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Harnessing yeast subcellular compartments for the production of plant terpenoids

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ABSTRACT

The biologically and commercially important terpenoids are a large and diverse class of natural products that are targets of metabolic engineering. However, in the context of metabolic engineering, the otherwise well-documented spatial subcellular arrangement of metabolic enzyme complexes has been largely overlooked. To boost production of plant sesquiterpenes in yeast, we enhanced flux in the mevalonic acid pathway toward farnesyl diphosphate (FDP) accumulation, and evaluated the possibility of harnessing the mitochondria as an alternative to the cytosol for metabolic engineering. Overall, we achieved 8- and 20-fold improvement in the production of valencene and amorphadiene, respectively, in yeast co-engineered with a truncated and deregulated *HMG1*, mitochondrion-targeted heterologous FDP synthase and a mitochondrion-targeted sesquiterpene synthase, i.e. valencene or amorphadiene synthase. The prospect of harnessing different subcellular compartments opens new and intriguing possibilities for the metabolic engineering of pathways leading to valuable natural compounds.

1. Introduction

Terpenoids, synthesized by all organisms, are the most widespread and structurally diverse class of biological compounds (Ajikumar et al., 2008; Bouvier et al., 2005). Among the numerous biological functions ascribed to these compounds are hormone signaling, electron transport, protein modification, and structural/functional integrity of biological membranes. Numerous specialized metabolites, such as antibiotics, phytoalexins, pigments, and aroma compounds are also terpenoids. Moreover, these molecules are widely used in commercial applications, from adhesive materials and pharmaceuticals to coloring agents, fragrances, and flavors (Ajikumar et al., 2008).

Terpenoids are biosynthesized by two distinct biochemical pathways (Maury et al., 2005). Most bacteria utilize the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway, whereas archaea and most eukaryotes utilize the mevalonic acid (MVA) pathway. In plants, both pathways are active, with the former being localized to the plastids. The MEP pathway starts with condensation of pyruvate and glyceraldehyde 3-phosphate and the MVA pathway starts with two molecules of acetyl-CoA. In both cases, high-energy carbon phosphate intermediates are converted to isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP)—the universal precursors of all natural terpenoids. These carbon units can then undergo condensation, cyclization, oxidation, glycosylation, halogenation, etc., yielding the tremendous structural diversity and chirality found in this class of chemicals (Aharoni et al., 2005).

Because chemical synthesis of most terpenoids of economic interest is inherently complex and often of low yield, and as in many cases, target terpenoids, such as paclitaxel and artemisinin, are produced in minute quantities in their native hosts, terpenoids have been the target of metabolic-engineering attempts (Ajikumar et al., 2008; Asadollahi et al., 2009; Majors et al., 2007; Yang et al., in press). One of the most attractive hosts for *de-novo* production of complex terpenoids is *Saccharomyces cerevisiae*, a eukaryote with a well-characterized MVA pathway (Maury et al., 2005). Several studies have demonstrated successful metabolic engineering of the MVA pathway in *S. cerevisiae* for enhanced production of terpenoids, mainly the C_{15} sesquiterpenes, based on the manipulation of genes related to the native yeast pathway: both their activation (e.g. *HMG1/HMG2* coding for 3-hydroxy-3-methylglutaryl-coenzyme A reductases, *ERG20* encoding farnesyl diphosphate synthase (FDPS) and the transcriptional regulator *UPC2*) and/or downregulation (e.g. *ERG9* coding

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for squalene synthase and *DPP1* encoding diacylglycerol pyrophosphate phosphatase) have been tested (Herrero et al., 2008; Jackson et al., 2003; Paradise et al., 2008; Ro et al., 2006; Takahashi et al., 2007). For example, early on, Jackson et al. (2003) found that co-expression of *ERG20* and the hyperactive *upc2-1* allele of *UPC2*, in a strain engineered whith epi-cedrol synthase, elevated sesquiterpene yield by 50%. Paradise et al. (2008) demonstrated that amorphadiene production can be enhanced up to fivefold by fine-tuning *ERG9* expression. A further demonstration of the key role of downregulating squalene synthase for enhanced production of sesquiterpenes in yeast was provided by Takahashi et al. (2007), who employed an *erg9* mutant strain with an additional mutation enabling aerobic sterol uptake. Interestingly, combining knockout of *dpp1* (a diacylglycerol pyrophosphate phosphatase that can act on farnesyl diphosphate) and *HMG1* overexpression in the *erg9Δ* strain only hampered sesquiterpene production (Takahashi et al., 2007). The common approach in these and other studies was to enhance metabolic flux toward the sesquiterpene precursor farnesyl diphosphate (FDP) or to increase its levels.

FDP stands at a major intersection of metabolic pathways leading to the production of, for example, sterols, dolichols, ubiquinone and prenylated proteins (Fig. 1), and enzymes that utilize FDP reside in different subcellular compartments. For instance Erg9p is localized to the membrane of the endoplasmic reticulum (ER), Bts1p (encoding geranylgeranyl diphosphate synthase) in the mitochondria and Srt1p (encoding cis-prenyltransferase) in lipid particles (Grabinskaa and Palamarczyk, 2002; Huh et al., 2003). Here we evaluated the possibility of harnessing intracellular compartments for the production of plant sesquiterpenes. We first generated yeast strains with enhanced flux in the MVA pathway by expressing a mutated, soluble, non-feedback-inhibited *HMG1* and a heterologous FDPS and analyzed the effects of blocking FDP metabolism toward hexaprenyl diphosphate and farnesol (FOH) on production of foreign terpenes, i.e. the plant sesquiterpenes valencene (a major citrus aroma constituent) and amorphadiene (an antimalarial drug precursor). Targeting of these sesquiterpenes to mitochondria revealed that yeast have a viable pool of FDP there, which can be efficiently redirected toward the production of plant terpenoids. Production levels could be further elevated by co-localizing FDPS and sesquiterpenes synthases to the mitochondria and using yeast with both cytosol- and mitochondrion-targeted foreign terpene synthases. The advantages of integrating various subcellular localizations for efficient metabolic engineering of terpenoids are discussed.

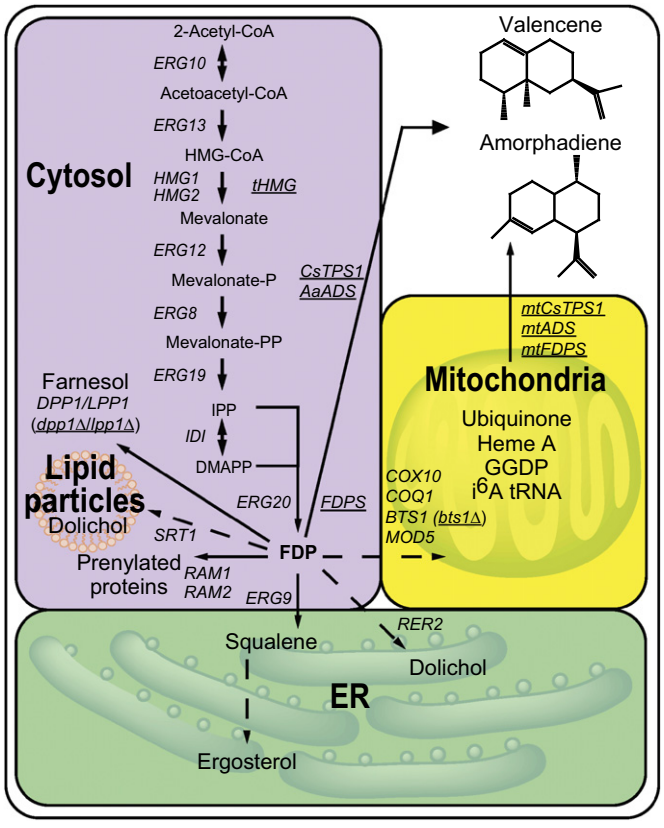

Fig. 1. The mevalonic acid (MVA) pathway in *S. cerevisiae*. Genes that were integrated into the pathway (underlined) and those that were deleted (underlined and marked with Δ) are indicated. *tHMG*—truncated 3-hydroxy-3-methylglutaryl-coenzyme A reductase, *FDPS*—heterologous farnesyl diphosphate synthase, *CsTPS1*—valencene synthase, and *AaADS*—amorpha-4,11-diene synthase; mt denotes mitochondrion-targeting sequence fused to the corresponding gene.

2. Materials and methods

2.1. Materials

Microbial growth medium was purchased from Difco Laboratories (Sparks, MD, USA). Molecular biology reagents, enzymes, and kits were from Fermentas International (Burlington, Ontario, Canada), and Promega (Madison, WI, USA). 5-Fluoroorotic acid (5-FOA) was obtained from Zymo Research (Orange, CA, USA). All other chemicals were purchased from Sigma-Aldrich (Rehovot, Israel).

2.2. Yeast and bacterial host strains

Escherichia coli XL1-Blue (Stratagene, La Jolla, CA, USA) was used for all routine cloning and plasmid propagation. Bacteria were grown in Luria–Bertani broth supplemented with 100 mg/ml ampicillin. *Saccharomyces cerevisiae* strains W303-1A (*MATa*, *ade2-1*, *trp1-1*, *leu2-3*, *112 his3-11*, *15 ura3-1*) and BDXe (a uracil auxotroph derivative of strain BDX, Lallemand, Rexdale, Ontario, Canada) were used as the parent strains.

2.3. Construction of yeast expression vectors

To enable genetically stable and controlled expression of several genes in yeast, plasmid pδE was constructed. First the promoter and 5′-UTRs of the copper-inducible promoter *CUP1* (P_{CUP1}) were amplified by polymerase chain reaction (PCR) from W303-1A yeast genomic DNA. Next the 3′-UTR and terminator of *CYC1* (T_{CYC1}) were PCR-amplified from yeast genomic DNA using primers that introduced multiple cloning sites (Labbé and Thiele, 1999). After digestion with *Not*I and *Xba*I, the two fragments were ligated with *Sac*I/*Xba*I-digested pδ-UB plasmid (Lee and Da Silva, 1997) to generate pδE. This vector allows strong copper-inducible expression, genomic integration into chromosomal δ sequences and recycling of the *URA3* selection marker by counter-selection on 5-FOA-supplemented medium. To generate a mutated form of yeast 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*HMG-R*), the catalytic domain of *HMG1* (*tHMG*; Polakowski et al., 1998) was PCR-amplified from yeast genomic DNA and cloned into pδE, and the resulting plasmid was termed pδ-tHMG.

Arabidopsis thaliana farnesyl diphosphate synthase (At*FDPS*, accession no. X75789) was PCR-amplified from cDNA using gene-specific primers designed to target the short cytosolic form (*FPS1S*; Cunillera et al., 1996) and which added 5′ *Not*I and 3′ *Sph*I restriction sites. The amplified fragment was cloned into pδE at the *Not*I and *Sph*I sites, generating vector pδE-AtFDPS. *Homo sapiens* FDPS (HsFDPS, accession no. BC010004) was cloned

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similarly using clone ID HsCD00045488, obtained from PlasmidID (http://plasmid.med.harvard.edu/PLASM ID).

The complete coding sequence of *Citrus sinensis* valencene synthase *CsTPS1* (accession no. AF441124) was PCR-amplified from pRSETa-CsTPS1 (Sharon-Asa et al., 2003) using primers that added 5′ *Not*I and 3′ *Sph*I restriction sites. The *Artemisia annua* terpene synthase, amorpha-4,11-diene synthase (ADS, accession no. Q9AR04; Mercke et al., 2000), was cloned using *A. annua* cDNA and gene-specific primers that added 5′ *Not*I and 3′ *Sph*I restriction sites. Amplified *CsTPS1* and *ADS* genes were cloned individually into pδE at the *Not*I/*Sph*I sites generating pδE-CsTPS1 and pδE-ADS. To generate plasmid pRS303N-CsTPS1, harboring CsTPS1 under P_{CUP1} in the antibiotic-selectable integration vector pRS303N (Taxis and Knop, 2006), the entire expression cassette (comprising P_{CUP1}–*CsTPS1*–T_{CYC1}) was mobilized from pδE-CsTPS1 using *BssH*II restriction and inserted at the *Asc*I restriction site of pRS303N.

To target enzymes of interest to the yeast mitochondria, the native yeast mitochondrial signal peptide from *COX4* (Hurt et al., 1985) was fused to *CsTPS1*, *AaADS*, and *AtFDPS*. The resultant mitochondrion-targeted constructs mtCsTPS1, mtADS, and mtFDPS were cloned into the pδE vector, yielding pδE-mtCsTPS1, pδE-mtADS, and pδE-mtFDPS, respectively.

2.4. Yeast transformation, strain construction, and cultivation

Yeasts were grown in YPD medium (1% yeast extract, 2% peptone, 2% glucose, w/v) or synthetic minimal medium (SD; 0.67% yeast nitrogen base, 2% glucose, w/v, and auxotrophic amino acids and vitamins as required).

All *S. cerevisiae* strains used in this study are listed in Table 1. Yeast were transformed by the lithium-acetate method; when pδE plasmids were used, they were linearized at the *Xho*I site in the δ-sequence prior to transformation. Colonies growing on uracil drop-out media were verified as harboring the relevant gene by colony PCR. To allow stacking of genes of interest into the yeast genome, the *URA3* selection gene of an integrated pδE plasmid was selected against by growing cells on medium containing 5-FOA. Integration of pRS303N-CsTPS1 at the *HIS3* locus was achieved by transformation of a *Bst*BI-treated vector and selection with medium supplemented with nourseothricin. Retention of the relevant genes following the selection scheme was verified by PCR and sequencing.

A *S. cerevisiae* mutant strain with deleted geranylgeranyl diphosphate synthase was constructed by disrupting the *BTS1*/*YPL069C* locus in strain W303-1A using the *HIS3* auxotrophic selection marker from pRS303 (Sikorski and Hieter, 1989), which was PCR-amplified using primers containing 40-bp sequences homologous to the *BTS1* gene. Following transformation with the resultant PCR fragment, the transformants were selected on SD medium lacking histidine. A *DPP1*/*YDR284C*-knockout strain, in the W303-1A background, was also obtained by the PCR gene-deletion method using *ADE2* amplified from BY4741 yeast strain genomic DNA. A double *dpp1Δ*/*lpp1Δ* deletion strain was obtained by transformation of the *dpp1Δ* strain with a *KanMX4* cassette flanked by homologous sequences of the *LPP1*/*YDR503C* (coding for a lipid phosphate phosphatase; Faulkner et al., 1999) locus. Correct replacement of the native allele was verified by PCR and sequencing.

For analysis of terpenoid production, precultures were grown overnight in test tubes containing 5 ml SD supplemented with 100 μM bathocuproine disulfonate (BCS). Precultures were diluted to an OD_{600} of 0.1 in 10 ml fresh medium supplemented with 100 μM $CuSO_4$. For *in-situ* removal of terpenoids, a two-phase partitioning batch culture was employed by adding 10% (v/v) n-dodecane as the organic phase (Ro et al., 2006). Cultures were grown for 6 days, and the organic layer was then sampled for gas chromatography-mass spectrometry (GC–MS) analysis. From each transformation event, several colonies were evaluated for FOH and plant terpenoid production; no yield differences were noted.

2.5. GC–MS analysis of terpenoids

Terpenoid identification and quantification were performed as described by us previously (Farhi et al., 2010). The system was composed of a TRACE GC 2000 gas chromatograph and a TRACE DSQ quadrupole mass spectrometer (ThermoFinnigan, Hemel, UK). Gas chromatography was run in a 30 m Rtx-5Sil MS column with 0.25-μm film thickness (Restek, Bad Homburg, Germany). The injection temperature was set at 250 °C, the interface at

Table 1
Saccharomyces cerevisiae strains generated in this study.

Strain no.	Host strain	Integrated constructs[a]
M144	W303-1A	δ::P_{CUP1}–*tHMG*, δ::P_{CUP1}–*AtFDPS*, δ::P_{CUP1}–*CsTPS1*
M208	W303-1A	δ::P_{CUP1}–*CsTPS1*
M212	BDXe	δ::P_{CUP1}–*CsTPS1*
M213	BDXe	δ::P_{CUP1}–*mtADS*
M241	BDXe	δ::P_{CUP1}–*mtCsTPS1*
M243	BDXe	δ::P_{CUP1}–*tHMG*, δ::P_{CUP1}–*mtCsTPS1*
M246	BDXe	δ::P_{CUP1}–*tHMG*, δ::P_{CUP1}–*mtFDPS*, δ::P_{CUP1}–*mtADS*
M263	BDXe	δ::P_{CUP1}–*ADS*
M277	W303-1A	*bts1*δ::*HIS3*, δ::P_{CUP1}–*CsTPS1*
M278	W303-1A	*lpp1*δ::*ADE2*, *dpp1*δ::*KanMX4*, δ::P_{CUP1}–*CsTPS1*
M280	W303-1A	*bts1*δ::*HIS3*, δ::P_{CUP1}–*tHMG*, δ::P_{CUP1}–*CsTPS1*
M281	W303-1A	*lpp1*δ::*ADE2*, *dpp1*δ::*KanMX4*, δ::P_{CUP1}–*tHMG*, δ::P_{CUP1}–*CsTPS1*
M287	W303-1A	δ::P_{CUP1}–*tHMG*, δ::P_{CUP1}–*CsTPS1*
M288	W303-1A	δ::P_{CUP1}–*tHMG*, δ::P_{CUP1}–*HsFDPS*, δ::P_{CUP1}–*CsTPS1*
M290	W303-1A	δ::P_{CUP1}–*AtFDPS*, δ::P_{CUP1}–*CsTPS1*
M291	W303-1A	δ::P_{CUP1}–*HsFDPS*, δ::P_{CUP1}–*CsTPS1*
M1058	BDXe	δ::P_{CUP1}–*tHMG*, δ::P_{CUP1}–*FDPS*, δ::P_{CUP1}–*mtADS*
M1059	BDXe	δ::P_{CUP1}–*tHMG*, δ::P_{CUP1}–*mtFDPS*, δ::P_{CUP1}–*ADS*
M1076	BDXe	δ::P_{CUP1}–*tHMG*, δ::P_{CUP1}–*FDPS*, δ::P_{CUP1}–*mtCsTPS1*
M1077	BDXe	δ::P_{CUP1}–*tHMG*, δ::P_{CUP1}–*mtFDPS*, δ::P_{CUP1}–*mtCsTPS1*
M1216	BDXe	δ::P_{CUP1}–*tHMG*, δ::P_{CUP1}–*FDPS*, δ::P_{CUP1}–*CsTPS1*
M1297	BDXe	δ::P_{CUP1}–*tHMG*, δ::P_{CUP1}–*FDPS*, *HIS3*::P_{CUP1}–*CsTPS1*, δ::P_{CUP1}–*mtCsTPS1*

[a] δ:: denotes integration into a δ element insertion site using pδE-based vector.

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250 °C, and the ion source adjusted to 230 °C. Helium was used as the carrier gas at a flow rate of 1 ml/min. The analysis (1 μl sample) was performed under the following temperature program: 1 min isothermal heating at 60 °C, followed by a 10 °C/min oven temperature ramp to 130 °C, then a 3 °C/min oven temperature ramp to 170 °C, followed by a 50 °C/min oven temperature ramp to 270 °C and final heating for 2 min at 270 °C. A scan range of 40–450 m/z was used. Metabolites were identified by comparing retention times and mass spectra with those in the NIST library and with authentic standards. Terpenoids were quantified using a valencene standard curve and a caryophyllene internal standard.

3. Results

3.1. Production of the Citrus sesquiterpene valencene in yeast is affected by deletion of BTS1 but not of DPP1 or LPP1

FDP is at a metabolic branching point in the terpenoid pathway in yeast (Fig. 1) and several enzymes, located in different cellular compartments, can compete for it (Grabinskaa and Palamarczyk, 2002). To test whether production of foreign sesquiterpenes can be enhanced through the elimination of putative competing reactions, we first generated yeast line M208 expressing *C. sinensis CsTPS1*, coding for valencene synthase, under the control of *CUP1* (Table 1). To trap terpenes generated by the yeast culture, the medium was overlaid with dodecane. GC–MS analysis of the organic layer confirmed that M208 was capable of synthesizing valencene following *CUP1* induction. Only the single product and no secondary modification of the hydrocarbon could be detected in the analysis. In culture in which expression from the *CUP1* promoter was inhibited by addition of the copper chelator BCS, only a negligible amount of valencene was detected (Fig. 2). Yeast's production of valencene was evaluated for 6 days in YPD medium. Valencene continued to accumulate throughout the culture period, reaching a maximal level of 38 μg/l on the sixth day, while cultures reached the stationary stage of growth within 48 h (Fig. 2b and c). The accumulation pattern of the endogenous sesquiterpene FOH was essentially the same as that of valencene (Supplementary information, Fig. S1a). Prolonged culture time without fresh medium supplementation did not yield any significant increase in valencene production (data not shown), and a 6-day growth period was therefore chosen for further experiments.

Geranylgeranyl diphosphate synthase, encoded in *S. cerevisiae* by *BTS1/YPL069C*, utilizes FDP as part of the ubiquinone biosynthesis pathway and the protein geranylgeranylation process; its mutant does not confer a significant growth phenotype (Fig. 1; Jiang et al., 1995). To evaluate whether this reaction competes with valencene production, we generated yeast with *bts1Δ*. Valencene production in a *bts1Δ* background, measured using a dodecane biphasic culture, decreased by half as compared to that in the WT background (Fig. 3), while cell density was not affected by the knockout.

To test whether a reduction in Dpp1p and Lpp1p phosphatase activity on FDP might enhance valencene production, *CsTPS1* was expressed in a yeast strain lacking *DPP1* and *LPP1* in its genome. Not only did knockout of the phosphatase genes not enhance valencene production, it actually led to a marginal reduction of ca. 10% compared to that in the WT background (Fig. 3). Specific production

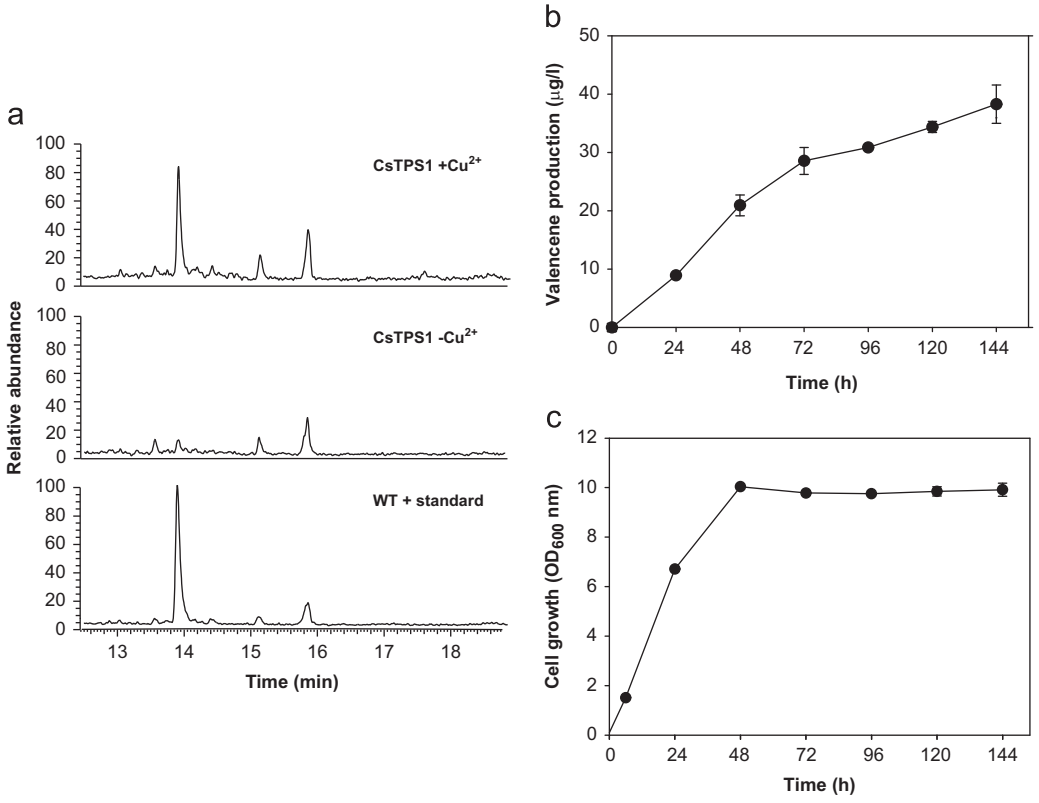

Fig. 2. Valencene production in yeast engineered with *CsTPS1*. Yeast transformed with *CsTPS1* or the parental strain W303-1A were grown on YPD medium overlaid with dodecane. Cells were supplemented with 100 μM $CuSO_4$ for induction of the CUP1 promoter (+Cu) or with BCS to inhibit induction (−Cu). Aliquots of dodecane and medium were collected and analyzed by GC–MS to determine levels of valencene and optical cell density, respectively. (a) Total ion chromatographs of dodecane extract from cell cultures following induction or inhibition of CUP1 promoter; WT—parental strain W303-1A supplemented with valencene authentic standard. (b) Valencene was measured at the indicated time points using GC–MS; data represent means of triplicate cultures ± standard error. (c) Changes in cell density of yeast culture expressing CsTPS1 over the 144 h growth period.

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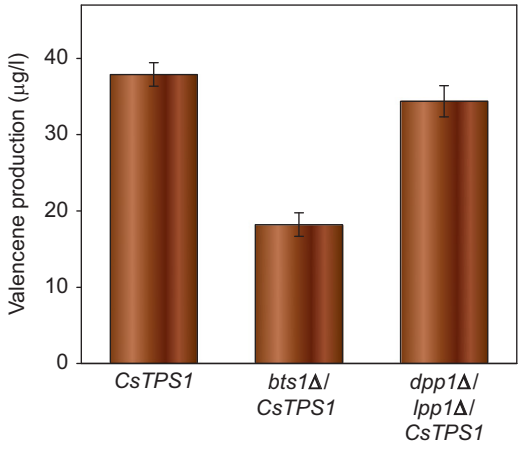

Fig. 3. Effect of *bts1Δ* or *dpp1Δ* and *lpp1Δ* on valencene production. W303-1A, *bts1Δ* or *dpp1Δ*, and *lpp1Δ* yeast strains were transformed with *CsTPS1* and grown on YPD medium overlaid with dodecane. The dodecane layer was analyzed by GC–MS. Data represent means of at least triplicate cultures ± standard error.

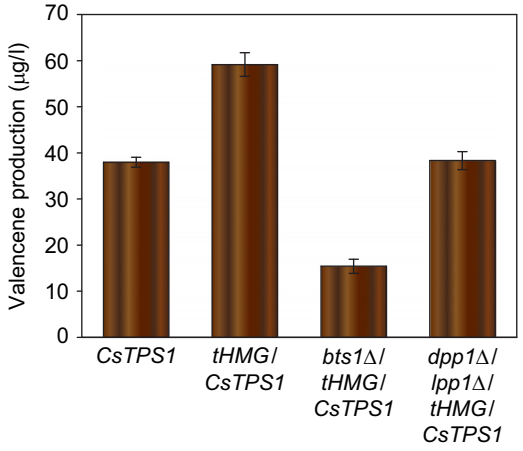

Fig. 4. Effect of *tHMG* expression in *bts1Δ* or *dpp1Δ* and *lpp1Δ* background on valencene production. W303-1A, *bts1Δ* or *dpp1Δ*, and *lpp1Δ* yeast strains were transformed with *CsTPS1* or *CsTPS1* and *tHMG* and grown on YPD medium overlaid with dodecane. The dodecane layer was analyzed by GC–MS. Data represent means of at least triplicate cultures ± standard error.

of valencene, when normalized to OD_{600} units, in *dpp1Δ*/*lpp1Δ* as well as in a *bst1Δ* background was similar to that calculated per total culture; increasing the mutant culture growth periods to up to 12 days did not affect production, similar to the WT.

3.2. Improved substrate availability enhances valencene production but does not overcome the negative effects of *bts1Δ* and *dpp1Δ*/*lpp1Δ*

To evaluate the effect of elevating metabolic flux in the MVA pathway on valencene production, we overexpressed an N-terminal-truncated form of Hmg1p (tHMG) from *S. cerevisiae*, which gives a soluble form of the enzyme that is relieved of inhibition by the pathway's products (Polakowski et al., 1998). Yeast engineered with *CsTPS1* and *tHMG* were able to produce approximately 1.6-fold more valencene than the parental strain with *CsTPS1* alone (Fig. 4). Similarly, production of FDP-derived FOH was also elevated in the *tHMG*-expressing strain (Supplementary information, Fig. S1b). In contrast, addition of *tHMG* to a strain engineered with *CsTPS1* and lacking *DPP1* and *LPP1* did not improve valencene production, maintaining a titer of ca. 40 μg/l. The *bts1Δ* background also led to a reduction in valencene production levels. Despite expression of *tHMG*, a ca. fourfold decrease was observed in this mutant background as compared to the WT background (Fig. 4), keeping production levels in the latter similar to those observed without tHMG in the mutant strain (Fig. 3).

To enhance substrate availability for foreign terpene production via an increase in FDPS levels, we cloned *A. thaliana* short cytosol-localized FDPS (AtFDPS) and expressed it in yeast (Cunillera et al., 1996). Expression of *AtFDPS*, similar to *tHMG*, had a positive effect, i.e. a ca. 30% increase, on valencene production. Replacing arabidopsis *FDPS* with a human-derived gene (*HsFDPS*) had a similar effect on valencene levels.

Co-expression of the genes enhancing flux in the MVA pathway, *tHMG*, and *FDPS*, had a synergistic effect, improving production of valencene fourfold compared to the WT background. Use of *AtFDPS* or *HsFDPS* had a comparable effect: 150 μg/l of valencene was measured after 144 h growth on YPD medium (Fig. 5). To further substantiate the effect of *tHMG* and heterologous *FDPS* on valencene production, the non-laboratory yeast line BDXe was engineered by genomic integration of *CsTPS1*, *tHMG*, and *AtFDPS*,

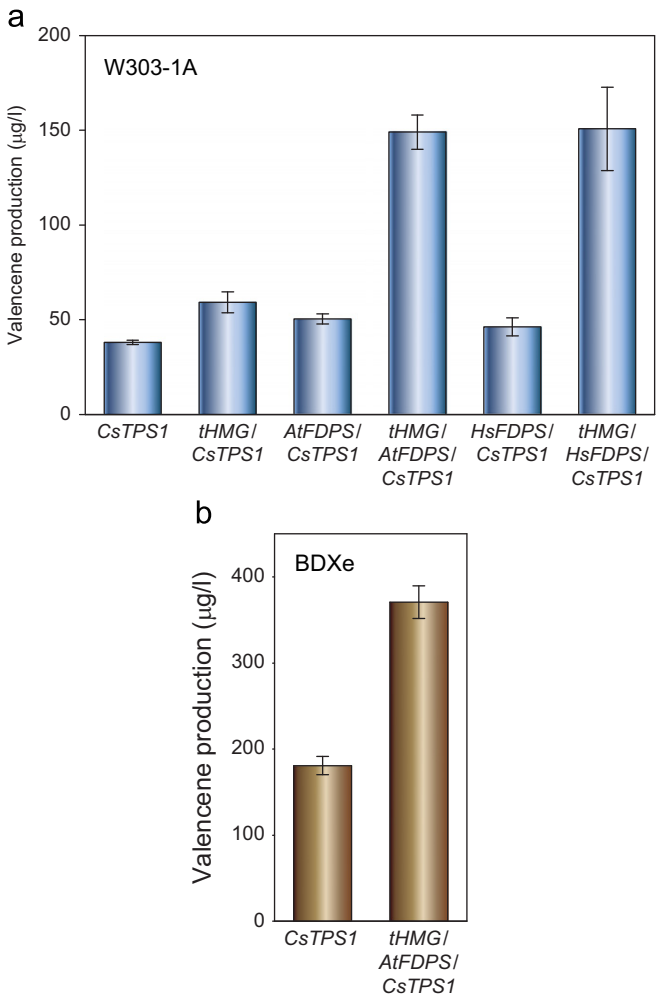

Fig. 5. Expression of heterologous FDPS enhances valencene production in yeast *CsTPS1*/*tHMG* strains. (a) GC–MS was used to measure valencene produced by yeast strain W303-1A engineered as indicated in the figure. *AtFDPS* and *HsFDPS*—*Arabidopsis thaliana* and *Homo sapiens* farnesyl diphosphate synthase, respectively. (b) GC–MS was used to measure valencene produced by yeast strain BDXe transformed with *CsTPS1* or with *CsTPS1*, *tHMG*, and *AtFDPS*. Data represent means of triplicate cultures ± standard error.

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driven by *CUP1* promoters. Strain BDXe expressing only *CsTPS1* produced 4.7 times higher valencene levels than *CsTPS1*-expressing W303-1A. Co-expression of *tHMG* and *FDPS* further doubled valencene production in the *CsTPS1*-expressing BDXe, bringing it to 370 μg/l (Fig. 5).

3.3. Shunting engineered terpenoid metabolism through yeast mitochondria results in a dramatic increase in plant sesquiterpene production

Several mitochondrial enzymes, e.g. Cox10p, Coq1p, and Bts1p (Fig. 1; Grabinskaa and Palamarczyk, 2002), utilize FDP. To examine whether the mitochondrial FDP pool can be harnessed to synthesize foreign terpenes, we fused the bona fide mitochondrial targeting signal peptide from the yeast *COX4* gene (Hurt et al., 1985) to *CsTPS1*, generating *mtCsTPS1*. Expression of *mtCsTPS1* in BDXe yeast led to a threefold rise in valencene titers compared to that generated via the use of cytosolic *CsTPS1* (Fig. 6a). Combining *tHMG* expression with *mtCsTPS1* enhanced valencene production by an additional 50%. Expression of *AtFDPS* in this background had no beneficial effect on valencene production levels. In contrast, when mitochondrion-targeted FDPS (*mtFDPS*) was used instead of *AtFDPS*, valencene production levels were further increased by 40%. Next, we tested whether both the mitochondria and the cytosol can be exploited simultaneously for valencene production. Addition of a cytosolic copy of valencene synthase, *CsTPS1*, to *tHMG*/*mtFDPS*/*mtCsTPS*-expressing strain BDXe further increased valencene production, yielding an overall 1.5 mg/l, a ca. eightfold increase over BDXe expressing only cytosolic *CsTPS1* (Fig. 6a). To substantiate the efficacy of using the mitochondrial compartment for production of plant sesquiterpenes, we cloned amorph-4,11-diene synthase (*ADS*) from *Artemisia annua* and fused it to the yeast mitochondrion-targeting signal, generating *mtADS*. Expression of *mtADS*, as compared to its cytosolic counterpart, strongly enhanced amorphadiene production. Co-expression of tHMG and mtFDPS with mtADS further elevated amorphadiene production, yielding ca. 20 mg/l of this sesquiterpene (Fig. 6b).

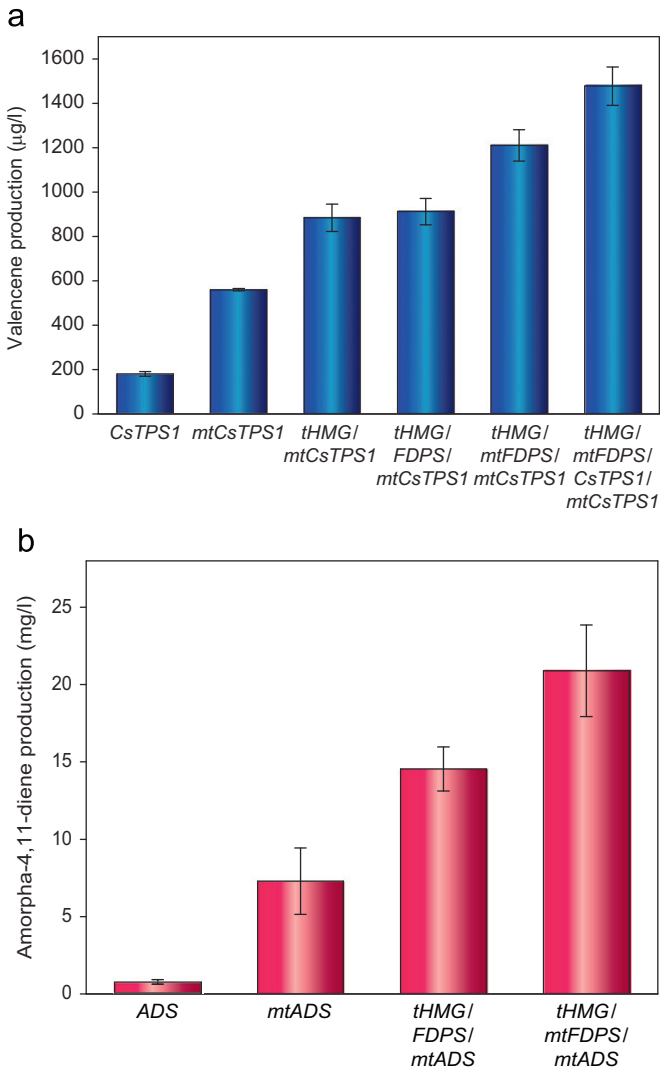

Fig. 6. Improved production of sesquiterpenes, valencene, and amorphadiene, by targeting CsTPS1, ADS, and FDPS to the yeast mitochondria. Cytosol- or mitochondrion-targeted CsTPS and ADS for valencene (a) and amorphadiene (b) production, respectively, were expressed in BDXe yeast engineered with tHMG and cytosol- or mitochondrion-targeted FDPS. GC–MS was used to measure valencene and amorphadiene production. Results are an average of three replicates with error bars indicating standard error from the mean.

4. Discussion

Enhancing terpenoid production by applying various metabolic-engineering strategies in the native host organism, or in a heterologous one, has enjoyed considerable interest in the last few years (Ajikumar et al., 2008). The use of microbial platforms for terpene production has significant advantages, such as the availability of data on metabolic networks, the ease of reconstructing and redesigning complete pathways, and the ability to scale up the fermentation process (Pfleger et al., 2007; Redding-Johanson et al., 2011). Indeed there are several examples of successful high-titer production of terpenoids such as amorphadiene, linalool, carotenoids, and taxadiene, in microbial systems such as *Escherichia coli*, *Lactococcus lactis*, and *S. cerevisiae* (Anthony et al., 2009; DeJong et al., 2006; Hernandez et al., 2007; Martin et al., 2003; Oswald et al., 2007; Verwaal et al., 2007). As a eukaryote, yeast has the advantage of being able to support post-translational modifications and host membrane-anchored and organelle-specific enzymes (Shiba et al., 2007; Szczebara et al., 2003). Some of the best examples of the biosynthesis of complex terpenoids in yeast include reconstruction of the mammalian hydrocortisone biosynthetic pathway, production of artemisinic acid (a precursor of the antimalarial drug artemisinin), and commercial bioproduction of β-carotene in the oleaginous yeast *Yarrowia lipolytica* (Ro et al., 2006, 2008; Szczebara et al., (2003); http://www.microbia.com/, 2010).

Most studies aimed at generating yeast-cell factories for the production of terpenoids have employed overexpression of key flux-regulating enzymes in the MVA pathway. Overproduction of the catalytic domain of HMG-R (tHMG) in *S. cerevisiae* was shown to enhance production of both native terpenoids (Donald et al., 1997; Tokuhiro et al., 2009) and plant-derived terpenoids when combined with expression of a heterologous terpene synthase (Ro et al., 2006). Overexpression of the native yeast FDPS *ERG20* resulted in a 28% increase in ergosterol but no significant increase in the production of plant-derived diterpenes or sesquiterpenes in yeast (Chambon et al., 1991; DeJong et al., 2006; Ro et al., 2006). We found that the use of a heterologous arabidopsis or human FDPS allowed enhanced production of valencene in *S. cerevisiae* (Fig. 5). It should be noted that valencene production levels were low compared to the levels of amorphadiene and to those reported for other plant sesquiterpenes produced in yeast, e.g. cubebol, and 5-epi-aristolochene (Asadollahi et al., 2010; Takahashi et al., 2007). Indeed, the activity of valencene synthase is low relative to that of other terpene synthases. Moreover, divergence in the coding sequences of various copies of genes encoding the enzyme in the same and different varieties (e.g., GenBank accession nos. AAM00426, AAQ04608, ACO36239, ACX70155 and CQ81350) might affect the productivity of plant

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sesquiterpenes in yeast (Takahashi et al., 2007; Yoshikuni et al., 2006). Here, enhancing flux in the MVA pathway through expression of tHMG increased valencene production levels, similar to the increase following FDPS expression, and combining overexpression of tHMG and FDPS had an additive effect. This was further validated using a different yeast strain, in which basal *CsTPS1*-driven valencene production was significantly higher. The differential ability of various yeast strains to generate foreign terpenoids has been described previously (Takahashi et al., 2007); in the case of BDXe as compared to W303-1A, the higher efficiency of the former might be due to its being a diploid, and not a laboratory strain that accumulated mutations, e.g. *hap1*, affecting MVA and terpenoid pathways (Tamura et al., 2004). In yeast, FDPS is under complex regulation; for instance, tRNA levels appear to regulate Erg20p levels (Kwapisz et al., 2002). It is therefore plausible that the use of a heterologous FDPS circumvents this control mechanism. Indeed, Wu et al. (2006) demonstrated that expression of FDPS derived from chicken in tobacco plants yields highly increased production levels of non-native sesquiterpenes such as patchoulol and amorpha-4,11-diene.

Another possible strategy for increasing the production of a selected terpenoid is limiting the use of the FDP pool for general cell metabolism. Several studies have demonstrated that limiting the FDP used for sterol biosynthesis by downregulating *ERG9* expression has a significant positive effect on sesquiterpene biosynthesis (Asadollahi et al., 2010; Paradise et al., 2008). We analyzed whether sesquiterpene biosynthesis can be enhanced by limiting the use of FDP for biosynthesis of ubiquinone and protein prenylation by deleting geranylgeranyl diphosphate synthase (*bts1Δ*), or by limiting FDP phosphatase activity by deleting endogenous phosphatases (*lpp1Δ* and *dpp1Δ*). In neither instance was sesquiterpene production improved, even when combined with flux enhancement in the terpenoid pathway generated by expression of *tHMG* (Fig. 4). It should be noted that the lack of a positive effect of *BTS1* deletion was not due to the intracellular location of Bts1p in the mitochondria, since valencene levels were not enhanced in this mutant background even when CsTPS1 was directed to the mitochondria (not shown). Dpp1p and Lpp1p have been shown to contribute to FDP phosphatase activity in yeast, and a cell-free extract of the double mutant was deficient in Mg^{2+}-independent FDP dephosphorylation activity (Faulkner et al., 1999). Yet, similar to our results, a yeast strain in which a single phosphatase (*DPP1*) was knocked out did not exhibit improved sesquiterpene production (Takahashi et al., 2007). Since Dpp1p and Lpp1p are localized to the vacuole and cellular membrane, respectively, they may not be directly involved in FDP dephosphorylation or in FOH or nerolidol production *in vivo*. Indeed, overexpression of *DPP1* did not lead to increased accumulation of either farnesol or nerolidol. The high levels of FOH observed by us and others in yeast can arise from the enzymatic or spontaneous degradation of FDP (Tokuhiro et al., 2009). On the other hand, it can be suggested that *lpp1Δ*/*dpp1Δ* positively affects foreign sesquiterpene production only above a certain threshold level of flux toward FDP.

Enzymes that use IPP, DMAPP, and FDP isoprene units in eukaryotes are compartmentalized within the cell (Fig. 1). In *S. cerevisiae*, the MVA pathway provides all terpenoid-backbone precursors for molecules destined to both cytoplasmic and intracellular compartments. Given that ubiquinone, heme A, and geranylgeranyl diphosphate are biosynthesized in the mitochondria, we reasoned that mitochondrial FDP might be harnessed for the production of selected terpenoids. We fused the yeast mitochondrial targeting sequence to two plant sesquiterpene synthases, valencene and amorphadiene synthase, and expressed them in yeast. In both cases, sesquiterpene biosynthesis was greatly improved relative to its production by the corresponding cytosolic forms of the terpene synthases. We also found that sesquiterpene production via mitochondrial targeting of the relevant terpene synthase can be further increased by enhancing metabolic flux in the MVA pathway via *tHMG* expression. The enhanced levels of sesquiterpenes produced following mitochondrial targeting of terpene synthases, as compared to cytosolic terpene synthases, suggest that the FDP pool is higher in the yeast mitochondria, FDP is more accessible for sesquiterpene biosynthesis, or the terpene synthases are more active in the yeast mitochondria. Lee and Chappell (2008) and Aharoni et al. (2004) isolated terpene synthases from magnolia and strawberry, respectively, with a dual chloroplast–mitochondrion-targeting signal peptide. Furthermore, there is one example from the plant kingdom in which mitochondrial targeting of a cytosolic strawberry sesquiterpene synthase, nerolidol synthase, led to elevated nerolidol production, in *A. thaliana* (Kappers et al., 2005). More recently, mitochondrial targeting sequences have been used by van Herpen et al. (2010) to produce artemisinin precursors in tobacco. Overall, it can be suggested that in yeast as well as in plants, the mitochondria possess strong potential as a factory for sesquiterpene production.

Mitochondrial targeting of FDPSs for the production of FDP in this organelle appears to be common to many eukaryotes, such as humans, plants, and insects (Martín et al., 2007). Since isopentenyl diphosphate isomerase 2 (IDI2) is also localized to the mitochondria in plants, pools of IPP and/or DMAPP are probably also present in those organelles (Phillips et al., 2008). In yeast, while there is no evidence of mitochondrial localization of FDPS or IDI, enzymes Bts1p and Mod5p, which utilize IPP and DMAPP, respectively, reside in the mitochondria (Dihanich et al., 1987; Huh et al., 2003). To test whether mitochondria have a significant pool of DMAPP and IPP and whether the amount of FDP available for sesquiterpene production can be boosted, we targeted FDPS to this subcellular compartment. Expression of *mtCsTPS1* or *mtADS* together with mitochondrion-targeted FDPS, as compared to its cytosolic form, in cells engineered with tHMG, led to increased levels of the respective sesquiterpenes. Expression of a cytosolic form of CsTPS1 in addition to mtCsTPS1 further elevated valencene production levels by approximately 25%, an amount (300 μg/l) that was similar to that produced by the strain engineered solely with tHMG and CsTPS1. The possibility of jointly harnessing different intracellular compartments, e.g. mitochondria and cytosol, for the production of terpenes of interest opens new and intriguing possibilities for the metabolic engineering of pathways leading to valuable natural compounds.

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Appendix A. Supplementary material

Supplementary materials associated with this article can be found in the online version at doi:10.1016/j.ymben.2011.05.001.

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