Production of benzylglucosinolate in genetically engineered carrot suspension cultures

Elena Kurzbach1,2, Matthias Strieker1,a, Ute Wittstock1,2,*

1Institute of Pharmaceutical Biology, Technische Universität Braunschweig, Braunschweig, Germany; 2 Center of Pharmaceutical Engineering (PVZ), Technische Universität Braunschweig, Braunschweig, Germany

*E-mail: u.wittstock@tu-bs.de Tel: +49-531-3915681 Fax: +49-531-3918104

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Abstract Glucosinolates, a group of sulfur-containing specialized metabolites of the Brassicales, have attracted a lot of interest in nutrition, medicine and agriculture due to their positive health effects and their involvement in plant defense. Their biological activities and the extensive knowledge of their biosynthesis have inspired research into development of crops with enhanced glucosinolate contents as well as their biotechnological production in homologous and heterologous systems. Here, we provide proof-of-concept for transgenic suspension cultures of carrot (Daucus carota, Apiaceae) as a scalable production platform for plant specialized metabolites using benzylglucosinolate as a model. Two T-DNAs carrying in total six genes of the benzylglucosinolate biosynthesis pathway from Arabidopsis thaliana as well as NPTIII and BAR as selectable markers were transferred to carrot cells by Agrobacterium tumefaciens-mediated transformation. Putative transformants selected based on their kanamycin and BASTA resistances were subjected to HPLC-MS analysis. Of 79 putative transformants, 17 produced benzylglucosinolate. T-DNA integration was confirmed for the five best producers. Callus from these transformants was used to establish suspension cultures for quantitative analysis. When grown in 60-ml-cultures, the best transformants produced roughly 2.5 nmol (g fw)−1 benzylglucosinolate, together with up to 10 nmol (g fw)−1 desulfobenzylglucosinolate. Only one transformant produced more benzylglucosinolate than desulfobenzylglucosinolate. The concentration of sulfate in the medium was not a major limiting factor. High production seemed to be associated with poor growth and vice versa. Therefore, future research should try to optimize medium and cultivation process and to separate growth and production phase by using an inducible promoter.

Key words: callus suspension cultures, carrot, glucosinolates, heterologous production.

Introduction

Plants produce a plethora of specialized metabolites whose enormous structural diversity has been shaped and optimized by selection pressures posed by the environment (Hartmann 2007). Mankind has made use of the biological activities and physicochemical properties of these metabolites for millennia, for example by using plant preparations or isolated plant compounds as medicines, stains, pesticides, spices, or perfumes (Appendino and Pollastro 2009; Dias et al. 2012). Besides these applications, specialized metabolites are endogenous components of plant-derived foods, equipping them with flavors, smells and colors as well as health-beneficial or, in some cases, adverse effects (Moreira et al. 2018; Traka and Mithen 2009; Zimmermann and Wagner 2021). Despite their long history of human usage, applications of specialized metabolites as pure compounds are often limited by their occurrence in small quantities and/or as complex mixtures. Sustainable supply of source materials can pose additional challenges, not only for the application of known compounds, but also for the exploration of the rich reservoir of optimized structures produced by plants as leads in drug discovery. Elucidation of biosynthetic pathways at the gene level have paved the way for exploiting bacteria, yeasts and plants as heterologous production platforms for the expression of a “foreign” biosynthetic pathway. Despite a number of successful examples, metabolic engineering for specialized metabolite production has remained challenging (Courdavault et al. 2021; Galanie et al. 2015; Lynch et al. 2021; Paddon et al. 2013; Schultz et al. 2019; Ye et al. 2000).

Glucosinolates, a relatively small group of sulfur-
containing specialized metabolites, which occur primarily in plants of the Brassicales order, have attracted a lot of interest in both fundamental and applied research (Blažević et al. 2020; Halkier and Gershenzon 2006). For example, their biosynthesis, transport, metabolism and biological roles can be studied in Arabidopsis, a natural glucosinolate producer, while potential health benefits associated with the ingestion of glucosinolate-containing vegetables such as broccoli have prompted the desire to breed high-glucosinolate varieties, to conduct clinical studies for evaluation of health effects, and to understand their mechanisms of action (Bednarek et al. 2009; Fahey et al. 2017; Frerigmann and Gigolashvili 2014; Nour-Eldin et al. 2012; Traka and Mithen 2009). As a consequence, heterologous production of glucosinolates has been studied intensively to provide a stable supply of structurally diverse glucosinolates or to use them in planta in pest management strategies or as functional foods (Petersen et al. 2018). The relatively simple biosynthetic pathways of benzylglucosinolate and indol-3-ylmethylglucosinolate as well as the more complex route to 4-methylsulfinylbutylglucosinolate and 2-phenylethylglucosinolate have been reconstituted in transgenic Escherichia coli, Saccharomyces cerevisiae, Solanum tuberosum, stably transformed Nicotiana tabacum and transiently transformed N. benthamiana, respectively, by transfer of up to 13 genes (Table 1). Yields ranged from 5 nmol per gram fresh weight (fw) to 1.8 µmol (g fw)^{-1} (2–740 µg (g fw)^{-1}) in plants and from 2 pmol ml^{-1} to 20 nmol ml^{-1} (0.7 µg l^{-1}–8 mg l^{-1}) in microbial hosts (Table 1). Of note, glucosinolate core structure biosynthesis and modifying reactions are catalyzed in cooperation of endoplasmatic reticulum (ER)-bound and cytosolic enzymes in nature while precursor modifications prior to core structure biosynthesis (i.e. side-chain elongation of protein amino acids) take place in plastids (Sønderby et al. 2010).

In the present study, we tested carrot callus suspension cultures as heterologous hosts for the expression of specialized metabolite biosynthetic pathways. We employed benzylglucosinolate as a model compound based on its relatively simple biosynthesis (Figure 1).

Table 1. Examples of glucosinolate production in heterologous hosts.

| Glucosinolate (side chain) | Host          | Yield^1                  | Reference                  |
|---------------------------|---------------|--------------------------|----------------------------|
| Benzyl-                   | E. coli       | 20 nmol ml^{-1} (8 mg l^{-1}) | (Petersen et al. 2019)    |
|                           | S. tuberosum  | 5 nmol (g fw)^{-1} (2 µg (g fw)^{-1}) | (González-Romero et al. 2021) |
|                           | N. tabacum    | 500 nmol (g fw)^{-1} (200 µg (g fw)^{-1}) | (Møldrup et al. 2012)    |
|                           | N. benthamiana (tr)^2 | 1,800 nmol (g fw)^{-1} (740 µg (g fw)^{-1}) | (Møldrup et al. 2011)    |
| Indol-3-ylmethyl-         | S. cerevisiae | 2 nmol ml^{-1} (1 mg l^{-1}) | (Mikkelsen et al. 2012)  |
| 4-methylsulfinylbutyl-    | E. coli       | 0.002 nmol ml^{-1} (0.0007 mg l^{-1}) | (Yang et al. 2020)       |
|                           | N. benthamiana (tr) | 40 nmol (g fw)^{-1} (18 µg (g fw)^{-1}) | (Mikkelsen et al. 2010)  |
| 2-phenylethyl-            | N. benthamiana (tr) | 40 nmol (g fw)^{-1} (17 µg (g fw)^{-1}) | (Wang et al. 2021)       |

^1Approximate yields are given based on the maximum value reported in the corresponding references (per volume for microbial hosts, yellow; per mass fw for plants, green). Glucosinolate contents in natural producers and vegetable crops are typically in the range of 100–5,000 nmol (g fw)^{-1}. ^2tr, transiently transformed plants.

Figure 1. Benzylglucosinolate biosynthesis. Scheme of the enzymatic steps leading from L-phenylalanine to benzylglucosinolate (Geu-Flores et al. 2011; Møldrup et al. 2012). Cofactors, cosubstrates and further products are omitted for clarity. Enzyme names refer to A. thaliana enzymes introduced to carrot cells in this study. GSH, glutathione; GGP1, γ-glutamylpeptidase 1; SUR1, enzyme encoded by Superroot1 (S-alkyl-thiohydroximate lyase); UGT74B1, UDP glucose-dependent glucosyltransferase 74B1; SOT16, sulfotransferase 16.
1) and proven suitability for metabolic engineering (Møldrup et al. 2012; Petersen et al. 2019). Although dedifferentiated, callus cells represent plant cells with the typical cellular compartments often required for plant specialized metabolite biosynthesis and the ability for post-translational modifications (Shaaltiel et al. 2007). They can be grown in a scalable fashion in bioreactors. This could help overcome the regulatory constraints associated with the large-scale growth of transgenic plants in many countries. The feasibility of such cultures for industrial production is evident from the drug ELELYSO®, which contains recombinant human glucocerebrosidase produced in transgenic carrot suspension cultures for enzyme replacement therapy of Gaucher’s disease (Shaaltiel et al. 2007) (https://www.eelyso.com/how-is-eelyso-made; last accessed on 7 March 2022).

Materials and methods

Wild-type carrot callus and suspension cultures

Seeds of D. carota “Sperling’s frühe Bund- und Waschmöhre, Nantaise 2” (Apiaceae; Samenhaus Knieke, Braunshweig, Germany) were treated sequentially with 70% (v/v) ethanol and 4% (v/v) sodium hypochlorite, washed with sterile water and germinated on solid modified Murashige & Skoog (MS) medium (Murashige and Skoog 1962) (Supplementary Table S1) at 20°C with 16 h light/8 h dark cycle. Plantlets were transferred to 100 ml Erlenmeyer flasks filled with 30 ml solid modified MS medium after 20–30 days and transferred to 100 ml Erlenmeyer flasks filled with 30 ml solid MS medium after 20–25 days and were subcultured every 28 days. Timentin was added to 10 ml YEP medium (1% (w/v) bacto peptone, 1% (w/v) yeast extract, 0.085 M sodium chloride) with 100 µg ml⁻¹ rifampicin (Duchefa, Harlem, The Netherlands), 20 µg ml⁻¹ gentamicin (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and 100 µg ml⁻¹ kanamycin (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) at 28°C and 220 rpm for 48 h. This culture was added to 100 ml YEP medium containing the same antibiotics to obtain an optical density (OD₆₀₀) of 0.15. The culture was grown at 28°C and 220 rpm until an OD₆₀₀ of 0.8–1.0 was reached. An aliquot of 10 ml bacterial culture was centrifuged at room temperature for 4,000 rpm for 20 min. The pellet was washed with DC medium and then gently suspended in 3 ml DC medium. The bacterial suspension was further diluted to an OD₆₀₀ of 0.3 and incubated with 100 µM acetosyringone (3,5-dimethoxy-4-hydroxycetophenone; Arcos Organics/Thermo Fisher Scientific, New Jersey, USA) in the dark at room temperature for 1 h.

A ten-day-old 50 ml carrot suspension culture was gently centrifuged in a 50 ml Falcon tube at 1,000 rpm for 2 min. The pellet was suspended in 50 ml DC medium and the suspension was divided into 6 ml aliquots. For transformation with a single construct (consecutive transformation), 300 µl of the prepared A. tumefaciens suspension (with either construct 1 or 2) were applied to the aliquot of carrot suspension and the mix was co-cultivated at 22°C and 110 rpm in the dark. For simultaneous transformation with both constructs, the two A. tumefaciens strains were mixed 1:1 before application to the aliquot of carrot suspension. In parallel, an aliquot of carrot suspension without addition of A. tumefaciens served as control culture. After three days, the carrot cells were harvested by centrifugation at room temperature for 3 min at 100×g. The pellet was washed three times in 6 ml DC medium with increasing duration of the centrifugation step (8, 5 and finally 3 min). The cell pellet (~5 ml) was taken up in 5 ml DC medium and volumes of 20 µl were dropped on solid DC medium supplemented with 300 µg ml⁻¹ timentin (Duchefa, Harlem, The Netherlands), 100 µg ml⁻¹ kanamycin or/and 6 µg ml⁻¹ BASTA. The drops were dried under the sterile bench for about 20–30 min. The petri dishes (94×16 mm, without vents, Greiner bio-one) were sealed with Parafilm® (Bemis Company, Neenah, USA) and kept at 22°C in half-shadow till callus formation was observed. Calli emerged after around 20–25 days and were subcultured every 28 days. Timentin was

Constructs for benzylglucosinolate production

Construct 1 (ORF1-GGP) (Møldrup et al. 2012) and construct 2 (ORF2) (Geu-Flores et al. 2011) for enhanced Cauliflower Mosaic Virus 35S promoter (eCaMV35S)-driven, polycistronic expression of the benzylglucosinolate biosynthesis pathway were kindly provided by Prof. Barbara Ann Halkier (University of Copenhagen) (Supplementary Figure S2A). Construct 1 contained the expression cassettes for CYP79A2, CYP83B1, GGP1 from A. thaliana and for the BAR gene (BASTA resistance). Construct 2 provided the expression cassettes for SUR1, UGT74B1, SOT16 from A. thaliana and for the NPTII gene (kanamycin resistance).

Transformation of carrot suspension cultures

Agrobacterium tumefaciens (C58C1::pMP90; Kaufholdt et al. 2016) transformed with either construct 1 or construct 2 was grown in 10 ml YEP medium (1% (w/v) bacto peptone, 1% (w/v) yeast extract, 0.085 M sodium chloride) with 100 µg ml⁻¹ rifampicin (Duchefa, Harlem, The Netherlands), 20 µg ml⁻¹ gentamicin (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and 100 µg ml⁻¹ kanamycin (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) at 28°C and 220 rpm for 48 h. This culture was added to 100 ml YEP medium containing the same antibiotics to obtain an optical density (OD₆₀₀) of 0.15. The culture was grown at 28°C and 220 rpm until an OD₆₀₀ of 0.8–1.0 was reached. An aliquot of 10 ml bacterial culture was centrifuged at room temperature for 4,000 rpm for 20 min. The pellet was washed with DC medium and then gently suspended in 3 ml DC medium. The bacterial suspension was further diluted to an OD₆₀₀ of 0.3 and incubated with 100 µM acetosyringone (3,5-dimethoxy-4-hydroxycetophenone; Arcos Organics/Thermo Fisher Scientific, New Jersey, USA) in the dark at room temperature for 1 h.

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omitted after the third subcultivation. Callus obtained from transformation with a single construct was used to generate suspension cultures for subsequent transformation with the second construct using the same procedure (consecutive transformation).

**Screening of calli for benzylglucosinolate production**

Callus material was supplemented with 50 µl 1 µM fraxin (Fluka/Sigma-Aldrich, St. Louis, Missouri, USA; internal standard) and freeze-dried. Freeze-dried material (20–50 mg) was homogenized in 1.4 ml methanol by vigorous shaking with five glass beads (2 mm in diameter, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) per sample using a Minimix MK4 paint shaker (Merris, Maidenhead, United Kingdom) for 2×90 s. After centrifugation at 14,000 rpm for 10 min, 700 µl supernatant were dried by a stream of compressed air and heating at 70°C in a sample concentrator (with Dri-Block heater, Techne, Staffordshire, United Kingdom). The sample was dissolved in 100 µl of 6% (v/v) formic acid in 60% (v/v) methanol and 10 µl were subjected to high performance liquid chromatography (HPLC)-tandem-mass-spectrometry (MS/MS) analysis.

**Transgenic carrot suspension cultures**

Callus recovered from solid DC medium with 100 µg ml⁻¹ kanamycin or/and 6 µg ml⁻¹ BASTA was disintegrated with a scalpel and submerged in 10 ml DC medium (50 ml Erlenmeyer flask). After approximately seven days at 22°C and 130 rpm in the dark, the whole suspension was transferred to a 100 ml Erlenmeyer flask with 20–30 ml DC medium and incubated at 22°C and 130 rpm in the dark for another 7–10 days. Then, half of one culture was added to 50 ml DC medium (in 200/250 ml Erlenmeyer flask) and grown at 22°C and 130 rpm in the dark. Subcultivation was conducted every 10–14 days by transferring 5–15 ml suspension (depending on the growth properties of the transformants) to 50 ml DC medium with the appropriate antibiotics. Suspension cultures (~60 ml) consisted of microscopically visible cell clusters with varying degree depending on the individual transformant. For general maintenance as callus or in suspension, cultures were grown in the presence of the appropriate antibiotics unless otherwise stated. For experiments on benzylglucosinolate production antibiotics were omitted.

**Genotyping of transformed carrot cells**

Genomic DNA was isolated from the frozen cell pellet of 1 ml suspension culture according to (Edwards et al. 1991). Primers designed to amplify specific T-DNA regions were used at a final concentration of 500 nM for PCR amplification in a total volume of 25 µl 1× DreamTaq buffer supplemented with 0.2 mM dNTPs, 0.13 µl DreamTaq polymerase (Thermofisher Scientific Inc., Waltham, USA) and 1 µl genomic DNA preparation. D. carota mitochondrial alternative oxidase 1 (DcAOX1, GenBank EU286573.2) was used as reference gene for carrot (Campos et al. 2016, 2009). Primers and temperature programs are given in Supplementary Table S2.

**Minicultures**

Suspension cultures were generated from sliced callus material as described above, that is sliced calli were submerged in 10 ml DC medium and cultivated at 22°C and 130 rpm in the dark. After 7 days, 20 ml DC medium were added. After another 7–17 days at 22°C and 130 rpm in the dark, 2 ml aliquots of this preculture were mixed with 10 ml DC medium (100 ml Erlenmeyer flask) to obtain a "miniculture". Cultivation was continued under the above conditions for 14–18 days. Samples of 1 ml were withdrawn before incubation, at day 5 and day 10 of cultivation (three samples per culture). Cell pellets obtained after centrifugation of the 1 ml aliquots at 14,000 rpm for 10 min were weighed before (fresh weight, fw) and after freeze-drying (dry weight, dw). Extraction was performed in the same way as described for the callus screening, but 5 µl were used for HPLC-MS/MS analysis.

**Sulfate supplementation**

The effect of increased sulfate concentrations in the medium was investigated with minicultures. Before inoculation with 2 ml preculture, 10 ml DC medium were supplemented with 24 or 60 µl of a 5 M (NH₄)₂SO₄ solution corresponding to an extra 10 or 25 mM sulfate in the medium.

**Yield determination**

Growth behaviour and metabolite production of 60 ml-suspension cultures of two selected transformants was monitored over a period of 10 days in DC medium. After 10 days of cultivation, suspension cultures were harvested by centrifugation at 4,000 rpm for 10 min (in 50 ml Falcon tubes, in two steps). Fw and dw of the pellets were determined and showed roughly a 20-fold difference. Samples of 20 mg lyophilized and ground material were extracted as described above for the callus screening and analyzed for benzylglucosinolate and desulfobenzylglucosinolate content by HPLC-MS/MS.

**HPLC-MS/MS analysis**

Analyses were performed with an Agilent 1200 HPLC instrument (Agilent, Waldbronn, Germany) coupled to an Applied Biosystems/MDS Sciex 3200 QTRAP MS/MS system (AB Sciex, Darmstadt, Germany). Glucosinolates were separated from salts and other compounds on a Chromolith Performance RP-18e 100-3 column (Merck, Darmstadt, Germany) at 25°C using 0.3% (v/v) formic acid in water as the mobile phase at a flow rate of 1 ml/min for 5 min (callus screening) or 3 min (suspension cultures). The column was washed with 0.3% (v/v) formic acid in acetonitrile at a flow rate of 1 ml/min for 3–6 min between runs. In the analysis of callus material, typical retention times were between 2.00 and 2.27 min for benzylglucosinolate, between 2.07 and 2.33 min for desulfobenzylglucosinolate and between 2.14 and 2.53 min for...
fraxin. For the analysis of suspension cultures typical retention times were 1.91 min for benzylglucosinolate and 1.88 min for desulfobenzylglucosinolate.

The compounds were identified by electrospray ionisation (ESI)-MS/MS analysis in negative mode with multiple reaction monitoring (MRM). The MS settings for the MRM experiments were: curtain gas: 40 psi; collision gas: high; ion spray voltage: −4500 V; temperature: 450 °C; ion source gas 1: 75 psi; ion source 2: 50 psi; interface heater: on. The total scan time (including pauses) was 0.3900 s for all MRM transitions. Each transition was performed with a dwell time of 60 ms and pause time of 5 ms. The collision cell exit potential was −1 V. For each analyte three MRM transitions were monitored: benzylglucosinolate (m/z): 408 to 97, 96 and 75 with a declustering potential (DP) of −45 V, an entrance potential (EP) of −10 V and a collision energy (CE) of −37 eV; desulfobenzylglucosinolate (m/z): 328 to 166, 119 and 75, DP was −28 V, EP −9.5 V and CE −24 eV; fraxin (m/z): 369 to 192, 206 and 207, DP was −40 V, EP −10 V and CE −32 eV. MRM peaks were automatically integrated (minimum peak height: 200 cps, time window: 30 s). In case of benzylglucosinolate and fraxin, the peak areas from the three recorded MRM traces were added, in case of desulfobenzylglucosinolate just two MRM traces (328 to 119 and 328 to 75) were used.

For callus screening, relative quantities of benzylglucosinolate were determined as normalized peak areas by dividing benzylglucosinolate peak area by that of the internal standard. For absolute quantification, external matrix-matched calibration was conducted using wildtype carrot cells spiked with defined amounts of benzylglucosinolate and desulfobenzylglucosinolate. Specifically, 50 µl stock solutions (eight concentrations in the range of 1–100 µM in methanol) of benzylglucosinolate (PhytoPlan, Heidelberg, or PhytoLab, Vestenbergsgreuth, Germany) and desulfobenzylglucosinolate (desulfated benzylglucosinolate, result of treatment with type H1 sulfatase from *Helix pomatia* (Sigma-Aldrich, St. Louis, Missouri, USA) for 30 min at room temperature) were added to an appropriate amount of lyophilized carrot cells from wildtype suspension cultures. The spiked samples were extracted and analyzed as described above for the screening of callus material. For each quantitative measurement, the experimental samples and calibration samples were run consecutively in the same sequence. The equations obtained upon construction of calibration lines by linear regression were used to calculate benzylglucosinolate and desulfobenzylglucosinolate contents in the experimental samples.

**Results**

The six relevant *A. thaliana* genes encoding for the benzylglucosinolate biosynthetic pathway enzymes were introduced in suspension cultures via *Agrobacterium*-mediated transformation. We conducted consecutive or simultaneous transformation with the constructs ORF1-GGP and ORF2. These harbored three biosynthetic genes each. Transformants were selected based on the functionality of the selection markers, i.e. resistance against kanamycin and BASTA, upon mounting of drops of cell suspension onto selection medium (Figure 2A). We did not analyze systematically if consecutive or simultaneous transformation differed in success rate, but simultaneous transformation was faster than consecutive transformation with no obvious drawbacks. Viable calli were subcultured several times on selection medium which was also supplemented with the antibiotic timentin for elimination of excessive *A. tumefaciens*. After three subcultures, timentin was omitted and viable calli were regarded as transformed. From a total of 1,689 drops, 281 calli emerged of which 79 were viable throughout the entire procedure. These were regarded as tentative transformants from independent T-DNA insertion events and subjected to further chemical and genetic analysis.

Callus material of the 79 selected transformants was extracted with methanol and extracts were analyzed for the presence of benzylglucosinolate by HPLC-MS/MS. In 17 transformants, benzylglucosinolate was detected while all remaining transformants did not contain detectable amounts of benzylglucosinolate. Benzylglucosinolate content in the producing transformants varied over more

![Figure 2](image-url)

**Figure 2.** Presence of benzylglucosinolate in kanamycin- and BASTA-resistant calli. A: Selection of kanamycin- and BASTA-resistant calli. A representative plate is shown as an example. B: Callus was extracted (left panel: transformants 1–7, right panel: transformants 7–17) and analyzed by HPLC-MS/MS (MRM). Peak areas were normalized to that of an internal standard. Results of transformant 7 are shown in both panels for scale comparison.
than four orders of magnitude based on normalized peak areas (Figure 2B). The six transformants with highest benzylglucosinolate content were chosen for further experiments. Their callus material was used to generate suspension cultures. After three rounds of subculturing in DC medium, homogenous suspensions were obtained with a tendency of aggregate formation in cultures of transformants 2 and 6. When the transformed material was grown in the presence of the appropriate antibiotics, it grew slower than the wildtype (grown in medium with no antibiotics). Typically, wildtype cultures reached the stationary phase ten days after subculture and had produced about twice as much biomass as transformed cultures (grown with antibiotics) at this time point. One of the transformants, transformant 3, did not grow well and lost its ability to produce benzylglucosinolate over time. Therefore, only five transformants (transformants 1, 2, 4, 5, 6) were subject to more detailed analyses.

Genomic DNA of transformants 1, 2, and 4–6 was analyzed by PCR for the inserted coding sequences of the benzylglucosinolate biosynthetic genes using primer pairs specific for a part of the introduced sequences (Supplementary Figure S2). The T-DNA insertion originating from the construct ORF1-GGP was proven by the amplification of \textit{CYP83B1} and \textit{BAR} fragments and that from the construct ORF2 by amplification of \textit{SUR1} and \textit{NPTII} fragments in all transformants (Supplementary Figure S2). As expected, no product was obtained when primer pairs for \textit{CYP83B1}, \textit{BAR}, \textit{SUR1} and \textit{NPTII} were used in PCR with genomic DNA from wildtype suspension cells. Together with kanamycin- and BASTA-resistance and benzylglucosinolate production, this confirmed that the five transformants had successfully been transformed with both constructs.

Growth behavior and benzylglucosinolate production of the five transformants were monitored over a period of 14–18 days in minicultures. Despite a lot of variation, growth and production for ten days delivered relatively reproducible results (Figure 3). High production seemed to be associated with poor growth and vice versa across the analyzed transformants. Highest benzylglucosinolate levels were found in transformant 1 (5–10 nmol (g fw)$^{-1}$; Figure 3B). Interestingly, threefold higher levels of the biosynthetic precursor desulfobenzylglucosinolate accumulated in the cells of transformant 1 (Figure 3B). While the total amount of both products was similar in transformant 1 and transformant 2, the proportion of benzylglucosinolate was even lower in transformant 2 in favor of desulfobenzylglucosinolate accumulation. Transformants 4 and 6 also contained higher levels of desulfobenzylglucosinolate than benzylglucosinolate. Only in transformant 5, the level of benzylglucosinolate exceeded that of desulfobenzylglucosinolate (factor 3). Taken together, this indicated that the final step in biosynthesis, transfer of sulfate, may represent a bottleneck of the pathway in the transgenic carrot cells.

To overcome the bottleneck at the sulfate transfer step, we tested if supplementation of the medium with additional sulfate increases the ratio of benzylglucosinolate-to-desulfo-glucosinolate content in minicultures. We did not find a general trend towards an improved product ratio upon addition of 10 or 25 mM ammonium sulfate to the DC medium (Figure 4). In transformants 2 and 6, addition of 10 mM sulfate—corresponding to an approximately sevenfold increase of the sulfate concentration in the medium—resulted in a higher total content of benzyl- as well as desulfobenzylglucosinolate when compared to no addition, with unchanged ratio (Figure 4B, E). In transformants 1 and 5, there was no effect (Figure 4A, D), while transformant 4 contained less of the products when 10 mM sulfate was added to the medium than with no addition (Figure 4C). These results suggested that the sulfate concentration of the medium is not the limiting factor for the sulfatation step in the transgenic carrot cultures.

To account for scaling effects and for comparison with reports from other heterologous systems, we determined the yield of benzylglucosinolate production in the two best producing transformants in flask cultures with 60 ml medium. Transformant 2 grew slightly less than transformant 1 and produced roughly 70% of the biomass of transformant 1 in ten days of cultivation (Figure 5A). However, the total product yield per
60 ml culture tended to be higher in transformant 2 than in transformant 1 in agreement with the above observation that growth and productivity seem to be inversely correlated (Figure 5B). The cultures produced two to four times more desulfobenzylglucosinolate than benzylglucosinolate. The yield of benzylglucosinolate varied around 35 nmol per 60 ml culture (0.58 nmol ml⁻¹, 240 µg l⁻¹) for both transformants, while approximately 75 and 120 nmol desulfobenzylglucosinolate were obtained per 60 ml culture of transformant 1 and transformant 2, respectively. This corresponds to roughly 47 nmol (g dw)⁻¹ (2.3 nmol (g fw)⁻¹) benzylglucosinolate for transformant 1 and 52 nmol (g dw)⁻¹ (2.6 nmol (g fw)⁻¹) benzylglucosinolate for transformant 2. When complete conversion of desulfobenzylglucosinolate to benzylglucosinolate was achieved, this would potentially yield roughly 150 nmol benzylglucosinolate per 60 ml culture of transformant 2 (corresponding to 2.5 nmol ml⁻¹, 1 mg l⁻¹, 255 nmol (g dw)⁻¹, 13 nmol (g fw)⁻¹).

Discussion

Glucosinolates have received great attention due to their presence in agriculturally important and nutritionally valuable crops. The idea to produce individual glucosinolates in a heterologous host organism was first realized in 2010 by the transfer of 13 coding sequences from Arabidopsis into N. benthamiana for the generation of 4-methylsulfinylbutylglucosinolate (glucoraphanin), the cancer-preventive agent naturally present in broccoli (Mikkelsen et al. 2010). Since then, many more attempts have been undertaken to produce this and other glucosinolates in plant or microbial hosts (Table 1; Petersen et al. 2018). The present study is the first to use a cell culture system derived from a non-glucosinolate-containing plant for reconstitution of the biosynthetic
pathway of a glucosinolate. We provide proof of concept for the feasibility of using carrot suspension cultures as a host for glucosinolate production.

Compared to other reports, the yield of benzylglucosinolate obtained in our study is relatively low. For example, the yield of benzylglucosinolate per liter carrot suspension culture was about 35 times lower than that in *E. coli* (Petersen et al. 2019). However, to achieve this production titer in *E. coli* a number of optimization steps had to be implemented such as *E. coli* strain selection, modification of the CYP79A2 membrane anchor, change of medium composition and choice of promoters (Petersen et al. 2019). In comparison to other plant systems, the yield in carrot suspension cultures was similar to that in transgenic potato plants, but more than 500-fold lower than that obtained in transiently transformed *N. benthamiana* on a fresh weight basis (González-Romero et al. 2021; Møldrup et al. 2011). Again, the high yield in *N. benthamiana* was the result of additional optimization steps. Specifically, introduction of an adenosine-5′-phosphosulfate kinase (APK), APK2, improved benzylglucosinolate production in *N. benthamiana* significantly (Møldrup et al. 2011). Nevertheless, the yield in all heterologous production systems tested so far is much too low for industrial use. In order to obtain 1 kg of benzylglucosinolate, almost 1.5 kg of transiently transformed leaves of *N. benthamiana* or 125 l of transgenic *E. coli* culture or 4,000 l of transgenic carrot suspension culture would have to be extracted. However, the levels gained in *N. benthamiana* and *N. tabacum* have reached the range of typical glucosinolate contents in natural producers and vegetable crops (Table 1).

The overall numbers illustrate that production in carrot suspension cultures needs optimization. We have so far tested supplementation with phenylalanine (data not shown) and sulfate (Figure 4) as benzylglucosinolate precursors. While phenylalanine supplementation did not increase benzylglucosinolate yield, the effects of sulfate addition were minor to moderate. Testing sulfate supplementation was inspired by the observation that most transformants produced four to more than twelve times more desulfobenzylglucosinolate than the target compound benzylglucosinolate. This indicated that the final step in benzylglucosinolate biosynthesis, sulfate transfer from adenosine-3′-phosphate-5′-phosphosulfate (PAPS) by sulfotransferase SOT16 (Klein et al. 2006), is limiting similar to the results obtained in other systems. This could be due to low efficiency of SOT16 or limited availability of PAPS. *A. thaliana* accumulates only minor amounts of benzylglucosinolate (Wittstock and Halkier 2000). Despite the broad substrate specificity of *A. thaliana* SOT16 used in the present study and its preference for tryptophan and phenylalanine-derived desulfoglucosinolates in vitro (Klein et al. 2006; Piotrowski et al. 2004), there might be more efficient desulfobenzylglucosinolate SOTs in other species with high benzylglucosinolate accumulation (e.g. *Tropaeolum majus* or *Lepidium sativum*). Simple addition of sulfate to the medium did not improve the benzylglucosinolate-to-desulfobenzylglucosinolate ratio in our experiments, at least not in the added concentrations. Therefore, future research should try to test other sulfate sources as supplements, to clone and characterize desulfoglucosinolate sulfotransferases from different species and evaluate their suitability for metabolic engineering, to increase SOT expression and to include an APK gene in the constructs to boost PAPS formation from adenosine-5′-phosphosulfate (APS) (Mugford et al. 2010). Introduction of APK2 to the pathway expressed in *N. benthamiana* enhanced benzylglucosinolate production about sixfold (Møldrup et al. 2011). In case of *E. coli* as host, attempts to increased PAPS availability by sulfate supplementation (up to 50 μM) and overexpression of adenylyl-sulfate kinase or sulfate adenylyltransferase from *E. coli* together with the benzylglucosinolate pathway did, however, not improve desulfobenzylglucosinolate conversion (Petersen et al. 2019).

In the present study, we have only quantified accumulation of desulfobenzylglucosinolate as last intermediate and substrate for the final step of biosynthesis. In order to optimize benzylglucosinolate production in carrot suspension cultures, future experiments should try to detect and quantify other intermediates in parallel with quantitation of transcript and protein levels of the introduced pathway. This information could be used to design constructs with adjusted expression levels and suggest further optimization steps with respect to the integration into the host’s general metabolism. Attention should also be paid to the involvement of glutathione-S-transferases (GSTs) (Piślewska-Bednarek et al. 2018; Zhang et al. 2022). The presently used constructs do not contain a GST gene as conjugation of the aci-nitro compound with glutathione (GSH) seems to be catalyzed by GSTs present in heterologous plant hosts (Mikkelsen et al. 2010; Møldrup et al. 2012). However, inclusion of *A. thaliana* GSTF11 upon engineering of the 4-methylsulfinylbutylglucosinolate pathway into *N. benthamiana* increased yield by about 20% (Mikkelsen et al. 2010).

In our experiments, we saw a tendency for lower biomass formation in transformants with higher production rates. Therefore, we assume that considerable improvement of yield can be achieved by optimizing the medium and the cultivation process by evaluating various carbon sources and concentrations, compositions of macro- and micronutrients, temperatures, bioreactor types, aeration and mixing techniques as well as different

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levels of shear stress. However, the negative impact on growth might also indicate metabolic constraints that have to be addressed at the metabolic level. For example, benzylglucosinolate biosynthesis introduces a new phenylalanine sink to the carrot cells. As phenylalanine biosynthesis is tightly regulated in plants together with tryptophan and tyrosine biosynthesis, the continuous withdrawal of phenylalanine from primary metabolism for benzylglucosinolate biosynthesis may cause metabolic perturbations beyond phenylalanine availability. Examples of metabolic crosstalk, revealed upon metabolic engineering of phenylalanine or tryptophan-derived pathways, have been described in the literature (reviewed by Lynch et al. 2021). Furthermore, intermediates and by-products of benzylglucosinolate biosynthesis (which have not been analyzed in the present study) may have direct or indirect negative effects on cell fitness. Thus, future research should aim at understanding the metabolic consequences of benzylglucosinolate production and at better integration of the new pathway into the metabolic networks of carrot cells. As an additional option, the use of inducible promoters might enable the separation of growth and production phases with the potential for separate optimization of these two phases for yield improvement.

Plant cell suspension cultures have been widely used to investigate biosynthesis of endogenous specialized metabolites (reviewed by Matsuura et al. 2018). In only few cases, it has been possible to exploit them as production platforms for such metabolites at a technical or industrial scale (Matsuura et al. 2018). The limitations observed for production of endogenous specialized metabolites in callus or cell suspensions are, in part, attributed to the undifferentiated nature of these cells which may lead to improper compartmentation of enzymes and intermediates, abnormal regulatory processes and limited availability of enzyme cofactors (Matsuura et al. 2018). These issues may also have to be considered when carrot suspension cultures are employed as a host for heterologous production.

Taken together, carrot suspension cultures are a promising platform for plant-based production of specialized metabolites. Production of benzylglucosinolate in recombinant carrot suspension cultures is a first important step in the development of such cultures as production platforms for various compounds of interest. It would be interesting to test the reconstitution of pathways located not only in the cytosol and ER, but involving steps catalyzed by plastidial enzymes, e.g. the biosynthesis of 4-methylsulfinylbutylglucosinolate. It is presently unknown if benzylglucosinolate produced in carrot suspension cultures is stored in vacuoles like it would be in its natural producer. Other specialized metabolites might require specialized storage compartments. Therefore, we expect that transport and storage of foreign metabolites in a heterologous production system need to be considered in the future to obtain high yields. In the present study we have reached production levels of about 500 nmol benzylglucosinolate per liter carrot suspension culture with simultaneous accumulation of about 2,000 nmol l⁻¹ desulfo benzylglucosinolate. As there are many options for genetic, metabolic and process optimization, there is a high potential for yield improvement.

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