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Evaluation of tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR1) hairy roots for the production of geraniol, the first committed step in terpenoid indole alkaloid pathway

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ABSTRACT

The terpenoid indole alkaloids are one of the major classes of plant-derived natural products and are well known for their many applications in the pharmaceutical, fragrance and cosmetics industries. Hairy root cultures are useful for the production of plant secondary metabolites because of their genetic and biochemical stability and their rapid growth in hormone-free media. Tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR1) hairy roots, which do not produce geraniol naturally, were engineered to express a plastid-targeted geraniol synthase gene originally isolated from *Valeriana officinalis* L. (*VoGES*). A SPME-GC–MS screening tool was developed for the rapid evaluation of production clones. The GC–MS analysis revealed that the free geraniol content in 20 hairy root clones expressing *VoGES* was an average of 13.7 μg/g dry weight (DW) and a maximum of 31.3 μg/g DW. More detailed metabolic analysis revealed that geraniol derivatives were present in six major glycoside forms, namely the hexose and/or pentose conjugates of geraniol and hydroxygeraniol, resulting in total geraniol levels of up to 204.3 μg/g DW following deglycosylation. A benchtop-scale process was developed in a 20-L wave-mixed bioreactor eventually yielding hundreds of grams of biomass and milligram quantities of geraniol per cultivation bag.

1. Introduction

Plants synthesize a vast range of low-molecular-weight compounds also described as natural products or secondary metabolites. Tropical rainforests are characterized by the richest species diversity on earth (up to 942 species/ha) including up to 298,000 species of higher terrestrial plants (Wilson et al., 2012; Mora et al., 2011). Many of the resulting secondary metabolites can be used as food, feed, flavors, fragrances, cosmetics, agrochemicals and pharmaceuticals. Secondary metabolites therefore contribute substantially to human wellbeing and can provide backbone structures or leads for new semi-synthetic or synthetic pharmaceutical compounds.

The complex stereochemical structure of many secondary metabolites means that total chemical synthesis is not feasible. Extraction from a natural source or biotechnology-based production may be considered, but the latter is required when the source species is rare or endangered. Key secondary metabolites often accumulate naturally in minute quantities, and the source species may also be difficult to cultivate and recalcitrant to genetic transformation. Therefore alternative approaches are required in which the corresponding metabolic pathways are engineered in more amenable hosts for the production of valuable secondary metabolites.

Hairy root cultures provide a useful platform for the production of plant secondary metabolites, particularly alkaloids and their derivatives, which are produced at high levels in hairy roots (Giri and Narasu, 2000). For example in *Hyoscyamus niger* hairy roots scopolamine contents of 4.1% of the DW have been recorded (Sevón and Oksman-Caldentey, 2002; Rischer et al., 2013). The advantages of hairy roots include relatively fast growth (0.1–2 g DW

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per liter and day), genetic and biochemical stability and growth in hormone-free medium (Shanks and Morgan, 1999; Sevón and Oksman-Caldentey, 2002). Hairy roots possess the full spectrum of biochemical activity required to produce the same secondary metabolites as the complete plants from which they are derived, but their capacity for the biosynthesis of natural products is often greater (Kim et al., 2002). Hairy roots therefore play an important role as a model system for the investigation of plant metabolism and genetic engineering (Shanks and Morgan, 1999).

Despite these advantages, it is more difficult to scale-up hairy root cultures than plant cell suspension cultures, which are therefore used more often in commercial production processes. The main challenge is the heterogenic morphology of hairy roots, as well as their irregular growth and high shear stress sensitivity. These issues have been addressed by the development of single-use (disposable) bioreactors as an alternative to glass or stainless steel cultivation systems such as spray reactors and bubble columns. Single-use wave-mixed systems such as BIOSTAT RM have been established that simplify the mass propagation of hairy roots by inducing waves within a cultivation bag, ideally operating in ebb-and-flow mode (Eibl and Eibl, 2006, 2009). Recent investigations have also shown that orbitally shaken single-use bags are also useful for the mass propagation of hairy roots (Lampart, 2013).

Terpenoid indole alkaloids (TIAs) are one of the major classes of natural products derived from plants. All terpenoids originate from isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) and the corresponding metabolic pathway has been characterized both at the genetic and biochemical levels (Phillips et al., 2008). The terpenoid indole alkaloids are then derived from tryptophan and the iridoid terpenoid secologanin, the latter originating from the triose phosphate/pyruvate or non-mevalonate pathway (Verpoorte et al., 1997; O'Connor and Maresh, 2006). Strictosidine synthase catalyzes the Pictet–Spengler reaction between tryptamine and secologanin to form strictosidine, the common precursor of TIAs (Stöckigt et al., 2008). The first committed step in the pathway is the formation of geraniol, an acyclic monoterpene alcohol that is synthesized in one step from geranyl diphosphate (GPP). Geraniol not only represents the first step of the TIA pathway, but also has important properties as a pharmaceutical (Carnesecchi et al., 2001) and fragrance (Chen et al., 2006). The geraniol is jointly transferred with a cytochrome P450 mono-oxygenase geraniol-10-hydroxylase (G10H) and NADPH:cytochrome P450 reductase (CPR) to 10-hydroxygeraniol (Meijer et al., 1993). The 10-hydroxygeraniol in turn undergoes dehydrogenation to 10-oxogeraniol which is further oxidized to 10-oxogeranial (Ikeda et al., 1991). It is postulated that 10-oxogeranial is first cyclized and reduced to form iridodial, which in turn undergoes hydroxylation, dehydrogenation and again a cyclisation. Loganic acid is then synthesized from the resulting iridotrial by hydroxylation, glucosylation and hydroxylation reactions (Verpoorte et al., 1997). Then two further steps lead to the formation of secologanin: a methylation and an acyclisation catalyzed by S-adenosyl-L-methionine:loganic acid methyltransferase (LAMT) and secologanin synthase (SLS), respectively (Murata et al., 2008; Irmler et al., 2000).

We previously showed that heterologous geraniol synthesis can be achieved by expressing the geraniol synthase from *Valeriana officinalis* L. (Valerianaceae) (*VoGES*) in tobacco (*Nicotiana tabacum* L. Samsun NN) leaves (Dong et al., 2013a). GC–MS and LC–MS analysis of leaf extracts revealed that the aerial parts of the plant produced free geraniol as anticipated. However, LC–MS analysis showed that endogenous tobacco enzymes oxidized the geraniol and conjugated these products into glycosides (Dong et al., 2013a). The monoterpenoid geraniol is produced in the plastids/chloroplasts so it is difficult to predict how hairy roots would perform, although they are known to contain plastids and do produce plastidial isoprenoids including monoterpenes (Lourenco et al., 1999) and diterpenes (Zhi and Alfermann, 1993). To determine the potential of hairy roots as a production platform for TIAs, we evaluated the capacity of hairy roots, generated by transformation with *Agrobacterium rhizogenes* containing the 35S::*VoGES* construct, to produce geraniol and its derivatives. We also investigated a range of scale-up systems to determine whether the tobacco hairy root production platform was in principle suitable for commercial applications.

2. Materials and methods

2.1. Generation of tobacco hairy roots

The hairy roots of *N. tabacum* (cv. Petit Havana SR1) were initiated by infecting leaves by *Agrobacterium rhizogenes* LBA9402carrying the pBIN2.4VoGES1 vector for the expression of VoGES. This vector contained a geraniol synthase gene isolated from *V. officinalis* (L.) augmented with an artificial plastid targeting signal, driven by the double-enhanced *Cauliflower mosaic virus* 35S promoter (www.pri.wur.nl/UK/products/ImpactVector/). The artificial plastid targeting was selected on the basis of data described in Dong et al. (2013a,b), where they compared different targeting signals with truncated *VoGES* and did not observe any difference between the native and the new artificial plastid transit signals. In addition, we generated wild-type (WT) hairy roots by transformation with wild-type *A. rhizogenes* LBA9402 and control hairy root clones by transformation with bacteria containing the empty vector (BIN). Leaves from tobacco plants grown *in vitro* were sliced into ~1 cm^2 pieces and placed mid-rib uppermost on hormone-free modified Gamborg's B5 medium as described by Häkkinen et al. (2007). The leaf mid-rib was inoculated with recombinant *A. rhizogenes* using a sterile needle and the leaves were incubated at 24 °C in the dark. After 48 h, the leaves were transferred to cultivation medium supplemented with 500 ppm cefotaxime to eliminate the bacteria. Hairy roots emerged approximately 2–3 weeks later and single root tips were placed on solid culture medium supplemented with 500 ppm cefotaxime. After one round of selection, hairy root clones originating from single root tips were transferred to solid culture medium without antibiotics. The hairy root clones were grown at 24 °C in the dark and subcultured at 4-week intervals. For metabolic analysis, 50 mg fresh weight (FW) of hairy root biomass was added to 50 ml of liquid modified Gamborg's B5 medium in 250-ml shake flasks and cultivated in a rotary shaker (70 rpm, 24 °C, in the dark) for 21 days. The biomass was then harvested by Büchner-filtration, frozen in liquid nitrogen and stored at −80 °C.

2.2. DNA analysis

Genomic DNA from tobacco hairy roots was isolated using the CTAB method of Murray and Thompson (1980). Putative transgenic hairy roots were screened by PCR, using forward primer 5′-CGA CAC TTA TGG CTC GTA TG-3′ and reverse primer 5′-ACC GAC TCG TTA CAA GAA GG-3′ to amplify a 850-bp fragment of *VoGES* and 100 ng of genomic DNA as the template. The presence of *rol*B was verified by PCR using forward primer 5′-ATG GAT CCC AAA TTG CTA TTC CTT CCA CGA-3′ and reverse primer 5′-TTA GGC TTC TTT CTT CAG GTT TAC TGC AGC-3′ to amplify a 780-bp fragment. To confirm the absence of *A. rhizogenes* in the hairy root clones, a 450-bp diagnostic fragment of the *vir*D gene was amplified by PCR using forward primer 5′-ATG TCG CAA GGA CGT AAG CCC A-3′ and reverse primer 5′-GGA GTC TTT CAG CAT GGA GCA A-3′. The pBIN2.4VoGES1 plasmid (1 ng) was used as a positive control, and for *rol*B and *vir*D a boiled preparation of WT *A. rhizogenes* LBA9402 was used as a control. PCR products were separated by 0.8% (w/v) agarose gel

electrophoresis and visualized by ethidium bromide staining under UV light, compared to a DNA molecular weight marker (Gene Ruler DNA Ladder 100 bp, Thermo Fisher Scientific Inc. Waltham, MA, USA).

2.3. RNA extraction and RT-PCR

Total RNA was extracted using the Qiagen RNA Plant Mini Kit (Qiagen, Venlo, Netherlands). Approximately 200 mg (FW) of tobacco hairy root material was frozen in liquid nitrogen and ground in a Retsch-mill in RNA extraction buffer. Reverse transcription was carried out using the Quanti Tect Reverse Transcription Kit (Qiagen) and the resulting cDNA was amplified using the *VoGES* primers (see Section 2.2). The products were analyzed by agarose gel electrophoresis (see Section 2.2).

2.4. Analysis of volatiles by SPME-GC–MS

We tested a solid-phase microextraction (SPME) system using Carboxen/PDMS fibers (Sigma-Aldrich, St. Louis, MO, USA) to increase the sensitivity of the analysis of geraniol and related compounds present in fresh or freeze-dried hairy root samples. The plant material (50 mg dry weight (DW) or 0.5 g FW) was ground and mixed with 1 ml of water in a 20-ml head-space bottle which was then sealed. The samples were incubated for 10 min at 45 °C, and the desorption time was 300 s. Each run was performed in splitless mode using the oven temperature program described below for GC–MS analysis.

2.5. Nuclear magnetic resonance (NMR) spectroscopy

The samples were extracted for NMR spectroscopy as described by Kim et al. (2010). A dried 50-mg sample was extracted in 1.5 ml methanol-d4:D_2O (1:1) in 50 mM KH_2PO_4 buffer, pH 6.0. The mixture was vortexed for 1 min, ultrasonicated for 30 min and centrifuged twice (20,800 × *g* at room temperature) before 600 μl was transferred to a 5-mm NMR tube for ^{1}H-NMR analysis. The deuterated methanol and D_2O were purchased from Sigma–Aldrich (St. Louis, MO, USA). The NMR experiments were carried out at 22 °C on a 600 MHz Bruker Avance III NMR spectrometer equipped with a QCIPN cryoprobe and SampleJet (Bruker Biospin) automated sample changer. The residual water signal was suppressed by 4 s pre-saturation using the Bruker noesygppr1D pulse sequence and 128 scans were accumulated from each sample.

The ^{1}H NMR spectral intensities were scaled to total intensity and the δ 0.50–10.00 region was automatically binned to 0.04 ppm integral regions using AMIX software (v.3.9, Bruker Biospin). The δ 4.7–5.0 and 3.28–3.34 regions were excluded from the analysis due to the residual water and methanol signals, respectively. SIMCA-P+ software (v.13.0, Umetrics, Umeå, Sweden) was used for principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA) with unit variance (in PCA) or Pareto-scaled (in PLS-DA) data.

A cloud tool for local intensity difference analysis (Locida) was developed for the analysis and visualization of NMR data and the pairwise comparison of sets of samples (Virkki et al., 2013; http://github.com/avirkki/Locida). Subsets of data were grouped as "treated" and "control". These groups were then inspected bin-by-bin for differences in the mean values. In the visual presentation, red shows higher values in the treated category and blue indicates the opposite. The monotone color intensity deepens according the (absolute or relative) size of the difference and the statistical significance was assessed using the Wilcoxon–Mann–Whitney *U*-test with a threshold of $p < 0.05$, and *p*-values smaller than the corresponding Bonferroni-corrected *p*-value used as an indication of full confidence.

2.6. Analysis of free geraniol and tobacco alkaloids by GC–MS

Freeze-dried hairy root samples (50 mg) were spiked with 20 μl of 2,4-dipyridyl prepared at a concentration of 1 mg/ml in dichloromethane (DCM). After mixing with 2 ml water, the samples were alkalized with 30 μl 10% (w/v) ammonia, stirred for 30 min and extracted with 2 ml DCM. The organic phase was separated after centrifugation (20,800 × *g* at room temperature) and the extraction was repeated. The combined DCM extracts were gently evaporated to dryness under nitrogen flow, dissolved in 100 μl DCM and transferred to GC vials. GC–MS analysis was carried out using an Agilent 7890A GC combined with a 5975C mass selective detector and an Rtx®-5MS silica capillary column (15 m × 0.25 mm, ID 0.25 μm film thickness; Restek, Bellefonte, PA, USA). The oven temperature was increased from 70 °C (1 min) at a rate of 10 °C/min to 270 °C (4 min). The split ratio was 25:1 and the samples were injected using a Gerstel Maestro MPS 2 sampling system (Gerstel GmbH&Co.KG, Mühlheim an der Ruhr, Germany). The data were collected at a mass range of 40–600 amu. Compounds were identified based on the retention times of pure substances, library comparison (NIST'08, Scientific Instrument Services, Inc., Ringoes, NJ, USA) and literature analysis.

2.7. Analysis of geraniol-derived conjugates by LC–QTOF-MS and LTQ-Orbitrap-MS^n

Non-volatile compounds in hairy root extracts were analyzed by liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (LC–QTOF-MS), essentially as previously described (De Vos et al., 2007; Dong et al., 2013a). Aliquots of frozen, powdered material (200 mg) were extracted with 0.6 ml 99.9% methanol supplemented with 0.133% formic acid, sonicated for 15 min and filtered through a 0.45-μm membrane (SRP4, Sartorius, Göttingen, Germany). We then analyzed 5 μl of the filtered extract using a Waters Alliance 2795 HPLC connected to a QTOF Ultima V4.00.00 mass spectrometer (Waters, MS technologies, Hertfordshire, UK). Eluent A (1:1000 (v/v) formic acid:ultrapure water) and eluent B (1:1000 (v/v) formic acid:acetonitrile) were pumped into the HPLC system at 190 μl/min with a linear increase from 5% to 35% eluent B in 45 min, followed by 15 min of column washing and equilibration. Measurements were taken in negative ionization mode and leucine encephalin ($[M-H]^-$ = 554.2620) was used as a lock mass for online mass calibration.

LC–MS data were acquired using MassLynx v4.0 (Waters) and processed using metAlign v1.0 (www.metalign.nl) to align the same masses and remove baseline and noise. Significant differences in signal intensity between transgenic and wild type samples were assessed using Student's *t*-test (level of significance set at 0.05). Metabolites were identified by determining the best fit elemental composition using C, H and O with MassLynx software (tolerance <5 ppm).

For further identification, selected compounds were targeted for fragmentation using an Accela HPLC tower connected to an LTQ-Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) as previously described (van der Hooft et al., 2011). The LTQ was programmed to use a window of 10 D to isolate the mass of interest in MS^1. The data-dependent fragmentation was set as follows: MS^2 fragmentation of most intense ion in MS^1; MS^3 fragmentation of the five most intense fragment ions in MS^2; MS^4 fragmentation of the five most intense fragment ions in each MS^3.

2.8. Enzymatic hydrolysis of geraniol glycosides

The treatment of geraniol glycosides with glycosidase and subsequent quantification was carried out as described by Dong et al. (2013a) with small modifications. Heptane was used as the

Table 1
Main process parameters and features of the single-use bag bioreactors.

Bioreactor	Parameters and specific features	
BIOSTAT® CultiBag RM 20 (Sartorius Stedim Biotech)	Cultivation bag	CultiBag RM 2L basic screw cap[a] CultiBag RM 20L basic screw cap[b]
	Platform	Basic for CultiBag RM 20L
	Rocking rate	8 rpm
	Rocking angle	6°
	Aeration	0.4 vvm
	Temperature	26 °C
	Sensors	None
	Feeding	Day 8[c], 15[c] and 22[c]
Multitron Cell (Infors HT)	Cultivation bag	CultiBag RM 2L basic screw cap[a]
	Platform	ShakerBag Option
	Shaking frequency	30 rpm
	Shaking diameter	50 mm
	Aeration	0.4 vvm
	Temperature	26 °C
	Sensors	None

[a] Initial culture volume of 200 mL.
[b] Initial culture volume of 1 L.
[c] Feeding of 40 mL in the CultiBag 2 L and 200 mL in the CultiBag 20 L.

main extraction solvent and the GC–MS analytical platform was a QP2010SE quadrupole mass spectrometer (Shimadzu, Duisburg, Germany).

2.9. Bioreactor description and experimental design

Two different bioreactor systems operating with a single-use 2D cultivation bag (CultiBag RM basic screw cap) were used to cultivate geraniol-producing tobacco hairy roots (clone VoGES 150210/25): (1) the wave-mixed bioreactor, the BIOSTAT® CultiBag RM 20 (Sartorius) and (2) the Multitron Cell with ShakerBag Option (Infors HT, Bottmingen, Switzerland). The main process parameters for cultivations are summarized in Table 1. All experiments were initiated by inoculating modified Gamborg's B5 medium with 5 g FW/l of 7-day-old viable root material (grown on solid medium). The culture conditions were maintained at constant values during the experiments, which were performed in batch or fed-batch mode. During fed-batch experiments, three feeding steps were introduced by adding 20% (v/v) of the initial culture volume of modified Gamborg's B5 liquid medium. The experiments lasted 21 days (batch mode) and 28 days (fed-batch mode).

2.9.1. In-process control

For sampling, 8 ml of the culture supernatant was taken with a 10 ml Luer lock syringe (Braun Medical Inc., Bethlehem, PA, USA) connected to the pre-assembled sampling port. After inoculation, and between days two and three, conductivity and pH were monitored offline (MC 226 and Five Easy, Mettler Toledo, Greifensee, Switzerland). In addition, carbohydrates (sucrose, fructose, and glucose) as well as ammonia and nitrate were measured by HPLC. Final fresh weight was determined by filtering the biomass for 3 min using a reusable filtration unit (Corning, NY, USA). For final dry weight determination, the filtered and frozen biomass was lyophilized using the ALPHA 2–4 freeze dryer (Martin Christ GmbH, Osterode am Harz, Germany).

2.9.2. Scale-up of geraniol production process

The fed-batch production process was scaled-up from the wave-mixed CultiBag RM 2L to the wave-mixed CultiBag RM 20L. With the exception of the feeding regime as shown in Table 1, the cultivation parameters of the 20-l bag were similar to those of the 2-l bag operating in batch mode.

3. Results

3.1. Transgenic tobacco hairy roots

To evaluate geraniol production in tobacco hairy roots, we generated 22 transgenic tobacco (*N. tabacum* L., cv. Petit Havana SR1) hairy root clones carrying *V. officinalis* geraniol synthase cDNA (*VoGES*) augmented with an artificial plastid targeting sequence (Supplementary Fig. S1). RT-PCR analysis (Fig. 1 and Supplementary Fig. S2) showed that 17 clones expressed the *VoGES* transgene. Preliminary screening by SPME-GC–MS analysis revealed that the clones produced volatile compounds: geraniol, geranial and its derivatives (Fig. 2). Furthermore, the SPME-GC–MS analysis proved that two hairy root clones, also not expressing the transgene, did not accumulate geraniol, and these were omitted from the more detailed metabolic analysis by NMR, GC–MS and LC–MS.

3.2. Analysis of transgenic hairy roots by NMR spectroscopy

NMR data derived from the hairy root clones was processed by PCA, PLS-DA and the Locida bioinformatics platform (Fig. 3). PCA did not separate transgenic hairy roots expressing *VoGES* from WT or BIN controls (Fig. 3A), and PLS-DA did not generate a model to distinguish between these groups, indicating that there were no

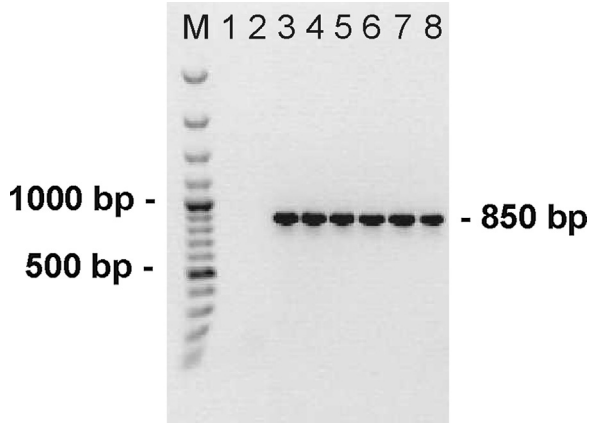

Fig. 1. RT-PCR showing expression of *VoGES* in transgenic tobacco hairy root clones (lanes 3–8) by amplifying an 850-bp fragment. The product was not present in WT counterparts (lanes 1, 2). The molecular weight standard is shown in lane M.

Table 2
LC–QTOF-MS and LTQ-Qrbitrap-MS^n analysis of geraniol-derived conjugates present in hairy roots expressing *VoGES*.

Retention time	Observed mass	Putative identity	Calculated mass	ΔMass (ppm)	Elemental formula	MS (n) fragment
21.00	493.2288	Dehexosyl hydroxygeraniol	493.2285	0.59	$C_{22}H_{37}O_{12}$	149, 191, 195, 237, 267, 279, 295, 313, 323, 331, 373, 447, 475
23.49	463.2184	Pentosyl-hexosyl hydroxygeraniol	463.2185	−0.15	$C_{21}H_{35}O_{11}$	149, 161, 191, 221, 251, 293, 311, 389, 445
31.89	639.2867	Trihexosyl geraniol	639.2864	0.44	$C_{28}H_{47}O_{16}$	305, 323, 381, 381, 425, 461, 477, 501, 525, 557, 609
36.38	477.2341	Dihexosyl geraniol	477.2331	2.10	$C_{22}H_{37}O_{11}$	161, 179, 193, 221, 281, 323, 341, 419
39.10	447.2240	Pentosyl-hexosyl geraniol	447.2235	1.12	$C_{21}H_{35}O_{10}$	149, 161, 191, 221, 251, 293, 311, 339, 417
39.80	447.2243	Pentosyl-hexosyl geraniol	447.2235	1.87	$C_{21}H_{35}O_{10}$	131, 143, 149, 161, 179, 191, 221, 239, 251, 275, 281, 293, 311, 315

statistically significant differences between the classes based on the NMR data. Furthermore, Locida analysis indicated only minor differences in areas 2–2.2 and 3.8–4.0, representing most probably changes in primary metabolism (Fig. 3B and C).

3.3. Analysis of transgenic hairy roots by GC–MS and LC–MS

GC–MS analysis of the extracts from transgenic hairy root clones expressing *VoGES* showed free geraniol levels of up to 31.1 μg/g DW (average, 13.7 μg/g DW), whereas no geraniol was detected in the WT or BIN clones (Fig. 4A). GC-MS analysis also revealed that the introduction of *VoGES* did not cause any statistically significant changes in major tobacco alkaloids (Student's *t*-test) (Fig. 4B). LC–QTOF-MS analysis of semi-polar non-volatile metabolites yielded six differential peaks which were present in *VoGES* but not WT hairy roots (Fig. 4C). To identify these putative geraniol-related products, the masses were targeted for fragmentation by LTQ-Orbitrap-MS^n. Interpretation of the MS^n spectra suggested that these six compounds were most likely hexose and/or pentose conjugates of geraniol and hydroxygeraniol (Table 2).

3.4. Bioreactor studies with wave-mixed and orbitally shaken bags

The bioreactor scale-up studies were carried out using the best-performing hairy root clone, VoGES 150210/25. At the 2-l scale, tobacco hairy roots were cultivated successfully regardless of the bioreactor type and process mode. There were no significant differences in biomass growth in batch mode when the roots were cultivated in wave-mixed or orbitally shaken bags (Fig. 5A). The final total biomass was 28.8 g FW in the wave-mixed bioreactor

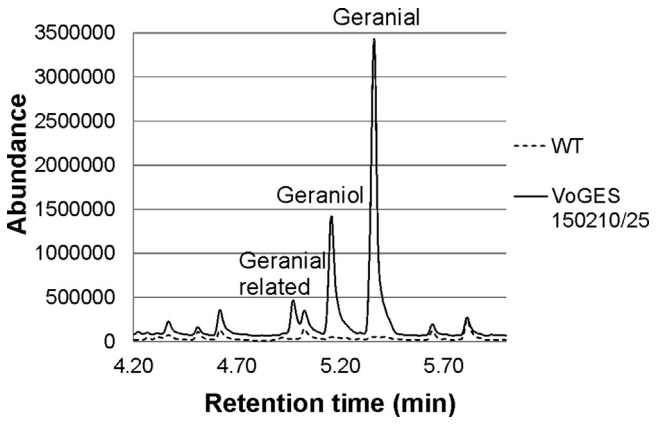

Fig. 2. Volatile geraniol, geranial and geranial derivatives analyzed by GC–MS–SPME from lyophilized wild type (WT) and transgenic (*VoGES* 150210/25) hairy root material. Ground samples were placed in water in a headspace vial and volatile compounds were collected to a speam and analyzed by GC–MS.

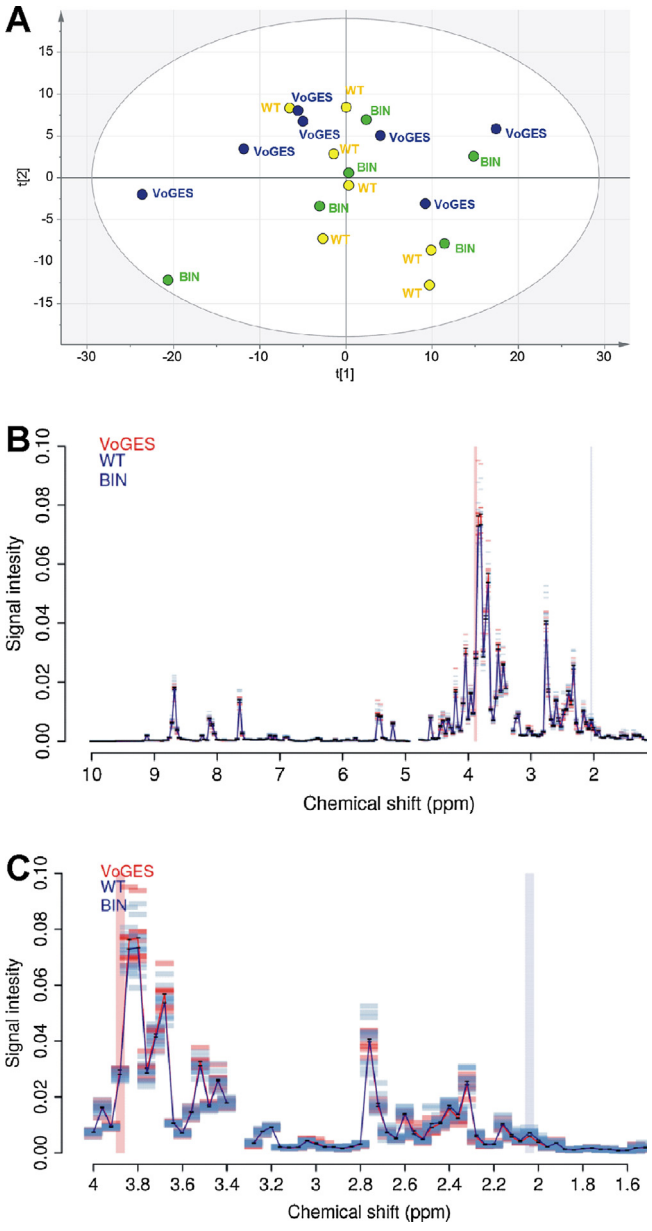

Fig. 3. NMR data showing no statistically significant differences between transgenic hairy root clones expressing *VoGES* and WT or vector backbone (BIN) controls. (A) PCA scores plot showing VoGES clones in blue, WT in yellow and BIN in green, (B and C) Locida bioinformatics tools showing the chemical shifts in the ranges 1–10 and 1.6–4.0, respectively. The red/blue color intensity of the highlighted areas in the Locida interpretations indicate the magnitude of the difference. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

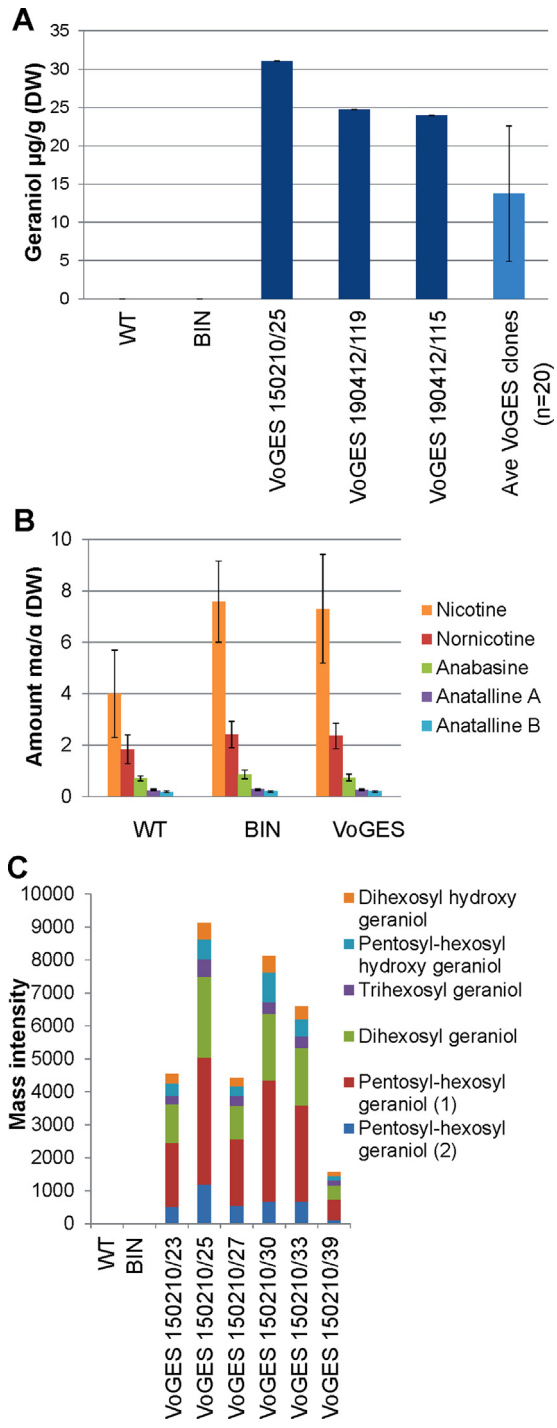

Fig. 4. Metabolite analysis of tobacco hairy roots overexpressing *VoGES* compared to WT and BIN controls. (A) GC–MS shows free geraniol content and (B) major tobacco alkaloids (Ave = average of 20 clones). (C) LC–QTOF-MS analysis of geraniol and hydroxygeraniol glycosides present in tobacco hairy roots.

and 25.4 g FW in the orbitally shaken bag. The final dry weight was 1.6 g in the wave-mixed bag and 1.4 g in the orbitally shaken bag. The total geraniol accumulation per cultivation bag was 162.6 µg/g DW in the wave-mixed bioreactor and slightly higher at 173.1 µg/g DW in the orbital bioreactor.

When the roots were cultivated in feeding mode, there was a positive influence on biomass propagation. The repeated addition of fresh medium resulted in 56% more dry biomass than batch cultivations and delivered an average total geraniol yield of 164.9 µg/g DW per bag (Fig. 5A). This was similar to batch mode, but because more biomass generated in feeding mode the total geraniol yield was 1.6-times higher.

Scale-up experiments demonstrated the successful transfer of the procedure established at the 2-l scale. Cultivation in wave-mixed 20-l bags resulted in a final total biomass of 185.3 g FW (10.7 g DW). The culture bag contained a viable, white and hairy biomass with a core of thick brown roots as shown in Fig. 5B and C. The biomass was taken from different areas (meristematic tip biomass, yellow fine roots and core biomass) for geraniol analysis, revealing a total geraniol concentration of 204.3 µg/g DW per bag, which was 23–25% higher to the levels measured in roots harvested from 2-l bags cultivated in batch or feeding modes.

4. Discussion

Tobacco hairy roots were evaluated as a production platform for terpenoid indole alkaloids (TIAs) by testing their ability to produce geraniol, which provides the first committed step into the TIA biosynthesis pathway (Simkin et al., 2013). The hairy roots were engineered with a geraniol synthase gene from *V. officinalis* L. (*VoGES*) under control of the constitutive CaMV 35S promoter. Geraniol accumulation was only detected in the transgenic VoGES hairy root clones and not in the WT or empty vector (BIN) controls. However, geraniol yields varied among the different clones, suggesting that *VoGES* expression levels may be affected by differences in genomic integration sites or somaclonal variation. NMR data indicated some minor differences in primary metabolism of the transgenic VoGES hairy root clones. There seemed to be some changes in the areas 2–2.2 and 3.8–4.0, representing most probably changes in aliphatic compounds and sugars, respectively. However, these changes were not statistically significant. In addition, no major differences were observed between transgenic and control hairy roots in the accumulation of tobacco alkaloids such as nicotine, nornicotine, anabasine and anatalline, indicating that the overexpression of *VoGES* did not affect other metabolic pathways. This was supported by previous flux studies showing clearly that the basic metabolism of tobacco hairy roots was resistant to perturbations (Maskapalli et al., 2014).

Monoterpene production positively correlates with photosynthesis (Maffei and Codignola, 1990; Ormeño et al., 2009; Porcar-Castell et al., 2009) because most of the carbon, ATP and NADPH required for monoterpene synthesis is generated by photosynthesis (Niinemets et al., 2002). Furthermore, continuous light has been shown to induce the expression of the monoterpene precursor gene encoding deoxy-D-xylulose-5-phosphate synthase in roots, compared to cultures grown in the dark (Souret et al., 2003). In this study, we cultivated the hairy roots in the dark, and the accumulation of geraniol derivatives indicated a substantial flux through the MEP pathway to provide substrate for VoGES enzyme activity. SPME-based headspace analysis was also carried out in the dark, confirming that geraniol is actively produced under these conditions. Our data therefore represent the production capacity of dark-grown hairy roots and show that although plastid development in non-green roots differs from that in green tissues, the plastids in dark-grown roots are nevertheless capable of geraniol synthesis. Furthermore, it is reported that IPP can be interchanged between the MVA and MEP pathways (Dudareva et al., 2005; Furumoto et al., 2011; Laule et al., 2003) and this cross talk is mediated by an as yet unidentified transporter localized in the plastid inner envelope membrane (Bick and Lange, 2003). It is currently unclear whether exposure to light could further activate the MEP pathway in root plastids, resulting in enhanced geraniol production.

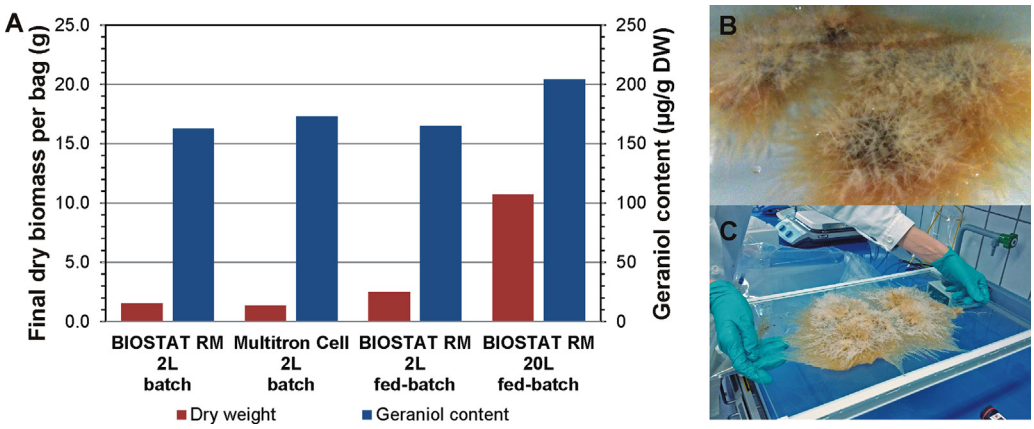

Fig. 5. Geraniol-producing tobacco hairy roots. (A) Final dry biomass of hairy roots grown in the bag bioreactors and total geraniol concentrations from the analysis of deglycosylated samples by GC–MS. (B and C) Harvested root biomass from a CultiBag 20L.

An alternative strategy to boost geraniol production in dark-grown hairy roots could be the re-localization of geraniol synthase to the MVA pathway. By removing the plastid target peptide from limonene synthase, Wu and co-workers (2006) have shown that the enzyme was successfully targeted to the cytosol in tobacco plants and yielded low but measurable levels of limonene, revealing a small cytosolic pool of GPP. Since there is a cytosolic pool of IPP and its isomer DMAPP, the direct precursors for GPP, the same group boosted GPP production in the cytosol by providing a cytosolic GPP synthase: co-expression of a cytosolic GPPS with cytosolic limonene synthase increased limonene accumulation on average by six-fold when compared to clones expressing limonene synthase alone (Wu et al., 2006). When a cytosolic pine GPPS and cytosolic VoGES were co-infiltrated into *Nicotiana benthamiana* leaves by Dong et al. (2013b), geraniol emission increased by 30-fold compared to infiltration with cytosolic VoGES alone. However, the stable engineering of tobacco hairy roots with cytosolic VoGES and cytosolic *Arabidopsis thaliana* GPPS did not result in the accumulation of geraniol. The major observation was that the abundance of labdanoid-type diterpenes increased at the expense of capsidiol-type sequiterpenoids in the transgenic lines compared to non-transformed controls (unpublished results). This finding demonstrates the indicative nature of transient expression studies, where pathway flux feedback may not play such a crucial role in the final metabolic profile and thus does not accurately predict the outcome of stable transformation.

Another approach to enhance the yield of geraniol is to inhibit its conversion into oxidized products such as geranial and its derivatives. These were twice as abundant as geraniol in the hairy root clones, as shown in Fig. 2. Oxidation products of geraniol have also been observed when *GES* from *Ocimum basilicum* was expressed in either *V. vinifera*, *N. benthamiana* or *A. thaliana* (Fischer et al., 2013). In addition, when a *GES* gene from *Lippia dulcis* was introduced into maize, no geraniol but geranyl-acetate was detected in the headspace of maize leaves (Yang et al., 2011). Furthermore, during pathway reconstitution in yeast and *N. benthamiana*, minor components such as 8-oxogeraniol, 8-oxogeranial and 8-carboxygeranial were identified but not geranial or its derivatives (Höfer et al., 2013). Combined, these results suggest that geraniol is easily converted to other products in plant cells. It is unclear whether the conversion of geraniol into oxidized geraniol products in hairy roots are from a specific (enzymatic) reaction or a spontaneous (non-enzymatic) process. If they are from enzymatic reactions inhibition of the genes encoding these geraniol oxidases could help increase geraniol yield. Further experiments are required to gain insight into this process. In addition, the ectopically produced geraniol that is potentially toxic for the cells need to be degraded or sequestered. In this study, we showed that most of the produced geraniol was stored inside the cell in the form of geraniol glycosides (Fig. 4), which presumably are located in the vacuole. Glycosylation may actually enhance geraniol (-derived) product accumulation and geraniol may be released by glucosidases.

A production platform based on hairy roots must be scaled up to increase efficiency, so we investigated the transfer of a 2-l to process to a 20-l cultivation bag. The 20-l bag produced 4.3-fold more dry biomass than the 2-l bag, which corresponds to the 5-fold increase in culture medium used at the 20-l scale. Furthermore, the total geraniol yield per 20-l bag was 5.3-fold higher than the 2-l bag. Interestingly, geraniol accumulation measured as µg/g DW was 1.2-fold higher in the 20-l bags than the 2-l bags, corresponding to an overall yield of 2.2 mg geraniol per 20-l bag. Geraniol is a non-polar compound that is probably sequestered into cell membrane, given that the capacity of plants to take up terpenoids seems to relate directly to the lipid content (Noe et al., 2008). Therefore, the geraniol extracted from hairy roots could reflect the hairy root lipid content which may limit the accumulation of free geraniol. In contrast, the glycosylated geraniol derivatives are probably sequestered into the vacuoles, which may have higher storage capacity. Indeed, in stably transformed tobacco plants, the glycoside portion of geraniol-related molecules was 7.5-fold higher than the free form of geraniol (Dong et al., 2013b). Furthermore, if geraniol secretion could be induced, it would be possible to extract the product from the culture medium continuously. This milking technology has already been reported for tropane alkaloids by Gontier et al. (2002), working with hydroponic cultures of *Datura innoxia* (Mill). By applying Tween-20 to the hairy roots, the accomplished permeabilization resulted in the secretion of 10–80 mg/l of hyoscyamine and scopolamine, and it remains to be seen if a similar process can be used to improve the extraction of geraniol.

5. Conclusions

This study provides proof of concept for the use of genetically engineered tobacco hairy roots expressing plastid-targeted GES for the production of geraniol. A detailed analysis of the best production clone revealed that geraniol was represented by at least six different glycoforms and thus the free geraniol content gave only a modest estimate of the total accumulation levels. A 20-l scale production batch was successfully completed, yielding milligram quantities of geraniol. Further experiments will investigate additional genetic modification of the TIA pathway to produce greater yields of known and novel metabolites.

Conflicts of interest

The authors declare no conflicts of interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2014.01.031.

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