Phytochemistry 175 (2020) 112371

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Hairy root transformation of *Brassica rapa* with bacterial halogenase genes and regeneration to adult plants to modify production of indolic compounds

Madeleine Neumann[a], Swantje Prahl[b], Lorenzo Caputi[c], Lionel Hill[d], Baldeep Kular[d], Antje Walter[a], Eugenio P. Patallo[e], Daniela Milbredt[e], Alfredo Aires[f], Maria Schöpe[b], Sarah O'Connor[c], Karl-Heinz van Pée[e], Jutta Ludwig-Müller[a,*]

[a] Faculty of Biology, Institute of Botany, Technische Universität Dresden, 01062, Dresden, Germany
[b] Vita34 AG, 04103, Leipzig, Germany
[c] Department of Natural Product Synthesis, Max Planck Institute for Chemical Ecology, 07745, Jena, Germany
[d] John Innes Centre, Norwich Research Park, Norwich, NR4 7UH, United Kingdom
[e] Biochemistry, Technische Universität Dresden, 01062, Dresden, Germany
[f] Centre for the Research and Technology for Agro-Environment and Biological Sciences, CITAB, University of Trás-os-Montes e Alto Douro, 5001-801, Vila Real, Portugal

ARTICLE INFO

Keywords:
Agrobacterium rhizogenes
Brassica rapa subsp. pekinensis
Brassicaceae
Chinese cabbage
Hairy root cultures
Plant regeneration
Cl-IAN
Cl-trp
Flavin-dependent tryptophan halogenases

ABSTRACT

During the last years halogenated compounds have drawn a lot of attention. Metabolites with one or more halogen atoms are often more active than their non-halogenated derivatives like indole-3-acetic acid (IAA) and 4-Cl-IAA. Within this work, bacterial flavin-dependent tryptophan halogenase genes were inserted into *Brassica rapa* ssp. *pekinensis* (Chinese cabbage) with the aim to produce novel halogenated indole compounds. It was investigated which tryptophan-derived indole metabolites, such as indole glucosinolates or potential degradation products can be synthesized by the transgenic root cultures. *In vivo* and *in vitro* activity of halogenases heterologously produced was shown and the production of chlorinated tryptophan in transgenic root lines was confirmed. Furthermore, chlorinated indole-3-acetonitrile (Cl-IAN) was detected. Other tryptophan-derived indole metabolites, such as IAA or indole glucosinolates were not found in the transgenic roots in a chlorinated form. The influence of altered growth conditions on the amount of produced chlorinated compounds was evaluated. We found an increase in Cl-IAN production at low temperatures (8 °C), but otherwise no significant changes were observed. Furthermore, we were able to regenerate the wild type and transgenic root cultures to adult plants, of which the latter still produced chlorinated metabolites. Therefore, we conclude that the genetic information had been stably integrated. The transgenic plants showed a slightly altered phenotype compared to plants grown from seeds since they also still expressed the *rol* genes. By this approach we were able to generate various stably transformed plant materials from which it was possible to isolate chlorinated tryptophan and Cl-IAN.

1. Introduction

The need for new remedies and industrial production of so-called phytochemicals continues to be a burning question. *In vitro* cultivation is a powerful tool, but conventional field production or even wild collection is mostly used for large-scale production of medicinal plants and supply of plant remedies. Therefore, in recent years the production of metabolites in so-called hairy roots as well as the modification of metabolites by means of enzymatic derivatization has been recognized as an attractive alternative. The infection of plants by *Agrobacterium rhizogenes* can be used to generate hairy root cultures (Nilsson and Olsson, 1997; Sevon and Oksman-Caldentey, 2002). Such root cultures are an interesting and profitable research system for biotechnological laboratories because of their simple cultivation and increased production of specialized metabolites compared to green plants (Hamill et al., 1987; Shanks and Morgan, 1999). Various reviews describe a variety of substances such as ginkgolide (aging disorders), coniferin (anti-cancer) or morphine (sedative), which can already be produced in transformed roots of various plant species (Guillon et al., 2006; Chandra and Chandra, 2011).

In addition to the bacterial *rol* genes necessary for generation of root cultures, other genes from plants or other organisms such as yeast or

* Corresponding author.
E-mail address: Jutta.Ludwig-Mueller@tu-dresden.de (J. Ludwig-Müller).

https://doi.org/10.1016/j.phytochem.2020.112371
Received 28 November 2019; Received in revised form 31 March 2020; Accepted 1 April 2020
0031-9422/

bacteria, can be stably integrated and expressed. In consequence, changes in the metabolite spectrum can occur. Pioneering work has been done by Hamill et al. (1990), who were able to increase nicotine accumulation in transgenic roots of tobacco by overexpressing a yeast gene encoding an ornithine decarboxylase. Other leading work used the downregulation of genes to alter the ratio of desired metabolites in the transgenic root cultures (Chintapakorn and Hamill, 2003). A more recent example related to this study is the generation of transgenic roots from *Catharanthus roseus* transformed with bacterial halogenase genes that are able to produce chlorinated alkaloids (Runguphan et al., 2010). The halogenases employed belong to the flavin-dependent tryptophan halogenases, which halogenate the indole ring of tryptophan regioselectively at different positions (Zhu et al., 2009). In addition, other halogenases and haloperoxidases such as non-haem iron halogenases, haem or vanadium haloperoxidases are known (Neumann et al., 2008; Butler and Sandy, 2009).

The particular interest in the mechanism of halogenation is based on the observation that halogenated metabolites show an increased bioactivity compared to non-halogenated ones. Illustrative examples are the increased effects of 4-Cl-IAA on the development of pericarp over IAA (Reinecke et al., 1995) and the decrease in the antibacterial effect of vancomycin as soon as the number of available chlorine atoms is reduced (Sun et al., 2004). Our previous work showed that expression of halogenase genes from different bacterial species (Hammer et al., 1997; Kirner et al., 1998; Zehner et al., 2005; Seibold et al., 2006) can lead to the production of Cl-Trp, Cl-IAN and Cl-IAA in *Arabidopsis thaliana* plants that were stably transformed (Patallo et al., 2017). Also, bacterial halogenase genes could be expressed transiently in tobacco also leading to chlorination of tryptophan derivatives (Fräbel et al., 2016).

Chinese cabbage (*Brassica rapa* spp. *pekinensis* (Lour.) Hanelt) produces a high number of indolic compounds from tryptophan (Radwanski and Last, 1995; Kim et al., 2010; Pedras and Yaya, 2010; Bhandari et al., 2015) and other specialized metabolites which provide the potential of pharmaceutical or food application. Beside its cultivation as an agricultural crop plant there are many reports on Brassicaceae (Murata and Orton, 1987), among them some on *B. rapa*, for which *in vitro* cultivation protocols are available (Cogbill et al., 2010; Khan et al., 2010). Because *B. rapa* offers natural production of valuable compounds and generates biomass easily, there have been attempts to genetically modify this species (Qing et al., 2000; Baskar et al., 2016) to unleash its potential as a bioreactor for the production of valuable molecules. Transformed roots are a well described tool for production of metabolites, but they are limited to *in vitro* cultivation. Furthermore, they represent only one specific type of plant tissue and additional potential for production of various metabolites might be restricted to other tissue types. Regeneration of whole plants from transformed root cultures followed by cultivation in the green house for large scale production is therefore a logical consequence. Regeneration of shoots from transformed roots is a well described technique for several plant species (Christey, 2001). However, in case of *B. rapa* only a few reports on regeneration of transgenic plants are available (Qing et al., 2000; Baskar et al., 2016).

The aim of this work was twofold. First, we wanted to integrate the bacterial flavin-dependent tryptophan *hal* genes *pyrH*, *thal* (or *thdH*) and *prnA* into the plant genome of *B. rapa* by generating transgenic root cultures (Fig. 1). Subsequently, it should be examined whether novel chlorinated indole-derived metabolites are produced and whether production could be enhanced by altering the growth conditions. In contrast to previous work using the genes of tryptophan halogenases we have chosen Chinese cabbage since this species is known to produce a variety of interesting bioactive indolic compounds such as indole glucosinolates and indole phytoalexins (Kim et al., 2010) that are different from those produced for example in *A. thaliana* (Rauhut and Glawischnig, 2009). This suggested that transgenic *Brassica* root cultures could form a variety of novel chlorinated indolic substances not yet produced before (see also Fig. 2). Second, we wanted to achieve the successful regeneration of mature plants from transformed *B. rapa* root cultures carrying a bacterial tryptophan halogenase gene and to demonstrate their stable transformation (Fig. 1), since these can be later stored as seeds. In addition, adult plants can be cultivated in bioreactors which gives higher biomass and in consequence possibly also yield of the desired phytochemical (Paek et al., 2001; Takayama and Akita, 2005).

2. Results and discussion

2.1. Which bioactive indole metabolites are synthesized in Brassica rapa plants and wild type root cultures?

So-called hairy roots are considered as stable and easy to cultivate variants for the production of specialized metabolites of value by transformation with wild type *A. rhizogenes* (Chandra and Chandra, 2011; Ludwig-Müller et al., 2014; Häkkinen et al., 2016). They contain a variety of compounds either in comparable or sometimes even higher concentrations than the mother plant (Georgiev et al., 2015). However, sometimes the metabolite levels are below the ones from the plant they stem from and then production can be increased by elicitation or genetically modifying such cultures (Chandra and Chandra, 2011; Ramirez-Estrada et al., 2016). Transgenic root cultures can be generated when genetically altered *A. rhizogenes* strains are used by addition of new genes of interest.

The indole glucosinolates (IGL) of *Brassica* species and their hydrolysis products have been implied to be important for flavor, smell and nutritional properties (Aires et al., 2019). We compared the production of metabolites between wild type root cultures (for the molecular verification of the transformed roots see Fig. 3) of *B. rapa* and parts of the host plant, because recent work showed that root cultures produce significant amounts of glucosinolates (Petersen et al., 2018; Aires et al., 2019). The amount of total IGL differs significantly between leaves (160–580 μmoles 100 g^{-1} dry wt) and roots (1740–1960 μmoles 100 g^{-1} dry wt) of mature plants, but between plant roots and transformed roots no significant difference was detected (Fig. S3A).

Over the years, there were reports that plant signals such as salicylic (SA) or jasmonic acids (JA) differentially induced IGL in leaves and/or roots of *B. rapa* ssp. *pekinensis* (e.g. Ludwig-Müller et al., 1997; Zang et al., 2015). In the study of Aires et al. (2019) SA and JA, in addition to using yeast extract as an elicitor, were employed. Furthermore, growth conditions were altered (light instead of darkness, altered temperatures, namely 8 °C and 37 °C). The induction of the IGL by elicitor treatment and altered growth conditions was possible, but the variation between individual transformed root lines was also very high (Aires et al., 2019). Lowering the temperature to 8 °C can increase indole derivatives including IGL as shown for some *A. thaliana* accessions (Kissen et al., 2016). Kastell et al. (2017) showed an increased IGL production in transformed roots of *Eruca sativa* after treatment with ethephon and JA and Guo et al. (2013) confirmed an induction of IGL by JA in *A. thaliana*. Cultivation in the light compared to darkness of the transformed root cultures decreased the IGL fraction, most likely because the light cultivation is a stress condition for transformed roots adapted to growth in darkness. In *A. thaliana* plants cultivated in darkness, however, the total glucosinolate content was reduced after several days most likely due to carbon starvation (Brandt et al., 2018). Such strategies can be further exploited to increase the respective fraction of compounds (see also 2.5).

In addition to the IGL fraction, *Brassica* species also contain a plethora of other bioactive compounds such as indole phytoalexins which are important defense compounds (Pedras and Yaya, 2010). However, we were never able to detect any indole phytoalexins. Since the transformed roots grow under sterile conditions, it was tested whether these antimicrobial compounds could be elicited as well, but this was not the case (data not shown).

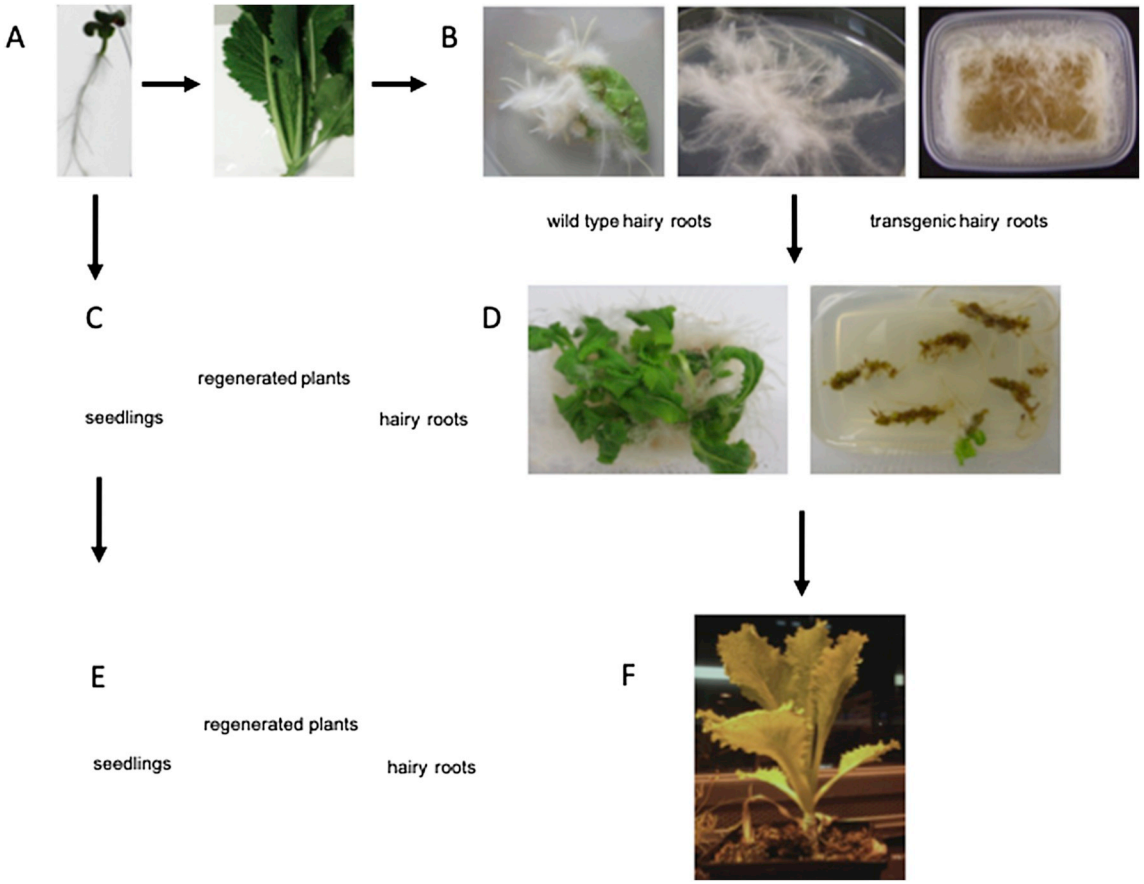


Fig. 1. Experimental scheme showing the different types of plant materials generated. A. Mature wild type plants/seedlings; B. Wild type and transgenic root cultures; C. Regenerated sterile plants from wild type seedlings; D. Regenerated sterile plants from wild type and transgenic root cultures; E. Adult plants in soil from wild type cultures; F. Adult plants in soil from transgenic root cultures.

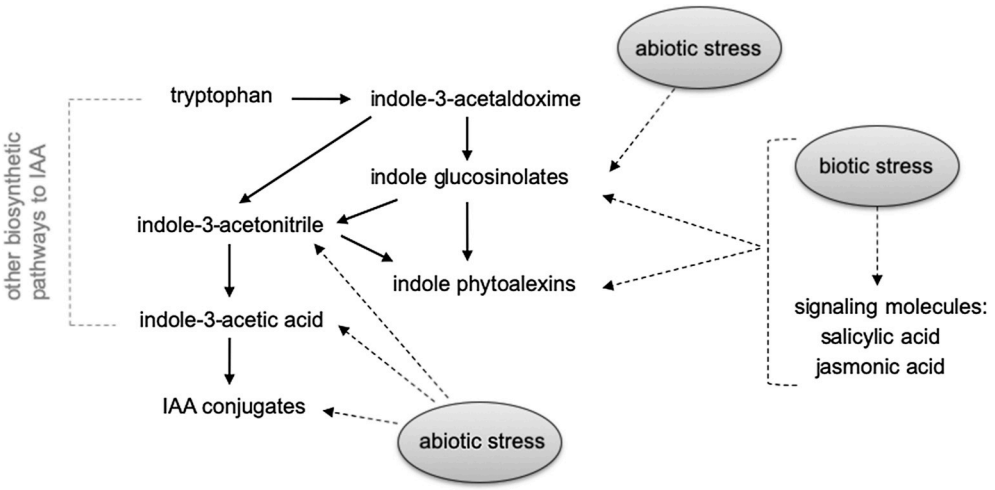
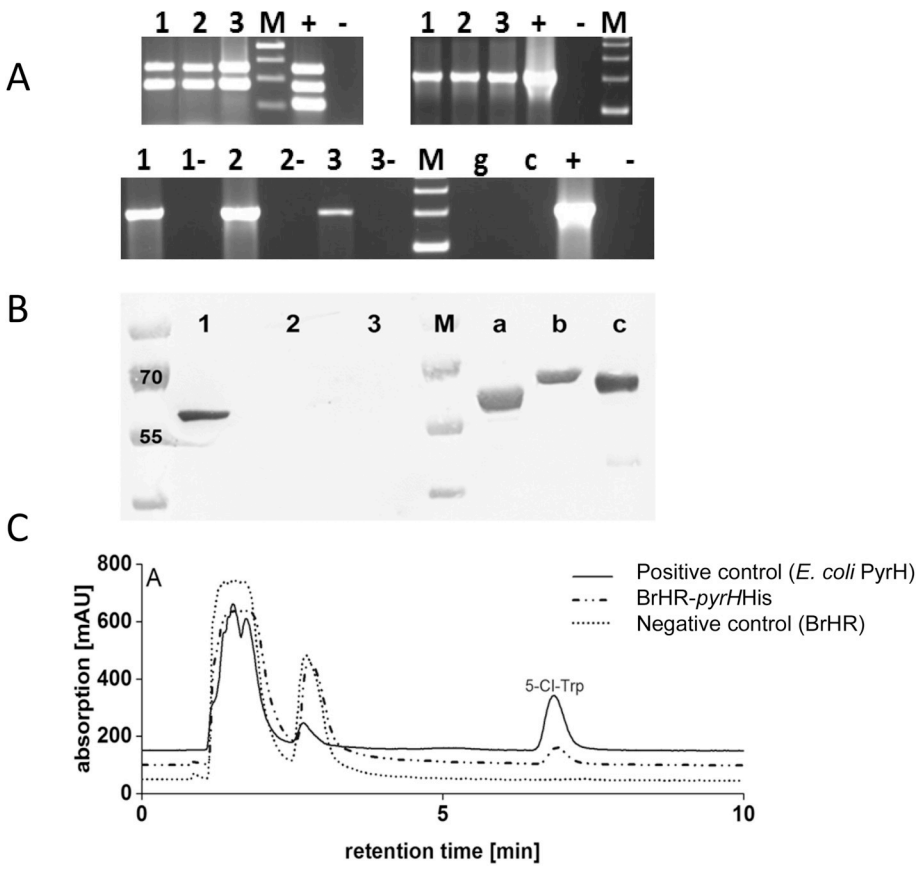


Fig. 2. Expected indole metabolites and their interconversion (in black) that could be derived from tryptophan via the indole glucosinolate/indole phytoalexin pathway. It is indicated (in grey) that there are alternative pathways to IAA. The possible induction (dashed arrows) of chlorinated metabolites by abiotic and biotic stress factors, the latter also via the signaling molecules salicylic acid and jasmonic acid, is shown.

2.2. Which chlorinated compounds can be synthesized by wild type root cultures from Cl-tryptophans?

The main possible biosynthetic indole compounds derived from Trp are shown in Fig. 2. The wild type root cultures can generally produce chlorinated compounds with Cl-Trp as substrate when incubated with 100 μM of 5-, 6- and 7-Trp (Table 1). Without addition of Trp derivatives and in samples after incubation with non-chlorinated tryptophan, no chlorinated compounds were detected. For all chlorinated Trp derivatives used as substrate we found a considerable uptake into the plant cultures and also the production of Cl-IAN. After the addition of 5-Cl-Trp, the highest amount of Cl-IAN was produced and with 6-Cl-Trp the lowest. The production of chlorinated IAA was also detectable after incubation with 5-, 6- and 7-Cl-Trp (Table 1). Similarly, Tivendale et al. (2012) showed the ability of pea plants to convert Cl-Trp (in this case the 4-Cl derivative) to the follow-up products of the IAA biosynthesis pathway. Also, Fräbel et al. (2016) showed that other pathway enzymes are able to use Cl-Trp as a substrate. They performed their experiments in a background that could not produce other Cl-derivatives from Cl-Trp (tobacco), but after additional transformation with a tryptophan



Halogenase	no. of lines	Cl-Trp found	Cl-IAN found
PyrHHis	5	5	3
PyrH	5	3	3
ThalHis	5	0	0
Thal	5	1	0
PrnAHis	5	3	0
PrnA	5	3	0

Fig. 3. A. All transgenic root lines were analyzed by the following experiments and the data are presented for selected lines transformed with the pMDC32 + 2xCaMV35S:*pyrH*:nosT construct (*pyrH* = 5-Cl-Trp-forming). Transformation of roots with *A. rhizogenes* was verified using gDNA and cDNA for a successful insertion and expression, respectively. A. Upper panel: Amplified *rolB* (423 bp) and *rolC* (626 bp) for three different lines (lanes 1–3) using gDNA. The *virG* gene (350 bp) was only detectable in the positive control (Ri-plasmid of *A. rhizogenes*) (lane +) "-" denotes a negative PCR control. Lower panel: Integration of full length *hal* gene (ca. 1.5 kb) using gDNA. Expression of full length *hal* gene (ca. 1.5 kb) using cDNA. "+": positive control (plasmid containing *pyrH* or the other *hal* genes), "-": negative PCR control, g: gDNA wild type, c: cDNA wild type, 1–3: three independent transgenic root culture lines with the pMDC32 + 2xCaMV35S:*pyrH*:nosT construct, "1-"-"3-": RT negative controls (containing no DNA). B. Western blot with the purified His-tagged proteins: 1: PyrH, 2: ThaI, 3: PrnA, a: PyrH synthesized in *E. coli*, b: positive control ThaI synthesized in *E. coli*, c: positive control PrnA synthesized in *E. coli*. Wild type protein as control did not show any signal (data not shown). C. Enzyme assay with the purified halogenase PyrH. The positive control is PyrH protein synthesized in bacteria. The negative control is purified protein from wild type root cultures. Since only for PyrH enzyme activity could be detected, the data for the other halogenases are shown in the supplement (Fig. S1). D. Production of chlorinated tryptophan (Cl-Trp) and indole-3-acetonitrile (Cl-IAN) in transgenic root lines. For each halogenase construct five independent lines were tested. Results for 5 lines per halogenase type with and without His-tag are indicated by the numbers of lines with the respective metabolites. The detailed results for all individual lines are shown in the supplement (Fig. S2).

Table 1
Indole derivatives produced by wild type root cultures of *Brassica rapa* ssp. *pekinensis* after incubation with different chloro-tryptophan derivatives. Amounts are given in μg g^{-1} dry wt and the range of concentrations found is given. '+' = present, but below limit for quantification.

Incubation with/Indole derivative detected	5-Cl-Trp	6-Cl-Trp	7-Cl-Trp	L-Trp	none
Cl-Trp	195 to 400	76 to 250	280 to 290	–	–
Cl-IAN	5 to 13	0.4 to 0.5	1 to 3	–	–
Cl-IAA	0.02 to 0.08	0.02 to 0.06	0.05 to 0.08	–	–
Cl-IAA-Ala	–	–	–	–	–
Cl-IAA-Asp	+	+	+	–	–
Cl-IAA-Glu	+	+	+	–	–
Cl-IAA-Gly	+	+	+	–	–

decarboxylase they detected chlorinated tryptamine. We also detected some chlorinated IAA conjugates but all were under the quantification limit (Table 1). Two of these compounds are IAA-aspartate and IAA-glutamate, amino acid conjugates thought to be involved in the degradation pathway of auxin, whereas the third is IAA-glycine, an amino acid conjugate that is a substrate for hydrolysis to free IAA (Ludwig-Müller, 2011). The chlorinated version of IAA-alanine, another conjugate that is often synthesized in higher plants, was not found.

2.3. Generation and verification of transgenic root cultures

Initially, for each halogenase construct 12 independent lines were obtained. Half of the constructs contained a His-tagged version for protein purification and enzyme assays. First root tips occurred on the explants after about 5 weeks and these were transferred to selective medium (Fig. 1). After 3 weeks growth on MS agar supplemented with hygromycin B, the positive lines were verified by PCR. The generation and verification of the transgenic root lines is shown as an example for three lines with a construct harboring the *pyrH* gene (5-Cl-Trp-forming). All transgenic root lines possess the genes *rolB* and *rolC* and the absence of *virG* indicates that no residual *A. rhizogenes* were in the roots (Fig. 3A). Therefore, the amplicons for the complete *hal* gene (*pyrH* 1536 bp, *thaI* 1614 bp, *prnA* 1617 bp) exhibit a successful integration of

the gene into the plant genome. Subsequently, the gene expression was verified for the full length *hal* gene using cDNA (Fig. 3A). In total, we obtained 5 transgenic lines for each construct that were confirmed in *hal* gene expression.

Protein was isolated and purified from transgenic roots harboring constructs with His-tag to perform an enzyme assay with PyrH, ThaI and PrnA. The amount and the purity were determined by SDS polyacrylamide gel electrophoresis (data not shown), followed by Western blot analysis. Detection with anti-His antibodies revealed only signals for protein preparations from PyrH-containing transgenic roots (Fig. 3B). The HPLC chromatograms after the enzyme assay showed that the purified PyrH from transgenic roots is active and produces 5-Cl-Trp (Fig. 3C). The negative control was extract from wild type root cultures, the positive control used purified PyrH heterologously produced in *E. coli* (Patallo et al., 2017). For lines containing ThaI and PrnA, it was not possible to isolate active enzymes from the transgenic roots (Fig. S1), even though some were able to produce halogenated compounds (Fig. 3D, Fig. S2).

2.4. Production of chlorinated compounds in transgenic root cultures

Halogenated compounds in general are chemically difficult to synthesize due to various reasons (Kolvari et al., 2013), so their bioactive potential is often unexplored. Therefore, the exploitation of biological halogenation has come strongly into focus (Weichold et al., 2016). Previously, it was possible to generate transgenic root cultures of *C. roseus* harboring bacterial *hal* genes and this resulted in the production of novel tryptophan-derived compounds within the terpene indole alkaloids (Runguphan et al., 2010). Tryptophan-derived metabolites are present in other plant species and we were able to stably transform *A. thaliana* with bacterial *hal* genes, which resulted in the production of chlorinated Trp, IAN and IAA (Patallo et al., 2017). Also, transient production of a Trp-halogenase in tobacco leaves resulted in the accumulation of Cl-Trp variants (Fräbel et al., 2016). Thus, it has been shown that the technique is applicable to a variety of different putative plant production systems.

We have applied this approach to generate transgenic root cultures of *B. rapa* ssp. *pekinensis* and analyzed the production of potential Cl-derivatives of Trp in these transgenic root lines. For each halogenase all confirmed, independent transgenic root lines were tested for the production of chlorinated compounds (Figs. 3D and S2). The lines containing the constructs with *pyrH* showed the highest amounts of chlorinated compounds with 8 out of 10 lines synthesizing 5-Cl-Trp and 6 in addition 5-Cl-IAN. For the *thaI*-containing lines just one transgenic root culture produced small amounts of 6-Cl-Trp and 7-Cl-Trp was quantified in six independent lines expressing *prnA*. For none of the two latter transgenic root lines the respective Cl-IAN derivative was detected (Fig. S2). We neither found IAA nor IAA conjugates as we did after incubation with Cl-tryptophan derivatives (Table 1).

Since the production of chlorinated compounds could affect the phenotype of the root cultures, we tested their growth on Cl-Trp derivatives in comparison to Trp (Fig. 4A). While the growth of wild type cultures was indeed strongly inhibited already at lower concentrations of 5-Cl-Trp, growth inhibition by 6- and 7-Cl-Trp was less pronounced compared to non-chlorinated Trp. Correlation of Cl-Trp concentrations and growth rates showed three distinct clusters (Fig. 4B). However, only one cluster was as expected if high concentrations of endogenous metabolites would inhibit growth, the two other clusters did not show such a correlation. Therefore, we assume that the lower production of 6- and 7-Cl metabolites is more likely due to preferences for 5-Cl-Trp by the converting enzymes (see 2.8).

Plants with potentially bioactive indole-derived metabolites such as the indole glucosinolates (IGL) and indole phytoalexins in Brassicaceae (Abdel-Farid et al., 2006) could be exploited to obtain additional chlorinated bioactive indole compounds. The indole glucosinolates are important for the plant defense system against pathogens (Aires et al., 2011; Pedras and Hossain, 2011; Witzel et al., 2013) and herbivores (Textor and Gershenzon, 2009; Kos et al., 2012), but they are also of interest as food additives for human health (Traka and Mithen, 2009; Mikkelsen et al., 2010; Rameeh, 2015). Whether they might be more active when halogenated has yet to be demonstrated. Since the wild type root cultures produced several IGL, we therefore investigated whether any of the transgenic roots expressing the gene for a certain halogenase showed an elevation in IGL since this might be a good candidate for the detection of other Cl-indole derivatives. However, there was no correlation between IGL and a specific transgene, so we concluded that the differences are line specific effects (Figs. S3B and C), as also found for wild type transformants (Aires et al., 2019). So far, we could not detect any chlorinated IGL derivatives.

Since halogenated compounds are often assumed to have an interesting and sometimes even higher bioactivity than the non-halogenated compounds (Wagner et al., 2009), the bioactivity of extracts from the transgenic roots and adult plants produced during this study should be tested. Since we found IAN and IAA derivatives during various experiments, promising tests can include growth assays but also antimicrobial activities. The strong growth promoting activity of Cl-derivatives of IAA has been described (Katayama et al., 2010), but any such application would first need authorization and biotoxicity assays. It was also shown that some altered auxin derivatives, including halogenated compounds showed strong antimicrobial activity (Matsuda et al., 1998). Furthermore, the potential on human cell lines might also be of interest due to the *Brassica*-specific compounds therein. However, to our knowledge there are no studies on the stability of the halogenated compounds synthesized in our study in humans. Experimental approaches using for example liver enzymes to study metabolism by cytochrome P450 enzymes, the major candidates for catabolism (Ashrap et al., 2017), are suitable to obtain such data and should be carried out in the future.

2.5. Production of chlorinated compounds under altered growth conditions

Root cultures can be cultivated in liquid medium, therefore it is possible to add elicitors and also relatively easy to subject the cultures to altered growth conditions. This approach is feasible since many phytochemicals are inducible by either abiotic or biotic stress conditions and/or stress hormones of which some examples are already discussed above (Fig. 2; see also 2.1). Therefore, we changed growth media composition, growth conditions or added potential stressors including plant hormones associated with biotic stress. For these experiments we used transgenic root lines expressing the 5-Cl-Trp halogenase gene pyrH, where the most consistent production of Cl-Trp and Cl-IAN was observed over all lines (Fig. S2).

Not only IGL as defense compounds can be induced by biotic stress factors and the hormonal signals involved (Fig. 2; Aires et al., 2011; Witzel et al., 2013; Zang et al., 2015), also abiotic stressors such as light and temperature changes can alter the levels of IGL (Kissen et al., 2016; Brandt et al., 2018; see also 2.1), but also of IAA (Mittag et al., 2015) and in consequence also possible intermediates of the pathways. In addition, a role for IAA conjugates in salt stress tolerance has reported (Junghans et al., 2006). Changing the composition of the growth media (for more details see legend to Fig. S1) did not result in significant alterations in the production of Cl-Trp or Cl-IAN (data not shown). However, the growth at 8 °C led to a significant increase in the 5-Cl-IAN production. Under light/dark cycles the 5-Cl-IAN concentration was, however, significantly decreased. The production of 5-Cl-Trp was not altered by either treatment (Table 2). Supplementing the culture media with 1 mM NaCl for the complete culture period led to a 6-fold higher concentration of 5-Cl-Trp compared to the control treatment and an increase by ca 2-fold of 5-Cl-IAN (Table 2). However, higher concentrations of NaCl (up to 100 mM) did not further increase the production of chlorinated compounds (data not shown). The increase might thus rather be an effect of chlorine supply than salt stress. Other

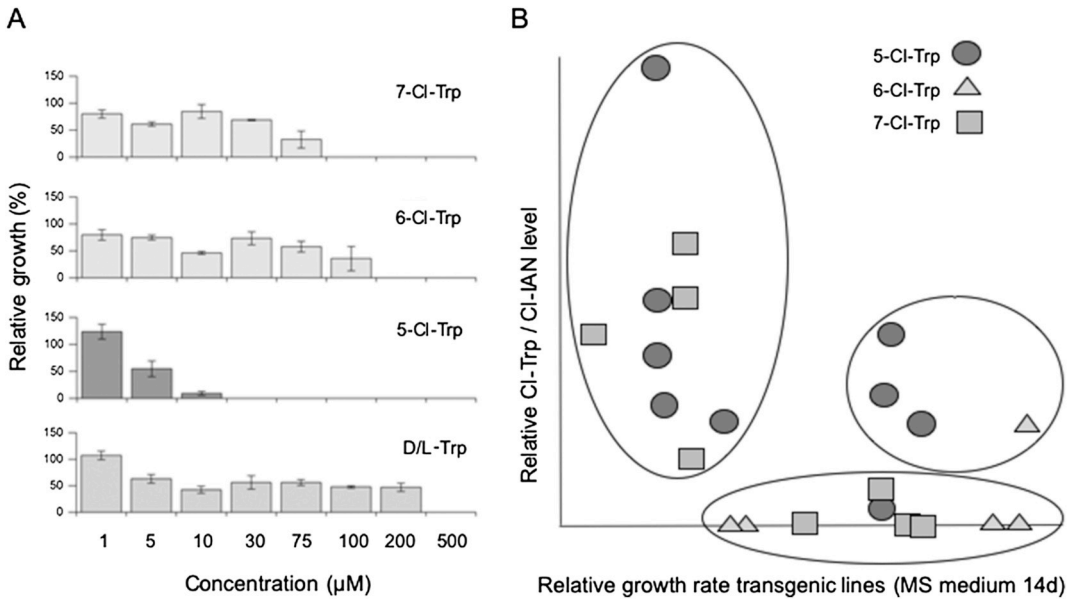


Fig. 4. A. Growth of wild type root cultures on different Trp derivatives as compared to growth on MS medium only. B. Correlation of the relative growth and the production of different Cl-Trp compounds.

Table 2
Production of chlorinated indole derivatives by transgenic root cultures of *Brassica rapa* ssp. *pekinensis* transformed with the 5-Cl bacterial halogenase gene *pyrH* under altered growth conditions (light, low temperature) and in the presence of 1 mM NaCl. The different treatments were performed during different experimental periods, so there are controls for each experiment. Amounts are given in µg g^{-1} dry wt. Values in bold indicate significant changes compared to the control.

	5-Cl-Indole derivative detected			
Condition	Cl-Trp	Cl-IAN	Cl-IAA	Cl-IAA conjugates
Control	1.12 ± 0.59	0.32 ± 0.12	–	–
Light	1.29 ± 0.89	**0.18** ± 0.07	–	–
Low temperature (8 °C)	0.58 ± 0.32	**0.79** ± 0.11	–	–
Control	2.64 ± 0.67	0.13 ± 0.04	–	–
1 mM NaCl	**11.95** ± 1.8	**0.22** ± 0.02	–	–

promising candidates for induction are microbial elicitors or the improvement of elicitation by plant hormones (e.g. Guo et al., 2013; Kastell et al., 2017), since an increase in non-chlorinated selected indole compounds after treatment with JA was found (Aires et al., 2019). However, neither treatment with the hormones JA and SA nor yeast extract showed an increase in an endogenous halogenated compound, but it was possible to increase their concentration by treatment with cell wall preparations from different phytopathogenic fungi of the genus *Fusarium* (M. Neumann and J. Ludwig-Müller, unpublished results). Since IGL have also a function in protecting plants against mechanical damage from chewing insects (Textor and Gershenzon, 2009; Kos et al., 2012), such a condition could also be used to elicit the IGL fraction in future research.

2.6. Regeneration of transgenic plants from root cultures

Root cultures have to be sub-cultivated over time, so that it is labor- and space-intensive to keep many cultures. Therefore, we have also aimed to regenerate the transgenic roots of which we knew that they produced chlorinated metabolites. Transgenic plants from root cultures are mostly not chimeric and therefore stable (Ohara et al., 2000). Such transgenic roots have been used to increase the production of valuable specialized metabolites by overexpression of additional genes (Liu et al., 2010). Shoot regeneration from transgenic root culture material is documented for a number of species including *Brassica* spp. such as *Brassica oleracea* and *B. campestris* (Christey, 1997, 2001), but media composition is extremely plant or even cultivar specific. Other examples besides *Brassica* Habibi et al., 2016; Inoue et al., 2003; Mei et al., 2001; Peres et al., 2001; Sharafi et al., 2014 are given as a summary in Table S1. Furthermore, the influence of light quality and period on shoot regeneration from transgenic roots has been demonstrated (Saitou et al., 1992).

2.6.1. In vitro cultivation

The cytokinin:auxin ratio is critical for shoot regeneration, but it is not yet clear whether the hormone mimetic effect of *rol* genes is also important in the regeneration process (Gunjan et al., 2013). Many media compositions were tried out, not only those described for *Brassica* but also other plant species (Table S1), until we found the composition of the media that allowed to regenerate mature plants from transgenic roots (detailed in Table S2; 4.1.2) and it never happened spontaneously like described in many publications for different plant species (Al-Barbawi and Atheel, 2003; Moriuchi et al., 2004; Gangopadhyay et al., 2010; Gunjan et al., 2013). Regeneration of shoots was successful for 17 out of 25 root lines including wild type. The quantity of regenerated shoots was medium-dependent and varied among the different genetic lines of *B. rapa* (Fig. 5, Tables S2 and S3). Only medium A contained exclusively cytokinins, while media B and C contained the same amount of cytokinin, albeit with different concentrations of auxin. Out of 17 regenerated lines in total, four lines exclusively regenerated on medium B and another four lines exclusively on medium C (Fig. 5). Seven lines regenerated on both media and another two lines regenerated additionally on medium A (Fig. 5A). In total, 50 shoots regenerated on 24 root culture explants on medium C, followed by 35 shoots on medium B. Medium A was less successful with 2 regenerated shoots per culture and none of the regenerated lines exclusively formed shoots on medium A (Fig. 5A). The arithmetic mean value of regenerated shoots on the 3 different media was as follows: 2.87 medium C > 2.02 medium B > 0.07 medium A.

Regeneration events mostly occurred within 4 weeks after transplanting the root cultures on regeneration media. In some cases, regeneration was observed 8 weeks after transplanting the root pieces. Examples of selected plants of the regeneration experiments are shown in Fig. 5B. The majority of regenerated shoots was of adequate quality

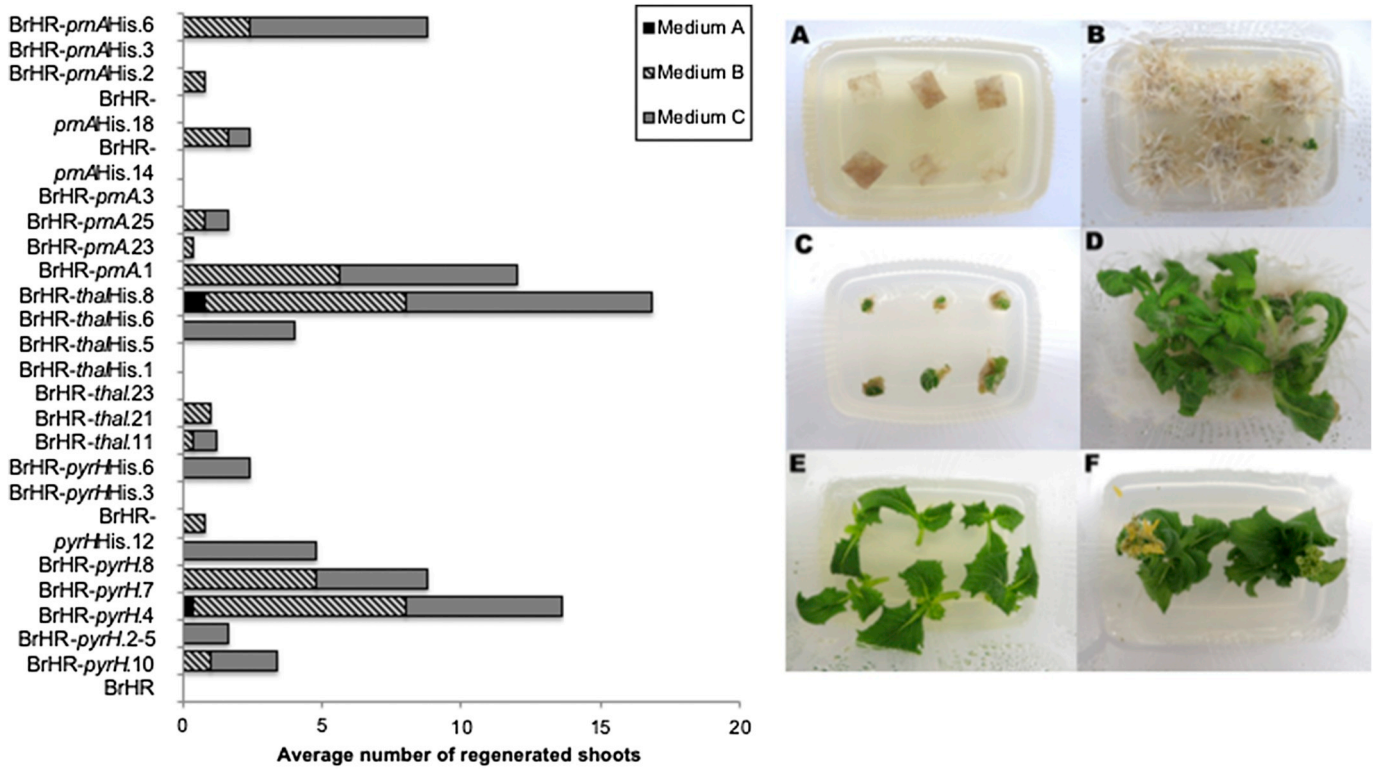


Fig. 5. Left: Average number of regenerated shoots for 25 *Brassica rapa* "hairy root" lines (denoted therefore as BrHR ….) on 3 media compositions (n = 24). Medium A: GB5 medium containing 8 g l^{-1} phytoagar, 20 g l^{-1} sucrose and 10 mg l^{-1} 6-BAP. Medium B: MS medium containing 8 g l^{-1} phytoagar, 30 g l^{-1} sucrose, 4 mg l^{-1} 6-BAP, 4 mg l^{-1} AgNO3 and 3 mg l^{-1} NAA. Medium C: MS medium containing 8 g l^{-1} phytoagar, 30 g l^{-1} sucrose, 4 mg l^{-1} 6-BAP, 4 mg l^{-1} AgNO3 and 0.5 mg l^{-1} NAA. Right: Shoot regeneration from *B. rapa* root cultures. Pieces from these root cultures were cut into pieces of approximately 1 cm^2 and placed on semisolid agar (A). Regeneration of shoots was visible after 4 weeks of cultivation (B). Regenerated shoots were separated and transferred to fresh media (C). Shoot growth was often accompanied by growth of transformed/transgenic roots (D). Shoots of adequate biomass quality were subcultivated (E). Some *B. rapa* lines displayed a shortened life cycle after regeneration and began flowering (F).

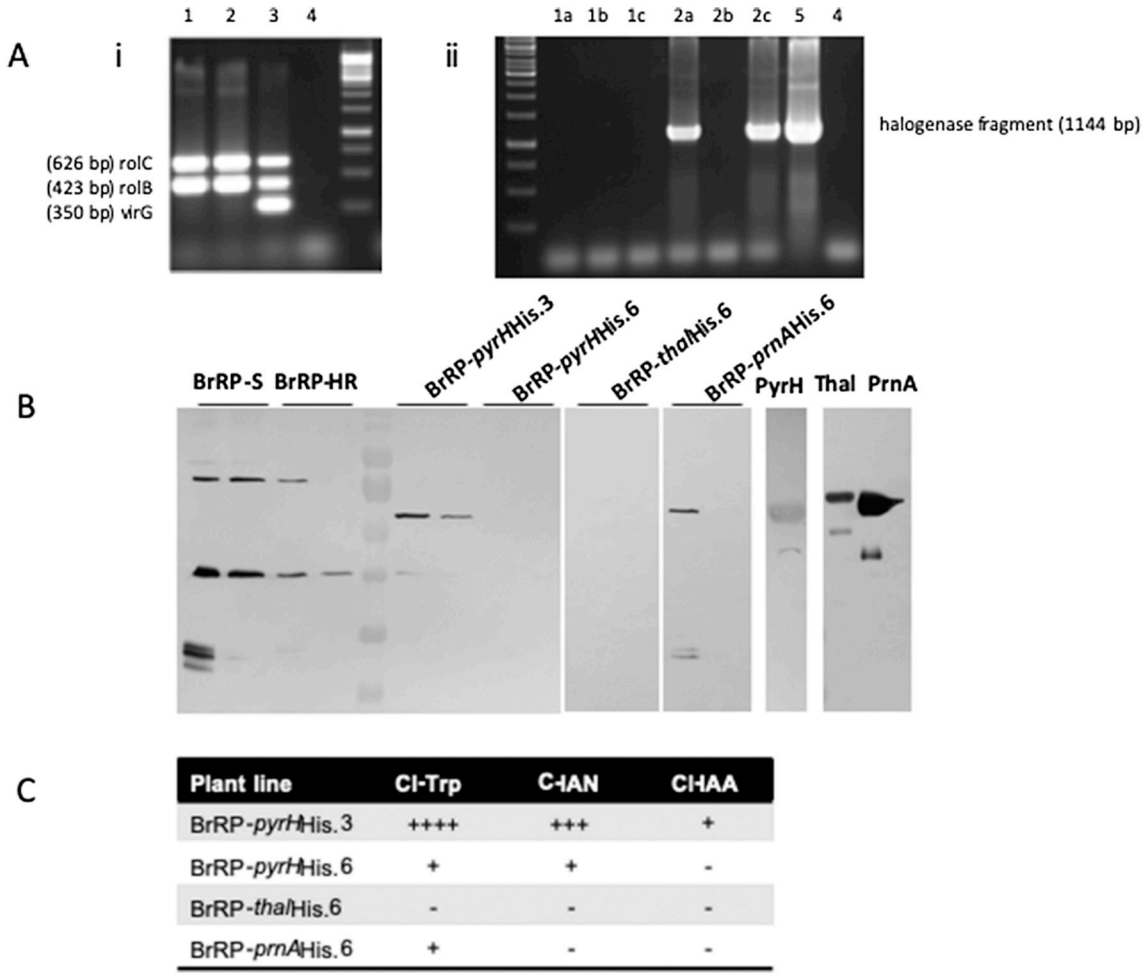
for further sub-cultivation (Fig. 5B) and only a few regenerated shoots showed a translucent and swollen morphology and were excluded from further cultivation. Occasionally, some shoots showed premature flower development. For *B. oleracea* also the formation of florets was reported on axenically cultured and from root culture regenerated plantlets (Al-Hamad, 2014). The stable transformation with *rol* and *hal* genes was confirmed by PCR (Fig. 6A; see 2.7). Regenerated shoots of 11 lines began flowering *in vitro* after 8–24 weeks of sub-cultivation. Further, there were phenotypic differences visible when regenerated shoots were compared to shoots grown from seeds. Leaves of regenerated shoots were bigger (Fig. 7), roots were longer and shoots were shorter (except flowering shoots, which displayed elongated shoots) in comparison to *in vitro* control shoot cultures.

2.6.2. Greenhouse cultivation

For further observation of phenotypes, plants derived from regenerated shoots and shoots grown from seeds were brought to the greenhouse for comparison. While multiple phenotypic traits after one month of growth in the greenhouse revealed no significant differences in number of shoots, leaves or roots between the three groups as well as for leaf width and fresh weight of the aerial parts of the plants, maximum plant height, leaf and root length and the fresh weight of roots were significantly different (Fig. 7). All greenhouse plants originated from *in vitro* shoots including two different lines of regenerated shoots, one with and one without insertion of bacterial *hal* genes, and shoots grown from sterile seeds. While it was not possible to include wild type plants pre-grown under exactly the same conditions, comparison of regenerated seed-derived shoots with wild type (*rol* only) and *hal* transformed regenerated plants from transgenic roots allowed to display phenotypical differences that could be attributed to the *rol* genes alone and to the combination of *rol* and *hal* gene expression.

The maximum plant height of wild type plants grown from seeds was significantly lower than the height of regenerated plants harboring the *rol* genes or regenerated plants harboring additionally one *hal* gene, but between the two groups of regenerated plants there was no significant difference detected (Fig. 7). Leaves of the wild type were significantly shorter than leaves of regenerated plants or regenerated plants transformed with *hal* genes, whereas no significant difference between the two groups of regenerated plants was detected (Fig. 7). Between 6 and 7 months after transfer to the greenhouse some plants showed signs of seed pod formation and it was possible to harvest viable seeds. The presence of the respective halogenase gene in these offspring was not yet determined.

For maximal root length no significant difference was detected when wild type plants were compared to the two groups of root culture derived plants, yet maximal root length of regenerated plants without *hal* genes was significantly higher than maximal root length of regenerated plants transformed with bacterial *hal* genes (Fig. 7). Since root growth is associated with the proper auxin concentrations, i.e. low concentrations are inducing while high concentrations are inhibiting root growth (Savić et al., 2009), one interpretation could be that the root growth inhibition in the *hal* containing plants could be due to higher levels of Cl-IAA. This is in accordance with observations that chlorinated IAA derivatives are more root growth inhibitory than the non-chlorinated variant (Walter et al., 2020). Also, the fresh weight of roots was significantly higher for regenerated plants without *hal* genes in comparison to the wild type and regenerated plants transformed with bacterial halogenases since roots from wild type plants were more delicate than roots of regenerated plants (Fig. 7). We have found Cl-IAA conjugates after feeding of IAA, but not in the transgenic lines, so their



Plant line	Cl-Trp	Cl-IAN	Cl-IAA
BrRP-*pyrH*His.3	++++	+++	+
BrRP-*pyrH*His.6	+	+	-
BrRP-*thal*His.6	-	-	-
BrRP-*prnA*His.6	+	-	-

Fig. 6. A. Confirmation of i) that the regenerated plants contain still the rol genes and ii) the integration of the *hal* gene into the genome and its transcription into cDNA (1: regenerated plants from wild type root cultures; 2: BrRP-*pyrH*HIS.6; 3: *A. rhizogenes* plasmid; 4: negative PCR control; 5: positive control - *hal* amplification from plasmid; a: cDNA, b: cDNA „no template control; genomic DNA. B. Western blot of His-tagged halogenase (PyrH, Thal, PrnA: purified enzymes from over-expressing *E. coli* strain as positive controls; BrRP-HR = WT, regenerated plants from wild type root cultures; BrRP-S: regenerated plants from Chinese cabbage seedlings; BrRP-*pyrH*, -*thal*, -*prnA*: regenerated plants from transgenic roots.). Always two different dilutions were applied. The gel strips were from the same gel, but due to large parts with samples without an immunosignal, the respective areas were cut out and are presented here. C. Relative amounts of chlorinated metabolites in the regenerated plants.

possible contribution to the growth phenotype cannot be assumed at the moment.

In many cases altered plant phenotypes have been observed on regenerated plants still harboring the *rol* genes. We and others (e.g. Piatczak et al., 2015) observed that the transgenic plants were larger in belowground and aboveground features (Fig. 7), but the leaves of regenerated plants were of normal color and shape. In other work, only the roots of explants were showing altered phenotypes (Zhou et al., 2009). Despite the long-lasting research on *Agrobacterium rhizogenes* - plant interactions the function of the *rol* gene products in the "hairy root" phenotype is still not clear up to now (Pavlova et al., 2014). Earlier work suggested the direct involvement of hormones, especially via the hydrolysis of hormone glucose conjugates (Chriqui et al., 1996), while today this phenotype is attributed to a more general effect of the *rol* gene products in the plant, e.g. for the *rolB* product a phosphatase activity has been demonstrated (Bulgakov, 2008). Whether any of the growth phenotypes can be directly linked to a specific *rol* gene product can therefore not been assessed.

2.7. The presence of halogenase results in Cl-Trp and Cl-IAN production in the regenerated plants

The presence of *rol* and *hal* genes in 20 lines of the regenerated plants was proven by PCR analysis using genomic DNA and expression was verified using cDNA and selected examples are shown (Fig. 6). For eight 5-Cl-Trp, six 6-Cl-Trp and six 7-Cl-Trp putative halogenase gene containing plantlets the integration of the halogenase gene was confirmed; we show an example for a *pyrH* transformant (Fig. 6A). Also, plantlets regenerated from wild type root cultures showed the presence of *rol* genes, but no *hal* genes, while regenerated plants from seedlings showed neither amplification product (data not shown). In selected regenerated plantlets (two per construct with His-tag) protein was extracted and analyzed by Western blot (Fig. 6B). While two lines BrRP-*pyrH*His exhibited a signal that corresponds to the respective protein size, only one line (BrRP-*prnA*His) showed a weak protein band. None of the tested lines BrRP-*thal*His displayed a signal using anti-His antibodies. Since we found also in treangenic root lines chlorinated compounds even though no protein was detected (Fig. 3), we subjected BrRP-*thal*His.6 to LC-MS analysis for chlorinated compounds (Fig. 6C). However, only the two lines harboring PyrH showed also the presence of chlorinated tryptophan in addition to Cl-IAN. In one line with a high production we also found minute amounts of Cl-IAA. In line BrPR-*prnA*His we found a small amount of Cl-Trp. Our work therefore showed that the production of chlorinated compounds is maintained throughout regeneration of adult plants from root cultures. This approach could lead to the generation of transgenic plants possibly

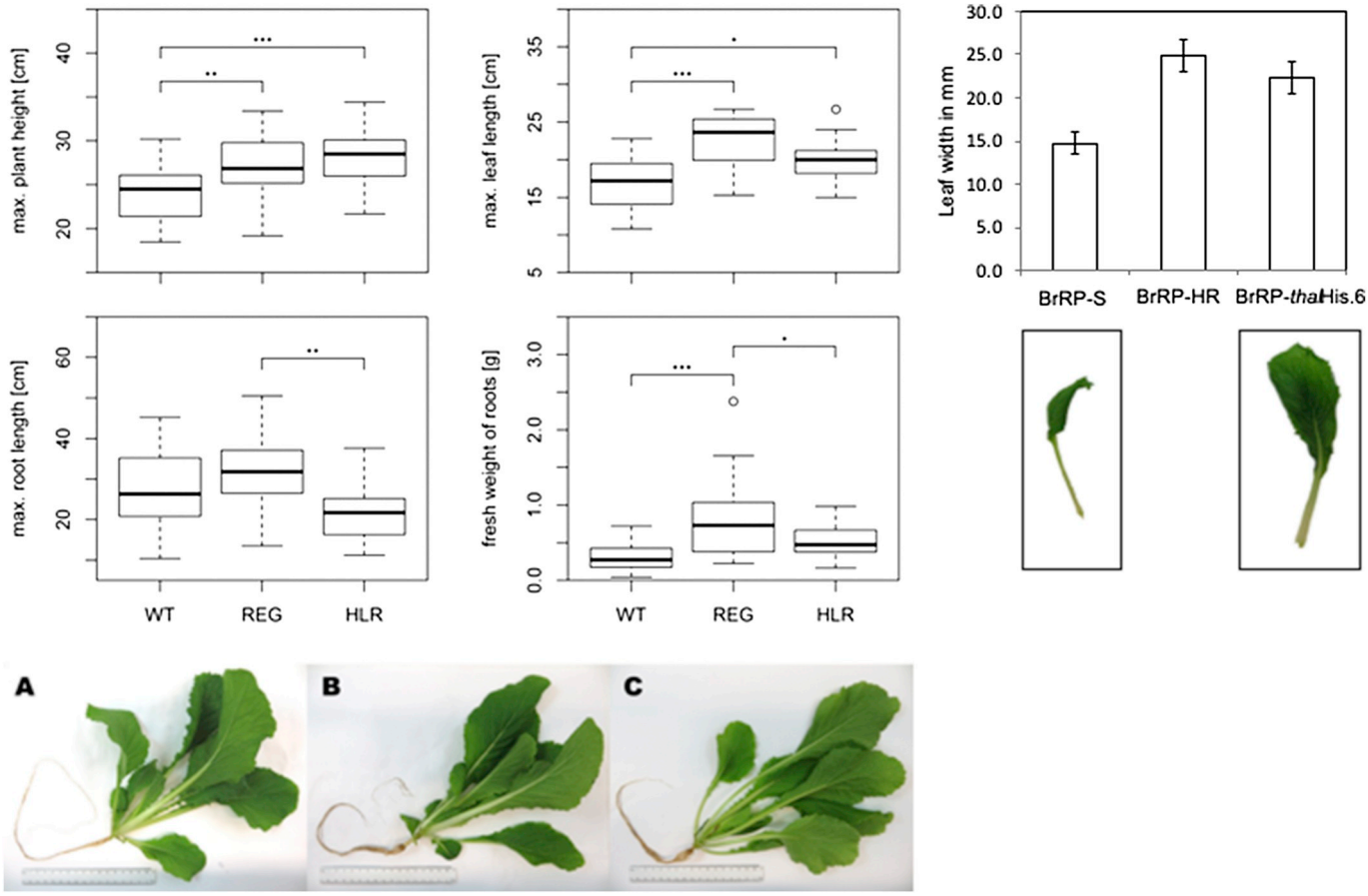


Fig. 7. Comparison of phenotypic traits for three groups of *Brassica rapa* grown in the greenhouse. WT – shoots from wild type plants grown from seeds (photo A); REG – regenerated shoots originated from transformed root cultures (photo B); HLR – regenerated shoots originated from transgenic roots transfected with bacterial *hal* genes (photo C). Significant differences between treatments are labeled as follows: 0 '***' 0.001 '**' 0.01 '*' 0.05 (with n = minimum of 20 individually potted plants). Leaves of *in vitro* shoots originated from seeds (left) or regenerated from root cultures (right) are shown.

producing also other indole-derived metabolites with high bioactive potential such as the IGL. To increase the production rate one possibility would be to use starting materials with higher IGL or other interesting indole levels. Since it is possible to tailor such halogenase proteins to result in altered enzymatic activities in transgenic roots (Glenn et al., 2011), this might be an additional experimental approach for the future to generate other indole derived halogenated metabolites.

2.8. Preference for 5-Cl derived metabolites?

The majority of transgenic root lines producing detectable amounts of Cl-Trp and derivatives were harboring PyrH, the 5-Cl-Trp halogenase. Lines harboring the 6-Cl-tryptophan halogenase Thal showed only in a few cases production of 6-Cl-Trp, but not 6-Cl-IAN. The 7-Cl-Trp halogenase PrnA-containing lines were better producers than 6-Cl-Trp halogenase-containing lines, but not all lines tested produced 7-Cl-metabolites. Like in *A. thaliana* (Patallo et al., 2017; Walter et al., 2020) we also found here a preference for 5-Cl-Trp formation in transgenic plants and its conversion to subsequent products such as Cl-IAN. In work by other authors not all regioselective halogenase types were used, so there is no comparison on the efficiency possible. Fräbel et al. (2016) employed Trp-dependent halogenases from the 6-Cl and 7-Cl type and found chlorinated metabolites from both variants. They also constructed a line with two halogenases that produced the 6,7-Cl-Trp derivative, but they did not give any concentrations, so a comparison is not possible. Further, while we used Thal for our experiments, in the work of Fräbel et al. (2016) SttH was the 6-Cl-Trp halogenase making conclusions about the efficiency of the enzymatic transformation difficult. In the transformation of *C. roseus* root cultures only the 5-Cl (PyrH) and 7-Cl-Trp (RebH) halogenases were used (Runguphan et al., 2010), but in the results also no information on the concentrations of halogenated metabolites are given.

The *in vitro* enzyme characteristics support the *in planta* observations that 6-Cl-Trp derivatives are less abundantly present in transgenic roots (Patallo et al., 2017). While all three heterologous produced halogenases converted IAA to the respective Cl-derivative *in vitro*, detailed kinetics showed that Thal had a much lower activity with IAA than the two other halogenase types (Patallo et al., 2017). However, Runguphan et al. (2010) examined the K_m values for the tryptophan decarboxylase of *C. roseus in vitro* and found a K_m for Trp of ca 50 μM and for 5-Cl- and 7-Cl-Trp K_m values of 540 and 500 μM, respectively. These kinetic data do not allow the conclusion that 5-Cl-Trp derivatives would be better converted *in planta*.

Another possibility could be that 5-Cl-Trp is less toxic to the root cultures or plants. We tested this possibility by growing wild type root cultures in the presence of the respective Trp derivatives. Only at higher (≤10 mM) Trp concentration a growth inhibition by-Trp 5-Cl in comparison to the other tryptophans was found. Concentrations of 1 and 5 mM of 5-Cl-Trp showed the same toxicity as the other Cl-Trp derivatives. We also did not find a correlation between the type of Cl-Trp formed in the plant and the growth of the respective transgenic root line (Fig. 4). Therefore, it can be assumed that the formation of 5-Cl-Trp and derivatives is more dependent on the preference of the endogenous enzymes or maybe also depending on the stability of the respective halogenase proteins. The former assumption is supported by feeding experiments with different chlorinated tryptophan derivatives to wild

type root cultures. While incubation with 5-Cl-Trp resulted in high amounts of 5-Cl-IAN, incubation with the other Cl-Trp derivatives resulted in the production of lower amounts of the respective Cl-IAN derivative. On the contrary, there was no such regioselective preference found for the formation of Cl-IAA derivatives and Cl-IAA amino acid conjugates (Table 1). Also, all Cl-Trp derivatives were taken up into the transformed root lines to the same extent. As long as in other work not the complete set of regioselective halogenases will be employed it has to remain speculative whether halogenation in the 5-position might be the most effective for the bacterial enzyme and subsequent plant proteins.

3. Conclusion

This work has extended previous work on the gene expression of bacterial Trp-dependent halogenases in plants by choosing plant species with potential novel pathways derived from tryptophan, i.e. indole glucosinolates/phytoalexins. Furthermore, it was possible to establish two types of plant materials with stable halogenase gene integration that can be used for different purposes, namely transgenic roots and adult plants regenerated from these root cultures. The latter did not show any negative phenotypic alterations that could hamper their usefulness. These two achievements are prerequisites to further optimize both types of plant materials for increased production of halogenated compounds derived from tryptophan.

Transgenic roots of *B. rapa* are able to produce some indole-derived compounds such as Cl-Trp and Cl-IAN, but we assume that the halogenases act preferably on Trp, whereas the other chlorinated indole derivatives are most likely produced by the enzymes of the following biosynthetic pathway to IAA. At the moment, the detection limit for such compounds seems to be the major problem, in addition to the lack of standard compounds for some possible chlorinated metabolites. It was possible to increase the production of Cl-IAN specifically when the transgenic roots were grown at 8 °C instead of 26 °C. In addition, adding 1 mM NaCl increased the level of Cl-Trp produced. Furthermore, changes in media such sulphur supply could be tested to increase levels of putative IGL.

In cases with high Cl-Trp also more Cl-derivatives could be detected, such as IAA in the regenerated plants with high Cl-Trp and Cl-IAN. After feeding of high concentrations of Cl-Trp derivatives to wild type root cultures also Cl-IAA was found and even some IAA amino acid conjugates with chlorine attached. So, if we can increase the amount of Cl-Trp in the plant or transgenic root line considerably, then we might be able to detect additional compounds. To circumvent the addition of compounds transgenic root cultures or plants with altered contents of compounds can be used as starting material. Alternatively, other *Brassica* species with a different natural indole derivative pattern can be used, i.e. *B. napus*, *B. oleracea* or *B. juncea*.

4. Experimental

4.1. Plant material

For the generation of transformed and transgenic root cultures *Brassica rapa* ssp. *pekinensis* (Lour.) Hanelt (Chinese cabbage; Brassicaceae) (Chinakohl Cantonner Witkrop, ISP Quedlinburg, Germany) was used because of the high content of indolic compounds (Kim et al., 2010).

4.1.1. Generation of wild type and transgenic root cultures of Brassica rapa

Wild type transformed roots were induced on *B. rapa* leaves using the strain *Agrobacterium rhizogenes* ATCC 15834. For *A. thaliana* codon-optimized (done by Life Technologies; Patallo et al., 2017) bacterial halogenase genes (*hal*) *pyrH* (Zehner et al., 2005), *thaI* (=*thdH*) (Seibold et al., 2006) and *prnA* (Hammer et al., 1997; Kirner et al., 1998) were inserted into the genome of *B. rapa* by generating transgenic roots. The bacterial *hal* genes were cloned with the Gateway™ Recombination Cloning Technology (Invitrogen) into the pMDC32 vector. For each halogenase we generated constructs with (pMDC32 + 2xCaMV35S:*hal*His:nosT) and without (pMDC32 + 2xCaMV35S:*hal*:nosT) His-tag. The constructs were transformed into *A. rhizogenes* ATCC 15834 by electroporation. To generate the transgenic roots a culture of *A. rhizogenes* with pMDC32-hal was grown for 1 day at 26 °C and 120 rpm in YEB media (0.5% peptone, 0.1% yeast extract, 0.5% meat extract, 0.5% sucrose, 2 mM $MgSO_4$, 1.5% agar with 50 μg ml^{1-} kanamycin; pH 6.8–7.2). This bacterial suspension was centrifuged at 5000 rpm for 20 min and resuspended in MS media supplemented with 3% sucrose (pH 5.8) and diluted to an OD_{600} of 0.5–1. Concurrently, Chinese cabbage leaves were sterilized with 70% EtOH (2 min), 8.5 g l^{-1} $Ca(OCl)_2$ (10 min) and washed in ddH_2O (2 × 5 min). These leaves were cut into small pieces of 2 × 2 cm and co-inoculated with the agrobacterium-MS-suspension by wounding the leaf veins. The explants were incubated for 3 day at 26 °C under light conditions (16 h light/8 h dark) on Murashige & Skoog (MS) agar medium (supplemented with 3% sucrose, 0.65% phyto agar, pH 5.8). Afterwards the leaf explants were transferred to MS agar including a 1x antibiotic and antimycotic solution (Sigma Aldrich, München, Germany) to eliminate the bacteria and to retain the explants without microbial contamination. After ca. 4 weeks the first transformed/transgenic roots emerged from the wounded areas. These roots were separated on MS agar including 250 mg l^{-1} cefotaxime (26 °C, dark). Transgenic roots were selected by growth on hygromycin B (100 μg ml^{-1}). All root lines contained the constitutive CaMV35S promoter in front of the respective halogenase construct. The transgenic root lines are denoted e.g. BrHR-*pyrH*His.3 (Br = *Brassica rapa*; HR = "Hairy Root"; e.g. *pyrH* = respective *hal* gene; His = with His-tag or alternatively without; dot followed by number = line number). Wild type lines are denoted as BrHR.x.

4.1.2. Regeneration of root cultures to adult plants

Experiments with 43 media compositions were conducted to optimize medium for regeneration. Detailed composition of all media is displayed in Table S2. Of these, 3 media compositions were used for regeneration of the transgenic lines (Medium A: GB5 medium containing 8 g l^{-1} phytoagar, 20 g l^{-1} sucrose and 10 mg l^{-1} 6-BAP; Medium B and C: MS medium containing 8 g l^{-1} phytoagar, 30 g l^{-1} sucrose, 4 mg l^{-1} 6-BAP, 4 mg l^{-1} $AgNO_3$ and either 3 or 0.5 mg l^{-1} NAA, respectively). Media were adjusted to pH 5.8 and autoclaved at 121 °C for 20 min. Roots from *in vitro* root cultures of *B. rapa* wild type and 24 transgenic lines were cut into pieces of approximately 1 cm^2 and placed on semisolid agar. Explants were checked for shoot formation twice a week. Medium was renewed every 4 weeks. Brownish material was cut off, regenerated shoots were separated and transferred to fresh media. Explants were photographed every 4 weeks and additionally in case of shoot regeneration. Number and time point of regeneration events was recorded. Root culture explants were completely renewed after 12 weeks, if no regeneration was observed. Growth conditions were a photoperiod of 16 h light/8 h dark with fluorescent lamp (Osram Fluora® L18 W/77) with photosynthetic photon flux density in the range of 40–56 μmol per m^2 s at an average temperature of 25 °C. Statistics were performed using Microsoft Excel 2007 and R version 3.3.1. Number of regeneration events was normalized for 24 root culture explants for comparison. Arithmetic mean value of regenerated shoots on the 3 different media was calculated for 24 root culture explants. For comparison of phenotypic traits (see below) a multifactor ANOVA was performed after checking for approximately normal distribution of the data, followed by pair wise *t*-test (p value adjustment method: bonferonni) for traits that differed significantly according to ANOVA. The regenerated plant lines are denoted e.g. BrRP-*pyrH*His.3 (Br = *Brassica rapa*; RP = regenerated plant; e.g. *pyrH* = respective *hal* gene; His = with His-tag or alternatively without; dot followed by number = line number). Lines regenerated from wild type "hairy" roots are denoted as BrRP-HR.x and the plants regenerated from seedlings as BrRP-S.x.

4.2. Cultivation of plants and root cultures

4.2.1. Indole metabolite extraction

Wild type root cultures were grown for 21 days under standard conditions. The Chinese cabbage plants were grown under sterile and non-sterile conditions. The non-sterile plants were grown for 5 weeks under defined conditions in the greenhouse (16/8 h day/night, 23/18 °C). For sterile conditions the seeds were incubated with 70% EtOH for 2 min, 0.02 g ml^{-1} $Ca(OCl)_2$ for 10 min and washed three times with ddH2O. These seeds were germinated and grown for 3 weeks on MS agar (supplemented with 1% sucrose, 0.65% phyto agar, pH 5.8) under defined greenhouse conditions (see above). The harvested material was freeze-dried (Christ, Alpha 1–4; Osterode, Germany) and analyzed for IGL.

4.2.2. Halogenated compounds extraction

Transgenic and WT root cultures were grown for 21 d in liquid MS media at 28 °C and 70 rpm in the dark. The materials from root cultures were freeze-dried (see 4.2.1) and ground to fine powder.

4.2.3. Feeding experiments

WT root cultures were grown for 18 d in liquid MS media at 28 °C and 70 rpm in the dark. At this time point 100 μM of the different Cl-Trp derivatives were added. Since all chlorinated Trp derivatives were in the D/L-form, we present the data for the D/L-form for non-chlorinated Trp. Results were the same for L-Trp, though (data not shown). The transformed roots were grown for additional 3 days before harvest.

4.2.4. Enzyme assays

Transgenic and wild type (as control) root cultures were grown for 21 days in liquid MS media (supplemented with 3% sucrose, pH 5.8) at 28 °C and 70 rpm in the dark.

4.2.5. Stress conditions

Growth conditions were altered for a possible induction of chlorinated metabolites by stress factors as described in detail in Table S4. As stress conditions two different additional temperatures were used (37 °C and 8 °C), growth in a light/dark cycle, and addition of stress signaling compounds (50 μM jasmonic acid, 100 μM salicylic acid, 5 g l^{-1} yeast extract) as well as different NaCl concentrations (between 1 and 200 mM). For all conditions, except NaCl treatment where NaCl was added over the complete cultivation period of 21 days, the compounds were added after 18 days of cultivation for another 3 days (Table S4) and the roots were harvested after 21 days in culture, freeze-dried (see 4.2.1) and analyzed for IGL or halogenated indole derivatives (see 4.5). Their growth was also recorded. The freeze-dried plant material was treated as described above.

4.2.6. Phenotype determination of regenerated plants

Regenerated *in vitro* plants with adequate shoot and root growth were transferred to the greenhouse to observe phenotypes of transgenic plants. Regenerated shoots of *B. rapa* root cultures with and without insertion of bacterial *hal* genes and shoots grown from seeds were planted in single pots, watered 3 times a week with tab water and grown for 4 weeks with 16 h light daily and temperatures of 25–27 °C. Whole plants were harvested and carefully cleaned with tap water. Fresh weight and phenotypic traits (plant height, shoot count, leaf count, root count, leaf width, leaf length, root length, fresh weight) were documented and photos were taken of each individual plant.

4.3. Verification of transgenic lines using gDNA and RNA/cDNA

The genomic DNA (gDNA) from transgenic roots was isolated as described by Doyle and Doyle (1987). The RNA was extracted with RNAzol®RT (Molecular Research Center, Inc., Cincinnati, OH, USA) according to the manufacturer's protocol. Verification of integration of the T-DNA by PCR was done using primers for the amplification of *rolB* (Grabkowska et al., 2010) and *rolC* (Grabkowska et al., 2010). These *rol* genes are necessary for the formation of the "hairy root" phenotype. The absence of *virG* (Sidwa-Gorycka et al., 2009) indicates that the transformed roots are free of living agrobacteria. The expected fragment sizes are 423 bp for *rolB*, 626 bp for *rolC* and 350 bp for *virG*. To verify the insertion of the whole *hal* gene PCR and RT-PCR were done with primers, which amplify the complete gene. All primers, their annealing temperatures and the expected fragment sizes are listed in Table S5. The amplified fragments were separated in a 1.5% agarose gel and stained with ethidium bromide. The gels were documented with a BioDocAnalyzer (Biometra, Göttingen, Germany). Similarly, gDNA and RNA was extracted from the regenerated plants. Integration of *rol* and *hal* genes and expression of the *pyrH*, *thaI* and *prnA* genes was investigated using the same primer set under the same conditions as for transgenic root cultures.

4.4. Western blot and enzyme assay with purified proteins from transgenic roots

4.4.1. Protein extraction

The protein purification and enzyme assays were carried out essentially according to Patallo et al. (2017). For the isolation of proteins for the enzyme assays, a minimum of 30 g of transgenic roots were harvested from the liquid cultures and ground with the following extraction buffer (1 ml per 500 mg material): 100 mM sodium phosphate (pH 6.8) containing 25 mM $Na_2S_2O_5$, 0.25 mM sucrose, 5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 1 mM $CaCl_2$, 2 mM DTT, 5 mM ascorbic acid and 0.2% (v/v) β-mercaptoethanol with a mortar and pestle on ice (Croteau and Winters, 1982). The suspension was centrifuged at 20,000 g and 4 °C for 30 min to remove insoluble material. The His-tagged halogenases were purified by immobilized metal affinity chromatography.

4.4.2. SDS-PAGE and Western blot

The production, the amount and purity of protein was analyzed on a 12% SDS polyacrylamide gel electrophoresis and Western blot using anti-His antibodies at a dilution of 1:10,000 (GE Healthcare, Little Chalfont, UK) for the lines containing a His-tagged construct. The proteins were blotted onto a PVDF membrane and visualization of signals was performed using alkaline phosphatase coupled secondary antibodies (GE Healthcare, Little Chalfont, UK) with either luminescent detection and CDP-*Star*® as substrate (GE Healthcare, Little Chalfont, UK) or color detection and nitroblue-tetrazolium/5-bromo-4-chloro-2-indoxylphosphate (NBT/BCIP) as substrate. As negative controls extracts from wild type plants were used, as positive controls purified halogenase from *E. coli* (Patallo et al., 2017).

4.4.3. Enzyme assay

The enzyme assay (Patallo et al., 2017) to determine halogenase activity contained hisFre (10.8 mU), FAD (10 μM), NaCl (12.5 μM), L-Trp (250 μM) and NADH (2.4 μM) and was filled up to a final volume of 200 μl with purified enzyme (in 20 mM potassium phosphate buffer, pH 7.2). The assay was incubated at 30 °C for 60 min. Reactions were stopped by incubation at 95 °C for 5 min. Precipitated proteins were removed by centrifugation and the supernatant was analyzed by HPLC. Analytical HPLC was performed with a Waters SymmetryShield™ column (RP18, 5 μm, 2.1 × 150 mm; Waters, Milford, MA, USA) at a flow rate of 0.3 ml min^{-1} was used. An isocratic solvent of MeOH/H_2O/TFA (40:60:0.1) was used and the absorbance was monitored at 220 nm.

4.5. Metabolite analysis

4.5.1. Analytics of non-halogenated indole compounds

IGL were measured in leaves and roots from Chinese cabbage under sterile and non-sterile conditions as well as in root cultures and the

individual IGL identified were those described in Aires and Carvalho (2017) and Aires et al. (2019). For IGL determination 200 mg dry wt of each sample was added to 5 ml 70% methanol:water, heated to 70 °C for 10 min and then centrifuged at 4000 g for 20 min. The method is described in detail in Aires and Carvalho (2017). Briefly, the supernatants were collected and the volume was adjusted with methanol 70% to a final volume of 10 ml. The methanolic samples were submitted to an enzymatic desulfation on a DEAE Sephadex A-25 anion exchange column. To each column 0.5 ml of pre-equilibrated resin was added, washed with 0.5 ml H_2O to remove ions. After adding 2 ml sample the columns were washed twice with H_2O, followed by 0.5 ml 0.02 M pyridine. Then 75 μL of aryl sulfatase (E.C.3.1.6.1) type H1 from *Helix pomatia* (Sigma-Aldrich, München, Germany) was loaded onto each column, followed by an incubation overnight at RT. The desulfated IGL were eluted 3 times with 0.5 ml H_2O and the combined eluates were stored at −80 °C prior to HPLC analysis. The determination of IGL by HPLC was performed on an Agilent 1200 HPLC-DAD-UV/Vis (Agilent Technologies, Santa Clara, CA, USA) equipped by a C18 (250 × 4.60 mm, 5 μm) column with a mobile phase of H_2O (solvent A) and 200 ml l^{-1} acetonitrile:water (v/v) (solvent B), with a flow rate of 1.5 ml min^{-1} and an injection volume of 10 μL, with a gradient starting with 1% of solvent B at 1 min, 99% of solvent B at 24 min and 1% of solvent B at 39 min. The IGL peak identification and quantitative estimations were made using benzyl GL at 1 mg ml^{-1} as internal standard (IS). The quantification of compounds was based on the internal standard method.

4.5.2. UPLC-QqQ-MS analysis of chlorinated compounds

Sixty mg freeze-dried material was extracted with 1 ml 100% MeOH. The extracts were stored at −20 °C prior UPLC-QqQ-MS analysis. UPLC-MS analysis of chlorinated compounds was carried out on a Waters Acquity UPLC system connected to a Xevo TQS (Waters, Milford, MN, USA) spectrometer. Chromatography was performed on an Acquity BEH C18 1.7 μm 2.1 × 50 mm column kept at 35 °C. Mobile phase A was water containing 0.1% formic acid; mobile phase B was acetonitrile containing 0.1% formic acid. The flow rate was 0.35 ml min^{-1}, and the gradient was 0 min, 1% B; from 0 to 6 min, linear gradient to 60% B; from 6 to 6.5 min, isocratic 60% B; from 6.5 to 6.6 min, linear gradient to 100% B; from 6.6 to 8.5 min, wash at 100% B; from 8.5 to 8.6 min, back to the initial conditions of 1% B and column equilibration until 10 min. The injection volume of both the standard solutions and the samples was 2 μl. Samples were kept at 10 °C during the analysis. MS detection was performed in positive ESI. Capillary voltage was 3.0 kV; the source was kept at 150 °C; desolvation temperature was 500 °C; cone gas flow, 100 L h^{-1}; and desolvation gas flow, 800 L h^{-1}. Unit resolution was applied to each quadrupole. Multiple reaction monitoring (MRM) signals, optimized using authentic standards, were used for detection and quantification of chlorinated tryptophans (239.2 > 222.2; CE 12 eV), chlorinated IAN (191.1 > 164.1 and 151.1; CE 10 eV) and chlorinated IAA (210.05 > 164.05; CE 18 eV).

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgements

This work was conducted under the ERA-IB project NBCPBH-EIB.13.008 entitled Production of New Bioactive Compounds by Plants and Bacteria Using New and Improved Halogenases. Individual support of the research within the ERA-IB project was by the Bundesministerium für Bildung und Forschung (BMBF) to M. Schöpe, K.-H. van Pée and J. Ludwig-Müller (031 A338); by the Fundação para a Cieˆncia e a Tecnologia I.P. (FCT), under the project ERA-IB-2/0004/2013 to Alfredo Aires; and by the Biotechnology and Biological Sciences Research Council (BBSRC) to S. O'Connor (ERA-IB BB/M004570/1). We thank Freia Benade and Dr. Linda Jahn (TU Dresden) for helping with experiments.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.phytochem.2020.112371.

References

Abdel-Farid, I.B., Choi, Y.H., Kim, H.K., van den Hondel, C.A.M.J.J., van der Meijden, E., Verpoorte, R., 2006. The role of secondary metabolites in *Arabidopsis* and *Brassica* in the interaction with fungi. Curr. Top. Plant Biol. 7, 47–73.

Aires, A., Carvalho, R., 2017. Rapid separation of indole glucosinolates in roots of Chinese cabbage (*Brassica rapa* subsp. *pekinensis*) by High-Performance Liquid Chromatography with Diode Array Detection. Int. J. Anal. Chem. 2017, 6. https://doi.org/10.1155/2017/5125329. Article ID 5125329.

Aires, A., Dias, C.S.P., Carvalho, R., Oliveira, M.H., Monteiro, A.A., Simões, M.V., Rosa, E.A.S., Bennett, R.N., Saavedra, M.J., 2011. Correlations between disease severity, glucosinolate profiles and total phenolics and *Xanthomonas campestris* pv. *campestris* inoculation of different Brassicaceae. Sci. Hortic. (Canterb.) 129, 503–510.

Aires, A., Neumann, M., Ludwig-Müller, J., Schöpe, M., van Pée, K.H., 2019. Induced production of indol-3-ylmethyl glucosinolates in hairy roots of Chinese cabbage (*Brassica rapa* subsp. *pekinensis*): perspectives to enhance the content of bioactive compounds. Acta Hortic. 1242, 49–56.

Al-Barbawi, N.I., Atheel, N.Y., 2003. Regeneration of transformed forage legume plants by *Agrobacterium rhizogenes* R1601. Qatar Univ. Sci. J. 23, 109–115.

Al-Hamad, H., 2014. Naphthaleneacetic acid solely induced extensive hairy root development that precedes extensive shoot regeneration from stalk explants of cauliflower (*Brassica oleracea* var. *botrytis*). J. J. Biotech. Bioeng. 1, 001.

Ashrap, P., Zheng, G., Wan, Y., Li, T., Hu, W., Li, W., Zhang, H., Zhang, Z., Hu, J., 2017. Discovery of a widespread metabolic pathway within and among phenolic xenobiotics. Proc. Natl. Acad. Sci. U.S.A. 114, 6062–6067.

Baskar, V., Gangadhar, B.H., Park, S.W., Nile, S.H., 2016. A simple and efficient Agrobacterium tumefaciens-mediated plant transformation of *Brassica rapa* ssp. *pekinensis*. 3 Biotech 6, 88. https://doi.org/10.1007/s13205-016-0402-1.

Bhandari, S.R., Jo, J.S., Lee, J.G., 2015. Comparison of glucosinolate profiles in different tissues of nine Brassica crops. Molecules 20, 15827–15841. https://doi.org/10.3390/molecules200915827.

Brandt, S., Fachinger, S., Tohge, T., Fernie, A.R., Braun, H.-P., Hildebrandt, T.M., 2018. Extended darkness induces internal turnover of glucosinolates in *Arabidopsis thaliana* leaves. PloS One 13, e0202153.

Bulgakov, V.P., 2008. Functions of *rol* genes in plant secondary metabolism. Biotechnol. Adv. 26, 318–324.

Butler, A., Sandy, M., 2009. Mechanistic considerations of halogenating enzymes. Nature 460, 848–854.

Chandra, S., Chandra, R., 2011. Engineering secondary metabolite production in hairy roots. Phytochemistry Rev. 10, 371–395.

Chintapakorn, Y., Hamill, J.D., 2003. Antisense-mediated down-regulation of putrescine *N*-methyltransferase activity in transgenic *Nicotiana tabacum* L. can lead to elevated levels of anatabine at the expense of nicotine. Plant Mol. Biol. 53, 87–105.

Chriqui, D., Guivarc'h, A., Dewitte, W., Prinsen, E., van Onkelen, H., 1996. *Rol* genes and root initiation and development. Plant Soil 187, 47–55.

Christey, M.C., 1997. Transgenic crop plants using *Agrobacterium rhizogenes*-mediated transformation. In: Doran, P.M. (Ed.), Hairy Roots: Culture and Applications. Harwood Acad. Publ., Amsterdam, pp. 99–110.

Christey, M., 2001. Use of Ri-mediated transformation for production of transgenic plants. In: Vitro Cell. Dev. Biol. Plant. vol. 37. pp. 687–700.

Cogbill, S., Faulcon, T., Jones, G., McDaniel, M., Harmon, G., Blackmon, R., Young, M., 2010. Adventitious shoot regeneration from cotyledonary explants of rapid-cycling fast plants of *Brassica rapa* L. Plant Cell Tiss. Organ Cult 101, 127–133.

Croteau, R., Winters, J.N., 1982. Demonstration of the intercellular compartmentation of l-menthone metabolism in peppermint (*Mentha piperita*) leaves. Plant Physiol. 69, 975–977.

Doyle, J.J., Doyle, J.L., 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. 19, 11–15.

Fräbel, S., Krischke, M., Staniek, A., Warzecha, H., 2016. Recombinant flavin-dependent halogenases are functional in tobacco chloroplasts without co-expression of flavin reductase genes. Biotechnol. J. 11, 1586–1594.

Gangopadhyay, M., Chakraborty, D., Bhattacharyya, S., Bhattacharya, S., 2010. Regeneration of transformed plants from hairy roots of *Plumbago indica*. Plant Cell Tissue Organ Cult. 102, 109–114.

Georgiev, M.I., Radziszewska, A., Neumann, M., Marchev, A., Alipieva, K., Ludwig-Müller, J., 2015. Metabolic alterations of *Verbascum nigrum* L. plants and SAArT transformed roots as revealed by NMR-based metabolomics. Plant Cell Tissue Organ Cult. 123, 349–356.

Glenn, W.S., Nims, E., O'Connor, S.E., 2011. Reengineering a tryptophan halogenase to preferentially chlorinate a direct alkaloid precursor. J. Am. Chem. Soc. 133, 19346–19349.

Grabkowska, R., Krolicka, A., Mielicki, W., Wielanek, M., Wysokinska, H., 2010. Genetic

transformation of *Harpagophytum procumbens* by *Agrobacterium rhizogenes*: iridoid and phenylethanoid glycoside accumulation in hairy root cultures. Acta Physiol. Plant. 32, 665–673.
Guillon, S., Trémouillaux-Guiller, J., Pati, P.K., Rideau, M., Gantet, P., 2006. Hairy root research: recent scenario and exciting prospects. Curr. Opin. Plant Biol. 9, 341–346.
Gunjan, S.K., Lutz, J., Bushong, A., Rogers, D.T., Littleton, J., 2013. Hairy root cultures and plant regeneration in *Solidago nemoralis* transformed with *Agrobacterium rhizogenes*. Am. J. Plant Sci. 4, 1675–1678.
Guo, R., Shen, W., Qian, H., Zhang, M., Liu, L., Wang, Q., 2013. Jasmonic acid and glucose synergistically modulate the accumulation of glucosinolates in *Arabidopsis thaliana*. J. Exp. Bot. 64, 5707–5719.
Habibi, P., Grossi de Sa, M.F., Lopes da Silva, A.L., Makhzoum, A., da Luz Costa, J., Borghetti, I.A., Soccol, C.R., 2016. Efficient genetic transformation and regeneration system from hairy root of *Origanum vulgare*. Physiol. Mol. Biol. Plants 22, 271–277.
Häkkinen, S.T., Moyano, E., Cusidó, R.M., Oksman-Caldentey, K.-M., 2016. Exploring the metabolic stability of engineered Hairy Roots after 16 years maintenance. Front. Plant Sci. 7, 1–9.
Hamill, J.D., Parr, A.J., Rhodes, M.J.C., Robins, R.J., Walton, N.J., 1987. New routes to plant secondary products. Biotechnology 5, 800–804.
Hamill, J.D., Robins, R.J., Parr, A.J., Evans, D.M., Furze, J.M., Rhodes, M.J.C., 1990. Over-expressing a yeast ornithine decarboxylase gene in transgenic roots of *Nicotiana rustica* can lead to enhanced nicotine accumulation. Plant Mol. Biol. 15, 27–38.
Hammer, P.E., Hill, D.S., Lam, S.T., van Pée, K.-H., Ligon, J.M., 1997. Four genes from *Pseudomonas fluorescens* that encode the biosynthesis of pyrrolnitrin. Appl. Environ. Microbiol. 63, 2147–2154.
Inoue, F., Sugiura, H., Tabuchi, A., Karasawa, D., Minami, M., 2003. Plant regeneration of peppermint, *Mentha piperita*, from the hairy roots generated from microsegment infected with *Agrobacterium rhizogenes*. Plant Biotechnol. 20, 169–172.
Junghans, U., Polle, A., Düchting, P., Weiler, E., Kuhlman, B., Gruber, F., Teichmann, T., 2006. Adaptation to high salinity in poplar involves changes in xylem anatomy and auxin physiology. Plant Cell Environ. 29, 1519–1531.
Kastell, A., Schreiner, M., Knorr, D., Ulrichs, C., Mewis, I., 2017. Influence of nutrient supply and elicitors on glucosinolate production in E. sativa hairy root cultures. Plant Cell Tissue Organ Cult. 132, 1–12.
Katayama, M.K., Saito, T.S., Katayama, K.K., 2010. 5,6-Dichloro-3-acetic acid and 4-chloroindole-3-acetic acid, two potent candidates for new rooting promoters without estrogenic activity. J. Pestic. Sci. 35, 134–137.
Khan, M.M.A., Arif Hasan Khan Robin, A.B.M., Nazim-Ud-Dowla, M.A.N., Talukder, S.K., Hassan, L., 2010. *In vitro* regeneration potentiality of Brassica genotypes in differential growth regulators. Bangladesh J. Agril. Res. 35, 189–199.
Kim, J.K., Chu, S.M., Kim, S.J., Lee, D.J., Lee, S.Y., Lim, S.H., Ha, S.H., Kweon, S.J., Cho, H.S., 2010. Variation of glucosinolates in vegetable crops of *Brassica rapa* L. ssp. *pekinensis*. Food Chem. 119, 423–428.
Kirner, S., Hammer, P.E., Hill, D.S., Altmann, A., Fischer, I., Weislo, L.J., Lanahan, M., van Pée, K.-H., Ligon, J.M., 1998. Functions encoded by pyrrolnitrin biosynthetic genes from *Pseudomonas fluorescens*. J. Bacteriol. 180, 1939–1943.
Kissen, R., Eberl, F., Winge, P., Uleberg, E., Martinussen, I., Bones, A.M., 2016. Effect of growth temperature on glucosinolate profiles in *Arabidopsis thaliana* accessions. Phytochemistry 130, 106–118.
Kolvari, E., Koukabi, N., Khoramabadi-zad, A., Shiri, A., Zolfigol, M.A., 2013. Alternative methodologies for halogenation of organic compounds. Curr. Org. Synth. 10, 837–863.
Kos, M., Houshyani, B., Wietsma, R., Kabouw, P., Vet, L.E.M., Van Loon, J.J.A., Dicke, M., 2012. Effects of glucosinolates on a generalist and specialist leaf-chewing herbivore and an associated parasitoid. Phytochemistry 77, 162–170.
Liu, X., Yang, C., Chen, M., Li, M., Liao, Z., Tang, K., 2010. Promoting scopolamine accumulation in transgenic plants of *Atropa belladonna* generated from hairy roots with over expression of *pmt* and *h6h* gene. J. Med. Plants Res. 4, 1708–1713.
Ludwig-Müller, J., 2011. Auxin conjugates: their role for plant development and in the evolution of land plants. J. Exp. Bot. 62, 1757–1773.
Ludwig-Müller, J., Schubert, B., Pieper, K., Ihmig, S., Hilgenberg, W., 1997. Glucosinolate content in susceptible and tolerant Chinese cabbage varieties during the development of the clubroot disease. Phytochemistry 44, 407–414.
Ludwig-Müller, J., Jahn, L., Lippert, A., Püschel, J., Walter, A., 2014. Improvement of hairy root cultures and plants by changing biosynthetic pathways leading to pharmaceutical metabolites: strategies and applications. Biotechnol. Adv. 32, 1168–1179.
Matsuda, K., Toyoda, H., Nishio, H., Nishida, T., Dohgo, M., Bingo, M., Matsuda, Y., Yoshida, S., Harada, S., Tanaka, H., Komai, K., Ouchi, S., 1998. Control of the bacterial wilt of tomato plants by a derivative of 3-indolepropionic acid based on selective actions on *Ralstonia solanacearum*. J. Agric. Food Chem. 46, 4416–4419.
Mei, W.Y., Wang, J.B., Lu, D., Jia, J.F., 2001. Regeneration of plants from callus cultures of roots induced by *Agrobacterium rhizogenes* on *Alhagi pseudoalhagi*. Cell Res. 11, 279–284.
Mikkelsen, M.D., Olsen, C.E., Halkier, B.A., 2010. Production of the cancer-preventive glucoraphanin in tobacco. Mol. Plant 3, 751–759.
Mittag, J., Gabrielyan, A., Ludwig-Müller, J., 2015. Knockout of *GH3* genes in the moss *Physcomitrella patens* leads to increased IAA levels at elevated temperature and in darkness. Plant Physiol. Biochem. 97, 339–349.
Moriuchi, H., Fujikawa, Y., Aly, M.A.M., Saneoka, H., Fujita, K., Yamashita, I., Tanaka, N., 2004. Hairy root-mediated transgenic plant regeneration in Egyptian clover (*Trifolium alexandrinum* L.). Plant Biotechnol. 21, 165–168.
Murata, M., Orton, T.J., 1987. Callus initiation and regeneration capacities in *Brassica* species. Plant Cell Tissue Organ Cult. 11, 111–123.
Neumann, C.S., Fujimori, D.G., Walsh, C.T., 2008. Halogenation strategies in natural product biosynthesis. Chem. Biol. 15, 99–109.
Nilsson, O., Olsson, O., 1997. Getting to the root: the role of the *Agrobacterium rhizogenes* *rol*-genes in the formation of hairy roots. Physiol. Plantarum 100, 463–473.
Ohara, A., Akasaka, Y., Daimon, H., Mii, M., 2000. Plant regeneration from hairy roots induced by infection with *Agrobacterium rhizogenes* in *Crotalaria juncea* L. Plant Cell Rep. 19, 563–568.
Paek, K.-Y., Hahn, E.-J., Son, S.-H., 2001. Application of bioreactors for large-scale micropropagation systems of plants. In: Vitro Cell. Dev. Biol. Plant. vol. 37. pp. 149–157.
Patallo, E.P., Walter, A., Milbredt, D., Thomas, M., Neumann, M., Caputi, L., O'Connor, S., Ludwig-Müller, J., van Pée, K.-H., 2017. Strategies to produce chlorinated indole-3-acetic acid and indole-3-acetic acid intermediates. Chemistry 2, 11148–11153.
Pavlova, O.A., Matveyeva, T.V., Lutova, L.A., 2014. *rol*-Genes of *Agrobacterium rhizogenes*. Russ. J. Genet.: Appl. Res. 4, 137–145.
Pedras, M.S.C., Yaya, E.E., 2010. Phytoalexins from Brassicaceae: news from the front. Phytochemistry 71, 1191–1197.
Pedras, M.S.C., Hossain, S., 2011. Interaction of cruciferous phytoanticipins with plant fungal pathogens: indole glucosinolates are not metabolized but the corresponding desulfo-derivatives and nitriles are. Phytochemistry 72, 2308–2316.
Peres, L.E.P., Morgante, P.G., Vecchi, C., Kraus, J.E., van Sluys, M.-A., 2001. Shoot regeneration capacity from roots and transgenic hairy roots of tomato cultivars and wild related species. Plant Cell Tissue Organ Cult. 65, 37–44.
Petersen, A., Wang, C., Crocoll, C., Halkier, B.A., 2018. Biotechnological approaches in glucosinolate production. J. Integr. Plant Biol. 60, 1231–1248.
Piatczak, E., Kuzma, L., Skała, E., Zebrowska, M., Balcerczak, E., Wysokinska, H., 2015. Iridoid and phenylethanoid glycoside production and phenotypical changes in plants regenerated from hairy roots of *Rehmannia glutinosa* Libosch. Plant Cell Tissue Organ Cult. 122, 259–266.
Qing, C.M., Fan, L., Lei, Y., Bouchez, D., Tourneur, C., Yan, L., Robaglia, C., 2000. Transformation of pakchoi (*Brassica rapa* L. ssp. *chinensis*) by *Agrobacterium* infiltration. Mol. Breed. 6, 67–72.
Radwanski, E.R., Last, R.L., 1995. Tryptophan biosynthesis and metabolism: biochemical and molecular genetics. Plant Cell 7, 921–934.
Rameeh, V., 2015. Glucosinolates and their important biological and anti-cancer effects: a review. Jordan J. Agric. Sci. 11, 1–13.
Ramirez-Estrada, K., Vidal-Limon, H., Hidalgo, D., Moyano, E., Golenioswki, M., Cusidó, R.M., Palazon, J., 2016. Elicitation, an effective strategy for the biotechnological production of bioactive high-added value compounds in plant cell factories. Molecules 21, 182. https://doi.org/10.3390/molecules21020182s.
Rauhut, T., Glawischnig, E., 2009. Evolution of camalexin and structurally related indolic compounds. Phytochemistry 70, 1638–1644.
Reinecke, D.M., Ozga, J.A., Magnus, V., 1995. Effect of halogen substitution of indole-3-acetic acid on biological activity in pea fruit. Phytochemistry 40, 1361–1366.
Runguphan, W., Qu, X., O'Connor, S.E., 2010. Integrating carbon-halogen bond formation into medicinal plant metabolism. Nature 468, 461–464.
Saitou, T., Kamada, H., Harada, H., 1992. Light requirement for shoot regeneration in horseradish hairy roots. Plant Physiol. 99, 1336–1341.
Savić, B., Tomić, S., Magnus, V., Gruden, K., Barle, K., Grenković, R., Ludwig-Müller, J., Salopek-Sondi, B., 2009. Auxin amidohydrolases from *Brassica rapa* cleave the alanine conjugate of indolepropionic acid as a preferable substrate: a biochemical and modeling approach. Plant Cell Physiol. 50, 1587–1599.
Seibold, C., Schnerr, H., Rumpf, J., Kunzendorf, A., Hatscher, C., Wage, T., Ernyei, A.J., Dong, C., Naismith, J.H., van Pée, K.-H., 2006. A flavin-dependent tryptophan 6-halogenase and its use in modification of pyrrolnitrin biosynthesis. Biocatal. Biotransform. 24, 401–408.
Sevon, N., Oksman-Caldentey, K.M., 2002. *Agrobacterium rhizogenes*-mediated transformation: root cultures as a source of alkaloids. Planta Med. 68, 859–868.
Shanks, J.V., Morgan, J., 1999. Plant "hairy root" culture. Curr. Opin. Biotechnol. 10, 151–155.
Sharafi, A., Sohi, H.H., Azadi, P., Sharafi, A.A., 2014. Hairy root induction and plant regeneration of medicinal plant *Dracocephalum kotschyi*. Physiol. Mol. Biol. Plants 20, 257–262.
Sidwa-Gorycka, M., Krolicka, A., Orlita, A., Malinski, E., Golebiowski, M., Kumirska, J., Chromik, A., Biskup, E., Stepnowski, P., Lojkowska, E., 2009. Genetic transformation of *Ruta graveolens* L. by *Agrobacterium rhizogenes*: hairy root cultures a promising approach for production of coumarins and furanocoumarins. Plant Cell Tissue Organ Cult. 97, 59–69.
Sun, L., Lindbeck, A.C., Nilius, A.M., Towne, T.B., Zhou, C.C., Paulus, T.J., 2004. Synthesis, isolation and antibacterial activities of monodechlorovancomycins. J. Antibiot. (Tokyo) 57, 691–694.
Takayama, S., Akita, M., 2005. Practical aspects of bioreactor application in mass propagation of plants. In: Hvoslef-Eide, A.K., Preil, W. (Eds.), Liquid Culture Systems for in Vitro Plant Propagation. Springer, The Netherlands, pp. 61–78.
Textor, S., Gershenzon, J., 2009. Herbivore induction of the glucosinolate–myrosinase defense system: major trends, biochemical bases and ecological significance. Phytochemistry Rev. 8, 149–170.
Tivendale, N.D., Davidson, S.E., Davies, N.W., Smith, J.A., Dalmais, M., Bendahmane, A.I., Quittenden, L.J., Sutton, L., Bala, R.K., Le Signor, C., 2012. Biosynthesis of the halogenated auxin, 4-chloroindole-3-acetic acid. Plant Physiol. 159, 1055–1063.
Traka, M., Mithen, R., 2009. Glucosinolates, isothiocyanates and human health. Phytochemistry Rev. 8, 269–282.
Wagner, C., El Omari, M., König, G.M., 2009. Biohalogenation: nature's way to synthesize halogenated metabolites. J. Nat. Prod. 72, 540–553.
Walter, A., Caputi, L., O'Connor, S., van Pée, K.-H., Ludwig-Müller, J., 2020. Chlorinated auxins - How does *Arabidopsis thaliana* deal with them? Int. J. Mol. Sci. 21, 2567.
Weichold, V., Milbredt, D., van Pee, K.-H., 2016. Specific enzymatic halogenation - from the discovery of halogenated enzymes to their applications *in vitro* and *in vivo*. Angew. Chem. Int. Ed. 55, 6374–6389.

Witzel, K., Hanschen, F.S., Schreiner, M., Krumbein, A., Ruppel, S., Grosch, R., 2013. Verticillium suppression is associated with the glucosinolate composition of *Arabidopsis thaliana* leaves. PloS One 8, 1–10.

Zang, Y.-x., Ge, J.-l., Huang, L.-h., Gao, F., Lv, X.-s., Zheng, W.-w., Hong, S.-b., Zhu, Z.-j., 2015. Leaf and root glucosinolate profiles of Chinese cabbage (*Brassica rapa* ssp. *pekinensis*) as a systemic response to methyl jasmonate and salicylic acid elicitation. J. Zhejiang Univ.-Sci. B (Biomed. & Biotechnol.) 16, 696–708. https://doi.org/10.1631/jzus.B1400370.

Zehner, S., Kotzsch, A., Bister, B., Süssmuth, R.D., Méndez, C., Salas, J., van Pée, K.-H., 2005. A regioselective tryptophan 5-halogenase is involved in pyrroindomycin biosynthesis in *Streptomyces rugosporus* LL-42D005. Chem. Biol. 12, 445–452.

Zhou, Y.Q., Duan, H.Y., Zhou, C.E., Li, J.J., Gu, F.P., Wang, F., Zhang, Z.Y., Gao, Z.M., 2009. Hairy root induction and plant regeneration of *Rehmannia glutinosa* Libosch. f. *hueichingensis* Hsiao via *Agrobacterium rhizogenes*-mediated transformation. Russ. J. Plant Physiol. 56, 224–231.

Zhu, X., De Laurentis, W., Leang, K., Herrmann, J., Ihlefeld, K., van Pée, K.-H., Naismith, J.H., 2009. Structural insights into regioselectivity in the enzymatic chlorination of tryptophan. J. Mol. Biol. 391, 74–85.