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Research Article

Recombinant flavin-dependent halogenases are functional in tobacco chloroplasts without co-expression of flavin reductase genes

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Halogenation of natural compounds in planta is rare. Herein, a successful engineering of tryptophan 6-halogenation into the plant context by heterologous expression of the *Streptomyces toxytricini* Stth gene and localization of its enzymatic product in various tobacco cell compartments is described. When co-expressed with the flavin reductase *rebF* from *Lechevalieria aerocolonigenes*, Stth efficiently produced chlorinated tryptophan in the cytosol. Further, supplementation of KBr yielded the brominated metabolite. More strikingly, targeting of the protein to the chloroplasts enabled effective halogenation of tryptophan even in absence of the partner reductase, providing crucial evidence for sufficient, organelle-specific supply of the $FADH_2$ cofactor to drive halogen integration. Incorporation of an alternative enzyme, the 7-halogenase RebH from *L. aerocolonigenes*, into the metabolic set-up resulted in the formation of 6,7-dichlorotryptophan. Finally, expression of *tryptophan decarboxylase* (*tdc*) in concert with *stth* led to the generation of 6-chlorotryptamine, a new-to-nature precursor of monoterpenoid indole alkaloids. In sum, the report highlights the tremendous application potential of plants as a unique chassis for the engineering of rare and valuable halogenated natural products, with chloroplasts as the cache of reduction equivalents driving metabolic reactions.

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1 Introduction

Amid the myriad of specialized metabolites found in nature, halogenated structures constitute but a limited proportion. Nonetheless, the total number of halometabolites reported so far reaches almost 4000 (3700 documented by Gribble [1]), with the antibiotics, vancomycin [2] and chloramphenicol, or the 20S proteasome inhibitor and potential anticancer agent, salinosporamide A [3] most prominent among them. Majority of the halogenated natural compounds derive from prokaryotes or marine organisms and contain, in the main, bromine or chlorine substituents [4]. Higher plants, principal contributors to the remarkable diversity of natural products, are (so far) considered relatively devoid of halogenated structures. The very few examples of plant halometabolites include fluoracetate from *Acacia georginae* [5] or 4-chloroindole-3-acetic acid, a halogenated auxin from pea [6].

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Abbreviations: BFP, blue fluorescent protein; **cp**, chloroplast; **ER**, endoplasmic reticulum; **FAD**, flavin adenine dinucleotide (quinone, oxidized form); **$FADH_2$**, flavin adenine dinucleotide (hydroquinone, reduced form); **FW**, fresh weight; **GB2.0**, GoldenBraid 2.0; **HPLC**, high-performance liquid chromatography; **KBr**, potassium bromide; **MIAs**, monoterpenoid indole alkaloids; **MS**, mass spectrometry; **NADH**, nicotinamide adenine dinucleotide (reduced form); **PyrH**, tryptophan 5-halogenase from *Streptomyces rugosporus*; **RebF**, flavin reductase from *Lechevalieria aerocolonigenes*; **RebH**, tryptophan 7-halogenase from *L. aerocolonigenes*; **Stth**, tryptophan 6-halogenase from *Streptomyces toxytricini*; **TDC**, tryptophan decarboxylase; **t_R**, retention time; **TU**, transcriptional unit; **UPLC**, ultra-performance liquid chromatography; **YFP**, yellow fluorescent protein

The importance of many halogenated products from prokaryotes and fungi lies in their often pronounced biological and pharmacological effects, driving progress in the investigation of their biosynthetic pathways. Recent identification of an array of halogenating enzymes and cloning of the corresponding genes enabled thorough characterization and grouping of the catalysts into distinct classes. Haloperoxidases, for example, utilize hydrogen peroxide and halide ions to produce hypohalous acids, freely diffusible halogenating agents able to attack susceptible organic molecules. These enzymes, therefore, lack substrate specificity and regioselectivity [7]. In contrast, the subsequently discovered class of flavin-dependent halogenases seems to play a vital role in the biosynthesis of a substantive number of natural products, with its representatives displaying high substrate specificity and/or regioselectivity [8]. In general, flavin-dependent halogenases require $FADH_2$ to introduce a halogen atom into an organic molecule, given that the structure of the latter encompasses a double bond susceptible to electrophilic substitution [8]. Supply of the cofactor, via NADH-driven reduction of FAD, falls to a cooperating enzyme, flavin reductase. Although functionally linked, the catalytic partners need not derive from the same organism or biosynthetic gene cluster. As reported by Zeng and Zhan, the in vitro catalytic activity of tryptophan 6-halogenase Stth from *Streptomyces toxytricini* was maintained while bolstered by Fre, a flavin-reductase from *E. coli*, confirming that a heterologous, yet similar, enzyme was sufficient to provide $FADH_2$ and efficiently augment halogenation of tryptophan [9].

The fact that many halogenating enzyme systems affording introduction of novel functionalities into amenable molecules are now at hand opens the possibility of combinatorial biosynthesis of new-to-nature compounds in heterologous contexts. In this light, the halogenases acting on tryptophan and its derivatives emerge as catalysts of considerable interest. Plants produce a plethora of tryptophan-derived metabolites, crucially exemplified by the vast array of indole alkaloids encompassing many potent pharmacological agents [10, 11]. Introduction of halides into these structures might result in altered plant products with modified activities or offer new prospects for subsequent synthetic customization steps [12]. In their ground-breaking work, the group of O'Connor integrated two flavin-dependent halogenases from soil bacteria, RebH and PyrH, into the biosynthetic pathway of monoterpenoid indole alkaloids in the medicinal plant, *Catharanthus roseus* [13].

To fully capitalize on the potential of plants to provide a versatile and compelling platform for synthetic biology and metabolic engineering, their inherent complexity as multicellular organisms with highly compartmentalized cells needs to be taken into account. Further, plant biosynthetic pathways are intricate networks regulated on temporal and spatial levels [14]. Hence, consolidation of biosynthetic enzymes into novel multi-faceted metabolic circuits might require individual fine-tuning of their localization within a plant cell. Consequently, metabolic engineering endeavors call for in-depth investigation of high numbers of permutations of combined genes, targeting sequences and promoters to establish the optimal context for production of the desired molecule(s). In recent years, modular cloning technologies for metabolic engineering, enabling fast and reliable assembly of multiple expression constructs from a limited set of standardized building blocks, have been proposed [15]. Among them, the intercompatible GoldenBraid (GB) and MoClo standards proved especially useful in plant synthetic biology approaches, spurring the development of effective promoter shuffling strategies [16] and CRISPR/Cas9-based genome editing tools [17] facilitating designer transcriptional regulation as well as helping to tackle the intricacies of plastome engineering [18].

In the present study, we set out to expand the repertoire of tryptophan halogenases amenable for plant synthetic biology-driven redesign. To this end, we elected to examine the engineering potential of the aforementioned, recently discovered tryptophan 6-halogenase from *S. toxytricini*, Stth [9]. To further expand the characterization of catalytic properties of the enzyme, we introduced it into the GoldenBraid context and investigated the effect of divergent subcellular localization on product formation. We further addressed the ability of Stth to provide precursors for monoterpenoid indole alkaloid formation in a combinatorial biosynthetic approach.

2 Materials and methods

2.1 Chemicals

5-DL-chlorotryptophan, 6-DL-bromotryptophan and 7-DL-bromotryptophan used as standards for identification of RebH and Stth products were purchased from Carbosynth (Berkshire, UK). 6-DL-chlorotryptophan and 5-DL-bromotryptophan were procured from Biosynth (Staad, Switzerland).

2.2 Genetic material

DNA constructs encompassing the coding sequences of RebH from *Lechevalieria aerocolonigenes* (GenBank AB071405.1, part of gene cluster) and TDC from *C. roseus* (GenBank X67662.1) were kindly provided by Prof. Sarah E. O'Connor from the John Innes Centre, Norwich, UK and Prof. Johan Memelink from the Leiden University, the Netherlands, respectively. In addition, GB-customized *stth* deriving from *S. toxytricini* (GenBank HQ844046.1) and *rebF* from *L. aerocolonigenes* (GenBank AB071405.1, part of gene cluster) were synthesized by Thermo Fisher Scientific (Waltham, MA, USA).

2.3 Bacterial strains and growth conditions

E. coli DH5α (New England Biolabs, Ipswich, MA, USA) or TOP10 cells (Thermo Fisher Scientific, Waltham, USA), used for cloning of DNA constructs, were cultivated at 37°C in lysogeny broth (LB) supplemented with relevant antibiotics and, when appropriate, 40 µg/mL of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside acid (X-Gal, Applichem, Darmstadt, Germany) [19]. Transient transformation of *Nicotiana benthamiana* plants was performed using *Agrobacterium tumefaciens* EHA105 cells (ICON Genetics, Halle, Germany) grown at 28°C in LB medium supplemented with appropriate antibiotics [19] as well as rifampicin (50 µg/mL) and 100 µM 3,5-dimethoxy-4-hydroxyacetophenone (acetosyringone, Sigma-Aldrich, St. Louis, MO, USA).

2.4 Cloning of DNA constructs

All DNA constructs used for transient transformation of *N. benthamiana* plants were assembled using the GoldenBraid 2.0 (GB2.0) cloning technique [19]. For domestication in the pUPD vector, the relevant sequences were amplified by means of PCR ascertaining addition of appropriate fusion sites and *Bsm*BI recognition sites as well as removal of internal *Bsm*BI and *Bsa*I recognition sites (Supporting information, Table S1). To afford chloroplast localization of recombinant enzymes, *stth*, *rebH* and *rebF* were fused to a chloroplast transit peptide sequence (*cp*), a consensus of dicot sequences, originating from the pICH20030 plasmid (ICON Genetics, Halle, Germany, [20]). For the transport of halogenases to the apoplast, *stth* and *rebH* were fused to the signaling peptide sequence of calreticulin (*er*) from *Arabidopsis thaliana*, as featured in the pICH17620 plasmid (ICON Genetics, Halle, Germany, [20]). Expression of *cp*- and *er*-fused *stth* and *rebH* was regulated by the cauliflower mosaic virus (CaMV) 35S promoter (P35S, GB0030) and the nopaline synthase terminator (TNos, GB0037) from *A. tumefaciens*, while the *cp*- and *er*-fused *rebF* was ligated to the polyubiquitin 3 promoter (PUbq3, GB0272) and the actin terminator (TAct2, GB0210) from *A. thaliana*. To simplify the cloning procedure of the respective genes encoding cytosol-bound catalysts, a modified P35S module, spanning positions 01–13 of the GoldenBraid TU build-up (Fig. 1, [19]), was generated. For verification of successful biosynthesis of recombinant enzymes, *stth* and *rebH* were fused with *yfp* (GB0024) and *rebF* was ligated to *bfp* (GB0043), with the fluorescent partner-protein coding regions occupying position 16 in the TU structure (Fig. 1). The GB2.0 α level-assembled transcriptional units (TUs) were further combined within Ω level vector backbones, yielding appropriate multigene constructs.

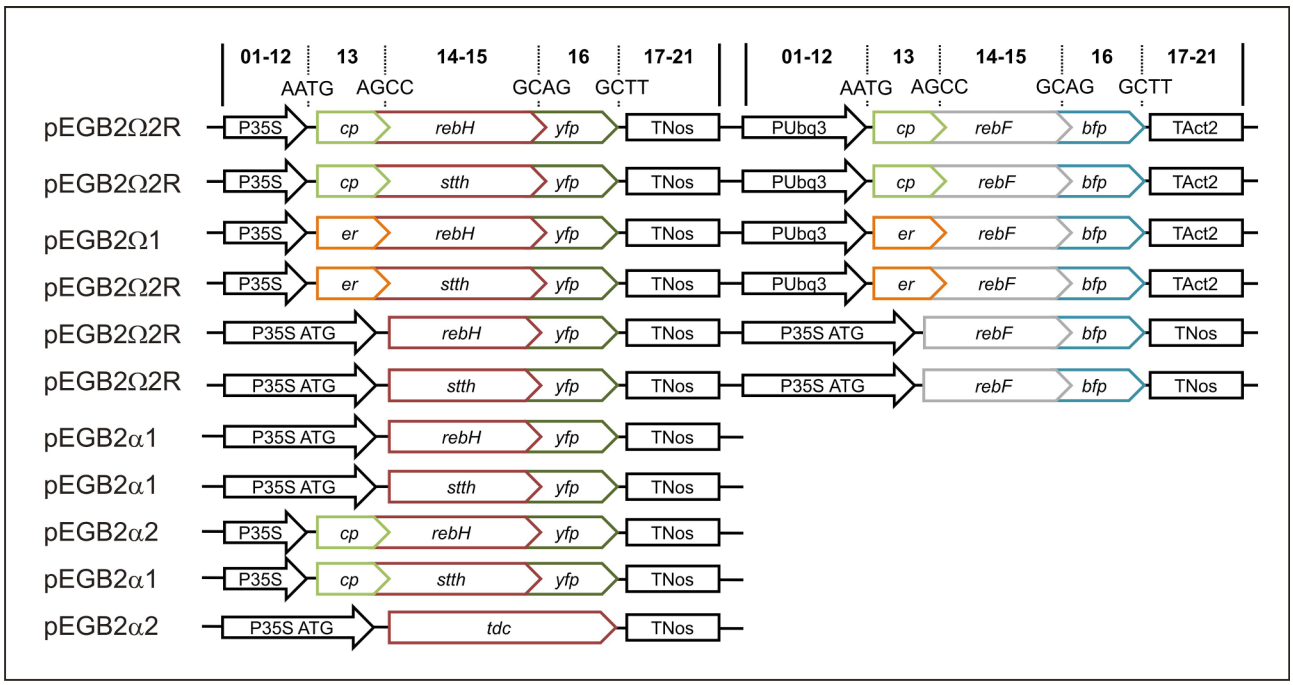

Figure 1. Schematic representation of the expression constructs used in this study. Numbers above the diagram represent standard GoldenBraid classes within the TU structure, while capital letter designations indicate four-nucleotide overhangs ascertaining seamless assembly of GBparts [19]. P35S, cauliflower mosaic virus (CaMV) 35S promoter; P35S ATG, cauliflower mosaic virus (CaMV) 35S promoter with the integrated start codon (spanning GB positions 01–13); *cp*, chloroplast transit peptide sequence; *er*, ER signal peptide sequence; TNos, terminator of the nopaline synthase gene from *A. tumefaciens*; PUbq3, promoter of the polyubiquitin gene from *A. thaliana*; TAct2, terminator of the actin 2 gene from *A. thaliana*.

2.5 Transient transformation of *N. benthamiana* plants

N. benthamiana plants (cultivated at 23°C, 60% humidity and 16/8 h day/night cycle) were used for transient transformation via *Agrobacterium*-mediated gene transfer. Overnight cultures of *A. tumefaciens* EHA105 cells carrying the genes of interest (as well as the pDGB2α2 empty vector, EV serving as a negative control) were centrifuged at 8000 ×*g* for 5 min and the cell pellet was resuspended in the infiltration buffer (10 mM MES, 10 mM $MgSO_4$, 100 µM acetosyringone, pH 5.5) to the final OD_{600} of 1. Bacterial suspensions were infiltrated into leaves (on their abaxial side) of four-week-old plants using a syringe without a needle; all infiltrations were performed in three biological replicates. In case of co-infiltration of alternative individual *Agrobacterium* cultures, the ratios of all relevant constructs were kept constant within each experiment. To analyze brominating potential of the investigated halogenases, the infiltration buffer was supplemented with 40 mM KBr (Carl Roth, Karlsruhe, Germany). The plants were incubated for four to six days and successful biosynthesis of recombinant enzymes was verified post-transformation by fluorescence microscopy (Axioskop 40, Zeiss, Oberkochen, Germany).

2.6 Chromatographic analysis of halogenated metabolites

For the extraction of metabolites from plant samples, 100 mg of frozen leaf powder were homogenized by sonication in 200 µL of 80% v/v methanol for 30 min. Subsequently, the extracts were purified two times from solid particles by centrifugation at 17 000 ×*g* for 10 min at 4°C. 10 µL of each leaf extract were injected onto a 150 × 4.6 mm, 5 µm analytical HPLC column (Zorbax 300SB-C18, Agilent, Santa Clara, CA, USA) embedded within the 1260 Infinity HPLC system (G1379B micro vacuum degasser, G1312B binary pump, G1329B autosampler, G1316A column thermostat, G4212B diode array detector, Agilent, Santa Clara, CA, USA). Metabolites were separated at the flow rate of 1 mL/min and column temperature of 30°C using 0.1% v/v formic acid (A) and acetonitrile (B) as a mobile phase. The following nonlinear mobile phase gradient (% B) was used for the analyses: 0–4 min, 10; 4–9 min, 10 to 30; 9–12 min 30. Finally, the column was flushed with 100% B for 3 min and re-equilibrated with 10% B for additional 3 min. Tryptophan derivatives were detected at the wavelength of 280 nm. Further investigation was conducted by means of the Acquity UPLC system (Waters, Milford, MA, USA) coupled to the Micromass Quattro Premier triple quadrupole mass spectrometer (Waters, Milford, MA, USA). For LC-MS analyses, 10 µL of plant extracts were injected onto the Acquity UPLC BEH C18 analytical column (100 × 2.1 mm, 1.7 µm, Waters, Milford, MA, USA) equipped with the BEH C18 VanGuard pre-column (2.1 × 5 mm, 1.7 µm, Waters, Milford, MA, USA). The column temperature was set at 40°C and the flow rate at 0.25 mL/min. The mobile phase was composed of 0.1% v/v formic acid (A) and acetonitrile (B) and the analyses were performed with the following nonlinear mobile phase gradient (% B): 0.0–2.0 min, 10; 2.0–4.5 min, 10 to 30; 4.5–6.0 min, 30; 6.0–6.5 min, 30 to 100. All remaining molecules were eluted with 100% B for 1.5 min and the column was re-equilibrated with 10% B for 2 min. Metabolites were detected in the positive ESI mode with a full scan MS experiment (m/z 50–1000). Nitrogen was applied as the desolvation gas (400°C, 800 L/h) and cone gas (10 L/h). The capillary voltage was 2.75 kV and the cone voltage was set to 25 V.

3 Results

To evaluate the functionality of Stth in planta, we anticipated screening a considerable number of clones, to wit, combinations of various genes as well as targeting sequences for subcellular localization. To alleviate the cloning effort and enable aggregation of a large number of pathway permutations, we applied the GoldenBraid standard for the design and assembly of expression constructs [19, 21]. Figure 1 depicts the general assembly scheme. Final vectors were mobilized into *Agrobacterium* for transient transformation of *N. benthamiana* plants. Expression of genes of interest in tobacco leaves was evaluated by monitoring the presence of fluorescent proteins serving as fusion partners of the target polypeptide molecules. Plant material was harvested, extracted and analyzed for metabolite formation by HPLC and UPLC-MS.

3.1 Stth is active in plants and forms 6-chlorotryptophan

To test our experimental set-up and the ability of Stth to perform tryptophan halogenation in tobacco leaves, we utilized the gene encoding RebH as a positive control. RebH was shown to function in *C. roseus* when its coding sequence was co-expressed with that of a corresponding flavin reductase, RebF [22]; the latter, essential for the generation of $FADH_2$ ascertaining halogenase activity. As the catalytic capacity of Stth was confirmed also in the presence of an unrelated flavin reductase [9], we incorporated *rebF* to our expression strategy to provide the recombinant Stth with the necessary partner reductase.

Transient expression of *rebH* together with *rebF* facilitating cytosolic localization of the encoded proteins resulted in the formation of a new metabolite, absent from control samples, that exhibited an HPLC t_R value of 7.9 min (Fig. 2B). UV-absorption spectrum of the molecule was similar to that of tryptophan (220 nm, 280 nm), featuring two maxima at 222 and 283 nm (Supporting informa-

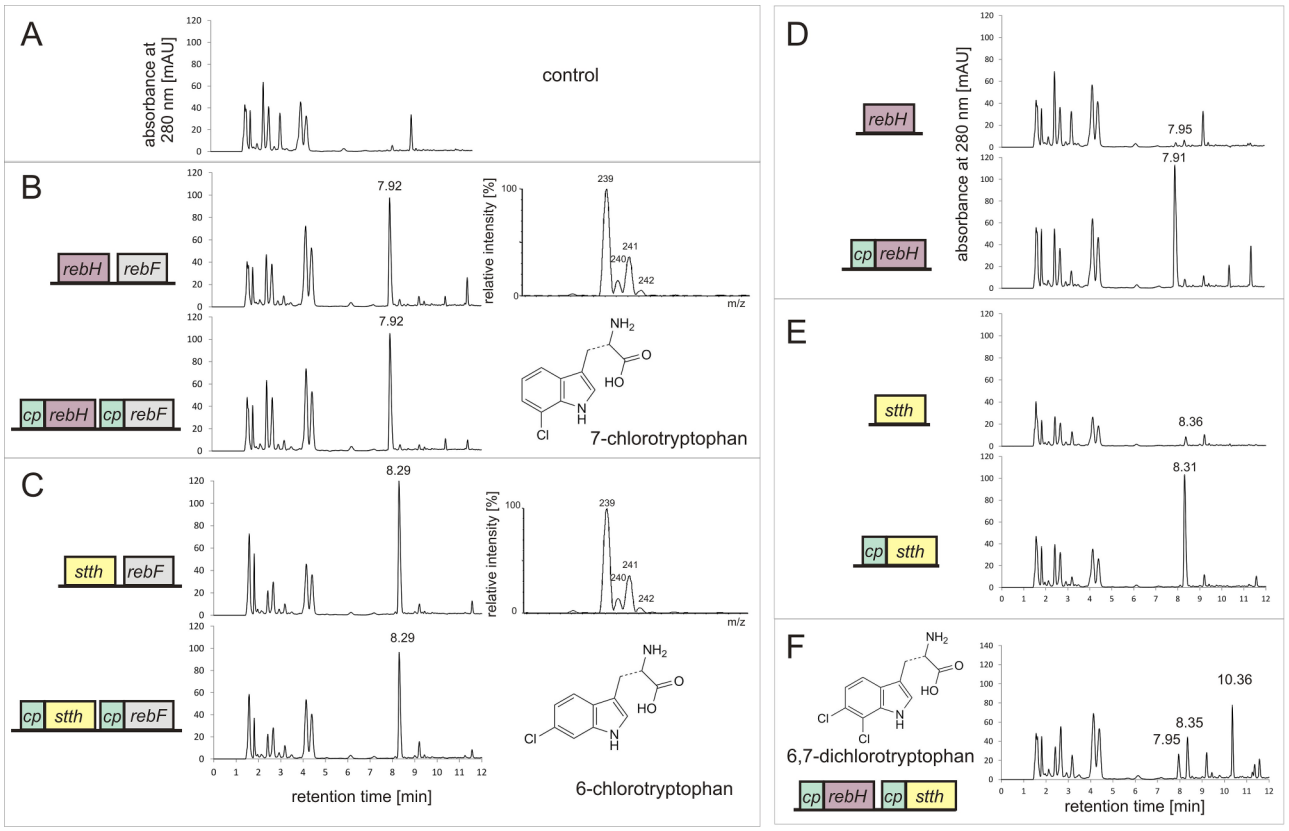

Figure 2. UPLC-MS analysis of plant extracts after infiltration with differentially targeted halogenase and reductase constructs. (**A**) Negative control, empty vector. (**B**) *RebH* and *rebF*, without (upper panel) or with (lower panel) chloroplast targeting (*cp*); mass analysis shows a pair of $[M]^+$ ions at m/z 239 and 241 in a 3:1 ratio. (**C**) *Stth* and *rebF*, without (upper panel) or with (lower panel) chloroplast targeting (*cp*) and $[M]^+$ ions at m/z 239 and 242. Comparative analysis of cytosolic and chloroplast-targeted halogenase expression constructs without co-expression of *rebF*: *rebH* and *cp:rebH* (**D**), *stth* and *cp:stth* (**E**). (**F**) Parallel expression of both *cp:rebH* and *cp:stth*.

tion, Fig. S1). The observed shift could be attributed to substitution of a chloride ion into the heteroaromatic ring system. Further LC-MS analyses revealed an m/z value of 239 in the positive ionization mode. Although unequivocal identification of the metabolite was impeded by the lack of the relevant standard compound, the obtained evidence emphatically pointed to it being 7-chlorotryptophan. This outcome was in clear accord with previous results obtained for *C. roseus*, confirming tobacco as a suitable host for functional recombinant RebH/RebF tandem.

Subsequent substitution of *rebH* with *stth* led to identification of an alternative metabolite eluting after 8.3 min, characterized by a mass spectrum exhibiting two m/z values of 239 and 241 consistent with those of monochlorinated tryptophan (Fig. 2C) and absorbing UV light at 227 and 284 nm (Supporting information, Fig. S2). Thus, detection of 6-chlorotryptophan, as synthesized by recombinant Stth, was corroborated.

For the conclusive authentication of tryptophan derivatives synthesized by Stth and RebH, retention time values of 6- and 7-chlorotryptophan, as detected in plant extracts, were compared to those of relevant standard compounds by means of LC-MS, using a modified nonlinear mobile phase gradient (Supporting information, supporting materials and methods). Due to the unavailability of the 7-chlorotryptophan reference metabolite, plant extracts characteristic of cytosolic RebH were spiked with 5- and 6-chlorotryptophan. However, as 7-, 5- and 6-chlorotryptophan derivatives were not separated efficiently under the applied LC conditions, revealing t_R values of 5.21 min, 5.31 min and 5.59 min, respectively (Supporting information, Fig. S3 upper panel), proof of regiospecific halogenation by Stth and RebH was obtained through comparative analysis with brominated tryptophan standard compounds (Section 3.2).

3.2 Bromination

Since both halogenases were reported capable of introducing bromide ions into the tryptophan scaffold in vitro [9, 22], we set out to examine whether the activity was heterologously translated into plants. Therefore, KBr solution (40 mM) was co-infiltrated along with *Agrobacterium*

cells harboring the respective halogenase expression constructs. As shown in Supporting information, Fig. S4, brominated tryptophan was detected upon analysis of extracts from *stth*- and *rebH*-infiltrated plants. Mass spectra of the newly generated 6- and 7-substituted amino acid molecule exhibited m/z values of 283 and 285, with identical signal intensities consistent with those characteristic of a mono-brominated tryptophan structure.

LC-separation of authentic 7-, 5- and 6-bromotryptophan was sufficient for effective metabolite identification, with their respective t_R values of 5.94 min, 6.11 min and 6.35 min (Supporting information, Fig. S3 lower panel) consistent with those recorded for the putative 7- and 6-bromotryptophan synthesized by RebH and Stth after KBr supplementation. Additionally, extracts of *stth*- and *rebH*-infiltrated plants, containing the putative 6-chloro-, 6-bromo- or 7-bromotryptophan, were enriched with the appropriate standard compounds and analyzed by LC-MS. The increase of peak areas in the spiked extracts further confirmed regioselective halogenation of the amino acid substrate by the investigated recombinant enzymes (Supporting information, Fig. S5–S7).

Quantification of halotryptophans (Supporting information, supporting materials and methods) revealed cytosolic 7- and 6-chlorotryptophan levels reaching 77.8 ± 16.6 ng/mg FW (fresh weight) and 70.3 ± 13.0 ng/mg FW, respectively, five days after infiltration. Following KBr supplementation, the respective 7- and 6-bromotryptophan concentrations amounted to 81.5 ± 2.9 ng/mg FW and 42.1 ± 21.4 ng/mg FW, within the same incubation period.

3.3 Cooperativity of bacterial halogenases with plant flavin reductases

Using the modular set-up of expression construct design, we further evaluated the effect of varied subcellular localization of RebH/RebF and Stth/RebF on their catalytic activity. When either of the halogenase/reductase combination was targeted to the chloroplasts, the respective specific products, 7- and 6-chlorotryptophan were detected (Fig. 2B and 2C). In contrast, directing them to the ER/apoplastic space yielded no significant amounts of chlorinated tryptophan (data not shown). To verify the correct subcellular localization of the halogenases, fusions of either RebH or Stth with YFP were visualized under a confocal laser scanning microscope (Supporting information, Fig. S8 and supporting materials and methods).

To determine whether plants contain endogenous flavin reductases able to cooperate with the investigated bacterial halogenases, or if free $FADH_2$ is present in sufficient amounts, we tested the functionality of RebH and Stth without co-expression of *rebF*. As shown in Fig. 2D and 2E, the specific products, 6- and 7-chlorotryptophan were detected after sole expression of *stth* and *rebH*, respectively, but only in trace amounts when the enzymes were located in the cytosol. On the other hand, targeting the halogenases to the chloroplasts resulted in significant yields of the relevant tryptophan derivatives, comparable to those augmented by the recombinant cytosolic RebF. The outcome points to plastids as a valuable cache of reduction equivalents driving metabolic reactions.

3.4 Concerted activity of Stth and RebH leads to formation of 6,7-halotryptophan

Next, we wished to examine if a combination of the two halogenases yielded di-halogenated tryptophan. Their capacity to introduce a second chloride atom to the chlorinated amino acid molecule was investigated by transient co-transformation of *N. benthamiana* with *rebH* and *stth*. To evaluate the biosynthesis of dichlorotryptophan in the cytosol, both halogenases were co-localized with the partner reductase, RebF. Additionally, tandem halogenation in chloroplasts was analyzed without RebF. Besides the formation of 6- and 7-chlorotryptophan, co-localization of Stth and RebH correlated with accumulation of a third metabolite, eluting after 10.36 min in HPLC analyses (Fig. 2F). Further LC-MS investigation revealed an m/z value of 273 in positive ionization mode, while the relevant isotope pattern gave proof of effective dichlorotryptophan biosynthesis in planta (Supporting information, Fig. S9). Interestingly, trace amounts of the molecule were also detectable in control samples representative of RebH alone, suggesting that the enzyme exhibits residual activity toward 6-halogenation and is, to some extent, capable of producing dichlorotryptophan [23]. This became evident upon analysis by UPLC-MS (Supporting information, Fig. S10). While Stth produced solely 6-chlorotryptophan, RebH-containing plants accumulated 6,7-dichlorotryptophan as well.

The tandem activity of RebH and Stth was also examined after co-transformation of both relevant gene constructs and subsequent infiltration of 40 mM KBr. Compared to negative control samples, co-expression of *rebH* and *stth* correlated with the formation of a novel metabolite that eluted after 5.97 min and revealed m/z values of 361, 363 and 365 with signal intensity ratios of 1:2:1, compatible with a di-brominated tryptophan molecule (Supporting information, Fig. S11). Moreover, an alternative metabolite exhibiting a t_R value of 5.83 min and m/z values of 317, 319 and 321 was observed (Supporting information, Fig. S11). According to the detected mass and the characteristic mass spectrum, this molecule was, most likely, that of a mono-chlorinated and mono-brominated tryptophan.

Finally, biosynthesis of the observed metabolites occurred independently of co-localization of both halogenases in chloroplasts or the cytosol, showing that subcellular context had no bearing on enzyme activity as long as the abundance of reducing equivalents was ensured.

3.5 Formation of halogenated tryptamine, a precursor of monoterpenoid indole alkaloids

Tryptophan is a vital precursor driving biosynthesis of numerous specialized plant metabolites. We, therefore, addressed the question of integration of its heterologously generated 6- or 7-chlorinated derivatives into pathways leading to the formation of highly valuable monoterpenoid indole alkaloids (MIAs). To provide the relevant answer, we included *tdc*, a gene encoding tryptophan decarboxylase (TDC), in our modular cloning set-up and expression approach. Analysis of extracts derived from plants expressing *tdc* together with either *rebH* or *stth* resulted in detection of 7- and 6-chlorotryptamine, respectively (Fig. 3). Again, once appropriate reducing conditions were ascertained, halometabolite accumulation proved independent of subcellular context, as no differences between the cytosolic and chloroplast-derived yields were recorded. It ought to be noted that TDC was located solely in the cytosol, as its targeting to the chloroplasts had an adverse impact on plant viability (data not shown), possibly due to tryptophan depletion. However, co-expression of *rebH* and *stth* together with *tdc* did not lead to the formation of detectable levels of dichlorotryptamine. Results thus obtained confirmed the potential of Stth to serve as a valuable tool for the production of new-to-nature, halogenated indole alkaloids.

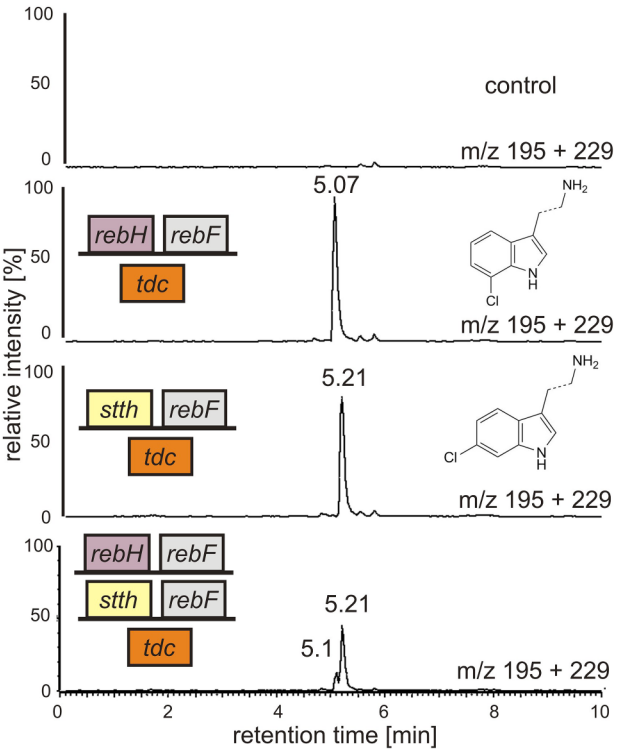

Figure 3. Product formation after co-expression of halogenase/reductase construct combinations together with tryptophan decarboxylase gene (*tdc*). While 7-chlorotryptamine (t_R, 5.07 min) and 6-chlorotryptamine (t_R, 5.21 min) could be detected, no double halogenation occurred.

4 Discussion

Our transient expression approach allowed us to demonstrate that the investigated tryptophan 6-halogenase, Stth was highly active in *N. benthamiana* and could serve as a valuable tool to efficiently generate novel metabolites in planta. Comparison with the tryptophan 7-halogenase, RebH pointed to analogous activity rates of both catalysts toward their primary amino acid substrate. The detected halometabolite signals represented the most prominent peaks in the chromatograms of the analyzed plant extracts, suggesting significant accumulation of the newly formed products. Further, the observed halotryptophan yields exceeded those of the amino acid itself, indicating no sizeable turnover or degradation of the engineered compounds. Thus, the factors determining the biosynthetic halotryptophan output levels seem to be the activity of involved enzymes as well as the precursor (Trp) and cofactor ($FADH_2$) availability.

Since flavin-dependent halogenases rely on the catalytic cooperation of flavin reductases, we initially used RebF from *L. aerocolonigenes* to determine in planta activity of Stth. The interchangeability of the augmenting enzymatic partners was previously demonstrated by Zeng and Zhan. [9]. Indeed, while sole expression of *stth* did not result in significant conversion of tryptophan in the cytosol, co-expression of *rebF* restored the activity of the enzyme, showing that flavin-dependent halogenases are promiscuous toward their partner reductase.

However, most striking in our studies was the observation that tryptophan halogenation took place when either RebH or Stth was translocated to the chloroplasts, even when the reductase, RebF was not co-integrated. For efficient halogenation, all relevant flavin-dependent catalysts require reduced FAD, chloride ions and oxygen as co-substrates [24]. While O_2 should be present in chloroplasts in sufficient amounts due to photosynthetic activity and chloride levels reach up to 100 mM [25], our findings corroborated the ubiquity of FAD and its reduction to diffusible $FADH_2$ in the organelles, providing the necessary reducing capacity for halogenation reactions. Since co-localization of RebF in the chloroplasts did not yield higher amounts of chlorinated tryptophan, it can be assumed that $FADH_2$ supply is not the rate-limiting factor of plastidal halogenation. Further, our contention is in perfect alignment with chloroplasts constituting original biosynthetic hubs of plant FAD, generated therein by virtue of monofunctional flavin synthases [26], rendering its conversion to $FADH_2$ by reductases or other proteins involved in the electron transfer of the photosystem highly likely.

Contrarily, dispensing with RebF co-incorporation in course of functionality studies of cytosolic RebH and Stth

abolished production of halogenated tryptophan, showing clearly that endogenous $FADH_2$ supply is a chloroplast-exclusive feature. Furthermore, no halogenase activity was detected after translocation of the investigated enzymes to the ER and, finally, to the apoplast. This observation could stem from the absence of tryptophan from the apoplast, demonstrated previously in four-week-old tomato plants [27]. Additionally, degradation of halogenases by proteases located in the apoplast might account for the loss of enzymatic activity [28].

Double halogenation of substrate molecules by RebH was previously observed by Payne *et al.* [23] in vitro assays, wherein long incubation periods facilitated binary substitution of a diverse assortment of aromatic metabolites. A corresponding effect was noted in case of plant-produced RebH; however, the amount of 6,7-halogenated tryptophan was significantly higher when *stth* was co-expressed.

The considerable catalytic activity of the investigated halogenases enabled efficient bromination of tryptophan, leading to high retrieval rates of the resulting amino acid derivatives in leaves of *N. benthamiana* after no more than five days of incubation. Quantification of the newly formed halometabolites by integration of HPLC peak areas, based on the analysis of serial dilutions of the relevant standard compounds, revealed that 6-bromotryptophan levels, after *stth* expression and KBr supplementation, were commensurate with those of concomitantly formed 6-chlorotryptophan. Moreover, upon KBr co-infiltration, RebH synthesized roughly double the amount of 7-bromotryptophan as compared to the 7-chlorinated amino acid derivative. According to published data, RebH brominates tryptophan with a k_{cat} value of 0.4, about three times lower than that characteristic of chlorination, indicating that bromide levels exceeded those of chloride within the cytosol post-infiltration [22]. The average chloride concentration in the cytoplasm of higher plants ranges between 3 and 10 mM [29]. Further, anions are actively transported into the cell by anion/proton symporters while being released by passive fluxes through anion channels located in the plasma membrane [29]. The observed increased rates of bromination of tryptophan suggest active transport of bromide ions into the cytoplasm after leaf infiltration, resulting in bromide levels that probably exceeded inherent cytosolic chloride concentrations, thereby promoting biosynthesis of bromotryptophan.

In summary, characterization of bacterial tryptophan halogenases, RebH and Stth, undertaken herein unveiled various possible applications of these enzymes in facilitating the biosynthesis of expensive and rare molecules, such as chlorotryptophan, dichloroptryptophan, chlorotryptamine, bromotryptophan and dibromotryptophan in planta. In concert with the described transient approaches, generation of stable transgenic tobacco plants would spur constitutive production of the aforementioned valuable halometabolites. Moreover, target-oriented biosynthesis of particular compounds could be achieved through establishment of relevant cell suspension cultures. Modification of culture medium composition through chloride elimination or bromide supplementation would then foster the generation of one specific molecule. Additionally, including tryptophan in the culture medium might further increase yields of final products of interest. Withal, the variety of halogenated metabolites could be magnified by virtue of heterologous integration of additional regiospecific tryptophan halogenases. While PyrH from *Streptomyces rugosporus* was shown to be functional in *C. roseus*, incorporating the halo-atom at position C5 of the amino acid scaffold [13, 30], no flavin-dependent tryptophan 4-halogenase has been described to date. Lastly, combination of diverse halogenases, bolstered by the catalytic activity of TDC and supplementation of bromide might facilitate the biosynthesis of a tremendous variety of mono-, di- or tri-halogenated tryptophan and tryptamine derivatives in planta, thus providing a compelling platform for the generation of indole-derived natural and non-natural compounds (i.a. MIAs) via metabolic engineering.

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5 References

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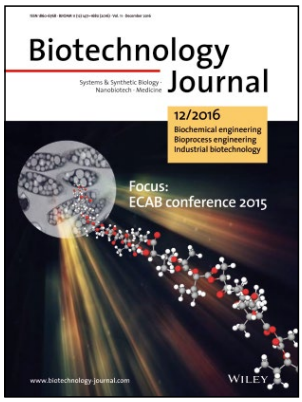

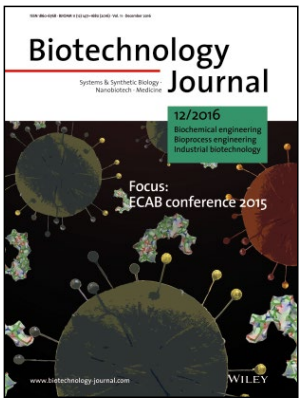

Cover illustration

This issue focuses on the ECAB conference and is edited by Professors Raquel Barros, Jochen Büchs, and Guilherme Ferreira. Transmission electron micrograph (circle) of metabolically engineered *E. coli* cells accumulating polyhydroxyalkanoates as white distinct granules. Poly(2-hydroxyisovalerate-co-lactate) structure out of the cell is schematically shown. The Image is provided by Jung Eun Yang, Je Woong Kim, Young Hoon Oh, So Young Choi, Hyuk Lee, A-Reum Park, Jihoon Shin, Si Jae Par, and Sang Yup Lee authors of "Biosynthesis of poly(2-hydroxyisovalerate-co-lactate)by metabolically engineered Escherichia coli". Cover image created by Jung Eun Yang and Sang Yup Lee (http://dx.doi.org/10.1002/biot.201600420).

Inside cover illustration

This issue focuses on the ECAB conference and is edited by Professors Raquel Barros, Jochen Büchs, and Guilherme Ferreira. Existing purification technologies for retroviral vectors result in very low yields of infective particles, often associated with the absence or loss of the envelope protein at the surface of viral particles. A peptide selective for amphotropic viral particles was discovered by panning a phage display peptide library, and employed to selectively capture and recover bound particles under gentle conditions. The Image is provided by Cláudia S. M. Fernandes, Inês Barbosa, Rute Castro, Ana Sofia Pina, Ana Sofia Coroadinha, Ana Barbas, and A. Cecília A. Roque authors of "Retroviral particles are effectively purified on an affinity matrix containing peptides selected by phage-display" (http://dx.doi.org/10.1002/biot.201600025).

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