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Research

Co-expression of peppermint geranyl diphosphate synthase small subunit enhances monoterpene production in transgenic tobacco plants

Jun-Lin Yin[1], Woon-Seng Wong[1], In-Cheol Jang[1] and Nam-Hai Chua[2]

[1]Temasek Life Sciences Laboratory, 1 Research Link, National University of Singapore, Singapore 117604, Singapore; [2]Laboratory of Plant Molecular Biology, The Rockefeller University, 1230 York Avenue, New York, NY 10065, USA

Authors for correspondence:
In-Cheol Jang
Tel: +65 6872 7409
Email: jangi@tll.org.sg

Nam-Hai Chua
Tel: +1 212 327 8000
Email: chua@mail.rockefeller.edu

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Summary

- Monoterpenes are important for plant survival and useful to humans. In addition to their function in plant defense, monoterpenes are also used as flavors, fragrances and medicines. Several metabolic engineering strategies have been explored to produce monoterpene in tobacco but only trace amounts of monoterpenes have been detected.
- We investigated the effects of *Solanum lycopersicum 1-deoxy-D-xylulose-5-phosphate synthase* (*SlDXS*), *Arabidopsis thaliana geranyl diphosphate synthase 1* (*AtGPS*) and *Mentha × piperita geranyl diphosphate synthase small subunit* (*MpGPS.SSU*) on production of monoterpene and geranylgeranyl diphosphate (GGPP) diversities, and plant morphology by transient expression in *Nicotiana benthamiana* and overexpression in transgenic *Nicotiana tabacum*.
- We showed that *MpGPS.SSU* could enhance the production of various monoterpenes such as (−)-limonene, (−)-linalool, (−)-α-pinene/β-pinene or myrcene, in transgenic tobacco by elevating geranyl diphosphate synthase (GPS) activity. In addition, overexpression of *MpGPS.SSU* in tobacco caused early flowering phenotype and increased shoot branching by elevating contents of GA_3 and cytokinins due to upregulated transcript levels of several plastidic 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway genes, *geranylgeranyl diphosphate synthases 3* (*GGPPS3*) and *GGPPS4*.
- Our method would allow the identification of new monoterpene synthase genes using transient expression in *N. benthamiana* and the improvement of monoterpene production in transgenic tobacco plants.

Introduction

A large number of plant secondary metabolites are released from leaves, flowers and fruits of plant as volatiles. Monoterpenes (C_{10}), such as limonene, linalool, myrcene and pinene, are major constituents of plant scent. Emitted monoterpenes play important roles in plant/insect relationship as they can either directly repel or deter feeding of herbivorous insects, or they may attract natural predators and parasitoids of herbivores (Lange & Ahkami, 2013). Therefore, modulation of monoterpene volatile profile by metabolic engineering may provide a strategy for pest management (Dudareva & Pichersky, 2008). In addition to playing an important role in plant defense (Degenhardt *et al.*, 2003), monoterpenes also are used as major ingredients in flavors and fragrances (Aharoni *et al.*, 2004) and as precursors of medicinal compounds (Rodriguez-Concepcion, 2004).

Two separate biosynthetic pathways, the cytosolic mevalonic acid (MVA) and the plastidic 2-C-methyl-D-erythritol-4-phosphate (MEP) pathways, are involved in plant terpene biosynthesis (Mahmoud & Croteau, 2002). Most monoterpenes are produced from geranyl diphosphate (GPP), which is synthesized from pyruvate and D-glyceraldehyde-3-phosphate (G3P) via the action of 1-deoxy-D-xylulose-5-phosphate synthase (DXS), 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), hydroxymethylbutenyl diphosphate reductase (HDR) and several other enzymes in the plastidic MEP pathway (Mahmoud & Croteau, 2002). The GPP can be further converted to geranylgeranyl diphosphate (GGPP) by geranylgeranyl diphosphate synthases (GGPPS), which serves as the precursor of carotenoids, GA_3, diterpenes, chlorophylls (Chl), tocopherols and strigolactones (Cordoba *et al.*, 2009).

Because tobacco (*Nicotiana tabacum*) has been used as an agricultural crop to produce biomass due to its low production cost, several attempts have been made to manipulate terpene production in transgenic tobacco plants. However, overexpression of monoterpene genes led to only a small increase in terpene concentrations (Lange & Ahkami, 2013). For instance, transgenic tobacco plants expressing three monoterpene synthase

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(monoTPS) genes, which encode γ-terpinene synthase, (+)-limonene synthase and β-pinene synthase from lemon (*Citrus limon*), under the control of a CaMV 35S promoter, emitted only trace amount of monoterpenes from its leaves and flowers (Lücker *et al.*, 2004).

Several strategies have been used to overcome low terpene production amounts in transgenic plants (Ohara *et al.*, 2003; Wu *et al.*, 2006). Differential localization of limonene synthase (LimS) in the cytosol, plastids or endoplasmic reticulum (ER) of tobacco cells was examined to identify the most optimal subcellular location needed for efficient metabolic engineering of monoterpene biosynthesis (Ohara *et al.*, 2003). Targeting of LimS to plastids produced tobacco plants with a three-fold increase of limonene production compared to plants with cytosol-localized LimS, whereas no enzyme activity was detected for ER-localized limonene synthase (Ohara *et al.*, 2003). However, the low limonene concentration caused by cytosolic expression of *LimS* could be overcome by co-expression of a cytosolic form of geranyl diphosphate synthase (GPS) and plants co-expressing both enzymes produced six-fold higher limonene concentrations compared to plants with plastid-targeted LimS alone (Wu *et al.*, 2006). Moreover, the limonene concentration was increased up to 10-fold when both LimS and GPS proteins were targeted to plastids of tobacco cells (Wu *et al.*, 2006). These results suggested that co-expression of MEP pathway genes with monoTPSs in plastids could provide an attractive alternative to the classical method of single gene expression.

GPP is formed from IPP and DMAPP by a reaction catalyzed by homodimeric GPSs or heterodimeric GPSs. Homodimeric GPSs are present in both angiosperms and gymnosperms (Bouvier *et al.*, 2000; van Schie *et al.*, 2007; Schmidt & Gershenzon, 2008), whereas heterodimeric GPSs have been found so far only in angiosperms (Burke *et al.*, 1999; Tholl *et al.*, 2004). Both subunits of homodimeric GPSs and the large subunit of heterodimeric GPSs (GPS.LSU) contain a conserved prenyl substrate binding domain, $DD(X)_{2\text{-}4}D$ (Wang & Dixon, 2009; Tholl & Lee, 2011). However, this motif is absent from the small subunit of heterodimeric GPSs (GPS.SSU) that is inactive by itself (Wang & Dixon, 2009; Tholl & Lee, 2011). The GPS.SSU has a variety of functions. For example, both GPS.LSU and GPS.SSU from *Mentha* × *piperita* are inactive by themselves, but co-expression of *M. piperita* GPS.LSU (MpGPS.LSU) with *M. piperita* GPS.SSU (MpGPS.SSU) in *Escherichia coli* is able to produce GPP (Burke *et al.*, 1999); the *Antirrhinum majus* (Snapdragon) GPS.LSU (AmGPS.LSU) is able to convert DMAPP and IPP to GGPP *in vitro*. However, the *A. majus* GPS.SSU (AmGPS.SSU) can modify the chain length specificity of AmGPS.LSU to produce GPP (Tholl *et al.*, 2004); in another case, *Humulus lupulus* GPS.LSU (HlGPS.LSU) produces both GPP and GGPP *in vitro* and the rate of formation of both products can be accelerated by the *H. lupulus* GPS.SSU (HlGPS.SSU) (Wang & Dixon, 2009). Snapdragon AmGPS.SSU was used to modulate monoterpene biosynthesis of tobacco through modifying the chain length specificity of GGPPS; however, along with the blocking of GGPP, transgenic tobacco plants expressing AmGPS.SSU showed leaf chlorosis, increased light sensitivity and dwarfism due to the decreased amount of Chl, carotenoids and GA_3 (Orlova *et al.*, 2009).

In order to optimize monoterpene production in transgenic tobacco plants, we investigated the effects of overexpression of *Solanum lycopersicum 1-deoxy-D-xylulose-5-phosphate synthase* (*SlDXS*), the *Arabidopsis thaliana geranyl diphosphate synthase 1* (*AtGPS*) or *MpGPS.SSU* on monoterpene concentrations and GGPP diversities by transient expression in *Nicotiana benthamiana* and stable transgene expression in *N. tabacum*. Here, we showed that *MpGPS.SSU* from peppermint was able to enhance the production of various monoterpenes in transgenic tobacco plants and promoted plant growth, flowering time and shoot branching owing to increased accumulation of GA_3 and cytokinins. Moreover, the transcript levels of several plastidic MEP pathway genes were upregulated in *MpGPS.SSU*-overexpressing tobacco plants. Our findings provide new technical advances for sustainable production of monoterpenes in tobacco plants.

Materials and Methods

Plant materials

Nicotiana benthamiana and *N. tabacum* SR1 plants were grown in a glasshouse under natural light conditions. *N. benthamiana* was used for *Agrobacterium*-mediated transient expression and *N. tabacum* for *Agrobacterium*-mediated transformation using a standard protocol (Schardl *et al.*, 1987).

Vector construction

Supporting Information Fig. S1 shows all vectors used in this study. A 634-bp DNA fragment corresponding to the *Ubiquitin 10* (*UBQ10*) promoter (GenBank: HQ693235.1) was amplified with UBQ-*Hin*dIII-F and UBQ-SpeI-R primers (Table S1) from *A. thaliana* genomic DNA. After digestion with *Hin*dIII and SpeI enzymes, the *UBQ10* promoter was inserted into pK7WG2D vector to generate pK7-UBQP. All cDNAs encoding *monoTPSs* (*PaLimS*, *Picea abies (−)-limonene synthase*; *PaLinS*, *P. abies (−)-Linalool synthase*; *PsPinS*, *P. sitchensis (−)-pinene synthase*; and *PaMyrS*, *P. abies myrcene synthase*) and cDNA for *SlDXS* (NM_001247743) were synthesized chemically by Genscript Ltd (Hong Kong, China). Full-length open reading frames (ORFs) of *Arabidopsis thaliana geranyl diphosphate synthase 1* (*AtGPS*) (NM_179903) and *Mentha* × *piperita geranyl diphosphate synthase small subunit* (*MpGPS.SSU*) (AF182827) were amplified by PCR from Arabidopsis and peppermint cDNAs. Primer sets listed in Table S1 were designed to amplify gene products for Gateway cloning (Invitrogen). Purified PCR products were cloned into pDONR221 (Invitrogen). The pDONR221 clone containing intermediate genes of the GPP biosynthetic pathway (*MpGPS.SSU*, *AtGPS* and *SlDXS*) or *monoTPSs* (*PaLimS*, *PaLinS*, *PsPinS* and *PaMyrS*) was integrated by LR clonase into the destination vector, pBA-DC or pK7-UBQP, which contained either a CaMV35S or a UBQ10 promoter (Invitrogen). Furthermore, full-length ORF of *MpGPS.SSU,* without a putative N-terminal plastidic transit peptide sequence, was cloned into pMBP-DC vector

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(Zhang *et al.*, 2005). The accession numbers of genes used in this study are listed in Table S2.

Agrobacterium-mediated transient expression of monoTPSs in *N. benthamiana*

Agrobacterium tumefaciens strain AGL1 containing different *monoTPS* constructs was inoculated in lysogeny broth media containing spectinomycin (50 mg l^{-1}) and rifampicin (50 mg l^{-1}), grown at 28°C with agitation (250 rpm) for 2 d and cultures were harvested by centrifugation at 4000 ***g*** for 10 min. The pelleted cells were resuspended in 20 ml infiltration medium (10 mM MES, pH 5.7, 10 mM $MgCl_2$, 100 μM acetosyringone) to a final OD_{600} of 1.0 and further incubated at room temperature for 2 h. *A. tumefaciens* AGL1 culture was mixed with an equal volume of *A. tumefaciens* AGL1 containing a plant expression vector for the viral-encoded protein P19 that suppresses transgenic silencing.

For co-infiltration assays, the underside of 3-wk-old *N. benthamiana* leaves were infiltrated with *A. tumefaciens* culture using a 1 ml needleless syringe. Infiltrated *N. benthamiana* plants were maintained in a growth chamber at 25°C under long day conditions (16 h : 8 h, light : dark) and GC-MS analysis was performed 3 d post-infiltration (dpi).

Headspace GC method

A push–pull headspace collection system was used to collect volatile compounds from 10 g of transgenic tobacco leaves in a growth chamber at 25°C under long day conditions (16 h : 8 h, light : dark) (Tholl *et al.*, 2006). Volatile compounds trapped by Porapak Q trap (80/100 mesh size; Sigma) for 24 h were eluted twice with 200 μl of dichloromethane. One microliter (100 mg l^{-1}) of camphor was added to serve as an internal standard. The eluted samples were analyzed by Agilent GC 7890A with 5975C inert mass selective detector, equipped with a HP-5MS column (30 m × 0.25 mm, 0.25 μm film thickness; Agilent Technologies, Santa Clara, CA, USA) according to the following program: injection of samples (2 μl) into the column at 250°C and a temperature gradient of 8°C min^{-1} from 50°C (1 min hold) to 300°C (5 min hold). Compounds were identified by comparison of their mass spectra with those in the NIST MS 2014 library.

Phytohormone treatment

Twenty microliters of GA_3 (500 μM) or 6-Benzylaminopurine (BA) (500 μM) in water containing 0.05% Tween-20 (v/v) was dropped directly onto leaf axillary buds with a micropipette. After 7 d of treatment, the length of shoot branch was measured (Ni *et al.*, 2015).

Chl, carotenoids, GA_3, *trans*-zeatin (tZ) and N^6-(Δ^2-isopentenyl) adenine (iP) content

Ten grams of young leaves in liquid nitrogen were dried using a freeze dryer. Five milligrams of the homogenized freeze-dried leaves were added to the tube containing 8.0 ml of 96% ethanol and the mixture was vortexed, wrapped with aluminum foil and incubated at room temperature overnight. After centrifugation, the absorbance of the extract was measured at 470.0 nm, 648.6 nm and 664.2 nm, and then Chl*a*, Chl*b* and total carotenoid contents were calculated (Lichtenthaler, 1987). For GA_3, tZ and iP content, 1 g of dried tobacco leaves was ground with liquid nitrogen using a mortar and pestle. One milliliter of extraction solvent (2-propanol : H_2O : concentrated HCl, 2 : 1 : 0.002 (v/v)) was added to each tube and the mixture was incubated on a shaker (100 rpm) at 4°C for 30 min. The mixture was further incubated under the same conditions by adding 1 ml of dichloromethane. After centrifugation at 4000 ***g*** for 10 min, 900 μl of the lower phase was transferred into a new tube and dried using a nitrogen stream evaporator. The final sample was dissolved in 200 μl of methanol, analyzed by Shimadzu Nexera X2 HPLC system using the Synergi™ 4 μm fusion-RP 80Å (Phenomenex, Torrance, CA, USA) and detected by a SPD-M30A diode array detector at 254 nm. GA_3, tZ and iP were identified by comparison to the chromatograph spectrum of standards (GA3, tZ and iP; Sigma). The amount of GA_3, tZ and iP was calculated quantitatively from the peak area of standards. Standard curves were generated using standard solutions with known GA_3, tZ and iP concentrations (Pan *et al.*, 2010).

Carotenoid analysis

One gram of fresh tobacco leaves was ground with liquid nitrogen using a mortar and pestle. Two milliliters of acetone were added to the leaf powder and the mixture was shaken for 30 min in darkness. After centrifugation, 1 ml of supernatant was analyzed by High Performance Liquid Chromatography (HPLC), and carotenoids were identified by comparison to standards (β-carotene and lutein; Sigma-Aldrich). The amount of carotenoids was calculated quantitatively from the peak area of each carotenoid. A standard curve was generated using solutions with known carotenoid concentrations (Mann *et al.*, 2000).

Prenytransferase assays

MBP-fusion protein bound to amylose resin was purified using a buffer containing 10 mM maltose in 20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.4. To extract total soluble protein of tobacco leaves, 1 g of leaf sample was ground with 1 ml of extraction buffer (25 mM MOPSO, pH 7.0, 10 mM $MgCl_2$, 1 mM DTT, 1% (w/v) polyvinylpyrrolidone (PVP-40), 1 mM methanesulfonyl fluoride and 10% (v/v) glycerol). After centrifugation at 12 000 ***g*** for 10 min, the supernatant was transferred into a new E-tube. Prenyltransferase assay was performed with total soluble protein (25 μg) of tobacco leaves with or without recombinant MBP-MpGPS.SSU (1 μg) in a final volume of 100 μl consisting of 40 μM [1-^{14}C]-IPP (50 mCi $mmol^{-1}$) and 40 μM DMAPP in assay buffer (25 mM MOPSO, pH 7.0, 10 mM $MgCl_2$, 1 mM DTT, and 10% (v/v) glycerol). The reaction mixture was overlaid with 100 μl of hexane and incubated at 30°C for 1 h. After incubation, 50 μl of 37% HCl was added to stop the reaction and to hydrolyze the allylic diphosphates to

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their corresponding alcohols. The final alcohol products were extracted by hexane, separated based on polarity by Thin Layer Chromatography (TLC), and visualized by autoradiography using a phosphor-imager (Typhoon 9200; GE Healthcare Life Sciences, Pittsburgh, PA, USA). Control assay using boiled proteins showed no IPP hydrolysis. For TLC analysis, 10 µl of geraniol, farnesol and geranylgeraniol hexane solution (1 mg ml^{-1}, Sigma) were added as standards and a solvent (methanol : water, 6 : 1 (v/v)) was used to separate the products on TLC plates (Orlova *et al.*, 2009).

RNA isolation and quantitative real-time PCR

Total RNA was isolated from tobacco plant using the RNeasy Plant Mini Kit (Qiagen). Purified RNA was used for cDNA synthesis with iScript RT Supermix (Bio-Rad). The quantitative real-time polymerase chain reaction (qRT-PCR) was performed using Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) in ABI 7900 RT-PCR system (Thermo Fisher Scientific). Gene-specific primers for qRT-PCR were listed in Table S3. *Elongation factor-1 alpha* gene (*EF1α*, GenBank: D63396.1) was used as an internal normalization in each qRT-PCR. All reactions were carried out in technical triplicates and with biological triplicates.

Results

Transient co-expression of peppermint *GPS.SSU* with *monoTPS* genes increased the production of monoterpenes in *N. benthamiana*

Plant monoterpenes are synthesized through the plastidic MEP pathway, which produces C5 precursors that are condensed by monoTPSs within plastids.

In order to see if co-expression of intermediate genes of the GPP biosynthetic pathway along with monoTPS may enhance monoterpene production, we performed transient co-expression of genes in *N. benthamiana* leaves. First, we tested the activities of three *limonene synthase* genes from different plant species (*Picea abies*, *Citrus limon* and *Mentha spicata*) by transient expression in leaves of *N. benthamiana* followed by GC-MS analysis of the infiltrated leaves at 3 dpi. We found that only the *P. abies (−)-limonene synthase* gene (*PaLimS*) (Martin *et al.*, 2004) was active in this assay system (Fig. S2). We next investigated the effects of co-expression of tomato *SlDXS*, Arabidopsis *AtGPS* or peppermint *MpGPS.SSU* on (−)-limonene production mediated by *PaLimS* (Fig. 1a). At 3 dpi, volatile organic compounds (VOCs) emitted from agroinfiltrated *N. benthamiana* leaves were collected for 24 h using a push–pull headspace collection system and the content was analyzed by GC-MS. Fig. 1a shows that no significant changes in (−)-limonene concentrations were detected when *PaLimS* was co-expressed with *SlDXS* or *AtGPS* compared to the lines expressing *PaLimS* alone, which was used as a control. However, co-expression of *MpGPS.SSU* with *PaLimS* clearly increased (−)-limonene concentrations by *c.* 22–35 times compared to control plants (Figs 1a, S3; Table 1).

Next, we asked whether co-expression of *MpGPS.SSU* could also be used to increase production of other monoterpenes. Three well-known *monoTPSs*, *P. abies (−)-linalool synthase* (*PaLinS*), *Picea sitchensis (−)-pinene synthase* (*PsPinS*) and *P. abies myrcene synthase* (*PaMyrS*) were selected as representative *monoTPSs* (Martin *et al.*, 2004; McKay *et al.*, 2003; Fig. S1) and these genes were tested by similar strategy described above. Fig. 1(b) shows that *N. benthamiana* leaves expressing *PaLinS*, *PsPinS* and *PaMyrS* with *MpGPS.SSU* were able to significantly increase (−)-linalool, (−)-α-pinene/β-pinene and myrcene production levels, respectively, compared to the plants expressing each *monoTPS* alone.

Although the fold-change of enhanced production of (−)-linalool, (−)-α-pinene/β-pinene and myrcene was not as high as in the case of (−)-limonene production, the effect of co-expression of *MpGPS.SSU* was evident (Figs 1, S3; Table 1). Overexpression of *MpGPS.SSU* alone did not increase concentrations of any endogenous monoterpene suggesting that *N. benthamiana* leaves might barely synthesize monoterpenes (Figs 1, S4). Taken together, our results show that overexpression of *MpGPS.SSU* can change or accelerate the metabolic flux towards GPP formation in *N. benthamiana* leaf cells.

Co-expression of *MpGPS.SSU* with *monoTPS* genes increases monoterpene production in transgenic tobacco plants

Results from transient expression of each *monoTPS* along with *MpGPS.SSU* provided us with preliminary evidence to investigate possible enhancing effect of *MpGPS.SSU* on monoterpene formation in transgenic plants. To avoid possible gene silencing mediated by promoter homology (Park *et al.*, 1996) in transgenic tobacco plants, we used the Arabidopsis *ubiquitin-10* promoter to express *monoTPSs* and a CaMV 35S promoter to express *MpGPS.SSU*. We generated more than 10 transgenic tobacco (*N. tabacum*) lines for each construct expressing 4 different *monoTPSs* (*PaLimS*, *PaLinS*, *PsPinS* and *PaMyrS*) with or without co-expression of *MpGPS.SSU* (Fig. S5). As controls, we selected three independent lines of each construct with comparable *monoTPS* transcript levels for further analysis (Fig. 2a,c,e,g). All transgenic lines expressing each *monoTPS* with or without *MpGPS.SSU* were able to produce viable seeds and their T1 progenies were used for further analysis.

In order to assess the quantitative impact of *MpGPS.SSU* on monoterpene production, we collected monoterpenes emitted from transgenic tobacco plants for 24 h. Ten grams of leaves (equivalent to two leaves) from 2-month-old tobacco plants of each transgenic line were used for headspace collection of VOCs using a push-pull headspace collection system.

Figs 2(b) and S6(a) show increased (−)-limonene concentrations in LG transgenic lines expressing both *PaLinS* and *MpGPS.SSU* compared to L lines expressing *PaLinS* alone. The peaks in GC-MS analysis were identified by comparison to authentic standard of (−)-limonene based on both retention time and mass spectra (Fig. S6a). The (−)-limonene content in L lines varied from 3.75 ± 0.37 to 6.79 ± 0.51 µg g^{-1} FW·24 h^{-1}

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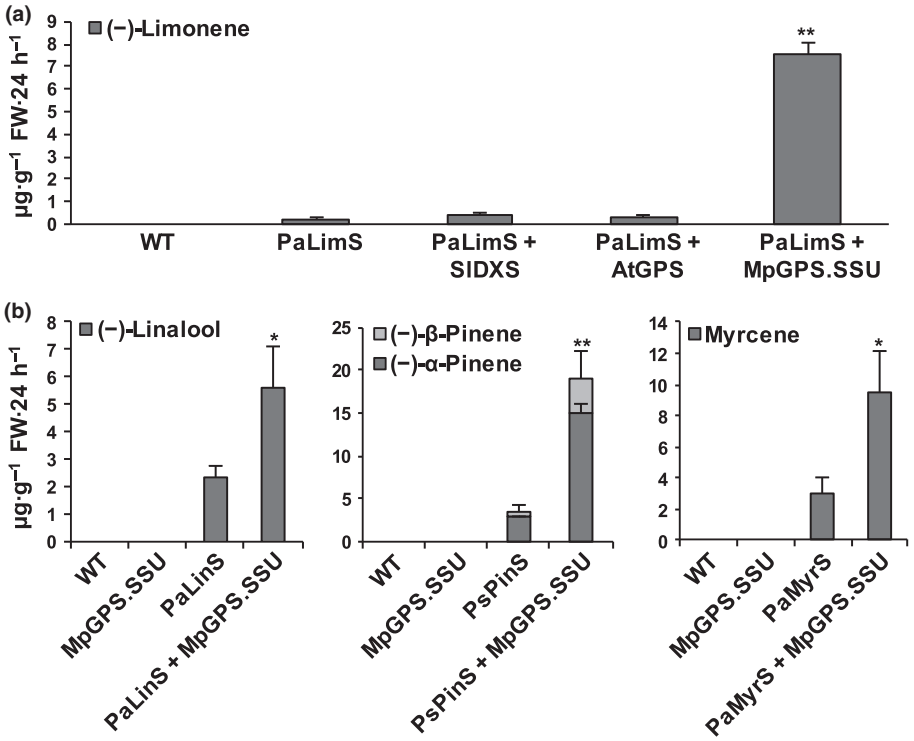

Fig. 1 Transient co-expression of peppermint *geranyl diphosphate synthase small subunit* (*MpGPS.SSU*) with genes encoding monoterpene synthases increased production of monoterpenes in *Nicotiana benthamiana*. (a) Effect on limonene production by co-expression of *SlDXS*, *AtGPS* or *MpGPS.SSU* with *PaLimS*. (b) Effect on monoterpene production by co-expression of *MpGPS.SSU* with *monoTPS* genes. *monoTPS* genes were transiently expressed in *N. benthamiana* leaves alone or with *MpGPS.SSU* by *Agrobacterium*-mediated infiltration. Chemical compounds were analyzed 3 d post-infiltration (dpi) by GC-MS. WT, wild-type; *AtGPS*, *Arabidopsis thaliana geranyl diphosphate synthase 1*; *SlDXS*, *Solanum lycopersicum 1-deoxy-D-xylulose-5-phosphate synthase*; *MpGPS.SSU*, *Mentha × piperita geranyl diphosphate synthase small subunit*; *PaLimS*, *Picea abies (−)-limonene synthase*; *PaLinS*, *P. abies (−)-Linalool synthase*; *PaMyrS*, *P. abies myrcene synthase*; *PsPinS*, *P. sitchensis (−)-pinene synthase*. Error bars represent standard deviation (SD) ($n = 3$). Asterisks indicate statistically significant differences from lines expressing single *monoTPS* gene based on Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$).

Table 1 Production of monoterpenes in *Nicotiana benthamiana* using agroinfiltration (µg g^{-1} FW 24 h^{-1})

	(−)-limonene	(−)-linalool	(−)-α-pinene	(−)-β-pinene	Myrcene
WT	nd	nd	nd	nd	nd
MpGPS.SSU	nd	nd	nd	nd	nd
PaLimS	0.23 ± 0.05[1]	nd	nd	nd	nd
PaLinS	nd	2.34 ± 0.35	nd	nd	nd
PsPinS	nd	nd	2.79 ± 0.80	0.73 ± 0.24	nd
PaMyrS	nd	nd	nd	nd	3.03 ± 1.04
PaLimS + *SlDXS*	0.33 ± 0.01	nd	nd	nd	nd
PaLimS + *AtGPS*	0.21 ± 0.06	nd	nd	nd	nd
PaLimS + *MpGPS.SSU*	7.52 ± 1.55	nd	nd	nd	nd
PaLinS + *MpGPS.SSU*	nd	5.55 ± 1.55	nd	nd	nd
PsPinS + *MpGPS.SSU*	nd	nd	15.03 ± 3.03	4.05 ± 1.05	nd
PaMyrS + *MpGPS.SSU*	nd	nd	nd	nd	9.56 ± 2.57

FW, fresh weight; WT, wild-type; nd, not detected.
[1]Mean ± SD for three biological replicates are shown.

(Table 2), whereas the (−)-limonene content in LG lines ranged from 24.33 ± 1.17 to 29.27 ± 5.82 µg g^{-1} FW·24 h^{-1}. Comparison of these results showed that co-expression of *MpGPS.SSU* increased the (−)-limonene production by *c.* 4–8 times.

Similar results were obtained with three other *monoTPSs* (Fig. 2c–h). By comparison to the corresponding authentic standard, we were able to detect (−)-linalool, (−)-α-pinene/β-pinene and myrcene in Ln, P and M lines which expressed *PaLinS*, *PsPinS* and *PaMyrS*, respectively (Fig. S6b–d). Monoterpene concentrations were increased up to 6–19 times in LnG lines, 2–48 times in PG lines and 2–14 times in MG lines by co-expression of *MpGPS.SSU* (Fig. 2d,f,h; Table 2). These results show that co-expression of *MpGPS.SSU* with *monoTPSs* can be

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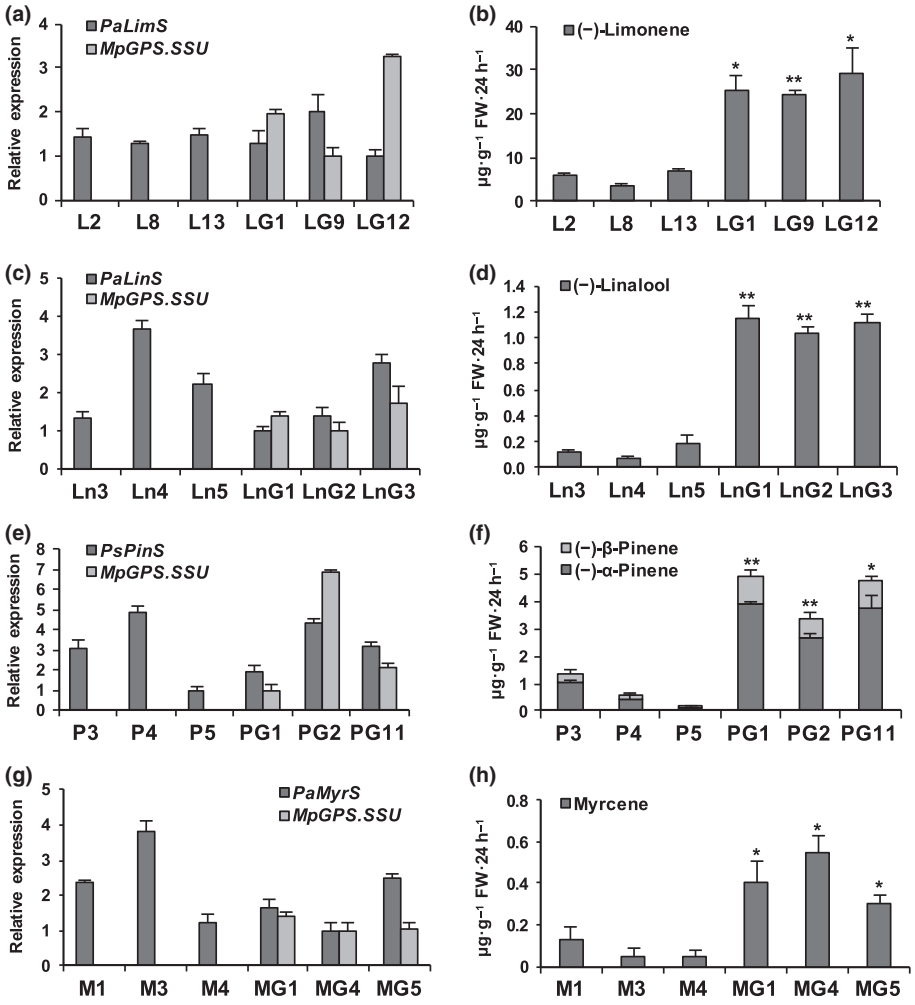

Fig. 2 Monoterpene production was increased by co-expression of peppermint *geranyl diphosphate synthase small subunit* (*MpGPS.SSU*) in transgenic *Nicotiana tabacum* plants. (a) Relative expression levels of *PaLimS* and *MpGPS.SSU* in transgenic tobacco plants. (b) Production of (−)-limonene in transgenic tobacco plants expressing *PaLimS* or *PaLimS* + *MpGPS.SSU*. (c) Relative expression levels of *PaLinS* and *MpGPS.SSU* in transgenic tobacco plants. (d) Production of (−)-linalool in transgenic tobacco plants expressing *PaLinS* or *PaLinS* + *MpGPS.SSU*. (e) Relative expression levels of *PsPinS* and *MpGPS.SSU* in transgenic tobacco plants. (f) Production of (−)-α-pinene/β-pinene in transgenic tobacco plants expressing *PsPinS* or *PsPinS* + *MpGPS.SSU*. (g) Relative expression levels of *PaMyrS* and *MpGPS.SSU* in transgenic tobacco plants. (h) Production of myrcene in transgenic tobacco plants expressing *PaMyrS* or *PaMyrS* + *MpGPS.SSU*. *PaLimS*, *Picea abies (−)-limonene synthase*; *PaLinS*, *P. abies (−)-Linalool synthase*; *PsPinS*, *P. sitchensis (−)-pinene synthase*; *PaMyrS*, *P. abies myrcene synthase*; *MpGPS.SSU*, *Mentha* × *piperita geranyl diphosphate synthase small subunit*; L, transgenic lines expressing *PaLimS*; LG, transgenic lines expressing *PaLimS* and *MpGPS.SSU*; Ln, transgenic lines expressing *PaLinS*; LnG, transgenic lines expressing *PaLinS* and *MpGPS.SSU*; P, transgenic lines expressing *PsPinS*; PG, transgenic lines expressing *PsPinS* and *MpGPS.SSU*; M, transgenic lines expressing *PaMyrS*; MG, transgenic lines expressing *PaMyrS* and *MpGPS.SSU*. Error bars represent standard deviation (SD) ($n = 3$). Asterisks indicate statistically significant differences from lines expressing single *monoTPS* gene based on Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$).

used to increase monoterpene production in transgenic tobacco plants.

Among the four monoterpenes tested here, only (−)-linalool possesses a hydroxyl group which potentially can be glycosylated by glucosyltransferase (Lücker *et al.*, 2001). To assess if (−)-linalool was indeed glycosylated in tobacco plants, we treated homogenized LnG leaf samples with either a saturated $CaCl_2$ solution or a glycosidase (Methods S1). The reaction mixture was extracted by hexane followed by GC-MS analysis. No (−)-linalool was detected in untreated, homogenized frozen leaf tissues of LnG line (Fig. S7a). Moreover, we were unable to obtain free (−)-linalool from homogenized LnG leaf samples after incubation with $CaCl_2$ or a glycosidase enzyme (Fig. S7b,c). These results suggest that (−)-linalool is not stored as a non-volatile linalyl-glycoside or other glycosylated forms in the leaf tissues or cells of tobacco plants.

Phenotypes of transgenic tobacco plants

An important consideration is whether *MpGPS.SSU* expression would lead to phenotypic changes of transgenic tobacco plants. Previous reports showed that transgenic tobacco plants expressing high and moderate levels of Snapdragon *AmGPS.SSU* were dwarf and displayed strong chlorosis with reduced Chl

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Table 2 Production of monoterpenes in transgenic *Nicotiana tabacum* (μg g^{-1} FW 24 h^{-1})

	(−)-limonene	(−)-linalool	(−)-α-pinene	(−)-β-pinene	Myrcene
WT	nd	nd	nd	nd	nd
MpGPS.SSU	nd	nd	nd	nd	nd
L2	5.81 ± 0.55[1]	nd	nd	nd	nd
L8	3.75 ± 0.37	nd	nd	nd	nd
L13	6.79 ± 0.51	nd	nd	nd	nd
LG1	25.37 ± 3.36	nd	nd	nd	nd
LG9	24.33 ± 1.17	nd	nd	nd	nd
LG12	29.27 ± 5.82	nd	nd	nd	nd
Ln3	nd	0.11 ± 0.02	nd	nd	nd
Ln4	nd	0.06 ± 0.02	nd	nd	nd
Ln5	nd	0.18 ± 0.06	nd	nd	nd
LnG1	nd	1.15 ± 0.10	nd	nd	nd
LnG2	nd	1.02 ± 0.06	nd	nd	nd
LnG3	nd	1.11 ± 0.07	nd	nd	nd
P3	nd	nd	1.07 ± 0.06	0.30 ± 0.09	nd
P4	nd	nd	0.45 ± 0.10	0.15 ± 0.04	nd
P5	nd	nd	0.08 ± 0.01	0.05 ± 0.02	nd
PG1	nd	nd	3.89 ± 0.11	1.00 ± 0.20	nd
PG2	nd	nd	2.69 ± 0.12	0.65 ± 0.22	nd
PG11	nd	nd	3.74 ± 0.48	0.97 ± 0.20	nd
M1	nd	nd	nd	nd	0.13 ± 0.05
M3	nd	nd	nd	nd	0.04 ± 0.04
M4	nd	nd	nd	nd	0.04 ± 0.03
MG1	nd	nd	nd	nd	0.40 ± 0.10
MG4	nd	nd	nd	nd	0.54 ± 0.08
MG5	nd	nd	nd	nd	0.29 ± 0.04

FW, fresh weight; WT, wild-type; nd, not detected.
[1]Mean ± SD for three biological replicates are shown.

contents (Orlova *et al.*, 2009). Interestingly, our transgenic lines expressing *MpGPS.SSU* (G), *PaLimS*+*MpGPS.SSU* (LG), *PaLinS*+*MpGPS.SSU* (LnG), *PsPinS*+*MpGPS.SSU* (PG) and *PaMyrS*+*MpGPS.SSU* (MG) showed a faster growth rate and the plants flowered early in mature stage compared to wild-type (WT) (Figs 3a,b, S8). However, there were no phenotypic changes in seedlings (Fig. 3c) and no significant difference in the total Chl content between WT and transgenic lines (G) expressing *MpGPS.SSU* (Fig. 3d). Orlova *et al.* (2009) found that leaf chlorosis and a delayed growth of transgenic tobacco plants expressing *AmGPS.SSU* were correlated to the reduction of GAs and carotenoid concentrations. Therefore, we measured GA_3 and total carotenoid contents in the leaves of transgenic lines expressing *MpGPS.SSU*. Fig. 3(d) shows that GA_3 concentrations in leaves of transgenic lines were slightly higher than in WT leaves. This result also was confirmed by the upregulation of *GA20ox* encoding GA20-oxidase that catalyzes the formation of active GAs in all three lines (Fig. 3e). However, total carotenoid content was not changed in leaves of transgenic lines, even though individual carotenoids tested showed slightly different accumulation patterns; lutein was increased in transgenic lines whereas β-carotene was a bit reduced (Fig. 3d). As a class of diterpenoid acids, GA_3 is produced from GGPP via *ent*-copalyl diphosphate and *ent*-kaurene (Hedden & Kamiya, 1997). We investigated transcript levels of tobacco *GGPPS* candidates (*GGPPS1-4*) in leaves of transgenic lines expressing *MpGPS.SSU*, and found that out of four *GGPPSs*, *GGPPS3* and *GGPPS4* were upregulated in transgenic lines (Fig. 3e). This result probably explained that the overexpressed MpGPS.SSU somehow affected the expression of *GGPPS3* and *GGPPS4* so that it could work together with GGPPS3 and/or GGPPS4 to increase both GPP and GGPP for monoterpenes and GA_3 biosynthesis in transgenic tobacco plants. Note that snapdragon AmGPS.SSU was able to interact with GGPPS1 and GGPPS2 to form functional GPS enzyme for the production of monoterpenes (Orlova *et al.*, 2009).

Another interesting phenotype observed in *MpGPS.SSU*-overexpressed lines was the development of shoot branching arising from the growth of axillary (lateral) buds. Fig. 4a shows that the overexpression of *MpGPS.SSU* promoted the growth of axillary buds in transgenic tobacco. This effect became increasingly clear during the growth period of transgenic lines (Fig. S9a). In general, auxin inhibits the growth of the axillary buds whereas cytokinin promotes it (Domagalska & Leyser, 2011). Therefore, we measured concentrations of the naturally occurring cytokinins, tZ and iP, in *MpGPS.SSU*-overexpressed lines. Fig. 4b shows that total tZ and iP content was increased up to three times in *MpGPS.SSU*-overexpressed lines suggesting that the increased growth of the axillary buds of transgenic tobacco may be due to the increased cytokinin content. To confirm if cytokinin indeed stimulates axillary bud growth in WT tobacco, we applied BA onto leaf axils and measured the length of shoot branches after 7 d. Fig. 4(c) shows that the growth of axillary buds was clearly promoted by BA treatment but not by GA_3 which served as a negative control. After BA treatment, the length

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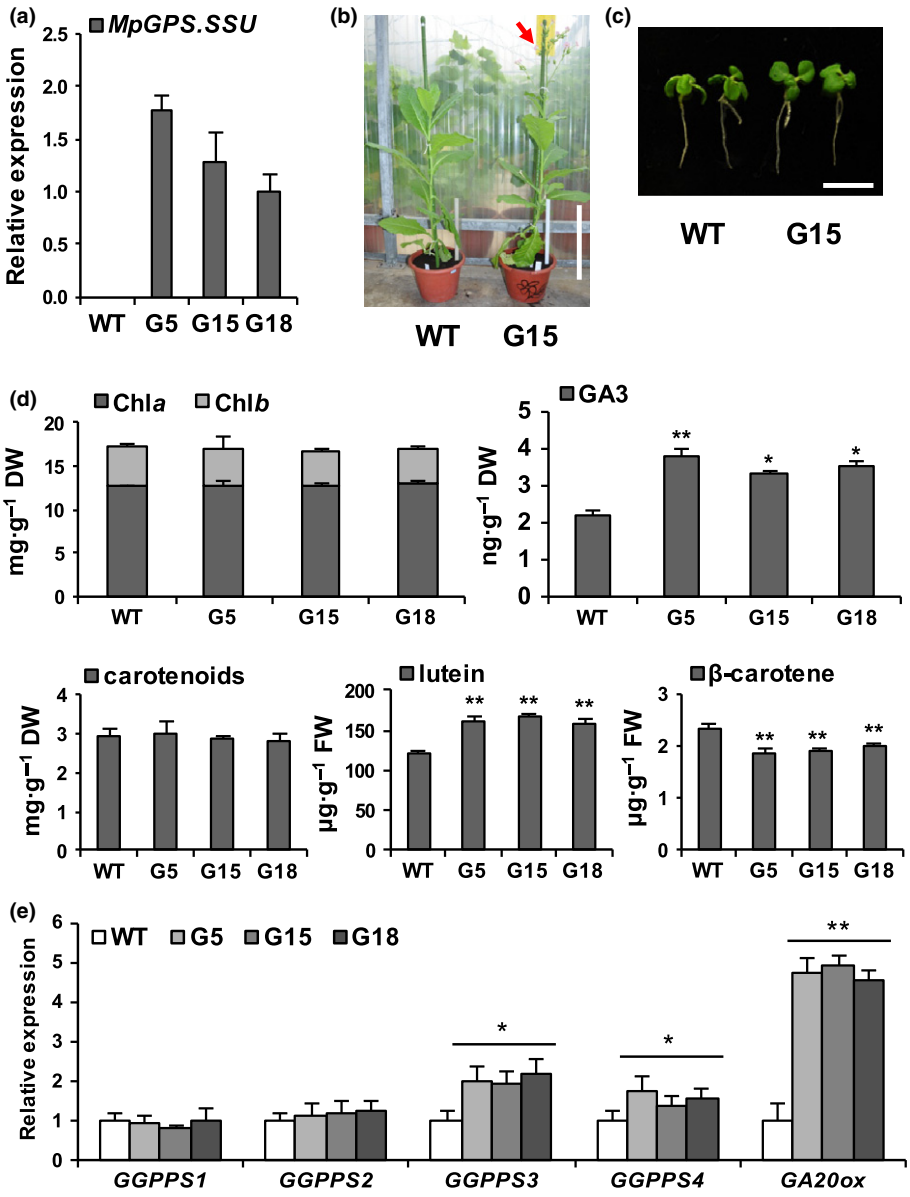

Fig. 3 Phenotypes of transgenic *Nicotiana tabacum* plants expressing peppermint *geranyl diphosphate synthase small subunit* (*MpGPS.SSU*). (a) Relative expression levels of *MpGPS.SSU* in transgenic lines expressing *MpGPS.SSU*. (b) An early flowering phenotype of transgenic tobacco plant expressing *MpGPS.SSU*; bar, 30 cm. The arrow indicates the complete inflorescences. (c) Phenotype of tobacco seedlings expressing *MpGPS.SSU*; bar, 10 mm. (d) Chl*a*, Chl*b*, GA_3, total carotenoids, lutein and β-carotene content in leaves of WT and transgenic lines expressing *MpGPS.SSU*. (e) Relative expression levels of *GGPPS* genes and *GA20ox* in transgenic lines expressing *MpGPS.SSU*. WT, wild-type; G, transgenic lines expressing *MpGPS.SSU*; Chl*a*, Chlorophyll a; Chl*b*, chlorophyll b; *GGPPS*, *N. tabacum geranylgeranyl pyrophosphate synthase*; *GA20ox*, *N. tabacum Gibberellin (GA) 20-oxidase*; *MpGPS.SSU*, *Mentha × piperita geranyl diphosphate synthase small subunit*. Error bars represent standard deviation (SD), $n = 3$ except GA3 ($n = 9$). Asterisks indicate statistically significant differences from WT based on Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$).

of the branches could be 10 times longer than in WT with or without GA_3 treatment (Fig. S9b). It has been known that cytokinins can be produced by the MEP pathway, because both DMAPP and hydroxymethylbutenyl diphosphate (HMBDP) that are synthesized through the MEP pathway are used as the isoprenoid side chain donors for the biosynthesis of cytokinins (Frébort *et al.*, 2011). We investigated transcript levels of representative intermediate genes involved in MEP pathway. Fig. 4d shows that transcript levels of *DXS*, *HDR* and 2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase gene (*MCS*) were increased in leaves of transgenic lines expressing *MpGPS.SSU* compared to WT leaves.

Effect of MpGPS.SSU on GPP/GGPP synthesis *in vitro*

Previous results from Orlova *et al.* (2009) showed that ectopic expression of snapdragon *GPS.SSU* altered the monoterpene profile of transgenic tobacco plants, which became dwarf and chlorotic. Here, we found that co-expression of *MpGPS.SSU* with *monoTPSs* was able to elevate monoterpene production in transgenic tobacco plants. To determine whether overexpression of *MpGPS.SSU* would lead to GPP formation in the presence of IPP and DMAPP, we performed *in vitro* prenyltransferase assays using MBP-fused MpGPS.SSU recombinant protein (MBP-MpGPS.SSU) and protein extracts from leaves of transgenic tobacco plants. Fig. 5(a) shows that total protein extract from leaves of transgenic tobacco plants (G15) expressing *MpGPS.SSU* increased GPP formation from IPP and DMAPP compared to WT protein extract. Moreover, addition of MBP-MpGPS.SSU to the reaction mixture of WT leaf protein extract enhanced only GPP formation from IPP and DMAPP, whereas addition of MBP alone had no effect. Under the same conditions, none of these reactions displayed any significant differences with respect to FPP and GGPP formation (Fig. 5a). Moreover, the GPP

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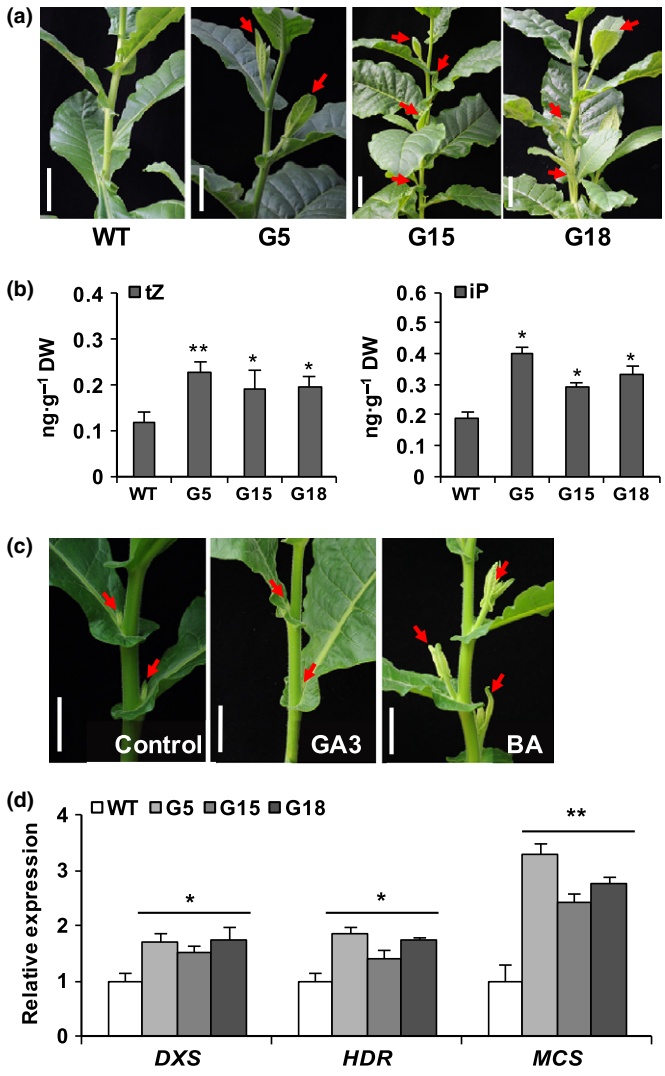

Fig. 4 Shoot branching phenotype of transgenic *Nicotiana tabacum* plants expressing peppermint *geranyl diphosphate synthase small subunit* (*MpGPS.SSU*). (a) The growth of axillary buds in transgenic lines expressing *MpGPS.SSU*; bar, 10 cm. Arrows indicate axillary buds. (b) *Trans*-zeatin and N^6-(Δ^2-isopentenyl) adenine contents in leaves of WT and transgenic lines expressing *MpGPS.SSU*. (c) Shoot branching of WT treated with water, GA3 or BA; bar, 10 cm. Arrows indicate axillary buds. (d) Relative expression levels of MEP pathway genes in transgenic lines expressing *MpGPS.SSU*. WT, wild-type; G, transgenic lines expressing *MpGPS.SSU*; tZ, *trans*-Zeatin; iP, N^6-(Δ^2-isopentenyl) adenine; BA, 6-Benzylaminopurine; *DXS*, *N. tabacum 1-deoxyxylulose-5-phosphate synthase*; *HDR*, *N. tabacum 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase*; *MCS*, *N. tabacum 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase*; *MpGPS.SSU*, *Mentha × piperita geranyl diphosphate synthase small subunit*. Error bars represent standard deviation (SD), $n = 9$ for (b) and $n = 3$ for (d). Asterisks indicate statistically significant differences from WT based on Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$).

production was increased by 2.2, 2.9 and 4.5 times following addition of 0.1 μg, 0.3 μg and 0.9 μg of MBP-MpGPS.SSU, respectively (Fig. 5b). These results indicate that the peppermint MpGPS.SSU may increase GPP formation *in vitro* and suggest that its expression may enhance monoterpene rather than diterpene biosynthesis in plastids of transgenic tobacco.

Discussion

In order to further optimize monoterpene production in tobacco plants, we evaluated the activity of three limonene synthases from *Picea abies*, *Citrus limon* and *Mentha spicata*, as well as the influence of *Solanum lycopersicum 1-deoxy-D-xylulose-5-phosphate synthase* (*SlDXS*), *Arabidopsis thaliana geranyl diphosphate synthase 1* (*AtGPS*) or *Mentha × piperita geranyl diphosphate synthase small subunit* (*MpGPS.SSU*) on the production of ectopic monoterpenes. We found that production of monoterpenes, (−)-limonene, (−)-linalool, (−)-β-pinene/α-pinene or myrcene, by exogenous *mono TPSs* could be enhanced by the co-expression of *MpGPS.SSU* (Figs 1, 2; Tables 1, 2). Furthermore, flowering and shoot branching were also promoted by increased contents of GA_3, *trans*-zeatin (tZ) and N^6-(Δ^2-isopentenyl) adenine (iP), respectively, owing to the upregulation of transcript levels of several 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway genes, *DXS*, *HDR* and *MCS*, which probably occurred by a feedback mechanism.

Overexpression of *MpGPS.SSU* produced high amount of monoterpenes in transgenic tobacco plants

Elevated accumulation of GGPP-derived products such as Chl, carotenoids, tocopherols and ABA have been found in transgenic Arabidopsis plants overexpressing *DXS* (Estevez *et al.*, 2001). However, a higher concentration of (−)-limonene was not detected in *N. benthamiana* leaves transiently co-expressing *Solanum lycopersicum 1-deoxy-D-xylulose-5-phosphate synthase* (*SlDXS*) and *PaLimS* compared to leaves expressing *PaLimS* alone. A major reason is that geranylgeranyl diphosphate synthase (GGPPS) directed most of Isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) into GGPP. Two lines of evidence support this hypothesis. First, only a certain amount of endogenous monoterpenes was emitted from wild-type (WT) tobacco leaves (Fig. S4). Second, in the prenyltransferase assay using crude protein extracts from WT leaves, most of the [1-^{14}C]-IPP and DMAPP were converted into GGPP with only a trace amount of GPP production (Fig. 5). We hypothesized that overexpression of *SlDXS* directed the MEP metabolic flux into GGPP production via the dominant GGPPS. This depleted the GPP pool for monoterpene biosynthesis (Table 1; Fig. 1a).

Despite the 10-fold increase in limonene production in transgenic tobacco plants co-expressing *limonene synthase* gene and *AtGPS* (Wu *et al.*, 2006), in our study, the formation of exogenous (−)-limonene was not affected by the transient co-expression of *AtGPS* with *Picea abies (−)-limonene synthase* (*PaLimS*). This result suggested that overexpression of *AtGPS* could not re-direct the metabolic flux from GGPP to GPP in tobacco plants (Fig. 1a). A recent study showed that *AtGPS* appears to have multiple functions and catalyzes the production of long-chain prenyl diphosphate rather than GPP (Hsieh *et al.*, 2011). Our results are consistent with this finding.

With respect to its function, GPS.SSU could be considered as a 'modifier' or an 'accelerator' (Wang & Dixon, 2009). On the

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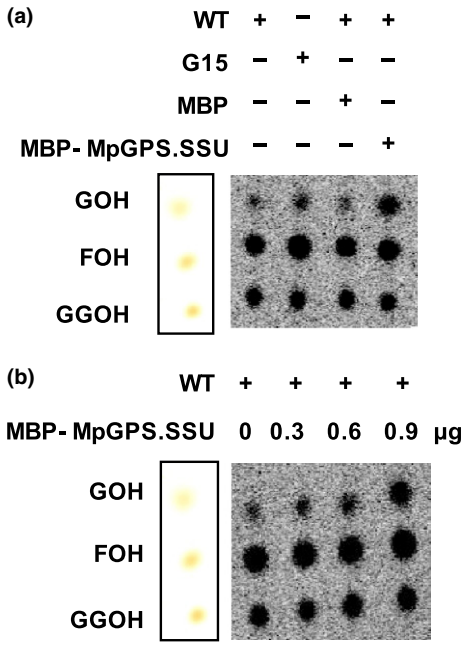

Fig. 5 Recombinant peppermint geranyl diphosphate synthase small subunit (MBP-MpGPS.SSU) protein stimulates GPP synthesis in total *Nicotiana tabacum* protein extract *in vitro*. (a) Recombinant MBP-MpGPS.SSU increases the biosynthesis of GPP *in vitro*. (b) The production of GPP has positive correlation with the amount of recombinant MpGPS.SSU. The reaction products GPP, FPP and GGPP were converted to GOH, FOH and GGOH, respectively, by HCl treatment. The final products were analyzed by TLC/autoradiography. WT, wild-type; G15, transgenic line #15 expressing *MpGPS.SSU*; MBP, maltose binding protein; *Mp*GPS.SSU, *Mentha* × *piperita* geranyl diphosphate synthase small subunit protein; GOH, geraniol; FOH, farnesol; GGOH, geranylgeraniol.

one hand, prenyltransferase assay showed the *Antirrhinum majus* geranyl diphosphate synthase small subunit (AmGPS.SSU) served as a 'modifier' to increase the production of GPP via modification of chain-length specificity of tobacco GGPPS and blocked GGPP production (Orlova *et al.*, 2009). On the other hand, GPS.SSU could also work as an 'accelerator'. For example, in the prenyltransferase assay of transgenic tomato fruits expressing *Antirrhinum majus* geranyl diphosphate synthase large subunit (AmGPS.LSU), GGPPS activity was not altered and GPS activity was upregulated (Gutensohn *et al.*, 2013). Both GPP and GGPP production of geranyl diphosphate synthase large subunit *Humulus lupulus* geranyl diphosphate synthase large subunit (HlGPS.LSU) could be accelerated by *Humulus lupulus* geranyl diphosphate synthase small subunit (HlGPS.SSU) *in vitro* (Wang & Dixon, 2009). In our prenyltransferase assay, addition of MpGPS.SSU recombinant protein enhanced the activity of GPS without affecting GGPPS activity in tobacco crude protein extracts (Fig. 5). These results suggested that the MpGPS.SSU seemed to play a role as an 'accelerator' rather than a 'modifier' in tobacco. Therefore, when *MpGPS.SSU* was co-expressed with several *monoTPS* genes, much higher amounts of exogenous monoterpenes were detected, either by transient expression in *N. benthamiana* or in transgenic tobacco plants (Figs 1, 2 and Tables 1, 2).

Phenotypes of transgenic tobacco plants overexpressing *MpGPS.SSU*

Snapdragon AmGPS.SSU could increase endogenous monoterpene production both by changing the tobacco GGPPS product specificity and by reducing the content of GGPP derivatives (carotenoids, Chl and GA_3 and so on). The latter resulted in transgenic tobacco plants with dwarfism, leaf chlorosis and other adverse effects (Orlova *et al.*, 2009). By contrast, different phenotypes were observed in transgenic tobacco plants expressing *MpGPS.SSU*. Figs 3(b), 4(a) and S8(a) show that transgenic lines expressing *Mp*GPS.SSU displayed accelerated flowering and promotion of shoot branching in adult stages.

Gibberellins play a very important role in promoting early flowering of Arabidopsis. For example, *GA20ox* overexpression amplifies the concentration of bioactive GA (Huang *et al.*, 1998) and transgenic plants display early flowering phenotype (Sun, 2008). Expression of the flowering genes *LEAFY* (*LFY*) and *SUPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*) is induced by GA (Blazquez *et al.*, 1998; Moon *et al.*, 2003). Therefore, we investigated the content of GA_3 in transgenic tobacco plants expressing *MpGPS.SSU*. Compared to WT plants, the MpGPS.SSU transgenic lines contained a higher amount of GA_3 (Fig. 3d) and this increased GA content may be responsible for the early flowering phenotype. Gibberellins in plants are biosynthesized from common GGPP catalyzed by *ent*-copalyl diphosphate synthase (CPS), *ent*-kaurene synthase (KS), *ent*-kaurene oxidase (KO) and *ent*-kaurenoic acid oxidase (KAO), GA 20-oxidases (GA20ox) and 3-oxidases (GA3ox) (Yamaguchi, 2008). It has been reported that GA homeostasis is maintained by upregulation of *GA20ox* and *GA3ox* which are controlled by feedback mechanism in GA-deficiency or mutations that reduce GA signaling (Matsushita *et al.*, 2007; Rieu *et al.*, 2008). In the present study, we found upregulation of *GGPPS3*, *GGPPS4* and *GA20ox* in transgenic lines expressing *MpGPS.SSU*. We hypothesized that when IPP and DMAPP are redirected to GPP via overexpression of *MpGPS.SSU*, GA homeostasis or elevation is achieved by upregulation of *GGPPS3*, *GGPPS4* and *GA20ox* (Fig. 3e), which is probably controlled by a feedback mechanism.

In many higher plants, shoot branching is regulated by auxin and cytokinin antagonism; growth of lateral buds is repressed by auxin but activated by cytokinin (Shimizu-Sato *et al.*, 2009; Muller & Leyser, 2011). To investigate the mechanism of shoot branching of transgenic tobacco plants, we compared tZ and iP contents in both transgenic tobacco and WT plants, and found higher amounts of tZ and iP in transgenic lines (Fig. 4b). This result suggests that the increased shoot branching probably was promoted by the increased cytokinins content in the transgenic lines. Because growth of lateral buds of plant is initiated by treating with cytokinin solution (Wickson & Thimann, 1958), to further investigate the effects of GA or cytokinin on shoot branching, GA_3 and 6-benzylaminopurine (BA) solution were applied directly to axillary buds of WT tobacco plant. One week after treatment, significant shoot branching was observed in BA-treated tobacco plants (Fig. 4c) suggesting that promotion of short branching was caused by an increase in cytokinin content.

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In higher plants, active cytokinins, tZ and iP are produced from DMAPP through the plastidial MEP pathway (Kasahara *et al.*, 2004). Because upregulation of several intermediate genes (*DXS*, *MCS* and *HDR*) of the MEP pathway were found in transgenic lines, we speculated that the elevation of tZ and iP in these plants was a result of the enhancement of MEP pathway probably achieved by a feedback mechanism induced through overexpression of *MpGPS.SSU*.

In summary, herein we describe a metabolic engineering strategy to improve monoterpene production in tobacco plants. We found that the peppermint *MpGPS.SSU* could increase the production of monoterpenes such as (−)-limonene, (−)-linalool, (−)-α-pinene/β-pinene and myrcene by enhancing the GPS activity in both *N. benthamiana* and *N. tabacum*. The general growth of transgenic plants, flowering and shoot branching were promoted by increased contents of GA_3, tZ and iP and by upregulation of *DXS*, *MCS*, *HDR*, *GGPPS3*, *GGPPS4* and *GA20ox*. This strategy can be used to aid the identification of new *monoTPS* genes using transient expression in *N. benthamiana*. It can also be applied to improve the production of exogenous monoterpenes in transgenic tobacco for basic biology research or commercial applications although further optimization of expression level is required.

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Author contributions

I-C.J. and N-H.C. planned the research; J-L.Y., I-C.J. and N-H.C. designed the experiments; J-L.Y. and W-S.W. performed the experiments; J-L.Y., I-C.J. and N-H.C. analyzed the data; and J-L.Y., I-C.J. and N-H.C. wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Vectors for monoterpene biosynthesis.

Fig. S2 Production of limonene in *Nicotiana benthamiana* leaves after *Agrobacterium*-mediated infiltration.

Fig. S3 Gas chromatography traces of agroinfiltrated *Nicotiana benthamiana* leaves expressing *PaLimS*, *PaLimS* and *MpGPS.SSU*, *PaLinS*, *PaLinS* and *MpGPS.SSU*, *PsPinS*, *PsPinS* and *MpGPS.SSU*, *PaMyrS*, and *PaMyrS* and *MpGPS.SSU*.

Fig. S4 Gas chromatography traces of agroinfiltrated *Nicotiana benthamiana* leaves expressing *MpGPS.SSU*.

Fig. S5 Relative expression levels of *monoTPS* transcripts in transgenic *Nicotiana tabacum*.

Fig. S6 Gas chromatography traces of transgenic *Nicotiana tabacum* expressing *monoTPS* or *monoTPS* with *MpGPS.SSU*.

Fig. S7 Gas chromatography traces obtained from LnG line after $CaCl_2$ or a glycosidase treatment.

Fig. S8 An early flowering phenotype of transgenic *Nicotiana tabacum* expressing *MpGPS.SSU* or *MpGPS.SSU+ monoTPS*.

Fig. S9 Shoot branching of transgenic *Nicotiana tabacum* expressing *MpGPS.SSU*.

Table S1 Oligonucleotides used in vector construction

Table S2 Gene name and GenBank reference number

Table S3 Primers used in qPCR

Methods S1 Terpene glycoside analysis.

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