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FEATURED ARTICLE

# Cytosolic monoterpene biosynthesis is supported by plastid-generated geranyl diphosphate substrate in transgenic tomato fruits

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## SUMMARY

**Geranyl diphosphate (GPP), the precursor of most monoterpenes, is synthesized in plastids from dimethylallyl diphosphate and isopentenyl diphosphate by GPP synthases (GPPSs). In heterodimeric GPPSs, a non-catalytic small subunit (GPPS-SSU) interacts with a catalytic large subunit, such as geranylgeranyl diphosphate synthase, and determines its product specificity. Here, snapdragon (*Antirrhinum majus*) *GPPS-SSU* was over-expressed in tomato fruits under the control of the fruit ripening-specific polygalacturonase promoter to divert the metabolic flux from carotenoid formation towards GPP and monoterpene biosynthesis. Transgenic tomato fruits produced monoterpenes, including geraniol, geranial, neral, citronellol and citronellal, while exhibiting reduced carotenoid content. Co-expression of the *Ocimum basilicum* geraniol synthase (*GES*) gene with snapdragon *GPPS-SSU* led to a more than threefold increase in monoterpene formation in tomato fruits relative to the parental *GES* line, indicating that the produced GPP can be used by plastidic monoterpene synthases. Co-expression of snapdragon *GPPS-SSU* with the *O. basilicum* α–zingiberene synthase (*ZIS*) gene encoding a cytosolic terpene synthase that has been shown to possess both sesqui- and monoterpene synthase activities resulted in increased levels of ZIS-derived monoterpene products compared to fruits expressing *ZIS* alone. These results suggest that re-direction of the metabolic flux towards GPP in plastids also increases the cytosolic pool of GPP available for monoterpene synthesis in this compartment via GPP export from plastids.**

**Keywords: geranyl diphosphate synthase, monoterpenes, compartmentalization, metabolic engineering, *Solanum lycopersicum*.**

## INTRODUCTION

Terpenoids represent one of the most diverse class of plant metabolites, being involved in numerous ubiquitous basic processes including photosynthesis, respiration, growth and development (Gershenzon and Kreis, 1999; Rodriguez-Concepción and Boronat, 2002). In addition to such vital molecules as sterols, carotenoids and the hormones gibberellins, strigolactones, abscisic acid and brassinosteroids, this class of metabolites includes monoterpenes, sesquiterpenes and diterpenes, which play important roles in plant defense against herbivores and pathogens, as well as in plant reproduction by attracting pollinators and seed dispersers (Dudareva *et al.*, 2006). All terpenoids are derived from the universal five-carbon building blocks isopentenyl diphosphate (IPP) and its isomer dimethylallyl

diphosphate (DMAPP), which are synthesized in plants by two alternative pathways that are localized in different subcellular compartments (Ashour *et al.*, 2010; Hemmerlin *et al.*, 2012). The classical mevalonic acid (MVA) pathway, which is localized in the cytosol and partially in peroxisomes, gives rise to IPP and via enzymatic isomerization to DMAPP, both of which serve as substrates (at a 2:1 ratio) for cytosolic farnesyl diphosphate (FPP) synthase (FPPS) to form FPP. In contrast, the plastidic methylerythritol phosphate (MEP) pathway directly produces both IPP and DMAPP (at a 6:1 ratio) for downstream formation of geranyl diphosphate (GPP) and geranylgeranyl diphosphate (GGPP) by the plastidic enzymes GPP synthase (GPPS) and GGPP synthase (GGPPS), respectively. While FPP in the cytosol serves as a precursor for sterols/brassinosteroids, plastidic GGPP is utilized for chlorophyll, carotenoid, strigolactone, abscisic acid and gibberellin biosynthesis. Independently, cytosolic sesquiterpene synthases use FPP and plastidic mono- and diterpene synthases use GPP and GGPP, respectively, as substrates. Although separated in two subcellular compartments, there is substantial evidence for metabolic interaction between the MVA and MEP pathways, with IPP exchange potentially in both directions (Kasahara *et al.*, 2002; Nagata *et al.*, 2002; Hemmerlin *et al.*, 2003a; Laule *et al.*, 2003; Schuhr *et al.*, 2003; Dudareva *et al.*, 2005; Furumoto *et al.*, 2011). It was shown that the metabolic flux through the MEP pathway often exceeds that of the MVA pathway (Dudareva *et al.*, 2005; Wu *et al.*, 2006), and export of MEP pathway-derived IPP is of particular importance for cytosolic terpene biosynthesis in some plants (Adam *et al.*, 1999; Steliopoulos *et al.*, 2002; Dudareva *et al.*, 2005; Hampel *et al.*, 2005a,b; Orlova *et al.*, 2009). Trafficking of IPP across the inner envelope membrane of plastids is mediated by an as yet unidentified metabolite transporter (Soler *et al.*, 1993; Bick and Lange, 2003; Flügge and Gao, 2005).

While plastidic GGPPSs are homodimeric (Dogbo and Camara, 1987; Laferrière and Beyer, 1991; Burke and Croteau, 2002), GPPSs have either homodimeric or heterodimeric architectures depending on the plant species (Nagegowda, 2010). Heterodimeric GPPSs, like the one found in snapdragon (*Antirrhinum majus*), contain a catalytically inactive small subunit (GPPS-SSU) that interacts with a *bona fide* GGPPS subunit to form a heterodimer that catalyzes GPP formation (Tholl *et al.*, 2004; Wang and Dixon, 2009). Moreover, inactive GPPS-SSUs interact *in vitro* and *in planta* with GGPPSs from distant plant species to form functional heterodimeric GPPSs (Burke and Croteau, 2002; Tholl *et al.*, 2004; Orlova *et al.*, 2009; Wang and Dixon, 2009). With the exception of *Lithospermum erythrorhizon* (Sommer *et al.*, 1995; Li *et al.*, 1998), all known plant GPPS enzymes appear to be plastidic. However, some cytosolic sesquiterpene synthases, in addition to FPP, can use GPP as a substrate to form monoterpenes (see the comprehensive list in Table S1). In cultivated strawberry (*Fragaria ananassa*) fruits, the cytosolic terpene synthase FaNES1 produces roughly similar amounts of the sesquiterpene nerolidol and the monoterpene linalool (Aharoni *et al.*, 2004). Moreover, over-expression in the cytosol of monoterpene synthases (Ohara *et al.*, 2003; Wu *et al.*, 2006) and a sesquiterpene synthase possessing monoterpene synthase activity (Davidovich-Rikanati *et al.*, 2008) resulted in formation of monoterpenes in transgenic plants, suggesting the existence of a GPP pool in this cellular compartment. These data raise questions about the origin of cytosolic GPP and its potential transport between plastids and the cytosol. To address these questions, a metabolic engineering approach was used to co-express snapdragon *GPPS-SSU* with the geraniol synthase gene *GES*, encoding a plastidic monoterpene synthase (Davidovich-Rikanati *et al.*, 2007), and the zingiberene synthase gene *ZIS*, encoding a cytosolic terpene synthase that has been shown to possess both sesqui- and monoterpene synthase activities (Davidovich-Rikanati *et al.*, 2008) in ripening tomato (*Solanum lycopersicum*) fruits under the control of the polygalacturonase (*PG*) promoter. A significant increase in monoterpenes was observed in both cases, suggesting that plastid-produced GPP is not only used for plastidic monoterpene formation, but is also exported to support cytosolic monoterpene biosynthesis.

## RESULTS

### Over-expression of snapdragon GPPS-SSU enhances monoterpene formation in tomato fruits

To increase the plastidic GPP pool available for monoterpene biosynthesis, we diverted the metabolic flux from carotenoid formation, which is highly active in ripening tomato fruits, by over-expressing the snapdragon *GPPS-SSU* gene under control of the fruit ripening-specific *PG* promoter (Nicholass *et al.*, 1995). This promoter was chosen to limit *GPPS-SSU* expression to ripening fruits and avoid potential negative effects on vegetative tissues and general plant development (Orlova *et al.*, 2009). Four independent lines with different *GPPS-SSU* expression levels, as determined by quantitative real-time PCR (Figure 1a) and protein gel-blot analysis (Figure 1b), were chosen for further characterization. *GPPS-SSU* transcript and protein levels were highest in line E9, intermediate in lines B3 and B10, low in line D8, and undetectable in the untransformed MP–1 control (Figure 1a,b). Consistent with the *GPPS-SSU* expression profiles, GPPS activity increased across transgenic fruits by 1.6- to 2.2-fold relative to controls containing endogenous GPPS activity (Table 1). To date, the exact architecture of tomato GPPS is still unknown; however, the tomato genome contains a gene encoding SI-GPPS-SSU [*Solanum lycopersicum* (SI)], which belongs to a recently described SSU II sub-family (Wang and Dixon, 2009), and

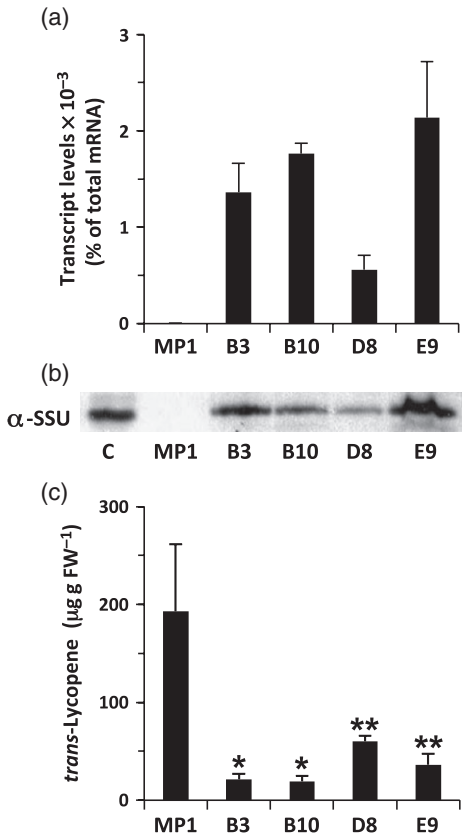


**Figure 1.** Profiling of selected transgenic tomato lines over-expressing snapdragon *GPPS-SSU* under the control of the fruit ripening-specific *PG* promoter.
(a) Transcript levels of *GPPS-SSU* in Br + 3 stage fruits of MP–1 (control), B3, B10, D8 and E9 *GPPS-SSU* transgenic lines determined by quantitative real-time PCR (means ± SE, $n = 3$ biological replicates).
(b) GPPS-SSU protein levels in MP–1 and transgenic tomato fruits. The representative protein gel shows the 30 kDa protein recognized by anti-AmGPPS-SSU antibodies. Protein extract from snapdragon flowers was used as a positive control (C).
(c) *trans*-lycopene levels in Br + 10 stage fruits of MP–1 and *GPPS-SSU* transgenic tomato lines (means ± SE, $n = 3$). Asterisks indicate statistically significant differences compared with control (*$P < 0.03$ and **$P < 0.05$, Student's *t* test). FW, fresh weight.

its expression did not change in transgenic fruits compared to control (Figure S1). An earlier proposed homodimeric tomato GPPS (van Schie *et al.*, 2007a) is highly homologous to the Arabidopsis At2g34630 gene product, which was recently identified as a *trans*-type polyprenyl pyrophosphate synthase (PPPS; Hsieh *et al.*, 2011; Ducluzeau *et al.*, 2012) and is not expressed in tomato fruits based on quantitative real-time PCR using gene-specific primers.

As expected from a *PG* promoter-driven expression, no *GPPS-SSU* transcripts were detected in fruits at the mature green (MG) stage (Figure S2a). *GPPS-SSU* transcripts began to accumulate at the breaker (Br) stage, reached the highest level 3 days after the Br stage (Br + 3), and declined in ripe fruits (Br + 10). *GPPS-SSU* over-expression reduced carotenoid accumulation, which was visible at various ripening stages (Figure S2b). Indeed, levels of *trans*-lycopene, the most abundant carotenoid in tomato fruits, decreased by 69–90% in transgenic fruits relative to control (Figure 1c and Figure S3b), showing negative correlation with *GPPS-SSU* expression. However, the levels of some lycopene-derived carotenoids, including β–carotene, zeaxanthin and lutein (Figure S3a), remained unaltered in the *GPPS-SSU* transgenic fruits (Figure S3b). The reduction in lycopene levels was not due to down-regulation of expression of the MEP pathway genes encoding 1–deoxy-D–xylulose 5–phosphate synthase (*DXS*) and 1–deoxy-D–xylulose 5–phosphate reductoisomerase (*DXR*), or those encoding the carotenoid biosynthetic enzymes phytoene synthase (*PSY–1* and *PSY–2*) and phytoene desaturase (*PDS*), whose transcript levels were similar in transgenic and control fruits (Figure S1). Of two *GGPPS* genes in the tomato genome, only one, *GGPPS–2,* is expressed in fruits (Ament *et al.*, 2006). Interestingly, its expression was up-regulated in the *GPPS-SSU* transgenic line (Figure S1), but there were no changes in GGPPS activity (Table 1).

To examine the effect of *GPPS-SSU* expression on the level of monoterpenes, volatiles were collected from ripe tomato fruits (Br + 10) for 24 h and analyzed by GC–MS. Marked differences in monoterpene profiles were observed between transgenic and control fruits (Figure 2a). Transgenic fruits produced geranial, neral and citronellol, and, to a lesser extent, geraniol, citronellal and linalool, but all these volatiles were absent in control fruits, except for a small amount of geranial (Figure 2a and Table S2). Internal

**Table 1** Prenyltransferase activities in crude extracts from control and *GPPS-SSU* tomato fruits (Br + 3)

| | | *GPPS-SSU* lines | | | |
|---|---|---|---|---|---|
| Activity | MP–1 | B3 | B10 | D8 | E9 |
| GPPS[a] | 1.70 ± 0.01 | 3.33 ± 0.66* | 3.22 ± 0.26** | 2.76 ± 0.01** | 3.69 ± 0.17** |
| FPPS[a] | 1.43 ± 0.38 | 1.47 ± 0.19 | 1.53 ± 0.19 | 1.53 ± 0.40 | 1.15 ± 0.24 |
| GGPPS[b] | 0.78 ± 0.06 | 0.90 ± 0.06 | 0.81 ± 0.05 | 0.91 ± 0.10 | 0.78 ± 0.07 |

Values are pkat $mg^{-1}$ protein (means ± SE, $n = 3$–6).
Asterisks indicate statistically significant differences compared with control (MP–1): *$P < 0.03$, **$P < 0.01$ by Student's *t* test.
[a]With IPP and DMAPP substrate.
[b]With IPP and FPP substrate.

 

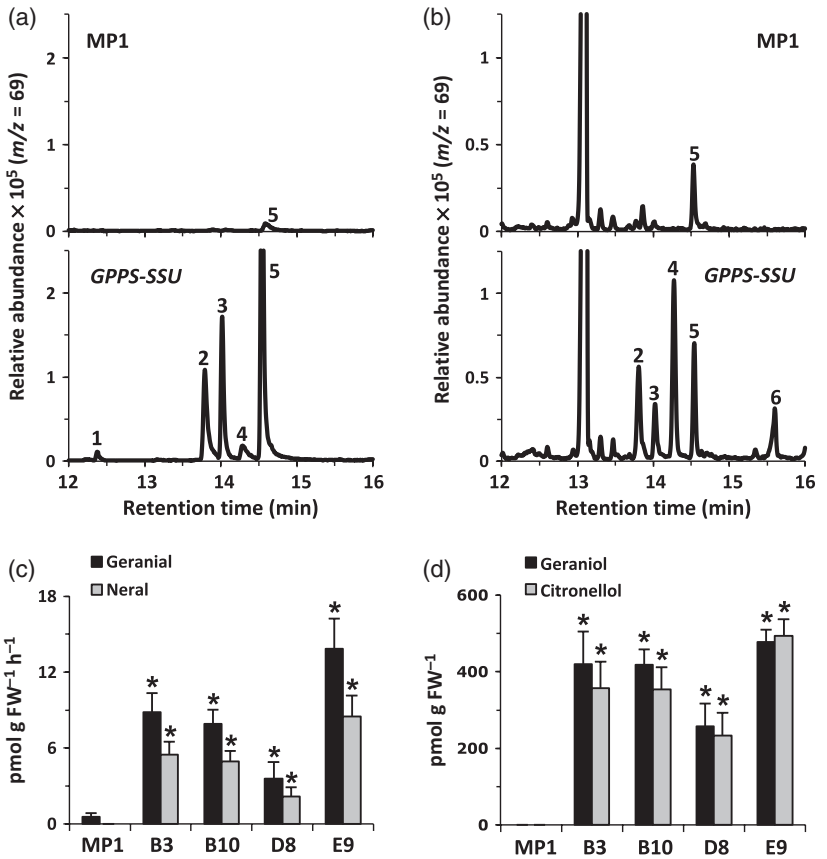


**Figure 2.** Effect of *GPPS-SSU* over-expression on the monoterpene profile in transgenic tomato fruits.
(a, b) Metabolic profiling of monoterpenes collected as emitted volatiles (a) and directly extracted (b) from ripe fruits of MP–1 and *GPPS-SSU* transgenic tomato lines. Volatiles were analyzed by GC–MS and traces obtained for $m/z = 69$ (characteristic for monoterpenes) are shown. Compounds were identified based on their mass spectra and retention time: 1, citronellal; 2, citronellol; 3, neral; 4, geraniol; 5, geranial; 6, geranic acid.
(c, d) Quantitative changes in abundant monoterpenes collected as emitted volatiles (c) and extracted (d) from fruits of MP–1 and *GPPS-SSU* transgenic tomato lines. Data are means ± SE ($n \geq 3$). Asterisks indicate statistically significant differences compared with control (*$P < 0.005$, Student's *t* test). FW, fresh weight.

pools of geraniol, citronellol, neral and geranic acid were also significantly increased in ripe *GPPS-SSU* transgenic tomato fruits, but the level of geranial increased only in the highly expressing E9 line (Figure 2b and Table S2). The amounts of monoterpenes, both those collected as emitted volatiles and directly extracted from fruits (Figure 2c,d and Table S2), positively correlated with the *GPPS-SSU* transcript and protein levels in the various transgenic lines (Figure 1a,b). These results indicate that the introduced snapdragon GPPS-SSU in tomato fruits diverted the metabolic flux of the plastidic terpenoid pathway from carotenoids towards GPP, which was subsequently converted to various monoterpenes.

### Co-expression of snapdragon GPPS-SSU with lemon basil GES significantly increases monoterpene accumulation in tomato fruits

Recently, it was shown that the tomato genome contains 44 terpene synthase (TPS) genes, 26 of which are functional (Bleeker *et al.*, 2011; Falara *et al.*, 2011). Among the six TPSs found to be expressed in ripe tomato fruits, only one monoterpene synthase, TPS5/MTS1, uses GPP as a substrate to produce linalool (van Schie *et al.*, 2007b). While a small amount of linalool was indeed found in *GPPS-SSU*-expressing tomato fruits (Table S2), the formation of other produced monoterpenes (Figure 2) cannot be attributed to TPS5/MTS1 action and is probably the result of the action of endogenous enzymes metabolizing GPP.

The low level of endogenous monoterpene synthases capable of utilizing GPP, and the presence of endogenous enzymes that use GPP in ripening tomato fruits, raise the question of whether the increased plastidic GPP pool in *GPPS-SSU* transgenic fruits is available for enzymatic conversion by introduced monoterpene synthases. Therefore, line E9 with the highest *GPPS-SSU* expression was crossed with a transgenic tomato line over-expressing *O. basilicum GES* under the control of the *PG* promoter (Davidovich-Rikanati *et al.*, 2007). In the resulting $F_1$ plants (*GPPS-SSU* × *GES*), the *GPPS-SSU* and *GES* transcript levels were comparable with those in fruits of parental lines (Figure 3a). Analysis of volatiles produced by ripe tomato fruits (Br + 10) of parental lines, as well as the cross, revealed the presence of the same monoterpene compounds, including citronellol, citronellal, geraniol, geranial and neral, (Figure 3b), with the exception of citronellyl acetate, which was not detected in *GPPS-SSU* fruits. While the composition of the monoterpene profile produced by fruits of the three analyzed lines was practically identical, the absolute amounts differed drastically between them, with the lowest amounts found with *GPPS-SSU* fruits and the highest amounts with *GPPS-SSU* × *GES* fruits (Figure 3b). Quantitative analysis of monoterpenes collected from *GPPS-*

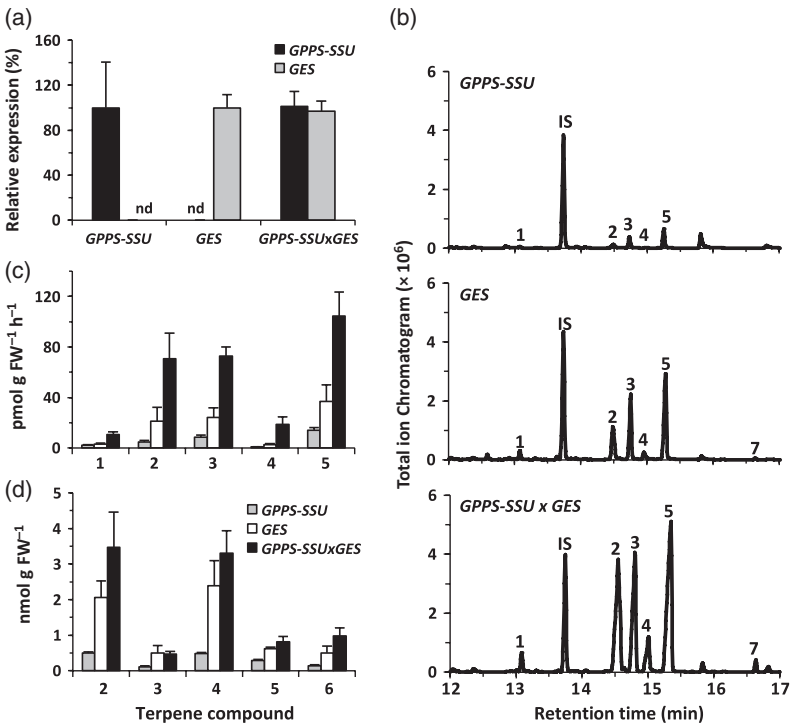


**Figure 3.** Effect of *GPPS-SSU* and *GES* co-expression on the monoterpene profile in transgenic tomato fruits.
(a) Transcript levels of *GPPS-SSU* and *GES* in Br + 3 stage fruits of *GPPS-SSU*, *GES* and *GPPS-SSU* × *GES* transgenic lines determined by quantitative real-time PCR (means ± SE, $n = 3$ biological replicates). Expression in *GPPS-SSU* × *GES* line is presented as a percentage of the corresponding value in the respective parental line, set as 100%.
(b) Metabolic profiling of volatiles collected from ripe fruits of *GPPS-SSU*, *GES* and *GPPS-SSU* × *GES* lines. Volatiles were analyzed by GC–MS, and total ion currents are shown. Compounds were identified based on their mass spectra and retention time: 1, citronellal; 2, citronellol; 3, neral; 4, geraniol; 5, geranial; 6, geranic acid, 7, citronellyl acetate; IS, internal standard (naphthalene).
(c, d) Quantitative changes in monoterpenes collected as emitted volatiles (c) and extracted (d) from fruits of *GPPS-SSU*, *GES* and *GPPS-SSU* × *GES* lines. Data are means ± SE ($n \geq 3$). The amounts of volatiles emitted from *GPPS-SSU* × *GES* fruits relative to both parental lines (c) and extracted from *GPPS-SSU* × *GES* fruits relative to *GPPS-SSU* fruits (d) are significantly different ($P < 0.02$ by Student's *t* test). FW, fresh weight.

*SSU* × *GES* fruits (Figure 3c and Table S2) revealed on average 6.9- and 19.2-fold increases in geraniol, 2.8- and 7.5-fold increases in geranial, 3.0- and 8.6-fold increases in neral, 3.3- and 14.4-fold increases in citronellol and 3.5- and 5.4-fold increases in citronellal when compared to fruits of the *GES* and *GPPS-SSU* parental lines, respectively. Likewise, quantitative analysis of internal monoterpene pools accumulated in *GPPS-SSU* × *GES* fruits (Figure 3d and Table S2) revealed on average 7-fold increase in geraniol, geranic acid and citronellol, as well as 2.9- and 4.4-fold increases in geranial and neral, respectively, relative to fruits of the *GPPS-SSU* parental line. However, when *GPPS-SSU* × *GES* fruits were compared with the *GES* parental line, no statistically significant differences in internal monoterpene pools were detected (Figure 3d). The *GES* parental line also produced a small amount of myrcene, limonene and ocimene of unknown origin (Davidovich-Rikanati *et al.*, 2007), the levels of which remained unchanged in *GPPS-SSU* × *GES* fruits (Table S2).

### Co-expression of snapdragon GPPS-SSU with lemon basil ZIS increases cytosolic monoterpene formation in tomato fruits

It is well established that the MEP pathway provides precursors not only for plastidic monoterpene formation, but also supports terpene biosynthesis in the cytosol via export of IPP across the plastid envelope membranes (Dudareva *et al.*, 2005; Hampel *et al.*, 2005a,b; Orlova *et al.*, 2009). As some cytosolic bifunctional terpene synthases exhibit both mono- and sesquiterpene synthase activities, we analyzed whether increased plastidic GPP formation also affects monoterpene biosynthesis in the cytosol. The *GPPS-SSU* line E9 was crossed with a transgenic tomato line over-expressing *O. basilicum ZIS*, encoding a cytosolic terpene synthase that not only uses FPP to form α–zingiberene and several other sesquiterpenes, but also accepts GPP as a substrate to produce a number of monoterpenes *in vitro* as well as *in planta* (Davidovich-Rikanati *et al.*, 2008). In the resulting $F_1$ plants (*GPPS-SSU* × *ZIS*), the *GPPS-SSU* transcript levels were not significantly different from those in the *GPPS-SSU* parental line (Figure 4a), while *ZIS* mRNA levels were reduced relative to the *ZIS* parental line (Figure 4a), probably due to the presence of several segregating copies of the *ZIS* transgene in the parental line. Analysis of volatiles collected from ripe tomato fruits (Br + 10 stage) of *ZIS* parental and *GPPS-SSU* × *ZIS* lines revealed that the sesquiterpene profiles were identical (Figure 4b), including 7–*epi*–sesquithujene, *E*–α–bergamotene, α–zingiberene and β–bisabolene. However, the amounts of sesquiterpenes emitted from *GPPS-SSU* × *ZIS* fruits, as well as the internal pools of sesquiterpenes, were reduced on average by 63–95% (Figure 4c and Table S3) and 42–83% (Figure 4d and Table S3), respectively, relative to the *ZIS* parental line, consistent with the 86% reduction in *ZIS* transcript levels in the *GPPS-SSU* × *ZIS* cross (Figure 4a). However, we cannot exclude the possibility that this reduction in sesquiterpene levels was partly due to a reduced plastidic IPP pool and its export to the cytosol, as observed

**Table 2** Subcellular fractionation of crude extracts from *GPPS-SSU* × *ZIS* tomato fruits

| Marker | Crude extract | Cytosolic fraction | Plastid fraction |
|---|---|---|---|
| ADH[a] | 16.13 ± 0.80 | 19.60 ± 1.10 | 0.25 ± 0.03 |
| GAPDH[b] | 784.95 ± 67.05 | 482.68 ± 118.42 | 1557.35 ± 95.38 |
| ZIS[b] | 0.48 ± 0.04 | 0.46 ± 0.07 | ND |
| GPPS[b] | 1.90 ± 0.08 | 0.98 ± 0.05 | 3.46 ± 0.15 |

Activities of marker enzymes, ZIS and GPPS were assayed in crude extracts, cytosolic and plastid fractions from tomato fruits. Alcohol dehydrogenase (ADH) and glyceraldehyde 3–phosphate dehydrogenase (GAPDH) were used as marker enzymes for the cytosol and plastids, respectively. Data are means ± SE ($n$ = 3). ND, not detected.
[a]Specific activities in nkat $mg^{-1}$ protein.
[b]Specific activities in pkat $mg^{-1}$ protein.

in transgenic *GPPS-SSU* tobacco (*Nicotiana tabacum*) plants (Orlova *et al.*, 2009).

Analysis of monoterpenes in these lines revealed that ripe *GPPS-SSU* × *ZIS* fruits produce comparable levels of geranial, neral and citronellol to those detected for fruits of the *GPPS-SSU* parental line, but these compounds were almost absent in the fruits of transgenic plants expressing *ZIS* alone (Figure 5a). In contrast, the levels of ZIS-derived monoterpenes, including α–thujene, α–pinene, sabinene and γ–terpinene, were significantly higher in *GPPS-SSU* × *ZIS* fruits than in *ZIS* fruits (Figure 5b,c and Table S3), despite an 86% decrease in *ZIS* mRNA abundance in the *GPPS-SSU* × *ZIS* cross. The increases in these monoterpenes, both those collected as emitted volatiles and directly extracted from fruits, ranged on average from 1.9- to 7.3-fold (Figure 5c and Table S3) and 2.1- to 4.6-fold (Figure 5d and Table S3), respectively, compared to fruits of the *ZIS* parental line. These results suggest that the increased GPP pool produced by over-expression of *GPPS-SSU* in plastids is available not only to monoterpene synthases (i.e. GES) present in the same subcellular compartment, but also to cytosolically localized terpene synthases, such as ZIS, which are able to use GPP as a substrate to form monoterpenes. To biochemically confirm the cytosolic localization of ZIS and the plastidic localization of GPPS, chromoplasts were isolated from *GPPS-SSU* × *ZIS* fruits, and enzyme activities were analyzed in crude extracts, cytosolic and plastidic fractions together with marker enzymes for assessing subcellular enrichment (Table 2). ZIS activity was found exclusively in the cytosol, while GPPS activity was detected in both plastids and cytosol. GPPS activity showed the same level of enrich-

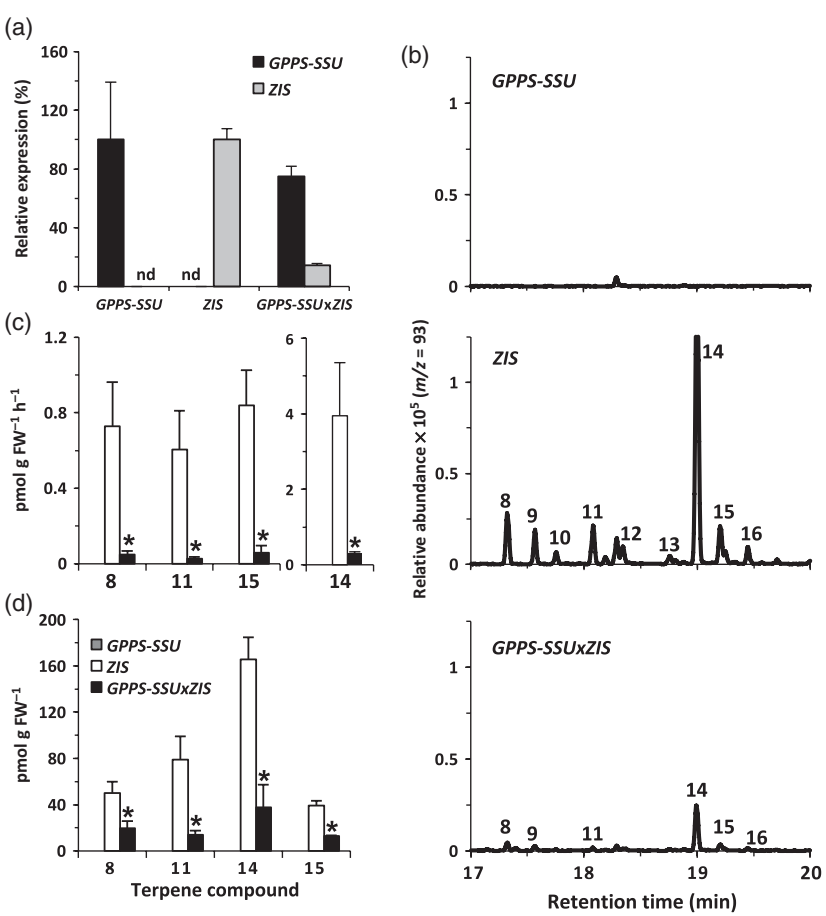


**Figure 4.** Effect of *GPPS-SSU* and *ZIS* co-expression on the sesquiterpene profile in transgenic tomato fruits.
(a) Transcript levels of *GPPS-SSU* and *ZIS* in Br + 3 stage fruits of *GPPS-SSU*, *ZIS* and *GPPS-SSU* × *ZIS* transgenic lines determined by quantitative real-time PCR (means ± SE, $n$ = 3 biological replicates). Expression in *GPPS-SSU* × *ZIS* is presented as a percentage of the corresponding value in the respective parental line, set as 100%.
(b) Metabolic profiling of volatiles collected from ripe fruits of *GPPS-SSU*, *ZIS* and *GPPS-SSU* × *ZIS* lines. Volatiles were analyzed by GC–MS, and traces obtained for $m/z$ = 93 (typical for the sesquiterpenes) are shown. The compounds were identified based on their mass spectra and retention time: 8, 7–*epi*–sesquithujene; 9, sesquithujene; 10, *Z*–α–bergamotene; 11, *E*–α–bergamotene; 12, β–farnesene; 13, α–curcumene; 14, α–zingiberene; 15, β–bisabolene; 16, β–sesquiphellandrene.
(c, d) Quantitative changes in sesquiterpenes collected as emitted volatiles (c) and extracted (d) from fruits of *GPPS-SSU*, *ZIS* and *GPPS-SSU* × *ZIS* lines. Data are means ± SE ($n \geq 3$). Asterisks indicate statistically significant differences (*$P < 0.02$ by Student's $t$ test) for line *GPPS-SSU* × *ZIS* relative to line *ZIS*. FW, fresh weight.

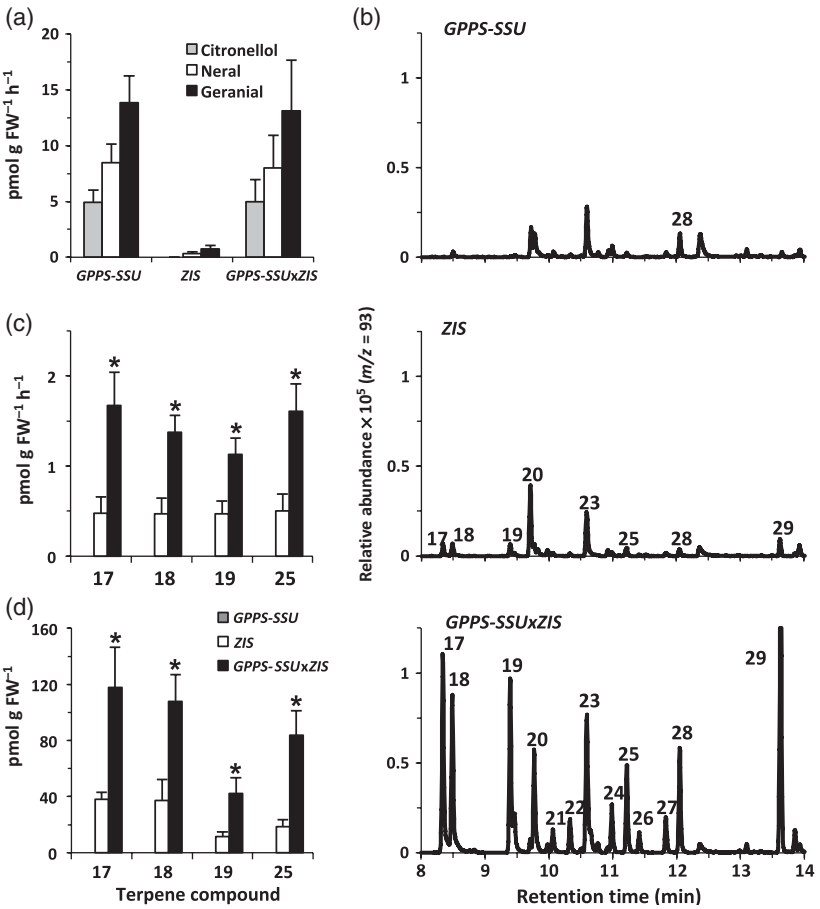


**Figure 5.** Effect of *GPPS-SSU* and *ZIS* co-expression on the monoterpene profile in transgenic tomato fruits.
(a) Quantitative analysis of GPPS-SSU-derived monoterpenes collected as emitted volatiles from fruits of *GPPS-SSU*, *ZIS* and *GPPS-SSU* × *ZIS* lines. Data are means ± SE ($n \geq 3$).
(b) Metabolic profiling of volatiles collected from ripe fruits of *GPPS-SSU*, *ZIS* and *GPPS-SSU* × *ZIS* lines. Volatiles were analyzed by GC–MS, and traces obtained for $m/z = 93$ (characteristic for monoterpenes) are shown. Compounds were identified based on their mass spectra and retention time: 17, α–thujene; 18, α–pinene; 19, sabinene; 20, β–myrcene; 21, α–phellandrene; 22, α–terpinene; 23, limonene; 24, β–ocimene; 25, γ–terpinene; 26, β–terpineol; 27; terpinolen; 28, linalool; 29, terpinen-4–ol.
(c, d) Quantitative changes in ZIS-derived monoterpenes collected as emitted volatiles (c) and extracted (d) from fruits of *GPPS-SSU*, *ZIS* and *GPPS-SSU* × *ZIS* lines. Data are means ± SE ($n \geq 3$). Asterisks indicate statistically significant differences (*$P < 0.02$ by Student's *t* test) for line *GPPS-SSU* × *ZIS* relative to line *ZIS*. FW, fresh weight.

ment in the plastidic and cytosolic fractions (1.82- and 0.52-fold, respectively) as the plastidic marker NADP-linked glyceraldehyde 3–phosphate dehydrogenase (GAPDH; 1.98- and 0.62-fold, respectively), indicating that GPPS activity exists in plastids and that the activity observed in the cytosolic fraction derives from plastids that are broken during fractionation.

## DISCUSSION

### GPPS-SSU over-expression provides substrate for monoterpene formation in plastids

GPPS is the key branchpoint enzyme leading to monoterpene biosynthesis, and is generally localized in plastids (Tholl *et al.*, 2004). We hypothesized that an increase in plastidic GPPS activity via over-expression of the corresponding prenyltransferase in transgenic plants would re-direct the metabolic flux of MEP pathway-derived IPP and DMAPP towards GPP formation, and thus enhance the availability of substrate for monoterpene biosynthesis. The catalytically inactive GPPS-SSU of heteromeric snapdragon GPPS was previously found to interact with phyllogenetically distant GGPPSs and change their product specificity from GGPP to GPP (Tholl *et al.*, 2004; Orlova *et al.*, 2009). Over-expression of snapdragon *GPPS-SSU* in transgenic tobacco plants significantly increased GPPS activities in chloroplasts, as well as the levels of monoterpenes emitted from leaves and flowers compared to control plants (Orlova *et al.*, 2009), indicating that the GPP pool available to endogenous monoterpene synthases limited overall monoterpene production. Unfortunately, *GPPS-SSU* tobacco lines displayed severe leaf chlorosis and dwarfism, probably due to reduced synthesis of GGPP required for production of chlorophyll, carotenoids and gibberellic acid (Orlova *et al.*, 2009).

Here, snapdragon *GPPS-SSU* was over-expressed in transgenic tomato under the control of the *PG* promoter in order to restrict expression to ripening fruits. In ripening wild-type tomato fruits, the plastidic MEP pathway is highly active to provide GGPP precursors for the massive accumulation of lycopene and other carotenoids (Lawrence *et al.*, 1997; Lois *et al.*, 2000; Botella-Pavia *et al.*, 2004; Paetzold *et al.*, 2010). However, only minute amounts of monoterpenes are formed (Buttery *et al.*, 1990; Baldwin *et al.*, 2000; Lewinsohn *et al.*, 2001; Davidovich-Rikanati *et al.*, 2007) due to the limited number of endogenous monoterpene synthases expressed in fruits (Bleeker *et al.*, 2011; Falara *et al.*, 2011). *GPPS-SSU* over-expression

in transgenic tomato plants had no obvious deleterious effects on vegetative tissues and general plant development, while lycopene levels in ripe fruits were reduced by up to 90% in some *GPPS-SSU* lines compared to controls (Figure 1c, and Figures S2b and S3b). In contrast, fruits of transgenic tomato plants expressing *GES* (Davidovich-Rikanati *et al.*, 2007) showed only a 50% reduction in lycopene levels despite even higher monoterpene production (Figure 3b–d). Therefore, the severe reduction of lycopene levels in *GPPS-SSU* fruits cannot be attributed solely to a re-direction of IPP/DMAPP metabolic flux from GGPP to GPP formation. The change in GGPPS product specificity via the interaction with introduced snapdragon GPPS-SSU may also contribute to the reduction of lycopene formation as shown in tobacco (Orlova *et al.*, 2009). However, in contrast to *GPPS-SSU*-expressing tobacco plants, GGPPS activity in *GPPS-SSU* tomato fruits was not reduced (Table 1), probably due to up-regulation of *GGPPS–2* expression (Figure S1). To date, the reason for the reduction in the lycopene level remains unknown, but it occurs post-transcriptionally (Figure S1), probably via feed-forward regulation of lycopene biosynthetic enzymes by one (or several) products formed in transgenic lines. Indeed, phytoene synthase from *Capsicum annum* was shown to be inhibited by inorganic pyrophosphate, one of the products of the GPPS-catalyzed reaction (Dogbo *et al.*, 1988). In addition, unaltered levels of β–carotene, lutein and zeaxanthin in *GPPS-SSU*-expressing lines suggest a complex regulation of carotenoid biosynthesis, which ensures the homeostasis of certain carotenoids.

The production of monoterpenes in ripe fruits of the *GPPS-SSU* tomato lines, both as emitted volatiles and endogenous internal pools (Figure 2 and Table S2), and their positive correlation with *GPPS-SSU* expression (Figure 1a,b), provides evidence for an increased plastidic GPP pool in transgenic fruits. However, the monoterpenes produced were not the result of action of endogenous monoterpene synthases, but of endogenous phosphatases (Perez *et al.*, 1980; Izumi *et al.*, 1996; Ganjewala and Luthra, 2009), reductase(s) and alcohol dehydrogenase(s) (Bicsak *et al.*, 1982; Davidovich-Rikanati *et al.*, 2007), which were previously shown to be present in tomato fruits (Davidovich-Rikanati *et al.*, 2007). Geraniol and linalool were the products of enzymatic hydrolysis of GPP by phosphatases. Geraniol was subsequently converted to citronellol by a reductase similar to that found in rose (*Rosa x hybrida*) (Dunphy and Allcock, 1972). Both geraniol and citronellol were probably further oxidized to the monoterpene aldehydes geranial (which reversibly isomerizes to neral) and citronellal, respectively, by alcohol dehydrogenase(s) as described in several plant species (Potty and Bruemmer, 1970; Bicsak *et al.*, 1982; Singh Sangwan *et al.*, 1993; Sekiwa-Iijima *et al.*, 2001; Luan *et al.*, 2005; Iijima *et al.*, 2006).

Over-expression of the monoterpene synthase gene *GES* in the *GPPS-SSU* background (*GPPS-SSU* × *GES* transgenic line) resulted in increased production of geraniol and its derivatives relative to parental *GPPS-SSU* and *GES* lines (Figure 3 and Table S2), suggesting that the increased plastidic GPP pool was available to GES for monoterpene formation. Interestingly, only the total amounts of monoterpenes detected in the headspace of *GPPS-SSU* × *GES* fruits were increased (Figure 3c and Table S2), but the internal monoterpene pools remained unchanged (Figure 3d and Table S2) compared to *GES* fruits. These results suggest that the overall metabolic flux through the engineered pathway was increased, with the internal monoterpene pools being saturated.

There are numerous successful examples of metabolic engineering via over-expressing plastidic monoterpene synthases in transgenic plants (Dudareva and Pichersky, 2008); however, to date a co-expression approach has only been used a few times. Wu *et al.* (2006) observed a roughly fivefold higher monoterpene accumulation by co-expressing a plastid-targeted *GPPS* and a lemon (*Citrus limon*) limonene synthase gene in transgenic tobacco plants compared with plants expressing the monoterpene synthase alone. Likewise, co-expression of a FPPS with a sesquiterpene synthase in tobacco lead to a drastic increase in product formation compared to plants only expressing the latter enzyme (Wu *et al.*, 2006). The data presented here, as well as those reported by Wu *et al.* (2006), demonstrate that simultaneous expression of prenyltransferases and TPSs results in significantly higher formation of terpenes compared to expression of either of the two enzymes alone.

### The increased plastidic GPP pool supports monoterpene formation in the cytosol

Recently, transgenic plants expressing a sesquiterpene synthase with monoterpene synthase activity (Davidovich-Rikanati *et al.*, 2008), and monoterpene synthases lacking their plastid-targeting transit peptide (Ohara *et al.*, 2003; Wu *et al.*, 2006), were found to produce some monoterpenes, suggesting the presence of a small GPP pool in the cytosol. Moreover, a cytosolic terpene synthase in cultivated strawberry (FaNES1) was shown to use GPP as well as FPP as substrates to produce roughly similar amounts of the monoterpene linalool and the sesquiterpene nerolidol, respectively (Aharoni *et al.*, 2004). While these discoveries further support the existence of a cytosolic GPP pool, they raise questions about the origin of GPP in this subcellular compartment.

GPPSs, irrespective of their homodimeric or heterodimeric architecture, are generally considered to be plastid-localized. The only known cytosolic GPPS exists in *Lithospermum erythrorhizon* and is involved in MVA pathway-dependent formation of the hemiterpenoid shikonin

(Sommer *et al.*, 1995; Li *et al.*, 1998). Even though GPP represents an intermediate in the biosynthesis of FPP catalyzed by cytosolic FPPS, it was assumed that GPP stays bound to the active site of the enzyme. However, small amounts of GPP have been observed as a product in *in vitro* assays using recombinant FPPSs (Burke and Croteau, 2002; Hemmerlin *et al.*, 2003b). Moreover, it has been hypothesized that the product specificity of FPPSs may be influenced by the relative concentrations of IPP and DMAPP substrates, with lower IPP concentrations leading to increased GPP formation (Szkopinska and Plochocka, 2005).

Despite the fact we cannot exclude the possibility that some cytosolic GPP may be formed as a side product of the reaction catalyzed by FPPS, our data obtained upon co-expression of the plastidic *GPPS-SSU* with the cytosolic terpene synthase *ZIS* suggest a different scenario for the origin of the cytosolic GPP pool. While transgenic tomato lines expressing *ZIS* alone accumulated high levels of α–zingiberene, as well as several other sesquiterpenes, in ripening fruits, they also contained small amounts of monoterpenes, including α–thujene, α–pinene, sabinene and γ–terpinene (Figure 5; Davidovich-Rikanati *et al.*, 2008), consistent with the ability of ZIS to use GPP as a substrate. However, the total amount of ZIS-derived monoterpenes in ripe transgenic tomato fruits was increased by more than 3.5-fold when cytosolic *ZIS* was co-expressed with plastidic *GPPS-SSU* (Figure 5 and Table S3). The observed increase in cytosolic monoterpene production is even more remarkable considering that *ZIS* expression was lower in the *GPPS-SSU* × *ZIS* fruits compared to the parental *ZIS* line (roughly one-fifth, Figure 4a). This unprecedented finding suggests that (i) the increased GPP pool in plastids due to *GPPS-SSU* over-expression also supports monoterpene formation in the cytosol, and (ii) GPP export from plastids to the cytosol occurs.

To date, ample evidence for metabolic exchange between MVA pathway-derived and MEP pathway-derived IPP pools in the cytosol and plastids has been collected. In particular, IPP export from plastids (Figure 6) was shown to be of importance for cytosolic sesquiterpene synthesis in several plants (Steliopoulos *et al.*, 2002; Dudareva *et al.*, 2005; Hampel *et al.*, 2005a,b; Orlova *et al.*, 2009). In addition, transporter-mediated trafficking of IPP across the inner envelope membrane of plastids has been demonstrated using isolated organelles as well as isolated plastid envelope proteins reconstituted into liposomes (Soler *et al.*, 1993; Bick and Lange, 2003; Flügge and Gao, 2005). However, substrate competition studies using proteoliposomes containing reconstituted inner envelope membrane proteins from spinach (*Spinacia oleracea*) chloroplast showed that this as yet unidentified transporter not only accepts IPP as a substrate, but also GPP, FPP and DMAPP (Bick and Lange, 2003). Analysis of its kinetic properties using the same system revealed that IPP and GPP are the preferred substrates for this unknown transporter, displaying similar $K_m$ and $V_{max}$ values (Bick and Lange, 2003). Our data support these *in vitro* assays, providing *in vivo* proof of GPP export from plastids to the cytosol for monoterpene synthesis (Figure 6).

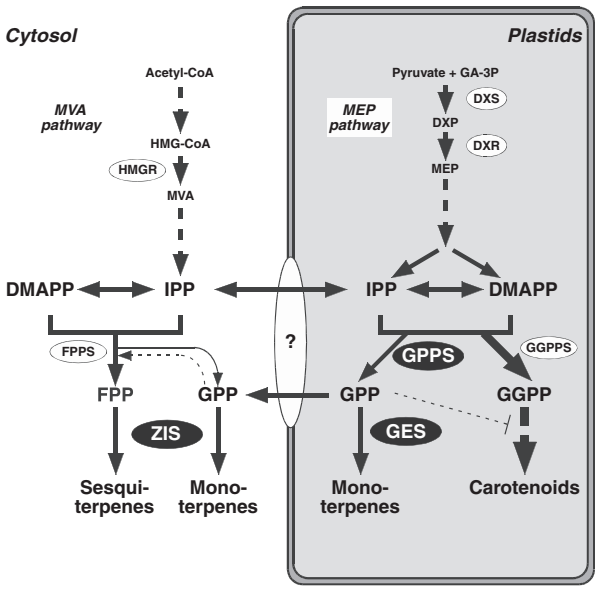


**Figure 6.** Plastidic and cytosolic monoterpene biosynthesis in plants.
The plastidic MEP and cytosolic MVA pathways lead to formation of IPP and DMAPP. Subsequent formation of cytosolic FPP and plastidic GPP as well as GGPP is catalyzed by FPPS, GPPS and GGPPS, respectively. Export of IPP and GPP from plastids to the cytosol is mediated by an as yet unidentified transporter localized in the inner envelope membrane (shown as a white oval with a question mark). The small cytosolic GPP pool may be a side product of FPPS, which accepts GPP as a substrate for FPP biosynthesis in some plants (shown by the dotted arrow). Enzymes over-expressed in tomato fruits are shown in black ovals. GPP serves as a substrate for cytosolic and plastidic monoterpene synthases (here ZIS and GES, respectively), while FPP is used by cytosolic sesquiterpene synthases (here ZIS). The proposed feed-forward regulation of carotenoid biosynthetic enzymes by products of the over-expressed GPPS-SSU enzyme is indicated by a dotted line. Dashed arrows indicate involvement of multiple enzymatic steps. DXP, 1–deoxy-D–xylulose 5–phosphate; DXR, 1–deoxy-D–xylulose 5–phosphate reductoisomerase; DXS, 1–deoxy-D–xylulose 5–phosphate synthase; GA–3P, D–glyceraldehyde 3–phosphate; HMG-CoA, 3–hydroxy-3–methyl-glutaryl CoA; HMGR, 3–hydroxy-3–methylglutaryl CoA reductase; MEP, 2–C–methyl-D–erythritol 4–phosphate; MVA, mevalonate.

The GPP export from plastids, and, as a consequence, the existence of a cytosolic GPP pool, shed new light on the function of cytosolic sesquiterpene synthases. To date, a diverse set of sesquiterpene synthases from various plant species, including gymnosperms and angiosperms (monocots and dicots), appears to be active with GPP in addition to FPP, and thus are able to produce monoterpenes (see Table S1). However, in the absence of a systematic characterization of all known plant sesquiterpene synthases with respect to their ability to use GPP, it is difficult to evaluate how widespread such cytosolic monoterpene synthase activity is. Thus, it should be considered that some monoterpenes produced in plant tissues may be synthesized by cytosolic (sesqui)terpene synthases using

plastid-derived GPP rather than by plastidic monoterpene synthases. Stable isotope labeling experiments using 1–[1–$^{13}$C]deoxy-D–xylulose and [1–$^{13}$C]glucose with subsequent analysis of labeling patterns and isotope abundances revealed that, in chamomile (*Matricaria recutita*) sesquiterpenes, the first two isoprene units are predominantly formed via the MEP pathway, while the third unit has mixed MVA/MEP origin, suggesting incorporation of plastidic GPP into cytosolically synthesized FPP and sesquiterpenes (Adam and Zapp, 1998; Adam *et al.*, 1999). Therefore, it is possible that export of MEP pathway-derived GPP from the plastids may also support cytosolic sesquiterpene synthesis in some plants.

## EXPERIMENTAL PROCEDURES

### Plant material and growth conditions

Tomato (*Solanum lycopersicum*) line MP–1 (Barg *et al.*, 1997) was used for generation of transgenic plants as well as a control for all analyses. Plants were grown under a 14 h photoperiod in standard greenhouse conditions. MP–1 plants, as well as transgenic *GES* and *ZIS* tomato lines (Davidovich-Rikanati *et al.*, 2007, 2008), were initially grown from seeds. Subsequently, all transgenic lines (*GPPS-SSU*, *GES* and *ZIS*) were propagated by re-rooting of cuttings on soil.

### Vector construction and plant transformation

The *A. majus GPPS-SSU* coding region was cloned into the binary vector pBIN19 (Fray *et al.*, 1994), which contains the tomato fruit-specific *PG* promoter (4.8 kb) and *PG* terminator (1.8 kb), and the kanamycin-resistance marker gene *NPTII*, driven by the CaMV 35S promoter (Nicholass *et al.*, 1995). The binary vector was introduced into *Agrobacterium tumefaciens* strain c58C1/pMP90 (Koncz and Schell, 1986), which was used for transformation. Tomato MP–1 plants were transformed as described previously (McCormick *et al.*, 1986) using kanamycin (100 mg $L^{-1}$) for selection. Transgenic plants rooted on kanamycin-containing medium were transferred to soil and adapted to greenhouse conditions.

### Molecular analysis of transgenic plants

Genomic DNA was extracted from tomato leaves as described previously (Ausubel *et al.*, 1994). Tomato lines were screened for the presence of transgenes by PCR on genomic DNA using gene-specific primers for snapdragon *GPPS-SSU* and *O. basilicum GES* and *ZIS* (Table S4).

### RNA isolation and quantitative real-time PCR

Total RNA was isolated from fruits of tomato plants as described previously (Eggermont *et al.*, 1996). For quantitative real-time PCR analysis, total RNA was pre-treated with RNase-free DNase (Promega, www.promega.com) and cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen, www.invitrogen.com). Gene-specific primers were designed using PrimerExpress software (Applied Biosystems, www.invitrogen.com) for snapdragon *GPPS-SSU*, *O. basilicum GES* and *ZIS*, and tomato *DXS*, *DXR*, *GPPS-SSU-II*, *GGPPS–2*, *PPPS*, *PSY–1*, *PSY–2*, *PDS* and *Actin-Tom52* (Table S4). All primers showed more than 90% efficiency at final concentrations of 300 nM (*Am-GPPS-SSU*, *Ob-GES*, *Sl-DXS*, *Sl-DXR*, *Sl-GPPS-SSU-II*, *Sl-PPPS*, *Sl-PSY–1* and *Sl-Actin-Tom52*), 500 nM (*Sl-GGPPS–2*, *Sl-PSY–2* and *Sl-PDS*) and 900 nM (*Ob-ZIS*). Quantitative real-time PCR reactions were performed, and transcript levels were determined as described previously (Orlova *et al.*, 2009) using the StepOne real-time PCR system (Applied Biosystems). Absolute transcript levels were determined for *GPPS-SSU*, *GES* and *ZIS*, and transcript levels of all tomato genes were analyzed relative to that of the reference gene *Actin-Tom52*. Each data point represents the mean of at least three independent biological samples with three technical replicates for each sample.

### Protein extraction and immunoblot analysis

Crude protein extracts were prepared from fruits of MP–1 control and *GPPS-SSU* transgenic tomato plants as described previously (Dudareva *et al.*, 1996). Total proteins (20 μg) were separated by SDS–PAGE, transferred to nitrocellulose membrane and probed using rabbit anti-GPPS-SSU polyclonal antibodies (Tholl *et al.*, 2004; 1:2500 dilution). Antigen bands were visualized using goat anti-rabbit IgG horseradish peroxidase conjugate (Sigma-Aldrich, www.sigmaaldrich.com; 1:30 000 dilution) as secondary antibodies and chemiluminescence reagents (Perkin Elmer, www.perkinelmer.com) according to the manufacturer's instructions.

### Prenyltransferase assays

Crude protein extracts were prepared from Br + 3 tomato fruits in extraction buffer (1:2 w/v tissue/buffer) as described by Nagel *et al.* (2012), followed by desalting on 2 ml Zeba Spin columns (7 K molecular weight cut-off; Thermo Scientific, www.thermoscientific.com). Prenyltransferase assays were performed as described previously (Orlova *et al.*, 2009) in the presence of 20 mM of the IPP isomerase inhibitor iodoacetamide (Sigma-Aldrich) using [1–$^{14}$C]-IPP (55 mCi $mmol^{-1}$) and DMAPP (for GPPS and FPPS activities) or FPP (for GGPPS activity). Each reaction containing 25 μg of protein was overlaid with 1 ml hexane and incubated for 30 min at 30°C. Assays were stopped by adding 3 N HCl, and additionally incubated for 20 min at 30°C. Hydrolysis products were extracted into the hexane phase, and aliquots were either counted in a liquid scintillation counter (GGPPS assays) or analyzed by TLC (GPPS/FPPS assays). For TLC analysis, 100 ng of geraniol, farnesol and gernylgeraniol were added to each sample as internal standard and carrier, and the hexane fractions were concentrated to 25 μl. The products were then separated on reversed-phase TLC plates (C18 silica TLC plates; Sorbent Technologies, www.sorbtech.com) using an acetone/water mobile phase (6:1 v/v). Radioactive spots were identified and quantified using a phosphor imager (Molecular Dynamics Typhoon 8600, www.gelifesciences.com). Internal standards were visualized by exposure to iodine vapor.

### Measurement of carotenoid levels in tomato fruits

All sample handling, extraction and analysis were performed under yellow lights to minimize photo-oxidative reactions. Extraction of carotenoids from Br + 10 tomato fruits was performed as described previously (Goltz *et al.*, 2012): 2.5 g of pericarp tissue were combined with 0.5 g sodium bicarbonate and 1 g Celite (Sigma Aldrich), and carotenoids were extracted using a 1:1 solution of acetone/petroleum ether (0.1% BHT) using a Polytron mixer (Kinematica, www.kinematica.ch). The suspension was filtered through Whatman (www.whatman.com) filter paper, and the remaining tissue homogenate was re-extracted three or four times. All acetone/petroleum ether fractions were combined and saponified using 40% w/w KOH in methanol. The mixture was transferred to a separatory funnel, and the ether layer was washed with 10 ml of saturated NaCl solution and distilled water. The

petroleum ether phase was collected, poured through a column of sodium sulfate to remove residual water, and brought to a total volume of 100 ml. Aliquots (4 ml) of the petroleum ether fraction were dried under a stream of nitrogen, re-solubilized in a MeOH/ethyl acetate (1:1) mixture, and filtered through a 0.45 μm filter. Extracted carotenoids were then analyzed and quantified by HPLC as described previously (Kean *et al.*, 2008).

### Collection, extraction and analysis of terpenoid volatiles from tomato fruits

Emitted volatiles were collected from ripe tomato fruits (Br + 10 stage) using a closed-loop stripping method under growth chamber conditions (21°C, 50% relative humidity, 150 μmol $m^{-2}$ per sec light intensity, and a 12 h photoperiod; Donath and Boland, 1995; Dudareva *et al.*, 2005). Volatile collections were performed for 24 h using Porapak Q traps (80/100 mesh size; Alltech Associates, www.alltechweb.com), and analyzed as described previously (Dudareva *et al.*, 2005). For analysis of internal pools of volatiles, approximately 30 g pericarp from fresh ripe tomato fruits (Br + 10) was cut into small pieces and extracted with 100 ml methyl *tert*-butyl ether as described previously (Davidovich-Rikanati *et al.*, 2007). The ethereal phase was separated off, dried with anhydrous $CaSO_4$, concentrated to 200 μl under gentle $N_2$ flow, and supplemented with 3.33 μg of naphthalene as an internal standard. A 2 μl aliquot of the concentrated methyl *tert*-butyl ether extract was analyzed by GC–MS as described previously (Dudareva *et al.*, 2005). Representative mono- and sesquiterpene standards were used to determine average response factors for both compound classes, which were used for quantification of the analyzed compounds.

### Isolation of chromoplasts and analysis of ZIS, GPPS and marker enzyme activities

Chromoplasts were isolated as described by Barsan *et al.* (2010): 100 g pericarp from tomato fruits of the *GPPS-SSU* × *ZIS* line was blended, and the resulting homogenate was filtered through Miracloth (www.emdmillipore.com). After centrifugation at 2000 ***g*** for 5 min, the supernatant (cytosolic fraction) was recovered. The plastid pellet was resuspended in buffer (50 mM HEPES, 330 mM sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA), and loaded on a Percoll (GE Healthcare Life Sciences, www.gelifesciences.com) step gradient as described by Siddique *et al.* (2006). After centrifugation at 7000 ***g*** for 30 min, intact plastids were recovered, washed in buffer and used for further analyses. All steps of plastid isolation and fractionation were performed at 4°C. Plastids were osmotically lysed, and the stromal fraction was recovered by centrifugation at 29 000 ***g*** for 15 min. Prior to enzyme assays, all obtained fractions were desalted. ZIS activities were analyzed as described by Davidovich-Rikanati *et al.* (2008) with 30 μM FPP, and volatile products were extracted by solid-phase micro-extraction and analyzed by GC-MS. GPPS activities were analyzed as described previously (Orlova *et al.*, 2009) using [1–$^{14}$C]-IPP (55 mCi $mmol^{-1}$). Enrichment of cytosolic and plastidic fractions was assessed by measuring the activities of marker enzymes: cytosolic alcohol dehydrogenase (ADH; Smith and ap Rees, 1979) and plastidic NADP-linked glyceraldehyde 3–phosphate dehydrogenase (GAPDH; Quinlivan *et al.*, 2003; Oostende *et al.*, 2008).

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

**Figure S1.** Expression levels of the MEP pathway genes *DXS* (AF143812) and *DXR* (AF331705), the endogenous tomato gene *GPPS-SSU-II* (AK325077), the tomato fruit-specific gene *GGPPS–2* (DQ267903) and the carotenoid biosynthesis genes *PSY–1* (EF534739). *PSY–2* (EF534738) and *PDS* (NM_001247166) in control and transgenic fruits.

**Figure S2.** Transgene expression and carotenoid accumulation during ripening of tomato fruits.

**Figure S3.** Analysis of carotenoid levels in fruits of control and transgenic tomato lines.

**Table S1.** Plant sesquiterpene synthases that use GPP as a substrate for monoterpene formation.

**Table S2.** Emission and internal pools of monoterpenes in *GPPS-SSU*, *GES* and *GPPS-SSU* × *GES* transgenic tomato fruits.

**Table S3.** Emission and internal pools of ZIS-derived monoterpenes and sesquiterpenes in *ZIS* and *GPPS-SSU* × *ZIS* transgenic tomato fruits.

**Table S4.** Primers for genomic PCR and quantitative real-time PCR analysis of control and transgenic tomato fruits.

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