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Monoterpene biosynthesis potential of plant subcellular compartments

Lemeng Dong, Esmer Jongedijk, Harro Bouwmeester and Alexander Van Der Krol

Laboratory of Plant Physiology, Wageningen University, Droevendaalsesteeg 1, 6708 PB, Wageningen, the Netherlands

Author for correspondence:
Alexander Van Der Krol
Tel: +31 317482448
Email: sander.vanderkrol@wur.nl

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Summary

- Subcellular monoterpene biosynthesis capacity based on local geranyl diphosphate (GDP) availability or locally boosted GDP production was determined for plastids, cytosol and mitochondria.
- A geraniol synthase (*GES*) was targeted to plastids, cytosol, or mitochondria. Transient expression in *Nicotiana benthamiana* indicated local GDP availability for each compartment but resulted in different product levels. A GDP synthase from *Picea abies* (*PaGDPS1*) was shown to boost GDP production. *PaGDPS1* was also targeted to plastids, cytosol or mitochondria and *PaGDPS1* and *GES* were coexpressed in all possible combinations.
- Geraniol and geraniol-derived products were analyzed by GC-MS and LC-MS, respectively. GES product levels were highest for plastid-targeted GES, followed by mitochondrial- and then cytosolic-targeted GES. For each compartment local boosting of GDP biosynthesis increased GES product levels.
- GDP exchange between compartments is not equal: while no GDP is exchanged from the cytosol to the plastids, 100% of GDP in mitochondria can be exchanged to plastids, while only 7% of GDP from plastids is available for mitochondria. This suggests a direct exchange mechanism for GDP between plastids and mitochondria. Cytosolic PaGDPS1 competes with plastidial GES activity, suggesting an effective drain of isopentenyl diphosphate from the plastids to the cytosol.

Introduction

Plants produce an array of different isoprenoids that are synthesized in plastids, mitochondria, and cytosol. The types of isoprenoids that are synthesized in each subcellular compartment correlate with the substrates, geranyl diphosphate (GDP), farnesyl diphosphate (FDP), and geranylgeranyl diphosphate (GGDP) that are available in these compartments. For example, in plastids the C10 GDP and C20 GGDP are present and are used for the biosynthesis of the C10 monoterpenes and C20 diterpenes, respectively. In the cytosol, the C15 FDP is used for C15 sesquiterpene and C30 triterpene and steroid (two FDP units) biosynthesis. In the mitochondria, GGDP is used for diterpene and ubiquinone and the FDP is used for sesquiterpene biosynthesis (Kappers *et al.*, 2005; Vranová *et al.*, 2012). The availability of these substrates is a function of the prenyltransferases localized within each organelle: geranyl diphosphate synthase (GDPS) in the plastids; geranylgeranyl diphosphate synthase (GGDPS) in the plastids, mitochondria and cytosol; and farnesyl diphosphate synthase (FDPS) in the mitochondria and cytosol. Despite their differential localisation, all three enzymes utilize isopentenyl diphosphate (IDP) and its isomer dimethylallyl diphosphate (DMADP) as substrates (Supporting Information Fig. S1). Isoprenoid biosynthesis capacity in the different subcellular compartments can be postulated to be a function of local GDPS, FDPS and GGDPS activity, local IDP and DMADP production, and the capacity of exchange of these substrates between the compartments.

Monoterpenoids play an important role in a broad range of ecological and biological processes, such as defence against insects and pathogens (Keeling & Bohlmann, 2006; Unsicker *et al.*, 2009), attraction of the enemies of herbivores (Arimura *et al.*, 2004; Poecke & Dicke, 2004) or attraction of pollinators (Borg-Karlson *et al.*, 1993). Monoterpenoids are generally volatile and can be stored in glandular trichomes or are emitted into the plant headspace. Monoterpenes may be modified by oxidation and/or glycosylation and monoterpene glycosides can be stored in the plant vacuole (Janson, 1993; Mateo & Jiménez, 2000; Lücker *et al.*, 2001; Nogués & Loreto, 2013). Monoterpenes are important components of flower fragrance (Hener *et al.*, 1990), wine (Câmara *et al.*, 2004) and foods (Lewinsohn *et al.*, 2001), are used as pesticide (Koul *et al.*, 2008; Yang *et al.*, 2012) or have useful pharmaceutical properties (Lappas & Lappas, 2012; Bezerra *et al.*, 2013). Exploration of the monoterpene biosynthesis potential of the different subcellular compartments in plants can give insight into the regulation of monoterpene production in plants and could provide useful information for the optimisation of metabolic engineering of terpenoids.

The biosynthesis of monoterpenes is controlled by three main steps: (1) building the basic units IDP and DMADP that have been reported to be present in plastids, cytosol and mitochondria (Aharoni *et al.*, 2005); (2) condensation of IDP and DMADP by GDPS to form GDP; GDPSs are mainly active in the plastids although a low GDP pool has been reported for the cytosol as well (Wu *et al.*, 2006; Vranová *et al.*, 2012); and (3) conversion of GDP to the monoterpene parent scaffold catalyzed by a monoterpene synthase; monoterpene synthases are mostly targeted to the plastids, but some were also shown to be active in the cytosol (Dong *et al.*, 2013). In this study we tested the monoterpene biosynthesis capacity of the different subcellular compartments by ectopic expression of a geraniol synthase from *Valeriana officinalis* (*VoGES*) targeted to plastids, cytosol, or mitochondria. To probe the potential IDP and DMADP precursor pool in these subcellular compartments we used overexpression of *GDPS* targeted to these different compartments. To explore the exchange of GDP between subcellular compartments, we assessed geraniol production by VoGES in one compartment with and without boosting of GDP availability in other compartments by coexpression of *GDPS*. We show that the highest potential for terpene biosynthesis resides in the plastids and that there is considerable (bi)directional GDP exchange between the different subcellular compartments. Implications for the metabolic engineering of terpene biosynthesis in plants are discussed.

Materials and Methods

Construction of different targeted GES and GDPS

A geraniol synthase from *Valeriana officinalis* was isolated and characterized as previously described (Dong *et al.*, 2013). To determine the potential of monoterpene biosynthesis in different subcellular compartments, *VoGES* was provided with a plastid, cytosol or mitochondrial targeting signal. First, a truncated version of VoGES lacking the first 56 amino acids (AA) (ΔNVoGES: bp 178–1785) was made using standard cloning techniques and then was cloned into impact vector pIV2B 2.1 (Table S1) under control of the CaMV 35S-promoter for cytosol targeting. In addition, the truncated versions of *VoGES* was provided with a heterologous plastid import signal by cloning into impact vector pIV2B 2.4 which carries an artificial plastid targeting signal (Table S1) (Loyola-Vargas *et al.*, 2007). The truncated *VoGES* was also cloned into impact vector pIV2B 2.5, which contains a yeast CoxIV secretion signal that was shown to deliver proteins to the mitochondrial matrix (Table S1) (Köhler *et al.*, 1997). The effect of the exchange of the native plastid targeting signal by an artificial one was assessed using transient expression in *Nicotiana benthamiana* Domin (see later) and was shown not to result in significant change in the total production of geraniol (Table S2). Full length cDNAs of *PaGDPS1* and *PaGDPS2* were isolated from *Picea abies* (Schmidt & Gershenzon, 2008; Schmidt *et al.*, 2010) and cloned into the pCAMGW (a pCAMBIA-derived vector) under the control of the CaMV 35S-promoter and a maize ubi1 promoter, respectively, using Gateway technology. Truncated *PaGDPS1* lacking the first 67 amino acids was cloned into impact vector pIV2A 2.1 (cytosolic targeting), pIV2A 2.4 (plastidic targeting), and pIV2A 2.5 (mitochondrial targeting) under the control of the CaMV 35S-promoter. *AtGDPS* (At2G34630.1) cDNA (Bouvier *et al.*, 2000) was cloned into impact vector pIV2A 2.4 (plastidic targeting). To test the targeting capacity under transient expression conditions in *N. benthamiana*, epi-fluorescence green fluorescent protein (eGFP) was cloned into the same impact vectors pIV2A 2.1, 2.4 and 2.5.

All the constructs above were sequenced to check integrity, before transferring the impact vector to the binary vector pBIN+ (van Engelen *et al.*, 1995) using LR recombination (Gateway technology) (Karimi *et al.*, 2007). Primer sequences for PCR amplification and restriction sites for each primer are listed in Table S3. Finally, the binary expression constructs were transformed into *Agrobacterium tumefaciens* strain AGL0 (Lazo *et al.*, 1991) which was used for transient expression in *N. benthamiana* as described later.

Transient expression in *N. benthamiana*

Transient expression of pathway genes using *A. tumefaciens* infiltration (agroinfiltration) of leaves of 5-week-old *N. benthamiana* plants was performed as described (Dong *et al.*, 2013). The concentration of each expression construct in the treatments was kept constant by adding *A. tumefaciens* carrying an empty vector, if appropriate. Leaves were harvested for GC-MS and LC-MS analysis 5 days post-agroinfiltration.

Volatile GC-MS analysis and nonvolatile liquid chromatography-quadrupole time of flight (LC-QTOF)-MS analysis

Headspace analysis of detached *N. benthamiana* leaves was done by volatile trapping for 2.5 h in the light. Samples were analyzed by GC-MS as described before (Dong *et al.*, 2013). For quantification, a geraniol calibration curve was made using an authentic standard. After headspace trapping, samples were frozen in liquid nitrogen and used for further analysis by LC-MS and GC-MS. LC-MS analysis of conjugates of geraniol and geraniol-derived compounds was performed as described previously (Dong *et al.*, 2013).

Glycosidase treatment followed by GC-MS analysis was performed to quantify glycoside conjugates of geraniol and geraniol-derived compounds. For this purpose, 1 ml of extraction buffer (0.1 M Na_2HPO_4, 0.04 M citric acid, pH 5.4) was added to 200 μg of leaf powder. The samples were vortexed briefly and sonicated for 15 min. Two hundred microlitres Viscozyme L (Sigma) was added and vortexed briefly to mix well. An overlay of 1 ml pentane with 2 μg ml^{-1} *cis*-nerolidol as internal standard was used to trap released volatiles. Samples were incubated at 37°C overnight without shaking. Total internal volatiles were extracted by vortexing 20 s, sonication for 5 min and centrifugation for 5 min at 3400 ***g***. The pentane layer was removed and the sample extracted with another 1 ml pentane containing 2 μg ml^{-1} *cis*-nerolidol. The combined organic phase was passed

through a small column containing anhydrous Na_2SO_4 to remove water and stored at −20°C until use. GC-MS analysis was performed on a 7890A gas chromatograph (Agilent Technologies, Amstelveen, the Netherlands), equipped with a mass selective detector (model 5975C; Agilent Technologies). Splitless injection of 1 µl sample was performed at 250°C on a Zebron ZB-5MS column (30 m × 0.25 mm, 0.25 µm thickness; Phenomenex, Utrecht, the Netherlands) at a helium flow rate of 1 ml min^{-1}. The temperature program was 2 min at 45°C, then 40°C min^{-1} until 300°C, then 3 min at 300°C. Compounds were identified by comparing the retention time and mass spectrum with authentic standards of citral (mix of neral and geranial), nerol, geraniol and geranic acid. The compounds were quantified using calibration curves.

Acquisition of headspace GC-MS data and LC-MS data was performed using Xcalibur (Thermo, Amsterdam, the Netherlands) and MassLynx 4.0 programs (Waters, Etten-Leur, the Netherlands), respectively. Data were processed using MetAlign v.1.0 (http://www.metalign.nl). Baseline and noise calculations were performed from scan number 500 to 9759 for GC-MS data and 70 to 2480 for LC-MS. The maximum amplitude was set to 10 000 000 for GC-MS data and 25 000 for LC-MS data and peaks below three times the local noise were discarded. Multiple mass signals derived from the same compound were grouped using MSClust (biotools.wurnet.nl). The selected mass intensities were normalized and analysed using GeneMath XT version 2.1 as described previously (Dong *et al.*, 2013). Mass spectra were compared with National Institute of Standards and Technology (NIST) library (http://www.nist.gov/) to acquire putative identification of selected compounds.

Results

Intrinsic monoterpene biosynthesis capacity of plastids, cytosol and mitochondria

To determine the potential of monoterpene biosynthesis in different subcellular compartments, the geraniol synthase from *Valeriana officinalis* (*VoGES*) (Dong *et al.*, 2013) was provided with a plastid, cytosol or mitochondrial targeting signal, each placed under control of the CaMV 35S promoter in a binary expression vector (see the Materials and Methods section). Each construct was transiently expressed using *A. tumefaciens* infiltration of *N. benthamiana* leaves. Headspace analysis showed similar geraniol emission by *N. benthamiana* leaves expressing cytosol (*C-VoGES*) and plastid-targeted VoGES (*P-VoGES*) and lower emission from the leaves expressing the mitochondrial targeted VoGES (*M-VoGES*) (Fig. 1a). However, LC-MS analysis of extracts of these leaves showed that there are several geraniol-derived glycosidic conjugates produced, as was described before (Dong *et al.*, 2013). These glycosides are produced by the action of *N. benthamiana* glycosyl transferases using the heterologously produced geraniol and geraniol-derived oxidation products such as geranic acid and carboxygeranic acid that are produced from geraniol by endogenous *N. benthamiana* enzymes, as substrates. The putative identification of these geraniol(-derived) glycosides is shown in Table 1. In contrast with the volatile emission, LC-MS analysis revealed a large difference in geraniol(-derived) glycosides between leaves expressing *C-VoGES* and *P-VoGES* (Fig. 1b), with much higher levels resulting from plastid targeting. The detection of free geraniol and geraniol-derived glycosides upon VoGES targeting to the different compartments suggests that all three subcellular compartments have some level of GDP available, but the level is clearly highest in the plastids. The high expression levels obtained during transient expression in *N. benthamina* could lead to saturation of the different organellar protein import pathways. To assess the protein targeting capacity under transient expression conditions in *N. benthamiana*, we performed localization studies with cytosolic eGFP and with eGFP fused to the mitochondrial or plastidic signal peptide. At 5 days post-agroinfiltration this showed the expected localization for each of the fusion proteins with little or no fluorescence in the cytosol for plastidial and mitochondrial targeted eGFP. Also, no mitochondrial targeting was observed for the plastidial-targeted eGFP and vice versa (Fig. S2).

Boosting GDP levels for geraniol production

VoGES activity in the different subcellular compartments may well be limited by the local conversion of IDP and DMADP to GDP, as local subcellular GDPS activity may differ. To maximize local conversion of IDP and DMADP to GDP, plastidic, cytosolic or mitochondrial targeted GDPS could be employed. We therefore set out to identify a functional GDPS. Two *GDPSs* from *P. abies*, *PaGDPS1* and *PaGDPS2* (Schmidt & Gershenzon, 2008; Schmidt *et al.*, 2010) and one *GDPS* from Arabidopsis, *AtGDPS* (Bouvier *et al.*, 2000) were cloned into a binary expression vector using the same plastid targeting signal as used for *VoGES* for *AtGDPS* and the native plastid targeting signal for the *PaGDPSs*. Each GDPS expression construct was coexpressed with *P-VoGES* in *N. benthamiana* leaves. Leaves were harvested 5 days post-agroinfiltration for headspace analysis by GC-MS and leaves were extracted for analysis of nonvolatile products by LC-MS. In order to also detect any unexpected products, we used an untargeted data analysis approach using MetAlign. This resulted in 6818 masses detected by GC-MS and 5057 masses detected by LC-MS. MSClust was used to combine masses belonging to the same compounds. The analysis revealed a large cluster of compounds that were upregulated in empty vector expressing leaves, presumably representing a metabolic response to the presence of *A. tumefaciens* in leaves (Fig. 2). The metabolite profile resulting from the expression of either *PaGDPS1*, *AtGDPS* or *PaGDPS2* did not reveal any metabolite increase compared with the empty vector control (Fig. 2a,b). However, the level of some metabolites was reduced, especially upon expression of *AtGDPS* (Fig. 2a,b), which may indicate competition for a common precursor. No endogenous *N. benthamiana* monoterpenes were boosted by agroinfiltration of these *GDPS* genes.

When *P-VoGES* was expressed in the *N. benthamiana* leaves, geraniol was the only new volatile in the headspace compared with leaves expressing the empty vector (Figs 2a, S3a). In extracts of *N. benthamiana* leaves expressing *P-VoGES*, a series of geraniol

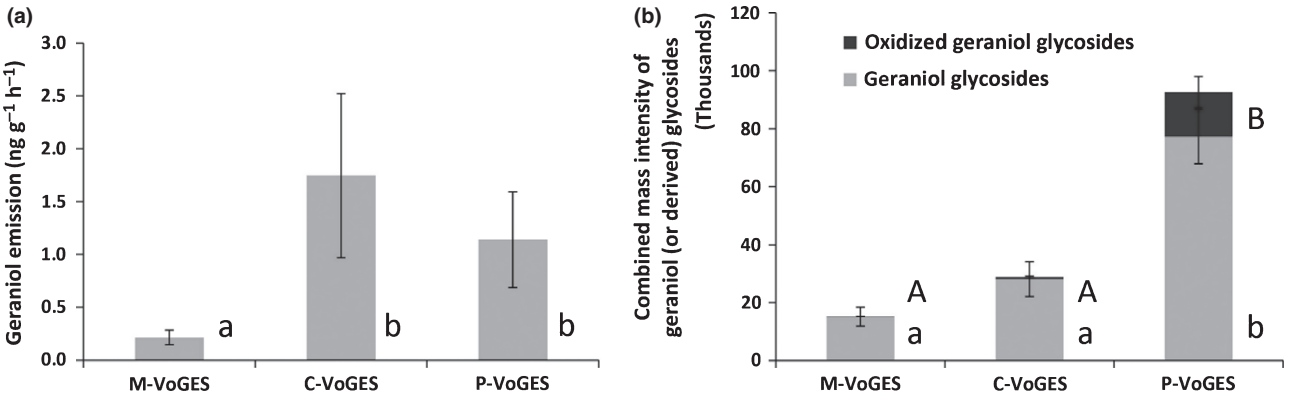

Fig. 1 Geraniol production upon transient expression in *Nicotiana benthamiana* of mitochondrial (M), cytosolic (C), and plastidic (P) targeted *Valeriana officinalis* geraniol synthase, *VoGES*. (a) GC-MS headspace analysis of geraniol emission. (b) Relative quantitative analysis of geraniol (grey bars) and oxidized geraniol (black bars) glycosides by LC-MS. Bars represent the total mass intensity of all geraniol and (or oxidized geraniol) glycosides, see Table 1. Bars with different letters (capital letters for oxidized geraniol glycosides, lowercase for free geraniol and geraniol glycosides) differ significantly ($P < 0.05$). Error bars are ± SE ($n = 4$).

(-derivative) glycosides was detected by LC-MS analysis (Table 1). Coexpression of *P-VoGES* with either *AtGDPS* or *PaGDPS2* did not result in increased geraniol or geraniol(-derivative) glycoside levels (Fig. 2). Upon coexpression of *P-VoGES* with *PaGDPS1*, in addition to geraniol, 45 other volatiles were significantly increased ($P < 0.05$) compared with *N. benthamiana* leaves agroinfiltrated with *P-VoGES* alone (Figs 2a, S3c). When the mass spectra of these 47 new volatiles were compared with spectra from the NIST library, 12 out of 45 matched geraniol-related compounds. While expression of *P-VoGES* alone or *PaGDPS1* alone did not significantly increase the level of other monoterpenes (e.g. myrcene, terpinene, limonene and ocimene), coexpression of *P-VoGES*+ *PaGDPS1* resulted in an increase in the production of myrcene, terpinene, limonene and ocimene compared with empty vector control (Figs 2a, S3c). We also note that after coexpression of *PaGDPS1* with *P-VoGES*, many alkaloids were upregulated, suggesting a complex response to the boosted ectopic production of geraniol in *N. benthamiana* (Fig. S3c).

In the *N. benthamiana* leaves which expressed *P-VoGES*+ *PaGDPS1*, the level of glycosides of geraniol and its further oxidized derivatives were significantly increased compared with those obtained by expression of *P-VoGES* alone (Figs 2b, S4), indicating that *PaGDPS1* can be used to boost production of geraniol by *VoGES*. Interestingly, when *P-VoGES* was coexpressed with *AtGDPS*, geraniol glycosides significantly decreased compared with *P-VoGES* expression alone (Fig. 2b), indicating a competition between *VoGES* and AtGDPS for substrates. *PaGDPS2* did not cause any change in geraniol(-derivative) glycosides when coexpressed with *VoGES* (Fig. 2b).

GDP flux in and to plastids: geraniol biosynthesis in the plastids in combination with GDP biosynthesis in different compartments

The coexpression of *PaGDPS1* apparently can increase the pool of GDP which is available for GES in the plastids, indicating that endogenous GDPS activity in the plastids is limiting for GDP production. To test the limitations in GDP biosynthesis also in the other compartments, we used VoGES activity in the different compartments as a proxy for the GDP flux and then assessed if this flux could be increased by coexpression of GDPS in the same or a different compartment. Hereto, we provided a truncated *PaGDPS1* (without chloroplast import peptide sequence) with a plastidic (*P-PaGDPS1*) or mitochondrial (*M-PaGDPS1*) targeting sequence, or no targeting sequence (cytosolic: *C-PaGDPS1*). All possible combinations of differentially targeted GES and differentially targeted GDPS were transiently expressed in *N. benthamiana* to determine how geraniol biosynthesis in each compartment is affected by GDP production in the same compartment and to determine how GDP production in one compartment affects GES activity in another compartments (thus assessing the exchange of GDP between compartments). Extracts from agroinfiltrated leaves were analyzed by LC-MS and total relative geraniol(-derivative) glycoside levels were semiquantified based on combined mass peak intensities of all geraniol-derived compounds.

Coexpression of *P-PaGDPS1* with *P-VoGES* resulted in a 118-fold increase in free geraniol in the headspace (Fig. 3a), a small increase in the geraniol-derived glycosides and an approximately five-fold increase in glycosides of oxidized geraniol derivatives (Fig. 3b). These results were similar as for *P-VoGES* with the native PaGDPS1 (Fig. 2) and confirm that GDPS activity in the plastid is limiting the substrate availability for P-VoGES. When this limitation is relieved by coexpressing *P-PaGDPS1*, the production capacity of the plastids becomes so large that the endogenous geraniol processing capacity (oxidation and glycosylation) become limiting. As a consequence, more of the geraniol is emitted to the headspace (Fig. 3a).

When expression of plastid-targeted *P-VoGES*, was combined with an increase in GDP production in the mitochondria by coexpression of *M-PaGDPS1*, free geraniol production was boosted 76-fold (Fig. 3a), suggesting that GDP produced in the mitochondria can be readily transported to the plastids. The increase in geraniol production in the plastids by *M-PaGDPS1* coexpression did not result in a significant increase in geraniol-derived

Table 1 Liquid chromatography-quadrupole time of flight (LC-QTOF)-MS analysis of geraniol(-derived) glycosides in *Nicotiana benthamiana* leaves transiently expressing *PaGDPS* and *VoGES*

	Retention time (min)	Putative identity	Mass
Oxidized geraniol glycosides	13.89	Dihexosyl carboxygeranic acid	521.1986
	23.22	Malonyl dihexosyl geranic acid (dimer)	1155.4382
	27.85	Malonyl-hexosyl geranic acid (dimer)	831.3294
	14.77	Hexosyl carboxygeranic acid	359.1049
	19.10	Hesoxyl hydroxy geranic acid formic acid	391.1982
	24.83	Dihexosyl carboxygeranic acid	521.2817
	17.09	Dihexosyl dihydro-8-carboxygeraniol	499.2000
	19.94	Malonyl dihexosyl pentosyl hydroxy geranic acid	725.2865
	15.53	Hexosyl carboxygeranic acid	359.1352
	23.01	Dihexosyl carboxygeranic acid	521.2249
	15.21	Hesoxyl hydroxy geranic acid formic acid	391.1602
	10.46	Hexosyl tetrahydro-carboxygeranic acid formic acid	409.1720
	20.97	Dihexosyl geranic acid	491.2139
	17.91	Malonyl-hexosyl hydroxy geraniol (dimer)	835.3620
	17.98	Trihexosyl geranic acid formic acid	699.2722
	18.18	Malonyl-hexosyl hydroxy geraniol	417.1781
	22.37	Pentosyl-hexosyl geranic acid formic acid	507.2076
	21.48	Dihexosyl geranic acid formic cid	537.2191
	27.30	Malonyl-hexosyl geranic acid (dimer)	831.3295
Geraniol glycosides	15.77	Hexosyl geraniol formic acid	377.1834
	18.78	Trihexosyl geraniol formic acid	685.2924
	21.36	Dihexosyl geraniol	477.2345
	20.06	Pentosyl dihexosyl geraniol formic acid	655.2819
	21.65	Dihexosyl geraniol	477.2344
	23.54	Acetyl dihexosyl geraniol	519.2450
	22.61	Pentosyl-hexosyl geraniol formic acid	493.2294
	26.89	Malonyl-hexosyl geraniol (dimer)	803.3709

glycosides, but glycosides of oxidized geraniol derivatives increased four-fold compared with expression of *P-VoGES* alone (Fig. 3b). Finally, when *P-VoGES* expression was combined with cytosolic GDP production by *C-PaGDPS1* expression, the free geraniol levels in the headspace did not significantly increase, while geraniol(-derivative) glycosides even decreased compared with leaves expressing *P-VoGES* alone (Fig. 3b). The decrease in geraniol(-derivative) glycosides suggests that C-PaGDPS1 activity in the cytosol lowers the IDP levels in the cytosol to such an extent that transfer of IDP from the plastids to the cytosol occurs, which then reduces the geraniol production capacity in the plastids.

GDP flux in and to the cytosol: geraniol biosynthesis in the cytosol in combination with GDP biosynthesis in different compartments

In the cytosol the mevalonate (MVA) pathway supposedly provides the bulk of the IDP and DMADP. Native conversion of cytosolic IDP and DMADP to GDP may be limiting geraniol production in the cytosol, as it was shown that there is just a small pool of cytosolic GDP (Wu *et al.*, 2006). Indeed, when *C-VoGES* was coexpressed with *C-PaGDPS1*, free geraniol levels increased 30-fold compared with *C-VoGES* alone (Fig. 4a). In addition, increased GDP production in the cytosol through expression of *C-PaGDPS1*, boosted the level of oxidized geraniol glycosides (Fig. 4b). When *C-VoGES* was combined with increased GDP production in the mitochondria by coexpressing *M-PaGDPS1*, geraniol levels in the headspace increased 12-fold. Apparently some GDP can be transferred from the mitochondria to the cytosol. By contrast, boosting GDP production in the plastid did not result in a significant increase in free geraniol and geraniol-derived glycosides produced by the cytosolic geraniol synthase (Fig. 4a,b), suggesting a minor transfer of GDP from the plastids to the cytosol. We note that in tomato fruit, cytosolic monoterpene biosynthesis can be supported by plastid-generated GDP (Gutensohn *et al.*, 2013). This difference in flux of GDP from plastid to cytosol may be due to the different cellular context (*N. benthamiana* leaf vs tomato fruit).

GDP flux in and to the mitochondria: geraniol biosynthesis in the mitochondria in combination with GDP biosynthesis in different compartments

When *M-VoGES* was coexpressed with *M-PaGDPS1*, geraniol production was increased 70-fold (Fig. 5a), while geraniol-derived glycosides increased 3.3-fold and oxidized geraniol glycosides 82-fold (Fig. 5b). This shows that conversion of IDP and DMADP to GDP in the mitochondria is limiting geraniol production. When *M-VoGES* expression was combined with *C-PaGDPS1* expression, free geraniol increased more than by coexpression with *M-PaGDPS1* (Fig. 5a), while geraniol-derived glycosides also increased compared with *M-VoGES* expression alone. This indicates that an efficient exchange of GDP from the

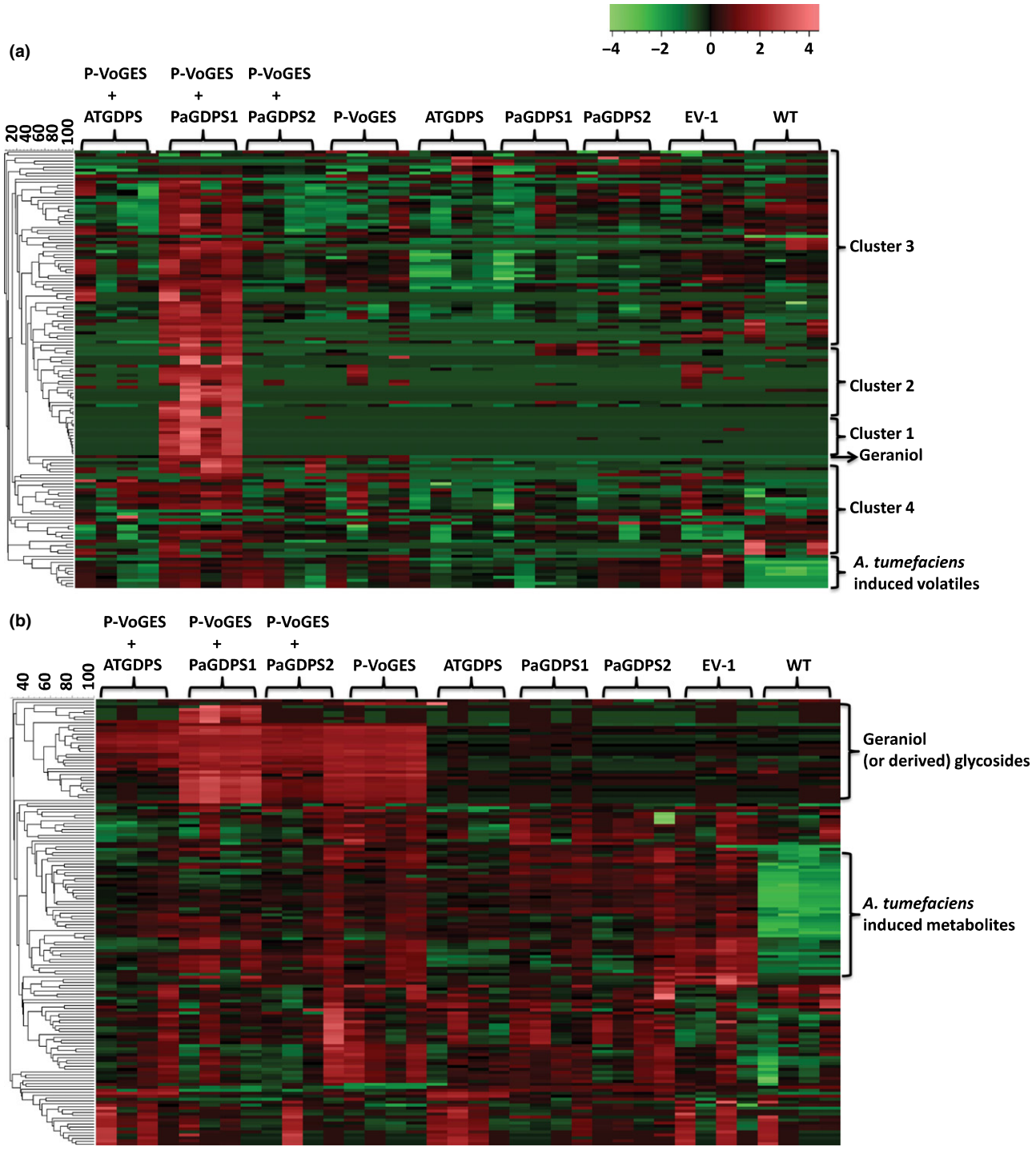

Fig. 2 Hierarchical cluster analysis of the metabolites in *Nicotiana benthamiana* leaves transiently expressing different geranyl diphosphate synthases, *AtGDPS*, *PaGDPS1* or *PaGDPS2* with and without plastid-targeted *Valeriana officinalis* geraniol synthase (P-*VoGES*). Metabolite profiles of leaves infiltrated with empty vector and noninfiltrated leaves are shown as controls. (a) Volatiles analysed by headspace GC-MS. All geraniol-derived volatiles, except geraniol itself, were only present in the leaves transiently expressing the combination of *PaGDPS1* and *P-VoGES*. Cluster 1, monoterpene and geraniol derivatives; cluster 2, compounds induced specifically by *PaGDPS1* + *P-VoGES* but not monoterpene; cluster 3 and 4, compounds did not show significantly different between *PaGDPS1* + *P-VoGES* either with empty vector or wild-type control. (b) Nonvolatile metabolites as analysed by LC-MS. Colour code indicates the quantitative change in mass intensity of all compounds.

cytosol to the mitochondria occurs. When GDP production was boosted in the plastids, geraniol production in the mitochondria also increased, although not as much as by boosting GDP production in cytosol or mitochondria (Fig. 5a,b). This finding indicates that a limited transport of GDP from plastids to mitochondria occurs. There seems to be no direct correlation between the free geraniol emission level and the level of the geraniol(-derived) glycosides. For instance, coexpression of *C-PaGDPS1* and *M-VoGES*

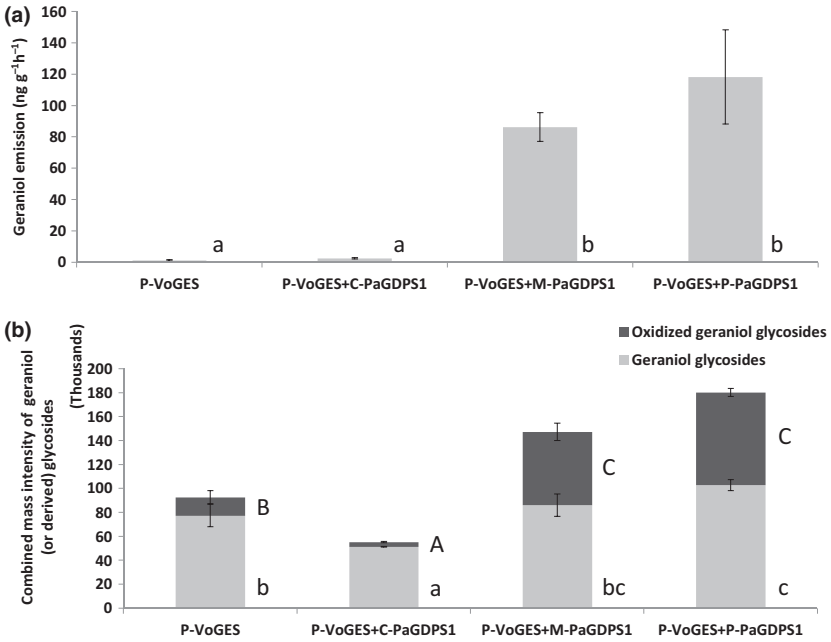

Fig. 3 Geraniol production by *Nicotiana benthamiana* leaves transiently expressing cytosolic (C), mitochondrial (M) or plastidic (P) *PaGDPS1* in combination with plastidic *VoGES*. (a) GC-MS headspace analysis of geraniol emission. (b) Relative quantitative analysis of geraniol (grey bars) and oxidized geraniol (black bars) glycosides by LC-MS. Bars represent the total mass intensity of all geraniol and (or oxidized geraniol) glycosides, see Table 1. Bars with different letters (capital for oxidized geraniol glycosides, lower case for free geraniol and geraniol glycosides) differ significantly ($P < 0.05$). Error bars ± SE ($n = 4$).

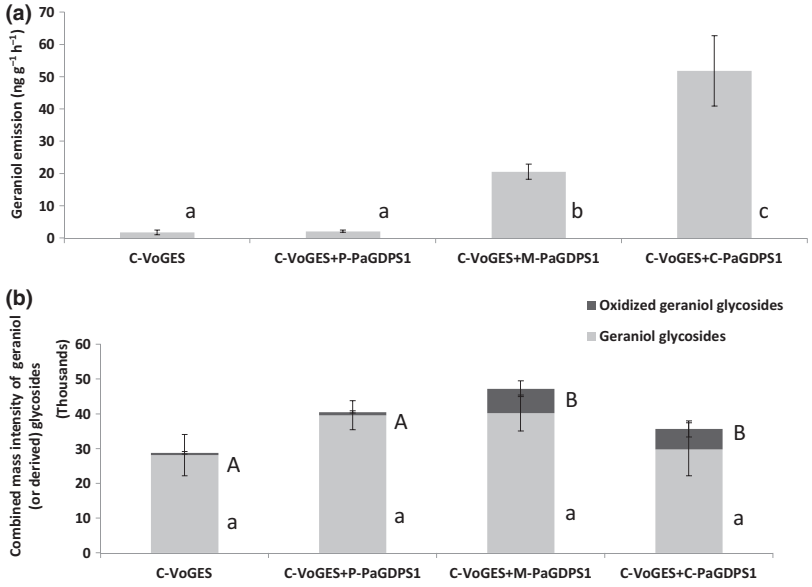

Fig. 4 Geraniol production by *Nicotiana benthamiana* leaves agroinfiltrated with cytosolic (C), mitochondrial (M) or plastidic (P) *PaGDPS1* in combination with cytosolic *VoGES*. (a) GC-MS headspace analysis of geraniol emission. (b) Relative quantitative analysis of geraniol (grey bars) and oxidized geraniol (black bars) glycosides by LC-MS. Bars represent the total mass intensity of all geraniol and (or oxidized geraniol) glycosides, see Table 1. Bars with different letters (capital for oxidized geraniol glycosides, lower case for free geraniol and geraniol glycosides) differ significantly ($P < 0.05$). Error bars ± SE ($n = 4$).

resulted in the highest level of emitted geraniol, while the highest geraniol(-derivative) glycoside level occurred in leaves expressing the combination of *M-PaGDPS1* and *M-VoGES* (Fig. 5).

Discussion

Monoterpene production capacity in *N. benthamiana*

Many of the primary monoterpene products produced by plants are emitted as volatiles (Janson, 1993; Nogués & Loreto, 2013). However, when ectopically produced in the heterologous host *N. benthamiana* the volatile geraniol is mostly converted into nonvolatile geraniol glycosides. In addition, geraniol is further oxidized to geranic acid, hydroxyl geraniol, hydroxyl geranic acid and carboxygeranic acid, which are glycosylated as well. Oxidation and glycosylation of geraniol in a heterologous host is likely a response to the phytotoxic geraniol and provides the means to keep free geraniol levels low. We assume that glycosylated geraniol products are sequestered into the vacuole and that therefore oxidation takes place on free geraniol and not on geraniol glycosides. After oxidation, the geraniol derivatives are then also further inactivated by glycosylation and stored in the vacuole. Judging from the high production of geraniol glycosides, this seems to be the preferred detoxification reaction.

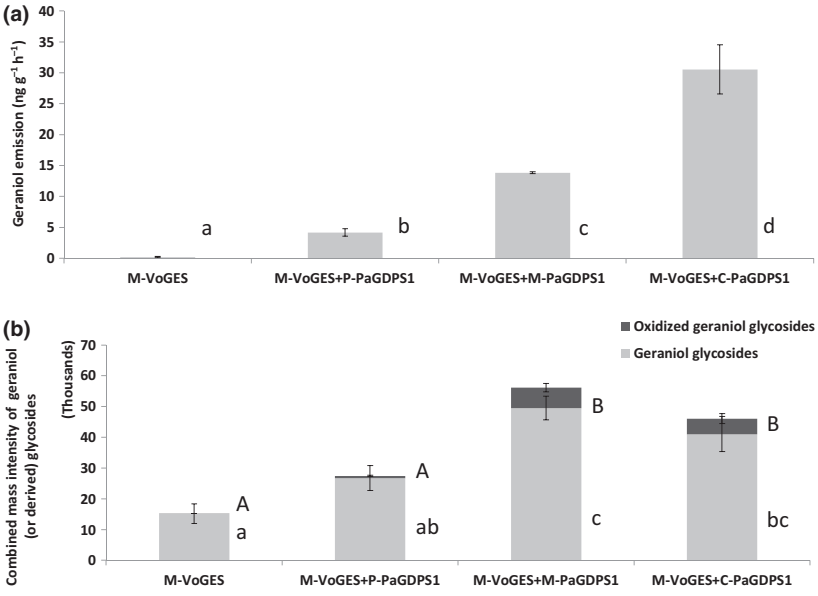

Fig. 5 Geraniol production by *Nicotiana benthamiana* leaves agroinfiltrated with cytosolic (C), mitochondrial (M) or plastidic (P) *PaGDPS1* in combination with mitochondrial *VoGES*. (a) GC-MS headspace analysis of geraniol emission. (b) Relative quantitative analysis of geraniol (grey bars) and oxidized geraniol (black bars) glycosides by LC-MS. Bars represent the total mass intensity of all geraniol and (or oxidized geraniol) glycosides, see Table 1. Bars with different letters (capital for oxidized geraniol glycosides, lower case for free geraniol and geraniol glycosides) differ significantly ($P < 0.05$). Error bars ± SE ($n = 4$).

Only *PaGDPS1* can boost geraniol production in *N. benthamiana*

In this study, a *GDPS* from Arabidopsis and *GDPS1* and *GDPS2* from *P. abies* were used. All three were shown to have some form of GDP synthase activity in *in vitro* assays (Bouvier *et al.*, 2000; Wu *et al.*, 2006; Schmidt & Gershenzon, 2008; Schmidt *et al.*, 2010). Hsieh *et al.* (2011), however, showed *AtGDPS1* to be a *trans*-type polyprenyl pyrophosphate synthase in *in vitro* biochemical and genetic complementation assays, rather than a GDPS. This finding likely explains why we did not find AtGDPS boost monoterpene production in our *N. benthamiana* transient expression assay.

PaGDPS1 was able to boost GES activity in the *N. benthamiana* transient expression assay and this gene was used in all our subsequent experiments. Under such boosted conditions, the capacity of geraniol glycosylation seems to be limiting and geraniol gets first mostly oxidized, resulting in the accumulation of oxidized geraniol glycosides (Figs 3–5). *Nicotiana benthamiana* leaves produce low levels of monoterpenes from endogenous monoterpene synthases. Plastid-targeted *PaGDPS1* alone or plastid-targeted *VoGES* alone had little effect on endogenous monoterpene levels (Figs S3a,b). By contrast, coexpression of plastid-targeted *PaGDPS1* with plastid-targeted *VoGES* resulted in a substantial increase (up to 28-fold) of multiple monoterpenes from endogenous enzyme activity (e.g. myrcene, terpinene, limonene and ocimene) (Fig. 2 (cluster 1), S3c; note log scale). In addition, 12 more geraniol-related compounds (e.g. geranic acid, citronellol, and epoxynerol) were detected in headspace analysis after coexpression with *PaGDPS1* (Fig. 2 (cluster 1), S3c). At present we cannot fully explain the lack of boosting activity by PaGDPS1 alone on endogenous monoterpene biosynthesis activity. However, the results could be an indication of endogenous monoterpene biosynthesis being organized in metabolons, so that enzymes and substrates outside the metabolon are of little influence (Jørgensen *et al.*, 2005). Results would then suggest that such metabolons are disrupted only upon coexpression of *PaGDPS* + *P-VoGES*. A third group of compounds, which are also induced specifically by *PaGDPS1* + *P-VoGES* (Figs 2 (cluster 2), S3c) are not monoterpene related but consist of alkaloids and other secondary metabolites. Induction of these compounds could signify a secondary response to the products from PaGDPS1 + P-VoGES activity.

Estimating production capacity of different cell compartments

To estimate the amount of geraniol(-derived) glycosylated products, leaf extracts were treated with a glycosidase mix and the captured released volatiles were quantified by GC-MS (Table S2). From this we estimated that *N. benthamiana*, transiently expressing native *VoGES*, produced *c.* 45 $\mu g\, g^{-1}$ fresh weight (FW) of geraniol-derived products during the 5 days post-agroinfiltration (Table S2), while VoGES with artificial plastid targeting produced *c.* 54 $\mu g\, g^{-1}$ FW of geraniol-derived products (Table S2). Boosting geraniol production with the plastid-targeted GDPS increased geraniol-derived product level to *c.* 129 $\mu g\, g^{-1}$ FW (Table S2). We note that the estimation of the highest geraniol production by *N. benthamina* leaves (*c.* 129 $\mu g\, g^{-1}$ FW) is an underestimation because not all of the geraniol-derived glycosides can be hydrolysed by the glycosidase (Viscozyme) treatment (e.g. glycosides capped with a malonyl group) and removal of the sugar groups from carboxygeranic acid-, tetrahydro-carboxygeranic acid-, hydroxyl geraniol-, hydroxyl geranic acid- and dihydro-carboxygeraniol-glycosides (all of which were found in LC-MS analysis of the leaves coexpressing *P-VoGES* and *P-PaGDPS1*) would result in free compounds which we do not detect by GC-MS. These geraniol-derived products are thus not included in the quantification. As these compounds form only a very small fraction of the total production (Fig. S5), the contribution of these products to geraniol production estimation was ignored.

When GDP production in the plastids is boosted, some geraniol is lost to the headspace (Fig. 2). However, compared with the amount of stored geraniol-derived products (*c.* 129 $\mu g\, g^{-1}$ FW after 5 days), the loss of volatile geraniol to the headspace is very limited (0.12–14.16 μg over 5 days based on geraniol emission varying between 0.001 and 0.12 $\mu g\, g^{-1}$ FW h^{-1} and assuming continuous emission for 5 days). The (boosted) production in the transient expression assay (*c.* 129 $\mu g\, g^{-1}$ FW) is higher than the geraniol-related product level in leaves of *Nicotiana tabacum* stably expressing *VoGES* (*c.* 36 $\mu g\, g^{-1}$ FW) (Dong *et al.*, 2013) and higher than in the leaves of *Arabidopsis thaliana* stably expressing an *Ocimum basilicum* geraniol synthase (*ObGES*) (19 $\mu g\, g^{-1}$) (Fischer *et al.*, 2013). The boosted geraniol production level in the *N. benthamiana* transient assay, however, is comparable to that obtained in *A. thaliana* stably transformed with a linalool synthase targeted to the plastids (*c.* 110 $\mu g\, g^{-1}$ FW linalool-derived products) (Aharoni *et al.*, 2003) and comparable with the results from stable transformation of *N. benthamiana with ObGES* (*c.* 83 $\mu g\, g^{-1}$ FW) (Fischer *et al.*, 2013). We note that there was no relationship between the amount of emitted free geraniol and the level of conjugated geraniol across the various combinations of differently targeted *VoGES* and *PaGDPS1* constructs. This could be an indication that the partitioning of geraniol between emission into the headspace and oxidation and/or conjugation may be different for plastidial, mitochondrial and cytosolically produced geraniol.

Highest monoterpene biosynthesis potential in the plastids

Here we show that in each subcellular compartment the level of monoterpene production (geraniol and geraniol-derived glycosides) can be boosted by PaGDPS1 activity (Fig. 6). This suggests that in each compartment the available GDP is limiting for GES activity. Upon overexpression of *PaGDPS1*, GDP production in each compartment may be limited by available IDP/DMADP (the precursor for GDP) (Fig. S1). In the plastids, IDP is synthesized through the MEP pathway (Rodríguez-Concepción & Boronat, 2002; Bick & Lange, 2003), while in the cytosol IDP is synthesized through the MVA pathway (Rodríguez-Concepción & Boronat, 2002; Bick & Lange, 2003). Some research also suggests that IDP in the cytosol may be (partially) provided by the MEP pathway and that trafficking of IDP occurs unidirectionally from plastids to cytosol (Dudareva *et al.*, 2005). For the mitochondria no IDP biosynthesis pathway has been described, however the mitochondrial localization of IDP isomerases (Phillips *et al.*, 2008; Guirimand *et al.*, 2012), FDPS (Martín *et al.*, 2007; Vranová *et al.*, 2013) and a GGDPS (Okada *et al.*, 2000) suggest that IDP is available in mitochondria to support terpene biosynthesis and it has been suggested that IDP is imported from the cytosol (Lütke-Brinkhaus *et al.*, 1984; Disch *et al.*, 1998). Overall both unboosted and boosted monoterpene production was highest in the plastids, followed by the mitochondria while lowest production occurred in the cytosol. As discussed earlier, it is assumed that isoprenoid production in the mitochondria depends on substrate (IDP) import from other compartments. However, the lower boosted production capacity of the cytosol than that of boosted production in the mitochondria intriguingly suggests that the capacity to boost production in the mitochondria is mostly derived from substrate (IDP) imported from the plastids rather than from the cytosol.

As the emission of geraniol is only a very small part (0.1–10% over 5 days) of the total geraniol production, the combined relative peak intensities of all geraniol(-derivative) glycosides are a good proxy for the available GDP in each compartment. The total capacity (including local boosting by GDPS) of the plastids is 182 units (104 relative units for glycosides of geraniol and 78 for glycosides of geraniol derivatives; see Figs 3–5, and summarized in Fig. 6). Measured in a similar way the overall capacity of the mitochondria is 56 units and of the cytosol 36 units (Figs 3–5, and summarized in Fig. 6). The increase in the geraniol glycosides upon coexpression of plastid-targeted *GDPS1* is *c.* 90 units, while this only results in a small increase in cytosolic or mitochondrial geraniol glycosides (both 12 units), suggesting that only a small fraction of the GDP produced in the plastids is available in the cytosol or the mitochondria. By contrast, when GDPS is targeted to the mitochondria, the production of geraniol-related glycosides by mitochondrial targeted GES is boosted by 41 units and almost all of the GDP produced in the mitochondria seems to be available for geraniol production in the plastids, suggesting a very efficient transfer of GDP from mitochondria to plastids. By contrast, when GDP production is boosted in the mitochondria, only 18 units of the potential 56 units in the mitochondria are available for geraniol production in the cytosol, suggesting that transfer of GDP from mitochondria to the cytosol is far less efficient than transfer to the plastids. When GDP production is boosted in the cytosol, almost all of the 36 units of GDP, namely 31 units, are available for geraniol production in the mitochondria, suggesting a very efficient transfer of GDP from the cytosol to the mitochondria. Finally, when GDP production is boosted in the cytosol, the geraniol production in the plastids decreased by 45% compared with expression of plastid-targeted *P-VoGES* alone (Fig. 3b). This result suggests that there is a drain of IDP from the plastids when high amounts of IDP are used in the cytosol, indicating a direct competition and preferential allocation of IDP to the cytosol.

GDP exchange between mitochondria and plastids

These results also suggest that when *GDPS* is expressed in the mitochondria, almost all produced GDP is available for geraniol production in the plastids and cytosol (Fig. 3). Because there is no exchange of GDP from the cytosol to the plastids (Fig. 6), this situation suggests that the boosting of plastidial geraniol production by GDP produced in the mitochondria is the consequence of direct exchange of GDP from mitochondria to plastids. It has been shown that plastids and mitochondria may closely associate with each other as some metabolites from the photorespiration pathway in the plastids require recycling in the mitochondria (e.g. glycolate is recycled to glycine in the mitochondria). Also ammonium, which is produced during photorespiration in the

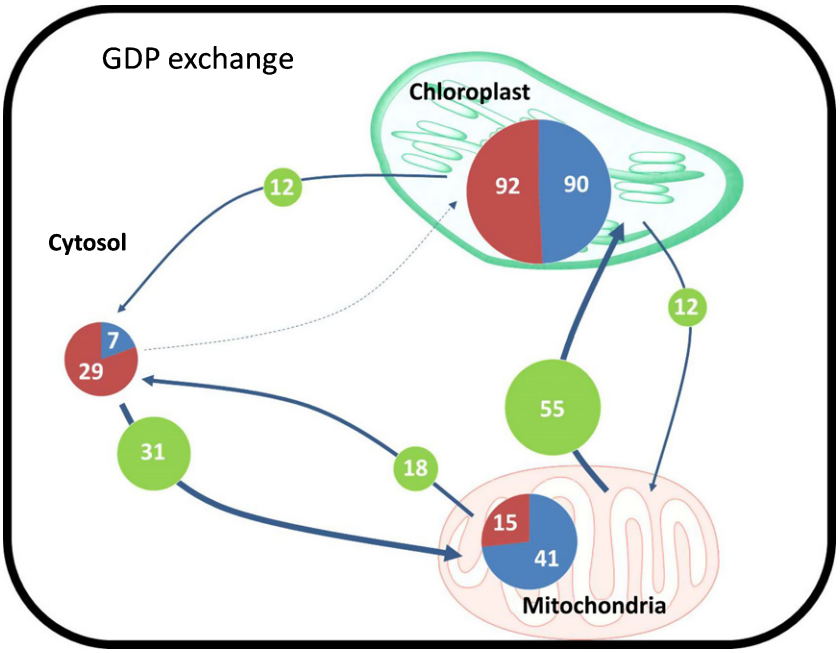

Fig. 6 Schematic representation of geranyl diphosphate (GDP) exchange between different subcellular compartments. The units of GDP in each compartment are based on quantification of the geraniol plus oxidized geraniol glycosides (relative units). The red section of the circles represents the endogenous local GDP units for each compartment without local boosting of GDP biosynthesis, the blue sector the additional GDP units produced by targeted *PaGDPS1*. Green circles show the relative units of GDP that are exchanged between compartments. Note that for transport of GDP of plastids to cytosol, plastids to mitochondria and mitochondria to cytosol actual estimated transport is just a small fraction of the potentially units of GDP available in these compartments. For the transport from mitochondria to plastids and cytosol to mitochondria almost all available GDP units are used, while there is no transport of GDP from the cytosol to the plastids.

mitochondria, must be reassimilated by the glutamine synthase–glutamate synthase cycle in the plastids, necessitating efficient transport between the two organelles (Hodges *et al.*, 2013).

It has been shown that plastids may display structures called stromules, dynamic tubular membranes that have been shown to interact with the membrane of the endoplasmic reticulum (ER) (Schattat *et al.*, 2011), nuclear envelope (Deslandes & Rivas, 2011) and with themselves (Gray, 2013). However, stromules may also interact with mitochondria (Gray, 2013; Hanson & Sattarzadeh, 2013). Gunning (2005) reported that also mitochondria can sometimes form transient tubular extensions and that these mitochondrial equivalents of the stromules interact with the streaming cytoplasm to develop a stromule-like protrusion. Presumably, at the contact side of stromules and mitochondrial tubular extensions, small molecules could be exchanged between these organelles, possibly including GDP.

Membrane contact sites (MCS) between different organelles of the cell have been described in the literature. These contact sites facilitate both signalling and the passage of molecules from one cellular compartment to another. Although discovered many decades ago, still very little information is known of the molecular machinery required to create MCS or their structure, function and regulation. The best characterized contact sites to date are the nucleus–vacuole junction, and the mitochondria–ER, plasma membrane–ER and plastid–ER contact sites (Elbaz & Schuldiner, 2011). In yeast, Kornmann & Walter (2010) identified a protein complex that is located at the interface of the ER and mitochondria, and serves to zip them together. The tethering complex is composed of proteins resident of both ER and mitochondria and is functionally connected to phospholipid biosynthesis and calcium-signalling genes. Such protein complexes have not been described for plants yet. Plastid–ER contact sites, however, have been described and are thought to be involved in the transfer of lipids between these organelles, especially under phosphate limitation when phospholipids of the plasma membrane are exchanged for plastid-derived galactolipids (Xu *et al.*, 2010). Because it has been shown that there is contact between the ER and both plastids and mitochondria, it could be that exchange of molecules between plastid and mitochondria occurs via the ER.

In conclusion, our results show that plastids have the highest potential for monoterpene production and that the monoterpene precursor GDP can be exchanged between different but not all subcellular compartments. When the diverse array of side reactions acting on the heterologous product – as occurred for geraniol – can be controlled in heterologous plant expression systems, the yield potential of ectopic expression in heterologous plant host for valuable terpene products can be substantial and may surpass production capacity of the original host from which the biosynthetic genes were isolated.

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Author contributions

L.D., H.B. and A.K. planned and designed the research. L.D. and E.J. performed experiments, analysed data and wrote the manuscript. H.B. and A.K. commented on the manuscript.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Schematic representation of terpenoid pathways and their subcellular localization and the metabolic engineering approaches in the different subcellular compartments applied in the present study.

Fig. S2 Visualization of subcellular compartments of *Nicotiana benthamiana* cells using transient expression of different targeting signal fused with epi-fluorescence GDP (eGDP) and empty vector without GDP.

Fig. S3 Upregulated volatiles in *Nicotiana benthamiana* leaves by coexpression of *PaGDPS1* and *P-VoGES* compared with expression of *P-VoGES*, *PaGDPS1* and empty vector (*EV*) alone.

Fig. S4 Nonvolatiles upregulated by the expression of *PaGDPS1* and *P-VoGES* in *Nicotiana benthamiana* leaves

Fig. S5 Geraniol(-derived) compound production in *Nicotiana benthamiana* leaves transiently expressing plastidic *PaGDPS1* in combination with plastidic *VoGES* analyzed by GC-MS after hydrolysis using a glycosidase mixture.

Table S1 Targeting signal and localization

Table S2 Estimation of geraniol production

Table S3 Primer sequences for PCR amplification and restriction site for impact vector

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