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Silencing of tryptamine biosynthesis for production of nonnatural alkaloids in plant culture

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Natural products have long served as both a source and inspiration for pharmaceuticals. Modifying the structure of a natural product often improves the biological activity of the compound. Metabolic engineering strategies to ferment “unnatural” products have been enormously successful in microbial organisms. However, despite the importance of plant derived natural products, metabolic engineering strategies to yield unnatural products from complex, lengthy plant pathways have not been widely explored. Here, we show that RNA mediated suppression of tryptamine biosynthesis in Catharanthus roseus hairy root culture eliminates all production of monoterpene indole alkaloids, a class of natural products derived from two starting substrates, tryptamine and secolloganin. To exploit this chemically silent background, we introduced an unnatural tryptamine analog to the production media and demonstrated that the silenced plant culture could produce a variety of novel products derived from this unnatural starting substrate. The novel alkaloids were not contaminated by the presence of the natural alkaloids normally present in C. roseus. Suppression of tryptamine biosynthesis therefore did not appear to adversely affect expression of downstream biosynthetic enzymes. Targeted suppression of substrate biosynthesis therefore appears to be a viable strategy for programming a plant alkaloid pathway to more effectively produce desirable unnatural products. Moreover, although tryptamine is widely found among plants, this silenced line demonstrates that tryptamine does not play an essential role in growth or development in C. roseus root culture. Silencing the biosynthesis of an early starting substrate enhances our ability to harness the rich diversity of plant based natural products.

Genetic manipulation of natural product biosynthetic pathways has emerged as a powerful strategy to rapidly access complex novel molecules (1). In one approach, the producer organism is supplemented with analogs of the naturally occurring starting materials where, ideally, these nonnatural starting materials are converted by the enzymes of the biosynthetic pathway into the corresponding unnatural products (2). The yields and purity of these unnatural products is typically improved if the biosynthesis of the natural starting material is genetically blocked, and the producing organism is forced to use exogenously supplied precursors for product biosynthesis. This strategy, termed mutasynthesis, was first applied several decades ago to yield novel antibiotics in the soil bacterium Streptomyces fradiae (3). Although mutasynthesis has proven to be highly successful in microbial systems (4, 5), the strategy has not been applied in more complex eukaryotes, notably plants. Here, we describe mutasynthesis of the monoterpene indole alkaloids in the medicinal plant Madagascar periwinkle (Catharanthus roseus).

Tryptamine 1 is the starting substrate for hundreds of monoterpene indole alkaloids produced by C. roseus (Fig. 1) (6, 7). This substrate is produced enzymatically from tryptophan 2 by tryptophan decarboxylase, an enzyme that serves as a key link between primary metabolism and natural product metabolism (8, 9). If tryptamine biosynthesis was blocked, alkaloid biosynthesis could be rescued by introducing exogenous tryptamine or tryptamine analogs to plant cell culture (Fig. 1). Precursor directed biosynthesis with wild-type C. roseus plants and tissue culture has already demonstrated that tryptamine analogs can be taken up and processed by the plant into a wide variety of alkaloids (10, 11). However, if tryptamine 1 is required for plant growth or survival, silenced lines would not be viable. No studies of down-regulating or suppressing tryptamine biosynthesis in plants have been described, and little information is available in the literature to support a definitive role for tryptamine, a metabolite that is found throughout the plant kingdom (12). Overproduction of tryptamine has been shown to deter insect feeding (13), but this role is nonessential in the controlled environment of plant cell culture. Additionally, 5-hydroxytryptamine (serotonin), which may be produced by hydroxylation of tryptamine, has been implicated in certain physiological roles such as flower development (14). Notably, tryptamine may also be involved in auxin biosynthesis (15).

Despite the uncertainty concerning the function of tryptamine in plants, suppression of tryptophan decarboxylase nevertheless provided an attractive entry point for introduction of new substrate analogs. In prokaryotic pathways, the gene of interest is usually deleted or “knocked out” using homologous recombination based strategies. However, although some success has been achieved with homologous recombination in higher plants (16), strategies to down-regulate or knockdown specific gene expression levels using RNA silencing are extremely well established for plants and these lines can be constructed on a relatively short time scale (~6–9 months) (17–19).

Here, we successfully demonstrate RNA silencing of tryptophan decarboxylase to suppress the biosynthesis of tryptamine in C. roseus culture. Alkaloid production almost completely disappeared in silenced cultures and could be rescued by the addition of exogenous tryptamine to the culture medium. A representative unnatural tryptamine analog, 5-fluorotryptamine 1a, was also incorporated into the pathway to yield several unnatural, fluorinated alkaloids. Disruption of this early biosynthetic gene did not impact the expression of known downstream alkaloid biosynthetic enzymes, indicating that changes can be made in the early part of this pathway without adversely affecting the alkaloid biosynthetic machinery. Moreover, these experiments demonstrate that tryptamine and monoterpene indole alkaloids do not play an essential role in growth or development in C. roseus hairy root culture.

Results

Alkaloid Production in Tryptophan Decarboxylase Silenced Lines. Tryptophan decarboxylase, a pyridoxal phosphate dependent enzyme that produces tryptamine 1 from tryptophan 2 (8, 9), was

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targeted for gene silencing (19–22) (Fig. 1). The plasmid designed to suppress tryptophan decarboxylase (pTDCi) was transformed into *Agrobacterium rhizogenes*, which was then used to infect *C. roseus* seedlings to generate hairy root culture as described in refs. 23 and 24. Hairy root lines harboring the pTDCi silencing plasmid were cultured in liquid media, and alkaloids were extracted from the plant tissue according to standard protocols. Gratifyingly, production of all major tryptamine derived alkaloids ajmalicine (1), serpentine (4), catharanthine (5), and tabersonine (6) was substantially decreased in the five silenced lines that were examined (Fig. 2A and SI). Suppression of alkaloid production appears to be stable, with little change in alkaloid production levels after seven subcultures performed thus far.

**Gene Expression in Silenced Lines.** Real time reverse transcription PCR confirmed that the tryptophan decarboxylase gene was significantly down regulated at the mRNA level in two representative lines harboring the pTDCi silencing plasmid (Fig. 3). Tryptophan decarboxylase is regulated by the transcription factor Orca3 (25), which also controls a number of other alkaloid biosynthetic genes including strictosidine synthase (26) and strictosidine glucosidase (27), enzymes that act immediately after tryptophan decarboxylase in the alkaloid biosynthetic pathway. The mRNA levels of strictosidine synthase, strictosidine glucosidase and Orca3 did not appear to be affected, indicating that the tryptophan decarboxylase gene could be silenced without perturbing the downstream pathway (Fig. 3). In contrast, down-regulation of a late stage morphine biosynthetic gene had an unpredictable effect on morphine biosynthesis, suggesting that suppression of a single metabolic enzyme can sometimes affect other pathway enzymes (28–30). Finally, the gene encoding the alpha subunit of antranilate synthase (31), an enzyme involved in tryptophan biosynthesis, showed modest decreases in expression levels in the silenced lines (Fig. 3). Increased concentrations of tryptophan inhibit antranilate synthase expression (32). Therefore, in the silenced lines, where tryptophan is no longer diverted to tryptamine biosynthesis, concentrations of tryptophan could increase, resulting in the decreased expression of antranilate synthase. A full transcriptional analysis of the monoterpene indole alkaloid pathway was not performed because the majority of the pathway enzymes are not known at the genetic level.

**Unnatural Alkaloid Production in Suppressed Lines.** Alkaloid biosynthesis could be rescued by feeding tryptamine or isotopically labeled d4-(deuterium) tryptamine to silenced cultures. We measured formation of the major alkaloids of hairy root culture, namely ajmalicine (3), serpentine (4), catharanthine (5) and tabersonine (6) (Fig. 1). The identity of these alkaloids was assigned by exact mass and coelution with authentic standards (SI). Isotopically labeled d5-L-tryptophan (500 μM) was also incubated with a wild-type and a representative silenced line. As expected, the wild-type culture could incorporate the exogenous isotopically labeled tryptophan into alkaloids whereas the suppressed line could not, clearly indicating that the required decarboxylation reaction did not occur when tryptophan decarboxylase was knocked down (SI).

A hairy root line that showed maximal suppression of alkaloid production was incubated with varying concentrations of tryptophan, and increased concentrations of tryptophan could increase, resulting in the decreased expression of antranilate synthase. A full transcriptional analysis of the monoterpene indole alkaloid pathway was not performed because the majority of the pathway enzymes are not known at the genetic level.
The production of ajmalicine was increased as more tryptamine was added to the tandem with high performance liquid chromatography. The representative silenced line. (Alkaloid production monitored in the total ion count of a wild-type and a representative silenced line. An authentic standard of secolaginan is also shown.

The production of ajmalicine, serpentine, catharanthine, and tabersonine were monitored by mass spectrometry in tandem with high performance liquid chromatography. The production levels increased as more tryptamine was added to the cell culture media, although catharanthine and tabersonine production was highest at 1,000 µM tryptamine. Notably, growth inhibition and browning of wild-type hairy roots was typically observed when exogenous tryptamine was added to the media. 

These suppressed C. roseus lines provided the first opportunity to explore the prospects of mutasynthesis—replacement of natural alkaloids with unnatural products—in plant culture. A representative tryptamine analog, 5-fluorotryptamine, has been shown in precursor directed feeding studies to be turned over by downstream biosynthetic enzymes to yield a variety of fluorinated monoterpene indole alkaloid analogs. A representative silenced line was incubated with varying concentrations of 5-fluorotryptamine. Two representative fluorinated alkaloids, fluoro-ajmalicine and fluoro-serpentine were identified by exact mass, UV spectrum, and by coelution with authentic standards that were characterized by NMR (10) (Fig. 5). When supplemented with natural tryptamine, silenced hairy root cultures produced catharanthine and tabersonine as the only observable compounds with m/z 337. When supplemented with 5-fluorotryptamine, silenced lines yielded two compounds at m/z (337 + 18), the expected molecular weight of fluoro-catharanthine (expected, 355.1816; observed, 355.1818) and fluoro-tabersonine (expected, 355.1816; observed, 355.1811). UV spectra of these two compounds matched authentic standards of catharanthine and tabersonine in the representative silenced line. The proportions of fluorinated alkaloids produced with the wild type were significantly greater than fluorinated ajmalicine, whereas catharanthine or tabersonine was not observed at all in the wild-type hairy root line for reasons that are not clear (24). Natural

The levels of natural (nonfluorinated) ajmalicine, serpentine, catharanthine, and tabersonine alkaloids were also quantified in wild-type cultures fed with 5-fluorotryptamine. The proportions of fluorinated alkaloids produced in the silenced cultures are different from the proportions of fluorinated alkaloids produced with the wild lines. As the genes involved in the biosynthesis of plagiolepidine glucosidase and Orca3. Levels of these genes are normalized to 100% in the wild-type line. The gene expression levels of two representative silenced lines relative to the wild-type levels are also shown. Levels represent the average of three trials, with error bars representing the standard deviation.

Fig. 2. Metabolite production in wild-type and tryptophan decarboxylase suppressed lines as evidenced by LC-MS analysis of C. roseus extracts. (A) Alkaloid production monitored in the total ion count of a wild-type and a representative silenced line. (B) Secologanin standard (m/z 389) production in wild-type and a representative silenced line. An authentic standard of secolaginan is also shown.

Fig. 3. Real time reverse transcriptase PCR of the mRNA encoding tryptophan decarboxylase, anthranilate synthase, strictosidine synthase, strictosidine glucosidase and Orca3. Levels of these genes were normalized to 100% in the wild-type line. The gene expression levels of two representative silenced lines relative to the wild-type levels are also shown. Levels represent the average of three trials, with error bars representing the standard deviation.

The complex mixture of alkaloid products was greatly simplified, because no natural alkaloids derived from endogenous tryptamine were present (SI). We also compared the yields of unnatural, fluorinated alkaloids in both wild-type and a representative silenced line fed with 5-fluorotryptamine. The levels of fluorinated ajmalicine and the compound assigned as catharanthine were greater in the representative silenced line, suggesting that some pathway branches could support increased production levels of the desired unnatural compounds when not challenged with competing natural substrate. Yields of fluorinated serpentine remained approximately the same in silenced and wild-type cultures, suggesting that serpentine biosynthesis may be tightly regulated. Surprisingly, the yield of the compound assigned as fluorinated tabersonine was approximately twofold higher in the wild-type line compared with the silenced line, suggesting that tabersonine biosynthesis was somewhat adversely affected in the wild-type line compared with the silenced line, suggesting that some pathway branches could support increased production levels of the desired unnatural compounds when not challenged with competing natural substrate. Yields of fluorinated serpentine remained approximately the same in silenced and wild-type cultures, suggesting that serpentine biosynthesis may be tightly regulated. Surprisingly, the yield of the compound assigned as fluorinated tabersonine was approximately twofold higher in the wild-type line compared with the silenced line, suggesting that tabersonine biosynthesis was somewhat adversely affected by suppression of tryptophan decarboxylase or the transformation process (Fig. 6A and B). The levels of fluorinated alkaloids produced in the silenced cultures are different from the proportions of fluorinated alkaloids produced with the wild lines. As the genes involved in the biosynthesis of strictosidine glucosidase and Orca3. Levels of these genes were normalized to 100% in the wild-type line. The gene expression levels of two representative silenced lines relative to the wild-type levels are also shown. Levels represent the average of three trials, with error bars representing the standard deviation.
serpentine 4 was produced at approximately the same level as fluorinated serpentine 4a in the wild-type line. At high concentrations of 5-fluorotryptamine 1a, levels of natural tabersonine 6 and the compound assigned as fluorinated tabersonine 6a were similar.

Finally, yields of secologanin 7 were estimated by mass spectrometry analysis of crude C. roseus extracts. Although secologanin 7, which reacts with tryptamine to form monoterpenoid alkaloids, is not normally found in large quantities in wild-type C. roseus plants or tissue, silenced lines showed a dramatic increase in 7 (Fig. 2B). ~0.15 g of secologanin per g dry weight in a representative silenced culture was observed, indicating that silencing of tryptophan decarboxylase led to dramatically increased secologanin 7 levels. When tryptamine 1 or a tryptamine analog was added to this silenced culture, the levels of secologanin decreased as this iridoid terpene was converted into alkaloids. Nevertheless, 0.11 g of secologanin 7 per g culture dry weight remained even when 500 μM tryptamine 1 was incubated with this silenced culture for 7 days. The levels of secologanin 7 in these silenced lines appeared to far exceed the capacity for downstream alkaloid biosynthesis (~75 mg of monoterpene indole alkaloid per gram of dry culture weight) (Fig. 4 and 6).

Although most of the genes involved in secologanin biosynthesis remain uncharacterized, real time reverse transcription PCR indicated that one or both of the two best characterized secologanin biosynthetic genes, secologanin synthase (SLS) (33, 34) and geraniol-10-hydroxylase (G10H) (35), were up-regulated in seven silenced lines (SI). As stated above, high concentrations of secologanin (0.11 g/g dry weight) were observed even after tryptamine was added to silenced lines, suggesting that the tryptamine metabolite does not repress secologanin production. Therefore, although the exact mechanism of how secologanin is up-regulated remains unclear, silencing of tryptophan decarboxylase clearly affects the expression of upstream biosynthetic enzymes. This observation sets the stage for further work to explore the complex regulatory network of alkaloid biosynthetic genes in C. roseus.

**Discussion**

Here, we show that down-regulation of tryptophan decarboxylase in C. roseus hairy root culture allowed the strategic incorporation of exogenously supplied tryptamine substrate 1 into the monoterpene indole alkaloid pathway. Using this strategy, natural alkaloid production was suppressed, and only unnatural alkaloids were produced when a fluorinated tryptamine analog 1a was introduced to the culture media. Silencing tryptophan decarboxylase did not impact the expression levels of either the key transcription factor Orca3 or the known downstream biosynthetic enzymes, strictosidine synthase and strictosidine glucosidase.

Secologanin 7, the partner starting material that normally reacts with tryptamine 1 (Fig. 1), accumulates in large quantities in silenced lines, whereas wild-type lines show negligible secologanin accumulation (Fig. 2B). Notably, although tryptophan decarboxylase silenced C. roseus lines produce >100 mg of...
rotyptamine nonfluorinated alkaloids produced in a wild-type line incubated with 5-fluorotryptamine. Moreover, the yields of certain alkaloids were improved; for example, when 5-fluorotryptamine were wounded with a scalpel at the stem tip, and transformed seedlings with the generated att B2 (reverse) was designed to amplify the complete STR gene, a region of the CaMV 35S promoter, the antibiotic resistance NPTII gene and the PDK intron (SI). The genomic DNA from transformed hairy roots was isolated (Qiagen DNeasy kit) and subjected to PCR amplification using T-DNA specific primers with STR primer: AGCTATCGATGGTACCTCATTTCTAATTCGGTGGCGGCTG). The resulting PCR product was then inserted into pENTR-TOPO (Invitrogen) vector via BP clonase mediated recombination using the manufacturer’s protocol. The gene fragment from the intermediate clone was subsequently inserted into pHELLSGATE12 using LR clonase mediated recombination. The final pTDCi construct was verified by sequencing to ensure plasmid integrity.

Generation of pTDCi Hairy Roots. The pTDCi vector was transformed into A. rhizogenes ATCC 15834 via electroporation (1-mm cuvette, 1.25 kV). Transformation of C. roseus seedlings with the generated Agrobacterium strain was performed as reported in ref. 23. Briefly, 250–300 C. roseus seeds (Vinca Little Bright Eyes, Nature Hills Nursery) were germinated aseptically and grown in a 16-hour light/dark cycle for 2–3 weeks on standard Gamborg’s B5 media (full strength basal salts, full strength vitamins, 30 g/L sucrose, pH 5.7). Seedlings were wounded with a scalpel at the stem tip, and transformed A. rhizogenes from a freshly grown liquid culture was inoculated on the wound. Hairy roots appeared at the wound site 2–3 weeks after infection. Root tips >5 mm were excised after 6 weeks and transferred to Gamborg’s B5 solid media (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) for removal of the remaining bacteria. Lines were cultured on media with and without tryptamine (50 μM), in case tryptamine proved to be essential for cell survival. Because tryptamine did not appear to be required for survival of these lines, all subsequent culture media used for second and third rounds of subculturing was not supplemented with tryptamine. All cultures were grown in the dark at 26 °C. After the selection process, hairy roots were subcultured at least once on solid media lacking both kanamycin and cefotaxime before adaptation to liquid culture.

To adapt hairy roots to liquid media, ~200 mg of hairy roots (typically five 3–4 cm long stem tips) from each line that grew successfully on solid media were transferred to 50 mL of half-strength Gamborg’s B5 liquid media (half strength basal salts, full strength vitamins, 30 g/L sucrose, pH 5.7). The cultures were grown at 26 °C in the dark at 125 rpm. All lines were maintained on a 14–21 day subculture cycle depending on the growth rate of each line.

Verification of Transferred DNA (T-DNA) Integration by Genomic DNA Analysis. The genomic DNA from transformed hairy roots was isolated (Qiagen DNAeasy kit) and subjected to PCR amplification using T-DNA specific primers with STR primers serving as a positive control. Specifically, primers for PCR amplification were designed to amplify the complete STR gene, a region of the CaMV 35S promoter, the antibiotic resistance NPTII gene and the PDK intron (SI).

Quantification of mRNA Production of Biosynthetic Genes by Real Time RT-PCR. mRNA from transformed hairy roots was isolated and purified from genomic DNA using Qiagen RNeasy Plant Mini Kit and RNase-free DNasel, respectively.
The resulting mRNA was then reverse-transcribed to cDNA using Qiagen QuantiTect Reverse transcription kit and subjected to PCR analysis with Qiagen SYBR Green PCR kit and a Bio-Rad DNA Engine Opticon 2 system. The threshold-cycle (Ct) was determined as the cycle with a signal higher than that of the background plus 10× standard deviation (SD). C. roseus 40S ribosomal protein S9 (Rps9), a housekeeping gene, was used to normalize the amount of the total mRNA in all samples (SI).

**Assessment of Alkaloid Production Rescue by Addition of Tryptamine.** Ten root tips from hairy roots transformed with pTDCi were subcultured in 50 mL of Gamborg’s B5 liquid media and grown at 26 °C in the dark at 125 rpm for 18 days before supplementing the media with tryptamine (Alfa Aesar) at 0, 62.5, 125, 250, 1,000 and 2,500 μM. After 1 week of cocultivation with the substrate, hairy roots were grown with a mortar, pestle and 100-μm glass beads in methanol (10 μL/g of fresh weight hairy roots) from the harvested tissue. The crude natural product mixtures were subjected to LC-MS analysis (Micromass LCT Premier TOF Mass Spectrometer). Peak integration and analysis was performed with MassLynx 4.1. To convert peak area to milligrams (Fig. 11), Bernhardt P, McCoy E, O’Connor SE (2007) Rapid identification of enzyme variants for alkaloid biosynthesis. Appl Microbiol Biotechnol 68:141–150.