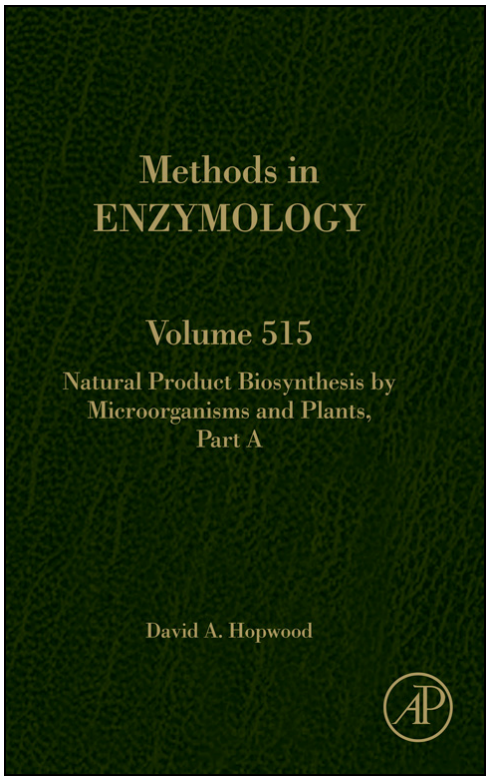

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CHAPTER NINE

Strategies for Engineering Plant Natural Products: The Iridoid-Derived Monoterpene Indole Alkaloids of *Catharanthus roseus*

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Contents

Abstract

The manipulation of pathways to make unnatural variants of natural compounds, a process often termed combinatorial biosynthesis, has been robustly successful in prokaryotic systems. The development of approaches to generate new-to-nature compounds from plant-based pathways is, in comparison, much less advanced. Success will depend on the specific chemistry of the pathway, as well as on the suitability of the plant system for transformation and genetic manipulation. As plant pathways are elucidated, and can be heterologously expressed in hosts that are more amenable to genetic manipulation, biosynthetic production of new-to-nature compounds from plant pathways will become more widespread. In this chapter, some of the key strategies that have been developed for metabolic engineering of plant pathways, namely directed biosynthesis, mutasynthesis, and pathway incorporation of engineered enzymes are highlighted. The iridoid-derived monoterpene indole alkaloids from *C. roseus*, which are the focus of this chapter, provide an excellent system for developing these strategies.

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1. INTRODUCTION

Alkaloids are a highly diverse group of natural products related only by the presence of a basic nitrogen atom at some position in the molecule. Even among biosynthetically related classes of alkaloids, the chemical structures are often highly divergent. The iridoid-derived alkaloids, which are derived from secologanin and tryptamine or tyramine, powerfully illustrate this point. The density of reactive functional groups on the iridoid terpene secologanin ensures that a wide range of rearrangements is chemically feasible (O'Connor & Maresh, 2006; van der Heijden, Jacobs, Snoeijer, Hallard, & Verpoorte, 2004). The biochemistry of iridoid-derived alkaloid biosynthesis is exceptionally diverse and largely uncharacterized.

The iridoid secologanin reacts with tryptamine to yield the monoterpene indole alkaloids, a class of about 3000 natural products with a variety of chemical structures (van der Heijden et al., 2004; Fig. 9.1). The monoterpene indole alkaloids also exhibit a wealth of biological activities and are used as anticancer, antimalarial, and antiarrhythmic agents. The bisindole alkaloids vinblastine and vincristine, produced exclusively by Madagascar periwinkle (*Catharanthus roseus*), are potent inhibitors of microtubule assembly. These compounds are used to treat leukemia, Hodgkin's lymphoma, and other cancers (Beljanski & Beljanski, 1986; Johnson, Wright, & Svoboda, 1959; Noble, 1990). Although synthetic methods have been developed to produce these valuable natural products (Ishikawa, Colby, & Boger, 2008; Kuboyama, Yokoshima, Tokuyama, & Fukuyama, 2004), which are derived *in planta* through the coupling of the monoterpene indole alkaloids vindoline and catharanthine (Costa et al., 2008), industrial production still relies on the low yields obtained through extraction and isolation from *C. roseus* leaves (Gueritte, Bac, Langlois, & Potier, 1980). As such, alternative production methods that improve the yields of these costly molecules would be widely beneficial. Additionally, the activity of many natural products can be modulated or improved by subtle changes in chemical structure. While synthetic chemistry can be used to introduce certain changes, hijacking the biosynthetic machinery to generate analogs provides an attractive strategy to ferment these "unnatural natural products."

Metabolic engineering efforts to develop more efficient production platforms, as well as strategies to manipulate the biosynthetic machinery to produce unnatural analogs, require a working knowledge of the biosynthetic pathway at the enzymatic and genetic level. Therefore, metabolic engineering

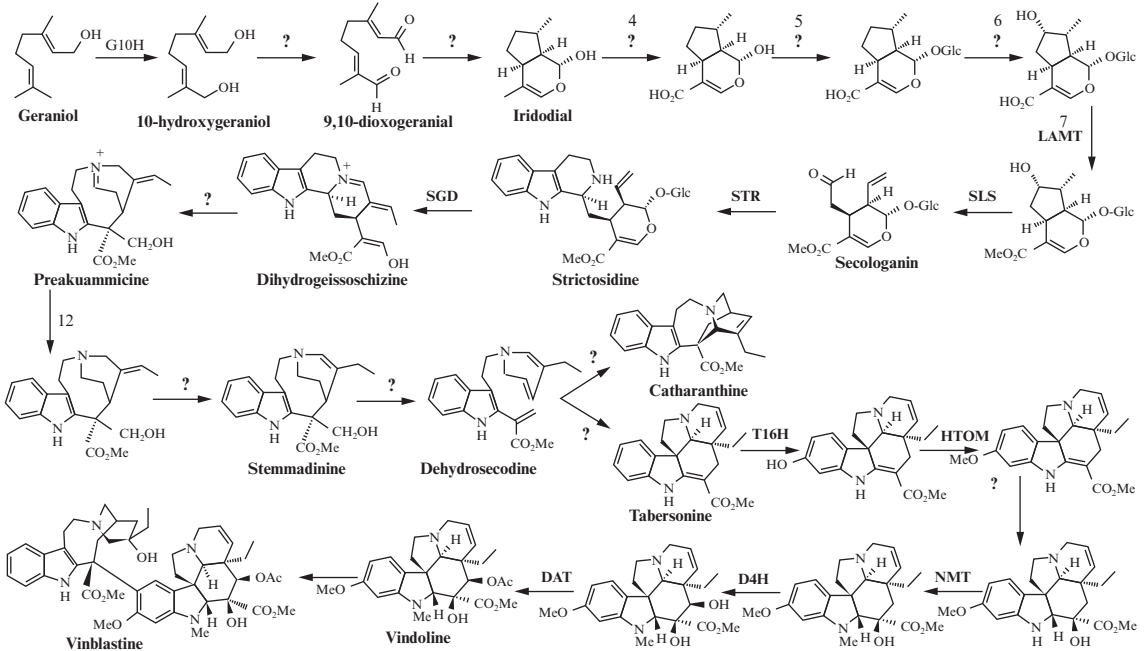

Figure 9.1 Scheme of major iridoid (secologanin) and alkaloid (vinblastine) biosynthetic pathways in *C. roseus*. Enzyme names are abbreviated as follows: G10H, geraniol 10-hydroxylase; LAMT, loganic acid methyltransferase; SLS, secologanin synthase; STR, strictosidine synthase; SGD, strictosidine glucosidase; T16H, tabersonine 16-hydroxylase; HTOM, 16-hydroxy tabersonine *O*-methyltransferase; NMT, 16-methoxy-2,3-dihydro-3-hydroxytabersonine *N*-methyltransferase; D4H, desacetoxyvindoline 4-hydroxylase; DAT, desacetylvindoline acetyltransferase.

of the iridoid-derived monoterpene indole alkaloids relies heavily on the identification and characterization of the genes and corresponding enzymes responsible for producing these compounds. The iridoid-derived monoterpene indole alkaloid biosynthetic pathways have been studied by chemical approaches, such as isotopic labeling experiments, since the middle of the twentieth century (Cordell, 1998). However, in the last decade, modern molecular biology and genetic methodologies have facilitated the identification of a number of biosynthetic genes responsible for the biosynthesis of these complex molecules (Ziegler & Facchini, 2008; Fig. 9.1). All monoterpene indole alkaloids are derived from the central precursor strictosidine, which is ultimately formed from the indole-containing tryptamine and the iridoid secologanin through the catalytic action of strictosidine synthase, which catalyzes a stereoselective Pictet–Spengler condensation between tryptamine and secologanin to yield strictosidine (Maresh et al., 2008). Tryptophan decarboxylase (TDC), a pyridoxal-dependent enzyme, generates tryptamine by decarboxylation of tryptophan (de Luca, Marineau, & Brisson, 1989; Facchini, Huber-Allanach, & Tari, 2000). A few genes involved in the biosynthesis of the iridoid secologanin, which is itself a natural product, have been characterized, though the biosynthetic pathway for this molecule has not been fully elucidated. Feeding studies indicate that secologanin is derived from the triose phosphate/pyruvate or "nonmevalonate" pathway (Contin, van der Heijden, Lefeber, & Verpoorte, 1998). Several enzymes involved in this "nonmevalonate" pathway have been cloned from *C. roseus* (Chahed et al., 2000; Veau et al., 2000). Several other genes involved in the later steps of secologanin biosynthesis, namely geraniol-10-hydroxylase (Collu, Alonso Garcia, van der Heijden, & Verpoorte, 2002; Collu et al., 2001), loganic acid methyltransferase (Murata, Roepke, Gordon, & De Luca, 2008), and secologanin synthase (Irmler et al., 2000), have been identified, but there are numerous steps that still remain to be elucidated (Ziegler & Facchini, 2008).

In *C. roseus*, one of the best-studied monoterpene indole alkaloid producers, strictosidine is deglycosylated by a dedicated β-glucosidase, converting it to a reactive hemiacetal intermediate (Geerlings, Ibanez, Memelink, Van der Heijden, & Verpoorte, 2000; Gerasimenko, Sheludko, Ma, & Stockigt, 2002). A variety of rearrangements subsequently act on deglycosylated strictosidine to yield the iboga-type alkaloid catharanthine and the aspidosperma-type vindoline precursor tabersonine (Qureshi & Scott, 1968). There is essentially nothing known about the enzymes responsible for the formation of tabersonine or catharanthine, though

more details are known about the six steps that catalyze the elaboration of tabersonine to vindoline (Liscombe, Usera, & O'Connor, 2010; Ziegler & Facchini, 2008). Vinblastine is derived from dimerization of vindoline and another terpenoid indole alkaloid, catharanthine.

Identification of these biosynthetic genes has facilitated a range of metabolic engineering strategies. However, the incompletely elucidated pathways limit this potential. In this chapter, we highlight some of the key strategies that have been developed for metabolic engineering of plant pathways. The iridoid-derived monoterpene indole alkaloids from *C. roseus*, which are the focus of this chapter, provide an excellent system for developing these strategies, because portions of the *C. roseus* monoterpene indole alkaloid pathway have been elucidated, providing an entry into metabolic engineering efforts. Additionally, *C. roseus* is a fast-growing plant susceptible to genetic transformation, thereby facilitating the development of a wide variety of plant-based engineering efforts.

2. METABOLIC ENGINEERING STRATEGIES

A number of monoterpene indole alkaloid analogs have improved or altered biological activity. For example, topotecan, a derivative of camptothecin, and vinorelbine and vinflunine, derivatives of vinblastine, are highly successful chemotherapies (Coderch, Morreale, & Gago, 2011; Cragg & Newman, 2005; Frampton & Moen, 2010; Ngan et al., 2000; Fig. 9.2). Manipulation of biosynthetic pathways is a powerful way to make unnatural natural products that are not easily accessible via total synthesis. The manipulation of pathways to make unnatural variants of natural compounds, a process often termed combinatorial biosynthesis, has been robustly successful in prokaryotic systems. The development of approaches to generate new-to-nature compounds from plant-based pathways is, in comparison, much less advanced (Pollier, Moses, & Goossens, 2011). Its success will depend on the specific chemistry of the pathway, as well as on

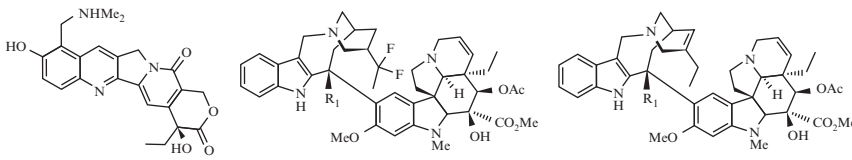

Figure 9.2 Representative analogs of monoterpene indole alkaloids used in the clinic.

the suitability of the plant system for transformation and genetic manipulation. Importantly, many metabolic engineering efforts must be performed within the native plant producer, since most plant biosynthetic pathways are incompletely elucidated at the genetic level. As pathways are elucidated, and plant-derived pathways can be heterologously expressed in hosts that are more amenable to genetic manipulation, biosynthetic production of new-to-nature compounds from plant pathways will become more widespread.

2.1. Precursor-directed biosynthesis

Precursor-directed biosynthesis describes the process in which an organism is cultivated with an analog of a natural precursor (starting substrate) for a natural product produced by that organism. The organism can take up the substrate, and incorporate it into the biosynthetic pathway, ultimately leading to the production of unnatural products derived from the unnatural substrate. This approach has been used widely in the fermentation of natural product derivatives from a variety of bacteria and fungal production hosts (Tsoi & Khosla, 1995).

2.1.1 Example of directed biosynthesis of monoterpene indole alkaloids

To assess the prospects for success of precursor-directed biosynthesis in the iridoid-derived alkaloid biosynthetic pathways, tryptamine and secologanin analogs were fed to *C. roseus* hairy root cultures (Galan, McCoy, & O'Connor, 2007; McCoy & O'Connor, 2006; Fig. 9.3). They do not produce the full complement of monoterpene indole alkaloids found in the differentiated plant; most notably, vindoline is not produced in root tissue. However, this tissue can be grown in liquid medium on a large scale, making feeding experiments practical. To assess the formation of unnatural alkaloids in *C. roseus*, hairy root culture was cultivated in liquid medium supplemented with the substrate analog (1 m*M*). Alkaloids were extracted from the roots after 3 weeks of growth and assessed by liquid chromatography–mass spectrometry (LC–MS). Comparison of cultures incubated with tryptamine and a deuterated tryptamine analog demonstrated that exogenous substrate was incorporated into many monoterpene indole alkaloids.

When hairy roots cultured in media supplemented with tryptamine analogs were analyzed, LC–MS identified alkaloid derivatives with molecular weights corresponding to the addition of a fluorine, hydroxyl, or methyl group on the indole ring of the unnatural tryptamine substrate, clearly indicating that the monoterpene indole alkaloid pathways can produce a broad range of tryptamine analogs. The intensities of the MS signals assigned to the

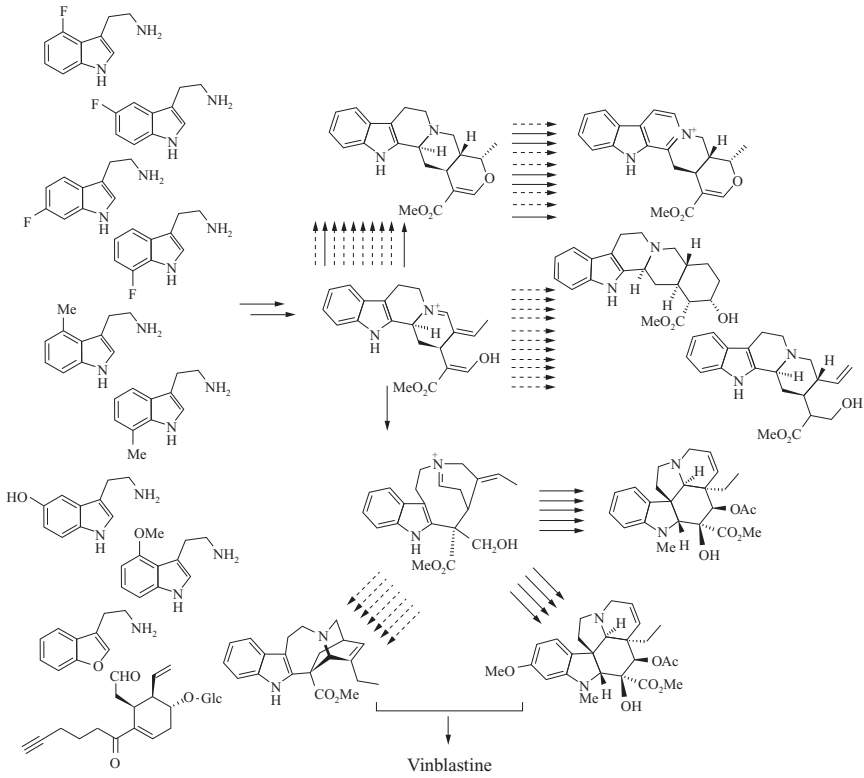

Figure 9.3 Precursor-directed biosynthesis of representative tryptamine and secologanin analogs in *C. roseus* tissues and seedlings. Solid arrows represent characterization by NMR while dashed lines indicate characterization by high-resolution mass spectrometry.

alkaloid derivatives suggest that the major products correspond to compounds derived from the unnatural starting material, with the parent (natural) alkaloids present in lower quantities for certain analogs at 1 m*M* concentration. Hairy root culture extracts could also be fractionated by preparative HPLC and several of the most abundant analogs were purified in milligram quantities and characterized by NMR. *C. roseus* seedlings could be aseptically germinated on solid medium containing 1 m*M* of the desired tryptamine analog; incorporation into the monoterpene indole alkaloid pathways was observed as evidenced by LC–MS.

Secologanin analogs derivatized at the ester moiety can be readily synthesized via a transesterification reaction of secologanin (Galan & O'Connor, 2006). They were also subjected to precursor-directed biosynthesis

studies. A secologanin pentynyl derivative was chosen for these studies since this analog contains an alkyne group that can be chemoselectively modified with an azide using Huisgen's 1,3-dipolar cycloaddition ("click chemistry") (Galan et al., 2007). This analog was incubated with *C. roseus* hairy root liquid cultures, and LC–MS analysis of the extracts suggested that the secologanin analog was incorporated into several alkaloid biosynthetic pathways, yielding compounds that could be purified from the crude plant extracts in milligram quantities. Furthermore, the alkyne moiety could be derivatized with an azide moiety via copper-catalyzed 1,3-dipolar cycloaddition. Azide biotin labeling reagent (Speers, Adam, & Cravatt, 2003) was incubated with the crude root extracts in the presence of copper sulfate and ascorbic acid, whereupon LC–MS analysis showed that the peaks corresponding to the alkaloid derivatives disappeared, and compounds exhibiting the expected 400 Da increase in mass correlating to the addition of the biotin moiety became apparent. Captavidin, a derivative of avidin that permits tight binding to biotin at low pH and dissociation of the biotin complex at basic pH, was used to purify biotinylated compounds from the crude extract. The success of this approach indicated that the alkaloids were modified as expected with biotin and that this is a viable way to isolate nonnatural metabolites from crude plant extracts.

In short, *C. roseus* can produce an array of iridoid-derived alkaloid analogs from unnatural starting materials. Furthermore, the electronic and steric properties of the nonnatural substrates impacted the way these substrates partitioned among the branches of the monoterpene indole alkaloid pathway of this medicinal plant. For example, secologanin analogs and tryptamine analogs with substituents at the 4-position, were primarily incorporated into heteryohimbine pathways; 5-substituted tryptamine analogs had an incorporation profile most similar to natural tryptamine; 6- and 7-substituted tryptamines favored incorporation into the strychnos-type alkaloids. This different partitioning among the branches of the pathway may lend insight into the mechanism or specificity of downstream enzymes.

2.1.2 *Methods for precursor-directed biosynthesis in* C. roseus *seedlings and hairy root cultures*

1. Dissolve tryptamine substrates in 0.27 N HCl (100 m*M*), sterile filter, and add to MS medium with vitamins (Sigma) (50 mL, pH = 5.7) with 2 g/L phytogel. The final concentration of analog in the medium is 1 m*M*. Sterilize *C. roseus* seeds (Horizon herbs) for 30 s in ethanol and for 10 min in 10% bleach, filter under vacuum, and wash three times

with filter-sterilized water. Transplant the seeds individually to the square-grid Petri dish (36 seeds per plate) containing MS medium with the indicated analog. Grow seedlings in the dark for 7 days and then under incandescent light with 16 h light and 8 h dark for 10 days, yielding approximately 1 g of seedlings per plate. In our study, no growth inhibition was seen for analog compared to the control plates.

2. Subculture *C. roseus* hairy root cultures in 25 mL of half-strength Gamborg's medium and vitamins (pH = 5.7) and grow for 7 days at 26 °C shaking at 50 rpm. Dissolve tryptamine analogs in DMSO and add to the medium to a final concentration of 1 m*M* on day 7. Culture flasks of roots (25 mL) for 14 more days. Add autoclaved water to the cultures weekly to compensate for evaporation.
3. To extract alkaloids, grind 1 g of fresh seedlings or roots in a mortar and pestle in methanol (3 × 30 mL). Filter the methanol extract and concentrate under vacuum to yield a yellow solid; sonicate for 30 min in 3% aqueous HCl and then extract with hexanes (3 × 30 mL) to remove hydrophobic material. Adjust the solution to pH 8 with NH_4OH and then extract with methylene chloride (3 × 30 mL). Concentrate the combined methylene chloride fractions to yield a yellow alkaloid extract (2–10 mg) and dissolve it in methanol (1 mL) for further analysis by LC–MS.
4. For LC–MS analysis, dilute the extracts 1/1000 with methanol. Analysis of alkaloids is best performed by ESI. As an example, samples were injected on a Micromass LCT Premier TOF Mass Spectrometer equipped with an Acquity Ultra Performance BEH C18, 1.7 μm, 2.1 × 100 mm column using a gradient of 10–60% acetonitrile/water 0.1% formic acid over 20 min at a flow rate of 0.25 mL/min. The capillary and sample cone voltages were 2000 and 30 V, respectively. The desolvation and source temperatures were 350 and 100 °C. The cone and desolvation gas flow rates were 20 and 700 L/h.
5. To purify individual alkaloids, load extracts onto a 10 × 20 mm Vydac reverse-phase column using a gradient of 20–50% acetonitrile/water (0.1% TFA or 0.1% formic acid) over 30 min. Monitor the mixture at 254 nm and combine and concentrate fractions containing the alkaloid analogs of interest.

2.2. Mutasynthesis

In precursor-directed biosynthesis, the producer organism is supplemented with analogs of the naturally occurring starting materials. These nonnatural

starting materials are, in turn, converted into the corresponding unnatural products. However, the yield and purity of these unnatural products is improved if the biosynthesis of the natural starting material is genetically blocked, and the producing organism is forced to utilize exogenously supplied precursors exclusively for product biosynthesis. This strategy, termed mutasynthesis, was first applied several decades ago to yield novel antibiotics in the soil bacterium *Streptomyces* (Shier, Rinehart, & Gottlieb, 1969) and has proven to be highly successful in microbial systems (Weissman, 2007).

2.2.1 Example of mutasynthesis for monoterpene indole alkaloids

Mutasynthesis can also be applied to the monoterpene indole alkaloids in *C. roseus* (Runguphan, Maresh, & O'Connor, 2009). Tryptamine, the starting substrate for all monoterpene indole alkaloids (O'Connor & Maresh, 2006; van der Heijden et al., 2004), is produced from tryptophan by TDC (de Luca et al., 1989; Facchini et al., 2000). If tryptamine biosynthesis is blocked, alkaloid biosynthesis could, in principle, be rescued by introducing exogenous tryptamine or tryptamine analogs to plant cell cultures. TDC was targeted for gene silencing (RNAi) to prevent formation of tryptamine. The plasmid designed to suppress TDC was introduced into *Agrobacterium rhizogenes*, which was then used to infect *C. roseus* seedlings to generate hairy root cultures (Hughes, Hong, Shanks, San, & Gibson, 2002; Runguphan et al., 2009). Hairy root lines harboring the silencing plasmid were cultured in liquid medium, where production of all major tryptamine-derived alkaloids was substantially decreased in the five representative silenced lines examined. RT-PCR indicated that the expression levels of TDC were substantially reduced in cultures harboring the silencing plasmid.

Alkaloid biosynthesis could be rescued by feeding tryptamine or isotopically labeled d_4-(deuterium) tryptamine to silenced cultures. A hairy root line was incubated with varying concentrations of tryptamine (62.5–2500 μM). The production levels of monoterpene indole alkaloids generally increased as more tryptamine was added. Notably, growth inhibition and browning of wild-type hairy roots were typically observed when exogenous tryptamine was added to the medium of wild-type cultures at concentrations of 1000 μM or greater, but this was not observed in the silenced lines where natural tryptamine production was suppressed.

The tryptamine analog 5-fluorotryptamine was chosen to illustrate the potential of mutasynthesis for the monoterpene indole class of alkaloids. A silenced hairy root culture was incubated with varying concentrations (250–2500 μM) of 5-fluorotryptamine. The complex mixture of alkaloid

products was greatly simplified, since no natural alkaloids derived from endogenous tryptamine were present (Fig. 9.4). The levels of certain fluorinated alkaloids (ajmalicine and catharanthine) were greater in the silenced culture, compared to precursor-directed biosynthesis with a wild-type hairy root line, suggesting that some pathway branches could support increased production levels of the desired nonnatural compounds when not challenged with competing natural substrates.

Producing a wide variety of natural product-based compounds by fermentation is an attractive way to obtain a diversity of natural product-inspired molecules. We envision that RNA silencing methods can also be used with additional unknown downstream biosynthetic enzymes, as they

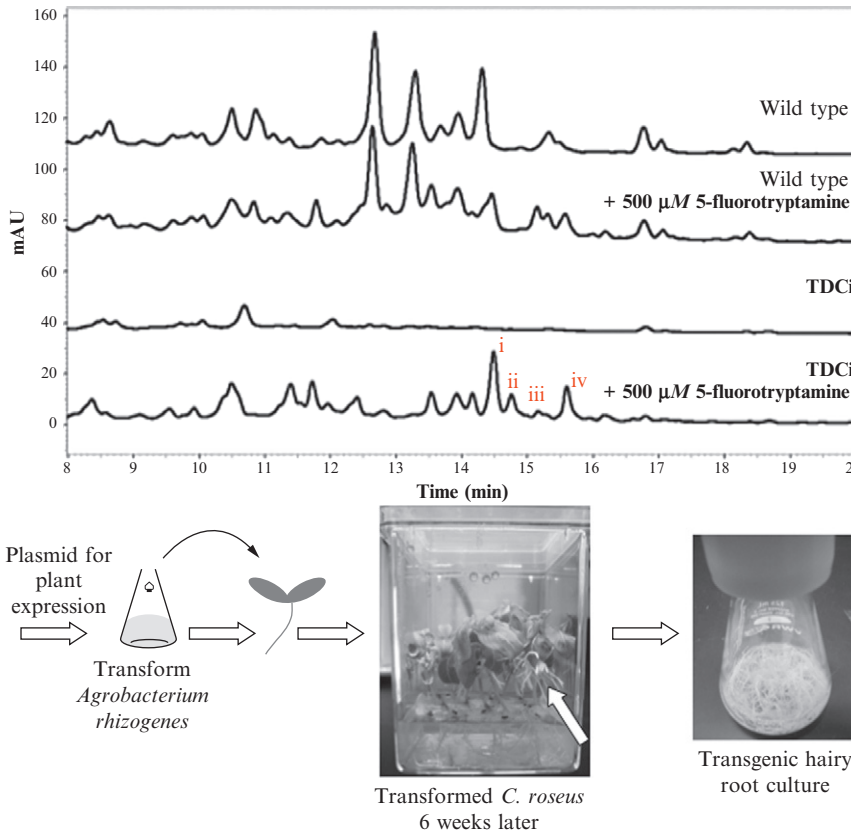

Figure 9.4 Metabolite production in wild type and tryptophan decarboxylase silenced line as evidenced by semipreparative HPLC analysis of *C. roseus* hairy root extracts. Alkaloids were monitored at 280 nm: (i) fluoro-ajmalicine; (ii) fluoro-catharanthine; (iii) fluoro-tabersonine; and iv, fluoro-serpentine.

become identified, to more effectively tailor the production of the desired unnatural alkaloid in plants and plant culture.

2.2.2 Methods for mutasynthesis in C. roseus

1. To construct the vector for silencing, place a 411-base pair fragment from the 5′ end of the TDC gene, flanked by *att*B1and *att*B2 sites, into pDONR201 (Invitrogen) vector via BP clonase-mediated recombination using the manufacturer's protocol. Then insert the gene fragment from the intermediate clone into pHELLSGATE12 (Helliwell & Waterhouse, 2003) using LR clonase-mediated recombination.
2. Introduce the silencing vector into *A. rhizogenes* ATCC 15834 via electroporation (1 mm cuvette, 1.25 kV). Germinate *C. roseus* seedlings (250–300) (Vinca Little Bright Eyes, Nature Hills Nursery) aseptically on standard Gamborg's B5 medium (full strength basal salts, full strength vitamins, 30 g/L sucrose, pH 5.7) and grow in a 16-h light/dark cycle for 2–3 weeks. Wound seedlings with a scalpel at the stem tip, and inoculate transformed *A. rhizogenes* from a freshly grown liquid culture on the wound. Hairy roots will appear at the wound site 2–3 weeks after infection. Excise root tips longer than 5 mm after 6 weeks and transfer to Gamborg's B5 solid medium (half-strength basal salts, full strength vitamins, 30 g/L sucrose, 6 g/L agar, pH 5.7) containing kanamycin (0.1 mg/mL), for selection, and cefotaxime (0.25 mg/mL), to remove the remaining bacteria. After the selection process, subculture hairy roots at least once on solid medium that lacks both kanamycin and cefotaxime. To adapt hairy roots to liquid medium, transfer approximately 200 mg of hairy roots (typically five 3–4-cm-long stem tips) from each line that grew successfully on solid medium to 50 mL of half-strength Gamborg's B5 liquid medium (half-strength basal salts, full strength vitamins, 30 g/L sucrose, pH 5.7). Grow the cultures in the dark at 26 °C at 125 rpm. Lines should be maintained on a 14–21 day subculture cycle, depending on the growth rate of each line.
3. To quantify mRNA levels of biosynthetic genes by real-time RT-PCR, isolate mRNA from transformed hairy roots from genomic DNA using the Qiagen RNeasy Plant Mini Kit and RNase-free DNaseI, respectively. Then transcribe the resulting mRNA to cDNA using Qiagen QuantiTect Reverse transcription Kit. Analyze this cDNA by PCR with the Qiagen SYBR Green PCR kit and a qPCR system. (We used a Biorad DNA Engine Opticon 2 system. The threshold-cycle (C_T) was determined as the cycle with a signal higher than that of the background

plus 10 × standard deviation (SD).) To normalize the amount of total mRNA in all samples *C. roseus* 40S ribosomal protein S9 (Rps9), a housekeeping gene, should be used.

4. To assess alkaloid production rescue by addition of tryptamine or tryptamine analogs, subculture 10 root tips from hairy roots transformed with the silencing plasmid in 50 mL Gamborg's B5 liquid medium and grow at 26 °C in the dark at 125 rpm for 18 days. Then supplement the medium with tryptamine analogs at 0, 62.5, 125, 250, 500, 1000, and 2500 μ*M*. After 1 week of cocultivation with the substrate, grind hairy roots in a mortar and pestle with 100 μm glass beads in methanol (10 mL/g of fresh weight hairy roots). Analyze the crude natural product mixtures using ESI LC–MS. As a representative example, we used a Micromass LCT Premier TOF Mass Spectrometer equipped with an Acquity Ultra Performance BEH C18, 1.7 μm, 2.1 × 100 mm column, using a gradient of 10–60% acetonitrile/water (0.1% TFA) over 13 min at a flow rate of 0.6 mL/min. The capillary and sample cone voltages were 1300 and 60 V, respectively. The desolvation and source temperatures were 300 and 100 °C. The cone and desolvation gas flow rates were 60 and 800 L/h. To convert peak area to milligrams, generate standard curves of the natural alkaloids.
5. To purify alkaloids from silenced hairy root cultures, use semipreparative HPLC equipped with a Hibar RT 250-4 prepacked reverse-phase column, and a gradient of 10–60% acetonitrile/water (0.1% TFA) over 25 min at 1 mL/min flow rate. Monitor alkaloids at 280 nm, collect the fractions, and then pool and concentrate.

2.3. Incorporation of engineered pathway enzymes in biosynthesis

Precursor-directed biosynthesis and mutasynthesis rely on the inherent ability of biosynthetic enzymes to accept nonnatural substrates with altered steric and electronic properties. Many biosynthetic genes, however, have tight substrate specificity and will not turn over the desired unnatural substrate analog. In these cases, alternative biosynthetic genes with altered substrate specificity can be incorporated into existing natural product pathways, enabling the production of unnatural compounds from nonnatural starting substrates. This approach has been applied to many microbial systems, but again, the application of this general strategy to plant-derived natural product pathways has been limited.

2.3.1 Example of incorporation of an engineered pathway enzyme in C. roseus

Precursor-directed biosynthesis studies in *C. roseus* have revealed that one key bottleneck in the production of unnatural monoterpene indole alkaloids in this plant is the stringent substrate specificity of strictosidine synthase, the enzyme that catalyzes formation of the biosynthetic intermediate strictosidine from tryptamine and the iridoid secologanin (Fig. 9.5). Notably, tryptamine analogs with substituents at the 5-position of the indole ring are not accepted by this enzyme. The recently reported crystal structure of strictosidine synthase (Ma, Panjikar, Koepke, Loris, & Stockigt, 2006) has enabled the design of enzyme mutants with broadened substrate specificities, allowing enzymatic production of a greater variety of strictosidine analogs (Bernhardt, McCoy, & O'Connor, 2007; Loris et al., 2007). Specifically, introduction of the point mutation Val214Met into strictosidine synthase (*C. roseus*) allows turnover of a variety of 5-subsituted tryptamine analogs in the corresponding strictosidine products.

The strictosidine synthase mutant gene containing the point mutation Val214Met was introduced into hairy root culture under the control of the strong, constitutive 35S CMV promoter (Runguphan & O'Connor, 2009). Transgenic hairy root lines harboring the Val214Met mutant enzyme were cultured in the presence of 5-chlorotryptamine, 5-methyltryptamine, or 5-bromotryptamine, which are only turned over by the Val214Met mutant enzyme, and are not recognized by natural strictosidine synthase (Bernhardt et al., 2007). After 1 week of culture, LC–MS analysis of the extracts of this plant tissue indicated the appearance of novel compounds derived from the exogenous substrates. Control experiments clearly indicated that these compounds were not present

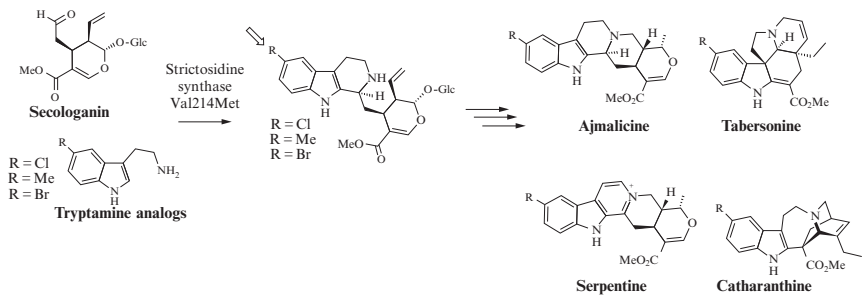

Figure 9.5 Reaction of secologanin and 5-substituted tryptamine analogs with strictosidine synthase Val214Met mutant to generate strictosidine and alkaloid analogs.

when the tryptamine analog was absent from the medium, or when *C. roseus* tissue was transformed with the wild-type strictosidine synthase gene.

Fermentation is a powerful strategy for large-scale production of natural product analogs. This example demonstrates that genetically reprogramming alkaloid metabolism can be achieved in medicinal plant cell culture, even when the genetic, biochemical and regulatory aspects of the pathway are incompletely characterized. Optimistically, this study sets the stage for further metabolic engineering efforts to improve the scope and practicality of unnatural product biosynthesis in plants.

2.3.2 Methods for incorporation of an engineered pathway enzyme in C. roseus

1. Isolate mRNA from *C. roseus* hairy root culture (Qiagen, Rneasy kit), and use reverse transcription PCR to amplify the native strictosidine synthase gene (*C. roseus*) with the complete signal sequence for correct localization. Use site-directed mutagenesis to introduce the Val214Met mutation using overlapping mutagenic primers (Stratagene, Quikchange). To create the plasmid for constitutive STR mutant expression, ligate the mutant STR gene into the *Nco*I/*Bst*EII site downstream of the CaMV 35S promoter in the pCAMBIA 1305.1 vector (http://www.cambia.org/daisy/cambia/585).
2. Introduce the pCAMBIA construct containing the Val214Met mutant strictosidine synthase gene (as well as an identical construct containing the wild-type strictosidine synthase gene as a negative control) into *A. rhizogenes* ATCC 15834 via electroporation (1 mm cuvette, 1.25 kV). Transform *C. roseus* seedlings with the generated *Agrobacterium* strains and generate transformed hairy roots as described in Section 2.2.2.
3. Add tryptamine analogs to the transformed hairy roots in liquid culture toward the end of the log phase and the beginning of the stationary phase (usually after 3 weeks). After 1 week of cocultivation with the substrate, grind the hairy roots in a mortar and pestle with 106 μm acid washed glass beads in methanol (10 mL/g of fresh weight hairy roots). Concentrate the crude natural product mixtures under vacuum and redissolve the dried mixture in 20% acetonitrile/water (0.1% TFA) (1 mL/g of fresh weight hairy roots). Filter through a 0.2 μ filter, and analyze this mixture by LC–MS as described earlier in Section 2.2.2.

3. CONCLUSIONS AND FUTURE DIRECTIONS

Engineering of metabolic pathways in plants poses significant challenges when compared to the extraordinary advances that have been achieved in prokaryotic systems. Nevertheless, plant metabolic engineering has made substantial strides over the last decade, and the work on the iridoid-derived monoterpene indole alkaloid systems has comprised a large part of these efforts. Moreover, the increasing speed at which plant pathways are being elucidated bodes well for the future of plant-derived natural products. As genomic technologies have improved, the genetic basis of the natural product chemistry catalyzed within medicinal plants has slowly—but surely—begun to emerge. This genetic information allows detailed mechanistic explorations of plant pathways, and also enables metabolic engineering efforts that could improve production of medically important plant natural products. This chapter highlights a number of methods that have been used to engineer plant biosynthetic pathways, with an emphasis on the well-studied monoterpene indole alkaloids of *C. roseus*. Finally, as heterologous reconstitution of plant pathways into tractable host organism such as tobacco or yeast become more routine, this will further enhance our ability to hijack the iridoid and downstream alkaloid pathways.

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