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Strategies to Produce Chlorinated Indole-3-Acetic Acid and Indole-3-Acetic Acid Intermediates

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Tryptophan and indole derivatives are common precursors in many natural biosynthetic pathways. In recent years it has been shown that the substrate specificity of tryptophan halogenases is much more relaxed than previously thought. Using the tryptophan 7-halogenase PrnA, the tryptophan 6-halogenase ThdH, and the tryptophan 5-halogenase PyrH we achieved the regioselective mono-halogenation of indole-3-acetic acid (IAA), which has not been reported as a substrate of tryptophan halogenases to date. The tryptophan 5-halogenase gene was introduced into *Arabidopsis thaliana* leading to the formation of 5-chlorotryptophan, 5-chloroindole-3-acetonitrile and 5-chloro-3-indole acetic acid by *A. thaliana*. PyrH activity could also be demonstrated for the plant-produced halogenase *in vitro*. These results show the potential of flavin-dependent halogenases to generate novel halogenated auxins or other secondary metabolites *in vitro* and *in vivo* by plants.

Introduction

There are many natural halogenated compounds produced by living organisms with a broad range of biological activities and in many cases, the activity of these compounds depends on the presence of a halogen atom[1]. Enzymatic halogenation reactions are well known since the first halogenated enzyme identified as a chloroperoxidase from the filamentous fungus *Caldariomyces fumago* was detected.[2] The reaction mechanism of this enzyme revealed the production of hypochlorous acid as halogenating agent which diffuses out of the active site and leads to chemical chlorination without regioselectivity.[3] However, regioselectivity is a desirable property in every chemical or biochemical reaction and especially important in halogenation reactions due to the easy formation of unwanted and often toxic by-products.[4,5] The flavin-dependent halogenases PrnA/RebH, ThdH, and PyrH catalyze the regioselective halogenation of free tryptophan at the positions 7, 6, and 5, respectively and require a flavin reductase as a second enzyme component to reduce FAD to $FADH_2$.[6–9] These halogenases are well characterized and the 3-dimensional structures of PrnA, RebH, and PyrH have been elucidated.[10–12] Recently, different authors have shown that these enzymes are able to halogenate other indolic and non-indolic substrates which demonstrates a much more relaxed substrate specificity then previously reported[13–16] (Figure 1). PrnA and RebH were shown to halogenate a variety of tryptophan derivatives and other indole ring-containing compounds such as tryptoline and also substituted naphthalenes. In many cases halogenation occurred exclusively at the C7 position.[13,14] In addition, variants of RebH, PrnA, and SttH, a new tryptophan 6-halogenase, were created by site directed mutagenesis or error-prone PCR which resulted in a few cases in modification of the regioselectivity.[15,17,19,20] However, although attempted,[16,21] successful chlorination of indole-3-acetic acid (IAA) was not reported for these halogenases so far.

It is well known that secondary metabolism of plants is a natural system to produce a huge variety of compounds with valuable properties.[22] Halogenation of natural products produced by terrestrial plants could be an alternative method to obtain new compounds with altered properties. For example, O'Connor and co-workers introduced the tryptophan 7-halogenase gene *rebH* and the flavin reductase gene *rebF*, isolated from *Lechevalieria aerocolonigenes*, into the medicinal plant *Catharanthus roseus*. Overexpression of these genes led to the production of the chlorinated tryptophan-derived alkaloid 12-chloro-19,20-dihydroakuammicine.[23] These experiments highlight the potential of combining the rich chemistry of plant metabolism with bacterial halogenases to generate unnatural products. Recently, the transient expression of another halogenase gene *stth*, coding for a tryptophan 6-halogenase from *Streptomyces toxytricini*, in tobacco was reported and the authors showed that co-expression of a tryptophan decarboxylase gene with this halogenase resulted in the production of chlorinated tryptamine.[24]

Indole-3-acetic acid (IAA) is the most widespread auxin in terrestrial plants and in nearly every process of plant develop-

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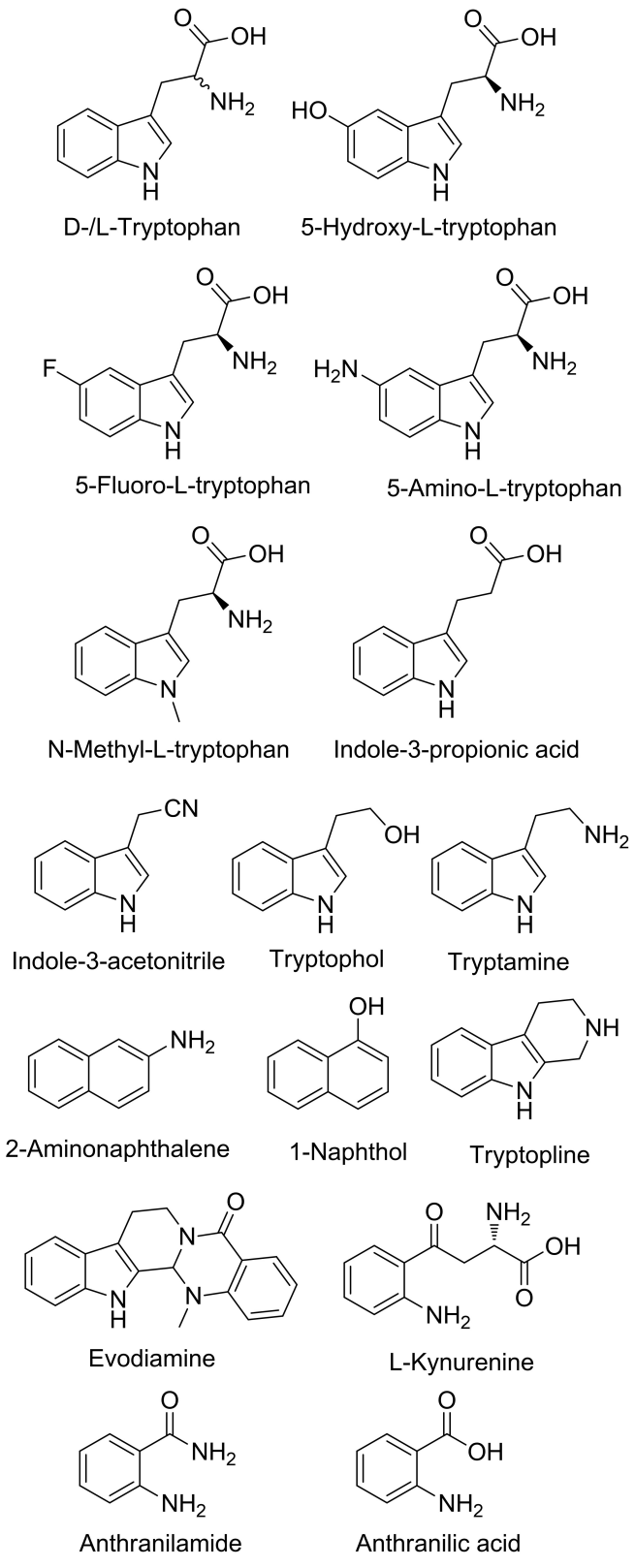


Figure 1. Examples of substrates of tryptophan halogenases.[13–16,19,21]

ment and growth, IAA plays an important role. Endogenous IAA is involved in root and shoot architecture formation,[25] cell division and elongation,[26] and tropic responses.[27] In seeds of *Pisum sativum* and other legumes, large quantities of a natural chlorinated auxin, 4-chloroindole-3-acetic acid (4-Cl–IAA) were found[28–31]. In bioassays, it was shown that this chlorinated auxin has a higher activity than IAA.[32] 4-Cl–IAA is the strongest natural auxin known in plants, so far.[33–36]

Inspired by the examples mentioned above and based on the well characterized heterologous expression of tryptophan halogenase genes in bacteria and plants,[23,24,37,38] we decided to investigate the *in vitro* chlorination of IAA by tryptophan halogenases and to assess the performance of one of these halogenases, PyrH, in *Arabidopsis thaliana* to evaluate the potential of flavin-dependent halogenases to generate novel halogenated auxins *in planta*.

Results and Discussion

Using *Pseudomonas fluorescens* BL915△ORF1-4, a mutant strain lacking the genes responsible for the biosynthesis of pyrrolnitrin[39] and the *Pseudomonas-E. coli* shuttle vector pCIBhis,[10] the three halogenase genes, *prnA*, *thdH*, and *pyrH*, were highly overexpressed and the His-tagged halogenases were purified by immobilized metal affinity chromatography (Figure S1). IAA was chlorinated by all three halogenases in the presence of a flavin reductase, NaCl, FAD, and NADH. HPLC analyses of the *tert*-butyl methyl ether extracts of the enzymatic reactions showed the formation of a single product which showed a mass consistent with monochlorinated IAA according to LC–MS (Figure 2). Andorfer *et al.* had also tried to obtain chlorinated

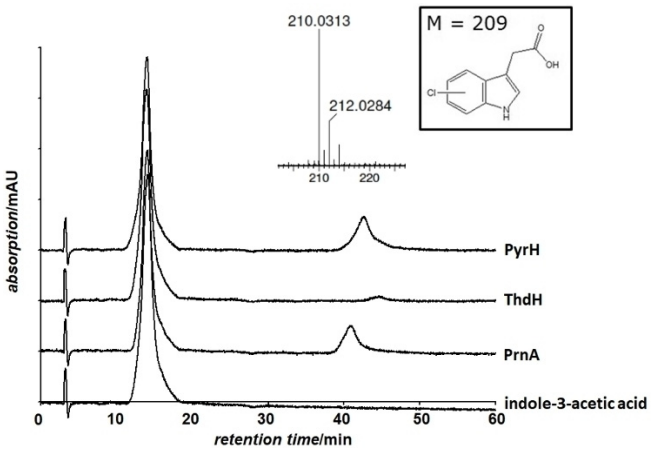


Figure 2. HPLC chromatograms of the reactions of the halogenases PyrH, ThdH, and PrnA with IAA as the substrate. The inset shows the parent M + H MS peak of the product with the characteristic isotope pattern of monochlorinated indole-3-acetic acid.

indole-3-acetic acid using eight flavin-dependent halogenases, but could neither detect the starting material, indole-3-acetic acid, nor chlorinated product.[21] Large-scale assays allowed the purification of the products by preparative HPLC and their characterization by NMR experiments. The NMR data clearly

showed IAA was regioselectively chlorinated by PrnA, ThdH, and PyrH in the positions 7, 6, and 5, respectively. The tryptophan 6-halogenase ThdH has a much lower activity with IAA as the substrate compared to the tryptophan 7-halogenase PrnA and the tryptophan 5-halogenase PyrH, which show similar activities (Figure 2).

The product formation was verified by more detailed kinetic analyses of the reactions using substrate concentrations between 1 and 5 mM. This rather narrow range had to be used, since for IAA concentrations below 1 mM the activities were too low for reliable quantification and IAA is not soluble in water above 5 mM. Specific activity values for the tryptophan 5-halogenase PyrH for indole-3-acetic acid were only 1/1000 of that of its natural substrate tryptophan (0.11 mU mg^{-1} and 113.7 mU mg^{-1}, respectively) using substrate concentrations of 0.25 mM. Higher tryptophan concentrations could not be used for comparison, since they inhibit the activity of the tryptophan 5-halogenase PyrH.

Incubation of IAA with PyrH (18 μM), ThdH (21.5 μM), and PrnA (17 μM) for 15 min resulted in the formation of 60.9 nmol, 6 nmol, and 11.2 nmol Cl–IAA, respectively.

The determination of k_{cat} values was not possible, since we could not reach substrate saturation due to the low solubility of IAA and the chlorination of IAA by the three halogenases clearly does not follow standard Michaelis-Menten kinetics (Figure S2). With their natural substrate tryptophan, the enzymes show Michaelis-Menten kinetics.[6,8,37] Obviously, efficient chlorination of IAA can only occur at higher IAA concentrations.

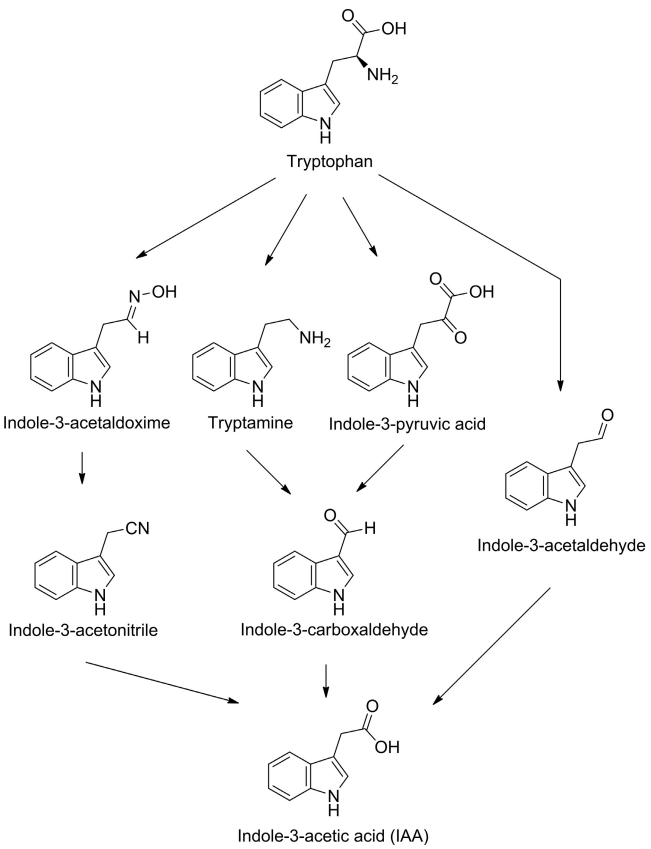
Since we could show that IAA is a substrate for the three tryptophan halogenases *in vitro,* we decided to explore the prospect of making chlorinated IAA derivatives *in planta*, where the halogenase would chlorinate tryptophan, which would then act as a substrate for the IAA pathway. Alternatively, the halogenase could halogenate IAA, but given the low catalytic efficiency of the halogenases with this substrate, this possibility seems much less likely.

In contrast to previous work with tobacco, where transient transformants were obtained,[24] *Arabidopsis thaliana* was stably transformed with a plant expression vector containing the His-tagged tryptophan halogenase gene *pyrH.* The tryptophan 5-halogenase gene was chosen because PyrH showed the highest activity of the three halogenases with IAA (Figure S2). In previously published transformations of plants with bacterial halogenase genes, codon optimized halogenase genes had been used.[23,24] In contrast to these experiments, we used native *pyrH* as well as *pyrH* with codons optimized for *A. thaliana.* We did not introduce a flavin reductase gene. Since it was known that no specific flavin reductase is required by the halogenase, it could be assumed that flavin reductases produced by *A. thaliana* would provide $FADH_2$ used by the halogenase.[40] Either native/bacterial or codon-optimized *pyrH* was ligated via the Gateway System into the pMDC32 vector under the control of the constitutive CaMV promoter. The final constructs were introduced into *Agrobacterium tumefaciens* for the stable transformation of *Arabidosis thaliana* via the floral-dip method.[41]

LC–MS analysis of extracts of several of these stably transformed plant lines revealed that *A. thaliana* containing the *pyrH* gene produced chlorinated tryptophan (Figure 3A). The amount of 5-chlorotryptophan in the different lines was very variable, in the range of 154 to 924 ng g^{-1} of lyophilized tissue. In addition, in some of the lines, we could also detect traces of a compound with an *m/z* value consistent with chlorinated indole-3-acetonitrile (IAN, Figure 3B and Figure S5). The MRM acquisition method for 5-Cl–IAN was set up based on the fragmentation pattern and collision energy of IAN (available as a standard). This compound (m/z 157.10) mainly fragments into m/z 130.1 (CE 10 eV) and m/z 117.1 (CE 16 eV). After chlorination on the aromatic ring we expected the pseudomolecular ion $[M + H]^+$ for 5-Cl–IAN to be m/z 191.1 and in the same fragmentation conditions used for IAN, which do not lead to cross fragmentation of the indole moiety, the main fragments were m/z 164.1 and m/z 151.1, respectively.

These transitions were used for its detection in the plant extracts.

IAN is an intermediate in the IAA pathway (Scheme 1). In early experiments, we could not detect chlorinated IAA. Later



Scheme 1. Possible tryptophan-dependent IAA biosynthetic pathways in *Arabidopsis thaliana* modified according to Nonhebel *et al.*[45]

we realized that this was probably due to the instability of IAA (Figure S22). When samples were analyzed shortly after their preparation, chlorinated IAA (70-90 ng g^{-1} plant material) could

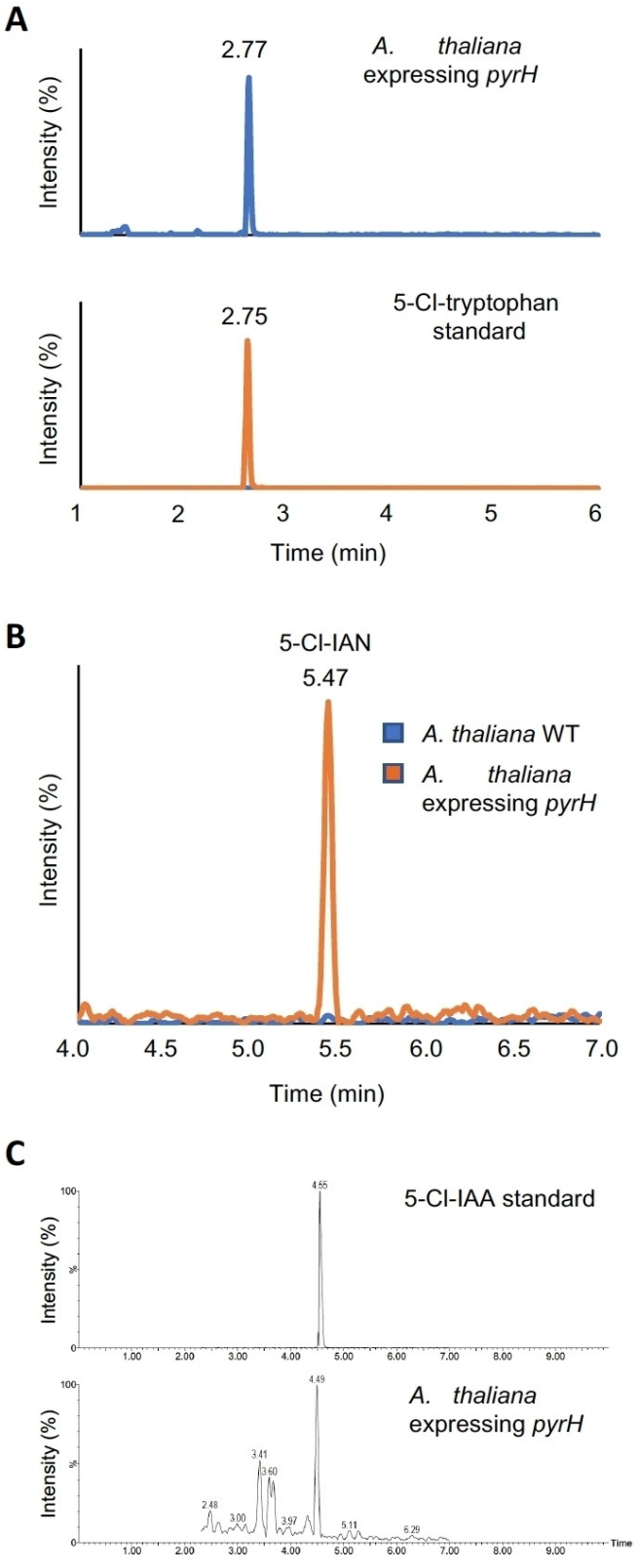


Figure 3. Detection of A, 5-chlorotryptophan, B, 5-chloroindole-3-acetonitrile (5-Cl–IAN) and C, 5-chloroindole-3-acetic acid (5-Cl–IAA) in recombinant *A. thaliana* in comparison with authentic standards.

be detected (Figure 3C). While the levels of 5-Cl–IAN were about 2.0-2.2 μg g^{-1} dry weight range, 5-Cl–IAA was found to be present in the lower ng range (70-90 ng g^{-1} plant material; Figure 4). Such concentrations also correspond to levels of non-chlorinated IAA measured in tissues (100-300 pmol g^{-1} fresh weight). This suggests that PyrH functions *in planta* to produce chlorinated tryptophan, which is subsequently converted to 5-Cl–IAA via 5-Cl–IAN as an intermediate.

Transformants harboring the codon-optimized *pyrH* gene contained less chlorinated tryptophan than those containing the native gene (Figure 3B). This is in agreement with the finding that plants transformed with the native gene produced substantially larger amounts of the halogenase transcript and protein than plants harboring codon-optimized *pyrH* (Figure 5).

Since LC–MS analysis only showed that chlorinated tryptophan was produced by the recombinant *A. thaliana* lines, but not at which position the chlorine atom was introduced, the halogenase was partially purified from plant extracts and used for *in vitro* reactions and the product was compared with authentic standards. Again, a much higher halogenating activity was detected in the corresponding plant extracts (enriched for the protein) that express native *pyrH* than in the case of plants transformed with codon-optimized *pyrH* (Figure 5C). Thus, it was possible to demonstrate that optimization of bacterial halogenase genes for expression in *A. thaliana*, as was done in previous work in *Catharanthus roseus* and tobacco,[23,24] is not an essential feature for *in planta* expression, and may even be detrimental.

Since non-chlorinated IAN is known to be an intermediate in one of the biosynthesis pathways of IAA from tryptophan in *A. thaliana* (Scheme 1),[42] the detection of chlorinated IAN in our study strongly suggests that Cl–IAA detected in *A. thaliana* is derived from tryptophan chlorinated by PyrH *in planta* via Cl–IAN and not by direct chlorination of IAA. This assumption is supported by the fact that chlorinating activity of PyrH with IAA as the substrate is very low and that the concentration of IAA in *A. thaliana* is very low (100-300 pmol g^{-1} fresh weight). In line with these results, we could detect 5-Cl–IAN and 5-Cl–IAA after incubating wild type *A. thaliana* plants with 5-Cl-tryptophan.

Since it is known that supplementing *A. thalian* with high levels of IAA results in the formation of large quantities of IAA amino acid conjugates,[43] we also looked for the presence of 5-chlorinated IAA amino acid conjugates, but could only find large amounts of various unchlorinated IAA amino acid conjugates (data not shown). Our discovery of low amounts of 5-Cl–IAA is due to improved extraction and timing of analysis. We found that not only IAA, but also 5-Cl–IAA was rapidly degraded under light during a time period of 17 days (Figure S23), while it was possible to protect both indoles from photooxidation by using yellow protection (for details see suppl. methods). These data on the relative instability of IAA and 5-Cl–IAA might also explain why in some reports neither chlorinated IAA nor IAA could be found.[21]

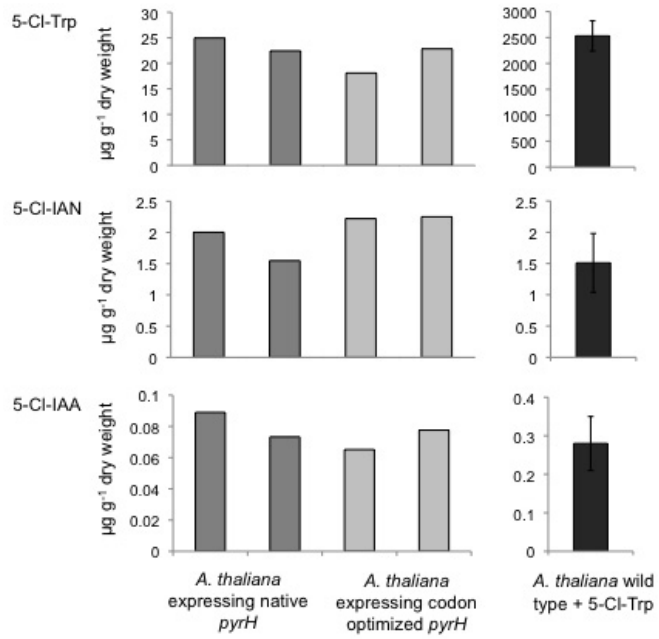


Figure 4. Quantitative analysis of 5-Cl-tryptophan, 5-Cl–IAN and 5-Cl–IAA formation in *A. thaliana* containing the codon optimized or the native tryptophan 5-halogenase gene *pyrH* given as ng / g dry weight and the same metabolites in Arabidopsis wild type incubated with 5-Cl-tryptophan. Single measurements of two different lines are shown for native and codon-optimized *pyrH,* respectively. Analysis was done by UPLC-MS.

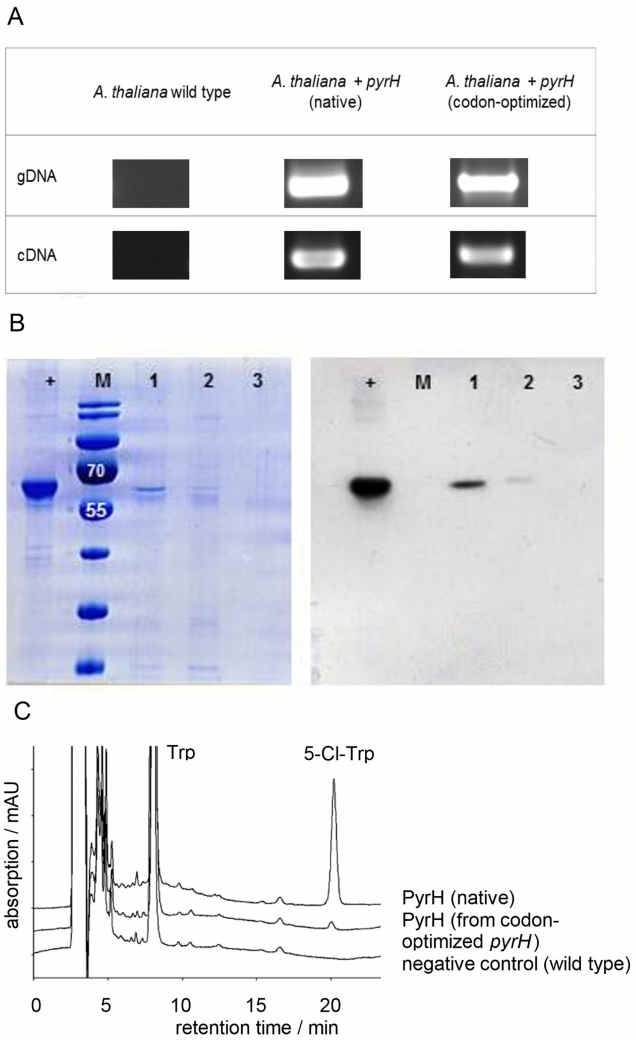


Figure 5. A, Verification of the integration and expression of native and codon-optimized *pyrH* in *Arabidopsis thaliana* with specific insert primers. **B,** SDS-PAGE showing purified PyrH from extracts of *A. thaliana.* M: prestained protein ladder. Lane 1, *A. thaliana* + native *pyrH*; lane 2, *A. thaliana* + codon optimized *pyrH*; lane 3, Arabidopsis wild type. Left site shows the purified proteins in a SDS gel with Coomassie brilliant blue staining. Right site shows a Western blot with anti-His antibodies. **C,** HPLC chromatograms showing the chlorinating activity of purified PyrH produced by *A. thaliana.*

Conclusions

Here we demonstrate that bacterial halogenases function *in planta*, as demonstrated by stable introduction of *pyrH* into *Arabidopsis thaliana.* Chlorinated tryptophan was observed showing that the enzyme is functional in this expression host. The detection of Cl–IAN and Cl–IAA strongly suggest that they are derived from chlorinated tryptophan and not by chlorination of IAN or IAA, respectively. A number of other metabolites of *A. thaliana* are also derived from tryptophan, most notably indole glucosinolates, though no other chlorinated metabolites were detected. Likely, improvements of the level of the Cl-Trp precursor could allow more widespread incorporation of this unnatural substrate into the *A. thaliana* metabolic pathways.

Supporting Information Summary

This section contains a detailed description of the materials and experimental procedures used and supporting data showing the results of the purification of the halogenase ThdH, kinetic data for the halogenases with indole-3-acetic acid as substrate, the characterization of products including NMR Data and an investigation of the stability of IAA and 5-Cl–IAA in solution.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: chloroindole acetic acid · chloroindole acetonitrile · chlorotryptophan · halogenase · indole acetic acid

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