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ORIGINAL PAPER

Comparison of plant-based expression platforms for the heterologous production of geraniol

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Abstract We compared the ability of different plant-based expression platforms to produce geraniol, a key metabolite in the monoterpenoid branch of the terpenoid indole alkaloid biosynthesis pathway. A geraniol synthase gene isolated from *Valeriana officinalis* (*VoGES*) was stably expressed in different tobacco systems. Intact plants were grown in vitro and in the greenhouse and were used to generate cell suspension and hairy root cultures. *VoGES* was also transiently expressed in *N. benthamiana*. The highest geraniol content was produced by intact transgenic plants grown in vitro (48 μg/g fresh weight, fw), followed by the transient expression system (27 μg/g fw), transgenic plants under hydroponic conditions in the greenhouse and cell suspension cultures (16 μg/g fw), and finally hairy root cultures (9 μg/g fw). Differences in biomass production and the duration of cultivation resulted in a spectrum of geraniol productivities. Cell suspension cultures achieved a geraniol production rate of 1.8 μg/g fresh biomass per day, whereas transient expression produced 5.9 μg/g fresh biomass per day (if cultivation prior to agroinfiltration is ignored) or 0.5 μg/g fresh biomass per day (if cultivation prior to agroinfiltration is included). The superior productivity, strict process control and simple handling procedures available for transgenic cell suspension cultures suggest that cells are the most promising system for further optimization and ultimately for the scaled-up production of geraniol.

Keywords Cell suspension cultures · Geraniol synthase · Hydroponics · Tobacco plants · *Valeriana officinalis*

Introduction

The terpenoids are the largest family of natural products, comprising more than 30,000 individual compounds with important and diverse physiological roles in plants (Dubey et al. 2003). This family includes the sterols, carotenoids, various hormones (gibberellins, strigolactones, abscisic acid and brassinosteroids), monoterpenoids, sesquiterpenoids and diterpenoids (Gutensohn et al. 2013).

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Monoterpenoids are a homogenous class of terpenoids containing two isoprene units (C_5H_8) in their chemical skeleton, and are often found as components of natural fragrances and flavors. The enzymes responsible for monoterpenoid synthesis from the precursor geranyl diphosphate are known as monoterpene synthases (Fischer et al. 2013). These enzymes have attracted attention because of their potential regulatory importance, their commercial significance in the production of essential oils and aromatic resins, and their ecological effects in plant defense (Colby et al. 1993).

Geraniol is an acyclic monoterpene alcohol which is valued in the agricultural, food and cosmetic industries. For example, the geraniol oxidation derivatives geranial, neral and citronellol (citral) are used as lemon flavors, and geraniol itself has antimicrobial (Ben Hsouna and Hamdi 2012; Misra et al. 2013), fungicidal (Boukhris et al. 2013), and nematicidal activities (Abdel-Rahman et al. 2013). Geraniol also suppresses several types of cancer, including colon, pancreatic, hepatic and prostate tumors (Kim et al. 2012). Geraniol inhibits mevalonate metabolism which may reduce serum cholesterol levels (Elson et al. 1989), and the lower HMG-CoA reductase levels might contribute to geraniol-mediated anticancer activity (Kim et al. 2012). Finally, geraniol restores the level of cytokines and chemokines in mevalonate kinase deficiency patients, and can therefore be considered a potential drug lead for this rare genetic autoinflammatory disease (Marcuzzi et al. 2008, 2013).

Geraniol is an intermediate metabolite in the monoterpenoid-secoiridoid pathway, which intersects with the indole pathway to form terpenoid indole alkaloids (TIAs). There are ~2,000 known TIAs, some of which are widely-used pharmaceutical products, such as vinblastine and vincristine for the treatment of cancer, and ajmaline for the treatment of cardiac arrhythmia (Ziegler and Facchini 2008). Although TIAs have great economic value, cell culture methods for their production are rarely reported (Guo et al. 2013).

In plants, geraniol is synthesized from the universal monoterpenoid precursor geranyl diphosphate by the enzyme geraniol synthase (GES) (Iijima et al. 2004). The heterologous expression of GES enzymes from species such as *Ocimum basilicum*, *Perilla citriodora*, *P. frutescens*, *Catharanthus roseus*, *Lippia dulcis* and *Valeriana officinalis* has been achieved in plants, yeast and bacteria (Dong et al. 2013; Fischer et al. 2011; Fischer et al. 2013; Gutensohn et al. 2013; Iijima et al. 2004; Ito and Honda 2007; Simkin et al. 2013; Yang et al. 2005). These studies reported extensive quantitative and qualitative differences in the production of geraniol and other monoterpenoids, reflecting varying GES expression levels, the influence of the microenvironment in different subcellular compartments (e.g. pH, redox status, access to substrate, transport/storage of the product), the impact of tissue-specific expression and the influence of light (Fischer et al. 2013). *Saccharomyces cerevisiae* and *Escherichia coli* produced up to 3 μg/ml geraniol following transformation with *O. basilicum GES*, whereas plants expressing the same gene produced up to 93 μg/g geraniol in the leaves. *Vitis vinifera* and *Nicotiana benthamiana* were more productive than *Arabidopsis thaliana* (Fischer et al. 2011, 2013). Tobacco plants expressing *V. officinalis GES* (*VoGES*) produced up to 32 μg of stored glycosylated geraniol per gram fresh weight (fw) of leaf tissue compared to 6.4 μg/g fw in flowers (Dong et al. 2013). The microbial and plant systems also demonstrated differences in the monoterpenoid profile, with plants accumulating higher levels of additional metabolites such as linalool, citronellol and nerol, whereas the microbes showed a clear species and strain dependency and lower product yields (Fischer et al. 2013).

GES has attracted considerable recent interest because the heterologous expression of this initial monoterpenoid biosynthesis step in plants could help to reconstitute the TIA pathway (Dong et al. 2013) thus addressing the economic demand for flavor and aroma ingredients such as geraniol, which are currently extracted from natural plant sources or produced by complete chemical synthesis. We therefore undertook a comparative study to evaluate the potential of different heterologous plant-based production systems expressing plastid-targeted VoGES for the enhanced production of geraniol.

Materials and methods

Cloning *V. officinalis GES* cDNA and the stable transformation of tobacco plants

The *VoGES* cDNA was prepared from *V. officinalis* leaf RNA and expressed in transgenic plants by *Agrobacterium*-mediated transformation using the expression construct 35S::VoGES pBIN+ as previously described (Dong et al. 2013). The GES coding region was preceded by the constitutive *Cauliflower mosaic virus* 35S promoter and an artificial plastid-targeting peptide (Wong et al. 1992). Transformed shoots from *Nicotiana tabacum* cv. Samsun NN were selected on medium containing 100 mg/l kanamycin. Primary transformed shoots were rooted on non-selective medium, checked by PCR for the presence of the expression construct, and positive T_0 shoots were transferred to soil and cultivated until seed set. T_1 plants were also cultivated until seed set, and three homozygous T_2 lines carrying a single insert were selected for further analysis. The work described herein was carried out on the best geraniol-producing line, VoGES#18.

Cultivation of transgenic plants in vitro and initiation of cell suspension cultures

Seeds of transgenic tobacco (*N. tabacum* cv. Samsun NN) T_7 plants expressing *VoGES* were germinated under illumination in sterile conditions on hormone-free MS medium (Duchefa, Netherlands) containing 100 mg/l kanamycin. The intact in vitro plants were propagated on the same medium without antibiotics in plastic transparent "Steri Vent" containers (Duchefa) with a 16-h photoperiod. Sterile seedlings were also used for the initiation of callus on MS medium with vitamins, supplemented with 0.1 mg/l kinetin and 1 mg/l 1-naphthaleneacetic acid (NAA). The resulting callus tissue was maintained under constant illumination. Cell suspension cultures were established by transferring of ~1 g of callus tissue to 50 ml TubeSpin® bioreactors (Techno Plastic Products AG, Switzerland) filled with 10 ml Gamborg's B5 medium plus vitamins, 0.1 mg/l kinetin and 1 mg/l NAA. Cell suspension cultures were incubated on a gyratory shaker (180 rpm) at 25 °C under illumination (35.6 µmol/s) and were subcultured every 2 weeks for more than 1 year until they reached a homogenous state. Following cultivation, the cell suspension biomass was harvested by vacuum filtration and stored at −80 °C.

Initiation and maintenance of hairy root cultures

Tobacco hairy roots (*N. tabacum* cv. Petit Havana SR1) were initiated by infecting the leaves of wild-type tobacco plants with *Agrobacterium rhizogenes* LBA9402 carrying the vector pBIN2.4VoGES1, driven by the double-enhanced *Cauliflower mosaic virus* 35S promoter (Dong et al. 2013). Hairy roots emerged approximately 2–3 weeks after infection, and single root tips were placed on solid modified Gamborg's B5 medium (Häkkinen et al. 2014) supplemented with 500 mg/l cefotaxime. Hairy root transformation was verified by PCR analysis using forward primer 5′-ATG GAT CCC AAA TTG CTA TTC CTT CCA CGA-3′ and a reverse primer 5′-TTA GGC TTC TTT CTT CAG GTT TAC TGC AGC-3′ to amplify a 780-bp fragment corresponding to the integrated *rol*B gene. In addition, to confirm the absence of *A. rhizogenes* in the hairy root clones, a 450-bp diagnostic fragment of the *vir*D_1 gene was amplified by PCR using a forward primer 5′-ATG TCG CAA GGA CGT AAG CCC A-3′ and a reverse primer 5′-GGA GTC TTT CAG CAT GGA GCA A-3′ (Fig. S1). In both cases, a boiled preparation of wild type *A. rhizogenes* strain LBA9402 was used as a positive control. The PCR products were separated by 0.8 % (w/v) agarose gel electrophoresis and visualized by ethidium bromide staining under UV light, relative to DNA molecular weight markers (Gene Ruler DNA Ladder 100 bp, Thermo Fisher Scientific Inc. Waltham, MA, USA).

After one round of selection, positive 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 suspension cultures, 50 mg fw of hairy root biomass was added to 50 ml 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.

Hairy root cultures were maintained during geraniol production on the aforementioned modified Gamborg's B5 medium containing 30 g/l sucrose (pH 5.8) and 4.5 g/l Gelrite at 26 °C in the dark. The roots were subcultured every 4 weeks. Geraniol production was carried out using the wave-mixed BIOSTAT® RM bioreactor (Sartorius Stedim Biotech, Germany) and a single-use CultiBag RM 2L in batch mode, rocking at 8 rpm at an angle of 6° with an aeration rate of 0.2 vvm and a temperature of 26 °C. Each CultiBag contained 200 ml medium and was inoculated with 1 g fw of a 7-day-old root culture grown in Petri dishes.

Transient expression in *Nicotiana benthamiana*

A truncated version of *VoGES* lacking the first 56 amino acids (ΔNVoGES: bp 178–1785) was prepared for transient expression in *N. benthamiana* leaves, which was carried out as previously described with minor changes (Dong et al. 2013; van Herpen et al. 2010). The expression construct pBIN+ *VoGES* was introduced by electroporation into *A. tumefaciens* strain LBA4404, and the bacteria were cultivated at 25–28 °C and 180 rpm for 24 h in YEB medium supplemented with 25 µg/ml rifampicin, 25 µg/ml kanamycin and 30 µg/ml streptomycin. After 3 days, the OD_{600} was adjusted to 1.0 with infiltration medium (100 g/l sucrose, 3.98 g/l glucose, 1 g/l Ferty-2 Mega fertilizer solution (Planta Düngemittel GmbH, Germany) and 200 µM acetosyringone). This was mixed with an equal volume of a bacterial suspension ($OD_{600} = 1.0$) transformed with the *Tomato bushy stunt virus* p19 silencing suppressor construct, kindly provided by Plant Bioscience Ltd (Norwich, England) to boost protein production (Voinnet et al. 2003).

Hydroponic and soil cultivation

Homozygous seeds from the *VoGES* transgenic tobacco Samsun NN plants (T_7 generation) were germinated on starter rockwool plugs (Grodan, Netherlands) soaked in 1.7 mS/cm of the hydroponics solution Ferty-2 Mega. Natural light in the greenhouse chambers was supplemented with artificial lightening automatically activated between 6.30 and 22.30 h when the natural light intensity fell below 35

klx. The plants were cultivated at 27/22 °C day/night temperature and 70 % relative humidity.

Two-week-old seedlings of equivalent size were divided into two groups. The first was transferred to cylindrical, non-transparent plastic containers (12 cm in diameter and 13 cm in height) containing 1,000 ml of hydroponic solution, with air supplied by forced aeration through an air-bubbler. A 2-cm hole was drilled in each lid to accommodate the stems and roots. Each container and lid was wrapped in aluminum foil to exclude light and thus prevent the growth of algae in the medium. Small pieces of foil were wrapped around the base of the stems to provide vertical support (Sundberg et al. 2003). The second group of seedlings was placed on "Plant Comfort" type rockwool (Cultilene, Netherlands) designated for the nutrient film technique (NFT). For plants cultivated in soil, pots were filled with standard soil supplied by Einheitserde Werksverband e.V., Germany, and the remaining conditions matched those used for hydroponic cultivation.

All soil-grown plants were irrigated and the hydroponic containers refilled with solution at least twice per week, depending on evaporation. Leaves were harvested before flowering from three vertical positions along the stem (upper, middle and lower) 6 h into the light period, and metabolic activity was quenched rapidly by snap freezing in liquid nitrogen (Lisec et al. 2006).

Extraction and quantitation of geraniol

Frozen plant material (200 mg) was ground in liquid nitrogen and 1 ml of citrate–phosphate buffer (pH 5.4) was added to the homogenous mixture in a glass tube (Dong et al. 2013). The samples were sonicated for 15 min in a water bath at room temperature before adding 0.5 ml of the cellulolytic enzyme mixture Viscozyme L (Sigma-Aldrich, Germany). Heptane (Fluka, Germany) was used as the organic solvent because it improves accuracy and reproducibility, and samples were overlaid with 1 ml heptane containing 10 µg/ml (*Z*)-nerolidol (Sigma-Aldrich) before incubating overnight at 37 °C. After brief vortexing, each sample was centrifuged for 10 min (1,750×*g*) at room temperature and the organic phase was removed. The sample was overlaid with heptane as above and the process repeated until three heptane extracts were obtained. The pooled extract was filtered through a glass Pasteur pipette containing a small plug of glass wool and ~1.5 cm of anhydrous sodium sulfate (Sigma-Aldrich).

The eluent was concentrated under nitrogen flow and the geraniol content was quantified by GC/MS using a QP2010SE quadrupole mass spectrometer (Shimadzu, Japan) following separation using a 30 m × 0.25 mm internal diameter Zebron ZB-5 ms column (Phenomenex, USA) containing 0.25 µm stationary phase, preceded by a 5-m guard column. The column was injected with 1 µl of each sample (split mode 1:10, injection port temperature 250 °C) and the ZB5 column was maintained at 45 °C for 1 min followed by a gradient of 10 °C per min until the temperature reached 300 °C, which was held for a further 7 min. The helium inlet pressure was checked by electronic pressure control to achieve a constant column flow rate of 1.0 ml/min. Geraniol was detected following electric ionization at 1 keV and scanning in SIM mode with diagnostic ion monitoring, based on reference spectra and the NIST library. Geraniol was quantified using the base peak ($m/z = 69$).

Statistical analysis

Significant differences between groups were determined using Student's *t* test and significant differences among several groups were determined by ANOVA with Tukey's post hoc analysis (GraphPad Software, Inc., USA).

Results

Characterization of transgenic plants and cultures

All transgenic plants regardless of the cultivation system showed normal growth and development, similar to wild-type controls in the same environment. *VoGES* transgenic plants cultivated using the NFT hydroponic system produced the greatest amount of biomass, with an average of 314.5 g fw per plant after 45 days. Plants cultivated in soil were marginally less productive (average biomass of 275.3 g fw per plant) but plants cultivated in stationary hydroponic vessels produced only one-third of the biomass of their NFT counterparts with an average of 114.3 g fw per plant. The in vitro grown plants were the least productive, accumulating an average of 9.9 g fw of biomass per plant after cultivation for 63 days. These data are summarized in Table 1.

The transgenic hairy roots produced 7.0 g fw of biomass after cultivation for 21 days in shake flasks, corresponding to 140 g fw per liter of medium. The productivity in 2-liter BIOSTAT® CultiBag RM disposable bioreactor bags was similar, corresponding to 144 g fw/l after 21 days. In both cases, the roots were cultivated in darkness. In contrast, transgenic cell suspension cultures were grown under constant illumination and produced 545 g fw biomass per liter of medium in 9 days (Table 1).

Geraniol production

Geraniol accumulates predominantly as geraniol glycosides in *VoGES* transgenic plants (Dong et al. 2013). We found that the intact plants grown in vitro accumulated the highest amount of geraniol (48 µg/g fw), followed by *N.*

Table 1 Biomass production of transgenic tobacco (*Nicotiana tabacum* cv. Samsun NN and cv. Petit Havana SR1) (n = 5) and wild type *N. benthamiana* plants (n = 5) expressing *VoGES*

Species/cultivar	System	Biomass	STDEV[a]	Cultivation (days)	Biomass productivity
N. tabacum cv. Samsun NN	Greenhouse plants/pots/	114.3[b]	9.4[b]	45	2.5 g/d
	Greenhouse plants/NFT/	314.5[b]	25.7[b]	45	7.0 g/d
	Greenhouse plants/soil/	275.3[b]	17.2[b]	45	6.1 g/d
	In vitro plants	9.9[b]	0.7[b]	63	0.2 g/d
	Suspended cells/shake flasks	10.9[c]	1.7[c]	9	60.6 g/l/d
N. tabacum cv. Petit Havana SR1	Hairy roots/BIOSTAT®	144[d]	7.1[d]	21	6.9 g/l/d
N. benthamiana (WT[e])	Greenhouse plants/soil/	92[b]	10.3[b]	54	1.7 g/d

[a] *STDEV* standard deviation

[b] (g fw/plant)

[c] (g fw/20 ml culture)

[d] (g fw/l culture)

[e] *WT* wild-type plants used for transient expression

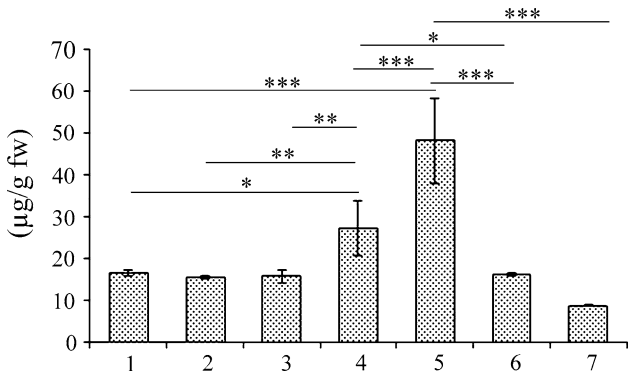

Fig. 1 Geraniol content achieved in each of the plant-based expression platforms (n = 5): *1* greenhouse plants grown in pots; *2* greenhouse plants cultivated in the NFT; *3* greenhouse plants grown in soil; *4* transient expression in *N. benthamiana*; *5* intact plants cultivated in vitro; *6* cell suspension cultures; *7* hairy roots. * *P* value <0.05; ** *P* value <0.01; *** *P* value <0.001

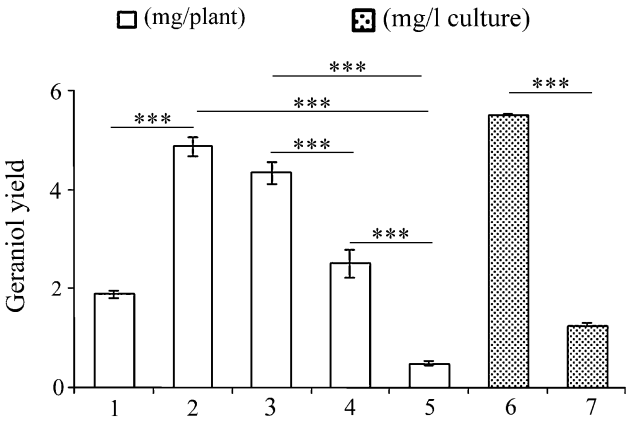

Fig. 2 Geraniol yield achieved in each of the plant-based expression platforms (n = 5): *1* greenhouse plants grown in pots; *2* greenhouse plants cultivated in the NFT; *3* greenhouse plants grown in soil; *4* transient expression in *N. benthamiana*; *5* intact plants cultivated in vitro; *6* cell suspension cultures; *7* hairy roots.*** *P* value <0.001

benthamiana plants transiently expressing *VoGES* (27 µg/g fw). The geraniol content of transgenic tobacco plants grown in the greenhouse and plant cell suspension cultures was similar (~16 µg/g fw). Hairy roots accumulated the lowest geraniol levels among the platforms we tested (8.8 µg/g fw) (Fig. 1). The smallest (youngest) leaves of the tobacco plants grown in the greenhouse contained more geraniol (17.5 µg/g fw) compared to the largest (oldest) leaves (14.9 µg/g fw) and the middle leaves (15.4 µg/g fw).

The productivity of each platform depends on both the biomass yield and the geraniol content per unit biomass. As shown in Fig. 2, transgenic tobacco plants grown in soil and in the NFT system had the highest geraniol yields of 4.9 and 4.3 mg/plant, respectively. The geraniol production per in vitro plant was approximately ten-fold lower than the above values, predominantly reflecting the comparatively low biomass yield. The cell suspension cultures were superior to the hairy root cultures, again reflecting the larger amount of biomass produced per liter of medium over a comparable cultivation period. The geraniol content of the cell suspension cultures was 5.5 mg/l compared to 1.26 mg/l for the hairy root cultures.

In addition to the biomass yield and geraniol content per unit biomass, the efficiency of a production platform depends on the length of the production cycle. The cell suspension cultures had the shortest cultivation period (9 days), which was also characterized by rapid biomass accumulation and the production of reasonable levels of the target monoterpenoid (Fig. 3). The overall geraniol productivity of the cell suspension cultures therefore reached

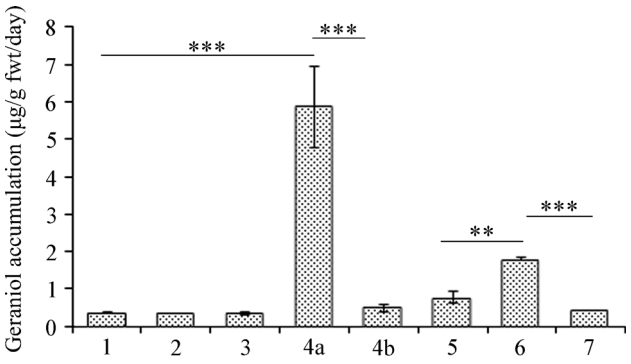

Fig. 3 Mean geraniol accumulation in each of the plant-based expression platforms (n = 5): *1* greenhouse plants grown in pots; *2* greenhouse plants cultivated in the NFT; *3* greenhouse plants grown in soil; *4a* transient expression in *N. benthamiana* excluding cultivation prior to agroinfiltration; *4b* transient expression in *N. benthamiana* including cultivation prior to agroinfiltration; *5* intact plants cultivated in vitro; *6* cell suspension cultures; *7* hairy roots. ** *P* value <0.01; *** *P* value <0.001

1.8 µg/g fw/day compared with 5.9 µg/g fw/day for transient expression in *N. benthamiana* if the pre-infiltration cultivation period is ignored (Fig. 3). However, if the cultivation period prior to agroinfiltration is included, then the geraniol productivity declines to 0.5 µg/g fw/day which is comparable to stably-transformed plants grown in the soil and in the NFT system, which achieved productivities of 0.34–0.37 µg/g fw/day, and to hairy root cultures, with a productivity of 0.42 µg/g fw/day (Fig. 3).

Discussion

Plants are ideal for the heterologous expression of monoterpenoid biosynthesis enzymes because this approach could reconstitute elements of the TIA pathway and facilitate the large-scale production of valuable flavor and aroma compounds such as geraniol (Dong et al. 2013). We explored the potential of diverse plant-based expression platforms for the production of geraniol because it is both valuable in its own right and a key precursor for the biosynthesis of downstream TIAs. We chose tobacco (*N. tabacum* L. cv. Samsun NN and cv. Petit Havana SR1) as a model system for the stable expression of plastid-targeted GES from *V. officinalis*, and used intact plants grown in the greenhouse or in vitro, as well as cell suspension cultures and hairy roots. We compared these platforms to transient expression in *N. benthamiana.* We quantified geraniol on the basis of fresh weight rather than dry weight because: (1) geraniol is a volatile compound and therefore freeze-drying may deplete the free aglycone fraction, thus reducing the accuracy of geraniol quantification and delaying the overall analytical process substantially; (2) rapid sample treatment and analysis is necessary to control the manufacturing process efficiently; and (3) it is better to describe the geraniol content in the same manner as previously reported to allow the meaningful comparison of our results with existing data (Davidovich-Rikanati et al. 2007; Dong et al. 2013; Fischer et al. 2013; Gang et al. 2001; Gutensohn et al. 2013; Höfer et al. 2013; Iijima et al. 2004). Direct comparisons between these diverse systems in terms of overall productivity can be challenging because there is no common frame of reference. Therefore, we determined the mean daily productivity of each system by measuring the output in micrograms of geraniol per gram fresh biomass per day, which can be applied to all systems from a product-orientated perspective. We undertook this comprehensive approach to evaluate the geraniol productivity of the different platforms systematically in order to make direct comparison among the systems.

All the intact plants expressing *VoGES* were similar in phenotype to wild-type controls growing under the same conditions. This remarkable vitality may reflect the efficient conjugation of geraniol with glycosyl side chains, producing highly-soluble geraniol glycosides that are stored in the vacuoles thus reducing the toxic effect of high geraniol levels in the plant cell. This is supported by previous reports showing the presence of high levels of geraniol glycosides in *N. benthamiana* leaves transiently expressing the same *VoGES* expression construct, reflecting the action of endogenous glycosyltransferases (Dong et al. 2013). The geraniol glycosides detected in our *VoGES* transgenic plants were mono-glycosides, di-glycosides and tri-glycosides with hexoside (glucopyranoside) or pentoside (arabinofuranoside, arabinopyranoside, apiofuranoside, xylopyranoside or rhamnopyranoside) monomers (Dong et al. 2013).

Geraniol productivity in plant-based expression platforms depends on three key factors: geraniol synthesis, biomass accumulation and cultivation time. Intact plants grown in vitro accumulated the highest levels of geraniol, but their slow growth produced relatively little biomass which made them less suitable as a competitive production platform. *In vitro* plants are cultivated in containment, so the volatile product geraniol is trapped and the accumulation of this compound may boost geraniol glycoside formation in the plant cell. Another potential explanation for the high geraniol content in plants grown in vitro is the accumulation of ethylene, which can act as an elicitor of secondary metabolism. Transcription factors of the plant-specific APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) family modulate secondary metabolism (De Boer et al. 2011). For example, ethylene response factor SIERF6 also plays an important role in carotenoid biosynthesis (Lee et al. 2012). Ethylene has also been shown to induce three genes from the MEP pathway which are responsible for the synthesis of the geraniol precursor

geranyl diphosphate (Papon et al. 2005). Plant cell suspension and hairy root cultures are also closed in vitro systems but they accumulated less geraniol than plants cultivated in vitro, perhaps reflecting the impact of tissue and organ differentiation on geraniol biosynthesis.

Transgenic tobacco plants in the greenhouse accumulated geraniol at levels similar to stably transformed *A. thaliana* plants expressing an *O. basilium* GES-GFP fusion construct (18.7 μg/g leaves) (Fischer et al. 2013). However, transgenic *V. vinifera* plants expressing *O. basilium* GES accumulated more geraniol (50.7 μg/g leaves) (Fischer et al. 2013) than our transgenic tobacco plants. This may reflect the different origin of the GES sequences and/or the presence of low levels of monoterpenols in the grapevine cultivar probably including geraniol precursors (Gunata et al. 1985). The transient expression of *O. basilium GES* and *GES-GFP* in *N. benthamiana* produced more geraniol than our transient expression platform (83.6 and 93 μg/g, respectively). However, the expression of *O. basilium GES* and *GES-GFP* was characterized by a relatively large standard deviation (45–57 %) compared to the overall biological and analytical variation in our transient expression platform of less than 7 %. Our cell suspension cultures expressing *VoGES* produced geraniol at levels similar to those achieved in mutant *ERG20* yeast cells expressing the *O. basilium GES*, i.e. 5 mg/l (Fischer et al. 2011).

The cell suspension cultures expressing *VoGES* accumulated large amounts of geraniol, which favors their use for process-scale production as long as productivity is maintained at larger scales. If so, cultivation in a 1000-l bioreactor for 45 days, corresponding to five batches each lasting for 9 days, would theoretically yield the same amount of geraniol as ~6,000 transgenic plants cultivated in the greenhouse for the same duration. The latter would require ~1,000 m^2 of greenhouse space and would involve much higher labor costs compared to cell suspension cultures growing in disposable bioreactors.

The hairy root platform was the only system cultivated in dark, and it accumulated the lowest amount of geraniol. Light is the key factor that synchronizes plant growth with the environment, and light also plays a significant role in the regulation of secondary metabolism. Monoterpene synthase transcript levels peaked after 9 h of illumination (Lu et al. 2002) and monoterpenoids in the flower headspace showed a clear diurnal emission profile with maximum levels during the day and minimum levels at night (Aharoni et al. 2003). This suggests precursor availability may also follow a diurnal rhythm with a peak during illumination. The induction of the otherwise constitutive CaMV 35S promoter by light (Schnurr and Guerra 2000) may also contribute to the activation of *VoGES*. However, the essential oil content of aromatic plants can be improved by exposure to UV-B radiation (Karousou et al. 1998). Therefore, cultivation under illumination combined with moderate exposure to UV-B radiation could help to boost geraniol levels even further, both in whole plants in the greenhouse and in contained cell suspension cultures. Geraniol produced by the different tobacco-based platforms is oxidized to produce geranial, hydroxygeraniol, geranic acid, hydroxygeranic acid and hydroxydihydrogeranic acid (Dong et al. 2013). The inhibition of such oxidation reactions could therefore be used as an additional strategy to maximize geraniol accumulation in the tobacco plants expressing *VoGES*.

In summary, our comparative study of plant-based expression platforms has shown that cell suspension cultures appear to be the most productive system for the manufacture of geraniol, which could be achieved in large-scale bioreactors under controlled environmental conditions. This approach is economically feasible, independent of seasonal and geographical variations and avoids laborious work in the greenhouse. Cell suspension cultures producing high levels of geraniol could be improved even further by medium and process optimization using a statistical design of experiments approach (Vasilev et al. 2013). Transgenic tobacco cell suspension cultures are currently the ideal plant-based expression platform for the production of monoterpenoids and the benefits of this platform could be extended to other terpenoids and different families of secondary products.

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