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Opportunities in metabolic engineering to enable scalable alkaloid production

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1 **Abstract**

2

3 **Numerous drugs and drug precursors in the current pharmacopoeia**
4 **originate from plant sources. The limited yield of bioactive compounds in**
5 **plant tissues, however, presents a significant challenge for large-scale**
6 **drug development reliant on whole plant extracts. Because of their**
7 **sophisticated molecular architecture, efficient chemical syntheses for**
8 **many plant-derived drugs are also seldom available. Metabolic engineering**
9 **has facilitated the development of plant cell and tissue systems for the**
10 **overproduction of high-value plant pharmaceuticals that can be**
11 **conveniently scaled-up in a controlled environment. Nevertheless, effective**
12 **metabolic engineering approaches, and the predictability of genetic**
13 **transformations are often obscured due to the myriad complexity of**
14 **cellular biology. Progress in systems biology has aided the understanding**
15 **of the genome-wide interconnectivity in plant-based systems. In parallel,**
16 **the bottom-up assembly of plant biosynthetic pathways in microorganisms**
17 **demonstrated the possibilities of a new production means. In this**
18 **Perspective, we discuss the advances and challenges of metabolic**
19 **engineering implementation in various scalable production platforms for**
20 **the bio-based synthesis of natural and unnatural plant alkaloids.**

21

22 Bioactive compounds with “privileged structures” are highly sought
23 paradigms in drug development. Functionally, a privileged structure is a
24 molecular scaffold that can accommodate various pharmacophores arranged to
25 promote interaction with biological targets{Costantino, 2006 #1; Evans, 1988
26 #74; Horton, 2003 #3; Maclean, 2000 #2}. While many have been synthetically
27 designed, nature remains the largest source of highly sophisticated biologically
28 active privileged compounds because presumably they play a key role in
29 increasing the survival fitness of an organism{Koehn, 2005 #75; Maplestone,
30 1992 #76; Williams, 1989 #77}. In fact, about one-third of the ~980 new
31 pharmaceuticals in the past two and a half decades originated from or were
32 inspired by natural products{Newman, 2007 #5}.

33 With over 10,000 structurally characterized members, plant alkaloids are
34 important privileged compounds from which many key clinical medicines are
35 derived (**Table 1**){Beghyn, 2008 #4; Facchini, 2008 #13; Kutchan, 1995 #6;
36 Verpoorte, 2000 #11}. The endogeneous role of alkaloids in plants has not been
37 fully elucidated. However, current evidence suggests that alkaloids are generally
38 involved in plant defense against pathogens, insects, and herbivores due to their
39 potent toxicity{Hartmann, 2004 #12}. For example, the indolizidine, indolizine,
40 and β -carbolines paradigmatic alkaloid backbone structures can exert over 25
41 biological activities, such as dopamine reuptake inhibitor, glucosidase inhibitor,
42 sodium channel blocker, and 5HT1D agonist{Beghyn, 2008 #4}. The bioactivities
43 of alkaloids have been recognized and exploited since ancient human civilization,
44 from the utilization of *Conium maculatum* (hemlock) extract containing the

45 neurotoxin alkaloid coniine to poison Socrates, to the use of caffeine in coffee
46 and tea as a mild stimulant{Kutchan, 1995 #6}. Today, numerous alkaloids are
47 pharmacologically well-characterized and used as clinical drugs, ranging from
48 cancer chemotherapeutics to analgesic agents (**Table 1**).

49 Despite their importance, the inefficiency of extracting some alkaloids
50 remains a significant barrier towards inexpensive bioprospecting for drug
51 development. The process of separating, purifying, and structurally
52 characterizing compounds of interest from a myriad of other metabolites is time-
53 consuming and expensive. Bioactive alkaloids are also usually present in small
54 quantities (**Table 1**). Furthermore, the yield consistency can not always be
55 guaranteed because it depends heavily on the source organisms as well as
56 geographical and climate conditions. The scarcity of some alkaloids in plants is
57 exemplified by the cancer chemotherapeutic compound vincristine, which is
58 found in *Catharanthus roseus* at concentrations that only reach 0.0003% by dry
59 weight{Kuboyama, 2004 #9}.

60 The field of organic synthesis has advanced tremendously in the past
61 decades in creating various methodologies suitable for constructing bisindole
62 alkaloids with multiple functionalities and stereocenters{Kuboyama, 2004 #9;
63 Miyazaki, 2007 #7; Uchida, 2006 #8; Yokoshima, 2002 #10}. Nevertheless, total
64 or semi-synthesis of many other alkaloids remains a daunting challenge that is
65 far from being practical at the industrial level. Plant tissue and cell cultures can
66 serve as alternative production platforms in which the biosynthesis of alkaloids
67 has been improved through various elicitation and culture manipulation

68 strategies. Additionally, supported by the availability of systems biology datasets,
69 metabolic engineering now has the potential to more effectively maximize the
70 capacity for alkaloid biosynthesis in cellular systems. For the most part, major
71 advancements in alkaloid metabolic engineering occurred within the last decade.
72 In this Perspective, we first focus on the milestones and challenges in
73 engineering plant tissue and cell lines for improving natural alkaloid production,
74 and for facilitating the synthesis of unnatural alkaloids. Recently, there has been
75 an increasing interest in the engineering of microorganisms for the synthesis of
76 high-value metabolites. To this end, we highlight the recent construction of
77 artificial alkaloid biosynthetic pathways in *Escherichia coli* and *Saccharomyces*
78 *cerevisiae* and discuss the potential for the use of microbes as novel alkaloid
79 production platforms.

80

81 **Alkaloid biosynthetic pathway elucidation and manipulation in plants**

82

83 The significance of alkaloids has motivated the characterization of their
84 biosynthetic pathways. Mechanistic elucidation of enzymatic steps typically
85 begins by tracking isotopically labeled metabolites in differentiated plants or plant
86 cell cultures. Further steps commonly involve reverse genetics where, following
87 plant enzyme isolation and purification, partial sequence data of the purified
88 protein is used to obtain the corresponding gene from a cDNA library. This allows
89 the identification of the starting substrates, and enables the proposal of a series
90 of logical biosynthetic transformations. Recently, genomic and transcriptomic

91 technologies have been used to rapidly identify biosynthetic steps. There are
92 currently over 40,000 expressed enzyme tags (ESTs) generated from alkaloid
93 producing plants that have been used to isolate genes involved in the alkaloid
94 pathway{Facchini, 2008 #13}. Nevertheless, the availability of genome
95 sequences of alkaloid producer plants is urgently needed to further speed the
96 elucidation of their biosynthesis. To date, the biosynthetic routes of four alkaloid
97 sub-classes have been partially characterized, namely the benzyloquinoline,
98 monoterpenoid indole, purine, and tropane alkaloids. Benzyloquinoline
99 alkaloids (BIAs) are derived from tyrosine and are comprised of ~2500 defined
100 structures found mainly in the *Papaveraceae*, *Ranunculaceae*, *Berberidaceae*,
101 and *Menispermaceae*{Liscombe, 2008 #16}. The first committed step of BIA
102 biosynthesis begins with the stereoselective Pictet-Spengler condensation of
103 dopamine and 4-hydroxyphenylacetaldehyde (4HPAA) to form norcoclaurine.
104 Through a series of methylations and hydroxylations, (S)-norcoclaurine is
105 converted into (S)-reticuline, the pivotal intermediate of many pharmaceutically
106 important BIAs in the downstream pathways (**Scheme 1a**). The second sub-
107 class, the monoterpenoid indole alkaloids (MIAs) are derived from tryptophan
108 metabolism. MIAs are some of the most structurally diverse natural products.
109 With over 2000 structures, they are mainly found in the *Apocynaceae*,
110 *Loganiaceae* and *Rubiaceae*{O'Connor, 2006 #15}. Similar to BIA, the committed
111 step of MIA biosynthesis begins with the condensation of tryptamine and
112 secologanin (derived from terpene biosynthesis) to form strictosidine{Maresh,
113 2008 #14}. Following the deglycosylation of strictosidine, equilibrium of the

114 unstable aglycon intermediates leads to the formation of 4, 21-
115 dehydrogeissoschizine, the branch-point precursor of MIAs (**Scheme 1b**).
116 Tropane alkaloids are the third sub-class whose biosynthetic pathways have
117 been investigated. They are found primarily in *Solanaceae*{Sato, 2001 #17}. The
118 first committed step of tropane biosynthesis is the N-methylation of putrescine to
119 form N-methylputrescine. Following the conversion to 1-methyl- Δ^1 pyrrolinium
120 cation, its condensation with nicotinic acid leads to nicotine synthesis while other
121 chemical conversions lead to the formation of tropinone, the branch-point
122 intermediate of many tropane alkaloids (**Scheme 1c**). The fourth alkaloid sub-
123 class is derived from purine nucleotides instead of amino acids. A well-known
124 example of a purine alkaloid is caffeine, whose biosynthetic route has been
125 largely studied in *Camellia*, *Coffea*, *Theobroma*, and *Ilex*{Ashihara, 2008 #18}. Its
126 upstream pathway involves four enzymatic steps which consist of three SAM-
127 dependent methyl transfers, and one nucleotide removal reaction to form
128 xanthosine, the first committed precursor of purine alkaloids (**Scheme 1d**).

129 While efforts to complete elucidation of alkaloid metabolism is
130 progressing, known enzymatic steps have been used as a basis for plant
131 metabolic engineering strategies to increase the biosynthesis of alkaloids of
132 interest or to eliminate undesired metabolites. For example, a transgenic coffee
133 cultivar with 70% reduction of caffeine content was created by the introduction of
134 RNAi constructs in order to down-regulate theobromine synthase (MXMT){Ogita,
135 2003 #19}. A more pest resistant tobacco cultivar has also been engineered by
136 expressing three N-methyltransferases from coffee to divert flux from xanthosine

137 to synthesize caffeine{Uefuji, 2005 #20}. Furthermore, the pharmaceutically
138 valuable scopolamine has been made available in *Atropa belladonna*, a plant that
139 normally accumulates hyoscyamine, by expressing *Hyoscyamus niger*
140 hyoscyamine 6 β -hydroxylase (H6H), the enzyme which converts hyoscyamine
141 into scopolamine{Yun, 1992 #21}.

142 Although there are many examples of successful attempts to achieve a
143 desired alkaloid production phenotype, the outcome of plant metabolic
144 engineering strategies is often unpredictable. For instance, consider *COR1* which
145 encodes for codeinone reductase, the final enzyme in morphine biosynthesis. By
146 only overexpressing this single gene, morphine and codeine content in
147 transgenic opium poppy was moderately increased by ~22% and ~58%,
148 respectively{Larkin, 2007 #22}. However, thebaine, an upstream metabolite in
149 the morphine branch pathway was also unexpectedly and significantly
150 amplified{Larkin, 2007 #22}. The outcome of the down-regulation of codeinone
151 reductase with RNAi was also puzzling. It was expected that this strategy would
152 lead to suppression of morphine formation and the accumulation of codeinone
153 and morphinone, the immediate precursors of codeinone reductase. Although the
154 amount of the morphinan alkaloids was decreased, biosynthesis of (*S*)-reticuline,
155 an early up-stream metabolite in the pathway was increased instead of
156 codeinone or morphinone{Allen, 2004 #24}. On the other hand, the
157 overexpression of another enzyme in the pathway, the cytochrome-P450
158 monooxygenase (*S*)-N-methylcoclaurine 3'-hydrolase (CYP80B3) resulted in an
159 up to 450% increase of total morphinan alkaloids without altering the product

160 distribution{Frick, 2007 #23}. This result suggests that although there are multiple
161 control points in the BIA pathway, CYP80B3 is an important target towards
162 improving morphine biosynthesis. The suppression of this gene by an antisense
163 construct which led to a reduced total alkaloid content in the transgenic opium
164 poppy supported this hypothesis{Frick, 2007 #23}.

165

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

167

168 The demand for highly abundant plant alkaloids, such as the morphinan
169 opiates, can be met through plant extraction{Ye, 1998 #39}. However, for some
170 scarcely available alkaloids, alternative production platforms are desirable. It was
171 discovered as early as the 1950s that undifferentiated plant cells have the
172 capacity to produce many of the same secondary metabolites as whole
173 plants{Angela M. Stafford, 1998 #63}. Today, several plant cell lines have been
174 developed to synthesize some important pharmaceuticals at industrial levels. For
175 example, concentrations of taxol as high as 0.5% of dry weight have been
176 achieved in plant cell culture with methyl jasmonate elicitation{Yukimune, 1996
177 #25}. This is a stark yield improvement over taxol concentrations in Pacific yew,
178 which account for as little as 0.01% of the dry weight{Witherup, 1990 #79}.

179 Shikonin, a naphthoquinone pigment used in cosmetics, has also been
180 successfully derived from *Lithospermum erythrorhizon* cell suspension
181 cultures{Touno, 2005 #26}.

182 Extensive efforts have focused on optimizing plant cell cultures for
183 improving the yield, controllability and reproducibility of several pharmaceutically
184 important alkaloids{Gamborg, 2002 #80}. However, because the activation of
185 many alkaloid biosynthetic pathways is tissue specific and a function of
186 developmental stage{Filner, 1969 #40}, plant tissue cultures are often utilized as
187 production platforms instead of cell lines{Shanks, 1999 #62}. For both tissue and
188 cell culture systems, the elicitation of alkaloid synthesis often involves the
189 utilization of certain small molecules{Kutchan, 1995 #6} and light{De Luca, 2000
190 #42; Vazquez-Flota, 2000 #41}. Aside from developing optimal culture
191 conditions, various metabolic engineering manipulations have also been explored
192 in plant tissue and cell lines to obtain alkaloid overproduction phenotypes. Similar
193 to whole-plants, however, the outcomes of chosen metabolic engineering
194 strategies in plant tissue and cell lines are often difficult to predict or control. In
195 one instance, while the overexpression of STR, the key enzyme in the MIA
196 pathway (**Scheme 1b**), in *C. roseus* cell lines improved the levels of ajmalicine,
197 serpentine, catharanthine and tabersonine, the highly productive lines were
198 deemed to be unstable{Canel, 1998 #27}. In another case, it was previously
199 known that tryptophan biosynthesis is feedback inhibited, hence tryptophan
200 availability might be a limiting factor in MIA biosynthesis. However, the
201 introduction of *Arabidopsis thaliana* feedback-resistant anthranilate synthase
202 (*AtAS*) and induction of tryptophan decarboxylase (TDC) in *C. roseus* hairy roots
203 did not significantly improve downstream MIAs even though the levels of early
204 MIA precursors tryptophan and tryptamine were increased{Hughes, 2004 #43;

205 Hughes, 2004 #44; Hong, 2006 #45}. This result suggested that the availability of
206 tryptophan and tryptamine are not limiting for MIA biosynthesis, confirming the
207 finding that secologanin availability was the important rate-limiting step in MIA
208 biosynthesis. Improvements in the secologanin precursor branch pathway
209 successfully increased MIA synthesis in suspension cells or hairy roots{Whitmer,
210 1998 #65; Whitmer, 2002 #64; Morgan, 2000 #67}. When the activity of the
211 terminal step of vindoline biosynthesis was amplified in *C. roseus* hairy roots by
212 the overexpression of deacetylvindoline-4-O-acetyltransferase (DAT), the
213 accumulation of horhammericine, an alkaloid not in the vindoline pathway was
214 elevated by four-fold{Magnotta, 2007 #68}. Further experiments revealed the
215 existence of cross-talk by DAT overexpression because this enzyme inhibited the
216 activity of minovincinine-19-O-acetyltransferase (MAT), the enzyme which turns
217 over horhammericine.

218 As with whole-plant systems, the intricate relationships among metabolic
219 pathways and regulatory schemes in plant cells and tissues are among the many
220 factors that limit the robustness of a metabolic engineering design to achieve a
221 specific overproduction target. Various systems biology approaches have been
222 developed to portray the complex metabolic interplay in plant tissue and cell
223 systems. In one instance, flux analysis using isotopomer{Schwender, 2004 #46;
224 Schwender, 2004 #47}, and 'bond-isomer'{Sriram, 2007 #48; Sriram, 2004 #49}
225 labeling and balancing has been employed to profile flux distribution in the
226 central metabolism of *C. roseus* hairy roots. Significant progress has also been
227 made towards elucidating the genome-wide interconnectivity among biological

228 functions resulting in datasets that contain gene-to gene and gene-to-metabolite
229 networks that reveal regulatory differences in key alkaloid pathways in *C. roseus*
230 cells{Rischer, 2006 #28}. Alkaloid metabolic regulatory machineries have also
231 been probed by using transcriptome analysis, leading to the identification of
232 several transcription factors in MIA biosynthesis{van der Fits, 2000 #30; Menke,
233 1999 #31}. All together, this information can potentially be useful in determining
234 metabolic engineering targets that can effectively deliver a desired improvement
235 in a specific alkaloid branch pathway. For instance, a metabolic engineering
236 strategy to increase MIA production was devised to exploit the utility of the
237 ORCA3 transcription factor to upregulate the expression of many MIA
238 biosynthetic genes simultaneously{van der Fits, 2000 #30}. However, initial
239 ORCA3 overexpression in *C. roseus* cell cultures did not significantly improve
240 MIA synthesis. It was discovered that even though ORCA3 positively regulates
241 the expression of many genes that lead to the synthesis of strictosidine, it does
242 not up-regulate the expression of geraniol 10-hydroxylase (G10H), the enzyme in
243 the terpenoid pathway that leads to the synthesis of secologanin. Upon
244 supplemental feeding of the secologanin precursor, loganin, the overexpression
245 of ORCA3 resulted in ~3-fold increase of MIA biosynthesis. Another
246 transcriptome study revealed the complexity of MIA biosynthetic control by a
247 variety of transcriptional regulators. In this case, it was discovered that although
248 the MIA biosynthetic enzymes that were upregulated upon ORCA3
249 overexpression in *C. roseus* hairy root lines is similar to those in cell lines, the
250 transcriptional repressors ZCT1 and ZCT2 were also upregulated{Peebles, 2008

251 #50}. This finding provided an explanation for the insignificant improvement of
252 MIA synthesis in *C. roseus* hairy roots upon ORCA3 overexpression.

253

254 **Mutasynthesis of novel alkaloid analogs using plant tissue culture**

255

256 Functional group substitution of natural alkaloids can lead to the
257 generation of compounds with improved pharmacological properties. For
258 example, Vinflunine (4'-deoxy-20', 20'-difluoro-C'-norvincalceukoblastine), a new
259 compound that is currently in clinical trials, was created by the introduction of two
260 fluoro groups into vinblastine{Chen, 2006 #52}. The current availability of novel
261 alkaloids, however, remains limited because they are still semi-synthetically
262 derived from naturally isolated precursors. Precursor-directed biosynthesis, or a
263 "mutasynthetic" approach, is a powerful strategy in increasing the availability of
264 alkaloid derivatives. The technology that harnessed whole-cell biocatalysts for
265 mutasynthetic purposes arose from the discovery that several fluorinated tropane
266 alkaloids could be produced by simply feeding fluorinated phenyllactic acid
267 analogs to *Datura stramonium* root cultures{David O'Hagan, 1999 #53}. Similarly,
268 a wide variety of tryptamine and secologanin analogs could be introduced into *C.*
269 *roseus* root cultures and seedlings in order to synthesize unnatural MIAs{McCoy,
270 2006 #56; McCoy, 2006 #55}. The apparent flexibility of downstream alkaloid
271 pathways opened the possibility of generating enzyme variants with increased
272 selectivity towards unnatural substrate analogs, thereby improving the efficiency
273 of precursor directed biosynthesis and increasing the number of unnatural

274 alkaloids. Several STR variants with altered substrate specificity have been
275 successfully engineered. In one instance, the structural elucidation of *Rauvolfia*
276 *serpentina* STR{Ma, 2006 #57} led to the identification of several amino acid
277 residues that form the binding pocket of *C. roseus* STR{Loris, 2007 #78}. Using
278 *in vitro* assays that incorporated secologanin derivatives, an enzyme variant
279 containing a D177A mutation that exhibited increased selectivity towards a
280 secologanin analog with a pentynyl group was identified. This strategy clearly
281 demonstrated the benefit of re-engineering STR plasticity for mutasynthetic
282 purposes. However, the ability to explore a widely diverse mutational space was
283 still limited due to the lack of a facile screening assay. In approaching this
284 challenge, a medium-throughput colorimetric assay was developed in order to
285 identify functional STR mutants that can accept tryptamine analogs{Bernhardt,
286 2007 #54}. The medium-throughput assay took advantage of the formation of
287 products downstream of STR that can be visualized when metabolized by
288 strictosidine glucosidase (SG). By applying a saturation mutagenesis strategy on
289 several residues that form the tryptamine binding pocket, two STR mutants
290 (V214M, F232L) that turned over unnatural tryptamine compounds to synthesize
291 β -carboline analogs were identified using the *in vitro* assay{Loris, 2007 #78}.
292 When the newly synthesized strictosidine analogs were fed to *C. roseus* hairy
293 root cultures, a number of novel MIA analogs were obtained (**Scheme 2**). This
294 finding set the stage for rational metabolic engineering of unnatural products
295 within the plant cell environment, or “plant metabolic reprogramming”. Indeed,
296 upon feeding with the tryptamine analogs that the reengineered enzyme was

297 designed to accept, transgenic *C. roseus* hairy roots expressing the V214M
298 mutant enzyme produced a variety of unnatural alkaloid compounds. These
299 results show the power of biocatalysis to facilitate the synthesis of unnatural
300 alkaloids, an approach that has now been demonstrated to be applicable to plant
301 cell culture{Runguphan, 2009 #83}.

302

303 **Engineering alkaloid biosynthetic pathways in microorganisms**

304

305 Due to the smaller genome size, the degree of complexity in
306 microorganisms is significantly lower than that of plants. Moreover,
307 microorganisms have fewer intracellular organelles compared to plant cells;
308 hence metabolite transport between enzymatic steps can be negligible. Indeed,
309 the relative simplicity and tractability of microorganisms has sparked significant
310 interest in their engineering for the synthesis of high-value plant metabolites. To
311 this end, the bacterium *E. coli* and yeast *S. cerevisiae* were recently explored as
312 production hosts of plant alkaloids. In both cases, the metabolic engineering
313 efforts in microorganisms entailed the re-construction of the plant biosynthetic
314 pathways (**Scheme 3a**). In plants, (S)-reticuline, the direct precursor of many
315 BIAs, is derived from the condensation of dopamine and 4-hydroxyphenyl-
316 acetaldehyde by norcoclaurine synthase (NCS) to form (S)-norcoclaurine.
317 Subsequently, (S)-norcoclaurine is methylated by norcoclaurine 6-O-
318 methyltransferase (6-OMT), and coclaurine-N-methyltransferase (CNMT),
319 hydroxylated by the cytochrome-P450 CYP80B3, and further methylated by 3'-

320 hydroxy-N-methylcoclaurine-4'-O-methyltransferase (4'-OMT) (**Scheme 3**). To
321 assemble an artificial pathway to achieve reticuline biosynthesis in *E. coli*,
322 *Micrococcus luteus* monoamine oxidase (MAO) was introduced together with
323 *Coptis japonica* NCS, 6-OMT, CNMT, and 4'-OMT in plamid-based expression
324 systems{Minami, 2008 #58} (**Scheme 3b**). In this strategy, the utilization of the
325 microbial MAO allowed the incorporation of the hydroxyl group early in the
326 reticuline pathway through the synthesis of 3,4-dihydroxyphenyl-acetaldehyde
327 from dopamine, hence obviating the need to express the plant P450 CYP80B3 in
328 the bacterium, which is often problematic. Upon induction of enzyme expression,
329 and supplementation with ~780 mg/L dopamine, ~11 mg/L (*R,S*)-reticuline could
330 be detected in the culture medium of the recombinant *E. coli*. Because plant NCS
331 exclusively synthesizes (*S*)-enantiomers, the generation of the racemic products
332 by the artificial pathway was confounding. Further investigation concluded that
333 when dopamine and 3,4-dihydroxyphenyl-acetaldehyde were sufficiently
334 available, a spontaneous chemical conversion that resulted in the synthesis of
335 racemic reticuline occurred. Nevertheless, the availability of (*R,S*)-reticuline
336 allowed the biosynthesis of downstream BIAs. By co-culturing the reticuline-
337 producing *E. coli* with *S. cerevisiae* expressing the *C. japonica* berberine bridge
338 enzyme (BBE) or CYP80G2 in the presence of dopamine, (*S*)-scoulerine or
339 magnoflorine could be detected at ~8 mg/L and ~7 mg/L, respectively after
340 incubation to 48 to 72 h.

341 The use of two microbial systems for pathway construction reduced the
342 efficiency of alkaloid synthesis due to necessary metabolite transport between

343 cells. In another study, *S. cerevisiae* was used as a sole host organism for the
344 assembly of artificial BIA pathways{Hawkins, 2008 #59} (**Scheme 3c**). In this
345 work, (*R,S*)-reticuline biosynthesis from (*R,S*)-norlaudanosoline was enabled by
346 expressing 6-OMT, CNMT, and 4'-OMT derived from either *Thalictrum flavum* or
347 *Papaver somniferum*. After stable insertion into the yeast genome under a
348 reduced-strength promoter variant (TEF7){Nevoigt, 2006 #61}, the heterologous
349 gene expression resulted in the creation of an artificial plant pathway with
350 reduced transcriptional activities, while maintaining high catalytic activities for the
351 synthesis of reticuline. Furthermore, the plasmid-based expression of *P.*
352 *somniferum* BBE together with *T. flavum* (*S*)-scoulerine 9-O-methyltransferase
353 (SMT) in (*R,S*)-reticuline-producing yeasts resulted in the synthesis of ~60 mg/L
354 (*S*)-tetrahydrocolumbamine from ~1 g/L (*R,S*)-norlaudanosoline in 48 h.
355 Additional plasmid-based expression of the *C. japonica* P450 enzyme CYP719A1
356 and the integration of *Arabidopsis thaliana* P450-redox partner protein ATR1 in
357 the genome gave rise to the accumulation of an estimated ~30 mg/L (*S*)-
358 canadine, the direct precursor of the pharmaceutically important berberine. The
359 synthesis of (*R*)-reticuline by the engineered yeast strain also enabled the
360 synthesis of salutaridine, an intermediate in the morphine branch pathway,
361 through a shorter route. In plants, the synthesis of salutaridine from (*S*)-reticuline
362 undergoes multiple enzymatic steps, many of which are not characterized.
363 However, by expressing a human cytochrome-P450 involved in morphine
364 metabolism, CYP2D6, together with human CPR1 reductase, in the reticuline-
365 producing yeasts, ~20 mg/L salutaridine could be synthesized from (*R,S*)-

366 norlaudanosoline. Yeast have also been engineered to accommodate the
367 biosynthesis of high-value MIAs from secologanin and tryptamine{Geerlings,
368 2001 #32}. Transgenic yeast were created by expressing *C. roseus* STR and
369 strictosidine β -glucoside (SGD) using a plasmid-based expression system. Upon
370 supplemental feeding of STR substrates, ~2 g/L strictosidine was detected in the
371 media where the heterologously expressed STR was exported. Permeabilization
372 of yeast cells to allow the diffusion of strictosidine into the cells was necessary for
373 its metabolism by SGD to result in the generation of cathenamine, also at the
374 yield of ~2 g/L.

375 The bottom-up assembly of artificial biosynthetic pathways in *E. coli* and
376 yeast enabled the biosynthesis of plant alkaloids in a short period of time (48-72
377 h). One advantageous feature of yeast is the ability to support the functionality of
378 plant membrane-bound cytochrome P450 enzymes that are rendered difficult in
379 *E. coli* due to the absence of endoplasmic reticulum required for anchorage.
380 However, protein engineering strategies to allow the functional expression of
381 plant P450s in *E. coli* have been reported recently{Chang, 2007 #83; Leonard,
382 2007 #87}. In general, the current technology of supplying alkaloids from
383 engineered microorganisms is not economical because it still relies on the
384 supplementation of expensive intermediate metabolites. However, the complete
385 elucidation of alkaloid biosynthetic pathways from the early amino acid
386 precursors could lead to the generation of inexpensive microbial production
387 platforms. In fact, the feasibility of high-level plant metabolite synthesis from
388 inexpensive precursors in both *E. coli* and *S. cerevisiae* has been demonstrated.

389 High-level synthesis of plant flavonoids (~400 mg/L) from engineered *E.*
390 *coli*{Leonard, 2007 #88} could be facilitated by redirecting various metabolic
391 fluxes from glucose towards malonyl-CoA (a flavonoid building block). This titer
392 was further improved up to ~700 mg/L by partially repressing fatty acid
393 metabolism in the *E. coli* hosts{Leonard, 2008 #86}. In the case of high-level
394 production of plant natural product in *S. cerevisiae*, the synthesis of ~100 mg/L
395 artemisinic acid from glucose could be achieved by the up-regulation of the
396 mevalonate pathway and the downregulation of a competing pathway (sterol
397 biosynthesis){Ro, 2006 #85}.

398

399 **Perspective**

400

401 Plant tissue and cell cultures are prospective scalable alkaloid production
402 platforms. The utility of these systems is exemplified by the industrial scale
403 production of scopolamine and berberines from cell culture by Sumitomo
404 Chemical Industries and Mitsui Petrochemical Industries{McCoy, 2008 #81;
405 Roberts, 2007 #73}. One major drawback of plant tissues and cell lines however,
406 is the inability to produce certain alkaloids due to the lack of specialized cell
407 types{St-Pierre, 1999 #70}. For instance, vincristine and vinblastine are not
408 significantly produced in *C. roseus* cell suspension and hairy root cultures
409 because the vindoline precursor pathway is only fully activated in aerial plant
410 parts{Bhadra, 1993 #72; Shanks, 1998 #71}. Metabolic reconstruction will
411 therefore be required for increasing the utility of plant cell lines and tissues for

412 commercial production systems, and necessitate the enrichment and
413 development of robust genetic tools for plant transformation. Similar to whole
414 plant systems however, the existence of multiple alkaloid biosynthetic pathways,
415 regulatory control mechanisms, and pathway compartmentalization are among
416 the factors that significantly increase the degree of unpredictability of metabolic
417 engineering efforts (**Fig. 1a**). The lack of complete understanding of the complex
418 alkaloid biosynthetic networks also hinders the determination of an effective
419 metabolic engineering strategy to achieve a specific production phenotype. The
420 development of mathematical models of plant metabolism{Morgan, 2002 #66}
421 together with systems biology analyses{Rischer, 2006 #29} can eventually be
422 used to aid in determining effective metabolic engineering strategies.
423 Additionally, because the inherent complexity of plant cellular systems often
424 causes single gene manipulations to be ineffective for altering a biosynthetic
425 phenotype, methods that are capable of effecting simultaneous changes in
426 multiple metabolic points, such as the use of transcription factors, are
427 promising{Gantet, 2002 #38}.

428 Microbes are even more scalable than plant tissue and cell cultures, with a
429 long and successful history as chemical factories for the large-scale production of
430 both bulk and specialized chemical products. The degree of complexity in
431 microorganisms is significantly less than that of plant systems (**Fig. 1b**), such
432 that the lack of preexisting branch alkaloid pathways and transcription factors in
433 microbes should also simplify the choice of metabolic engineering targets and
434 approaches. The recent demonstrations of engineering alkaloid pathways in

435 microbes are promising, but are limited by the need to provide expensive
436 intermediate precursors exogenously. The complete elucidation of alkaloid
437 biosynthesis from simple precursors can facilitate the construction of the
438 upstream pathway in microbes, hence avoiding the necessity of providing
439 expensive intermediates. However, this strategy will likely entail the implantation
440 of numerous biosynthetic steps, which is not trivial. For example, the
441 reconstruction of the plant (S)-reticuline biosynthetic pathway from tyrosine will
442 involve at least 7 enzymes. There are several other challenges that must be met
443 before microorganisms can be used as an industrial alkaloid production platform.
444 For example, because many steps in alkaloid biosynthesis require methylation,
445 high-level production in microbial systems will likely be limited by the intracellular
446 availability of S-adenosyl-L-methionine (SAM). Therefore, this bottleneck
447 motivates further metabolic engineering efforts to increase the SAM pool in the
448 microbial host. The cytotoxicity of alkaloids in yeast has also been
449 implicated{Geerlings, 2001 #32}, and is presumably a factor in other microbes as
450 well. Therefore, practical and effective strategies need to be devised to mitigate
451 toxicity in order to generate alkaloid overproducing microbes. A transcriptomic
452 approach has been recently used to diagnose the effect of metabolite toxicity,
453 and resulted in a strategy to dampen the impact of the toxicity on growth
454 inhibition{Kizer, 2008 #82}. Moreover, a new strategy to increase microbial
455 tolerance towards toxic metabolites by engineering transcription factors{Alper,
456 2007 #89} can also potentially be applied in alkaloid producer microbes.

457 In conclusion, although both plant cell/tissue and microbial systems offer
458 tremendous advantages as scalable alkaloid production platforms, many
459 opportunities still lie in cellular and metabolic engineering sectors to create the
460 multifaceted phenotypic traits (e.g. high productivity, product tolerance, stability)
461 required for use in industrial bioprocesses. Moreover, because the characteristics
462 between plant cell/tissue and microbial systems are inherently different, they can
463 serve as complimentary unit operations when cleverly integrated together in
464 biomanufacturing in order to solve the long-standing problem of robust alkaloid
465 production.

466

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468

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477

478 **Competing interest statement**

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480 The authors declare no competing financial interests.

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487 **Figure legend**

488

489 **Table 1.** Examples of medicinally important alkaloids.

490

491 **Scheme 1.**

492 The general biosynthetic schemes of four alkaloid sub-classes. Some important

493 alkaloid products are represented. **(a)** BIA (NCS, norcoclaurine synthase). **(b)**

494 MIA (TDC, tryptophan decarboxylase; STR, strictosidine synthase). **(c)** Tropane

495 alkaloid (ODC, ornithine decarboxylase; PMT, putrescine N-methyltransferase).

496 **(d)** Purine alkaloid (XMT, xanthosine N-methyltransferase/7-methylxanthosine

497 synthase; XN, 7-methylxanthosine nucleotidase; MXMT, 7-methylxanthine N-

498 methyltransferase/theobromine synthase; DXMT, dimethylxanthine N-

499 methyltransferase/caffeine synthase).

500

501 **Scheme 2.**

502

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

510

511 **Scheme 3.**

512

513 Reconstruction of BIA pathway in microorganisms. (a). Native plant pathway. (b).
514 Microbial biocatalysts employing both *E. coli* and *S. cerevisiae*. (c). A microbial
515 biocatalyst solely employing *S. cerevisiae*. Enzymatic or metabolite modifications
516 are indicated in red. NCS, norcoclaurine synthase; 6-OMT, norcoclaurine 6-O-
517 methyltransferase; CNMT, coclaurine-N-methyltransferase; 4'-OMT, 3'-hydroxy-
518 N-methylcoclaurine-4'-O-methyltransferase; DRS, 1,2-dehydroreticuline
519 synthase; DRR, 1,2-dehydroreticulene reductase; BBE, berberine bridge
520 enzyme; SMT, scoulerine 9-O-methyltransferase; MAO, bacterial monoamine
521 oxidase; CYP2D6, human cytochrome-P450 enzyme; CYP80G2; CYP719B1;
522 CYP719A1, plant cytochrome-P450 enzyme.

523

524 **Figure 1.**

525

526 Metabolic engineering (ME) of **(a)** plant system and **(b)** microorganism. Multiple
527 branch pathways exists (A-F) in plant cells that lead to the formation of diverse
528 alkaloid products (P1, P1, P3). These pathways are also fragmented in different
529 intracellular compartments such as the vacuole (blue triangle), plastid (orange
530 square), or endoplasmic reticulum (red curve). Moreover, alkaloid biosynthesis in
531 plant cells is also regulated by transcription factors (tf). Microorganisms, on the
532 other hand, have fewer (or no) intracellular organelles, and are devoid of
533 preexisting alkaloid pathways and transcription factors. Rational metabolic
534 engineering strategies (overexpression; orange arrow, deletion; red cross) to
535 increase a particular alkaloid product (e.g. P1), often lead to unexpected
536 outcomes (e.g. the significant amplification of P3 and P4) due to the inherent
537 complexities of plant cellular biology and the lack of understanding of alkaloid
538 biosynthetic networks. Microorganisms can facilitate the biosynthesis of a sole
539 alkaloid product (e.g. P1) by the construction of an artificial biosynthetic pathway.
540 However, synthetic intermediates (X) have to be provided.

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559 **References**

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