis. Improvements in the secologanin precursor branch pathway successfully increased MIA synthesis in suspension cells or hairy roots (Whitmer, 1998 #65; Whitmer, 2002 #64; Morgan, 2000 #67). When the activity of the terminal step of vindoline biosynthesis was amplified in C. roseus hairy roots by the overexpression of deacetylvindoline-4-O-acetyltransferase (DAT), the accumulation of horhammericine, an alkaloid not in the vindoline pathway was elevated by four-fold (Magnotta, 2007 #68). 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 which turns over horhammericine.

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 (Schwender, 2004 #46; Schwender, 2004 #47), and ‘bond-isomer’ (Sriram, 2007 #48; Sriram, 2004 #49) labeling and balancing has been employed to profile flux distribution in the central metabolism of C. roseus hairy roots. Significant progress has also been made towards 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\cite{Rischer, 2006 #28}. Alkaloid metabolic regulatory machineries have also been probed by using transcriptome analysis, leading to the identification of several transcription factors in MIA biosynthesis\cite{van der Fits, 2000 #30; Menke, 1999 #31}. 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\cite{van der Fits, 2000 #30}. 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 strictosidine, it does not up-regulate the expression of geraniol 10-hydroxylase (G10H), the enzyme in the terpenoid pathway that leads to the synthesis of secologanin. Upon supplemental feeding of the secologanin precursor, loganin, the overexpression of ORCA3 resulted in ~3-fold increase of MIA biosynthesis. 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 is similar to those in cell lines, the transcriptional repressors ZCT1 and ZCT2 were also upregulated\cite{Peebles, 2008 #29}.\footnote{Rischer, 2006 #28; Menke, 1999 #31; van der Fits, 2000 #30; Peebles, 2008 #29}
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 using plant tissue culture**

Functional group substitution of natural alkaloids can lead to the generation of compounds with improved pharmacological properties. For example, Vinflunine (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{Chen, 2006 #52}. The current availability of novel alkaloids, however, remains limited because they are still semi-synthetically derived from naturally isolated precursors. Precursor-directed biosynthesis, or a "mutasynthetic" approach, is a powerful strategy in 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{David O'Hagan, 1999 #53}. Similarly, a wide variety of tryptamine and secologanin analogs could be introduced into C. *roseus* root cultures and seedlings in order to synthesize unnatural MIAs{McCoy, 2006 #56; McCoy, 2006 #55}. The apparent flexibility of downstream alkaloid pathways opened the possibility of generating enzyme variants with increased selectivity towards 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{Ma, 2006 #57} led to the identification of several amino acid residues that form the binding pocket of *C. roseus* STR{Loris, 2007 #78}. Using *in vitro* assays that incorporated secologanin derivatives, an enzyme variant containing a D177A mutation that exhibited increased selectivity towards a secologanin analog 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 due 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 tryptamine analogs{Bernhardt, 2007 #54}. The medium-throughput assay took advantage of the formation of products downstream of STR that can be visualized when metabolized by strictosidine glucosidase (SG). By applying a saturation mutagenesis strategy on several residues that form the tryptamine binding pocket, two STR mutants (V214M, F232L) that turned over unnatural tryptamine compounds to synthesize β-carboline analogs were identified using the *in vitro* assay{Loris, 2007 #78}. When the newly synthesized strictosidine analogs were fed to *C. roseus* hairy root cultures, a number of novel MIA analogs were obtained (Scheme 2). This finding set the stage for rational metabolic engineering of unnatural products within the plant cell environment, or "plant metabolic reprogramming". 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. 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 (Runguphan, 2009 #83).

**Engineering alkaloid biosynthetic pathways in microorganisms**

Due 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 re-construction of the plant biosynthetic pathways (Scheme 3a). In plants, (S)-reticuline, the direct precursor of many BIAs, is derived from the condensation of dopamine and 4-hydroxyphenyl-acetaldehyde by norcoclaurine synthase (NCS) to form (S)-norcoclaurine. Subsequently, (S)-norcoclaurine 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 reticuline 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 (Minami, 2008 #58) (Scheme 3b). 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-dihydroxyphenyl-acetaldehyde from dopamine, 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 dopamine, ~11 mg/L (R,S)-reticuline 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 dopamine and 3,4-dihydroxyphenyl-acetaldehyde were sufficiently available, a spontaneous chemical conversion that resulted in the synthesis of racemic reticuline occurred. Nevertheless, the availability of (R,S)-reticuline allowed the biosynthesis of downstream BIAs. By co-culturing the reticuline-producing *E. coli* with *S. cerevisiae* expressing the *C. japonica* berberine bridge enzyme (BBE) or CYP80G2 in the presence of dopamine, (S)-scoulerine or magnoflorine could be detected at ~8 mg/L and ~7 mg/L, respectively after incubation to 48 to 72 h.

The use of two microbial systems for pathway construction reduced the efficiency of alkaloid synthesis due 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{Hawkins, 2008 #59} (Scheme 3c). In this work, *(R,S)*-reticuline biosynthesis from *(R,S)*-norlaudanosoline 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){Nevoigt, 2006 #61}, the heterologous gene expression resulted in the creation of an artificial plant pathway with reduced transcriptional activities, while maintaining high catalytic activities for the synthesis of reticuline. 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 *(S)*-tetrahydrocolumbamine from ~1 g/L *(R,S)*-norlaudanosoline in 48 h. Additional plasmid-based expression of the *C. japonica* P450 enzyme CYP719A1 and the integration of *Arabidopsis thaliana* P450-reduct partner protein ATR1 in the genome gave rise to the accumulation of an estimated ~30 mg/L *(S)*-canadine, the direct precursor of the pharmaceutically important berberine. The synthesis of *(R)*-reticuline by the engineered yeast strain also enabled the synthesis of salutaridine, an intermediate in the morphine branch pathway, through a shorter route. In plants, the synthesis of salutaridine from *(S)*-reticuline undergoes 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 salutaridine could be synthesized from *(R,S)*-
norlaudanosoline. Yeast have also been engineered to accommodate the biosynthesis of high-value MIAs from secologanin and tryptamine\cite{Geerlings, 2001 #32}. Transgenic yeast were created by expressing \textit{C. roseus} STR and strictosidine \(\beta\)-glucoside (SGD) using a plasmid-based expression system. Upon supplemental feeding of STR substrates, \(\sim 2\) g/L strictosidine was detected in the media 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, also at the yield of \(\sim 2\) g/L.

The bottom-up assembly of artificial biosynthetic pathways in \textit{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 \textit{E. coli} due to the absence of endoplasmic reticulum required for anchorage. However, protein engineering strategies to allow the functional expression of plant P450s in \textit{E. coli} have been reported recently\cite{Chang, 2007 #83; Leonard, 2007 #87}. 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 \textit{E. coli} and \textit{S. cerevisiae} has been demonstrated.
High-level synthesis of plant flavonoids (~400 mg/L) from engineered *E. coli* [Leonard, 2007 #88] could be facilitated by redirecting various metabolic fluxes from glucose towards malonyl-CoA (a flavonoid building block). This titer was further improved up to ~700 mg/L by partially repressing fatty acid metabolism in the *E. coli* hosts [Leonard, 2008 #86]. In the case of high-level production of plant natural product in *S. cerevisiae*, the synthesis of ~100 mg/L artemisinic acid from glucose could be achieved by the up-regulation of the mevalonate pathway and the downregulation of a competing pathway (sterol biosynthesis) [Ro, 2006 #85].

**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 scopolamine and berberines from cell culture by Sumitomo Chemical Industries and Mitsui Petrochemical Industries [McCoy, 2008 #81; Roberts, 2007 #73]. One major drawback of plant tissues and cell lines however, is the inability to produce certain alkaloids due to the lack of specialized cell types [St-Pierre, 1999 #70]. For instance, vincristine and vinblastine are not significantly produced in *C. roseus* cell suspension and hairy root cultures because the vindoline precursor pathway is only fully activated in aerial plant parts [Bhadra, 1993 #72; Shanks, 1998 #71]. Metabolic reconstruction will therefore be required for increasing the utility of plant cell lines and tissues for
commercial production systems, and necessitate the enrichment and development of robust genetic tools for plant transformation. Similar to whole plant systems however, the existence of multiple alkaloid biosynthetic pathways, regulatory control mechanisms, and pathway compartmentalization are among the factors that significantly increase the degree of unpredictability of metabolic engineering efforts *(Fig. 1a)*. 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\cite{Morgan,2002} together with systems biology analyses\cite{Rischer,2006} 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\cite{Gantet,2002}.

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 less 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. The complete elucidation of alkaloid biosynthesis from simple precursors can facilitate the construction of the upstream pathway in microbes, hence avoiding the necessity of providing expensive intermediates. However, this strategy will likely entail the implantation of numerous biosynthetic steps, which is not trivial. For example, the reconstruction of the plant (S)-reticuline biosynthetic pathway from tyrosine will involve at least 7 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\cite{Geerlings, 2001 #32}, 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 impact of the toxicity on growth inhibition\cite{Kizer, 2008 #82}. Moreover, a new strategy to increase microbial tolerance towards toxic metabolites by engineering transcription factors\cite{Alper, 2007 #89} can also potentially be applied in alkaloid producer microbes.
In conclusion, although both plant cell/tissue and microbial systems offer tremendous advantages as scalable alkaloid production platforms, many opportunities still lie in cellular and metabolic engineering sectors to create the multifaceted phenotypic traits (e.g. high productivity, product tolerance, stability) required for use in industrial bioprocesses. Moreover, because the characteristics between plant cell/tissue and microbial systems are inherently different, they can serve as complimentary unit operations when cleverly integrated together in biomanufacturing in order to solve the long-standing problem of robust alkaloid production.

Acknowledgements

The authors are grateful for the constructive discussion and contribution of Mr. Michael Tackett (Whitehead Institute for Biomedical Research and Department of Biology, MIT). Research in the Prather laboratory is supported by the US National Science Foundation/Synthetic Biology Engineering Research Center (SynBERC; Grant No. 0540879) and the MIT Energy Initiative (Grant No. 6917278). Research in the O'Connor laboratory is funded by the US National Institutes of Health (GM074820) and the US National Science Foundation (MCB-0719120).

Competing interest statement
The authors declare no competing financial interests.

Figure legend

Table 1. Examples of medicinally important alkaloids.

Scheme 1.
The general biosynthetic schemes of four alkaloid sub-classes. Some important alkaloid products are represented. (a) BIA (NCS, norcoclaurine synthase). (b) MIA (TDC, tryptophan decarboxylase; STR, strictosidine synthase). (c) Tropane alkaloid (ODC, ornithine decarboxylase; PMT, putrescine N-methyltransferase). (d) 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).

Scheme 2.
Mutasynthetic strategy for generating unnatural alkaloids. STR mutants (identified from the development of colorimetric medium throughput assay) that can efficiently turn-over tryptamine analogs were used to synthesize unnatural strictosidines from unnatural tryptamines and secologanin. Unnatural complex alkaloids can be generated from feeding the unnatural strictosidines into *C. roseus* hairy root culture. Tryptamine analogs can also be directly converted into complex alkaloid by metabolically reprogramming *C. roseus* hairy root.

**Scheme 3.**

Reconstruction of BIA pathway in microorganisms. (a) Native plant pathway. (b). Microbial biocatalysts employing both *E. coli* and *S. cerevisiae*. (c). A microbial biocatalyst solely employing *S. cerevisiae*. Enzymatic or 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; CYP719A1, plant cytochrome-P450 enzyme.

**Figure 1.**
Metabolic engineering (ME) of (a) plant system and (b) microorganism. Multiple branch pathways exists (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 (orange square), or 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; orange arrow, deletion; red cross) to increase a particular alkaloid product (e.g. P1), often lead to unexpected outcomes (e.g. 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 sole alkaloid product (e.g. P1) by the construction of an artificial biosynthetic pathway. However, synthetic intermediates (X) have to be provided.
References