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Integrating Carbon-Halogen Bond Formation into Medicinal Plant Metabolism

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Abstract

Halogenation, once considered a rare occurrence in nature, has now been observed in many natural product biosynthetic pathways[1]. However, only a small fraction of halogenated compounds have been isolated from terrestrial plants[2]. Given the impact that halogenation can have on the biological activity of natural products[1], we rationalized that introduction of halides into medicinal plant metabolism would provide the opportunity to rationally bioengineer a broad variety of novel plant products with altered, and perhaps improved, pharmacological properties. Here we report that chlorination biosynthetic machinery from soil bacteria can be successfully introduced into the medicinal plant *Catharanthus roseus* (Madagascar periwinkle). These prokaryotic halogenases function within the context of the plant cell to generate chlorinated tryptophan, which is then shuttled into monoterpene indole alkaloid metabolism to yield chlorinated alkaloids. A new functional group– a halide– is thereby introduced into the complex metabolism of *C. roseus*, and is incorporated in a predictable and regioselective manner onto the plant alkaloid products. Medicinal plants, despite their genetic and developmental complexity, therefore appear to be a viable platform for synthetic biology efforts.

Numerous halogenase enzymes from soil bacteria have been identified and characterized extensively[1, 3–5]. Two of these flavo-enzymes, PyrH[6, 7] and RebH[8–11], chlorinate the indole ring of tryptophan in the 5 and 7 positions, respectively. Transferring these enzymes into other natural product pathways would allow site-specific incorporation of halogens onto a range of tryptophan-derived alkaloid products[12], provided that the downstream enzymes could accommodate the chlorinated tryptophan precursor.

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One sentence summary: Periwinkle plant tissue can be genetically engineered to halogenate the medicinal natural products that it produces.

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Author Contributions All authors contributed to experimental design and data analysis. X. Q. initiated the project and its design and performed steady state kinetics. W. R. developed and implemented the transformation strategy and performed steady state kinetics and metabolite analysis. All authors contributed to preparation of the manuscript.

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Catharanthus roseus produces a wide variety of monoterpene indole alkaloids (Figure 1a)[13]. This metabolic pathway begins with the conversion of tryptophan **1** to tryptamine **2** by tryptophan decarboxylase[14]. Tryptamine then condenses with the iridoid terpene secologanin **3** to form a biosynthetic intermediate strictosidine **4**, which is subsequently functionalized in *C. roseus* to form over 100 alkaloids, including the anticancer agent vinblastine[13]. Previous work has shown that when *C. roseus* cell culture is supplemented with a variety of halogenated tryptamines, the corresponding halogenated alkaloid analogs are produced in isolable yields[15],[16]. If prokaryotic halogenases could function in the eukaryotic plant cell, and if tryptophan decarboxylase could convert halogenated tryptophan into halogenated tryptamine, then *C. roseus* would produce chlorinated alkaloids *de novo* (Figure 1b).

Since RebH and PyrH do not turnover tryptamine **2**, this strategy requires that tryptophan decarboxylase from *C. roseus* recognize halogenated tryptophan. Tryptophan decarboxylase (*C. roseus*) was assayed *in vitro* with tryptophan **1** (K_m = 51.7 ± 9.2 μM, k_{cat} = 5.1 ± 0.1 min^{-1}, k_{cat}/K_m = 0.099 $\mu M^{-1}min^{-1}$), 7-chlorotryptophan **1a** (K_m = 499 ± 74 μM, k_{cat} = 1.6 ± 0.04 min^{-1}, k_{cat}/K_m = 0.00327 $\mu M^{-1}min^{-1}$) and 5-chlorotryptophan **1b** (K_m = 538 ± 48 μM, k_{cat} = 2.5 ± 0.08 min^{-1}, k_{cat}/K_m = 0.00455 $\mu M^{-1}min^{-1}$) (Supplementary Fig. 1, 2). The activity of the enzyme suggested that halogenated tryptophan could be decarboxylated *in vivo*.

When considering how to merge the prokaryotic biosynthetic machinery with the plant alkaloid pathway, we chose to transfer the halogenase enzymes into *C. roseus*, rather than move the plant biosynthetic enzymes into a microbial host. Most of the monoterpene indole alkaloid biosynthetic genes have not been identified, making heterologous expression of this pathway impossible at this time. Moreover, we note that reconstitution of plant alkaloid pathways continues to be a challenging endeavor[17], [18]. Many alkaloids use complex starting materials (such as secologanin **3**) that are only produced by a few specialized plants, so reconstitution of plant alkaloid pathways must also include biosynthesis of these precursors. For example, ajmalicine **6** (Figure 1a), one of the simplest of the monoterpene indole alkaloids, requires an estimated 14 discrete enzymes for biosynthesis from tryptophan **1** and the terpene geraniol[13]; reconstitution of a pathway of this length constitutes a significant engineering problem. Therefore, we believe that exploring approaches in the host plant is an important aspect of alkaloid metabolic engineering efforts.

To produce 7-chlorotryptophan **1a** *in planta*, we generated an expression construct containing codon-optimized cDNA encoding the 7-tryptophan chlorinase RebH, and its required partner flavin reductase, RebF, in a plant expression vector (**pCAMBIA1300**), both under the control of constitutive CaMV 35S promoters. For production of 5-chlorotryptophan **1b**, an expression construct encoding the 5-chlorinating enzyme PyrH, along with RebF as the partner reductase, was generated. No signal sequence was added to the halogenase genes to ensure that RebH, PyrH and RebF would produce chlorinated tryptophan in the cytosol, where it would most readily encounter the decarboxylase, also localized to the cytosol (Supplementary Fig. 3–5)[19].

Agrobacterium rhizogenes was utilized to generate hairy root culture of *C. roseus* transformed with the halogenase genes[20]. One of the early biosynthetic enzymes, strictosidine synthase, cannot turn over 5-chlorotryptamine **2b**[21]. Therefore, when transforming *C. roseus* with *pyrH* and *rebF*, we also introduced a mutant of strictosidine synthase (STRvm) that can convert 5-chlorotryptamine **2b** to 10-chlorostrictosidine **4b**[16, 22]. After a selection process, we cultivated the transformed root culture on standard Gamborg's B5 plant media, and monitored chlorinated alkaloids using mass spectrometry (LC-MS). We were gratified to observe formation of chlorinated tryptophan **1a** and **1b** and chlorinated alkaloids in both the RebH/RebF and PyrH/RebF/STRvm hairy root lines (Figure 2, Supplementary Fig. 6–15). These results indicate that RebH, PyrH and the partner reductase function productively in the plant cell environment, clearly demonstrating that the flavin halogenases are highly transportable among kingdoms. Since chlorinated alkaloid production was observed in the transformed lines, we conclude that tryptophan decarboxylase can competently turn over halogenated tryptophan substrates *in vivo*.

Hairy roots transformed with RebH and RebF, which produce 7-chlorotryptophan **1a**, yielded a major chlorinated product at *m/z* 359 (Figure 2a). An authentic standard of 12-chloro-19,20-dihydroakuammicine **5a** co-eluted with this compound. Natural products containing the akuammicine scaffold display a variety of pharmacological activities[23–25]. Though the parent compound, 19,20-dihydroakuammicine **5** has been isolated in good yields from other plants[26], it is not a major alkaloid in *C. roseus* hairy root culture. However, when wild type *C. roseus* cell lines were incubated with 7-chlorotryptamine **1a**, 12-chloro-19,20-dihydroakuammicine **5a** was also the major chlorinated product (Supplementary Fig. 16). Therefore, the predominance of **5a** in RebH/RebF is likely due to substrate specificity of downstream enzymes for 7-chlorotryptamine **1a**. A hairy root line transformed with the 5-chlorotryptophan **1b** enzyme system, PyrH, RebF and STRvm, produced a variety of chlorinated alkaloids (Figure 2bcd). Two representative chlorinated alkaloids, 10-chloroajmalicine **6b** and 15-chlorotabersonine **7b**, were identified by co-elution with authentic standards[21].

Chlorinated alkaloid production appeared to be stable over the course of at least 6 subcultures. The alkaloid 12-chloro-19,20-dihydroakuammicine **5a** was produced at 26 ± 4 μg per gram of fresh root weight of a representative cell line averaged over six subcultures. For comparison, wild type cell lines produced approximately 25 μg per gram fresh weight tissue of chlorinated alkaloids when the media was supplemented with 200 μM 7-chlorotryptamine **2a**. Similarly, 10-chloroajmalicine **6b** and 15-chlorotabersonine **7b** were produced at 2.8 ± 0.9 and 4.0 ± 1.0 μg per gram of fresh root weight, respectively, for a representative cell line averaged over 4 subcultures (Supplementary Fig. 12, 14). Different concentrations of KCl (3 μM – 20 mM) were added to the media, but increasing amounts of exogenous chloride salt did not significantly affect the yields of chlorinated alkaloids (Supplementary Fig. 17, 18).

Previous reports demonstrated that RebH can utilize bromide to yield brominated tryptophan **1c**[8]. To assess the capacity of RebH for bromination *in vivo*, we supplemented a low chloride cell culture media with KBr. The *in vitro* halide specificity of RebH correlated with the products generated *in vivo*, as we observed the formation of a compound that co-eluted

with an authentic standard of 12-bromo-19,20-dihydroakuammicine **5c** (Figure 3) (21± 8 μg and 49± 20 μg per gram of fresh root with 10 mM and 20 mM KBr supplementation, respectively). In contrast, supplementation of the media with KI failed to yield either iodinated tryptophan or iodinated alkaloids. Again, this correlated with *in vitro* studies showing that RebH does not accept iodide as a substrate (Supplementary Fig. 19–22)[8].

We also measured the transcript levels of the heterologous enzymes by real time reverse transcription PCR. Production of halogenated compounds depended on the expression of both RebF and RebH or PyrH. Notably, when the strictosidine synthase mutant STRvm was not expressed in the PyrH/RebF hairy root lines, we observed accumulation of 5-chlorotryptophan **1b** (representative cell line, 9 ± 1 μg per gram of fresh root weight) and 5-chlorotryptamine **2b** (representative cell line, 20 ± 9 μg per gram of fresh root weight), but no downstream alkaloids were observed (Supplementary Fig. 23, 24).

Tryptophan **1** does not appear to accumulate in either wild type or transformed hairy roots. However, accumulation of 7-chlorotryptophan **1a** (50 ± 12 μg per gram of fresh root weight for a representative RebH/RebF cell line) and 5-chlorotryptophan **1b** (8 ± 2 μg per gram of fresh root weight for a representative PyrH/RebF/STRvm cell line) was observed, suggesting that decarboxylation of chlorinated tryptophan is a bottleneck *in vivo*, a step that could potentially be subjected to future engineering efforts. This is consistent with the 30 fold lower catalytic efficiency of the decarboxylase enzyme for halogenated tryptophan *in vitro*. The morphology of the halogen producing lines were thicker and slower growing than wild type lines (Supplementary Fig. 25). Since tryptophan serves as the precursor for other small molecule metabolites, we speculate that chlorinated tryptophan may be diverted into other pathways such as auxins. Notably, 4-chloro indole acetic acid, which is found in several species of pea, has altered activity compared to the auxin indole acetic acid[27, 28].

Medicinal plants produce a wide range of complex natural products, but generate relatively few halogenated compounds; chlorinated or brominated compounds are not found among the approximately 3000 known monoterpene indole alkaloids produced by plants in the Apocynaceae, Rubiaceae and Loganiaceae families. Halogenation of natural products often has profound changes on the bioactivity of the compound, and can also serve as a useful handle for further chemical derivatization[1, 29]. Despite the metabolic and developmental complexity of plant tissue, transformation of these prokaryotic genes led to the regioselective incorporation of halides into the alkaloid products of the existing plant pathway. Notably, the yield of chlorinated alkaloids in the highest producing lines (~26 μg/g fresh weight of plant tissue) is only 15 fold lower than the yield of total natural alkaloids (compounds **5** + **6** + **7** + **8**) from wild type tissue (~ 420 μg/g fresh weight of plant tissue) (Supplementary Fig. 26). The ease with which we could engineer the successful production of chlorinated alkaloids in *C. roseus*, a plant with limited genetic characterization, indicates that medicinal plants can provide a viable platform for synthetic biology.

Methods Summary

More detailed methods are provided in the online Methods. Structural characterization is shown in Supplementary Fig. 27–32 and Supplementary Table 1, 2.

Generation of Transgenic C. roseus Hairy Root Cultures

The expression construct pCAMRebHRebF was transformed into *Agrobacterium rhizogenes* ATCC 15834 via electroporation (1mm cuvette, 1.25 kV). pCAMPyrHRebF and pCAMSTRvm were co-transformed into *Agrobacterium rhizogenes* ATCC 15834 via electroporation. Transformation of *C. roseus* seedlings with the generated *Agrobacterium* strains was performed as previously reported ([20]).

Evaluation of Alkaloid Production in Transgenic C. roseus Hairy Roots

Every transgenic hairy root line that survived hygromycin selection media was evaluated for alkaloid production. Transformed hairy roots were grown in Gamborg's B5 solid media (half strength basal salts, full strength vitamins, 30 g/L sucrose, 6 g/L agar, pH 5.7). The total chloride concentration in Gamborg's B5 formulation is ~ 1mM. Three-week-old hairy roots were ground with a mortar, pestle and 106 μm acid washed glass beads in methanol (10 mL/g of fresh weight hairy roots). The crude natural product mixtures were filtered through 0.2 mm cellulose acetate membrane (VWR) and subsequently subjected to LC-MS analysis. Additionally, hairy roots transformed with wild-type *A. rhizogenes* lacking the plasmid were also evaluated.

Brominated Alkaloid Production in Transgenic C. roseus Hairy Roots

A selected transformed hairy root line was grown for two weeks in low chloride solid media (67 mg/L $(NH_4)_2SO_4$, 353 mg/L $Ca(NO_3)_2.4H_2O$, 61 mg/L $MgSO_4$, 1250 mg/L KNO_3, half strength Murashige and Skoog's micronutrient salts and full strength Murashige and Skoog's vitamins, 3 μM total chloride concentration). Hairy roots were transferred to the same media supplemented with either potassium bromide or potassium iodide (10 – 20 mM final concentration) and cultivated for an additional two weeks. Hairy roots were then processed and alkaloid production analyzed as described above (Figure S12–S15). Experiments were performed in duplicate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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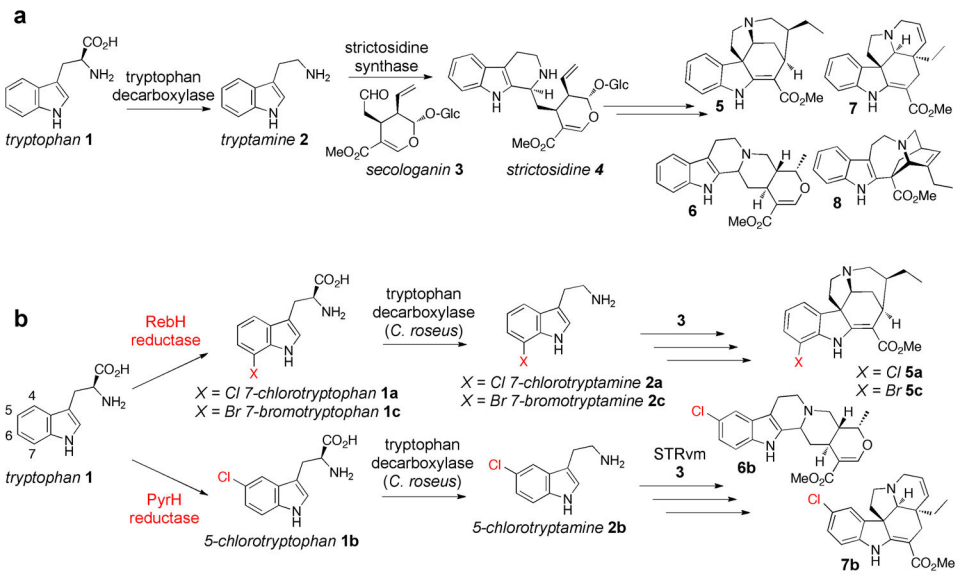

Figure 1.

Monoterpene indole alkaloid biosynthesis. **A.** Tryptophan **1** is decarboxylated by tryptophan decarboxylase to yield tryptamine **2**, which reacts with secologanin **3** to form strictosidine **4**. After numerous rearrangements, strictosidine **4** is converted into a variety of monoterpene indole alkaloids, such as 19,20-dihydroakuammicine **5**, ajmalicine **6**, tabersonine **7** and catharanthine **8**. These compounds display a variety of pharmacological activities[24–26, 30]. **B.** RebH and PyrH, along with a partner reductase, halogenate the indole ring of tryptophan **1** to yield chloro-tryptophan. Here we show that after transformation of these enzymes into *C. roseus*, halogenated tryptophan **1a** and **1b** can be decarboxylated by tryptophan decarboxylase (*C. roseus*) to form chlorotryptamine **2a** and **2b**, and then converted into chlorinated monoterpene indole alkaloids.

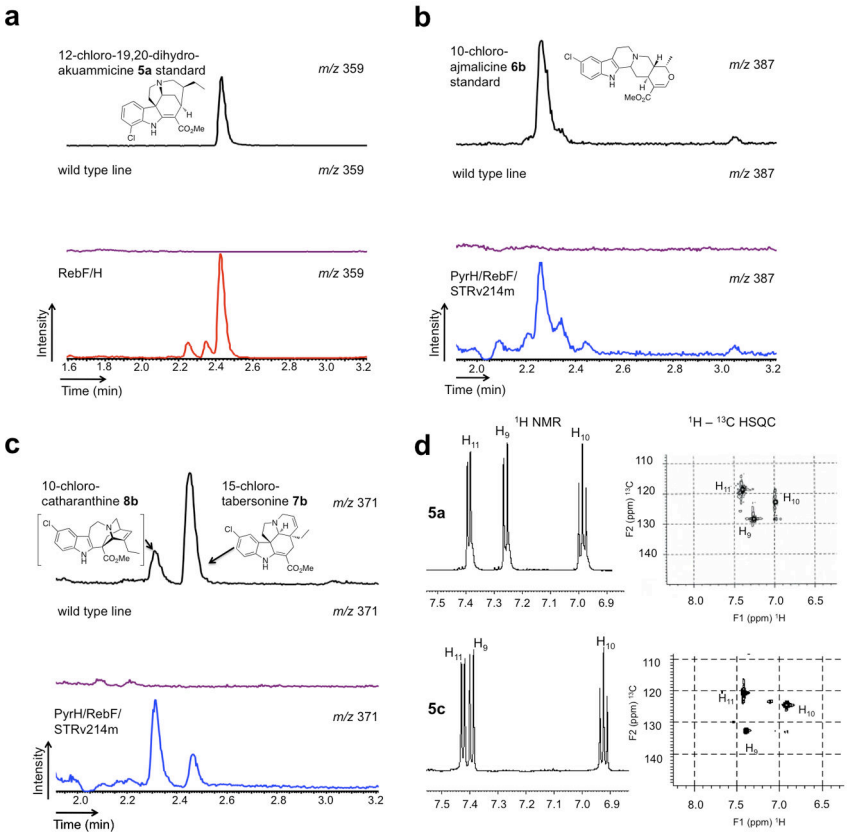

Figure 2.

Chlorinated alkaloids in *C. roseus* hairy root culture. **A.** Liquid chromatography-mass spectrometry (LC-MS) chromatograms showing 12-chloro-19,20-dihydroakuammicine **5a** (*m/z* 359) in RebF/H hairy roots (red trace), contrasted with control cultures transformed with no plasmid (pink trace). An authentic standard of **5a** validated the structural assignment (black trace, Supplemetary Fig. 19, 20). **B.** Chromatograms showing 10-chloroajmalicine **6b** in RebF/PyrH/STRvm hairy roots (purple trace), contrasted with control cultures (pink trace). An authentic standard of **6b** is shown (black trace)[22]. **C.** Chromatograms showing 15-chlorotabersonine **7b** in RebF/PyrH/STRvm hairy roots (purple trace) contrasted with control cultures (pink trace). An authentic standard of **7b** is shown (black trace)[22]. The other major peak at *m/z* 371 had an exact mass and UV spectra consistent with a chlorinated catharanthine **8** analog (Supplementary Fig. 18)[22]. **D.** ^{1}H NMR and ^{1}H-^{13}C HSQC spectra of **5a** and **5c**.

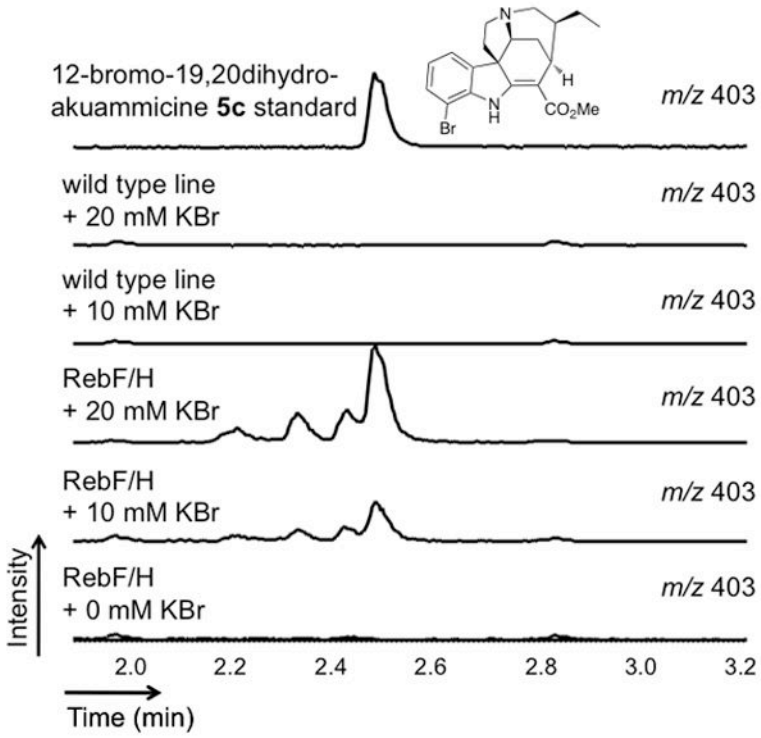

Figure 3.

Extracted LC-MS chromatograms showing the presence of 12-bromo-19,20-dihydroakuammicine **5c** (*m/z* 403) in RebF/H hairy roots. Hairy roots are grown in media supplemented with KBr (0 – 20 mM final concentration) for two weeks prior to alkaloid extractions. 12-bromo-19,20-dihydroakuammicine **5c** is not observed in control cultures transformed with no plasmid after incubation in potassium bromide supplemented media. An authentic standard of 12-bromo-19,20-dihydroakuammicine **5c** is used to validate the structural assignment (Supplementary Fig. 18).