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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.

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 benzylisoquinoline,

98 monoterpenoid indole, purine, and tropane alkaloids. Benzylisoquinoline

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 deglucosylation 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 threobromine 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 6β-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   (*At*AS) 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'-norvincaleukoblastine), 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

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

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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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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